Towards an understanding of the role of DNA methylation in rheumatoid arthritis: therapeutic and diagnostic implications

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***Abstract*:** The term 'epigenetics' loosely describes DNA-templated processes leading to heritable changes in gene activity and expression, which are independent of the underlying DNA sequence. Epigenetic mechanisms comprise of post-translational modifications of chromatin, methylation of DNA, nucleosome positioning as well as expression of noncoding RNAs. Major advances in understanding the role of DNA methylation in regulating chromatin functions have been made over the past decade, and point to a role of this epigenetic mechanism in human disease. Rheumatoid arthritis (RA) is an autoimmune disorder where altered DNA methylation patterns have been identified in a number of different disease-relevant cell types. However, the contribution of DNA methylation changes to RA disease pathogenesis is at present poorly understood and in need of further investigation. Here we review the current knowledge regarding the role of DNA methylation in rheumatoid arthritis and indicate its potential therapeutic implications.

Keywords: DNA methylation, epigenetics, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a debilitating chronic inflammatory condition of the joints that affects approximately 1% of the world's population. It is a systemic inflammatory disease with characteristic features of joint swelling, pain and stiffness [Feldmann *et al.* 1996]. Major advancements in understanding the development of RA originate from studies investigating the expression and regulation of pro-inflammatory cytokines within the affected synovial tissue. One of these pro-inflammatory cytokines, tumour necrosis factor (TNF)-α, is a pivotal factor in the inflammatory cascade and led to its identification as a target for therapeutic intervention [Brennan *et al.* 1992]. Subsequently, in 1992, the first open-label trial of a TNF-α blocking agent was initiated at the Charing Cross Hospital, United Kingdom [Elliott *et al.* 1993]. Anti-TNF therapy is one of the most successful treatments for RA and has paved the way for other biological therapies, targeting CTLA-4, CD20 and IL-6 (e.g. abatacept, rituximab and tocilizumab) [Malottki *et al.* 2011]. Despite their successes, these numerous new medicines for inflammatory arthritis all have broadly similar efficacy and benefit only a proportion of patients treated. Undoubtedly it is a difficult task to develop new treatment options, however it is hoped that an understanding of the complexity underpinning the regulation of gene expression in different cell types that are critically involved will yield an improved understanding of the causes of the disease. In this review we aim to collate the current knowledge on DNA methylation in autoimmunity with a particular focus on RA, its role in altering gene expression in different cells that contribute to the pathogenesis of RA, and discuss its therapeutic and diagnostic potential.

Genetic and nongenetic factors influencing rheumatoid arthritis onset

The etiological paradigm of RA is that an environmental trigger in a genetically predisposed individual can induce autoimmune responses. Most attention thus far has been given to determination of genetic factors governing the susceptibility of an individual to developing RA. A key observation made decades ago was that specific alleles of the class II major histocompatibility

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complex (MHC) confer susceptibility to RA [Winchester, 1994]. In particular, susceptibility to RA was found to be associated with certain HLA-DRB1 alleles, which encode for a similar amino acid sequence (QKRAA, QRRAA or RRRAA) providing the rationale for the aptly named 'shared-epitope' hypothesis (reviewed by Ollier and Thompson [1992] and Orozco *et al.* [2006]). Subsequently, with the advent of genome wide association studies (GWAS) a number of other non-HLA susceptibility alleles have also been implicated, such as PTPN22, PADI4, CD40 and CTLA-4, many of which are implicated in T cell signalling and function [Suzuki *et al.* 2011].

Although it is undeniable that genetic factors play a major role in the susceptibility to RA, the low concordance rate (12–20%) observed in RA between monozygotic twins suggests that environment factors play a significant role in the pathogenesis of RA [Aho *et al.* 1986; Silman *et al.* 1993; Svendsen *et al.* 2002]. Indeed, a number of potential environmental risk factors have been implicated, including smoking and periodontitis [De Pablo *et al.* 2008]. *Porphyromonas gingivalis*, the cause of periodontitis, expresses an enzyme with peptidylarginine deiminase (PAD) activity (termed PPAD in *P. gingivalis*), leading to citrullinated peptide residues. Autoantibodies against citrullinated peptides are present in the majority of RA patients and it has been suggested that PPAD activity, which enzymatically regulates the conversion of peptidyl-arginine to peptidyl-citrulline, can mediate the generation of autoantibodies that drive autoimmunity in RA [Lundberg *et al.* 2010]. Similarly, cigarette smoking, which is also associated with periodontitis, can influence not only disease onset but can contribute to the severity of RA in a dose-dependent fashion [Saag *et al.* 1997]. In addition to smoking, other known environmental factors include latent viral infections [Costenbader and Karlson, 2006], sex hormones [Cutolo *et al.* 2002] and vitamin D [Merlino *et al.* 2004]. It is thought that these environmental factors influence epigenetic modifications, which in concert with the individual genetic susceptibility status result in the development of RA symptoms. Since these environmental factors play a significant role in RA pathogenesis, understanding epigenetic modifications that constitute the underpinning link between environmental cues and disease will provide a better understanding of how to treat RA.

Epigenetics and chromatin modification

The British developmental biologist Conrad Waddington originally coined the term 'epigenetics' in 1942 when he attempted to link genotypic and phenotypic changes during embryogenesis [Waddington, 1942, 2012]. Epigenetics was initially broadly defined as the 'causal mechanisms' that link the genetic information of an organism and how it interacts with the environment in order to produce observable traits. More recently the Epigenetic Roadmap Project has defined epigenetics as referring to both DNA-independent heritable changes in gene activity and expression, and also stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable, thereby including much broader chromatin-templated processes. DNA is wrapped around an octamer of histone proteins H3, H4, H2A and H2B, thus forming the complex structure of chromatin [Li, 2002]. Posttranscriptional modification of the histone tails and methylation of cytosine bases in the DNA can modify the accessibility of chromatin and alter gene transcription by allowing transcription factors to bind at promoter sites and initiate transcription [Ernst *et al.* 2011; ENCODE Project Consortium *et al.* 2012]. The dynamic nature of these modifications means that these changes are not always stable, and they can be altered in response to stimuli [Bird, 2007; Lund *et al.* 2012]. Moreover, the sequence and modification-specific context of chromatin modifications, and the particular composition and recognition potential of regulatory factors led to the hypothesis of a 'histone code' that controls specific DNA-templated systems such as gene transcription, genome stability, imprinting and X-chromosome inactivation [Jenuwein and Allis, 2001]. The complexity, dynamics and specific roles of these post-translational modifications on histones and other nuclear proteins, and the associated recognition systems in genome and chromatin biology is subject of current studies, revealing a multitude of covalent modifications whose significance is only partially understood (reviewed by Rothbart and Strahl [2014]). Since epigenetic modifications are less stable than genome sequences, these processes are subject to variable responses and hence can lead to altered development and disease. Epigenetics and the study of chromatin modifications has gained increased interest because it has been realized that the study of epigenetic modifications specific to disease may give a mechanistic insight into age-related autoimmunity [Hirst and

Marra, 2009]. Therefore, when investigating RA in the context of epigenetics it may provide an indication as to why genetically predisposed individuals develop disease while others do not, and may also explain the heterogeneity of symptoms and therapeutic responses observed [Oppermann, 2013].

DNA methylation

Methylation of the carbon 5 position of cytosine (5mC) is one of the best-studied and mechanistically understood epigenetic mechanisms, however it is clear that knowledge of DNA methylation is still incomplete [Smith and Meissner, 2013]. In mammals, cytosine methylation occurs largely in the context of CpG dinucleotides, which are often clustered in 5' regulatory regions termed CpG islands [Deaton and Bird, 2011], typically encompassing the first exons of genes [Yoder *et al*. 1997]. In mammals, most CpG dinucleotides are unmethylated and are often clustered in 5' regulatory regions termed CpG islands [Deaton and Bird, 2011], typically encompassing the first exons of genes [Yoder *et al.* 1997]. Typically, an area is termed a CpG island when, on average, a region of 1000 base pairs long shows an elevated C+G base composition and an absence of DNA methylation [Deaton and Bird, 2011]. The precise mechanism of how CpG islands are protected against methylation is not completely understood, but transgenic mice experiments suggest that protection may be directed by recognition of common *cis*-acting sequences located in CpG islands [Siegfried *et al.* 1999]. However, when methylated, CpG islands can constitute a powerful gene silencing mechanism (described below).

Other regions such as the gene body show a high degree of methylation, which is often associated with a higher level of gene expression in a dividing cell [Hellman and Chess, 2007]. In contrast to gene bodies, promoter regions are typically lacking DNA methylation [He *et al.* 2011]. IN addition, DNA methylation also plays a major role in suppressing transposable elements, by providing long-term heritable gene silencing [Wu and Zhang, 2010]. Around 45% of the mammalian genome consists of transposable and viral elements [Schulz *et al.* 2006]. These viral insertions require long-term silencing because if expressed they are potentially harmful because their replication and insertion can lead to aberrant gene expression.

The process of cytosine methylation is catalysed by DNA methyltransferases (DNMTs), which use S-adenosylmethionine (SAM) as the methyl donor [Chiang *et al.* 1996]. DNMTs are divided into two classes based upon their physiological function. One category comprises the *de novo* methyltransferases DNMT3A and DNMT3B, which establish methylation patterns in both unmethylated and hemimethylated DNA with equal efficiencies [Okano *et al.* 1998, 1999; Yanagisawa *et al.* 2002; Chen and Li, 2004]. The other category comprises the 'maintenance' methyltransferase DNMT1, which recognizes and copies methylated CpGs to newly synthesized DNA [Bestor and Ingram, 1983; Bestor *et al.* 1988]. DNMT1 is upregulated during S phase and is recruited to DNA replication forks and is preferentially active on hemi-methylated CpG substrates [Pradhan and Esteve, 2003]. DNMT1 then methylates CpG sites on new daughter strands, thereby maintaining the methylation patterns that are complementary to the parent strand [Wilson *et al.* 2005]. The importance of these enzymes is demonstrated by the loss of DNMT1 or DNMT3b in mice resulting in embryonic lethality around day (E) 9 and loss of DNMT3a leads to severe runting and death around 1 month after birth [Li *et al.* 1992; Okano *et al.* 1999]. Moreover, in humans mutations in DNMT3B are responsible for the immunodeficiency, centromere instability and facial abnormalities (ICF) disorder [Hansen *et al.* 1999; Xu *et al.* 1999; Jin *et al.* 2008], while mutations in DNMT3A have been reported in acute myeloid leukaemia [Ley *et al.* 2010; Yan *et al.* 2011].

DNA demethylation

Although DNA cytosine methylation has been regarded in the past as a stable epigenetic mark in adult vertebrates, more recent studies have revealed that this mark is not as static as previously thought. In particular, evidence that efficient DNA demethylation systems exist is highlighted during development, where DNA methylation following fertilization is nearly completely erased, and specifically re-installed during embryonic development. DNA demethylation can be reversed through passive and active processes. Passive DNA demethylation refers to the relative lack of DNMT activity across cell divisions, leading to a gradual reduction in global DNA methylation levels [Monk *et al.* 1987], whereas active DNA demethylation refers to the enzymatic process through

which the methyl group on the cytosine is removed. Several mechanisms have been proposed to explain the process of active DNA demethylation. The simplest of these mechanisms is the direct enzymatic removal of the methyl group from 5mC. Although this reaction was initially reported for methyl-CpG-binding domain protein 2 (MBD2) [Bhattacharya *et al.* 1999], its proposal as a DNA demethylase enzyme is controversial and other laboratories have not been able to reproduce the reported enzymatic activity [Wu and Zhang, 2010]. Regardless of this, it is still conceivable that a DNA demethylase enzyme exists, however at present no other enzyme has been described to fit this role.

Another proposed mechanism by which DNA demethylation occurs is through oxidative modification followed by removal of the base. Two seminal papers published in 2009 described the presence of 5-hydroxymethylcytosine (5hmC) and gave the first insights into the process of active DNA demethylation in both mouse and human [Kriaucionis and Heintz, 2009; Tahiliani *et al.* 2009]. The ten–eleven translocation (TET) enzymes, belonging to a large family of Fe2+ and alpha-ketoglutarate dependent dioxygenase enzymes have now been demonstrated as the DNA hydroxylases responsible for the conversion of 5mC to 5hmC [Johansson *et al.* 2014]. Furthermore, TET enzymes have been described as being responsible for the further oxidation of 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (reviewed by Shen *et al.* [2014]). Following this, an unmodified C can be generated through either a replication dependent dilution of the 5hmC or through base excision repair (BER) processes, mediated by the excision of 5fC or 5caC by thymine DNA glycosylase (TDG) (Figure 1) [Kohli and Zhang, 2013].

Genome-wide mapping has revealed that 5hmC plays a role in both transcriptional activation and silencing [Wu and Zhang, 2011]. Recent progress in identification of different types of protein modules that recognize unmethylated CpG dinucleotides (by CxxC domain containing proteins), 5mC and/or its oxidation products 5hmC, 5fC, 5caC (such as MeCp binding domains, Zn-finger and SET and RING-associated (SRA)) sheds new light on the complexity of DNA methylation (reviewed by Rothbart and Strahl [2014]) as well as its interaction with posttranslational histone modifications in chromatin regulation [Rose and Klose, 2014].

Although there have been some major advancements made into investigating the role of DNA methylation in disease, little is known about the role of 5hmC, 5fC or 5caC. However, the processes that regulate DNA demethylation have been implicated in cancer. For example, fusion of *MLL* to *TET1* is important for acute myeloid leukaemia [Ono *et al.* 2002], and inactivating *TET2* mutations are frequently found in myeloid lineage malignancies [Delhommeau *et al.* 2009; Langemeijer *et al.* 2009]. Interestingly, in nonmyeloid cancers, downregulation of TET expression has been observed in human breast, liver, pancreatic and prostate cancer [Yang *et al.* 2013]. These disruptions in TET are associated with a decrease in 5hmC, which is in keeping with findings from combined TET1 and TET2 knockout mice that show decreases in 5hmC and increased 5mC expression [Dawlaty *et al.* 2013].

DNA methylation and gene regulation

It is well established that hypermethylation of promoter elements results in transcriptional silencing [Nakano *et al.* 2013]. This inverse correlation between DNA methylation and transcriptional activation has been verified in numerous experiments in which plasmid DNA is *in vitro* methylated and transfected into target cells [Yisraeli *et al.* 1988; Kass *et al.* 1997]. Similarly, increased gene expression is also demonstrated following culture with the demethylating agent 5-azacytidine [Christman, 2002]. There are two proposed hypotheses for explaining the mechanism for transcriptional silencing mediated by DNA methylation. One hypothesis that has been proposed is that methylation can directly inhibit binding of transcriptional regulators. The use of reporter constructs in which *in vitro* methylation has been shown to reduce the binding of a number of different transcription factors [Eden and Cedar, 1994]. This interference has been demonstrated for a number of transcription factors such as c-Myc [Prendergast and Ziff, 1991], E2F [Di Fiore *et al.* 1999; Campanero *et al.* 2000], nuclear factor of activated T cells (NFAT) [Cribbs *et al.* 2014] and NF-κB [Bednarik *et al.* 1991], all of which have a CpG dinucleotide within their core binding motif. A second hypothesis proposes that methylation-specific binding proteins (MBP) interact with methylated DNA and bind to prevent transcriptional initiation complexes [Boyes and Bird, 1991]. The family of MBP proteins is composed of five members, methyl-CpG-binding protein 2 (MeCp2), MBP1, MBP2, MBP3 and MBP4. With the exception of MBP4, which is involved in the

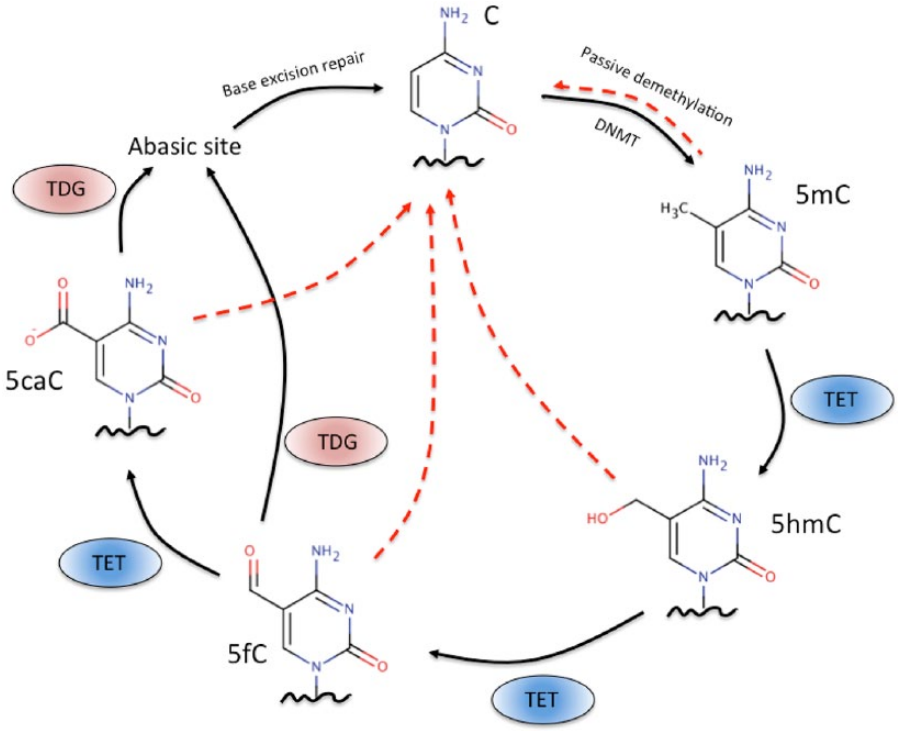

Figure 1. The DNA demethylation cycle. Cytosine methylation is catalyzed by DNA methyltransferases (DNMTs). 5mC is hydroxylated by the ten–eleven translocation enzymes (TETs) to yield 5-hydroxymethylcytosine (5hmC), which in turn is oxidized to 5fC and 5caC. During passive demethylation, 5hmC, 5fC and 5caC are diluted in a replication dependent manner to regenerate an unmodified C. In a process of active restoration, 5fC and 5caC can be excised by TDG generating an abasic site as part of the base excision repair (BER) process that regenerates unmodified C. Red arrows denote passive DNA demethylation mechanisms and black arrows denote active DNA demethylation.

DNA repair process, these proteins associate with histone deacetylases (HDACs) and result in transcriptional silencing through altered chromatin structure [Wade *et al.* 1999; Momparler, 2003]. DNA methylation also directs dimethylation of histone 3 lysine 9 (H3K9me2), a repressive chromatin mark, through the interaction of DNMT1 with the replication complex [Hashimshony *et al.* 2003; Esteve *et al.* 2006]. In addition, there is also evidence to suggest that DNA methylation inhibits histone 3 lysine 4 (H3K4) methylation, a mark indicative of active transcription, however the precise mechanism underpinning this is currently unknown [Hashimshony *et al.* 2003; Okitsu and Hsieh, 2007]. Therefore, it seems likely that a DNA methylation profile acts as a scaffold to maintain transcriptional repression patterns through modification of active marks on histone tails.

DNA methylation in RA

Whole genome analysis of aged individuals has highlighted a number of hypomethylated regions that may contribute to age-related diseases, including some autoimmune diseases such as RA [Heyn *et al.* 2012]. The first evidence to suggest that DNA methylation may play a role in ageing and autoimmunity came from studies investigating the effect of the DNA methyltransferase inhibitor 5-azacytidine that can induce symptoms associated with autoimmunity [Richardson, 1986; Richardson *et al.* 1986]. Moreover, there is now a large body of evidence to suggest that patients with certain autoimmunity syndromes such as systemic lupus erythematosus (SLE) display a globally hypomethylated genome [Liu *et al.* 2011a, 2011b]. Specifically for RA, differential DNA methylation has been demonstrated in the IL-6 promoter [Nile *et al.* 2008]. In this paper, Nile and colleagues isolated DNA from peripheral blood mononuclear cells (PBMCs) and determined the methylation status of the IL-6 promoter. They found that IL-6 promoter methylation reduced transcriptional activity and identified a single CpG within the IL-6 promoter that was key to regulating IL-6 gene expression.

Similarly, another study identified an association between smoking and hypomethylation of a CpG motif in the IL-6 promoter of chronic periodontitis patients [Ishida *et al.* 2012]. However, a note of caution needs to be made when interpreting studies where the data has been generated using DNA isolated from whole PBMC populations. A large proportion of RA patients can present with leukopenia, with altered PBMC cellular frequencies and compositions. Since methylation patterns are cell-type specific, and the balance of T cells in the RA patients can be significantly altered, results from unsorted cell populations may skew results and interpretations from whole blood methylome analyses [Martens *et al.* 1997; Lawson *et al.* 2006].

More recently, studies have begun to utilize new technologies and take into account that some of these differences could be attributed to the altered frequency of cell populations. Liu and colleagues performed a genome-wide epigenome analysis on PBMC populations using the Illumina 450K methylation bead array and used a series of statistical algorithms to reduce the confounding factors that have hampered previous analysis [Liu *et al.* 2013]. Using this approach they were able to filter out methylation changes that are likely to cause disease, rather than those changes that are a consequence of disease. The consequence of this analysis was the identification of two clusters within the MHC region whose differential methylation pattern potentially mediates genetic risk for RA. In the general context of disease pathogenesis, this study is important because it suggests that DNA methylation is a critical mechanism for conferring disease in already identified associated genes such as the MHC region. However, one limitation of this study is that the contribution of other disease correlated cell types, such as synovial fibroblasts are missing from their analysis. Moreover, since the study relies on the use of computational approaches to correct for differences in cell type, the results from this study should be validated in isolated populations. Therefore, although this study gives us the first glimpse of the relationship between DNA methylation and genetic susceptibility, further investigations exploring the functional consequences of these changes need to be conducted.

DNA methylation in RA fibroblasts

Epigenetic investigations into synovial fibroblasts isolated from RA patients have so far provided the best evidence that epigenetic modifications contribute to the disease pathology of RA. RA synovial fibroblasts (RASF) are intensively studied cells primarily because they are abundant in the joint and are key contributors to the high levels of cytokine expression seen in RA. RASF display a DNA methylome pattern distinctive from osteoarthritis synovial fibroblasts (OASF) that contributes to their pathogenic function [Nakano *et al.* 2013]. Early reports have suggested that RASF display a hypomethylated genome [Karouzakis *et al.* 2009]. This was specifically shown in a number of key genes regulating cell migration, adhesion and extracellular matrix interactions. Intriguingly, DNMT1 expression was reduced in RASF compared to OASF and may provide a mechanism for the reduction in methylation [Karouzakis *et al.* 2009]. However, despite the apparent global hypomethylation seen in RASF from early studies, later studies have not confirmed this finding [Takami *et al.* 2006; De La Rica *et al.* 2013; Nakano *et al.* 2013; Whitaker *et al.* 2013]. Specifically, a number of differentially methylated regions, including both hypomethylated and hypermethylated regions were observed. In addition, the reduction of DNMTs shown in the earlier studies has also not been confirmed in later studies [Nakano *et al.* 2013]. Notwithstanding these results, a note of caution should be made regarding the findings from RASFs because many studies remove the RASF cells from their environmental niche and passage them *in vitro*. However, although Nakano and colleagues have shown that the methylation pattern in RASF are stable following multiple passages [Nakano *et al.* 2013], more work is required to determine whether some of these observed differences are also present in freshly isolated RASFs.

DNA methylation in RA T cells

The role of T cell DNA hypomethylation in the autoimmune disease SLE is well established (reviewed by Zhang *et al.* [2013]), however little is currently known about the role of DNA methylation in T cells from RA affected individuals. The first evidence to suggest that RA patients exhibit dysregulated T-cell methylation patterns was provided by Richardson and colleagues who showed global hypomethylation in CD4+ RA T cells when compared to healthy controls [Richardson *et al.* 1990]. Looking at gene-specific methylation patterns at critical factors, it was more recently discovered that $CD4^{+}CD28^{-}$ T cells, a population of pro-inflammatory T cells found frequently in

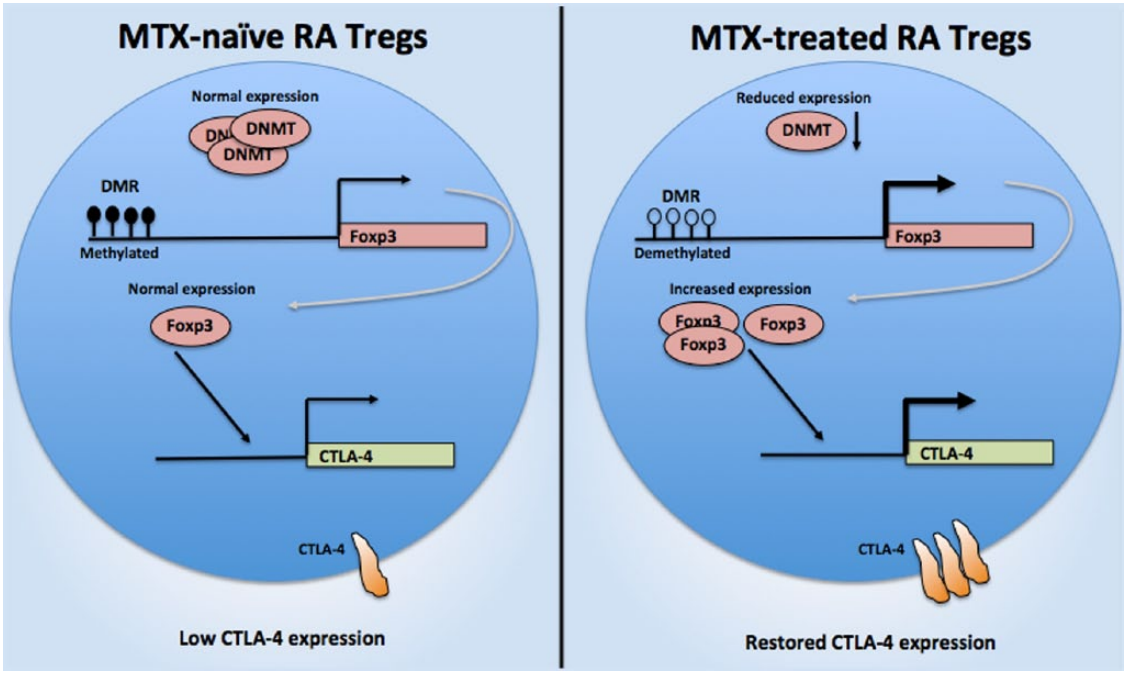

Figure 2. Methotrexate (MTX) treatment restores regulatory T cell (Treg) function in rheumatoid arthritis (RA) patients. In MTX naïve RA Tregs, a newly identified differentially methylated region (DMR) is methylated and this is associated with normal Foxp3 expression but reduced CTLA-4 expression. In patients treated with MTX, a reduction in DNMT1 expression correlates with reduced methylation of the DMR in Tregs. This leads to increased Foxp3 expression and a consequential increase in CTLA-4 expression that restores suppressive function. Overall, this suggests that MTX can induce epigenetic modifications in RA Tregs that lead to normal suppressive function and highlight a newly identified mechanism of action for MTX.

RA, have a significantly hypomethylated *IFNG* promoter than conventional $CD4^+CD8^+$ T cells and produced higher levels of IFN-γ following TCR stimulation [Pieper *et al.* 2014]. DNA demethylation also plays a role in the regulation of the X linked gene *CD40L* in $CD4^+$ T cells and may in part explain the female prevalence of RA [Liao *et al.* 2012]. Similarly, DNA methylation dynamics plays a key role in the differentiation of different Th cell subsets [Cohen *et al.* 2011]. Janson and colleagues demonstrated that $CD4^+$ T cells in the synovial joint are committed towards a Th1 and a regulatory T-cell phenotype. They demonstrated that *IL17A* gene expression was regulated by promoter methylation however Th17 commitment was not a common feature found in RA synovial joints [Janson *et al.* 2011]. Work from our own laboratory has shown that DNA methylation contributes to dysregulated regulatory T-cell (Treg) function [Cribbs *et al.* 2014]. In untreated RA patients, we found that reduced CTLA-4 protein expression was a consequence of increased methylation of a single CpG dinucleotide in the *CTLA4* promoter. Methylation of this site prevented the binding of the transcription factor, NFAT, which in turn led to a reduction in the transcriptional activity of the *CTLA4* gene. [Bernard, 2014; Cribbs *et al.* 2014]. Subsequently, we showed that in a cohort of RA patients treated with methotrexate (MTX), demethylation of the upstream enhancer in the *FOXP3* locus in RA patients is correlated with decreased Dnmt1 expression and increased expression of Foxp3 protein levels [Kennedy *et al.* 2014; Cribbs *et al.* 2015]. A consequence of this is that CTLA-4 protein expression normalizes and Tregs are subsequently able to suppress effector T-cell responses (Figure 2). Overall, this suggests that MTX can induce DNA methylation changes in the *FOXP3* locus that regulate the expression of Foxp3 in established RA disease, leading to increased Treg function and reduced disease activity.

Therapeutic implications of altered DNA methylation patterns in RA

In malignant cells, global DNA hypomethylation is a commonly observed feature [Feinberg and Vogelstein, 1983], and the most important DNA methylation changes occur in CpG rich promoter

regions. Despite the global hypomethylation pattern observed, around 5–10% of normally unmethylated CpG island promoters often display abnormal methylation in cancer genomes [Dawson and Kouzarides, 2012], affecting a set of critical tumour suppressors that are aberrantly silenced [Herman *et al.* 1994]. This has led to the implementation of strategies designed to reduce the overall DNA methylation in these regions, primarily through targeting DNMT activity. Currently, 5-azacytidine and decitabine are two FDA-approved therapies for use in myelodysplastic syndrome, a treatment that targets changes in DNA methylation patterns. Several studies have shown that 5-azacytidine and decitabine therapy can significantly extend survival time and improve quality of life, even in patients with late-stage disease [Kantarjian *et al.* 2006; Fenaux *et al.* 2009]. However, it is unlikely that those DNMT inhibitors will be of any significant benefit in RA since treatment of RASF with 5-azacytidine leads to increased activation, suggesting that hypomethylating treatments may result in increased disease [Karouzakis *et al.* 2009]. Moreover, 5-azacytidine and another DNMT inhibitor, procainamide, can induce self-reactivity in $CD4^+$ T cells and are both sufficient to induce lupus-like disease [Richardson, 1986; Richardson *et al.* 1986; Quddus *et al.* 1993]. However, the recently described DNA methylation inhibitor, zebularine, has been used in combination with IFN-γ *in vitro* to synergistically induce the immunomodulatory indoleamine 2,3-dioxygenase (IDO) pathway [Zhou *et al.* 2002; Xue *et al.* 2012]. This suggests that in order for DNMT inhibitors to be successful and provide superior outcomes over established therapies they need to be given in the right disease context. Likewise, DNMT inhibitors may be more successful when supplied in combination with other epigenetic therapies such as HDAC inhibitors [Oppermann, 2013], a finding also reported in oncology [Gore, 2011].

Another promising therapeutic approach that may have a benefit for RA and is indirectly related to DNA methylation was suggested from the results of Karouzakis and colleagues. The authors found that increased expression of S-adenosyl methionine decarboxylase (AMD), spermidine/spermine N1-acetyltransferase (SSAT-1) and polyamine-modulated factor binding protein 1 (PMFBP-1) led to reduced levels of the methyl-donor SAM, which was associated with hypomethylation in RASF [Karouzakis *et al.* 2012]. It was suggested that the deficiency of SAM in RASFs could be overcome with dietary supplementation with SAM or L-methionine [Klein and Gay, 2013]. However, the authors suggest that the high levels of SSAT-1 expression would make this an unattractive therapeutic option, since the high levels of SSAT-1 expression would constantly consume the SAM. More recently, it has been shown that diminazene aceturate (DA), an inhibitor of SSAT-1, can restore both the levels of DNA methylation and the expression of DNMT1, leading to reduced RASF invasive function [Neidhart *et al.* 2014]. These results open up the possibility of using pharmacological therapy to restore the DNA methylation patterns in RASF, and subsequent efforts are being made to develop a more specific inhibitor of SSAT-1 [Onuora, 2014].

Despite the aforementioned advantages and disadvantages of targeting DNA methylation pathways for treating RA, there is indeed evidence to suggest that current therapies can actually modulate their anti-inflammatory effects through modification of DNA methylation. MTX, a therapy used successfully in high doses in oncology and at low dose in RA for several decades, is one example of a routinely used drug having epigenome modifying effects. For example, it has been known for a number of years that in human tumours, administration of MTZ induces profound DNA hypermethylation [Nyce, 1989]. Similarly in RA, MTX was found to reverse global DNA hypomethylation [Kim *et al.* 1996]. However, the mechanism underpinning this phenomenon is currently unknown and certainly warrants further investigation. Intriguingly, this raises the possibility that other drugs used to treat RA may also act long-term by altering and reversing causative epigenetic changes underlying chronic disease.

DNA methylation as a biomarker for RA

Despite the challenges associated with DNA methylation as a therapeutic target, its biggest potential impact may come from its use as a biomarker to determine response to therapy. An important issue that still needs to be addressed in epigenetic research is whether any identified disease-associated epigenome change precedes disease onset, or is a result of the ongoing disease process. Identification of aberrant DNA methylation changes before disease onset may lead to a better understanding of the risk factors that contribute to disease development and thus result in the identification of biomarkers for disease. However, this is currently very challenging to

achieve because of the limited sample availability in predisease states.

A more realistic approach to using DNA methylation as a biomarker would be to identify responders and nonresponders to therapy. There have been a number of successful therapies for RA, however despite this, a proportion of patients fail to respond to conventional therapy. Ideally, it would be useful to identify this fraction of nonresponders earlier, in order to provide better treatment regimes that are tailored towards the individual patient. It is becoming clear that RA patients display a differentially methylated genome when compared to healthy individuals. This raises the possibility that measuring DNA methylation patterns of responders and nonresponders may lead to the use of DNA methylation as a predictive biomarker for treatment response. This approach has been validated in colorectal cancer, where circulating methylated DNA in peripheral blood can be measured using a simple polymerase chain reaction (PCR) assay [Lofton-Day *et al.* 2008; Lange *et al.* 2012]. More recently, reverse transcriptase (RT)-PCR assays have been developed to quantify the number of $Foxp3^+$ cells within tissue samples [Wieczorek *et al.* 2009]. Collectively, these techniques have revealed the potential for DNA methylation to be used as a biomarker for disease status and suggest that it may be useful for identifying patient response to therapy.

Conclusion and future direction

In recent years, a major advancement has taken place in understanding the role of DNA methylation in the pathogenesis of RA. It is hoped that the progress made in identifying epigenetic mechanisms occurring in cancer can also be exploited in inflammatory disease. The substantial clinical progress obtained by introducing highly effective biological treatments for autoimmune diseases will also lead to a better understanding of epigenetic factors that contribute or control the phenotype and clinical responses.

However, a consideration regards to the complexity of DNA methylation regulation in different cell types needs to be made. The main thrust of epigenetic research in RA is currently focused on synovial fibroblasts, not just because they are an important cell type that contributes to RA pathogenesis, but because they can be easily grown in sufficient quantities, thereby facilitating epigenome investigations that frequently rely on large numbers of cells to be analysed. However, if we are to completely understand the contribution of epigenetic modifications to disease then we need novel approaches that allow us to investigate all populations of cells from RA patients. Therefore, it is imperative to employ strategies that use cell sorting techniques to isolate cells to homogeneity and employ technologies that allow analysis of epigenetic modifications with small cell numbers. Technically, this has been hampered by the requirement for large quantities of DNA to perform techniques such as DNA sequencing and chromatin immunoprecipitation (CHIP) technology. However, the progress made in miniaturizing these technologies, and importantly the reduction in cost, will allow more precise and detailed genome wide analysis of epigenetic modifications in all cell populations in RA [Shankaranarayanan *et al.* 2011] and will constitute a further important step towards personalized medicine. One other area of epigenetics that still needs to be explored is the relationship in our understanding between the specific environmental factors and the influence on DNA methylation profiles [Javierre *et al.* 2011], which could provide the ultimate link between environmental factors and manifestation of nongenetic, but epigenomic effects leading to disease. It is hoped that in the near future in combination with technological advances some of these questions will be adequately addressed.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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