UNIVERSITAT DE BARCELONA

Molecular mechanisms for active DNA demethylation in cellular immune models

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MOLECULAR MECHANISMS FOR ACTIVE DNA DEMETHYLATION IN CELLULAR IMMUNE MODELS

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"*What is a scientist? … We give the name scientist to the type of man who has felt experiment to be a means guiding him to search out the deep truth of life, to lift a veil from its fascinating secrets, and who, in this pursuit, has felt arising within him a love for the mysteries of nature, so passionate as to annihilate the thought of himself.*"

Maria Montessori

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SUMMARY

Epigenetic factors, such as histone post-translational modifications and DNA methylation, are critical determinants of gene expression, which ultimately defines cell identity and function. Despite intense research in DNA methylation over the past twenty years, there are still many open questions in relation to the mechanisms underlying its targeting to different genomic sites, its specific functional roles and the identity of enzymes involved in its establishment and removal. Immune cells are extraordinarily diverse and require of many differentiation and activation steps in response to cytokines, growth factors and many other extracellular signals, including exposure to pathogenic molecules. The complexity of immune differentiation and responses provides excellent models to investigate questions about epigenetic mechanisms, including DNA methylation. In this thesis, we aimed at investigating the molecular mechanisms of active DNA demethylation and its genomic targeting through the use of two different immune models.

Firstly, we have explored the potential role of activation-induced cytidine deaminase (AID) in mediating active DNA demethylation through its deaminase activity. To this end, we performed whole genome bisulfite sequencing of naïve and memory B cells isolated from healthy controls and patients with hyper-IgM syndrome type 2 (HIGM2), a rare human primary antibody deficiency characterized by loss-of-function of AID. Comparison between the methylomes of B cell subsets from controls and HIGM2 patients, revealed global DNA methylation alterations associated with AID loss. In fact, the observed alterations include a blockage of demethylation during the transition from naïve to memory B cells. In addition, AID deficiency also results in alterations in naïve B cells. The analysis of the HIGM2-associated alterations occurring in the transition from naïve to memory B cell rules out the direct involvement of AID in active demethylation and suggests the participation of TET enzymes. Finally, the exploration of the DNA methylation alterations in HIGM2 naïve B cells revealed a role for AID in early stages of B cell development removing autoreactive B cells with a DNA methylation signature characteristic of BCR-stimulated cells.

Secondly, we also explored the role of DNA demethylation in the regulation of immunosuppressive phenotype of monocyte- (MO) derived dendritic cells (DC) differentiated in presence of vitamin D, focusing on the interplay among different signal-dependent transcription factors (TF) and the DNA demethylation machinery. In this study, we performed high-throughput DNA methylation screening of both inflammatory DCs and DCs differentiated in presence of vitamin D (TL). DNA methylation analysis revealed extensive condition-specific DNA demethylation events associated with differential immune properties. As expected, we observed that demethylation occurs in enhancer regions and displays an inverse correlation with gene expression. We proved that, vitamin D receptor (VDR) can bind to closed chromatin and correlates with TL-specific demethylation. Interestingly, we observed that tolerogenic properties in DCs are acquired together with activation of the IL-6/JAK/STAT3 pathway. In fact, VDR directly binds the IL-6 gene and JAK2-mediated STAT3 phosphorylation is specific to vitamin D-stimulation. In addition, we also observed that VDR interacts with both STAT3 and TET2. Finally, we reported that pharmacological inhibition of STAT3 phosphorylation reverts the vitamin–induced tolerogenic properties of DCs.

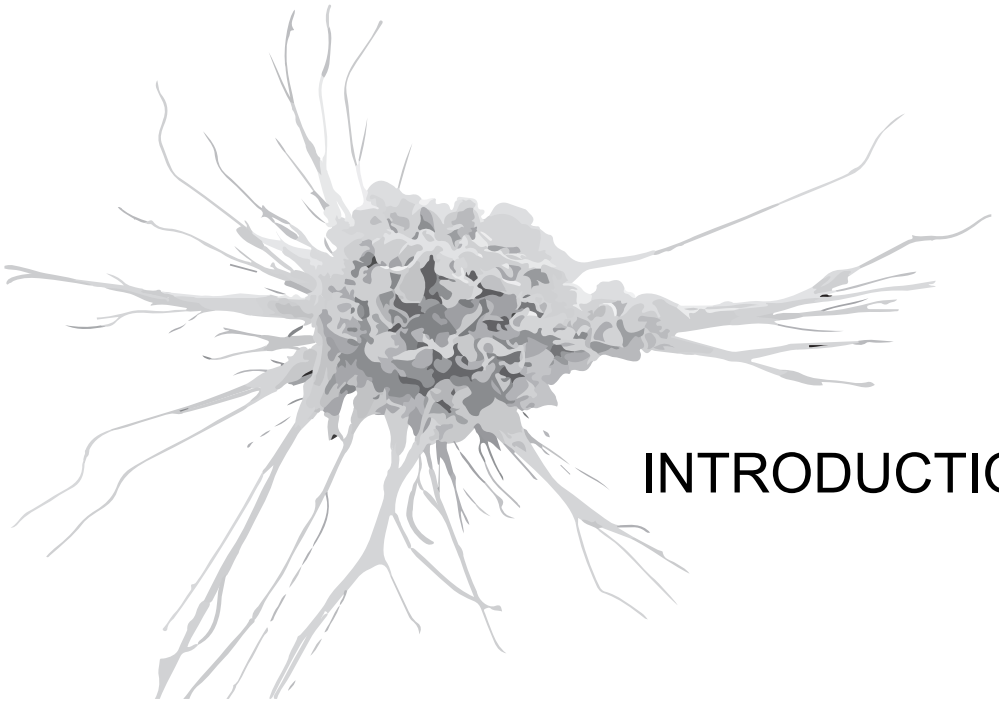
ABBREVIATIONS

5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ADD	ATRX-DNMT3-DNMT3L domain
AID	Activation-induced cytidine deaminase
α-KG	Alpha-ketoglutarate
BER	Base-excision repair
CGI	CpG island
CLP	Common lymphoid progenitors
CR	Cysteine-rich
csMBC	Class-switched memory B cell
CSR	Class-switch recombination
dC	Deoxycytosine
DC	Dendritic cell
DMR	Differentially methylated region
DNMT	DNA methyltransferase
DSBH	Double-strand β-helix
dUs	Deoxyuracil
ESC	Embryonic stem cell
TF	Transcription factor
FOB	Follicular B cell
GC	Germinal center
G-MCSF	Granulocyte-macrophage colony-stimulating factor 1
H3K9me2/3	Histone H3 Lysine 9 di- and tri-methylation

Abbreviations

H3ub	Histone H3 ubiquitination
HDAC	Histone deacetylase
HIGM2	Hyper IgM syndrome type 2
HMD	Highly methylated domain
HSC	Hematopoietic stem cell
ICGC	International Cancer Genome Consortium
ICR	Imprinting control region
iPSC	Induced pluripotent stem cell
JAK	Janus kinase
LMPP	Lymphoid-primed multipotent progenitor
MACs	Macrophage
M-CSF	Macrophage colony-stimulating factor 1
MO	Monocyte
MZB	Marginal zone B cell
NBC	Naïve B cell
ncsMBC	non-class switched memory B cell
NES	Nuclear export signal
NF-kB	Nuclear factor k-light-chain-enhancer of activated B cells
NK	Natural killer cell
NLS	Nuclear localization signal
OC	Osteoclast
PGE2	Prostaglandin E2
PMD	Partially methylated domain
pre-B	B cell precursor
pro-B	B cell progenitor
PWWP	Proline-Tryptophan-Tryptophan-Proline domain
RAG2	Recombination-activating gene 2
RANKL	Receptor Activator for Nuclear Factor-kB ligand

RNA Pol II	RNA polymerase II
SHM	Somatic hypermutation
SPT5	Transcription elongation factor 5
SRA	RING/SER domain
STAT	Signal transducers and activators of transcription
TDG	Thymine DNA glycosylase
TET	Ten-eleventranslocation methylcytosine dioxygenase
Tfh	Follicular T helper
TL	Tolerogenic dendritic cell
t-SNE	T-distribution stochastic neighbor embedding
TSS	Transcription start site
TTD-PHD	TUDOR-PHD domain
VDR	Vitamin D receptor
1,25(OH)2D3	1-alfa,25-dihidroxicolecalciferol



INTRODUCTION

INTRODUCTION

1 DNA methylation

1.1 Overview

In 1942, Conrad Hal Waddington neologized the term epigenetics to refer to '*the causal mechanism by which the genes of genotype bring about a phenotype*' (Waddington, 1942). Currently, a widely accepted definition of epigenetics is '*the layer of information that exists beyond that encoded in the DNA sequence, thereby making the genome function distinctively in different cell types*'. This definition covers DNA and chromatin modifications as well as other transcriptional regulators in the context of chromatin (Greally, 2018).

DNA methylation, one of the best-studied and understood epigenetic modifications, involves the addition of a methyl group to the fifth carbon of a cytosine (5mC). DNA methylation was originated in bacteria and was present in the first eukaryotes (Zemach, McDaniel, Silva, & Zilberman, 2010). This modification is conserved across Evolution among most fungi, plants and animals (Feng, Jacobsen, & Reik, 2010). In mammals, 5mC is mostly restricted to the context of symmetrical CpG dinucleotides, which are generally methylated in a symmetric manner (Doskočil & Šorm, 1962; Feng, Cokus, et al., 2010; Ramsahoye et al., 2000; Zemach et al., 2010; Ziller et al., 2011), allowing the propagation of 5mC throughout DNA replication (Song, Rechkoblit, Bestor, & Patel, 2011). Its deposition and maintenance is catalyzed by DNA methyltransferases (DNMTs) (E. Li, Bestor, & Jaenisch, 1992; Okano, Bell, Haber, & Li, 1999).

DNMT1 is the enzyme responsible for the maintenance of DNA methylation patterns across cell divisions. The mechanism of DNMT1-mediated methylation is well known. Firstly, E3 ubiquitin-protein ligase UHRF1 binds hemimethylated DNA through the interaction between unmethylated CpGs and histone H3 lysine 9 di- and tri-methylation (H3K9me2/3) through their respective RING/SER (SRA) and TUDOR-PHD (TTD-PHD) domains and recruits DNMT1. After binding, UHRF1 induces the ubiquitination of histone H3 (H3ub), a necessary step to remove the auto-inhibitory interaction of DNMT1.

Finally, activated DNMT1 binds H3ub and methylates DNA through its catalytic domain. As expected, UHRF1 knockout mice present the same phenotype as DNMT1 mutant mice (Edwards, Yarychkivska, Boulard, & Bestor, 2017; Greenberg & Bourc'his, 2019) (Figure 1C).

De novo DNA methylation is mediated by DNMT3A and DNMT3B with the help of DNMT3L. The three proteins share the ATRX-DNMT3-DNMT3L (ADD) and 'Proline-Tryptophan-Tryptophan-Proline' (PWWP) interaction domains, but the catalytic domain is not functional in DNMT3L. As for DNMT1, *de novo* DNMTs are inhibited by an auto-allosteric interaction between the ADD and the MTase domain (Greenberg & Bourc'his, 2019). This inhibition can be impaired by two mechanisms. On the one hand, the ADD domain recognizes unmethylated H3K4 residues, characteristic of inactive promoters leading to DNMT activation and the subsequent methylation. Interestingly, H3K4 methylation, present in CpG islands (CGI) associated with active promoters and enhancers, has a repulsive effect on the ADD domain (Ooi et al., 2007; Otani et al., 2009; Y. Zhang et al., 2010) (Figure 1A). On the other hand, a second mechanism for *de novo* DNMT genomic targeting involves the interaction between the PWWP domain and H3K36me present in transcribed regions, such as gene bodies (Baubec et al., 2015; Dhayalan et al., 2010; Krogan et al., 2003) (Figure 1B). In fact, intragenic DNA methylation is the most highly conserved in evolution supporting a relevant function.

Despite its origins in bacteria and presence in Eukaryotes, 5mC has been lost in many species, including fission yeast and certain invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster*, among others (Raddatz et al., 2013; Zemach & Zilberman, 2010). In fact, the inherent mutagenicity of 5mC, that can spontaneously deaminate to yield T, (Holliday & Grigg, 1993) is the most frequent mutation in mammals. Thus, organisms with DNA methylation present a 5-fold lower CpG content than expected (Bird & Taggart, 1980; Cooper & Krawczak, 1989). This observation supports the role of 5mC as a source of genetic variation driving evolution.

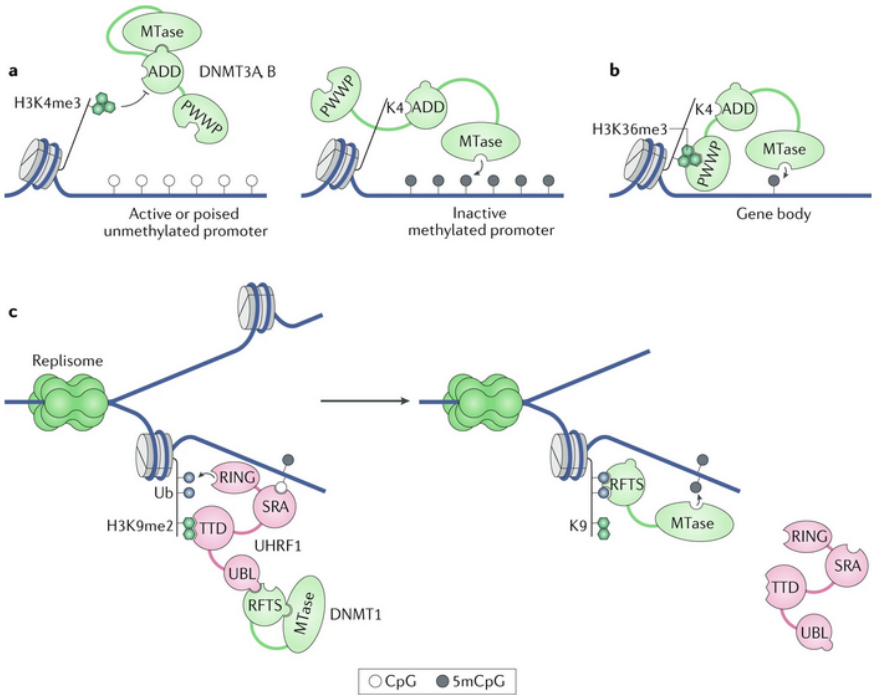


Figure 1: Mechanisms of DNA methylation. *(**a**) Representation of mechanisms of DNA methylation at promoter regions. Left: Inhibition of DNMT3A and DNMT3B activation by H3K4me3. Right: Methylation of inactive promoters by the absence of H3K4me3 and the subsequent loss of auto-inhibition interaction of DNMT3 enzymes. (**b**) Gene body methylation through the recruitment of DNMT3 enzymes by H3K36me3. (**c**) Molecular mechanism of DNA methylation maintenance during DNA replication* (Greenberg & Bourc'his, 2019).

In any case, mammalian genomes are highly methylated. For instance, in humans, 70-80% of the 28 million CpG sites are methylated (E. Li & Zhang, 2014). The methylation status of CpG sites is uneven. For instance, CpG sites located in CG-dense regions known as CGI, that are mainly associated with transcription start sites (TSS) of housekeeping and key development regulatory genes, are predominantly unmethylated (Deaton & Bird, 2011) with notable exceptions (Smallwood et al., 2011).

The role of methylation is highlighted by the effects of loss of DNMTs. For instance, loss of DNMTs entails embryonic lethality in mice (E. Li et al., 1992; Okano et al., 1999) and their dysregulation is observed in a wide range of cancer types (Baylin & Jones, 2016), supporting an important role in cell biology and differentiation.

1.2 Functions and molecular mechanisms of *de novo* methylation deposition

DNA methylation has predominantly been associated with transcriptional repression, although it does not confer silencing *per se* (Ben-Hattar & Jiricny, 1988; Iguchi-Ariga & Schaffner, 1989; Watt & Molloy, 1988). In addition, DNA methylation also has other functions depending on the genomic context. Also, for 5mC associated with gene silencing, different mechanisms can account for this transcriptional repression depending on whether 5mC occurs in repetitive sequences, promoters or gene bodies.

1.2.1 X-chromosome inactivation

In mammalian embryos following implantation, one X chromosome is randomly selected and repressed by the non-coding RNA X-inactive specific transcript. During the final steps of X chromosome inactivation, CGIs in this chromosome are methylated after the genes have already been repressed (Grant, Zuccotti, & Monk, 1992; Keohane, O'Neill, Belyaev, Lavender, & Turner, 1996; Lock, Takagi, & Martin, 1987; Singer-Sam et al., 1990). It has been described that DNMT3B is responsible for this process and that the other DNMTs are dispensable. However, the recruitment of DNMT3B to X-linked CGI-promoters is not well understood (Gendrel et al., 2012). Recent advances in other fields of DNA methylation suggest that it could be due to the loss of activating histone marks (Greenberg & Bourc'his, 2019) (Figure 2A).

1.2.2 Genomic imprinting

Another important process that involves DNA methylation during embryogenesis is genomic imprinting that leads to monoallelic gene expression in a parent-of-origin-specific manner (Ferguson-Smith, 2011; Sha, 2008). Interestingly, several imprinted genes are grouped in clusters. Around 20 genomic loci known as imprinting control regions (ICRs) have been described in both humans and mice, where mono-allelic expression of the associated

genes is determined (Lin et al., 2003; Proudhon et al., 2012; Williamson et al., 2006).

The majority of ICRs are methylated in the maternal alleles and coincide with CGI-associated promoters, such methylation entails gene silencing (Higashimoto, Soejima, Saito, Okumura, & Mukai, 2006) (Figure 2B), whereas paternal ICRs in general are located in CpG-poor intergenic regions and methylation avoids allele repression by DNA protein binding exclusion (Beygo et al., 2013; Thorvaldsen, Duran, & Bartolomei, 1998). Importantly, disruption of imprinting regions can cause neurological and metabolic disorders (Beygo et al., 2013; Fitzpatrick, Soloway, & Higgins, 2002; Higashimoto et al., 2006; Yang et al., 1998).

1.2.3 Germline-specific gene silencing

As previously mentioned, DNA methylation of CGIs leads to transcriptional repression and is involved in X-chromosome inactivation and imprinting. However, CGI methylation is also functionally relevant in other contexts. Normally CGI-associated promoters are only repressed by histone H3 lysine 27 trimethylation (H3K27me3). However, some genes with promoter CGIs can also be regulated by DNA methylation. These are mainly germline-specific genes that need to be inactivated in somatic cells. However, in differentiation processes or pathological conditions such as cancer, H3K27me3-marked CGI are more sensitive to undergo *de novo* DNA methylation (Figure 2C). Furthermore, unmethylated CGIs are able to acquire H3K4 methylation which can exclude the recruitment of *de novo* DNMTs (Greenberg & Bourc'his, 2019).

1.2.4 Inactivation of repetitive genomic regions

Roughly, half of the genome is occupied by retrotransposons. In this sense, DNA methylation plays a major role in controlling their expression. In fact, retrotransposon control has been proposed to be a major driver of the evolution of DNA methylation (Barau et al., 2016; Yoder, Walsh, & Bestor, 1997). In mouse embryos, it has been observed that DNMT1 knockout leads to

derepression of retrotransposons of the intracisternal A-particle family (Walsh, Chaillet, & Bestor, 1998).

Two independent recent works have reported the existence of a murine-specific member of the DNMT family termed DNMT3C. Originated from a tandem duplication of the DNMT3B, it has a specific role in controlling mobile elements. During evolution, DNMT3C maintained its ADD domain and kept its *de novo* methylation activity but it has lost the PWWP targeting domain. DNMT3C expression is restricted to male fetal germ cells, unlike other *de novo* DNMTs that are more ubiquitously expressed (Barau et al., 2016).

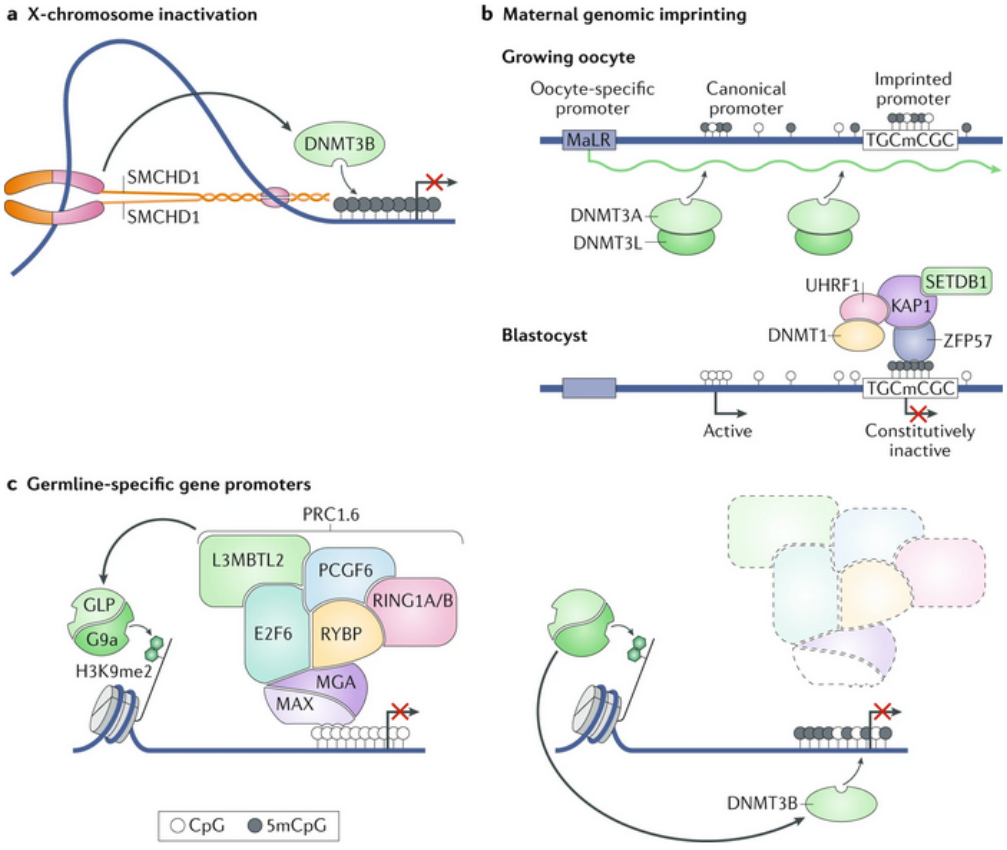


Figure 2: DNA methylation at CGI. *(**a**) SHMCHD1-dependent de novo DNA methylation at CGIs during X chromosome inactivation. (**b**) Mechanism of inhibition of sex-specific gene expression by DNA methylation of promoter associated CGI. (**c**) Model for germline-specific gene silencing by de novo methylation of promoter associated CGI* (Greenberg & Bourc'his, 2019).

In addition to the relatively temporal repression of retrotransposons, DNA methylation also has the potential to induce their permanent inactivation through mutagenic deamination and by limiting chromosomal rearrangements mediated by recombination of high sequence similarity (Greenberg & Bourc'his, 2019).

1.2.5 Gene bodies

As mentioned above, intragenic DNA methylation is highly conserved from an evolutionary standpoint, and it is still poorly understood at all levels. Currently, there are two models that can explain at least in part the functionality of methylation in this specific genomic context: one proposes a role for gene body methylation in negatively regulating spurious transcription initiation, the second model presents, a role in controlling splicing (Greenberg & Bourc'his, 2019; Smith & Meissner, 2013).

During transcription elongation, RNA polymerase II (Pol II) recruits histone methyltransferase SETD2, that simultaneously methylates H3K36 in gene bodies. This histone modification can be recognized by the PWWP domain of *de novo* DNMTs with the subsequent activation relaxing its auto-inhibitory interaction (Baubec et al., 2015; Dhayalan et al., 2010; Krogan et al., 2003) (Figure 1B). Two recent studies have reported that DNMT-associated activation leads to the methylation of alternative promoters controlling spurious transcriptional initiation (Neri et al., 2017; Weinberg et al., 2019). Although some authors argue against this hypothesis because the observed effect only occurs in a small proportion of cells within a population, it is consistent with the observation that methylation increases during cell differentiation processes and the reported chromatin compaction by histone deacetylases (HDACs) recruited by H3K36me of highly expresses genes (Greenberg & Bourc'his, 2019).

The second hypothesis, which proposes that 5mC controls splicing, is based in the reported enrichment in methylation of constitutive exons with respect to introns and alternative exons (Laurent et al., 2010; Lister et al., 2009). However, although there are examples of exclusion and inclusion of

exons regulated by DNA methylation, this phenomenon only accounts for a small fraction of all splicing events.

1.3 Functions and molecular mechanisms of DNA demethylation

Cytosine methylation, despite its chemical and genetic stability, can be removed in different ways and, in many cases, its elimination correlates with transcriptional activation. Firstly, functional disruption or inefficient DNA methylation maintenance can lead to passive DNA demethylation in proliferative cells. Secondly, demethylation can be achieved through active mechanisms, such as those mediated by ten-eleven translocation (TETs) methylcytosine dioxygenases or, alternatively, through the activity of other enzymes with potential DNA demethylase activity.

1.3.1 Passive DNA demethylation

In one of the first genome-wide DNA methylation studies, it was reported the existence of long genomic blocks of several kilobase pairs with highly variable methylation levels, that the authors termed partially methylated domains (PMDs) (Lister et al., 2009). In more recent studies, these regions were characterized and it was found that PMDs are associated with repressive histone marks, occur in regions with low gene and CG density coinciding with nuclear lamina domains, and are characterized by late replication timing (Aran, Toperoff, Rosenberg, & Hellman, 2011; Berman et al., 2012; Kasper D. Hansen et al., 2014; Kasper Daniel Hansen et al., 2011; M. Weber et al., 2005; Zhou et al., 2018).

Initially, PMDs were reported in highly proliferative cells such as long-term cultured cell and some cancers (Kasper D. Hansen et al., 2014; Lister et al., 2009; M. Weber et al., 2005). However, with the improvement of next-generation sequencing and the optimization of methods for PMD detection, they have also been identified in primary cells, such as memory B and T cells (Durek et al., 2016; Kulis et al., 2015; Schroeder et al., 2013). Interestingly, it

has been shown that there is an association between PMD demethylation and mitotic age, which generally correlates with chronical age (Zhou et al., 2018).

The molecular model of this phenomenon is not yet well defined. However, the most accepted hypothesis proposes that loss of methylation in PMDs is due to the inefficient activity of DNMT1 during the late stages of replication (Zhou et al., 2018). Interestingly, H3K36me2/3-marked CpGs within PMDs are resistant to passive demethylation and it has been suggested that their methylation status is maintained by DNMT3B, in a similar way to gene body methylation (Zhou et al., 2018).

1.3.2 Ten-eleven translocation methylcytosine dioxygenases

Unlike other enzymes proposed as potential demethylases of DNA, there are compelling evidences and a general consensus in relation to the role in active demethylation mediated for members of the TET family (TET1, TET2 and TET3). TET-mediated demethylation process consists in the iterative oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (He et al., 2011; Shinsuke Ito et al., 2010, 2011; Tahiliani et al., 2009). All three oxidative intermediates can be eliminated in a replication-dependent manner, by the inability of DNMT1 to recognize and maintain these cytosine modifications. Alternatively, 5fC and 5caC can be excised by TDG to generate an abasic site that it is then repaired by the base excision repair (BER) complex leading to cytosine demethylation (He et al., 2011; A. R. Weber et al., 2016) (Figure 3A).

5hmC is the most abundant intermediate towards active demethylation generated by TET enzymes. Interestingly, it has been observed that its presence at gene bodies correlates with gene expression in a positive manner (Tsagaratou et al., 2014). This suggests a relationship between TETs and RNA Pol II supported by the enrichment of 5hmC with H3K36me3 at gene bodies (Hashimoto et al., 2014). This evidence, together with those mentioned above about DNMT targeting, entail a colocalization of TETs and DNMT3B at gene bodies that would explain the stability of 5hmC at these regions. Moreover, TETs have been shown to interact with DNMT1. In addition, TET2 also interacts

with *de novo* DNMTs (Y. W. Zhang et al., 2017). However, a biological function for 5hmC is not yet well-known.

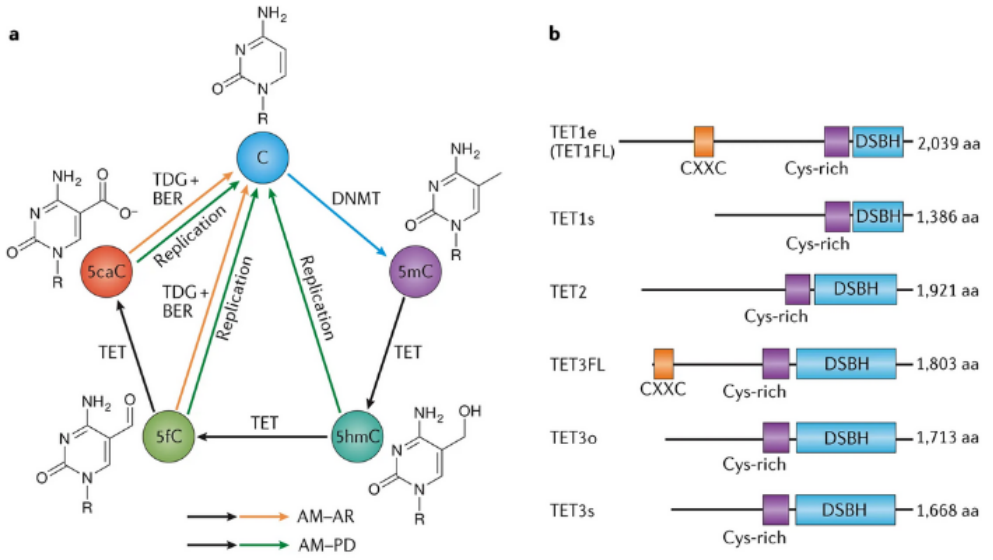


Figure 3. TET-mediated active DNA demethylation. *(**a**) Representation of the active DNA demethylation cycle. TET enzymes are able to oxidize 5mC to 5hmC, 5fC and 5caC, followed by its elimination by replication passive mechanisms and/or through a TDG/BER dependent mechanism. (**b**) Structure of TET enzymes. The N-terminal domain of TET1 and TET3 contains a CXXC DNA binding domain that recognizes un-methylated CpGs. In the case of TET2, the IDAX protein is required to bind DNA through its CXXC domain. The C-terminal domain contains the catalytic core of the protein that displays oxygenase activity and is positioned as DSBH and a Cys-rich region that is characteristic of this family of proteins* (X. Wu & Zhang, 2017).

TET enzymes share two domains, the cysteine-rich (CR) and a domain double-strand β-helix (DSBH). The CR domain plays an important role in stabilizing DSBH-DNA interaction, while the DSBH domain is able to bring closer 5mC with Fe(II) and alpha-ketoglutarate (α-KG) resulting in the oxidation of 5mC and the subsequent conversion of the α-KG into succinate (Hu et al., 2015). Interestingly, DSBH recognition does not depend on the methyl group and, therefore, TET proteins cannot only oxidize 5mC but also the subsequent oxidative intermediates (Hu et al., 2015). TET1 and TET3 also have a CXXC domain with a function not fully understood, but some evidences suggest that may be important for their targeting to specific genomic sites. During evolution, an ancestral TET2 underwent a chromosomal gene inversion during evolution,

thus separating its CXXC domain from the catalytic domain and becoming an independent gene known as IDAX, that can interact and regulate TET2 (Ko et al., 2013).

As any enzymatic reaction, oxidation of 5mC is not only highly influenced by the abundance of its participating substrates (oxygen, α-KG and Fe(II)), but also by its products (succinate and CO_2) (X. Wu & Zhang, 2017). For example, downregulation of isocitrate dehydrogenase enzymes (IDHs) in melanoma reduces the amount of α-KG, and therefore the global levels of 5hmC. On the other hand, IDH overexpression is associated with high levels of 5hmC (Figueroa et al., 2010; Lian et al., 2012; W. Xu et al., 2011). Oxygen availability is also essential for TET activity and correlates with low levels of 5hmC (Thienpont et al., 2016). In addition to the ratio substrate/product, there are some elements that can improve or decline TET activity. In this regard, it has been reported that vitamin C has an activity enhancing effect on TETs by direct interaction with the DSBH domain (Blaschke et al., 2013; Minor, Court, Young, & Wang, 2013; Sasidharan Nair, Song, & Oh, 2016; Yin et al., 2013). In all, the aforementioned evidences highlight the importance of metabolism on active DNA demethylation. The relevance of the metabolism can be extended to other epigenetic mechanisms that involve enzymatic reactions.

TET enzymatic activity is also highly regulated by a subset of molecular mechanisms, including post-translational modifications. For instance, under oxidative conditions it is known that acetylation of TET2 is important to preserve its stability (Y. W. Zhang et al., 2017). Additionally, the protein levels of all TETs are regulated by microRNAs. For example, the family of miR-29 is important to repress negative regulators of TET1 during specific phases of mouse development (X. Wu & Zhang, 2017). TET2 also presents a complex regulation mediated by several microRNAs, and its dysregulation is associated with hematological malignancies (Cheng et al., 2013). Finally, regulation of TET3 by miRNAs is proposed to participate during neurogenesis, preserving neural progenitor cells (Lv, Jiang, Liu, Lei, & Jiao, 2014)

Genomic targeting of TET enzymes is essential for their function but remains poorly understood. *In vitro* experiments have shown that the CXXC

domain of TET1 and TET3 can bind CpG-rich regions, preferentially when unmethylated (Ko et al., 2013; Y. Xu et al., 2012). According to these results, chromatin immunoprecipitation of TET1 shows that binds preferentially to CGIs and that CXXC mutation impairs its binding (H. Wu et al., 2011). However, this targeting mechanism is not valid for TET2 since it lacks the CXXC domain (Figure 3B).

Another proposed molecular mechanism for targeting TETs to specific genomic regions consists of their recruitment through the interaction with signal specific transcription factors (TFs). In fact, it has been described the interaction of TET proteins with different TFs like NANOG, SALL4A, WT-1, PU.1, E2A, C/EBPα, KLF4 and TFCP2L1 (Costa et al., 2013; de la Rica et al., 2013; Guilhamon et al., 2013; C. W. Lio et al., 2016; Sardina et al., 2018; Yiping Wang et al., 2015; Xiong et al., 2016). In addition, histone acetyltransferase EP300, which interacts with hundreds of TFs (Dyson & Wright, 2016), can also interact with TET proteins (Y. W. Zhang et al., 2017). This is consistent with the enrichment of TET2 binding sites at accessible regions with enhancer features, like EP300 binding and histone activation marks (Kasper D Rasmussen et al., 2019). A model that can explain all aforementioned findings would consist of a signal specific pioneer TFs binding at enhancers regions with the subsequent EP300-TET complex recruitment. This EP300-TET complex, by active histone mark deposition and DNA demethylation, would result in chromatin opening and 5mC binding protein disruption that facilitates binding of subsequent TFs. In fact, it has been observed that TET2 loss affects TF activity in enhancer regions (Kasper D Rasmussen et al., 2019).

Despite sharing its enzymatic activity, there is evidence suggesting the non-redundant functionality of TET enzymes, in some cases linked to their variable expression patterns in different cell contexts and genomic targeting mechanisms. For example, TET expression patterns are highly dynamic during mouse development. While TET3 is expressed during oocytes and preimplantation, in the embryoblast phase and in embryonic stem cells (ESC), TET1 and TET2 augment their expression in detriment of TET3 levels. During the next steps of mouse development, TET1 is progressively downregulated, whereas TET2 and TET3 gain relevance in the specification of three germ lines.

In adult tissues, TET2 and to a lesser extent, TET3 play a relevant role, especially in the hematopoietic system and brain (Kasper Dindler Rasmussen & Helin, 2016).

Individual loss of any of the three TET proteins leads to profound dysregulation of hematopoietic development. Specifically, TET2 disruption in mice increases hematopoietic stem cell (HSC) survival and proliferation and blocks differentiation of T, B and erythroid lineages (Ko et al., 2011). In humans, TET2 gene silencing reduces the global levels of 5hmC, blocks lymphoid and erythroid lineage differentiation and promotes myeloid differentiation (Pronier et al., 2011). Interestingly, even though TET2 loss favors the myeloid compartment, it is well established that TET2 is crucial during human monocyte differentiation (reviwed in Álvarez-Errico, Vento-Tormo, Sieweke, & Ballestar, 2015). Globally, these and other findings suggest a relevant role of TET2 and DNA demethylation in the hematopoietic system and its dysregulation is associated with a plethora of immunological disorders such as liquid tumorigenesis, autoimmunity and immunodeficiencies.

1.3.3 Activation-induced cytidine deaminase: an active demethylase?

As Steen Ooi and Tim Bestor commented in the past decade, '*The search for active DNA demethylation in mammals has been characterized by a parade of putative 5mC demethylases, each very different from the next. Indeed, the existence and nature of mammalian DNA demethylases have been the recurrent subject of uncertainty and controversy*' (Ooi & Bestor, 2008). The potential role in active DNA demethylation of one of these putative candidates, activation-induced deaminase (AID), still has not been solved (Ramiro & Barreto, 2015).

In 1999, Honjo and colleagues identified for the first time the upregulation of a non-described gene that they referred to as AID (Muramatsu et al., 1999) by comparing activated and non-activated murine B lymphoma cells (CH12F3-2). One year later, the same authors found that AID it is involved in two essential biological processes in B cells, specifically somatic hypermutation (SHM) and class-switch recombination (CSR) (Muramatsu et al.,

2000). AID is able to convert deoxycytosines (dCs) into deoxyuracils (dUs), producing dU:dG mismatches that are removed by mismatch repair and base-excision repair complexes (BER) (Di Noia & Neuberger, 2002; Poltoratsky, Goodman, & Scharff, 2000; Rada, Di Noia, & Neuberger, 2004; Rada, Ehrenstein, Neuberger, & Milstein, 1998; Rada et al., 2002). Deaminase activity is required for SHM and CSR of immunoglobulin genes, which are necessary processes for affinity maturation and antibody diversification of B cells within germinal centers (Arakawa, HauschiLd, & Buerstedde, 2002; Muramatsu et al., 2000; Revy et al., 2000).

AID is a relatively small protein (24 KDa), that has five relevant functional domains. First, it contains a nuclear localization signal (NLS) domain in its N-terminus for its nuclear import by the alpha and beta importin (Freitas & Cunha, 2009; Satomi Ito et al., 2004). The AID protein also has a conformational nuclear signal that provides a positive charge to the surface of the folded protein (Patenaude et al., 2009). The catalytic domain is localized between the amino acid 55 and 94 and shows homology with the cytidine deaminase motif present in members of the APOBEC protein family. Another important region of AID gene is the nuclear export signal (NES) domain, that in an exportin-dependent mechanism helps to extract AID from the nucleus (Barreto & Magor, 2011; Satomi Ito et al., 2004). Finally, AID has two regions that are associated with dimerization and tetramerization capacity of AID (Prochnow, Bransteitter, Klein, Goodman, & Chen, 2007).

In addition to its role in CSR and SHM, AID has been proposed to participate in active DNA demethylation through deamination of 5mC, leading to a C:T mismatch that is converted to G:C by thymine DNA glycosylase (TDG), followed by BER. In principle, AID could act directly on methylated CpG or downstream to TETs, whose activity has been discussed above. The model proposing a direct role for AID on 5mC, involve the formation highly mutagenic intermediary as T and do not reconcile to the fact that AID's affinity for 5mC is ten-fold lower than for C (Nabel et al., 2012). On the other hand, downstream model require the action of AID on oxidized forms of C (5hmC, 5fC 5caC), which is in conflict with biochemical data on AID (Nabel et al., 2012). In fact, during the past decade conflicting reports have both supported and discounted such

a role for AID in different cells types and contexts (Ramiro & Barreto, 2015) (Figure 4).

Although initially it was described that the AID expression is restricted to the B cell compartment (Muramatsu et al., 2000). Recent evidences have shown that it has been detected in a plethora of normal tissues with an unknown function. In non-physiological contexts, AID expression has been reported in lymphomas, leukemias and, surprisingly, epithelial cancer cells. Additionally, it has been described that chronic inflammation leads to ectopic AID expression mediated by aberrant nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling activation (Orthwein & Di Noia, 2012).

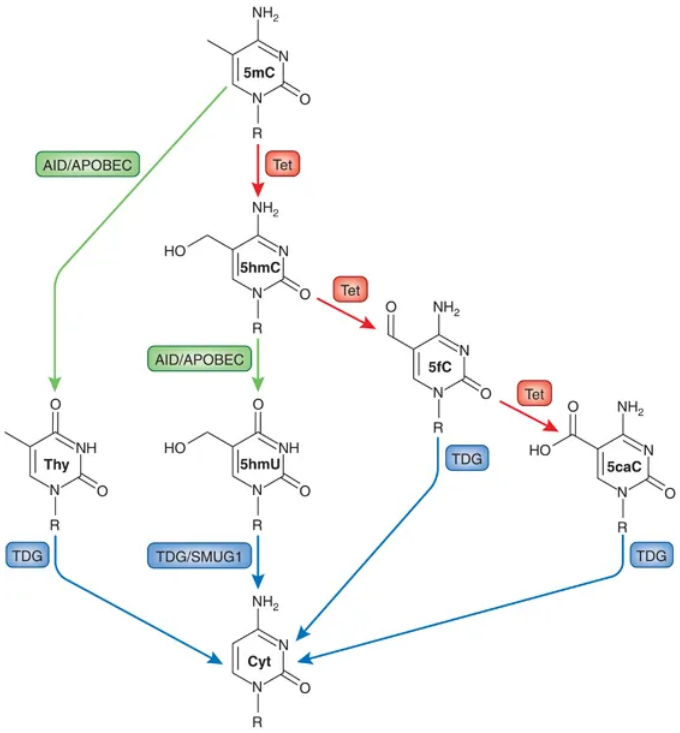


Figure 4. Proposed mechanisms of AID-dependent DNA demethylation. *AID could deaminate the amine group of 5mC leading to T. 5hmC could also be a substrate for AID, converting 5hmC into 5hmU. T and 5hmU are eliminated by TDG/BER dependent mechanism. The affinity of AID for 5mC and 5hmC is 10- and 100-fold lower than for non-modified cytosine* (Moore, Le, & Fan, 2013).

Despite that the highest AID expression levels are found in lymphoid cells, the first reports describing AID as a DNA demethylase corresponded to non-lymphoid cells. For example, during reprogramming of heterokaryons, it was reported that AID demethylates the OCT4 promoter (Bhutani et al., 2010; Sabag et al., 2014). However, a subsequent study found that AID is not expressed in heterokaryons and its overexpression does not affect reprogramming (Foshay et al., 2012). In induced pluripotent stem cells (iPSCs), there are also discrepancies about the role of AID in actively demethylation (Bhutani et al., 2013; Habib, Habib, Do, Moon, & Chung, 2014; Kumar et al., 2013).

In B cells, three independent studies using B cells from AID-deficient mice have interrogated the demethylase activity of AID. Two of them reported the absence of DNA methylation changes (Fritz et al., 2013; Hogenbirk et al., 2013), whereas the third study found DNA methylation differences relative to wild type mice (Dominguez et al., 2015). However, methodological aspects could explain the discrepancies between these studies. In parallel, the discovery of alternative enzymatic pathways that lead to *bona fide* active DNA demethylation through TET-mediated oxidation of methylcytosines (Shinsuke Ito et al., 2010; Kriaucionis & Heintz, 2009; Tahiliani et al., 2009) raised more doubts about the possibility that AID redundantly plays such a role. In fact, there is currently no consensus about whether AID is involved in mediating DNA demethylation in specific cell contexts.

An important aspect that could help to understand the relationship between AID and DNA demethylation is the study of AID genomic targeting. Two parallel studies found that AID mutations outside the immunoglobulin loci (AID off-target regions) are associated with highly expressed genes. Specifically, the authors showed an enrichment for intergenic super-enhancers that present high levels of convergent transcription (Meng et al., 2014; Qian et al., 2014). A more recent study found similar results for AID off-targeting, and conclude that the best predictor for AID off-target activity is the presence of high density binding for RNA polymerase II (RNA Pol II) and transcription elongation factor 5 (SPT5), coinciding with top expressed genes (Álvarez-Prado et al., 2018).

2 Immune models to investigate DNA methylation dynamics and mechanisms

2.1 B cells

B lymphocyte generation is the result of several cell lineage choices and differentiation steps which are tightly regulated at the transcriptional level. Differentiation starts in the bone marrow with the generation of lymphoid-primed multipotent progenitors (LMPPs) from HSCs, which can give rise to both myeloid and lymphoid lineages (Pieper, Grimbacher, & Eibel, 2013). Also, in the bone marrow, LMPPs generate common lymphoid progenitors (CLPs) which can differentiate into B cells, T cells and natural killer cells (NKs). During early B cell development, CLPs differentiate into B cell progenitors (pro-B). pro-B cells will further differentiate into B cell precursors (pre-B), which in turn give rise to immature B cells. Then, immature B cells leave the bone marrow and migrate to secondary lymphoid organs (spleen and lymph nodes) in order to complete B cell differentiation (Busslinger, 2004). Briefly, resting naïve B cells are activated and become germinal center cells (GCs) by generating high-affinity antibodies in response to antigens presented by T-helper cells. Finally, B cells exit the GC and differentiate into memory B cells or antibody-secreting plasma cells (Victora & Nussenzweig, 2012).

Differentiation across the B cell lineage provides an exceptional system to study the role of DNA methylation and other epigenetic mechanisms. On the one hand, B cell development has been well studied and its different cell stages are well defined and characterized at the transcriptional and surface marker levels. This allows the purification by flow cytometry of the different subpopulations of peripheral blood B cells, tonsils and bone marrow. On the other hand, the complexity of B cell development with diverse cellular properties facilities the study of diverse epigenetic processes. For instance, B cell maturation within the germinal center, characterized by clonal proliferation, is a good model to study passive DNA demethylation in physiological conditions. In addition, long-lived memory B cells enable the study of molecular mechanisms responsible for age-associated *de novo* DNA methylation. Finally, specific transcriptional mediated cell differentiation processes within B cell lineage are

useful for understanding TF-guided enhancer and promoter DNA demethylation.

2.1.1 B cell differentiation and maturation

B cells are major players of humoral immunity thanks to their capacity to produce antibodies against virtually all possible antigens. In addition, B cells are also capable of presenting antigens and secreting cytokines to promote immune responses in other cell types. The development of B cells begins with HSCs in the bone marrow and ends in the germinal centers, after a highly regulated multi-step sequential process. During each differentiation step, the activation of specific signaling cascades, TFs, epigenetic complexes and cell contacts are coordinated to give rise a functional cell capable of continuing the maturation process after an appropriate signal (Pieper et al., 2013) (Figure 5).

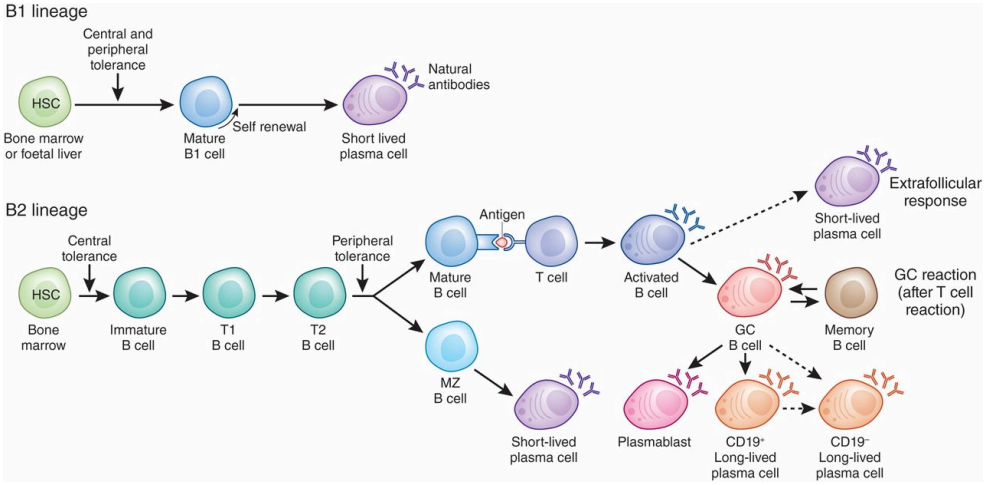


Figure 5. Representation of normal B cell development. *Top panel, The non-conventional B cell (B1) lineage ontogeny. Bottom panel, classical B cell lineage (B2) development, which can take place in the bone marrow (HSC-T2 B cell) and peripheral organs. B cell activation in germinal centers (GC) can be T cell and non-T cell dependent leading to a bifurcation in B cell development* (Schrezenmeier, Jayne, & Dörner, 2018).

Within the bone marrow, HSCs differentiated into immature B cells. During this process, B cells undergo arrangements in the loci of immunoglobulin H and L chain in a process known as V(D)J recombination, which is mediated by RAG enzymes (Schrezenmeier, Jayne, & Dörner, 2018). This process is important to develop and expose a functional BCR in the membrane surface. This process is regulated by positive and negative selection mechanisms. Positive selection occurs during pre-BCR stage, where B cells with functional BCRs are selected in an antigen-independent survival signaling. On the other hand, negative selection, also known as central B cell tolerance, consists in the elimination of B cells with self-reactive BCRs by overactivation that leads to apoptosis. Finally, resulting immature B cells migrate to the spleen where they differentiate to marginal zone B (MZB) cells or follicular B (FOB) cell depending on the received signals through the BCR. (Pieper et al., 2013).

Once differentiated, mature B cells are ready to become activated by the appropriate signal, which can be T cell-dependent or -independent. The T cell-dependent mechanism is specific for FOB cells and leads to the generation of antibodies with higher affinity than those generated from T cell-independent activation. Once T cells present the antigen to FOB cells, is taken up into B cells, processed and then presented to the follicular T helper (Tfh) cells through MHC-II on the cell membrane. Tfh – FOB cells interaction leads to the secretion of different signals for FOB cells promoting its cell proliferation, CSR, and SHM. This maturation process yields with the generation of short- and long-lived plasmablasts, and memory B cells for immediate and time persistent protection respectively (Crotty, 2015; Pieper et al., 2013).

Alternatively, B cells can be activated in a T cell-independent manner, specially MZB cells. The typical antigens for this type of B cell activation include foreign polysaccharides and unmethylated CpG DNA. T cell-independent activation leads to a rapid generation of antibodies but presents lower affinity and less versatility. Moreover, this type of activation is characterized by B cell proliferation without the formation of the germinal centers and the generation of short-lived IgM plasmablasts (Crotty, 2015).

Finally, memory B cells can be reactivated through the binding with their specific antigens during a secondary infection. Upon activation, memory B cells presents their antigens to memory follicular T helper cells via MCH-II-TCR interaction. This interaction leads to B cell differentiation to plasma cells and more memory B cells. Typically this reactivation is characterized by a more intense and rapid immune response (Kurosaki, Kometani, & Ise, 2015).

2.1.2 B cell tolerance

The main function of B cells as humoral mediators is the production and secretion of specific antibodies against foreign antigens. This highly regulated process leads to the generation of a highly diverse set of BCRs that virtually recognize all possible antigens, including those against self-antigens. Under normal conditions, autoreactive B cells are eliminated or inactivated in several ways and different stages during B cell development. B central tolerance refers to the first checkpoint for the elimination of autoreactive B cells and it is carried out in the bone marrow during the first steps of B cell development. The second group of molecular mechanisms is intended to eliminate potential evasions from the first tolerance checkpoint or new generated self-reactive BCR during diversification into the germinal centers (Nemazee, 2017) (Figure 6).

In many occasions, pathological conditions have revealed new functions for known genes. This is for instance the case of AID. Hyper IgM syndrome type 2 (HIGM2), a rare primary antibody deficiency, is characterized by loss-of-function mutations in AID. One of the most notable alterations in HIGM2 patients is the absence of CSR and SHM, characterized by normal or elevated serum IgM levels with absence of IgG, IgA, and IgE resulting in a profound susceptibility to bacterial infections. Moreover, HIGM2 patients are more prone to develop autoreactivity, which suggests a relevant role of AID in the elimination of autoreactive B cells (Revy et al., 2000). Recent evidence suggests that AID effectively has a role in this process (Cantaert et al., 2015; Kuraoka et al., 2011; Meyers et al., 2011). The proposed mechanism for central tolerance would be achieved during a secondary recombination process mediated by RAG enzymes that are induced by the activation of autoreactive

BCRs (Goodnow, 1996; Meffre, Casellas, & Nussenzweig, 2000; Nemazee, 2006). RAG positive cells into the bone marrow also exhibit expression of BCL6 a typical marker of germinal center B cells (Basso & Dalla-Favera, 2012; Duy et al., 2010). Moreover, BCL6 upregulation is associated with the amplification of BCR signaling and the inhibition of BCL2, an antiapoptotic factor (Meffre et al., 2000). Furthermore, RAG and AID positive cells in bone marrow present caspase 3 activation in a p53 dependent signaling, suggesting that those cells are in an elimination process by apoptosis (Cantaert et al., 2015; Crouch et al., 2007). The p53-mediated apoptotic process is suggested that is activated by the genomic instability produced by the high levels of RAG and AID expression, therefore the loss of AID would explain the autoreactive episodes of some HIGM2 patients (Cantaert et al., 2015; Revy et al., 2000). Importantly, this evidence fits with the translocation events detected in pre-B acute lymphocytic leukemia (Swaminathan et al., 2015; Tsai et al., 2008). Hence, B cell tolerance impairment might lead to a leukemic transformation (Figure 6).

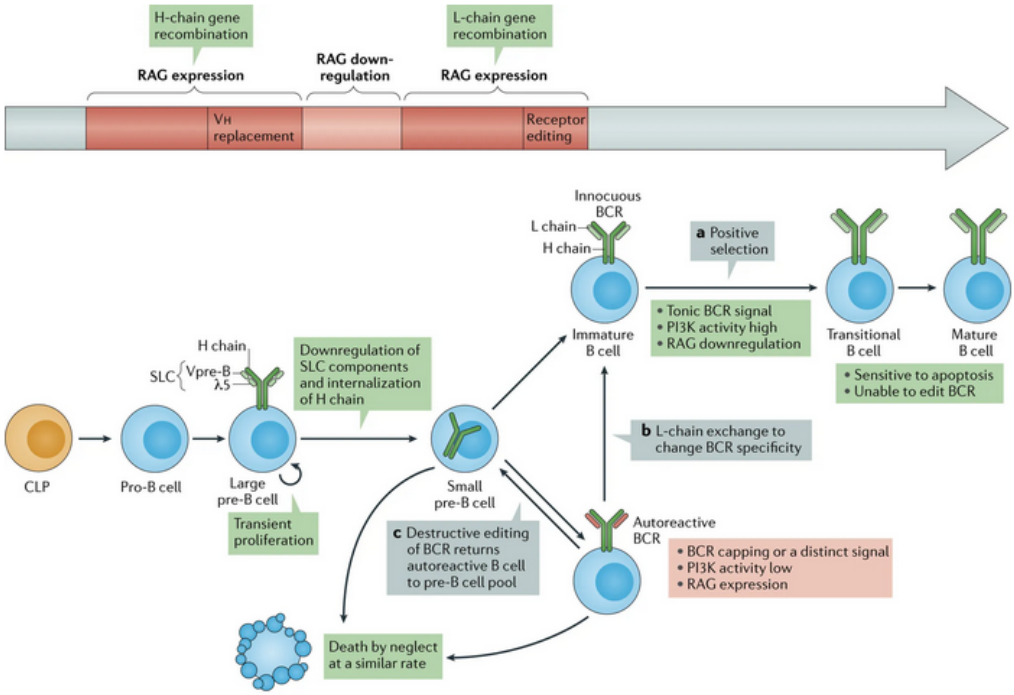


Figure 6. Central B cell tolerance. *During the first steps of B cell development, cells with self-reactive BCRs are activated to re-edit the BCR with the subsequent*

upregulation of mutagenic proteins (RAG and AID) and the downregulation of the antiapoptotic factor BCL2. Cells that are not able to revert the interaction with the self-antigen accumulate genomic instability that can lead to cell death by apoptosis (Nemazee, 2017).

AID loss has also been associated with peripheral B cell tolerance produced outside the bone marrow. One of the phenotypes of HIGM2 patients is hyperplasia of the lymphatic nodules (Revy et al., 2000) and *in vitro* experiments using different AID mutants have demonstrated that SHM and non-CSR are responsible for this cellular expansion (Cantaert et al., 2016). These results are supported by the fact that high-affinity BCRs generated by SHM are associated with downregulation of the germinal center reaction. Moreover, AID loss leads in an accumulation of follicular T cells in conjunction with the increment of IL-4 and IL-21 protein levels in the germinal center microenvironment, affecting the tolerogenic activity of regulatory T cells (Cantaert et al., 2016).

2.1.3 DNA methylation in the B cell compartment

During B cell differentiation, progressive demethylation takes places throughout different stages, especially during the transition from naïve to memory cells that occurs in the germinal center reaction following infection (Almamun et al., 2014; Barwick, Scharer, Bally, & Boss, 2016; Caron et al., 2015; Kulis et al., 2015; Lai et al., 2013; Lee et al., 2012; Oakes et al., 2016; Shaknovich et al., 2011). Genome-wide epigenetic studies have shown that around 30% of the genome undergoes DNA methylation changes during B cell differentiation (Kulis et al., 2015; Oakes et al., 2016), which makes this lineage among the most dependent on DNA methylation changes, ahead of T and myeloid linage (Schuyler et al., 2016).

Most of the changes in the DNA methylome of B cells during their development occur in nuclear lamina domains (Kulis et al., 2015). As mentioned above, DNA methylation changes in these regions are mainly associated with passive demethylation in a cell division dependent manner, due to the failure of DNMT1-dependent remethylation in late replicating regions. In

this sense, a recent study has shown that naïve to memory B cell transition is accompanied by passive DNA demethylation (Durek et al., 2016) coinciding with the highest proliferative event within B cell lineage in physiological conditions.

Despite the fact that 60-70% of DNA demethylation events are linked to passive loss, active losses of methylation are key during B development. Each transition process between a subpopulation of B cells to another involves the activation of different signaling pathways that lead to nuclear binding of a specific set of TFs to regulatory regions. This DNA-TF interplay is associated with the occurrence of epigenetic and conformational changes in promoter and enhancer regions (Corces et al., 2016; González, Setty, & Leslie, 2015; Javierre et al., 2016; Lara-Astiaso et al., 2014). Interestingly, TF binding correlates negatively with DNA methylation levels (Kulis et al., 2015) and it has been proposed that one of the consequences of TF binding is the recruitment of epigenetic machinery, including TET enzymes (C. W. J. Lio & Rao, 2019; Martin-Subero & Oakes, 2018).

Enhancer DNA demethylation is especially relevant during B cell commitment in the bone marrow and involves TET2 and TET3. In fact, knockout mice for these proteins result in a blockage of DNA demethylation of Igκ enhancers and a generalized loss of B cell enhancer accessibility. The proposed molecular mechanism for this process consists of the recruitment of TETs by pioneer TF PU.1 (C. W. Lio et al., 2016). TET recruitment mechanism by PU.1 has also been described during osteoclast differentiation from monocytes (de la Rica et al., 2013). TET proteins also regulate the expression of key TF for Igκ rearrangement such as IRF4 and IRF8 (C. W. Lio et al., 2016).

Another remarkable contribution of DNA methylation in B cell development is the reported regulation of AID expression levels by TET proteins during B cell maturation. Specifically, the authors of this work described that TET2 and TET3 double knockout mice present low levels of AID expression affecting class switch recombination. The proposed mechanism consists in the recruitment of TET proteins to AID super-enhancer by BATF transcription factor, promoting the hydroxymethylation of TET-responsive

elements and the subsequent increase of AID expression (C. W. J. Lio et al., 2019). TET2 in cooperation with CREBBP is also important during plasma cell differentiation promoting enhancer activation of BLIMP1, the master regulator of plasmablast differentiation process in the germinal center (Qian et al., 2014). Finally, additional TFs that also might have a relevant role in DNA methylation dynamics during B cell maturation are NF-kB, OCT2, IRF4, AP-1, EBF1 and RUNX3 given the relevant enrichment of their binding motifs in regions undergoing dynamic demethylation (C. W. J. Lio & Rao, 2019).

As mentioned in section 1.3.3, AID knockout mice present defects in the methylome of memory B cells. These have been directly related to the deaminase activity of AID. However, this connection between AID and DNA methylation in B cells is based on indirect approaches, where only the significance and not the percentage of overlap between methylation changes and known AID off-target features were taken in account. In fact, the significant overlap found by the authors between off-target genes and methylation changes represents less than 1% of the total of altered CpG (Dominguez et al., 2015). The presence of hypermethylation events in AID knockout mice does not help either to support a direct role of AID in DNA demethylation. Altogether, these results do not exclude the potential role of AID in demethylation, but further evidences are needed and it is still an open question that needs to be addressed.

Although during B cell development the majority of methylation events correspond to erasure of the methyl group, *de novo* gains of methylation are also observed. These events are enriched in repressed CGI by histone H3K27me3 deposited by the polycomb repressive complex II (Kulis et al., 2015). The redundant repression of these regions is suggested could help to reduce the plasticity of mature B cells protecting them against transdifferentiation events in pathological conditions such as cancer (Gal-Yam et al., 2008). Curiously, it has been observed that these hypermethylation events present certain stochasticity, increasing epigenetic diversity. This heterogeneity is proposed that might lead to phenotypic diversity of memory and plasma B cells. However, single cell experiments are needed to confirm it (Martin-Subero & Oakes, 2018).

2.2 *In vitro* models for monocyte terminal differentiation

Monocytes (MOs) are a heterogeneous population that represents around 10% of nucleated cells in blood tissue. These cells are part of the myeloid lineage and after adequate signals are able to infiltrate to the tissue, where they terminally differentiate in different myeloid cell types such as dendritic cells (DCs) and macrophages (MACs). MOs have been considered for years as fleeting precursors of the mononuclear phagocytic system formed by DCs and MACs. However, recent investigations have shown not only that MOs are important for the generation of new phagocytic cells, but also constitute *per se* a functional cell type with relevant functions in both physiological and pathological conditions.

As mentioned, MOs are a heterogeneous population composed by three subsets and are defined by the expression of two surface markers, classical ($CD14^{high}CD16^{+}$), intermediate ($CD14^{+}CD16^{+}$) and non-classical ($CD14^{dim}CD16^{+}$) MOs (Krüger, Büning, & Schriever, 2001; Passlick, Flieger, & Loms Ziegler-Heitbrock, 1989; Zawada et al., 2011; Ziegler-Heitbrock et al., 2010). Labelling assays in humans suggested that classical monocytes are the precursors of intermediate and non-classical MOs (Patel et al., 2017). In fact, mathematical modeling of single-cell mRNA-seq (scRNA-seq) data demonstrated a linear trajectory from classical to non-classical MOs (Tak et al., 2017). At the functional level, the three subsets have slight differences. While classical MOs are primed for phagocytosis and migration, the intermediate MOs display higher potential for antigen presentation and non-classical MOs are more specialized in Fc gamma-mediated phagocytosis (Gren et al., 2015; Wong et al., 2011).

After infiltrating the tissue, MOs can differentiate into DCs or MACs depending on the signals that they receive. These signals are highly variable and depend on a wide range of factors including the type of infiltrated tissue, the type and stage of inflammation and the presence of other immune cells, among others. For instance, in early stages of inflammation, there is a high concentration of proinflammatory cytokines that entail the differentiation of MOs to DCs and MACs with immunogenic phenotype. However, in later stages

where it is necessary to stop the inflammation and promote the tissue regeneration, differentiation of MOs is favored towards the generation of MACs with tolerant phenotype (Sica & Mantovani, 2012).

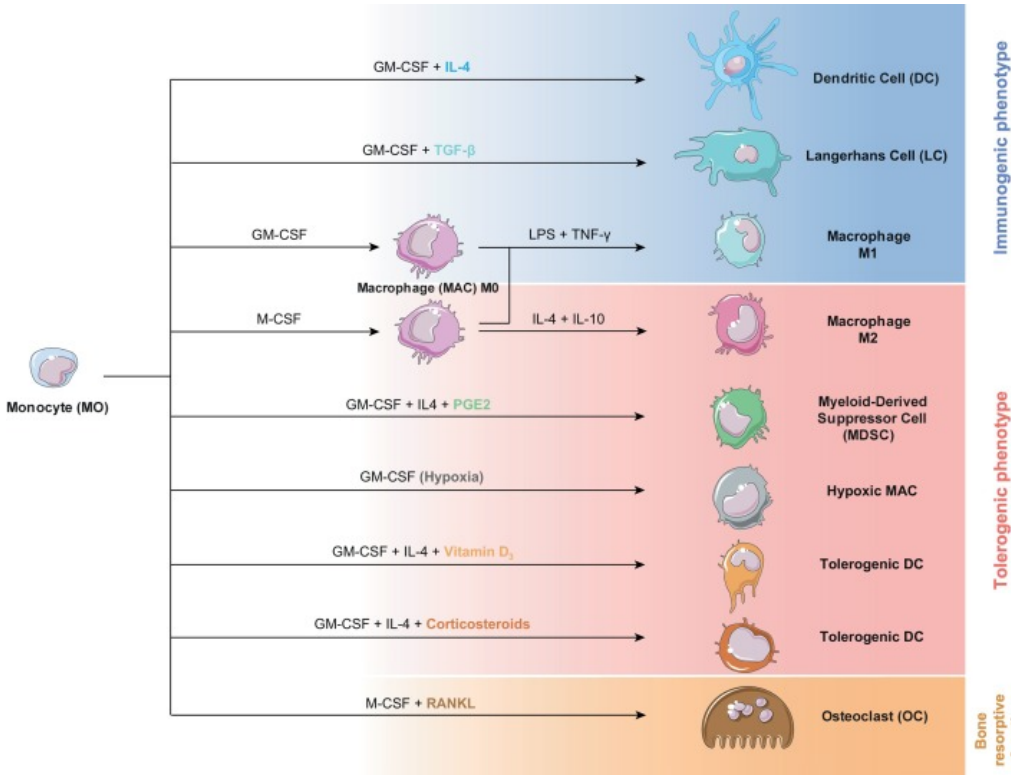


Figure 7. in vitro monocyte differentiation models. *Human monocytes can be differentiated in vitro towards cells with different phenotypical and functional properties* (Garcia-Gomez, Rodríguez-Ubreva, & Ballestar, 2018).

The relatively easy isolation of MOs from human blood, their ability to differentiate in different cell types, their plasticity and susceptibility to a multitude of external signals affecting their functionality, and the absence of cell division during the terminal differentiation from MOs, make these cells an extraordinary system for *in vitro* studies of signal-dependent DNA methylation dynamics and other epigenetic marks (Figure 7).

MOs isolated from peripheral blood can differentiate *in vitro* to MACs in presence of macrophage colony-stimulating factor 1 (M-CSF) during typically 4 to 6 days. After differentiation, MACs can be activated or polarized with the addition of different combinations of cytokines, hormones and other substances. The presence of these compounds leads to changes at the transcriptional and epigenetic level altering their functionality (Sica & Mantovani, 2012). Alternatively, MACs can be generated by the addition of Granulocyte-macrophage colony-stimulating factor 1 (G-MCSF), presenting in these cases more marked immunogenic properties (Verreck et al., 2004).

MOs can also be differentiated to osteoclasts (OCs) that are a bone-specific subtype of MACs. OCs have a specialized role in bone destruction and plays a relevant role in bone homeostasis under physiological conditions, but also under diseases such as multiple myeloma and rheumatoid arthritis. Osteoclastogenesis *in vitro* can be achieved by the exposing MOs to M-CSF and nuclear factor-kB ligand (RANKL) (Boyle, Simonet, & Lacey, 2003)

Finally, DCs can be generated from MOs exposed to GM-CSF and IL-4. These two cytokines are able to activate the pathways to produce a DCs with an immature phenotype. After the addition of the activating factor, generally lipopolysaccharide (LPS), immature DCs are activated acquiring a mature phenotype (Sallusto & Lanzavecchi, 1994). Alternatively, the immunophenotype of DCs can also be modulated in the presence of molecules such as the active form of vitamin D, prostaglandin E2 (PGE2) and dexamethasone. In all of these three cases, the resulting cells exhibit an immunosuppressive phenotype, antagonistic to the ones presented by conventional DCs.

2.2.1 DNA methylation changes during *in vitro* monocyte differentiation

Epigenomic regulation during human monocyte differentiation has been extensively studied during the past few years (Álvarez-Errico et al., 2015). During *in vitro* differentiation to MACs, DCs and OCs, all models exhibited cytokine-specific demethylation events, which can be attributed to active mechanisms given the absence of cell division during such differentiation

processes (de la Rica et al., 2013; Klug et al., 2010; Klug, Schmidhofer, Gebhard, Andreesen, & Rehli, 2013; Rodríguez-Ubreva et al., 2017; Vento-Tormo et al., 2016). Specifically, DCs generated from MOs in the presence of GM-CSF and IL-4 exhibited the activation of STAT6 in an IL-4 dependent manner. STAT6 activation induces TET2-dependent DNA demethylation necessary for the proper expression of DC-specific genes (Vento-Tormo et al., 2016). In another study, Garcia-Gomez and colleagues showed that demethylation mediated by TET2 during OCs and MACs differentiation is also assisted by TDG (Garcia-Gomez et al., 2017). In the same study, the authors also found that TET2-TDG-mediated demethylation is necessary for the subsequent recruitment of histone H3K4 methyltransferase SETD1A and increasing the H3K4me3 levels (Garcia-Gomez et al., 2017) at genes differentially expressed between MACs and OCs. Most importantly, H3K4 methylation prevents the binding of the ADD domain of *de novo* DNMTs and therefore avoids their activation (Ooi et al., 2007; Otani et al., 2009; Y. Zhang et al., 2010).

These differentiation models are also useful to investigate gains in DNA methylation. For instance, during MO differentiation to OCs or MACs, PU.1 not only interacts with TET2 but also with DNMT3B with the subsequent association with both DNA demethylation and hypermethylation (de la Rica et al., 2013). During MAC differentiation, it has also been described that NAD-dependent HDACs SIRT1/2 are important to prevent the premature activation of inflammatory genes. The proposed mechanism consists of gains in DNA methylation of inflammatory genes by the recruitment of SIRT1/2 and DNMTB (T. Li et al., 2019). These findings are consistent with the reported antagonism between histone acetylation and *de novo* DNMTs. DNA hypermethylation also has a relevant role in the acquisition of immunosuppressive phenotype during differentiation of DCs in the presence of a high concentration of PGE2 (Rodríguez-Ubreva et al., 2017). In this work, it was determined that DNMT3A-dependent hypermethylation of a set of genes is essential for the suppressive capabilities of PGE2-exposed DCs over cytotoxic T cells. However, the specific molecular mechanism of this phenomenon remains unknown.

2.2.2 Vitamin D and dendritic cell differentiation

Although the most recognized role of the active form of vitamin D ($1\alpha,25(OH)_2D_3$) is in the regulation of calcium and phosphate homeostasis and bone mineralization, during the year 2000, four independent works reported the inhibitory role of calcitriol (the active form of vitamin D) in DC differentiation from both, human and murine MOs (Berer et al., 2000; Griffin et al., 2000; Penna & Adorini, 2000; Piemonti et al., 2000). Subsequent studied showed that the presence of calcitriol redirected differentiation towards the generation of a functional cell type characterized by a tolerogenic phenotype. This exhibited phenotype is the result of a set of alterations that include changes at surface protein levels, cytokine secretion, metabolic activity and migration ability (Barragan, Good, & Kolls, 2015).

Tolerogenic dendritic cells (TLs) generated in presence of vitamin D are characterized by decreased levels of co-stimulation molecules such as CD80 and CD86 and of the antigen-presenting MHCII complex (Penna & Adorini, 2000). These proteins of the cell membrane are important for DC-mediated T cell activation and therefore, low levels of this would be associated with less T cell activation. On the other hand, TLs exhibit higher levels of CD14 expression suggesting a more immature phenotype (Bartels, Hvas, Agnholt, Dahlerup, & Agger, 2010).

Exposure to vitamin D also leads to alterations in the cytokine secretion of the TLs. It has been demonstrated that vitamin D inhibits the production of IL-12. Interestingly, IL-12 is important to induce Th1 responses via inhibition of TNF-α (Bartels et al., 2010). vitamin D also induces the expression of anti-inflammatory cytokine IL-10. In fact, IL-10 production in DCs promotes the development of Treg cells in humans (Van Der Aar et al., 2011).

Recently, the tolerogenic phenotype of TLs has been linked to metabolic reprogramming (Ferreira et al., 2015). In this regard, it was shown that vitamin D induces the expression of genes directly related to glucose metabolism, oxidative phosphorylation and tricarboxylic acid cycle. The authors of this work also observed that it is necessary the availability of glucose and its metabolization via glycolysis to induce and maintain the immunosuppressive

phenotype. They also showed that activation of PI3K/Akt/mTOR pathway is essential for the acquisition of the tolerogenic properties (Ferreira et al., 2015).

The active form of vitamin D requires the conversion of vitamin D to vitamin D_3 through a chemical reaction catalyzed by ultraviolet radiation that takes place in the skin. Alternatively, vitamin D_3 can be obtained directly from the diet. Next, vitamin D_3 also known as cholecalciferol, is converted within the liver in 25-hydroxycholecalciferol ($25(OH)D_3$) by the action of the enzyme encoded by the gene *CYP27A1*. After that, 25-hydroxycholecalciferol goes to the kidneys where it is hydroxylated through cytochrome P450 encoded by *CYP27B1* to become $1\alpha,25(OH)_2D_3$, the most active form of vitamin D. Importantly, MOs and their terminal differentiated cells (DCs and MACs), present expression levels of *CYP27A1* and *CYP27B1*, and therefore convert them the most susceptible cells to the action of vitamin D (Barragan et al., 2015).

Once $1\alpha,25(OH)_2D_3$ is produced, it is recognized by its receptor called vitamin D receptor (VDR). VDR is a member of the nuclear receptor family which acts as a receptor but also as a TF after heterodimerization with nuclear receptors of the retinoid X receptor (RXR) family. The VDR protein is comprised of three differentiated regions, a DNA binding domain in the amino-terminal region formed by a dual zinc-finger structure, a ligand-dependent activity domain in the C-terminal and an unstructured region that links the N- and C-terminal domains. $1\alpha,25(OH)_2D_3$ binding to the carboxyl terminus leads to a conformational change of VDR protein facilitating the interaction with RXR and also with coregulatory complexes required for VDR-mediated gene regulation (Carlberg, 2019). Apart from the nuclear action of VDR as a TF, it has also been described the role of VDR as a cytoplasmatic regulator of NF-κB activation. The proposed mechanism of action consists of the interaction of VDR with IKKB. This interaction would affect the correct assembly of the IKK complex with the consequent blockage of the IκBα degradation that retains NF-κB in the cytoplasm (Carlberg, 2019; Fetahu, Höbaus, & Kállay, 2014).

VDR binding has been linked to epigenomic changes. In fact, VDR can interact with different histone modification enzymes and chromatin remodelers,

such as bromodomain containing 7 (BRD7) and lysine demethylase 6B (KDM6B) (Pereira et al., 2011; Z. Wei et al., 2018). This can explain the correlation between VDR binding and the presence of activation marks like H3K27ac and H3K4me3 in human monocytic leukemia cells (THP1) (Nurminen, Neme, Seuter, & Carlberg, 2018, 2019). However, unliganded VDR can also form a complex with corepressor proteins with deacetylase activity as ALINE (Polly et al., 2000), or associated with NCOR1 and SMRT. Therefore, VDR has a dual role in transcriptional regulation acting as a repressor or activator depending on its partners.

In THP1 cells, vitamin D stimulation induces responses in more than 3000 loci including promoters and enhancer regions. In fact, chromatin accessibility assays have demonstrated that $1\alpha,25(OH)_2D_3$ treatment changes the accessibility at genomic level at direct and indirect manner (Seuter, Neme, & Carlberg, 2016). However, vitamin D exposure of differentiated cells shows that induced epigenetic changes are temporary, and the epigenomic effects of vitamin D have not yet been tested during differentiation processes (Carlberg, 2019).

The genome-wide binding pattern of VDR has been determined using ChIP-seq in different cells types and contexts: hepatic cells, prostate cells, colorectal cancer cells, lymphocytes, macrophage cells and leukemic monocytes (Ding et al., 2013; Fleet et al., 2019; Heikkinen et al., 2011; Meyer, Goetsch, & Pike, 2012; Neme, Seuter, & Carlberg, 2017; Ramagopalan et al., 2010; Tuoresmäki, Väisänen, Neme, Heikkinen, & Carlberg, 2014). The analysis of these datasets has shown that VDR genomic binding increases from 2 to 10 times in the presence of ligand. Interestingly, the majority of VDR binding is cell type-specific suggesting that the interaction of VDR with DNA is complex and dependent on other factors such as the epigenomic context or the interaction with other TF or cofactors. In this sense, it has been determined that VDR and PU.1 binding have high correspondence (Seuter, Neme, & Carlberg, 2017), as well as, with CEBPA (Nurminen et al., 2019), GABPA (Seuter, Neme, & Carlberg, 2018) and CTCF (Neme, Seuter, & Carlberg, 2016).

2.2.3 JAK/STAT signaling pathway

The Janus kinase/ signal transducers and activators of transcription (JAK/STAT) signaling pathway is highly relevant to integrate a multitude of extracellular stimuli in both, physiological and pathological contexts such as immunity and tumorigenesis. For instance, activation of the JAK3-STAT6 pathway is key for DC differentiation from MOs (Vento-Tormo et al., 2016). A wide variety of hormones and cytokines use this signaling pathway upon binding to receptors associated with kinases of the JAK family (JAK1, JAK2, JAK3 and TYK2). Following engagement by a ligand, JAKs become activated and phosphorylated and undergo conformational changes that allow the interaction and phosphorylation of STATs and its subsequent activation. Finally, activated STATs translocate to the nucleus, where they act as TFs regulating the expression of their target genes, characterized by the presence in the major part of the cases of interferon-γ activated sequence (GAS) motif (Villarino, Kanno, & Shea, 2017).

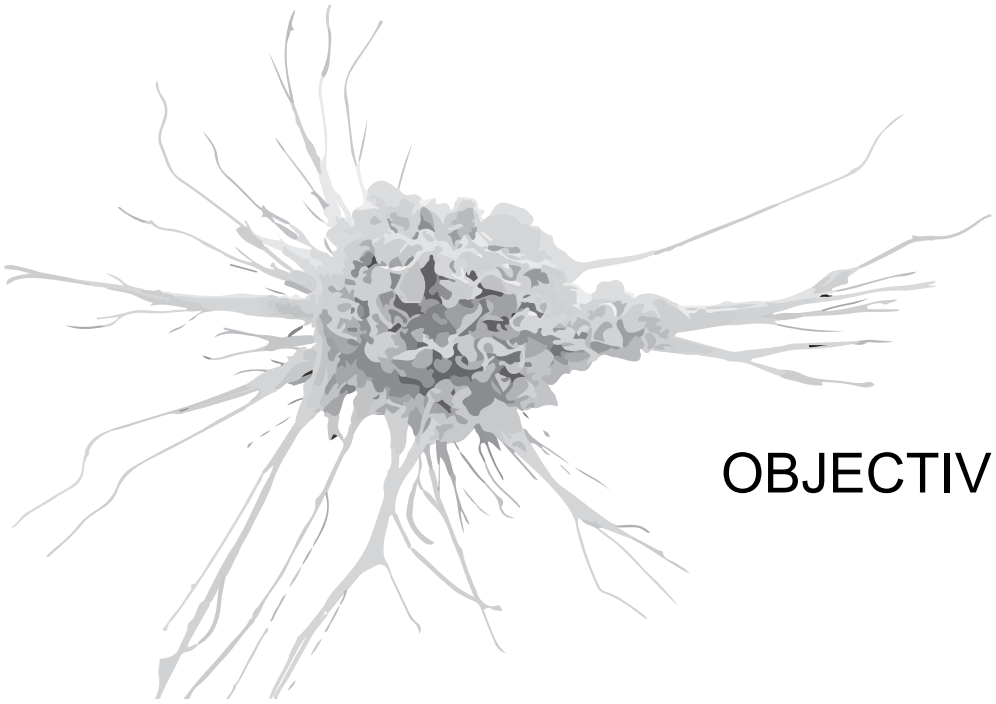
In mammals, the STAT protein family is composed of seven members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 (Yaming Wang & Levy, 2012). All of them act as TFs in the nucleus forming homodimers and heterodimers with other STATs. The integration of epigenetic profiles, TF-binding patterns and genomic physical interactions has shown that STATs typical bind distal enhancer regions (Ostuni et al., 2013; Qiao et al., 2013; Vahedi et al., 2012). It has also been shown that STATs act upstream of lineage-specific TFs, suggesting a role of signal-dependent TFs in priming cell-specific enhancers (Ostuni et al., 2013; Vahedi et al., 2012).

STAT protein binding can promote permissive and repressive epigenetic mark deposition (Durant et al., 2010; Qiao et al., 2013; Qiao, Kang, Giannopoulou, Fang, & Ivashkiv, 2016; L. Wei et al., 2010). The mechanisms underlying this process are not well established but the described interactions of STATs with CBP (p300) and EZH2 (Bhattacharya et al., 1996; Mandal et al., 2011) highlight the relevance of STATs as a epigenetic mediators. In addition, the integration of multiple ChIP-seq experiments suggests the association of STATs with various histone modification-containing multiprotein complexes. In

fact, it has been reported the interaction of STAT1-STAT2 with IRF9 (Veals et al., 1992), STAT3 with JUN (Schaefer, Sanders, & Nathans, 1995) and STAT5 with GR (Stocklin, Wissler, Gouilleux, & Groner, 1996) and the colocalization of STAT1 with NF-κB (Farlik et al., 2010).

Signaling dosage is always a critical aspect to maintain cell homoeostasis. In this regard, the JAK/STAT pathway presents different mechanisms for regulating its activity. Tyrosine dephosphorylation by JAKs, STATs and the associated receptors is one of the key regulatory modules inactivating the signaling. Another mechanism involves the inhibitory interaction of STATs with proteins of PIAS (protein inhibitor of activated STAT) family (Villarino et al., 2017). Finally, latent cytoplasmatic STATs proteins are a limiting resource and therefore changes in their concentrations have important consequences. For instance, overactivation of STAT1 ends with an increase in the activation of STAT3 by decreasing the available levels of STAT1 in the cytoplasm (Costa-Pereira et al., 2002; Qing & Stark, 2004).

Interestingly, it has been reported that the JAK/STAT signaling pathway has a key role during differentiation of DCs from MOs. In fact, STAT1 cooperation with IRF1, IRF8 and NF-κB is essential for correct differentiation (Garber et al., 2012). Moreover, inhibition of the JAK3/STAT6 signaling impairs the acquisition of DNA methylation changes of DC-specific genes and leads to aberrant gene expression patterns (Vento-Tormo et al., 2016).



OBJECTIVES

OBJECTIVES

DNA methylation and histone post-translational modifications are key epigenetic factors in the regulation of gene expression patterns that define cell identity and function. However, although DNA methylation has been intensely studied, there are still many controversies and unsolved questions. The complexity and variety of immune differentiation processes provide excellent models to investigate a plethora of molecular mechanisms related to DNA methylation. In this thesis, we aimed at investigating the molecular mechanisms of active DNA demethylation and its genomic targeting through the use of two different immune models.

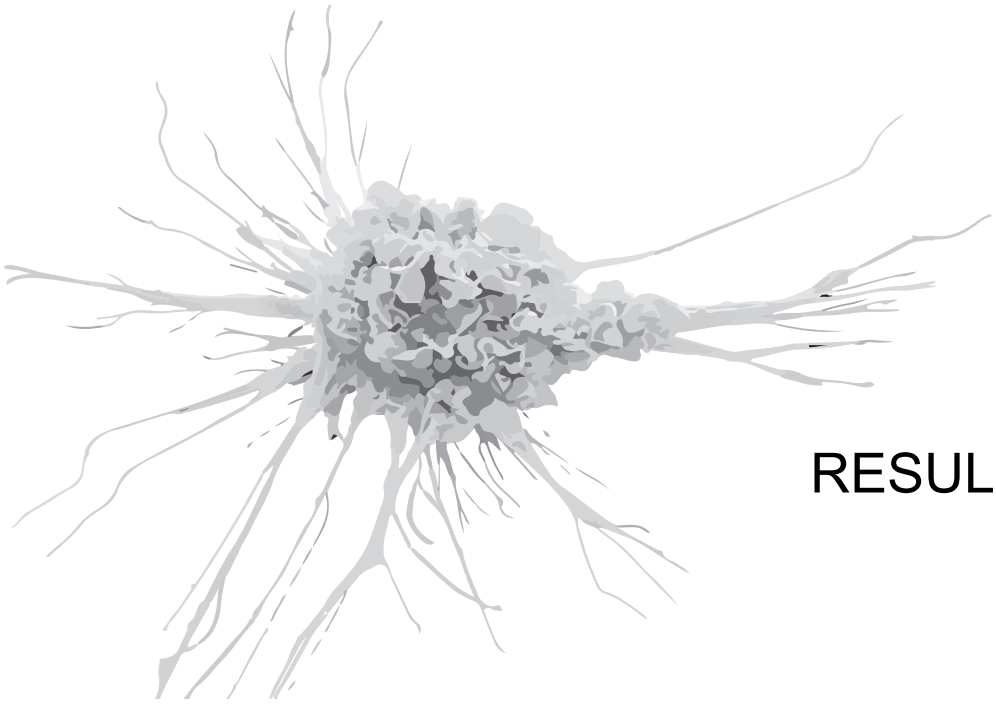
This general objective of the thesis was tackled by the proposal of the following specific objectives:

1. **To investigate the potential role of AID in mediating active DNA demethylation through its deaminase activity.**

   HIGM2 patients are characterized by loss-of-function mutations of AID which results in deficient production of class-switched memory B cells. This is due to the critical role of AID during B cell maturation in the germinal center, where it is highly upregulated. Therefore, the study of DNA methylomes of naïve and memory B cells from HIGM2 patients in comparison with controls is an optimal system to interrogate the role of AID in active demethylation *in vivo*. Moreover, this study not only will represent the first attempt to address this question in human cells but also the first epigenetic study of HIGM2.

2. **To explore the role of active DNA demethylation in the regulation of the tolerogenic phenotype of dendritic cells induced by vitamin D, focusing on the interplay among different TFs and the demethylation machinery.**

   Vitamin D exposure during monocyte differentiation in presence of IL-4 and GM-CSF leads to the generation of dendritic cells characterized by a stable tolerogenic phenotype. In this regard, DNA methylation may represent a potential contributor to the phenotypic stability of the tolerogenic properties induced by vitamin D and its receptor (VDR). Therefore, the study of this model focusing in DNA methylation can help us understand the potential role of VDR in the establishment of specific DNA demethylation and the interplay with signal dependent TFs.



RESULTS

RESULTS

ARTICLE 1

Title

Activation-induced deaminase is critical for the establishment of DNA methylation patterns prior to the germinal center reaction

Authors

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Abstract

Mutations in activation induced deaminase (AID) lead to hyper-IgM syndrome type 2 (HIGM2), a rare human primary antibody deficiency. AID-mediated cytosine deamination has been proposed as mediating active demethylation, although evidences both support and cast doubt on such a role. We here made use of HIGM2 B cells to investigate direct AID involvement in active DNA demethylation. HIGM2 naïve and memory B cells both display widespread DNA methylation defects, of which approximately 25% of these defects correspond to active events. For genes that undergo active demethylation that is impaired in HIGM2 individuals, our analysis discards AID involvement and supports the implication of TET enzymes. DNA methylation alterations in HIGM2 naïve B cells are related to premature overstimulation of the B-cell receptor prior to the germinal center reaction. Our data supports a role for AID in B cell central tolerance in preventing the expansion of autoreactive cell clones, affecting the correct establishment of DNA methylation patterns.

Introduction

Hyper-IgM syndrome type 2 (HIGM2) is a rare primary antibody deficiency characterized by loss-of-function mutations in activation-induced deaminase (AID) (Revy et al., 2000), an enzyme required for several crucial steps of B cell terminal differentiation. AID converts deoxycytosines (dCs) into deoxyuracils (dUs), producing dU:dG mismatches that are removed by mismatch repair and base-excision repair (Ramiro & Barreto, 2015). Deaminase activity is required for somatic hypermutation (SHM) and class-switch recombination (CSR) of immunoglobulin genes, which are necessary processes for affinity maturation and antibody diversification within the germinal centers (Arakawa, HauschiLd, & Buerstedde, 2002; Muramatsu et al., 2000; Revy et al., 2000). AID deficiency results in the absence of CSR and SHM, and leads to lymphoid hyperplasia (Revy et al., 2000). HIGM2 patients have normal or elevated serum IgM levels with severe reduction of IgG, IgA, and IgE, resulting in considerable susceptibility to bacterial infections (Revy et al., 2000).

In addition to its role in CSR and SHM, AID has been proposed as participating in active DNA demethylation through deamination of 5-methylcytosine, leading to a mismatch that is converted to G:C by thymine DNA glycosylase (TDG), followed by base-excision repair. During the past decade, conflicting reports have both supported and discounted such a role for AID [reviewed in (Ramiro & Barreto, 2015)]. For instance, in three independent studies using B cells from AID-deficient mice, two reported the absence of DNA methylation changes (Fritz et al., 2013; Hogenbirk et al., 2013), whereas the third study found DNA methylation differences relative to wild type mice (Dominguez et al., 2015). However, methodological aspects could explain the discrepancies between these studies. In parallel, the discovery of alternative enzymatic pathways that lead to *bona fide* active DNA demethylation through ten-eleven translocation methylcytosine dioxygenase (TET)-mediated oxidation of methylcytosines (Ito et al., 2010; Tahiliani et al., 2009) raised more doubts about the possibility that AID redundantly plays such a role. There is currently no consensus about whether AID is involved in mediating DNA demethylation in specific cell contexts.

Whole-genome analysis has shown the occurrence of a vast amount of demethylation associated with B cell differentiation. Changes occur mostly during naïve B cell activation, yielding memory B cells (Kulis et al., 2015; Oakes et al., 2016) that coincide with the highest peak of AID expression (Muramatsu et al., 2000; Revy et al., 2000). Naïve B cells start to proliferate upon activation by antigen encounter. Then they express AID which triggers the secondary diversification of antibodies by SHM and CSR. This is followed by affinity maturation which finally leads to a) a new cycle of SHM or b) terminal differentiation into memory or plasma B cells depending on the affinity of the B cell receptor (BCR) for the cognate antigen (Victora & Nussenzweig, 2012).

In this study, we took advantage of the exceptional possibility to address the direct role of AID in active demethylation by comparing the complete DNA methylomes of naïve and memory B cells of HIGM2 patients with those of healthy individuals. By studying two sibling patients with a homozygous mutation for AID that results in a severely truncated enzyme we were able to determine its direct link with DNA methylation defects and infer its catalytic activity in relation to active DNA demethylation.

Our results show that the absence of AID catalytic activity affects DNA methylation in naïve and memory B cells. The majority of the changes observed in the transition from naïve to memory B cells arise from passive demethylation and are linked to late-replicating domains. However, for those potentially associated with active demethylation, we found no evidence of direct involvement of AID, and our analysis indicates that TET enzymes are responsible for DNA methylation changes in this cell context. The increased DNA demethylation noted in naïve B cells of HIGM2 patients is associated with premature demethylation of BCR downstream genes prior to the germinal center reaction. Indeed, we found that these changes are related to the expansion of autoreactive clones, which suggests a major role for AID in preventing the expansion of such clones under normal conditions.

Results

Study strategy

We obtained peripheral blood from two HIGM2 sibling patients, both with the same homozygous mutation for AID, and two healthy controls. Specifically, the patients carried a deletion (Exon 2 c.22_40del19) that generated a frameshift variant (p.Arg8Asnfs*19) that affects the majority of AID, including its catalytic domain. We inspected the peripheral B cell compartment by flow cytometry. As previously described, HIGM2 patients are characterized by the absence of class-switched memory B cells ($CD19^+CD27^+IgM^-IgD^-$, csMBC)(Revy et al., 2000). Nevertheless, classic non-class switched memory B cells ($CD19^+$ $CD27^+$ IgM^+ IgD^+, ncsMBC) and naïve B cells ($CD19^+$ $CD27^-$ IgM^+ IgD^+, NBC) are present in patients (**Fig. 1A**). Under physiological conditions, ncsMBC cells display certain levels of SHM at the immunoglobulin locus (Oakes et al., 2016), which supports the expression of AID during their maturation in germinal centers (Muramatsu et al., 2000; Revy et al., 2000). Therefore, the comparison between the DNA methylation profiles from NBC and ncsMBC of healthy and HIGM2 individuals are an adequate model for testing the potential role of AID in demethylation.

HIGM2 patients display an aberrant methylation profile in naïve and unswitched memory B cells

We performed tagmentation-based whole-genome bisulfite sequencing (T-WGBS), a version of the WGBS method that allows analysis of limited DNA amounts (Wang et al., 2013), for two biological replicates of each of the two aforementioned B cell subsets of the HIGM2 and of healthy controls (from now on referred to as “naïve” and “memory” cells) (**Fig. 1B**). Pearson correlation and t-distribution stochastic neighbor embedding (t-SNE) between samples was highly reproducible between replicates (correlation coefficient > 0.9, **Fig. S1A,B**). We also compared our DNA methylation data from healthy controls with public data from the International Cancer Genome Consortium (ICGC) (Hudson et al., 2010) and Oakes et al. (Oakes et al., 2016), thereby confirming the robustness of our data (**Fig. S1C,D**).

Figure 1

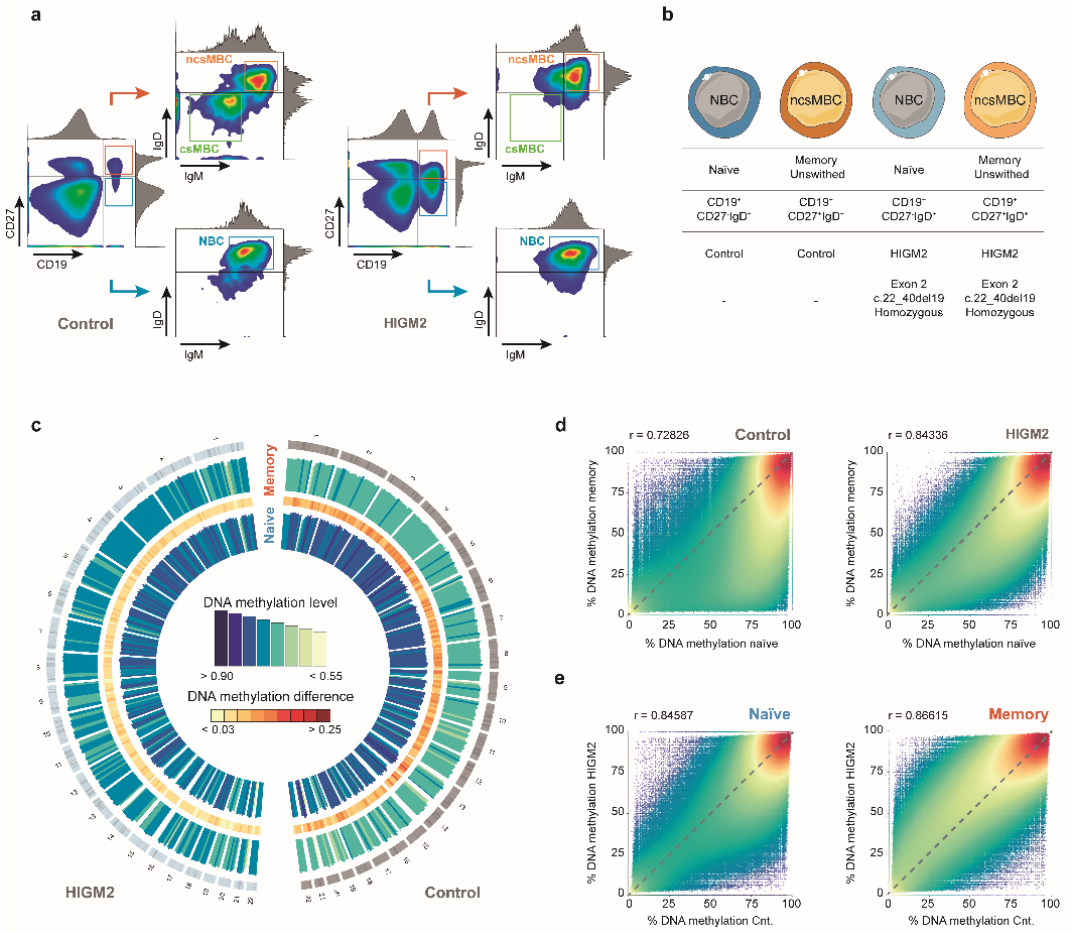

Figure 1: DNA methylome of HIGM2 B cell subpopulations determined by TWGBS. *(**a**) A representative example of a strategy for sorting B cell populations (naïve B cells, NBC; classic non-class-switched memory B cells, ncsMBC; class-switched memory B cells, csMBC). (**b**) Description of B cell subpopulations analyzed. (**c**) Circular representation of DNA methylation levels for naïve B cells (inner circle) and unswitched memory B cells (outer circle) for controls (right) and HIGM2 patients (left). Histogram tracks represent the average methylation levels over 10 Mb windows. Heatmap shows the DNA methylation differences between naïve and memory B cells (**d**) Scatter plots with density information and color scale for the pairwise comparisons of control naïve to control memory B cells and HIGM2 naïve to HIGM2 memory B cells. (**e**) Scatter plots with density information and color scale of the pairwise comparisons of HIGM2 naïve to Control naïve B cells and HIGM2 memory to Control memory B cells.*

Global inspection of DNA methylation confirmed that, as reported (Kulis et al., 2015; Oakes et al., 2016), transition from naïve to memory B cells is accompanied by global demethylation of the genome (**Fig. 1C**). However, the same comparison in HIGM2 patients showed a partial impairment of

demethylation during B cell differentiation (**Fig 1C,D**), compatible with a potential role of AID as a demethylating enzyme. Unexpectedly, we also observed that naïve B cells were more demethylated in HIGM2 patients than in healthy controls (**Fig. 1E**). Taken together, these global observations suggest that AID loss not only affects the DNA methylation patterns in the transition from naïve to memory B cells, but also has a significant role in establishing the B cell methylome in earlier stages of development.

A high proportion of DNA demethylation events identified in HIGM2 are due to passive demethylation of late-replicating domains

Recent studies have shown that a high proportion of the demethylation events occurring in cancer and in differentiation processes are associated with high proliferation rates. Such demethylation takes place in regions known as 'partially methylated domains' (PMDs), rather than 'highly methylated domains' (HMDs). PMDs are characterized by late replication, and their demethylation is a passive event, as a result of inefficient DNA remethylation during DNA replication (Hansen et al., 2014; Lister et al., 2009; Zhou et al., 2018). Recent reanalysis of the B cell lineage DNA methylation profiles published by the BLUEPRINT consortium (Kulis et al., 2015) has shown the occurrence of demethylation of PMDs in the transition towards memory cell and antibody-secreting plasma cells (Durek et al., 2016). This highlights how critical it is to separate the analysis of DNA methylation changes produced inside and outside PMDs when examining the occurrence of active demethylation processes to exclude those changes due to passive demethylation.

To address this matter, we examined the overlap of the differentially methylated regions (DMRs) corresponding to all comparisons with the PMDs and HMDs obtained by Zhou et al. (Zhou et al., 2018). We found that the majority of DMRs overlap with PMDs (72.5%, **Fig. 2A**). DMRs were therefore classified into two sets: the first comprised those DMRs that coincided with common PMDs (PMD-DMRs), and the second contained all the other DMRs (non-PMD-DMRs). Global inspection of the methylation values showed that the two groups of DMRs had intermediate methylation values in memory B cells,

Figure 2

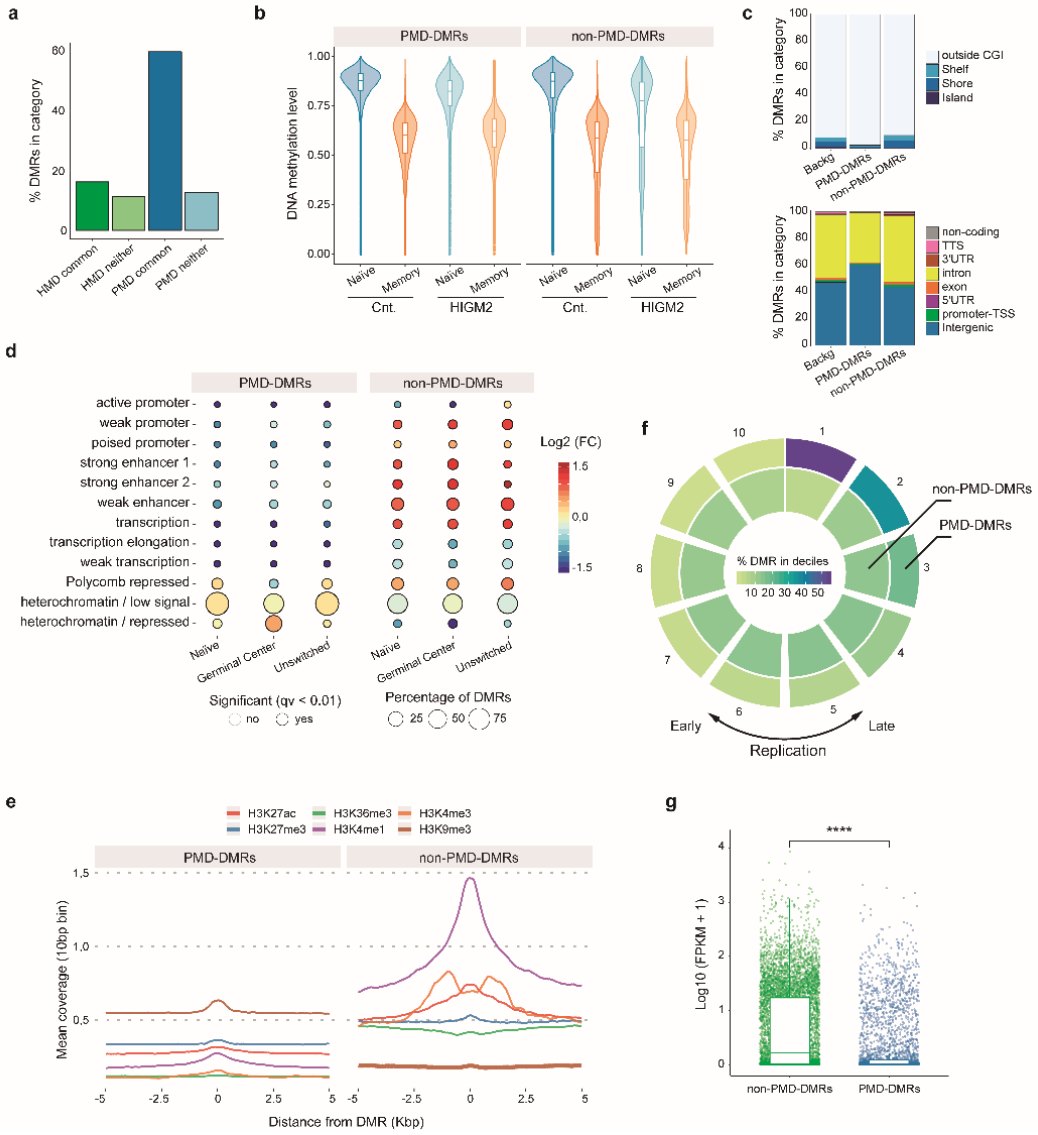

Figure 2: Detection and characterization of partially methylated regions. *(**a**) Bar plot showing the percentage of DMRs in PMD-HMD (partially and highly methylated domains, respectively) regions annotated by Zhou and colleagues (Zhou et al., 2018). 'Common' refers to those PMD-HMD regions common to all cell types analyzed by Zhou and colleagues. 'Neither' refers to those regions detected only in a fraction of the cell types analyzed. (**b**) Box and violin plots summarizing the distribution of DNA methylation levels per sample group of DMRs inside and outside PMDs. (**c**) Location proportions of PMD-DMRs and non-PMD-DMRs in the context of CpG islands (CGIs) and gene-related regions. (**d**) Bubble chart depicting the enrichment (red) or depletion (blue) of chromatin states. The dot fill represents the logarithmic-fold change, dot size indicates the percentage of DMRs in the chromatin state, and the edge indicates the statistical significance of the enrichment (black: significant, none: not significant; q < 0.01). (**e**) Composite plots of patterns of different histone marks ChIP-seq signals ± 5 kb around the midpoints of PMD-DMRs (left) and non-PMD-DMRs (right) of PMDs. (**f**) Circular representation of the proportion of DMRs in deciles of replication timing data of*

*GM12878 cell line. Color scale represents the proportion of DMRs in each decile. The first and last deciles correspond to the regions of latest and earliest replication, respectively. (**g**) Box and dot plots showing the distribution of expression values of DMRs associated genes inside and outside PMDs. Statistical tests: two-tailed Fisher's exact (**d**) and unpaired Wilcoxon's (**g**) tests. (FPKM= fragments per kb of transcript per million mapped reads).*

although non-PMD-DMRs had lower methylation levels (**Fig. 2B**), suggesting the presence of active demethylation events.

Next, we analyzed the functional genomic features of the two groups of DMRs to confirm whether it is appropriate to use the PMD/HMD annotation with our data. We found that the PMD-DMRs were enriched in intergenic regions (**Fig. 2C**). Considering the connection between the two groups of DMRs and the chromatin states, we found that DMRs occurring in PMDs were mainly associated with heterochromatic regions, while non-PMD-DMRs were highly enriched at enhancers and active promoters, which were mainly associated with active demethylation (Wu & Zhang, 2017) (**Fig. 2D,E**).

PMDs are characterized by their association with late-replication domains (Zhou et al., 2018). To test this property in our data, we divided the GM12878 Repli-seq data into deciles and measured the percentage of DMRs in each category. We confirmed that, indeed, the DMRs annotated as PMDs were mainly found in late-replicating regions (**Fig. 2F**) and were accompanied by lower expression levels of associated genes in germinal center B cells (**Fig. 2G**). Taking all these observations into account, our results indicated that most of the DNA methylation changes in all the comparisons occur in PMDs. However, the existence of a set of non-PMD-DMRs (~27%) located in highly active regions suggests the potential participation of active demethylation events, which could now be interrogated for the potential direct participation of AID.

HIGM2-associated defects in DNA methylation in the transition from naïve to memory B cells do not have the features of AID targets

Given all the previous considerations, including the removal of DNA methylation changes related to DNA replication (PMD-DMRs), our model allows us to

examine whether AID has a direct role in mediating demethylation in the B cell lineage. In this context, germinal center B cells in the transition from naïve to memory displayed the highest levels of AID mRNA expression (**Fig. S2A**) (Muramatsu et al., 2000; Revy et al., 2000). This makes the comparison between naïve and memory B cells the most suitable to explore the direct role of AID in active demethylation.

To this end, we selected those DMRs with methylation dynamics consistent with potential demethylation mediated by AID (P-AID DMRs). These are defined as DMRs that are demethylated in the transition from naïve to memory B cells in healthy controls, not occurring in the HIGM2 patients and having similar methylation levels in naïve cells of controls and HIGM2 patients (**Fig. 3A**). A total of 522 DMRs (containing 450 different genes) fulfilled these conditions.

We first investigated the overlap between the genes contained in the P-AID DMRs and 271 described off-target AID genes (AID targets outside the locus of immunoglobulins) (Álvarez-Prado et al., 2018) and found a low correspondence (only 6 out of 271, 2.2 %) (**Fig. 3B**). We also tested the presence of described AID hot spots (Rogozin & Kolchanov, 1992) and found a significant increase for the WRCY hotspot with respect to the background, although the increase in hotspots in both subsets of DMRs respect the background appeared too low (<1 hotspot per 100 bp) to be of biological relevance (**Fig. 3C**). On the other hand, we found that DMRs associated with off-target AID genes underwent demethylation in HIGM2 patients (**Fig. S2B**).

Two recent studies have characterized the genomic and epigenomic features of AID off-target regions. Independently, they found that AID targets regions with convergent transcription from intragenic super-enhancers (Meng et al., 2014; Qian et al., 2014). In this sense, although there were no significant differences regarding gene localization between the DMRs associated with AID off-targets and P-AID DMRs (**Fig. S2c**), the former exhibited greater enrichment of enhancer regions (**Fig. 3D and Fig. S2D**) that was associated with more transcriptional activity of associated genes in germinal center B cells (**Fig. 3E**). We also found that, although the two DMR groups had a similar

Figure 3

Figure 3: Indirect involvement of AID in DNA demethylation dynamics during B cell activation. *(**a**) Box and violin plots summarizing the distribution of DNA methylation levels per sample group of regions potentially demethylated directly by AID (P-AID). (**b**) Venn diagram showing the overlap between P-AID DMRs and human orthologues of the described mouse AID off-target genes. (**c**) Box and violin plots displaying the frequency of WRCY/RGYW (W = A/T; R = G/A; Y = C/T) hot spots per 100 bp in DMRs of each subset. (**d**) Bubble chart depicting the enrichment (red) or depletion (blue) of chromatin states. The dot fill represents the logarithmic-fold change, the dot size shows the percentage of DMRs in the chromatin state, and the edge indicates the statistical significance of the enrichment (black: significant, none: not significant, q < 0.01). (**e**) Circular representation of the proportion of DMRs in deciles of germinal center B cells. Color scale represents the proportion of DMRs in each decile. The first and last deciles correspond to the least and most highly expressed genes, respectively. (**f**) Venn diagram of the overlap between P-AID and AID off-target DMRs with germinal center super-*

*enhancers. (**g**) Box plots showing H3K27ac signal of AID off-target (red) and P-AID (green) associated super-enhancers. The one-sided unpaired Wilcoxon's test was used to examine signal intensity differences. (**h**) DNA methylation and H3K27ac profiles in the vicinity of two representative genes. DMR color indicates the type: P-AID (green) and AID off-target DMRs (red). At the bottom, the H3K27ac ChIP-seq signal in germinal center B cells is shown in dark orange. Identified super-enhancers are depicted below with dark orange bars. Enhancer regions are represented with light orange bars. Statistical tests: two-tailed unpaired Wilcoxon's (c, g) and Fisher's exact (d) tests.*

percentage of overlap with super-enhancers (P-AID 21%, AID off-target 33.3%; **Fig. 3F**), the super-enhancers of the AID off-targets had a stronger signal for H3K27ac (**Fig. 3G,H and Fig. S2E**) and greater transcriptional activity of their associated genes than P-AID (**Fig. S2F**). Finally, we hypothesized that if AID had a role mediating active DNA demethylation, we would expect to see differences in CpG sites in a WRCY context at the super-enhancers of AID off-target genes. However, no such differences were observed (**Fig. S2G)**. Taken together, our results suggest that, despite the differences in DNA methylation associated with B cell activation between wild type and AID-deficient B cells, such demethylation is not directly associated with AID catalytic activity.

Demethylation during activation of B cells involves TET family proteins

Our findings ruled out a role for AID in directly mediating DNA demethylation in human B cells and contradicted those of a previous study addressing this hypothesis in mouse B cells (Dominguez et al., 2015). However, the conclusions of that study were based on there having been a significant enrichment with SHM target genes and AID-associated dsDNA breaks (as was also the case in the present study), but with an overlap between the two of less than 10%.

While the removal of methyl groups of cytosines mediated by TET enzymes involves the generation of oxidation intermediates (Tahiliani et al., 2009), the proposed mechanism of DNA demethylation by AID implies that the 5mC conversion in a thymine could be repaired and replaced with an unmethylated cytosine (Ramiro & Barreto, 2015) (**Fig. 4A**). Given these considerations, we hypothesized that if AID deaminates 5mC and yields thymine in mouse B cells at the population level, AID-dependent demethylation events would be associated with lower levels 5mhC than those that are TET-

dependent. To address this possibility, we merged the methylation data of Dominguez and colleagues (Dominguez et al., 2015) with public hydroxy-meDIP-seq data of mouse B cell activation (Lio et al., 2019). We selected a set of CpGs demethylated in wild type but not in $AID^{-/-}$ mice (mouse potential AID targets, P-AID), a second CpG set that is significantly demethylated under both conditions and more likely therefore to be TET-dependent (positive control), and a third group of CpGs without demethylation (negative control; delta DNA methylation < 0.05) (**Fig. 4B**). Next, we determined the hydroxymethylation status of the three groups of CpGs, and found that mouse P-AID CpGs presented similar levels of hydroxymethylation to those that are TET-dependent (positive control), and significantly higher levels than CpGs selected as negative controls (**Fig. 4C**). On the other hand, we found that mouse P-AID CpGs overlapped little with the described AID off-target genes (< 8%, **Fig. 4D**). Taken together, these results indicate that demethylation changes occurring during B cell maturation are mediated by TET proteins, and if AID has a role it should be only in an indirect manner.

AID deficiency results in premature demethylation of the BCR pathway of naïve B cells

Our initial analysis suggested that alterations occur in DNA methylation in naïve cells of HIGM2 individuals in comparison with healthy controls. Specifically, HIGM2 naïve B cells appeared to be more demethylated than those of healthy controls. AID expression has customarily been associated with the germinal center reaction (Muramatsu et al., 2000; Revy et al., 2000). However, more recent evidence suggests that AID might have a role in earlier stages of B cell development (Cantaert et al., 2015; Kuraoka et al., 2011).

Comparing HIGM2 and control naïve B cells, we detected 2152 hypomethylated DMRs (**Fig. 5A**) and 127 hypermethylated DMRs (**Fig. S3A**), PMDs having been excluded. Both groups of DMRs were mostly found in intergenic regions and introns (**Fig. S3B**). However, while hypomethylation was associated with enhancer regions, hypermethylation was mainly enriched in promoters (**Fig. S3C**), in agreement with its reported regulatory role in the ‘spurious’ initiation of transcription (Neri et al., 2017).

Figure 4

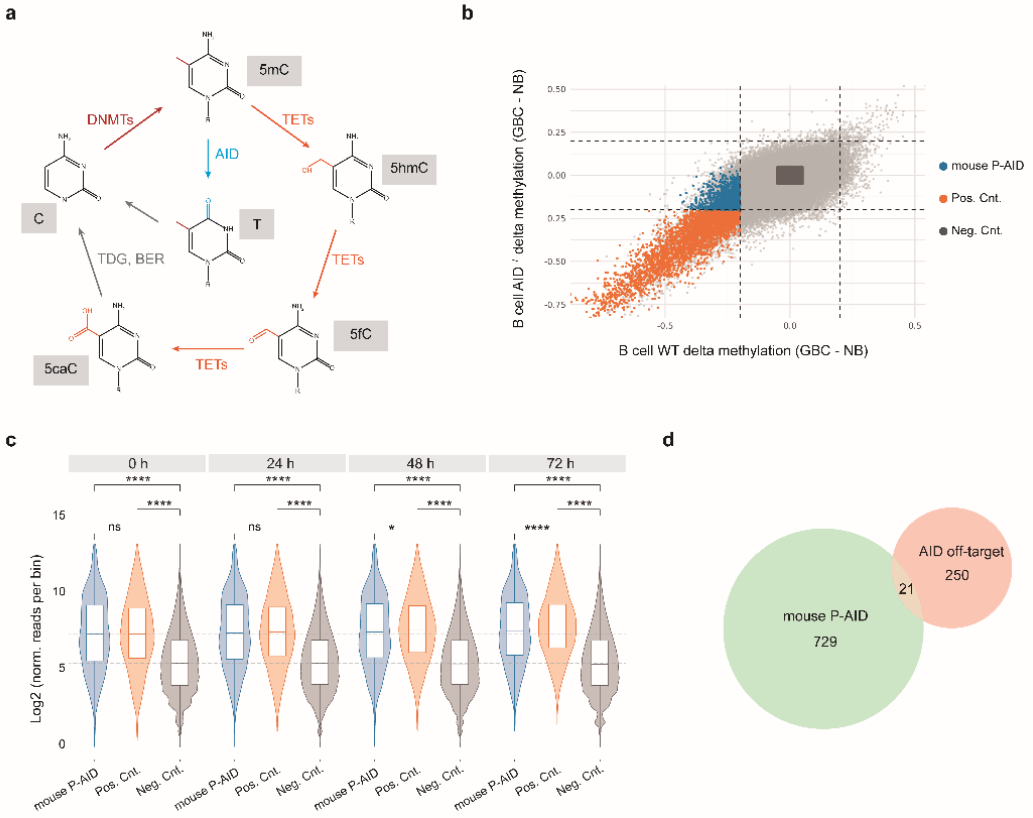

Figure 4: TET proteins are responsible for DNA demethylation events. *(**a**) Scheme depicting the potential dynamics of demethylation according to the oxidation reaction catalyzed by TET enzymes or the deamination-mediated reaction catalyzed by AID (**b**) Scatter plot showing a pairwise comparison of DNA methylation differences between naïve and germinal center B cells of WT and AID$^{-/-}$ mouse. Blue dots indicate CpGs potentially demethylated by AID (mice P-AD). Orange dots indicate those demethylated in both WT and AID DKO (Positive control). Gray dots show those without methylation changes in either condition (Neg. Cnt.). (**c**) Box and violin plots depicting the distribution of hydroxymethylation values of the sets of CpGs at different times after LPS/IL-4 B cell activation. Statistical test: Two-tailed unpaired Wilcoxon's test (d). **(d)** Venn diagram showing the overlap between mouse P-AID CpGs and human orthologues of the described mouse AID off-target genes.*

We observed that DMRs in this comparison, i.e. differentially methylated between naïve cells of HIGM2 patients and healthy controls, underwent a similar change in DNA methylation during the transition from naïve to memory cells in controls, suggesting that the DNA methylation alterations in HIGM2 naïve cells correspond to changes that occur later in normal differentiation as if these cells were pre-activated outside the germinal center (**Fig. 5A**). This is

consistent with the finding that genes associated with these DMRs became upregulated during the activation of naïve to memory cells in the germinal center (**Fig. 5B**) and were associated with functional categories related to B cell activation via BCR (**Fig. 5C**). Some genes that have altered DMRs, such as *BATF* (**Fig. 5D**) (Lio et al., 2019) or *MEF2A* (**Fig. S3D**) (Herglotz et al., 2016), are crucial to B cell development.

To explore the possibility that the changes between naïve B cells of HIGM2 patients and controls are due to pre-activation outside the germinal center, we tested the enrichment for TF binding motifs in the DMRs. Some of the most enriched TFs are downstream of the BCR pathway (Kandasamy et al., 2010) (**Fig. S3E**). We then validated these results through enrichment analysis of the ChIP-seq data available for GM12878 cells from the ENCODE consortium (Feingold et al., 2004) and found them to be in accordance with those of the motif enrichment analysis and showed enrichment of downstream TFs of the BCR (**Fig. 5E**). In addition, some of these TFs were associated with altered DMRs such as BATF, MEF2A, NFATC1, BCL11A and IRF4.

The type III latency state of the Epstein-Barr virus (EBV) is characterized by the constitutive activation of the BCR and CD40 pathways (Cahir-McFarland et al., 2004; Mancao & Hammerschmidt, 2007), both of which are major signaling pathways that function during B cell activation (Luo, Weisel, & Shlomchik, 2018). In this sense, the B lymphoblastoid cell line GM12878 presents a type III latency state (Ma et al., 2017) and is, therefore, a good model for testing if the changes at the altered DMRs are indeed produced via BCR/CD40. To test this hypothesis, we checked the methylation status of altered DMRs in public DNA methylation data of EBV-transformed B cells and CD40L/IL-4-activated B cells(Hansen et al., 2014). We observed that EBV transformation effectively reproduced methylation changes of the altered DMRs. Conversely, such changes did not take place with CD40/IL-4 activation, suggesting that the BCR pathway has a significant role (**Fig. 5F and Fig. S3F**). We confirmed these results by transforming naïve B cells with EBV. After 30 days, we used pyrosequencing to test a selection of genes that were aberrantly demethylated in HIGM2 naïve B cells. We found that these genes underwent

Figure 5

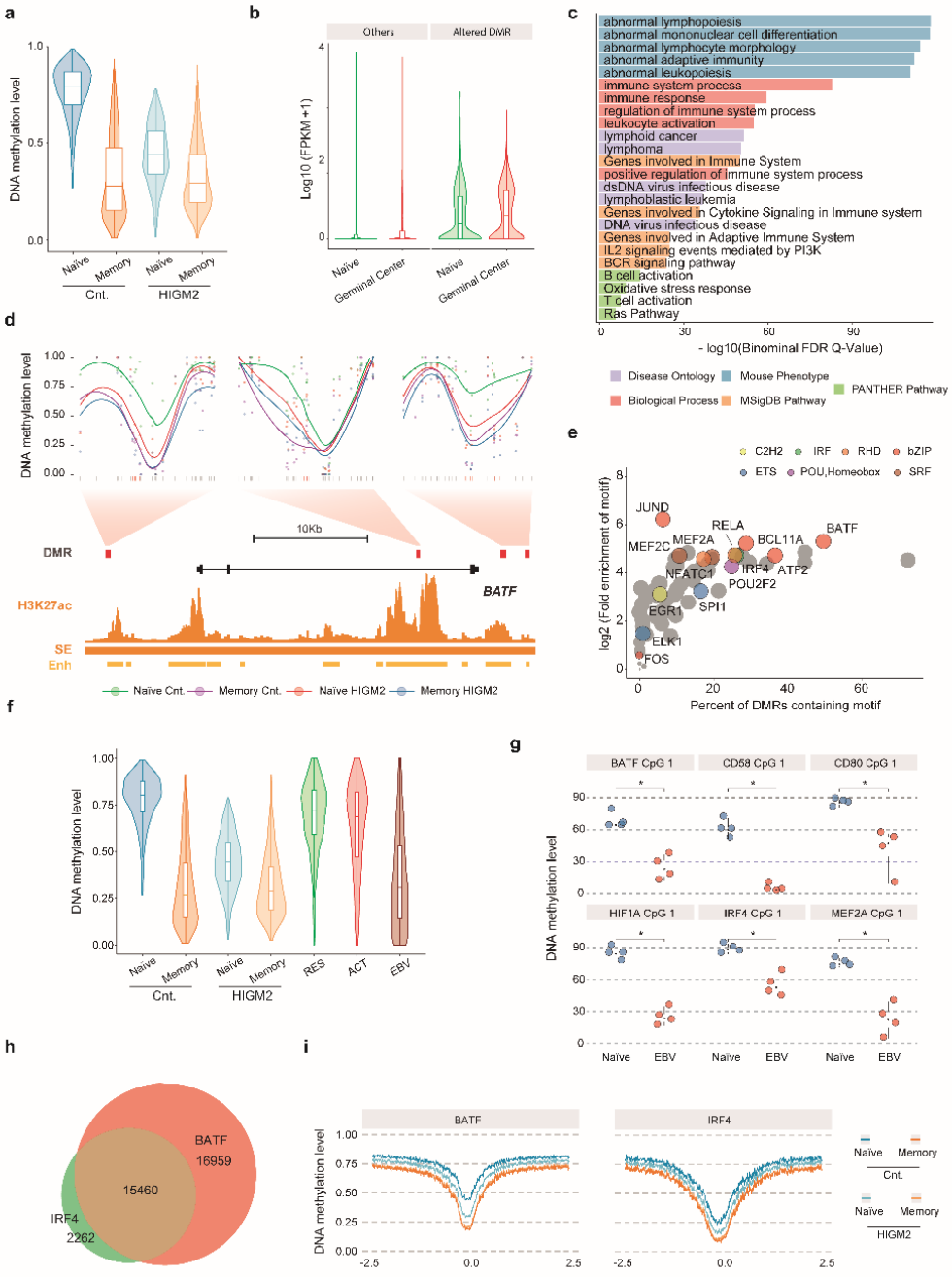

Figure 5: Role of AID in early B cell development. *(**a**) Box and violin plots summarizing the distribution of DNA methylation levels per sample group of differentially hypomethylated regions between control and patient naïve B cells. (**b**) Box plot showing the distribution of expression values of altered DMR-associated genes and all other genes in naïve (green) and germinal center (red) B cells. (**c**) Results of gene set enrichment analysis using GREAT software. The plot depicts the top five enriched terms for five annotation databases, based on P values from the binominal distribution. (**d**) Smoothed DNA methylation data of BATF-associated altered DMRs and H3K27ac ChIP-seq signal in germinal center B cells. (**e**) Bubble scatter plot of transcription factor ChIP-seq peaks determined in GM12878 lymphoblastoid cell line in altered DMRs. Only transcription factors downstream of BCR signaling are colored according to the*

*transcription factor family. Bubble size corresponds to the logarithm of adjusted values of P. (**f**) Box and violin plots summarizing the distribution of DNA hypomethylation levels of altered DMRs in resting B cells (RES), B cells activated with CD40L/IL-4 (ACT) and B cells infected with Epstein-Barr virus (EBV). (**g**) Dot plot showing the DNA methylation values determined by pyrosequencing of naïve B cells (naïve B cells) and EBV-infected naïve B cells (EBV). (**h**) Venn diagram representing the overlap between BATF and IRF4 ChIP-seq peaks in the GM12878 cell line. (**i**) Composite CpG methylation levels surrounding transcription factor ChIP-seq peaks (± 2.5 kb). Statistical tests: two-tailed Wilcoxon's test (g). (FPKM= fragments per kilobase of transcript per million mapped reads, FDR = false discovery rate).*

demethylation following EBV-mediated transformation of naïve B cells (**Fig. 5G and Fig. S3G**). Taken together, our findings showed that the changes between HIGM2 naïve B cells and those of the controls are due to the aberrant pre-activation of the BCR at some point of B cell development prior to the germinal center reaction.

The analysis of motif enrichment and ChIP-seq data in GM12878 indicated that BATF might be the main potential mediator of demethylation, perhaps facilitating the recruitment of TET enzymes and leading to the generation of altered DMRs in HIGM2 naïve B cells (~50% overlap with the DMRs). BATF is a regulator of B and T cell activation (BCR and TCR pathways, respectively), in cooperation with IRF4 (Ise et al., 2011; Ochiai et al., 2013) . We observed that 87% of the ChIP-seq peaks of IRF4 overlapped with BATF peaks in GM12878 (**Fig. 5H**). As expected, we were able to confirm that regions with BATF and IRF4 binding had lower DNA methylation levels in naïve cells of HIGM2 patients than in controls (**Fig. 5I**), as well as JUND, another TF downstream of BCR (**Fig. S4A**). This did not occur in regions enriched for other B cell-intrinsic TF binding motifs (**Fig. S4A**). Using mRNA transcription data from IRF4 and BATF knockouts from GM12878 cells (Ma et al., 2017), we determined that genes with binding motifs for both TFs displayed expression changes for many of these genes associated with altered DMRs (**Fig. S4B**). All these results suggest that a significant fraction of the altered DMRs in HIGM2 naïve B cells may be associated with the recruitment of the BATF/IRF4 complex to these genomic sites. Such recruitment might facilitate TET protein-mediated hydroxymethylation and the consequent demethylation of those regions, as recently reported (Lio et al., 2019).

AID deficiency causes blockade of central B cell tolerance with an expansion of pre-activated autoreactive B cells

Our results suggest that naïve B cells are pre-activated in AID deficient patients. However, the stage of B cell differentiation at which this alteration is produced remains to be established. Two independent studies reported a potential role for AID in removing autoreactive B cells during the central B cell tolerance process in the bone marrow. Specifically, the immature B cells with auto-reactive BCR were activated and went into a secondary receptor editing process with an increase in AID and recombination-activating gene 2 (RAG2). However, if the autoreactive BCR did not lose self-antigen affinity the genomic instability induced by the overexposure to high levels of AID led to apoptosis. However, AID deficiency reduces the genomic damage that causes the expansion of autoreactive B cells (Cantaert et al., 2015; Kuraoka et al., 2011). With that in mind, we hypothesized that the presence of naïve B cells with a pre-activation methylation signature in HIGM2 patients is a consequence of the impairment of central B cell tolerance that causes autoreactive naïve B cells to accumulate. In fact, it has been reported that 21% of HIGM2 patients suffer some kind of autoimmune disease (Mao et al., 2004).

To assess this hypothesis, we first checked whether there is an expansion of naïve autoreactive B cells in HIGM2 patients with respect to controls. To this end, we used a commercial antibody against 9G4+ IgG used to detect autoreactive clones in autoimmune diseases like systemic lupus erythematosus and rheumatoid arthritis (Moura et al., 2012; Richardson et al., 2013). We observed an expansion of the naïve B cell compartment in HIGM2 patients in comparison with healthy controls (**Fig. 6A,B)**. We did not find an expansion of $9G4^{+}$ in HIGM2 patients with respect to controls (**Fig. 6C**). However, we observed a significant increase of mean fluorescence intensity for 9g4 staining (**Fig. 6A,D**), as well as, an expansion of high $9g4^{+}$ naïve B cells (**Fig. 6E**). Next, we determined the methylation status of a selection of genes by pyrosequencing of $9G4^{-}$ (non-autoreactive) and $9G4^{+}$ (autoreactive) naïve B cells and found that autoreactive B cells had lower levels of DNA methylation than their non-autoreactive counterparts (**Fig. 6F**). Overall, our results suggest that the demethylation in naïve B cells of HIGM2 patients compared with control

donors is associated with an expansion of pre-activated autoreactive naïve B cells as a consequence of central B cell tolerance impairment mediated by AID deficiency.

Figure 6

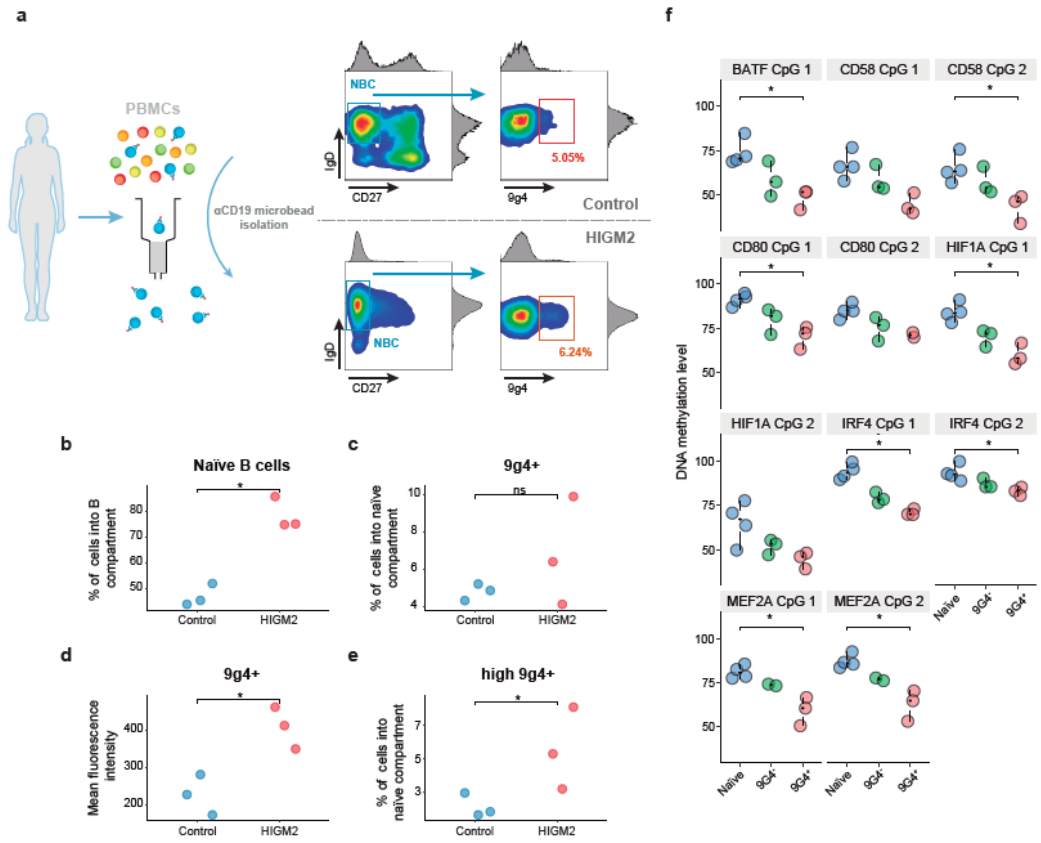

Figure 6: Central B cell tolerance impairment by AID deficiency. *(**a**) A representative example of a strategy for sorting naïve autoreactive B cells. (**b**) Dot plot showing the percentage of naïve B cells into B cell compartment in HIGM2 patients and controls. (**c**) Dot plot showing the percentage of autoreactive naïve B cells into naïve B cell compartment in HIGM2 patients and controls. (**d**) Dot plot showing the mean fluorescence of 9g4$^+$ gate. (**e**) Dot plot showing the percentage of high 9g4$^+$ naïve B cells into naïve B cell compartment in HIGM2 patients and controls. (**f**) Dot plot showing the DNA methylation values determined by pyrosequencing of control naïve B cells (naïve), HIGM2 patient 9g4$^-$ naïve B cells (9g4$^-$) and HIGM2 patient 9g4$^+$ naïve B cells (9g4$^+$).*

Discussion

Our results show that AID deficiency in HIGM2 syndrome results in the acquisition of aberrant DNA methylation profiles in naïve and memory B cells. Two major conclusions emerge from the study of this phenomenon. First, the analysis of the HIGM2-associated alterations occurring in the transition from naïve to memory B cell rules out the direct involvement of AID in active demethylation. Second, the comparison of naïve B cells in HIGM2 and healthy controls shows premature demethylation of genes downstream of the BCR in AID-deficient individuals, which is associated with the expansion of autoreactive B cell clones, prior to the germinal center reaction. This reinforces a novel role for AID in preventing the expansion of autoreactive B cell clones, affecting the DNA methylation profiles of naïve B cells.

Our study unequivocally demonstrates that AID does not play a direct role through its catalytic activity in mediating active demethylation in the transition from naïve to memory B cells. This transition is that associated with the highest proportion of DNA methylation changes of the entire B cell differentiation process (Kulis et al., 2015; Oakes et al., 2016) and also coincides with the highest peak of AID expression. Previous studies addressing the potential participation of AID in demethylation had not considered late-replicating domains or the relationship between DNA methylation changes and the genomic features of AID targets. In our study, we determined that most of the changes taking place during the transition from naïve to memory B cells occur through passive demethylation. It is possible that memory B cells of healthy controls undergo more divisions than the memory cells of HIGM2 patients, similarly to what has been described for AID-/- mice (Zaheen et al., 2009). This could explain the partial impairment of passive demethylation in HIGM2 patients. For active changes, no associations with AID targets were found. These findings are also in line with those by Álvarez-Prado and colleagues (Álvarez-Prado et al., 2018), who have indicated that AID-mediated mutation frequencies are too low. Such low frequency would be unlikely to produce a perceptible effect at the level of DNA methylation. In relation to the results obtained by Dominguez and colleagues (Dominguez et al., 2015), it is remarkable that even without the removal of PMDs in their analysis, the overlap

between the DNA methylation changes and AID-associated dsDNA breaks was very small, and our analysis suggests that TET proteins are involved in the observed DNA demethylation.

A second major conclusion of our study concerns the identification of DNA methylation defects in naïve B cells from HIGM2 patients in relation to healthy controls. Customarily, AID expression has been regarded as being restricted to germinal center B cells, but some evidence suggests that AID may also have a role in central B cell tolerance (Cantaert et al., 2015; Kuraoka et al., 2011; Meyers et al., 2011). In keeping with this, during B cell development, these cells not only become activated in the germinal center but also in previous stages of differentiation in the bone marrow. In that location, self-reactive immature B cells are activated in a process characterized by the upregulation of both AID and recombination-activating gene 2 (RAG2) and the downregulation of the anti-apoptotic MCL-1 (Nemazee, 2006). In this context, AID activity increases the probability of genomic damage with the subsequent activation of apoptosis through p53, which is also enhanced by the inhibition of the anti-apoptotic proteins BCL2 and MCL-1 (Cantaert et al., 2015). At that point, self-reactive immature B cells that are unable to correct their affinity for self-antigens by receptor editing are eliminated. In patients with AID deficiency, this mechanism of cell removal is impaired and autoreactive cells accumulate (Cantaert et al., 2015). Indeed, we noted that HIGM2 patients accumulated more autoreactive B cells than healthy donors, a finding that is compatible with the previously described high frequency of autoimmune disorders in this type of patient (Quartier et al., 2004). The failure in AID function in these patients could be responsible for the smaller degree of genomic damage that promotes the expansion of autoreactive naïve B cells. These self-reactive B cells, owing to the persistent activation of their BCR during negative selection in the bone marrow, display a more demethylated profile in genes downstream of the BCR compared with non-autoreactive naïve B cells. Our results therefore indicate that the enhanced demethylation of BCR downstream targets in HIGM2 naïve B cells may be the result of the expansion of autoreactive B cell clones as a consequence of the absence of AID.

Material and Methods

Human samples. Patients who fulfilled the diagnostic criteria for hyper-IgM syndrome type 2 were included in the study based on ESID clinical diagnostic criteria (Seidel et al., 2019) and genetic confirmation of *AICDA* mutation and exclusion of other primary and secondary causes of immunodeficiencies. Samples come from the Medical Center of the University Hospital, University of Freiburg, Freiburg, Germany and Hospital Universitari Vall d'Hebron, Barcelona, Spain. The Committees for Human Subjects of the local hospitals approved the study, which was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. All samples were in compliance with the guidelines approved by the local ethics committee and all donors (and/or their parents) received oral and written information about the possibility that their blood would be used for research purposes.

Isolation of B cell populations. Peripheral blood mononuclear cells (PBMCs) were obtained from blood. After Ficoll-Isopaque density centrifugation (Rafer, Zaragoza, Spain), collected cells were washed twice with ice-cold PBS, followed by centrifugation at 2000 rpm for 5 min. Next, cells were labeled with antibodies to CD19 – FITC (Miltenyi Biotec, clone LT19), CD27 – APC (Miltenyi Biotec, clone M-T271), IgD – PE (SouthernBiotech, Cat. No. 2032-09) and IgM – PerCP/Cy5.5 (BioLegend, clone MHM-88) for 20 min on ice in staining buffer (PBS with 4% FBS and 2 mM EDTA). Naïve B cells ($CD19^+$ $CD27^-$ IgD^+) and unswitched memory B cells ($CD19^+$ $CD27^+$ IgD^+) were obtained by FACS sorting on a MoFlo Astrios (Beckman Coulter). Purified samples were pelleted and stored at −80°C.

For isolation of naïve autoreactive B cells. Total B cells were isolated from PBMCs using positive selection with MACS CD19 microbeads (Miltenyi Biotec). Next, cells were stained with CD27-APC (Miltenyi Biotec, clone M-T271), IgD – PE (SouthernBiotech, Cat. No. 2032-09), HLA-DR – PE-Cy7 (eBioscience, clone LN3), 9g4 primary ab (igm Bioscience) and donkey ant-rat IgG (H + L) – Alexa Fluor 488 (invitrogen). 9g4+ naïve B cells ($CD27^-$ IgD^+ $9g4^+$) and 9g4- naïve B cells ($CD27^-$ IgD^+ $9g4^-$) were obtained by FACS sorting on a

BD FACSAria II (BD Biosciences). Purified samples were pelleted and stored at −80°C.

Genomic DNA extraction. For whole-genome bisulfite sequencing, DNA was extracted with a QIAamp DNA micro kit (Qiagen) according to the manufacturer's protocol. For pyrosequencing experiments, DNA was extracted with a Maxwell RSC Cultured Cells DNA kit (Promega).

Tagmentation-based whole-genome bisulfite sequencing. For whole-genome bisulfite sequencing, 30 ng of genomic DNA was used to produce four independent barcoded sequencing libraries per DNA sample using the tagmentation method (Wang et al., 2013). Sequencing of the TWGBS libraries was done on a HiSeq 2000, PE 125 bp mode. Bisulfite sequencing reads were processed by the DKFZ bisulfite analysis workflow. In brief, the reads were trimmed using Trimmomatic, pre-processed and aligned using MethylCTools, with default parameters (V. Hovestadt, S. Picelli, B. Radlwimmer, M.Z. and P.L., unpublished data), which uses the Burrows-Wheeler alignment algorithm (Li & Durbin, 2010). Following quality control of bisulfite conversion (>99.5% in all samples) and of read-mapping (80-90% could be mapped on average), we performed methylation calling using methylCtools. A summary of the sequencing data for each sample is provided in Table S1

DMR calling. Differentially methylated regions were detected with the DeNovoDMR algorithm included in the Specific Methylation Analysis and Report Tool (SMART2) (Liu et al., 2016) using all the default parameters except for the segment CpG number threshold, which was set to 4, the absolute mean methylation difference, which was set to 0.2, and a threshold value of *P* of 0.01. Only those CpGs with a coverage of ≥5 in all samples were considered in the construct of the SMART input matrix. DMR calling was performed or all possible comparisons between naïve and memory B cells for both control and HIGM2 patients.

Bisulfite pyrosequencing. 500 ng of genomic DNA was converted with an EZ DNA Methylation-Gold kit (Zymo Research), following the manufacturer's instructions. Bisulfite-treated DNA was PCR amplified using primers (see Table S2) designed with PyroMark Assay Design 2.0 software (Qiagen). Finally, PCR

amplicons were pyrosequenced with the PyroMark Q24 system and analyzed with PyroMark CpG software (Qiagen).

ChIP-seq data processing. Sequencing reads from ChIP-seq experiments from the BLUEPRINT consortium (Kulis et al., 2015) were mapped to the hg19 assembly of human reference genome using Burrows-Wheeler Aligner (BWA) v0.7.13 (with parameters -q 5, -l 32, -k 2). After removing reads with MAPQ < 30 with Sequence Alignment/Map (SAMtools) v1.2, PCR duplicates were eliminated using the Picard function available in MarkDuplicates software v1.126. Peak calling was performed using macs2 (with parameters -p 1e-2 --nomodel --shift 0 -B --SPMR). Only peaks with an overlap of ≥ 0.5 between replicates were considered. Histone mark signals around DMR sets were extracted with the annotatePeaks.pl algorithm available in Hypergeometric Optimization of Motif EnRichment (HOMER) software v4.10.3 (with parameters: size = 10000, hist = 10).

Super-enhancer identification. H3K27ac ChIP-seq data were used to identify the super-enhancer regions, as described previously (Whyte et al., 2013) using Rank-Ordering of Super-Enhancers (ROSE) software. An enhancer stitching distance of 15 kb was used along with a 2.5 kb transcriptional start site (TSS)-exclusion window.

Data analysis. Hierarchical clustering was carried out based on Pearson correlation distance metrics and average linkage criteria. For low-dimensional analysis we used the t-distributed stochastic neighbor embedding (t-SNE) method implemented in the Rtsne v0.15 package.

Transcription factor motifs were enriched for each set of DMRs using HOMER software v4.10.3. Specifically, we used the findMotifsGenome.pl algorithm (with parameters -size given -cpg) to search for significant enrichment against a background sequence adjusted to have similar CpG and GC contents.

Transcription factor binding analysis was performed interrogating the overlap between the different sets of DMRs with ChIP-seq data for transcription factors available for GM12878 cell line from the ENCODE Project (Feingold et al., 2004). The enrichment factor was calculated against random regions as a

background, and *P* values were calculated using Fisher's exact test. Finally, the transcription factors downstream of the BCR signaling pathway were manually annotated from a curated database (Kandasamy et al., 2010).

Chromatin states and histone mark enrichments analysis for NBC, germinal center B cells and ncsMBC were assessed using a custom adaptation of the EpiAnnotator R package (Pageaud, Plass, & Assenov, 2018) using BLUEPRINT data (Kulis et al., 2015). DMRs were converted to hg38 assembly with the liftOver function in the rtracklayer v1.42 R package.

Replication timing data in the GM12878 lymphoblastoid cell line were obtained from the UW Repli-seq track of the UCSC Genome Browser. Replication timing values were binned in deciles to perform the overlap with the DMR groups.

DMR annotation for genetic context location was performed using the annotatePeaks.pl algorithm in the HOMER software v4.10.3. For determine the location relative to a CpG island (CGI), we used 'hg19_cogs' annotation in the annotatr v1.8 R package.

GREAT software (McLean et al., 2010) was used to enrich downstream pathways and gene ontologies. We used the single nearest gene option for the association between genomic regions with genes.

All statistical analysis (excluding T-WGBS and ChIP-seq analyses) were done in R v3.5.1. Data distributions were tested for normality. Normal data were tested using two-tailed unpaired Student's t-tests; non-normal data were analyzed with the appropriate non-parametric statistical test. Levels of significance are indicated as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Non-significance ($P \geq 0.05$) was indicated as 'ns'.

Public RRBS of B cell activation. Data of EBV and CD40/IL-4 B cell activation were downloaded from the NCBI Gene Expression Omnibus (GSE49629) (Hansen et al., 2014). Methylation calls from RRBS data were filtered, so that only those CpGs with a minimum of five reads per position in all samples were retained. Since RRBS genomic coverage is significantly lower than T-WGBS we only tested the methylation status of positions common to two datasets.

EBV infection. For naïve B cell EBV infection experiments, we obtained buffy coats from anonymous donors through the Catalan Blood and Tissue Bank (CBTB). The CBTB follows the principles of the World Medical Association (WMA) Declaration of Helsinki. Before providing the first blood sample, all donors received detailed oral and written information and signed a consent form at the CBTB. PBMCs were isolated using Ficoll-Paque gradient centrifugation. Total B cells were isolated from PBMCs using positive selection with MACS CD19 microbeads (Miltenyi Biotec). Next, cells were stained with CD27-APC (Miltenyi Biotec, clone M-T271) and IgD – PE (SouthernBiotech, Cat. No. 2032-09) and naïve B cells were sorted as $CD27^{-}IgD^{+}$. Pure naïve B cells were incubated with B95-8 cell supernatant for 3 h at 37ºC in order to infect them with EBV. Finally, cells were collected after 30 days.

Data access: The data that support the findings of this study are available from the NCBI Gene Expression Omnibus (GEO). Code and data processing scripts are available from the corresponding author upon request.

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Author contributions

F.C.-M. designed and performed experimental experiments and bioinformatics analysis; F.C.-M., D.W. and C.P. generated TWGBS data; P.L. supervised some bioinformatics analysis; F.C.-M and A.F.Á.-P performed AID hot spot analysis; F.C.-M and J.R.-U. design sorting strategies; C.K., C.S, H.A., M.M.-G, R.D., P.S.-P., S.K., L.H., A.D., and B.D. contributed with clinical material and clinical interpretation of the results; E.B. conceived and supervised the study; F.C.-M. and E.B. wrote the manuscript; All authors participated in discussions and interpretation of the data and results.

Competing interests

The authors declare that they have no competing interests

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Supplementary Figures

Supplementary Fig. 1

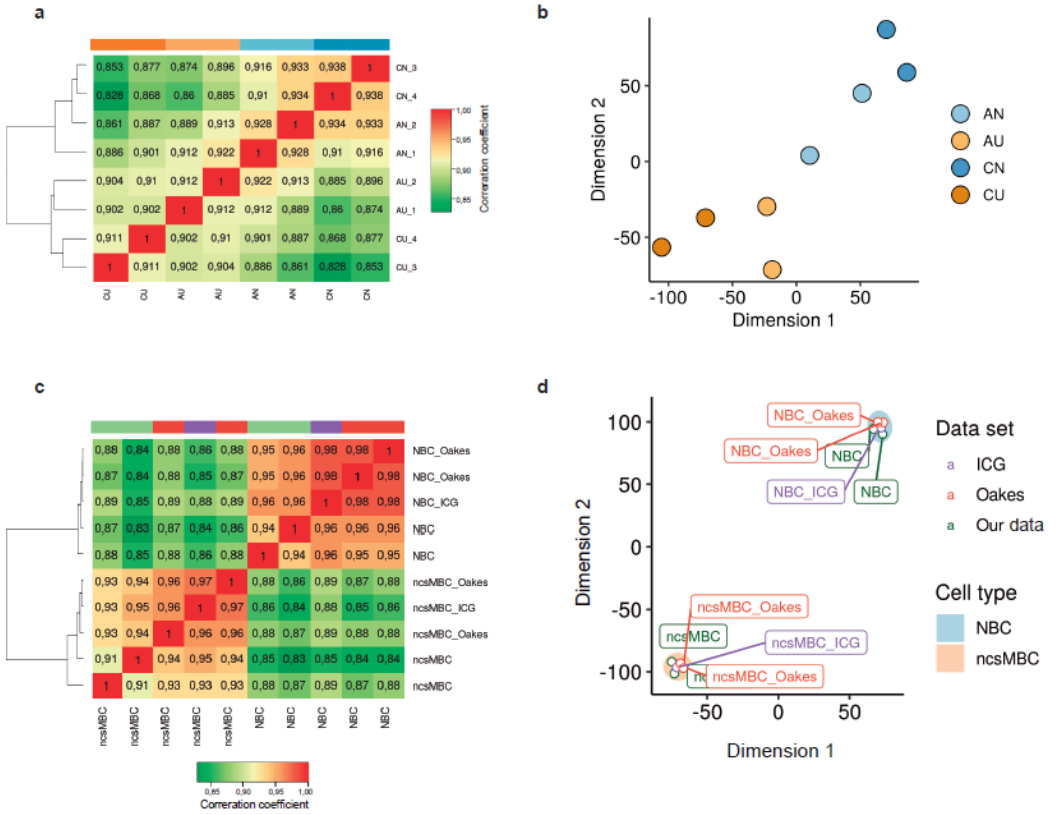

Figure S1: Reproducibility of DNA methylation data. *(**a**) Heatmap showing the Pearson correlation coefficient matrices of pair-wise comparisons using informative windows as described in Oakes et al 2016 of T-WGBS data for two biological replicates per cell subpopulation. Specifically, after TILLING the genome into 500-bp windows, only the methylation averages of those with > 4 CpGs were used (CN = control naïve B cells, CU = control unswitched memory B cells, AN = HIGM2 naïve B cells, AU = HIGM2 unswitched memory B cells). (**b**) t-SNE projection for two biological replicates per cell subpopulation. (**c**) Heatmap showing the Pearson correlation coefficient matrices using informative windows for our control data and previously published data (NBC = naïve B cells, ncsMBCs = non-class-switched B cells. Oakes refers to the Oakes et al 2016 data, ICG refers to International Cancer Genomic Consortium methylation data). (**d**) Unsupervised t-SNE projection comparing our T-WGBS control data with published datasets.*

Supplementary Fig. 2

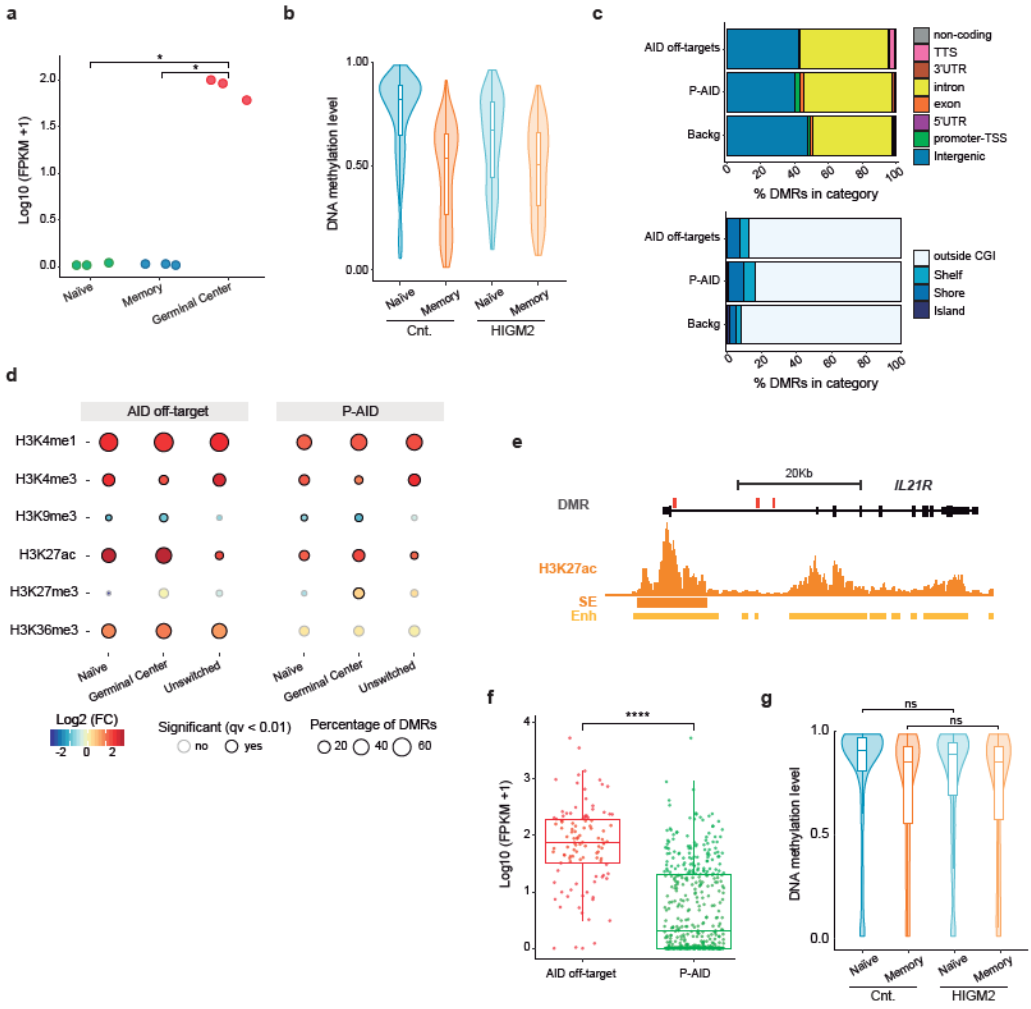

Figure S2: Indirect involvement of AID in DNA demethylation dynamics during B cell activation. *(**a**) Box and dot plots showing the distribution of expression values of the AICD gene in naïve, memory and germinal center B cells. (**b**) Box and violin plots summarizing the distribution of DNA methylation levels per sample group of AID off-target DMRs (DMRs associated with genes described as AID off-target). (**c**) Location proportions of P-AID, AID off-target DMRs in the context of CpG islands (CGI) and gene-related regions. (**d**) Bubble chart depicting the enrichment (red) and depletion (blue) of significant peaks of histone mark. The dot fill represents the logarithmic-fold change, dot size indicates the percentage of DMRs in the histone mark peaks, and the edge indicates the statistical significance of the enrichment (black: significant, none: not significant; q < 0.01). (**e**) DNA methylation and H3K27ac profiles in the vicinity of representative genes. DMR color indicates the type: P-AID (green) and AID off-target DMRs (red). At the bottom, the H3K27ac ChIP-seq signal in germinal center B cells is shown in dark orange; identified super-enhancers (SE) are depicted below with dark orange bars. Enhancer (Enh) regions are represented by light orange bars. (**f**) Box and dot plots showing the distribution of expression values of P-AID and AID off-target DMR-associated genes. One-sided unpaired Wilcoxon's test was used to test for differences in expression. (**g**) Box and violin plots summarizing the distribution of DNA methylation levels per sample group of CpG sites within the WRCY context at super-enhancers of AID off-target genes. Statistical tests: two-tailed Wilcoxon's (a, f, g) and Fisher's exact (d) tests. (FPKM= fragments per kilobase of transcript per million mapped reads).*

Supplementary Fig. 3

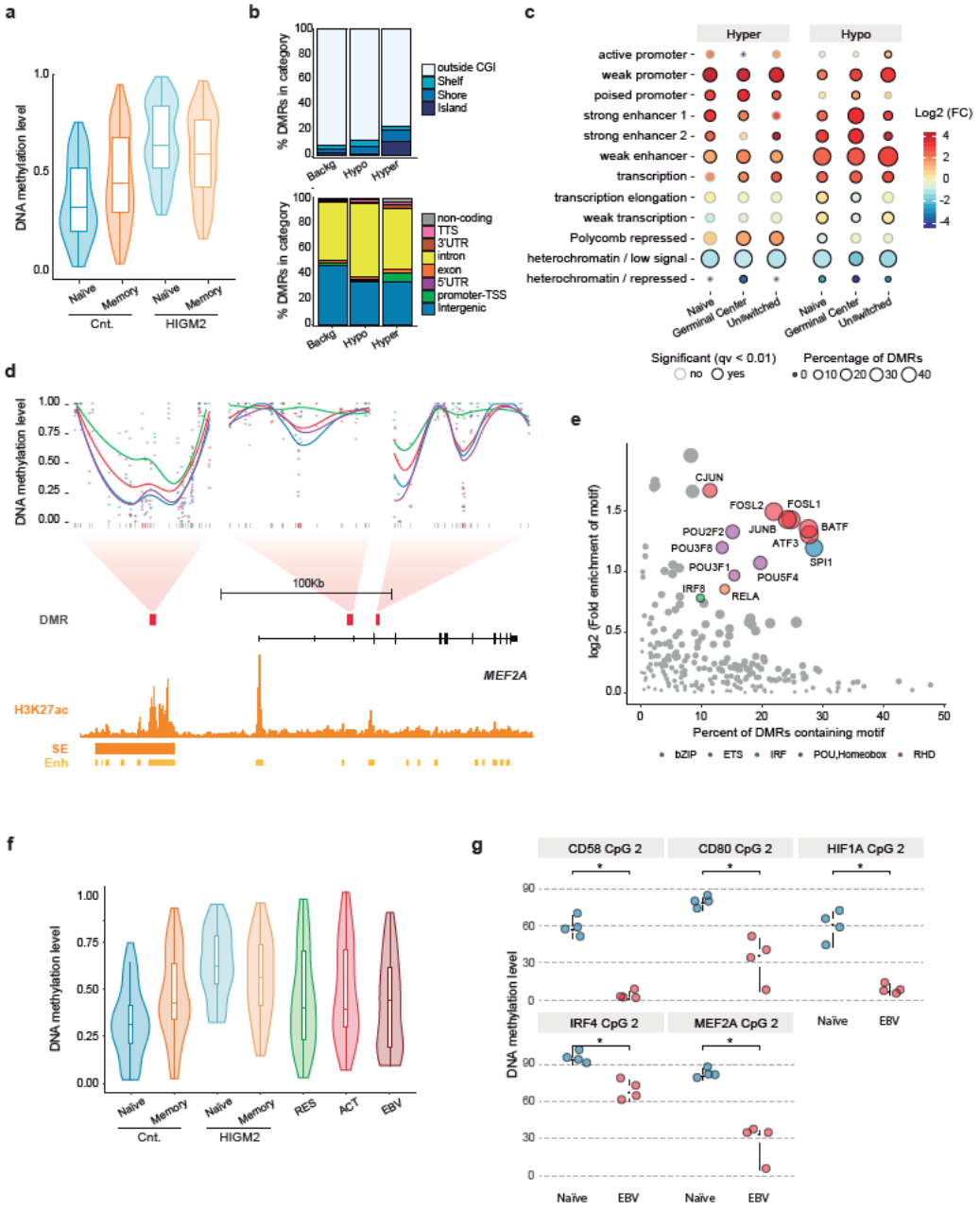

Figure S3: Role of AID in early B cell development. *(**a**) Box and violin plots summarizing the distribution of DNA methylation levels per sample group of differentially hypermethylated regions between control and patient naïve B cells. (**b**) Location proportions of DMRs between controls and patient naïve B cells in the context of CpG islands (CGIs) and gene-related regions. (**c**) Bubble chart depicting the enrichment (red) or depletion (blue) of chromatin states. The dot fill represents the logarithmic-fold change, dot size indicates the percentage of DMRs in the chromatin state, and the edge indicates the statistical significance of the enrichment (black: significant, none: not significant; q < 0.01). (**d**) Smoothed DNA methylation data of MEF2A-associated altered DMRs and H3K27ac ChIP-seq signal in germinal center B cells. (**e**) Bubble scatter plot of transcription factor motif enrichment in altered DMRs. Only transcription factors downstream of BCR signaling are colored according to the transcription factor family.*

*Bubble size corresponds to the logarithm of adjusted values of P. (**f**) Box and violin plots summarizing the distribution of DNA hypermethylation levels of altered DMRs in resting B cells (RES), B cells activated with CD40L/IL-4 (ACT) and B cells infected with Epstein-Barr virus (EBV). (**g**) Dot plot showing the DNA methylation values determined by pyrosequencing naïve B cells (naïve B cells) and EBV-infected naïve B cells (EBV). Statistical tests: two-tailed Fisher's exact (c) and two-tailed Wilcoxon's (g) tests.*

Supplementary Fig. 4

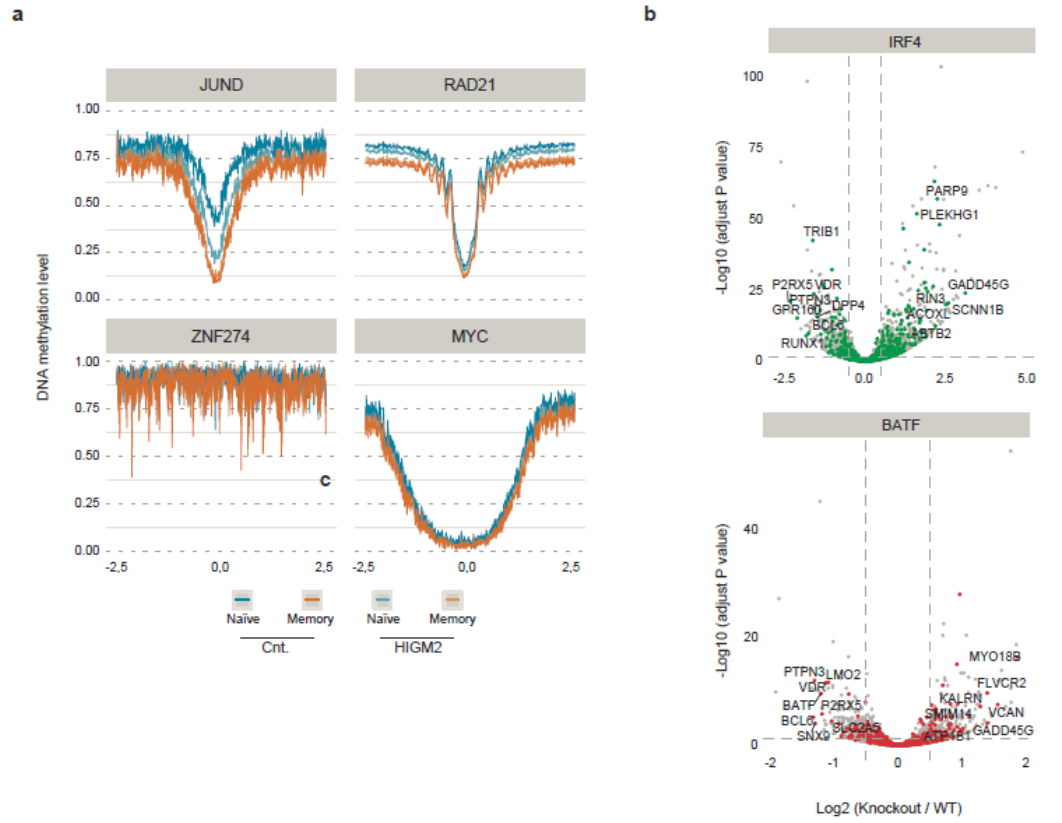

Figure S4: BATF / IRF4 complex-mediated part of methylation events. *(**a**) Composite CpG methylation levels surrounding transcription factor ChIP-seq peaks (2.5 kb). (**b**) Volcano plot showing the effect of the knockout of IRF4 or BATF at the transcriptional level of genes with co-binding of IRF4 and BATF. Dot colors indicates whether the gene has an altered DMR.*

ARTICLE 2

Title

Interplay between the vitamin D receptor and STAT3 in TET2-mediated DNA demethylation in the acquisition of tolerogenesis

Authors

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Abstract

The active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25(OH)2D3), induces stable tolerogenesis in dendritic cells (DC). This process involves the vitamin D receptor (VDR) which translocates to the nucleus, binds its cognate genomic sites and promotes epigenetic and transcriptional remodeling. In this study, we investigated the interplay between the vitamin D receptor (VDR) and transcription factors to induce DNA methylation changes, which might provide phenotypic stability to the tolerogenic phenotype of DCs. Our study reveals the occurrence of vitamin D-specific DNA demethylation and transcriptional activation at VDR binding sites associated with the acquisition of tolerogenesis. Tolerogenic properties in DCs are acquired together with activation of the IL-6/JAK/STAT3 pathway. In fact, VDR directly binds the IL-6 gene and JAK2-mediated STAT3 phosphorylation is specific to vitamin D-stimulation. VDR and the phosphorylated form of STAT3 interact with each other following vitamin D treatment. Interestingly, both VDR and phospho-STAT3 interact with methylcytosine dioxygenase TET2. Most importantly, pharmacological

inhibition of STAT3 phosphorylation reverts the vitamin–induced tolerogenic properties of DCs. Our results uncover an interplay between VDR and STAT3 for the acquisition of a DNA demethylation-dependent induction of tolerogenesis by vitamin D.

Introduction

Myeloid cells are not only responsible for innate responses but also participate in initiating adaptive responses. The immunological properties of myeloid cells vary depending on the environment. In fact, terminal myeloid cell differentiation is highly dependent on the activation of specific signaling pathways in response to extracellular signals, such as inflammatory cytokines, hormones, vitamins and other factors (Álvarez-Errico, Vento-Tormo, Sieweke, & Ballestar, 2015), which determine the immunogenicity of the resulting myeloid cells. The activation of signaling pathways leads to the activation of specific sets of transcription factors (TFs). Sequence-specific DNA binding of TFs is a pivotal process for the establishment of gene expression patterns in concert with the epigenetic machinery that determines cell identity and function (Monticelli & Natoli, 2017). Recent evidence has shown that pioneer TFs are associated with DNA demethylation and increased genomic accessibility of its binding regions facilitating the binding of subsequent TFs (Rasmussen et al., 2019). In this regard, methylcytosine dioxygenase ten-eleven translocation (TET) 2, the most relevant TET enzyme in the myeloid compartment can interact with multiple pioneer TFs such as PU.1, C/EBPα, KLF4 and others, which target it to different genomic regions (Costa et al., 2013; de la Rica et al., 2013; Guilhamon et al., 2013; Lio et al., 2016; Sardina et al., 2018; Wang et al., 2015; Xiong et al., 2016). Recently, it has been demonstrated that TET2 mutations, frequent in myeloid leukemias, result in enhancer DNA hypermethylation and changes in the subsequent binding of TFs, particularly member of the basic helix-loop-helix (bHLH) TF family. This suggests that TET2 recruitment by pioneer TFs leads to an epigenetic remodeling facilitating the binding of other TFs (Rasmussen et al., 2019). However, the mechanisms by which this process takes place and how the acquisition and stabilization of gene expression patterns in primary cell differentiation occurs is poorly studied.

Calcitriol (1,25(OH)2D3), the active form of vitamin D3, is a major modulator of the immune system (Barragan, Good, & Kolls, 2015; Carlberg, 2019; Mora, Iwata, & Von Andrian, 2008). Myeloid cells such as antigen-presenting dendritic cells (DCs) are the most susceptible target to vitamin D in a mixed immune population (Mora et al., 2008). In these cells, calcitriol can generate *in vitro* a stable maturation-resistant tolerogenic phenotype, with low expression of immunogenic molecules such as HLA-DR, CD80 or CD86, and increased interleukin 10 (IL-10)/IL-12p70 ratios that are maintained even after removal of the compound (Van Halteren et al., 2002). After ligand recognition, vitamin D receptor (VDR) translocates to the nucleus and acts as a TF controlling the expression of a set of immune and metabolic genes (Carlberg, 2019; Ferreira, Overbergh, Verstuyf, & Mathieu, 2013), but also act as a k-light-chain-enhancer of activated B cells (NF-kB) repressor at different levels (Carlberg, 2019; Fetahu, Höbaus, & Kállay, 2014). Several studies have shown the capacity of VDR to interact with different TFs including PU.1, GABPA, but also with chromatin remodeling factors and histone modification enzymes such as BRD7 and KDM6B (Pereira et al., 2011; Seuter, Neme, & Carlberg, 2017, 2018; Wei et al., 2018). Some evidences suggest a potential role of vitamin D inducing DNA methylation alterations (Doig et al., 2013; O'Brien et al., 2018). However, the molecular mechanism that leads to the acquisition of differential methylation patterns and how autocrine activation of secondary TFs can participate in this process remains unexplored.

Vitamin D supplementation is generally used to prevent or as a co-adjuvant of diseases with underlying autoimmune or pro-inflammatory states (Bscheider & Butcher, 2016; Dankers, Colin, van Hamburg, & Lubberts, 2017). As initiators of immune responses, DCs represent an excellent target of vitamin D to dampen autoimmunity and inflammation, however, the role of DC in vitamin D-mediated immunomodulation is not fully understood. The stability of the tolerogenic phenotype suggests the participation of regulatory mechanisms that allow the maintenance of stable changes of gene expression. In this sense, DNA methylation is a major epigenetic modification mainly involved in the acquisition or stable transcriptional states (Luo, Hajkova, & Ecker, 2018).

In this study, we investigated the involvement of VDR in the acquisition of DNA methylation changes in relation with the acquisition of tolerogenic properties during DC differentiation in the presence of vitamin D. We found that a specific DNA methylation signature that is dependent on VDR its direct physical interaction with TET2 and involves the activation of the IL-6/JAK3/STAT3 pathway. Our study demonstrates an interplay between VDR and STAT3 in the generation of a TET2-dependent DNA methylation signature that stabilizes tolerogenic properties of DCs in the presence of vitamin D.

Results

Vitamin D3 induces the acquisition of a specific DNA methylation profile associated with tolerogenesis during DC differentiation

To investigate the participation of VDR, and its potential interplay with other TFs, in targeting functional DNA methylation changes involved in the acquisition of tolerance, we differentiated *in vitro* monocytes (MOs) to dendritic cells (DCs) for 5 days using GM-CSF and IL-4 in the absence and presence of vitamin D3 (Figure 1A). As previously described (Penna & Adorini, 2000; Piemonti et al., 2000), DCs differentiated in presence of vitamin D3 (tolerogenic DCs, noted as TLs from here on) had higher levels of surface markers CD14 and CD11b and lower levels of HLA-DR, CD1a and CD86 in comparison with DCs (Supplementary Figure 1A). In addition, $CD8^+$ T cell proliferation assays in coculture with both DCs and TLs, confirmed the immunosuppressive properties of the latter (Supplementary Figure 1B). We also determined that under these conditions vitamin D3 induces the translocation of the VDR to the nucleus (Supplementary Figure 1C).

We then performed DNA methylation profiling of MOs and the resulting DCs and TLs. Principal component analysis (PCA) showed that, although most of the variability observed at the methylation level is explained by common events in both differentiation processes, the second principal component is capable of clustering separately DCs and TLs (Figure 1B). Differentiation mainly resulted in DNA demethylation, although gains of methylation did also occur (Supplementary Figure 2A), where both common and condition-specific

Figure 1

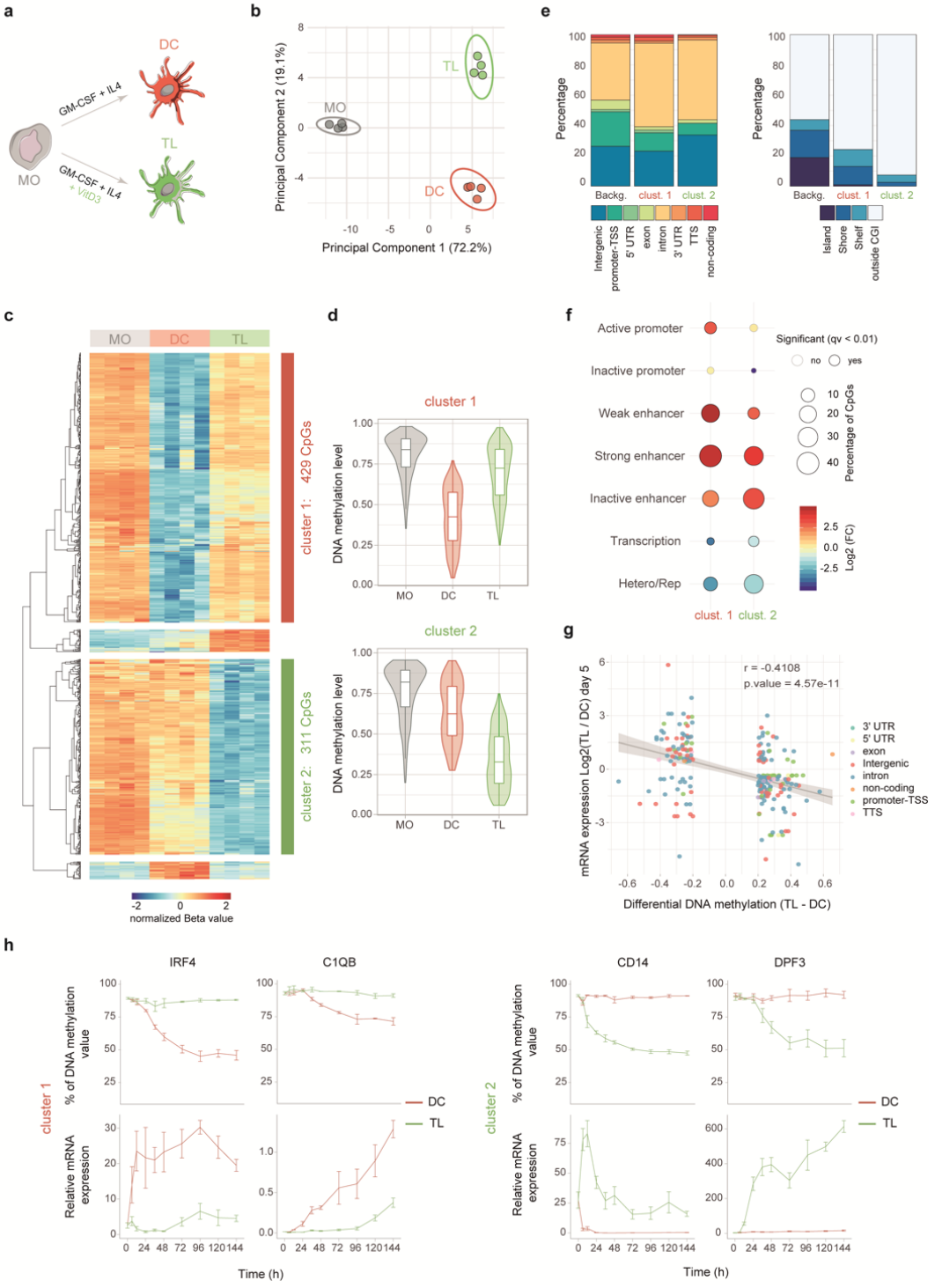

Figure 1. DNA methylation dynamics throughout vitamin D exposed dendritic cells differentiation. *(**a**) Schematic overview of differentiation model. (**b**) Principal component analysis of differentially methylated CpGs. (**c**) DNA methylation heatmap and cluster analysis of four paired samples of MOs and their derived DCs and TLs at day 5 of differentiation. The heatmap includes all CpG-containing probes displaying*

*significant methylation changes (∆ beta value >= 0.2 & q value < 0.05) in only TL-DC comparison. The color annotation marks the membership to cluster 1 (DC-specific DNA demethylation) or cluster 2 (TL-specific DNA demethylation). (**d**) Box and violin plots summarizing the distribution of DNA methylation levels per cell type of cluster 1 (top) and cluster 2 (bottom). (**e**) Location proportions of CpGs of cluster 1 and cluster 2 in the context of CpG islands (CGIs) (right) and gene-related regions (left). (**f**) Bubble chart depicting the enrichment (red) or depletion (blue) of chromatin states on dendritic cells. The dot fill represents the logarithmic-fold change, dot size indicates the percentage of CpGs in the chromatin state, and the edge indicates the statistical significance of the enrichment (black: significant, none: not significant; q < 0.01). (**g**) Scatter plot showing the correlation between DNA methylation and gene expression (at day 5 of differentiation). Only differentially methylated CpGs are represented. Dot color refers to gene-related associations. (**h**) DNA methylation (top) and mRNA expression (bottom) kinetics of two representative examples of cluster 1 and cluster 2. Statistical tests: two-tailed Fisher's exact test (f) and Pearson correlation (g).*

DNA methylation were observed. Hierarchical clustering of differential methylated CpGs between DCs and TLs (adjusted P value < 0.05 & absolute Δ Bvalue ≥ 0.2) revealed four main groups of CpG sites: a group of CpGs that undergo specific demethylation in DCs (cluster 1, 429 CpGs); a second group that specifically demethylate in TLs (cluster 2, 311 CpGs); and two small groups with DC- and TL-specific gains in DNA methylation (Figure 1C, D). Given the low number of CpGs in the hypermethylation clusters, we decided to focus our subsequent analyses on condition-specific demethylation events (i.e., on cluster 1 and 2), to investigate how vitamin D treatment leads to changes in the DNA methylome.

Functional Gene Ontology analysis revealed that CpGs in cluster 1 are associated with immunological categories such as defense and immune response, whereas those in cluster 2 have a higher enrichment in cell activation, cell-matrix adhesion and positive regulation of immune system process (Supplementary Figure 2B). In both clusters, the majority of changes occurred in introns and intergenic regions with underrepresentation of promoter-TSS regions. However, while cluster 1 presents a marked enrichment for intronic regions respect to the background, cluster 2 enriches in intergenic locations (Figure 1E, left). In accordance with this, CpG island annotation showed that CpGs of both clusters are outside CpG islands, which is more marked for the cluster 2 (Figure 1E, right). Next, we mapped the chromatin states of the CpG sites undergoing demethylation in the 2 clusters using

chromatin segmentation data corresponding to DCs (Pacis et al., 2015) (Figure 1F). We observed an enrichment in enhancer regions for the two clusters. Moreover, whereas cluster 1 (DC-specific demethylation) is enriched in weak and strong enhancers, cluster 2 (TL-specific demethylation) is more enriched in inactive enhancers in DCs, suggesting that these inactive regions in DCs are activated in TLs. To validate this hypothesis, we integrated our DNA methylation dataset with available expression data (Széles et al., 2009). The integration of the two datasets led to the identification of a significant inverse relationship between DNA methylation and mRNA expression at 12 h (r = -0.5926, *P value* = 4.90e-14) and 5 days of differentiation (r = -0.4108, *P value* = 4.57e-11) (Figure 1G and Supplementary Figure 2C). As expected, we validated that the genes associated with CpGs of cluster 2 located at DCs inactive enhancers presented higher expression levels in TLs (Supplementary Figure 2D).

Figure 2

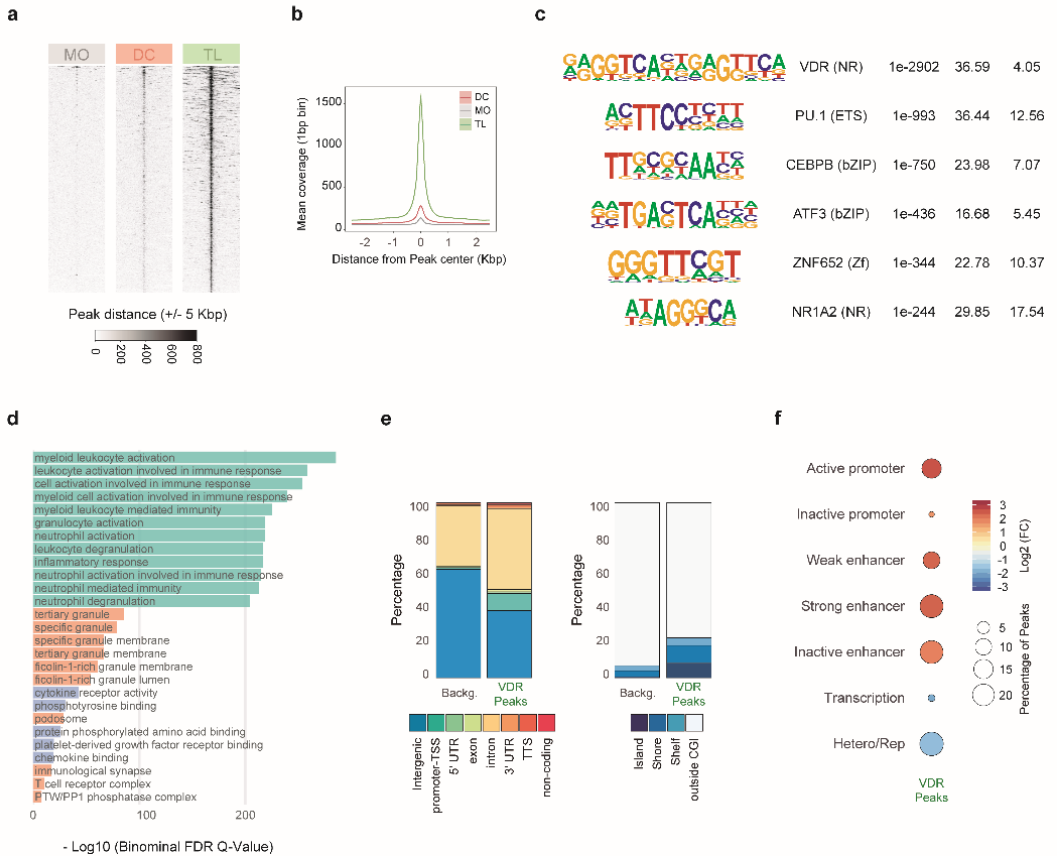

Figure 2. Genomic occupancy of vitamin D receptor. (a) *Heatmaps showing signal of vitamin D receptor (VDR) ChIP-seq at ± 2.5 Kbp window of VDR significant peaks.* ***(b)*** *Composite plots of VDR ChIP-seq distribution ± 2.5 Kbp around CpGs in MO (grey), DC (red) and TL (green) for VDR significant peaks. Smooth represents the confidence intervals (CI).* ***(c)*** *Motif discovery analysis using homer software showing qvalues, percentage of regions with motif and percentage of background sequences with motif.* ***(d)*** *Results of gene set enrichment analysis using GREAT software. The plot depicts the top enriched terms for biological process (green), molecular function (orange) and cellular component (purple) categories, based on adjusted P values from the binominal distribution.* ***(e)*** *Location proportions of CpGs of cluster 1 and cluster 2 in the context of CpG islands (CGIs) (right) and gene-related regions (left).* ***(f)*** *Bubble chart depicting the enrichment (red) or depletion (blue) of chromatin states on dendritic cells. The dot fill represents the logarithmic-fold change, dot size indicates the percentage of CpGs in the chromatin state, and the edge indicates the statistical significance of the enrichment (black: significant, none: not significant; q < 0.01).*

To explore the dynamics of the relationship between DC- (cluster 1) or TL-specific demethylation (cluster 2), we performed bisulfite pyrosequencing and qRT-PCR of a small selection of genes of a set of samples over time. Bisulfite pyrosequencing showed high concordance (r = 0.978, P value < 2.2e-16) with the data obtained with the EPIC arrays (Supplementary Figure 2E). These analyses not only confirmed the specificity of the changes but also their early occurrence and the concomitant changes between DNA methylation and gene expression (Figure 1H).

VDR binding associate with targeted DNA demethylation in TLs

We then explored the relationship between VDR and the observed DNA methylation changes in DC and TL differentiation. To this end, we performed a ChIP-seq experiment for VDR in our model. The analysis showed that exposure to vitamin D during DC differentiation leads to an increase in VDR genomic binding (Figure 2A,B). Interestingly, motif discovery analysis revealed promiscuity of VDR with respect to its genomic binding preferences, with only 37% of regions with the canonical VDR binding motif (Figure 2C). Functional annotation of associated genes an enrichment for immunological and signaling related categories (Figure 2D). In fact, a number of genes potentially related with the tolerogenic properties of TLs are direct targets of VDR. Global inspection of VDR genomic occupancy showed that VDR preferentially binds to promoters and introns in comparison with the background (Figure 2E, left). We also observed an enrichment of VDR binding in CpG islands, shores and

shelves, compatible with the enrichment observed for promoters (Figure 2E, right). Annotation with the DC chromatin states of VDR peaks confirmed preference of VDR to bind promoter regions, however it also showed enrichment in enhancer regions (Figure 2F).

Figure 3

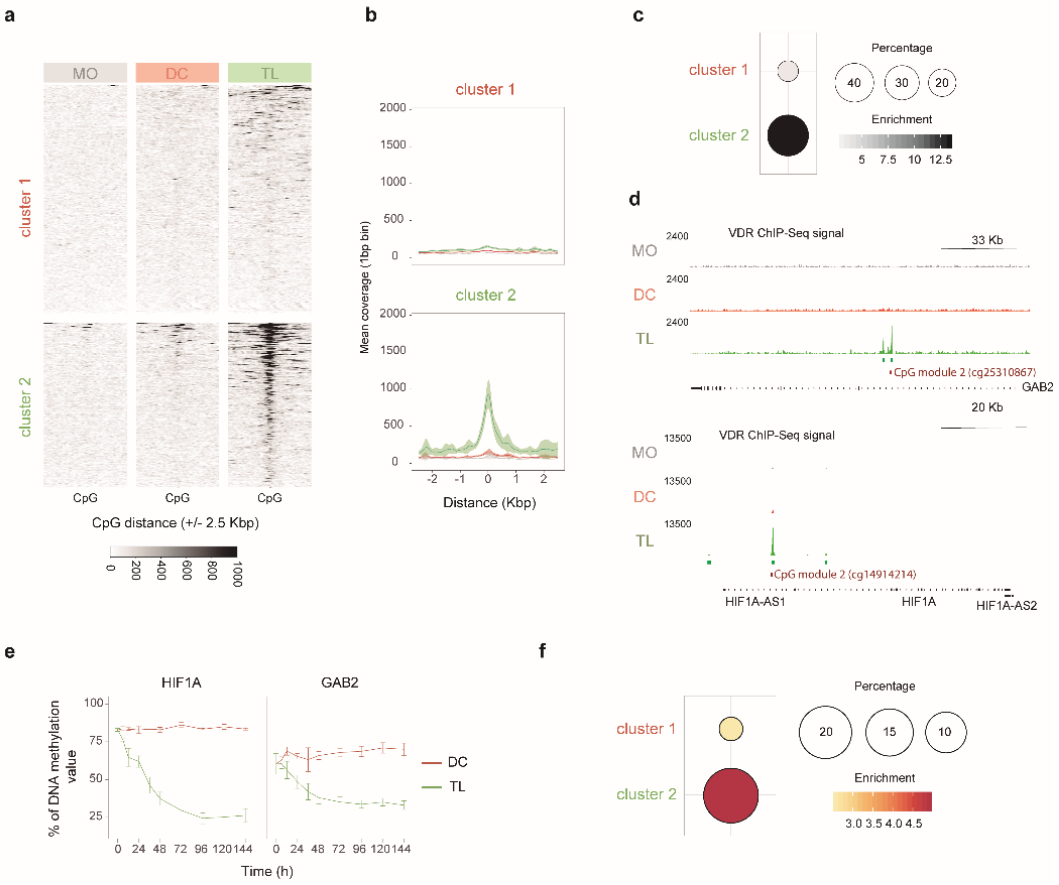

Figure 3. Binding of vitamin D receptor correlates with TL-specific DNA demethylation. (a) *Heatmaps showing signal of vitamin D receptor (VDR) ChIP-seq at ± 2.5 Kbp window of CpGs of cluster 1 (top) and cluster 2 (right). (b) Composite plots of VDR ChIP-seq distribution ± 2.5 Kbp around CpGs in MO (grey), DC (red) and TL (green) for cluster 1 (top) and cluster 2 (bottom). Smooth represents the confidence intervals (CI). (c) Bubble plot of VDR significant binding enrichment in each cluster of CpGs. Dots are colored according enrichment value. Bubble size correspond to percentage of CpGs overlapping with significant VDR peak for each cluster. Presence of black border indicates the significance of the enrichment (q value < 0.01). (d) VDR ChIP-seq signal profiles in the vicinity of the representative genes with CpGs of cluster 2. VDR signal are colored in function of the cell type. At the bottom, the significant VDR binding sites are shown in green. CpG position is depicted below in red. (**e**) DNA methylation kinetics of the CpGs represented in panel e.* ***(f)*** *Bubble plot of MO DNase*

significant binding enrichment in each cluster of CpGs. Dots are colored according enrichment value. Bubble size correspond to percentage of CpGs overlapping with significant VDR peak for each cluster. Presence of black border indicates the significance of the enrichment (q value < 0.01).

Integration of the DNA methylation and VDR ChIP-seq datasets revealed a specific association between VDR binding and TL-specific demethylation (cluster 2) (Figure 3A,B). In fact, we observed significant enrichment for VDR binding in cluster 2 with an overlap between the two data sets of more than 40% (Figure 3C). We obtained VDR ChIP-seq signal profiles for several representative genes with CpGs of cluster 2. In general, VDR peaks were found to be close to the CpGs that become demethylated in TLs (Figure 3D). Bisulfite pyrosequencing revealed kinetics associated with the active demethytation of these sites (Figure 3E).

Finally, we explored the possibility that VDR directly binds to closed chromatin, which may subsequently induce epigenetic remodeling, perhaps through the recruitment of TET2 or/and other epigenetic modifiers. To this end, we performed enrichment analysis comparing DNase-seq datasets from MOs and differentially methylated regions during differentiation to DCs and TLs. Our analysis confirmed that over 75% of TL-specific demethylated CpG sites occur in closed regions in MOs (Figure 3F), supporting a potential role for VDR as a pioneer TF.

VDR induces IL-6 upregulation in TLs leading to STAT3 activation and both form a complex with TET2.

Vitamin D3, through its receptor VDR induces changes in cytokine production and a profound metabolic reprograming (Ferreira et al., 2015). For this reason, we hypothesized that the autocrine activation of secondary signaling pathways during differentiation could lead to the activation of a set of TFs that can cooperate with VDR to mediate changes at the transcriptional and epigenetic levels. To explore this possibility, we adapted a tool initially designed to explore intercellular communication in both bulk and single-cell expression data to test autocrine signal activation (Browaeys, Saelens, & Saeys, 2019). With this approach, and using genes associated to both demethylation clusters with

significant expression differences (abs(log2(FC)) >= 1 & adjusted P value < 0.05) as an input, we inferred potential ligands that can explain those differences in expression (Figure 4A). Under these conditions, IL-6 stands out for its reported role in tumor immune suppression (Park et al., 2017). In fact, IL-6 is more expressed in TLs than DCs (Figure 4B) and such upregulation can interact with the major part of differential expressed target genes (Figure 4C).

As a validation, gene set enrichment analysis (GSEA) of differentially expressed genes also showed an enrichment for the IL-6/JAK/STAT3 signaling pathway (Figure 4D). In fact, VDR binds in several regions upstream of IL-6 gene suggesting that VDR directly regulates its expression (Figure 4E). To investigate whether autocrine IL-6 signaling induction leads to STAT3 activation, we tested its activity using DoRothEA (Discriminant Regulon Expression Analysis), a manually curated human regulon to estimate single sample TF activities (Garcia-Alonso, Holland, Ibrahim, Turei, & Saez-Rodriguez, 2019). We observed a specific increase for STAT3 activity in TLs after 5 days (Figure 4F). We then tested the phosphorylation levels of STAT3 in parallel with other STATs (STAT1 and STAT5) 3 d after differentiation in the absence or presence of vitamin D3. Interestingly, STAT3 showed specific phosphorylation in TLs and not in DCs. This behavior differed from the one observed for STAT1 and STAT5 which are phosphorylated in both conditions (Figure 3G). Thus, our results indicate that vitamin D induces an autocrine activation of IL-6 signaling with the subsequent STAT3 activation.

It is likely that the interplay between VDR and STAT3 involves a physical interaction. We therefore, performed co-immunoprecipitation experiments in TLs. Our analysis revealed a specific interaction between VDR and phospho-STAT3 (Figure 4H). We also explored the possibility that these factors interact with TET2, to target demethylation to their cognate sites. Interestingly, both VDR and STAT3 form a complex with TET2 in TLs (Figure 4I), supporting the notion that both factors act in a complex to induce the specific demethylation observed in TLs.

Figure 4

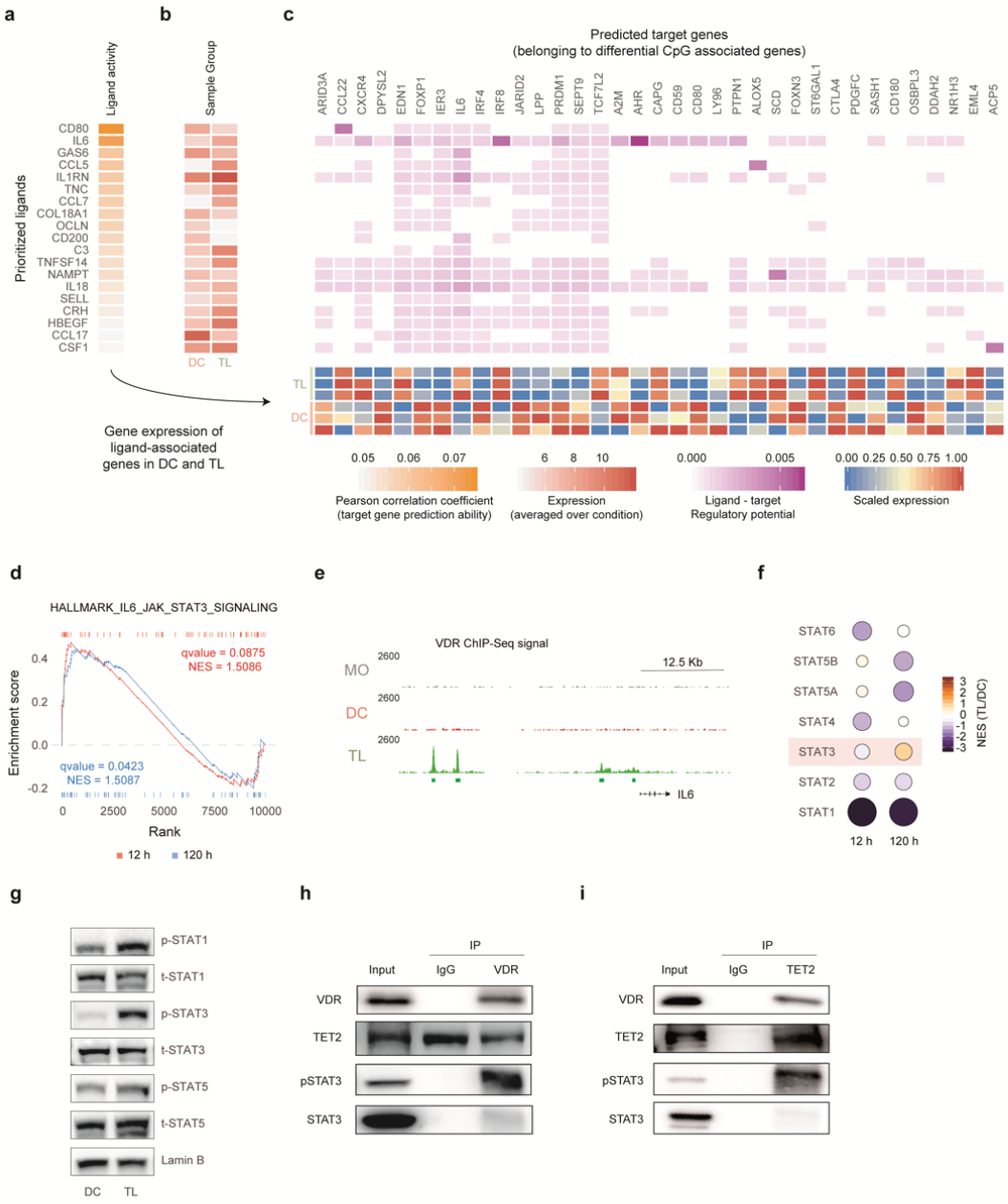

Figure 4. Vitamin D dependent autocrine activation of IL-6/JAK2/STAT3 pathway. *(**a**) Heatmap showing ligand activity based in the correlation with its target genes (**b**) Heatmap displaying averaged gene expression of ligands for DCs and TLs at day 5. (**c**) Heatmap showing the regulatory potential for each ligand on the target genes (up panel) and its expression levels in each sample (bottom). (**d**) Gene set enrichment analysis (GSEA) of differential expressed genes (abs(log2(FC)) >= 1 & q value < 0.05) at 12h (red) and 120h (blue). Results for IL-6/JAK/STAT signaling pathway are shown. (e) VDR ChIP-seq signal profiles in the vicinity of the IL-6 gene. VDR signal are colored in function of the cell type. At the bottom, the significant VDR binding sites are shown in green. (**f**) Schematic representation of IL-6/JAK2/STAT3 pathway. (f) Bubble chart depicting the TF activity predicted form mRNA expression with DoRothEA v2.0. The dot*

*fill represents the normalizes enrichment score (NES) (blue: more activity in DCs, red: more activity in TLs). Bubble size corresponds to the logarithm of adjusted P values. (**g**). WB showing the phosphorylated and total protein levels of STAT1, STAT3 and STAT5 at day 3 of differentiation process. (**h, i**) Co-immunoprecipitation assays were performed in MOs differentiated to TLs for 3 days. Protein extracts were immunoprecipitated utilizing anti-VDR, and anti-TET2 antibodies, in which IgG was used as a negative control and total protein extract was used as input.*

Inhibition of STAT3 activation impacts the acquisition of vitamin D-dependent tolerogenesis

We then investigated the consequences of inhibiting the IL-6/JAK/STAT3 pathway. To this end, we used TG101348, a pharmacological inhibitor for JAK2, during DC and vitamin D-dependent TL differentiation. Following TG101348 treatment, we confirmed the inhibition of STAT3 phosphorylation by western blot (Figure 5A). TG101348 treatment also resulted in a sharp decrease in the production of IL-10 (Figure 5B), an archetypical anti-inflammatory cytokine which is also a *bona fide* target for STAT3 (Schaefer et al., 2009; Ziegler-Heitbrock et al., 2003). We also tested the effects of JAK2 inhibition on surface markers. JAK2 inhibition resulted in an increase of CD14 and CD86 protein levels and downregulation of CD1a and CD11b (Figure 5C). In parallel, we investigated the effects of JAK2 inhibition on the DNA methylation and expression levels of TL-specific demethylated genes. Interestingly, we did not observe differences in DNA methylation (Figure 5D). In contrast, however, we observed alterations at the transcriptional level. Changes were observed not only in genes in cluster 2 (TL-specific) but also in genes in cluster 1. (Figure 5E). This is likely to be the result of the inhibition of phosphorylation of STAT1 and STAT5 which are also implicated in the activation other DC and TL genes.

Most importantly, JAK2 inhibition resulted in loss of the tolerogenic properties of DC differentiated in the presence of vitamin D. In fact, TL differentiated in the presence of TG101348 stimulated $CD8^+$ cell proliferation at similar extent than DCs, in contrast to TL in the absence of TG101348, reinforcing the coordinated activity of both VDR and the JAK2/STAT3 pathway in the acquisition of tolerogenic properties (Figure 5F).

Figure 5

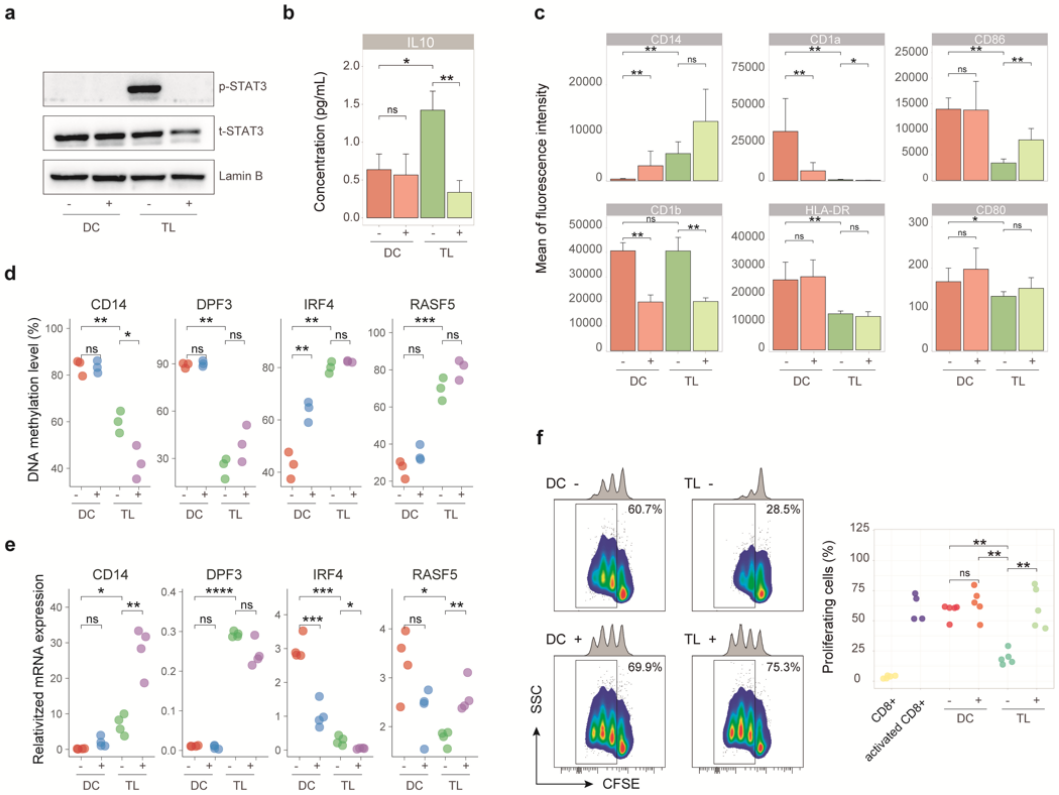

Figure 5: Phosphorylation inhibition of STAT3 reverts immunosuppressive proprieties of vitamin D exposed dendritic cells *(**a**) Effects at protein phosphorylation level of STAT3 after pharmacological inhibition of JAK2 with TG101348. (**b**) Effect of JAK2 inhibition on IL-10 released by DCs and TLs (n = 3). (**c**) Bar plots showing the impact of JAK2 inhibition on membrane receptor expression (n = 4). Protein levels are measured with flow cytometry. (**d**) Bisulphite pyrosequencing of four example CpGs displaying the consequence of JAK2 inhibition (n = 3). (**e**) mRNA expression of four example genes from cluster 1 and cluster 2, showing the effect of JAK2 inhibition (n = 4). Expression was relativized respect RPL38 gene expression. (**f**) Representative example and dot plot showing the effect on $CD8^+$ cell proliferation by DCs and TLs generated from MO in presence or absence of TG101348 (n = 5). Statistical tests: Two-tailed unpaired Wilcoxon's test (b-f).*

Discussion

In this study, we demonstrate that vitamin D is able to induce tolerogenesis in DCs through a mechanism that involves VDR-specific demethylation and activation of key immune genes in a coordinated manner with STAT3 activation. VDR is not only able to orchestrate a direct response on key immune targets but also to mediate a IL-6/JAK/STAT-mediated response. This not only involves the direct binding and activation of the *IL-6* gene but also the recruitment of TET2 by both VDR and the phosphorylated form of STAT3 to demethylate and activate genes. The essential role of the JAK2-STAT3 pathway in the acquisition of tolerogenesis is demonstrated by proving the functional impact of the pharmacological inhibition of this pathway.

Our results prove the direct role of VDR to direct TET2-mediated DNA demethylation to specific genomic sites during TL differentiation, i.e. in the presence of vitamin D3. We have shown that VDR forms a complex with TET2 promoting TL-specific demethylation that are associated with closed chromatin regions in MOs. These two findings support the role of VDR as a pioneer TF and is supported by the chromatin remodeling. This finding argues against a previous work done on the human monocytic THP-1 cells, where the authors found that VDR only binds to open regions before vitamin D3 exposure. However, it is easy to envision that the accessibility profile of immortalized cells could be substantially different from that in normal cells. In addition, the described interactions of VDR with different chromatin remodelers (Nurminen, Neme, Seuter, & Carlberg, 2018, 2019; Pereira et al., 2011; Wei et al., 2018) supports the potential role of VDR as a pioneer factor.

A recurrent question in the DNA methylation field is whether DNA methylation is causally involved in shaping gene expression profiles, rather than passively reflecting transcriptional states (Schübeler, 2015). Data support both possibilities and some DNA methylation changes appear to be more causative of subsequent changes than others. In our study, we present data in support of a role of TET2-mediated demethylation as a mechanism facilitating subsequent participation of other TFs, in this case STAT3. In fact, the absence of interference with DNA demethylation, while activation is impeded following

pharmacological inhibition of STAT3 phosphorylation, suggests that VDR-dependent demethylation is necessary and precedes STAT3-mediated activation of gene. This proposed mechanism was consistent with the alterations in TF activity reported in TET2 knockout mice (Rasmussen et al., 2019). TET2-associated functions may ensure the binding of some TFs and contribute in this manner to enhancer-dependent activity and gene expression.

Our study identifies a crucial role for the IL-6/JAK/STAT3 pathway in the acquisition of tolerogenesis in innate immunity. STAT3 has also been involved to be relevant in the context of myeloid derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), also characterized for their tolerogenic properties. This pathway has been reported to play a role in the tumor microenvironment-induced dysregulated immune responses (Park et al., 2017), where IL-6/JAK/STAT3 activation associates with adverse host inflammatory responses and reduced survival. It has been suggested that this pathway is an important immunosuppressive event blocking effective T-cell immune responses (Yao et al., 2016), in gliomas. Our data sheds light on how this pathway is directly activated in the context of vitamin D-mediated immunosuppression, in the context of DCs, which are essential in T cell mediated immunity. VDR directly targets the activation of the *IL-6* gene and vitamin D results in the specific phosphorylation of STAT3. We prove that the pharmacological impairment of STAT3 phosphorylation, by inhibiting JAK2, directly results in the loss of the tolerogenic properties of TLs, which are able to allow T cell proliferation, demonstrating the essential role of this pathway. In this sense, our results open up the possibilities of reverting the tolerogenic properties, not only in the context of vitamin D but also perhaps in other contexts.

Material and Methods

Differentiation of TLs and DCs from peripheral blood mononuclear cells. For *in vitro* differentiation experiments, we obtained buffy coats from anonymous donors through the Catalan Blood and Tissue Bank (CBTB). The CBTB follows the principles of the World Medical Association (WMA) Declaration of Helsinki. Before providing the first blood sample, all donors received detailed oral and written information and signed a consent form at the CBTB. PBMCs were isolated using Ficoll-Paque gradient centrifugation. MOs were isolated from PBMCs using positive selection with MACS CD14 microbeads (Miltenyi Biotec). Cells were resuspended in RPMI Medium 1640 + GlutaMAXTM-1 (Gibco, Life Technologies) containing 10% fetal bovine serum, 100 unit's/mL penicillin, and 100 µg/mL streptomycin. For TL differentiation, the medium was supplemented with 10 ng/mL human IL-4, 10 ng/mL GM-CSF (PeproTech), and 10 nM of vitamin D3 or calcitriol (Sigma Aldrich). For DCs, the medium did not contain vitamin D3. In some cases, differentiation was performed in the presence of a JAK2 inhibitor (TG101348, STEMCELL) at 500 nM.

$CD8^+$ cell proliferation assay. Allogenic $CD8^+$ T cells were isolated by negative selection using Dynabeads Untouched Human CD8 T Cells Kit (Invitrogen), labeled with carboxyfluorescein succinimidyl ester (CFSE) and seeded in 96-well plates at 200,000 cells/well alone, with TLs or DCs at different ratios (TL/DC:CD8+ T cell ratios: 1:2, 1:4, or 1:6). $CD8^+$ cells were then stimulated with anti-CD3/CD28 Dynabeads 5 µL/mL (Invitrogen) and cultured for 3 days. $CD8^+$ T cell proliferation was analyzed by FACS and determined by considering the proliferating $CD8^+$ T cells those where a CFSE staining had decreased compared to unstimulated $CD8^+$ T cells.

Cytokine measurements. For *in vitro* experiments, the concentration of cytokines was measured from the cell culture supernatants using an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (BioLegend, San Diego, CA, USA).

Genomic DNA extraction. For whole-genome bisulfite sequencing, DNA was extracted with a QIAamp DNA micro kit (Qiagen) according to the

manufacturer's protocol. For pyrosequencing experiments, DNA was extracted with a Maxwell RSC Cultured Cells DNA kit (Promega).

Bisulfite pyrosequencing. 500 ng of genomic DNA was converted with an EZ DNA Methylation-Gold kit (Zymo Research), following the manufacturer's instructions. Bisulfite-treated DNA was PCR amplified using primers (see Table S2) designed with PyroMark Assay Design 2.0 software (Qiagen). Finally, PCR amplicons were pyrosequenced with the PyroMark Q24 system and analyzed with PyroMark CpG software (Qiagen).

Co-immunoprecipitation (Co-IP). Co-IP assays were performed using TLs differentiated from CD14+ monocytes for 3 days. Cell extracts were prepared in lysis buffer [50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Triton-X-100, protease inhibitor cocktail (cOmplete™, Merck)] with corresponding units of Benzonase (Sigma) and incubated at 4°C for 4 h. 50 µl of supernatant was saved as input and diluted with 2× Laemmli sample buffer (4% SDS, 20% glycerol, 120 mM Tris–HCl, pH 6.8). Supernatant was first incubated with PureProteome™ Protein A/G agarose suspension (Merck Millipore) for 1 h to remove background signal. Samples then incubated overnight at 4°C with corresponding antibodies against VDR (12550, Cell Signaling), TET2 (ab124297, Abcam), STAT3 (79D7, Cell Signaling) and pSTAT3 (D3A7, Cell Signaling) according to the specifications of each antibody. Negative controls were incubated with rabbit (12-370, Merck Millipore) and mouse (12-371, Merck Millipore) IgGs. Subsequently, samples were incubated with magnetic beads at 4°C for 2 h, and beads were then washed three times with lysis buffer. For sample elution, 100 µl of 1× Laemmli sample buffer was added to beads. Samples and inputs were denatured at 95°C in the presence of 1% β-mercaptoethanol.

DNA methylation profiling. Infinium MethylationEPIC BeadChip (Illumina, Inc., San Diego, CA, USA) array were used to analyze DNA methylation. This platform allows > 850,000 methylation sites per sample to be interrogated at single-nucleotide resolution, covering 99% of reference sequence (RefSeq) genes. The samples were bisulfite-converted using EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA) and were hybridized in the array

following the manufacturer's instructions. Image processing and intensity data extraction software and procedures were as previously described. Each methylation data point was obtained from a combination of the Cy3 and Cy5 fluorescent intensities from the methylated and unmethylated alleles. Background intensity computed from a set of negative controls was subtracted from each data point. For representation and further analysis, we used beta and M values. The beta value is the ratio of the methylated probe intensity to the overall intensity (the sum of the methylated and unmethylated probe intensities). The M value is calculated as the log2 ratio of the intensities of the methylated versus unmethylated probe. Beta values range from 0 to 1 and were used to derive heatmaps and to compare DNA methylation percentages from bisulphite-pyrosequencing experiments. For statistical purposes, the use of M values is more appropriate by its normal distribution.

Methylation raw data were preprocessed with minfi package (Aryee et al., 2014). Data quality was assed using both minfi and RnBeads packages (Aryee et al., 2014; Assenov et al., 2014; Müller et al., 2019). After Snoob normalization, data were analyzed using an eBayes moderate t-statistical test in the limma package. Several criteria have been proposed to represent significant differences in methylated CpGs. In this study, we considered a probe to be differentially methylated if it had a methylation differential of 20% and its significant (q-value < 0.05).

ChIP-seq analysis. Chromatin immunoprecipitation was performed using iDeal ChIP-seq kit for Transcription Factors (Diagenode) according to the manufacturer's instructions. Briefly, cells at day 3 of differentiation were cross-linked with 1% formaldehyde for 15 min and glycine added to quench (final concentration 125 mM, incubated for 5 min at room temperature). Cells were washed once with cold PBS, scraped off the plates and pelleted. To obtain a soluble chromatin extract, cells were resuspended in 1 ml LB1 (50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100 and 1× complete protease inhibitor) and incubated rotating at 4 °C for 10 min. Samples were centrifuged, resuspended in 1 ml LB2 (10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA and 1× complete protease inhibitor) and incubated rotating at 4 °C for 10 min. Finally, samples were centrifuged,

resuspended in 1 ml LB3 (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.5% *N*-lauroylsarcosine, 1% Triton X-100 and 1× complete protease inhibitor). Chromatin extracts were sonicated for 12.5 min using a Covaris M220 focused ultrasonicator at a peak power of 75, a duty factor of 10 and 200 cycles per burst. The lysates were incubated with anti-VDR antibody (12550, Cell Signaling) bound to 30 µl protein A or protein G Dynabeads and incubated overnight at 4 °C with 5% kept as input DNA. Magnetic beads were sequentially washed with low-salt buffer (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM EDTA and 50 mM Tris-HCl), high-salt buffer (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM EDTA and 50 mM Tris-HCl), LiCl buffer (150 mM LiCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 1 mM EDTA and 50 mM Tris-HCl) and TE buffer (1 mM EDTA and 10 mM Tris-HCl). For ChIP–seq, beads were resuspended in elution buffer (1% SDS, 50 mM Tris-HCl pH 8.0, 10 mM EDTA and 200 mM NaCl) and incubated for 30 min at 65 °C. After centrifugation, the eluate was reverse-cross-linked overnight at 65 °C. The eluate was then treated with RNaseA for 1 h at 37 °C and with Proteinase K (Roche) for 1 h at 55 °C and the DNA was recovered using a Qiagen PCR purification kit.

Sequencing reads from ChIP-seq experiments were mapped to the hg19 assembly of human reference genome using Bowtie2 Aligner v2.2.6 (Langmead, Trapnell, Pop, & Salzberg, 2009). After removing reads with MAPQ < 30 with Sequence Alignment/Map (SAMtools) v1.2 (Li et al., 2009), PCR duplicates were eliminated using the Picard function available in MarkDuplicates software v1.126 (Broad Institute, 2018). Peak calling was performed using SPP (with parameters –npeak=300000 –savr –savp -rf). Irreproducible discovery rate (IDR) was used to filter peaks (IDR < 0.05). To visualize individual ChIP-seq data on Integrative Genomics Viewer (IGV), we converted bam output files into normalized bigwig format using the bamCoverage function in deepTools (v2.0).

Data analysis. Hierarchical clustering was carried out based on Pearson correlation distance metrics and average linkage criteria. For low-dimensional analysis, we used the principal component analysis (PCA). Transcription factor motifs were enriched for each set using HOMER software v4.10.3. Specifically,

we used findMotifsGenome.pl algorithm (with parameters -size 200 -cpg) to search for significant enrichment against a background sequence adjusted to have similar CpG and GC contents. Genomic regions annotation for genetic context location was performed using the annotatePeaks.pl algorithm in the HOMER software v4.10.3. For determine the location relative to a CpG island (CGI), we used 'hg19_cpgs' annotation in the annotatr v1.8 R package. GREAT software (McLean et al., 2010) was used to enrich downstream pathways and gene ontologies. We used the single nearest gene option for the association between genomic regions with genes. Chromatin states analysis for DCs were assessed using the EpiAnnotator R package (Pageaud, Plass, & Assenov, 2018). Inference of TF activities from expression values were calculate using DoRothEA (Garcia-Alonso et al., 2019). For ligand activity prediction we used nichenetr package (Browaeys et al., 2019).

Statistical analysis. All statistical analysis were done in R v3.5.1. Data distributions were tested for normality. Normal data were tested using two-tailed unpaired Student's t-tests; non-normal data were analyzed with the appropriate non-parametric statistical test. Levels of significance are indicated as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Non-significance ($P \geq 0.05$) was indicated as 'ns'

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Author contributions

F.C.-M. designed and performed experiments and bioinformatics analysis; F.C.-M. and T.L. performed co-immunoprecipitation experiments; L.C. gave technical support; J.R.-U and E.B. supervised the study. E.B. conceived the study. F.C.-M. and E.B. wrote the manuscript; All authors participated in discussions and interpretation of the data and results.

Competing interests

The authors declare that they have no competing interests

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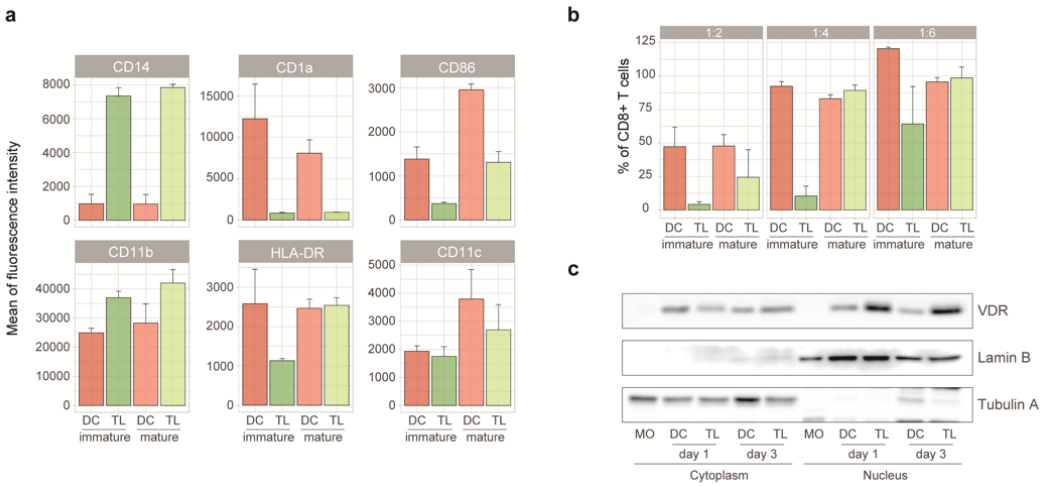
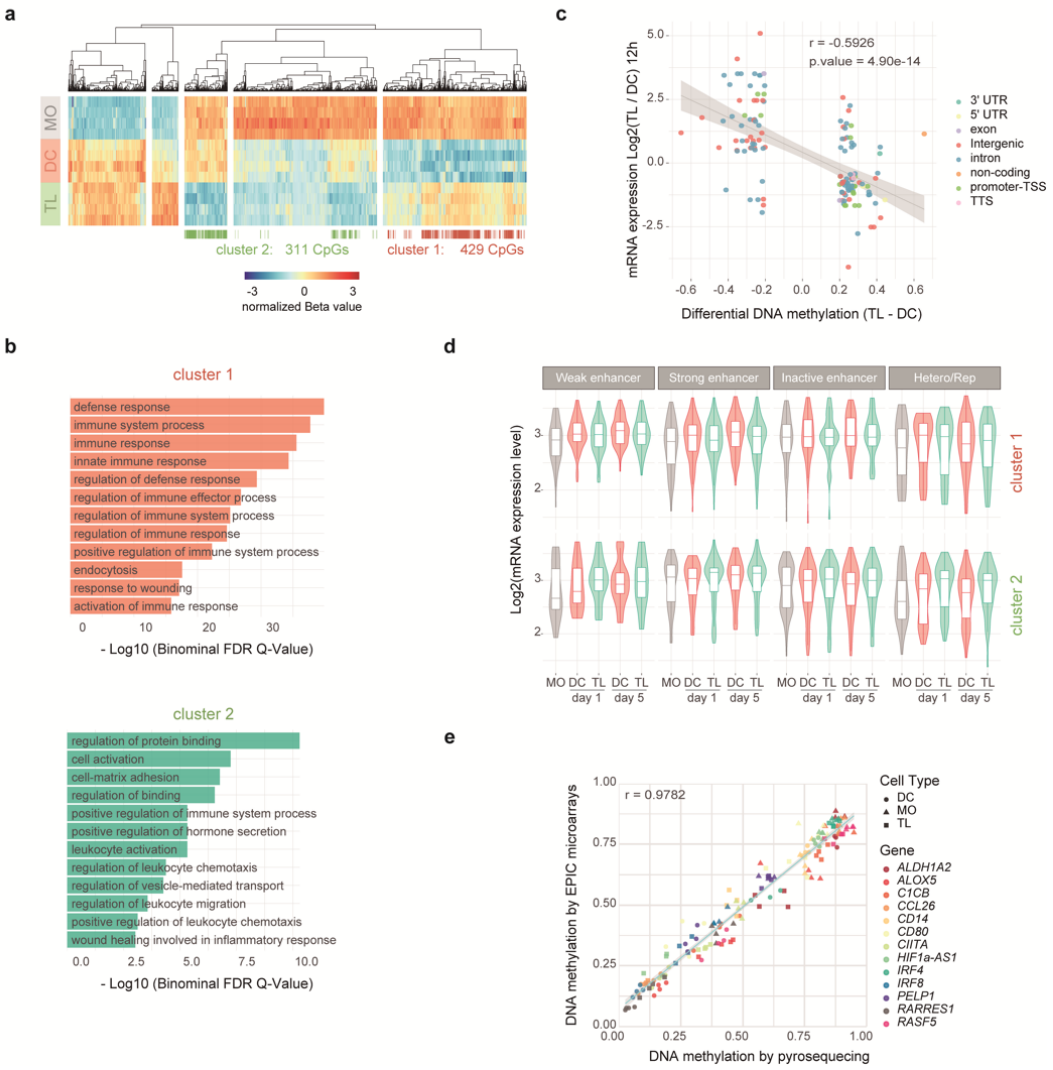
Supplementary Figures

Supplementary Figure 1

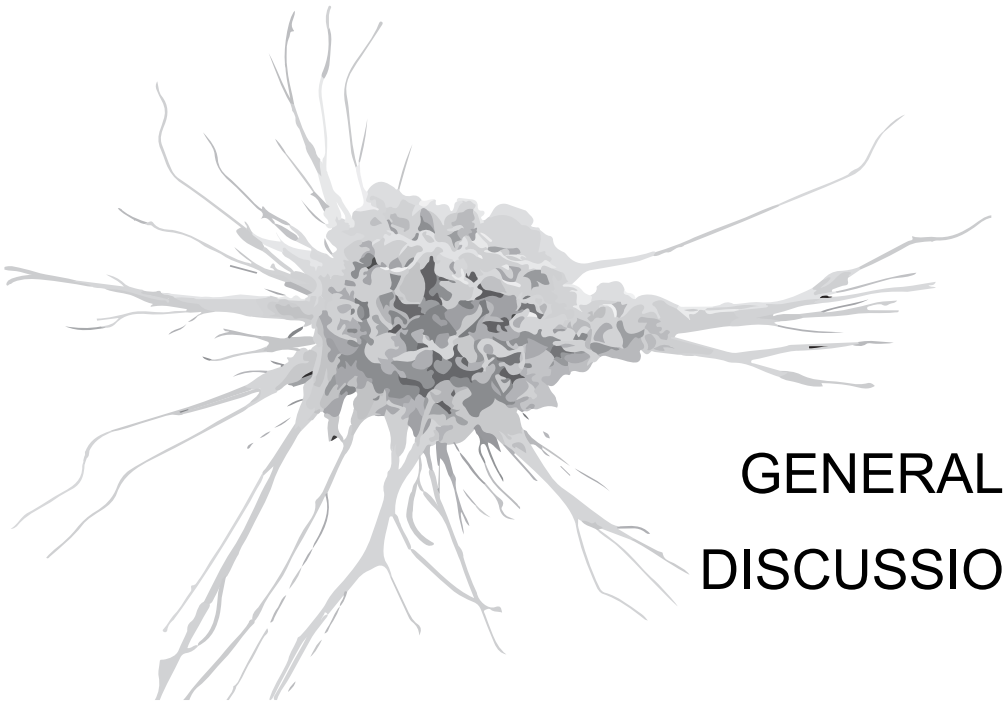


Supplementary Figure 1: Model characterization. *(**a**) Analysis of DC and TL surface markers analyzed by flow cytometry with (mature) and without (immature) LPS activation (n = 4). (**b**) Effects on allogenic CD8+ proliferation of in vitro differentiated DCs and TLs (DC or TL:CD8+ ratios 1:2, 1:4 and 1:6) before (immature) and after (mature) LPS activation. CD8+ T cell proliferation was analyzed by flow cytometry considering CD8+ T proliferating cells those in which CFSE staining is decreased (n = 4). Protein levels of vitamin D receptor in the cytoplasm and nucleus of MO, DCs and TLs at different time points. Tubulin A and Lamin B are used as cytoplasmic and nuclear markers respectively.*

Supplementary Figure 2



***Supplementary Figure 2*: DNA methylation dynamics throughout vitamin D exposed dendritic cells differentiation.** *(**a**) DNA methylation heatmap of four paired samples of MOs and their derived DCs and TLs. The heatmap includes all CpG-containing probes displaying significant methylation changes (Δ beta value >= 0.2 & q value < 0.05) in all of three possible comparisons (DC-MO, TL-MO and TL-DC). The lateral color annotation marks the membership to cluster 1 (DC-specific DNA demethylation) or cluster 2 (TL-specific DNA demethylation). Cluster assignation was done by clustering analysis of differential methylated CpGs of DC-TL comparison (Δ beta value >= 0.2 & q value < 0.05, Fig. 2c). (**b**) Results of gene set enrichment analysis using GREAT software by CpG cluster. The plot depicts the top twelve enriched terms for biological process annotation, based on P values from the binominal distribution. (**c**) Scatter plot showing the correlation between DNA methylation and gene expression (at day 12h of differentiation). Only differentially methylated CpGs are represented. Dot color refers to gene-related associations. (**d**) Box and violin plots summarizing the mRNA expression levels per cell type of cluster 1 (top) and cluster 2 (bottom) divided by chromatin state annotation of associated CpG. (**e**) Scatter plot showing correlation between array and bisulphite pyrosequencing DNA methylation values. Statistical tests: Pearson correlation (b, e).*



GENERAL DISCUSSION

General Discussion

In the present thesis, we have studied two different immune models to address outstanding questions related to active demethylation. It is well established that DNA methylation is a key regulatory mechanism in the biology and function of immune cells (Álvarez-Errico et al., 2015). Specifically, we have investigated the potential role of AID as a DNA demethylase, through its deaminase activity (Ramiro & Barreto, 2015), by obtaining the DNA methylomes of B cells from individuals with HIGM2 syndrome, which display loss-of-function for AID. We have also investigated how DNA methylation changes are targeted during the acquisition of a tolerogenic phenotype in MO differentiation to DCs exposed to vitamin D3.

1. A role of AID in active DNA demethylation

B cells represent an excellent model for the study of DNA methylation for several reasons: on the one hand, the profound knowledge about the development of the B cell lineage enables the isolation of different subpopulations. On the other hand, the complexity and diversity of the biological processes necessary for the different events of differentiation, allow the study of a plethora of epigenetic mechanisms (Oakes & Martin-Subero, 2018). The study of the DNA methylation changes during the transition from naïve to memory B cells is ideal to address the question of whether AID is able or not to demethylate *in vivo* (**Article 1**), given that during this transition in the CG AID displays its highest levels of expression, coinciding with SHM and CSR (Muramatsu et al., 2000; Revy et al., 2000). To interrogate this question in humans, HIGM2 patients provide an optimal model, since these patients harbor mutations in AID.

As previously described, the analysis of the flow cytometry data revealed a complete block in the formation of csMBC (Muramatsu et al., 2000), as well as alterations in the distribution of the intensity values of the different surface markers used, which may impact the composition of the other B cell subpopulations (NBC and ncsMBC). The absence of csMBC is due to the

inability to undergo CSR of HIGM2 patients, which results in the accumulation of IgD^+ cells. It is less clear what causes the observed alterations in the intensity values. A question that arises from these observations is whether the B cell subpopulations isolated from healthy and HIGM2 individuals are equivalent. In this sense, it is expected that the loss of function of AID, a protein with a very important role for the maturation of B cells (Muramatsu et al., 2000), causes alterations in the markers used, which in themselves are markers of maturation. Since there are no optimal alternatives to identify fully equivalent cell subpopulations we just have to rely on the accepted markers. A single cell transcriptomics approach may also help delineating the impact of AID mutations in B cell subpopulations. However, in such approach the assumption would be that mRNA levels are equal to protein levels, which is also an oversimplification (Granja et al., 2019; Nusinow et al., 2020) and may not reflect the actual phenotypic features of the entire B cell compartment.

Similarly to the findings in other primary antibody deficiencies (Rodríguez-Cortez et al., 2015), the comparison of the DNA methylomes of HIGM2 patients and healthy controls revealed the occurrence of alterations. This observation makes the integration of DNA methylation profiles of B cells from different primary antibody deficiencies interesting in order to find similarities and differences between them. Although this comparison has not been addressed in this work because it would require the recruitment of different cohorts of patients and, in the end, it was not the primary goal of this thesis. This type of study would open up the possibility of stratifying patients in relation to their DNA methylation profiles. This could be relevant considering that often patients are misdiagnosed and it sometimes takes years to provide them with a final diagnosis.

The genome-wide analysis of the DNA methylation profiles in HIGM2 has shown, on the one hand, a general blockage of demethylation during the transition from naïve to memory in patients. In principle, this result demonstrates the importance of AID for the correct establishment of DNA methylation patterns during the activation of B cells. However, these initial findings do not necessarily demonstrate a direct role of AID by demethylating through its deaminase activity. On the other hand, we have also observed

alterations in the comparison between the naïve cells and those of the patients, revealing a possible role of AID in the early stages of B cell development.

Our computational approach discards a direct role for AID in active DNA demethylation through its deaminase activity. This was demonstrated by first selecting those DMRs that are not PMDs, by identifying DMRs that take place in the naïve-to-memory B cell transition and whose demethylation is blocked in HIGM2 individuals and by merging these data with AID off targets. The conclusion obtained with our analysis contradicts the most recent study addressing the same question in AID knockout mice (Dominguez et al., 2015). There are different possible causes to explain such discrepancy, including the potential differences between different organisms and the different interpretation of similar results. It is unlikely that humans and mice use different mechanisms for active demethylation. Dominguez et al., like us, identified a group of CpGs that cease to become demethylated in $AID^{-/-}$ mice during the activation of naive B cells in the germinal center. However, they concluded that AID is responsible for these alterations directly, based on the observation of a significant overlap of these CpGs with SHM target genes and AID-associated dsDNA breaks. However, they did not take into consideration that the overlap, although significant, is less than 1% in both cases, without providing an explanation on how demethylation is blocked in the remaining 99% CpG sites. In addition, through the re-analysis of their data and the integration with hydroxymethylation data (C. W. J. Lio et al., 2019), we observed that these CpGs are potentially demethylated by TETs since they show an increase in hydroxymethylation levels during B cell activation, not compatible with an AID-mediated demethylation model (Ramiro & Barreto, 2015).

Interestingly, recent studies have shown that the genes with the highest transcription rates display co-localization of de novo DNMTs and TETs in their gene bodies (Baubec et al., 2015; Dhayalan et al., 2010; Hashimoto et al., 2014). The presence of DNMTs and TETs translates into increased levels of cytosine methylation and hydroxymethylation that has been linked to the regulation of spurious transcription initiation (Neri et al., 2017; Tsagaratou et al., 2014; Weinberg et al., 2019; Wen et al., 2014). In addition, the interaction between TETs and DNMTs has been reported (Y. W. Zhang et al., 2017). If we

take into account that a high proportion of these regions are also AID targets (Álvarez-Prado et al., 2018; Meng et al., 2014; Qian et al., 2014), we can propose a model where the increased levels of methylation and hydroxymethylation may reduce or inhibit the mutagenic potential of AID, considering that the affinity of AID for these modifications is 10 and 100 times lower respectively, than for unmodified cytosine (Nabel et al., 2012).

As already mentioned, the comparison of the DNA methylomes of naive control cells with those isolated from HIGM2 patients suggested a potential role of AID in early stages of development B. In this sense, although the expression of AID has been classically restricted to germinal centers, several studies have shown that discrete populations of B cells also express AID in the bone marrow (Cantaert et al., 2015; Kuraoka et al., 2011; Meyers et al., 2011; Orthwein & Di Noia, 2012). Through the annotation and functional characterization of alterations at the level of DNA methylation in HIGM2 naïve B cells, we have demonstrated that these changes are due to a pre-activation of B cells outside the germinal center. These results are validated by the *in vitro* activation of naive B cells with EBV characterized by activation of the BCR and CD40 pathways (Cahir-McFarland et al., 2004; Mancao & Hammerschmidt, 2007). While it is true that this is not the best *in vitro* system for activating B cells, it is the only method that allows long culture times, since the viability and proliferative capacity of human B cells activated with IgM, CD40L and IL21 are compromised after few days (data not shown). By integrating the DNA methylation data with multiple public data, we observe that the recruitment of the methylation machinery to these regions would be mediated at least in part to the BATF and IRF4 factors in a similar manner to previous works (C. W. J. Lio et al., 2019; C. W. Lio et al., 2016). In fact, the knockout of these proteins in B cells affects the expression of a large part of the genes that show alterations between naive controls compared to patients.

Finally, our study indicates that the observed DNA methylation changes in the naïve cell subset of patients is due to an expansion of autoreactive naive cells. These results are consistent with the observation that patients with HIGM2 have a higher susceptibility to autoimmune processes (Revy et al., 2000). In addition, recent work has shown that AID plays a relevant

role in the elimination of autoreactive clones both centrally and in peripheral organs (Cantaert et al., 2015, 2016; Kuraoka et al., 2011; Meyers et al., 2011). The proposed mechanism of tolerance in the bone marrow consists of continuous activation of the clones with autoreactive BCRs. This activation would be followed by an increase in AID and RAG levels, increasing genomic instability, which together with a decrease in BCL2 levels would end the elimination of these clones via apoptosis (Cantaert et al., 2015). However, in the absence of AID, the efficient elimination of autoreactive clones would decrease and consequently, the levels of B cells with a pre-activation signature at methylation level would increase in the bloodstream (Figure 8).

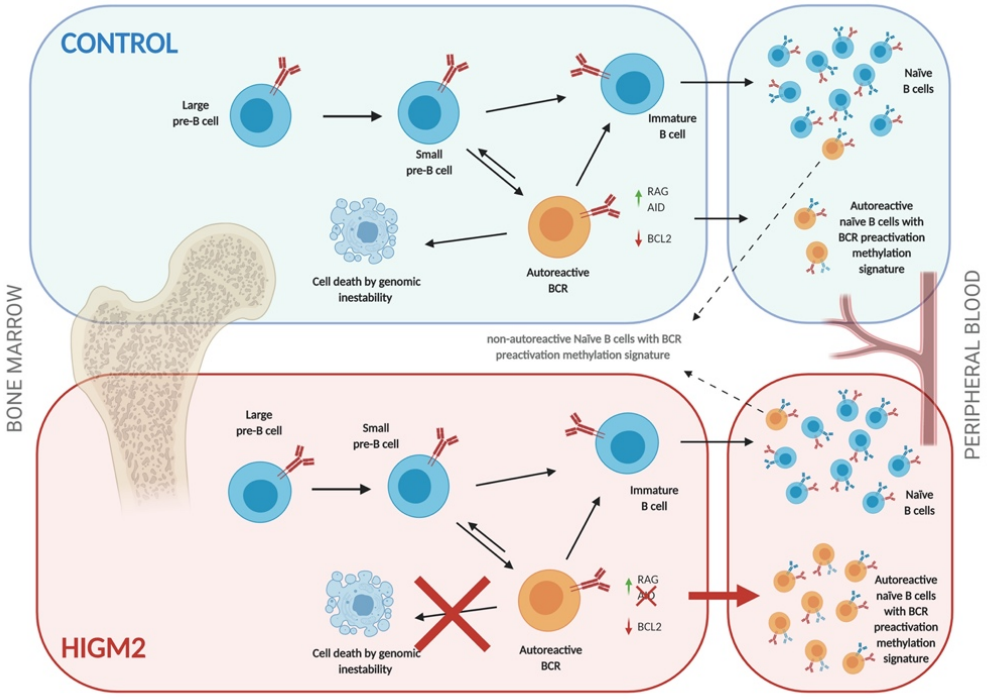


Figure 8: Impairment of central B cell tolerance associated with loss-of-function of AID. *In healthy controls, autoreactive Small pre-B cells undergo BCR-dependent activation that leads to AID and RAG upregulation and downregulation of the antiapoptotic factor BCL2. If cells cannot lose the interaction with self-reactive antigen, they are eliminated through apoptosis induced by the genomic instability promoted by AID and RAG. In HIGM2 patients, loss-of-function of AID results in impairment in the elimination of autoreactive B cells with subsequent expansion of autoreactive naïve B cells with a BCR-activated DNA methylation signature.*

To summarize this part of the thesis, our results represent the first characterization of epigenomic alterations associated with HIGM2 syndrome. Moreover, we demonstrate that DNA demethylation is not mediated by the catalytic activity of AID. In fact, our analysis support that these alterations are mediated by TET enzymes also in the context of B cell activation. Finally, our results show that AID loss of function leads to an expansion of autoreactive clones with a BCR pre-activation DNA methylation signature.

2. Role of VDR and STAT3 in targeting active demethylation in tolerogenic dendritic cells

Although the role of TET enzymes in active demethylation is widely accepted, little is known about the specific mechanisms of their recruitment to different genomic regions (Greenberg & Bourc'his, 2019). In the second part of this thesis (**Article 2**), we investigated specific targeting of DNA demethylation through the use of an *in vitro* model of terminal myeloid differentiation. Specifically, we studied the differentiation process from human MOs to DCs in the presence or absence of vitamin D3, which results in DCs with an immunogenic and tolerogenic phenotype respectively. Interestingly, a previous study had shown the stability of the tolerogenic properties upon withdrawal of vitamin D3 (Van Halteren et al., 2002), suggesting the participation of epigenetic mechanisms that stabilize such immunophenotype. In this sense, based on the higher stability of DNA methylation in comparison with histone modifications (Cedar & Bergman, 2009), as well as its relevant role in terminal myeloid differentiation models (de la Rica et al., 2013; Garcia-Gomez et al., 2017; Klug et al., 2010, 2013; T. Li et al., 2019; Rodríguez-Ubreva et al., 2017; Vento-Tormo et al., 2016), we decided to analyze the methylome in this process.

Our study has revealed that differentiation of MOs to DC and TLs is accompanied by DNA methylation changes. Although most of the observed changes correspond to common events, we also found DC- and TL-specific hypermethylation and hypomethylation dynamics, being the latter predominant. These results are in accordance with those already described by our laboratory,

showing that myeloid differentiation processes are highly sensitive to external signals, with repercussions not only at the phenotypic level, but also at DNA methylation level (T. Li et al., 2019; Rodríguez-Ubreva et al., 2017; Vento-Tormo et al., 2016).

To explore the mechanisms involved on DNA demethylation during the acquisition of a tolerogenic phenotype, we focused on condition-specific demethylation clusters: DC-specific demethylation (cluster 1, 429 CpGs); TL-specific demethylation (cluster 2, 311 CpGs). Functional annotation of the demethylation events showed a significant enrichment in enhancer and promoter regions, which were mainly associated with active demethylation (X. Wu & Zhang, 2017). Interestingly, we also found that whereas DC-specific demethylation is mainly associated with weak and strong enhancers, TL-specific demethylation is rather associated with inactive enhancers in DCs, suggesting that these inactive regions in DCs are activated in TLs. In fact, we found that effectively, the expression of the nearest genes of those CpGs were more expressed in TLs.

In agreement with the most common relationship between DNA methylation and expression (Greenberg & Bourc'his, 2019; C. W. J. Lio & Rao, 2019; Smith & Meissner, 2013), the integration of these datasets in our system revealed an inverse correlation between them. However, as in many other studies, we observed many exceptions to this inverse relationship. Given the limited coverage of our methylation datasets and the absence of genomic interaction data for our specific model, we cannot obtain more conclusions on the specific biological meaning of the observed methylation changes in relation to expression.

Vitamin D3 is recognized by its receptor VDR in the cytoplasm, where upon ligand binding it is translocated to the nucleus acting as a transcription factor. To test whether VDR is directly associated with the observed DNA methylation alterations, we performed ChIP-seq. We observed a notable increase of VDR genomic binding consistent with the increase in its nuclear translocation. We determined that VDR mainly binds to regions with the canonical motif. However, as previously reported we observed a certain degree

of VDR binding promiscuity (Heikkinen et al., 2011; Penvose, Keenan, Bray, Ramlall, & Siggers, 2019). Interestingly, the two top non-specific binding motifs detected were associated with PU.1 and CEBP proteins that are known to interact with VDR (Nurminen et al., 2019; Seuter et al., 2017). Therefore, we suggest that VDR can indirectly bind to these regions through the interaction with these two TFs.

After integrating DNA methylation with VDR genomic occupancy, we observed an increase of the signal levels for the binding of VDR in the TL-specific DNA demethylated positions. In fact, we found a significant enrichment with VDR peaks with an overlap of around 40%. The absence of non-significant VDR binding in more than 50% of TL-specific demethylated CpG sites could be simply explained by insufficient depth in the ChIP-seq data, which could affect peak calling in regions with low signal. Another plausible explanation could be that a fraction of the demethylated CpG sites in cluster 2 are associated with the binding of alternative TFs that are activated under vitamin D3 conditions. In any case, our results demonstrate a role of VDR in mediating a large fraction of TL-specific demethylation, confirming the previous observations that linked vitamin D3 exposition with a loss of global DNA demethylation levels (Doig et al., 2013; O'Brien et al., 2018).

Another interesting finding of our work is the capacity of VDR to bind to genomic regions characterized by closed chromatin in MOs. This finding argues against a previous work done on THP-1 cell line where the authors found that VDR only binds open regions prior to vitamin D3 exposure. However, it is easy to envision that the accessibility profile of immortalized cells could be substantially different from that in normal cells. In addition, the described interactions of VDR with different chromatin remodelers (Nurminen et al., 2018, 2019; Pereira et al., 2011; Z. Wei et al., 2018) supports the potential role of VDR as a pioneer factor.

Vitamin D3 exposure, through its receptor VDR, induces changes in cytokine production and a profound metabolic reprogramming (Ferreira et al., 2015). Therefore, we cannot rule out the activation of additional signaling pathways as a result of the drastic changes occurring in these cells. To explore

this possibility, we used a new computational approach to infer the activity of different ligands. We found and validated the activation of STAT3 via the IL-6/JAK2 pathway. Our results also highlight the usefulness of this approach to infer autocrine signaling in differentiation models, beyond single cell analysis where only cell-to-cell communication was taking into account.

The most accepted model for signal dependent gene activation in immune cells involves rapid activation and nuclear translocation of pioneer TFs. Once in the nucleus, pioneer TFs recruit chromatin remodelers to facilitate the binding of secondary TFs, that activate (or repress) gene expression (Monticelli & Natoli, 2017; Natoli & Ostuni, 2019; Kasper D Rasmussen et al., 2019). To explore whether VDR could act as a pioneer TFs we performed co-IP experiments that indicated not only the existence of interactions between VDR and STAT3, but also with TET2, the most relevant methylcytosine dioxygenase, involved in active demethylation, in the myeloid compartment (C. W. J. Lio & Rao, 2019). Furthermore, inhibition of STAT3 phosphorylation did not show impacted TL-specific demethylation but it altered mRNA and protein levels of relevant genes to the tolerogenic phenotype. These results suggest that while VDR is important for DNA methylation remodeling, STAT3 is downstream to such change and a subsequent determinant of changes at the transcriptional level.

Altogether, the results of this second part are potentially relevant in the immunotherapy field, since the modulation of these mechanisms could be used to both promoting immune responses against cancer, or attenuating them in the contexts of autoimmunity and organ transplantation. It has been described that tumors with higher IL-6 levels have lower immune infiltration (Johnson, O'Keefe, & Grandis, 2018). In addition, our results shown how STAT3 activation via IL-6 results in the acquisition of the immunosuppressive phenotype. We propose that boosted DCs could have lower effects in tumors with more IL-6 secretion that their counterparts. Therefore, a plausible option to reduce the susceptibility to IL-6 of boosted DCs against tumors could consist in the co-treatment with pharmacological IL-6/JAK2/STAT3 pathway inhibitors (i.e. IL-6 antibodies, Jak2 and/or STAT3 inhibitors). On the other hand, to promote immune tolerance in autoimmunity and organ transplantation the activation of

the IL-6/JAK2/STAT3 pathway could be a good strategy. In fact, oral and cutaneous administration of vitamin D3 is used extensively in the treatment of lupus and other autoimmune diseases.

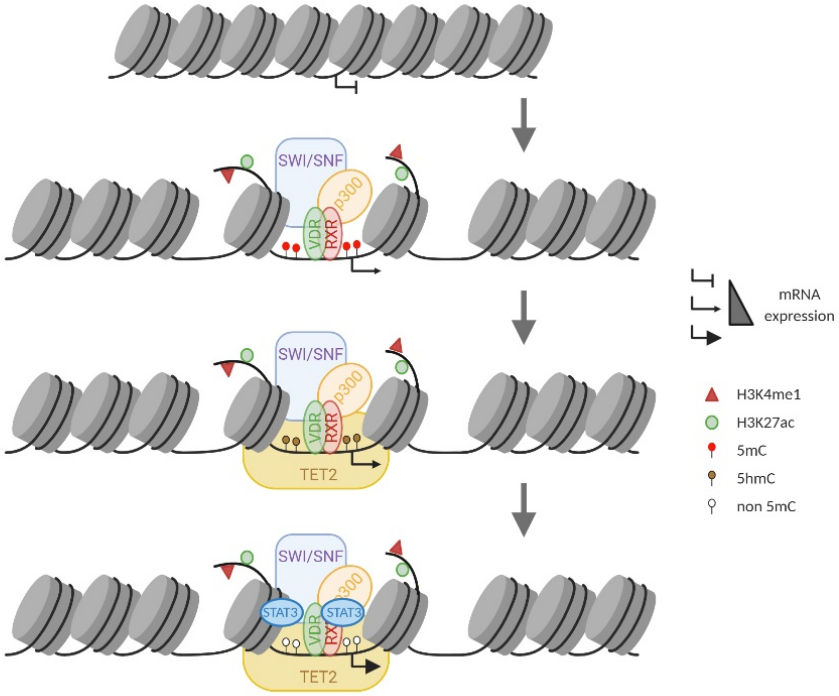
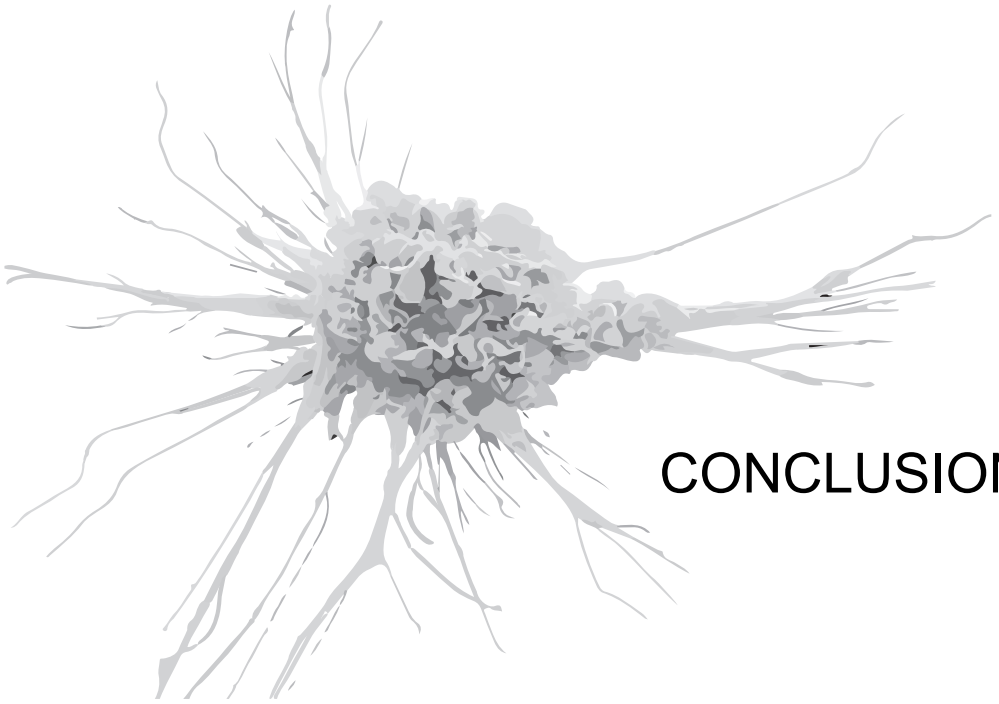


Figure 9: Proposed model for VDR-mediated epigenomic remodeling. *Following ligand recognition, VDR interacts with RXR, translocates to the nucleus and binds to open and closed chromatin. After binding to its genomics targets, VDR-RXR can interact with various epigenetic remodelers, with the subsequent deposition of permissive histone modifications and DNA demethylation. Finally, chromatin opening and DNA demethylation promote the recruitment of secondary TFs, which determine the activation of associated genes.*

To summarize the second part of the thesis, we have demonstrated that vitamin D3, through its receptor VDR is able to drive TL-specific DNA demethylation by recruiting TET2. Moreover, VDR binding induces IL-6 expression leading to STAT3 phosphorylation and activation via JAK2. Most importantly, STAT3 activation, although does not seem to be involved in DNA methylation changes, is crucial for the acquisition of the tolerogenic phenotype regulating in part the gene expression of VDR remodeled regions. Finally, our findings open up a number of possibilities from the translational perspective in relation to potential immunotherapy strategies.



CONCLUSIONS

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The conclusions obtained during this doctoral thesis can be summarized as follows.

1. Comparison of whole genome bisulfite sequencing profiles of naïve and memory B cells of both HIGM2 patients and healthy individuals, reveals global DNA methylation alterations in association with AID loss-of-function mutations.

2. Defects in DNA methylation in HIGM2 patients are characterized by a blockage of demethylation during the transition of naïve to memory B cells. In addition, AID loss also results in DNA methylation alterations in naïve B cells.

3. Both HIGM2 naïve and memory B cells display widespread DNA methylation defects, of which approximately 25% associate with alterations in active demethylation. The remaining DMRs occur in PMDs and are mainly associated with passive demethylation events.

4. Aberrantly hypermethylated sites in memory B cells from HIGM2 patients meeting the requirements to be considered potential targets for a putative AID demethylase activity show a low correspondence with *bona fide* AID off-target genes and are produced in genomic regions with epigenomic features not compatible with AID targeting mechanisms.

5. Demethylation events that are blocked in memory B cells from AID deficient mice are mediated by an oxidation dependent mechanism, suggesting the participation of TET enzymes rather than an implication of a deaminase-dependent demethylation process.

6. Alterations in DNA methylation in HIGM2 naïve B cells support a role for AID in early stages of B cell development and are characterized by a BCR pre-activation DNA methylation signature.

7. AID loss-of-function leads to an expansion of autoreactive B cells with a BCR pre-activation signature suggesting an impairment of the central B cell tolerance.

8. *In vitro* terminal differentiation from MOs to DCs in absence and presence of vitamin D3 results in condition-specific DNA demethylation events associated with differential immune properties.

9. During differentiation from MOs to DCs and TLs, specific DNA demethylation events preferentially occur in enhancer regions and display an inverse correlation with gene expression.

10. Vitamin D3 exposure during DC differentiation leads to an increase in the genomic occupation of VDR characterized by high binding promiscuity.

11. VDR can bind to closed chromatin regions in MOs correlating with TL-specific DNA demethylation and deposition of active histone marks, suggesting a function as a pioneer TF.

12. VDR induces IL-6 expression and subsequent STAT3 activation through JAK2-dependent phosphorylation.

13. VDR interacts with both STAT3 and TET2, probably in the same complex.

14. Impairment of STAT3 phosphorylation by JAK2 inhibition induces alterations at mRNA and protein levels without interfering with DNA demethylation, resulting in a reversion of the tolerogenic phenotype of TLs.

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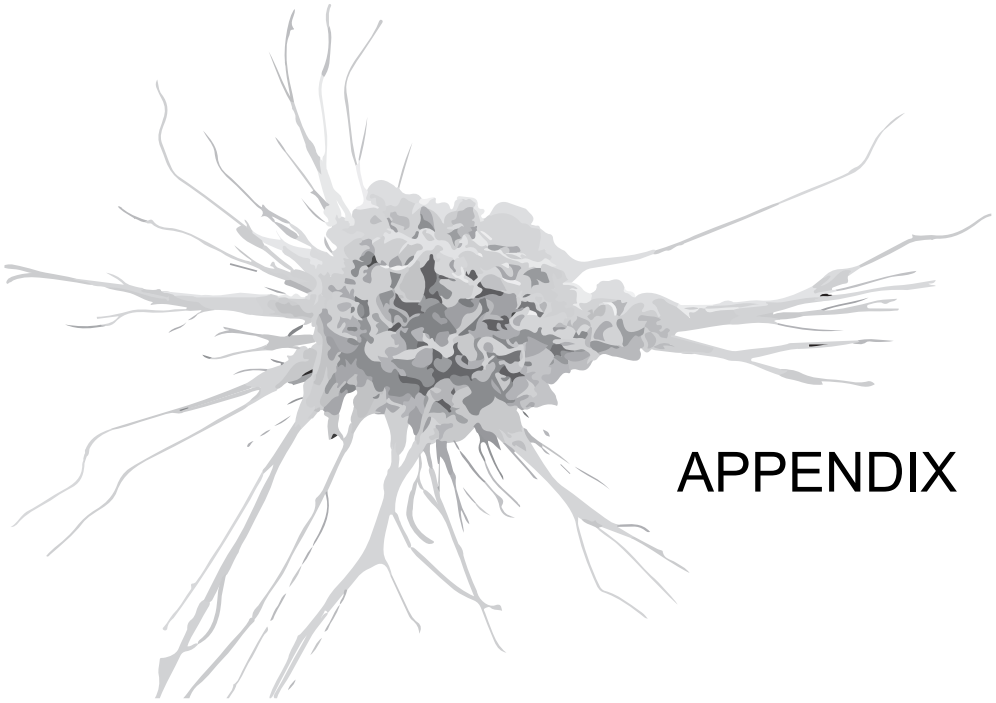

APPENDIX