



Review

Innate immunity, epigenetics and autoimmunity in rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by the progressive and irreversible destruction of joints.

RA remains an incurable condition, although a new class of drugs, biologicals, have made a major breakthrough in targeting and/or eliminating the immune cells, including T cells, B cells and monocytes/macrophages from the joints. That we cannot (yet?) cure the disease is most likely due to the lack of therapeutic targeting the endogenously activated RA synovial fibroblasts (RASf).

Most interestingly, RASf express Toll-like receptors (TLRs) 1–6 rendering them prone to activation by exogenous and endogenous TLR ligands and resulting in the production of numerous powerful chemokines and cytokines. These factors are responsible for the repopulation of immune cells in the joints after ceasing cell depleting therapies.

To characterize the molecular mechanisms of synovial activation, a new approach studying the epigenetic characteristics of RASf has been recently undertaken. Thereby, the pattern of histone acetylation, DNA methylation and gene expression regulating microRNA are being explored.

Since auto-antibodies have the most predictive and diagnostic value for RA, it is challenging to study more comprehensively the contribution of auto-antibodies to the disease. A new screening technique, serological analysis of recombinant human cDNA expression library (SEREX), adapted from cancer research allowed for the identification of novel auto-antibodies in RA, including anti-serpin E2 auto-antibodies. The serpin E2 auto-antibodies were found to inhibit the activity of serpin E2 and have potentially a functional role in the disease. The recent findings in the field of innate immunity, epigenetics and autoimmunity related to the pathogenesis of RA are in the scope of this review.

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1. Introduction

Rheumatoid arthritis is a chronic autoimmune disease of which the main characteristic is irreversible joint destruction (Harris et al., 1970). Even though it is known that persistent activation of the immune system in RA leads to autoimmunity, the trigger activating the immune response remains unclear (Gorony and Weyand, 2005). Before the turn of the century it was taken as a fact from clinical studies that non-steroidal anti-inflammatory drugs (NSAIDs) as well as disease modifying anti-rheumatic drugs (DMARDs) did not result in long-term effectiveness in patients with RA. Most patients still suffered from functional decline, radiographic progression, work disability and premature mortality. Now, biological-response modifiers including recombinant inhibitors of TNF α (infliximab, etanercept, adalimumab), IL-1 (anakinra), T cell co-stimulation (abatacept) and B cell targeted antibodies (rituximab) are powerful drugs for the majority of patients. However, since not all patients

respond to these therapies and the disease cannot be cured yet, research has focused on the cells at the site of joint destruction. More attention is now paid, for example, on targeting the bone resorbing osteoclasts (Bull et al., 2008; Herman et al., 2008). Surprisingly, no interest was raised so far on the notion that, next to the development of autoimmunity and activation of the cytokine driven pathway of joint destruction, a cytokine-independent pathway driven by intrinsically activated RASf is another important part in the pathogenesis of RA (Gay et al., 1993; Ospelt et al., 2004). In this review we focus on the stimulating work done in the past years on innate immunity, epigenetic modulations in synovial cells and autoimmunity in RA. These new results should be inspiring for the development of innovative strategies in the treatment of RA.

2. Innate immunity

Unlike adaptive immunity, innate immunity has a role in non-specific recognition of pathogens. The innate immune system constitutes the first defence against microbial particles such as viruses or bacteria, by which it is activated via pathogen associated molecular patterns (PAMPs) ligating to pattern recognition recep-

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tors (PRRs). Activation might also occur by other stress factors such as heat, fractures, necrosis, apoptosis, hypoxia or carcinogenesis via diverse molecules expressed on stressed, injured, infected, or transformed human cells that also act as PAMPs (Hietbrink et al., 2006; Rius et al., 2008; Wallin et al., 2002). The role of innate immunity in the organism is the identification of foreign substances and their removal by phagocytosis or nitric oxide (NO) production and the recruitment of immune cells to sites of infection or inflammation by producing cytokines. Thereby, activation of the complement cascade and the adaptive immune system by antigen presentation results in the inflammatory response. We summarize here what is currently known about PRRs and the possible ligands to shed more light on the pathogenesis of RA and on possible targets for novel therapies.

2.1. Toll-like receptors (TLR) in RA

In our body, the cells of the innate immune system are equipped with PRRs to be ready for an immediate response to an offensive event. In this regard, it is interesting that synovial fibroblasts, especially when derived from RA and other inflammatory conditions, express on their surface a range of TLRs belonging to the group of membrane associated PRRs. These cells have therefore the ability to react immediately or within few hours to a wide range of appearing pathogens but also to react to interactions with human cells expressing TLR ligands and induce an immune response. Since RASF express increased levels of TLR2, 3 and 4, they are more prone to activation by diverse TLR ligands when compared with osteoarthritis (OA) SF (Brentano et al., 2005b; Kim et al., 2007).

TLR1 mainly signals in a complex with TLR2 (Takeuchi et al., 2002). This complex recognizes tri-acetylated lipopeptides. TLR1 has also been found to heterodimerize with TLR10 (Hasan et al., 2005). The ligand of this complex is, however, not yet known. TLR1 is expressed in leukocytes and in non-immune cells such as astrocytes, fibroblasts, keratinocytes, endothelial and epithelial cells (Ospelt and Gay, in press). It was found to be expressed also by synovial fibroblasts, including RASF (Ospelt et al., 2008).

TLR2 has been identified to bind Gram-positive bacterial cell wall proteins such as peptidoglycans (PGN) and bacterial lipopeptides (Brightbill et al., 1999; Underhill et al., 1999). Most importantly, TLR2 has been reported to be present at higher levels in RASF than in OASF as well as in CD14+ macrophages from RA joints when compared to controls, like in vitro differentiated macrophages or peripheral blood monocytes from the same patients (Huang et al., 2007; Kim et al., 2007). Stimulation of TLR2 in RASF up regulates the production of pro-inflammatory cytokines IL-6 and IL-15, chemokines, as well as matrix degrading metalloproteinases (MMPs) and adhesion molecules (Jung et al., 2007; Kyburz et al., 2003; Pierer et al., 2004). Both of the ligands for TLR2 and 4 induce the production of RANKL by RASF and subsequent osteoclastogenesis (Kim et al., 2007). The heat shock proteins Hsp60, Hsp70, Gp96 and high mobility group box 1 protein (HMGB1) function as host derived ligands for TLR2 and have been described to play a role in the pathogenesis of RA (Prakken et al., 1997; van Beijnum et al., 2008). Moreover, HMGB1, in contrast to heat shock proteins, was already described to stimulate the TLR pathways in RA (van Beijnum et al., 2008).

TLR3 is a receptor for single-stranded RNA. This receptor is expressed in the endosome membrane and is the only known TLR that acts independently from the MyD88 signalling pathway. Brentano et al. (2005b) in our group found TLR3 to be at higher levels in RA synovial tissues than in OA. TLR3 was present mainly in the synovial fibroblasts of the lining layer and stimulation of TLR3 signalling in RASF was shown to induce the expression of IFN- β , CXCL-10, CCL-5, and IL-6. Most interestingly, necrotic cells from synovial fluid delivered unidentified ligand binding and

stimulating TLR3, subsequently leading to the production of the aforementioned pro-inflammatory cytokines. Furthermore, during acute inflammatory events, TLR3 was shown to serve as an endogenous sensor of necrosis and to play a role as a regulator of the amplification of immune response (Cavassani et al., 2008).

Another TLR over-expressed by RASF is TLR4. TLR4 is present on the surface of cells and binds lipopolysaccharides (LPS), a component of the outer membrane of Gram-negative bacteria. Several endogenous host ligands binding TLR4 were identified. Among them was fibrinogen, fibronectin extra domain A, hyaluronan, HMGB1 and several heat shock proteins (Brentano et al., 2005a). Even though heat shock proteins, fibrinogen and hyaluronan have not yet been demonstrated to play a functional role as a ligand for TLR4 in RA, they are present in the synovial fluid in RA patients and await investigation whether they stimulate TLR4 signalling. It has been reported that the TLR4 pathway was activated by incubation of CHO/CD14/HuTLR-4 reporter cells with synovial fluids and sera of RA patients (Roelofs et al., 2005). In our laboratory Sanchez-Pernaute et al. (2007) could show that fibrin stimulates the expression of pro-inflammatory cytokines in RASF via TLR4. Since fibrin clots are abundantly present in the synovium of patients with RA and free fragments of fibrin appear in the synovial fluid (Gormsen et al., 1971; Sanchez-Pernaute et al., 2003), fibrin could contribute to the stimulation of RASF via the induction of the TLR4 pathway. Furthermore, citrullinated fibrin found in RA synovial tissues could possibly signal in a similar manner.

TLR5 is a receptor for flagellin, the major structural protein of the flagella of Gram-negative bacteria. TLR5 is expressed mainly on monocytes and T cells. Nevertheless, on synovial fibroblasts, including RASF low levels of TLR5 were detected (Ospelt et al., 2008). TLR5 expressed on the surface of RASF was shown to be functional since stimulation of RASF with flagellin resulted in the induction of the pro-inflammatory cytokine IL-6.

TLR6 forms heterodimers with TLR2 and this complex is a receptor for di-acetylated lipopeptides. TLR6 is expressed on monocytes, macrophages, B cells, T cells, DCs, PMN, NK cells as well as on non-immune cells. It was also found to be expressed in synovial fibroblasts, including RASF (Ospelt et al., 2008).

Similarly to TLR3, also TLR7 has been found at higher levels in the RA synovium comparing to OA or healthy synovial tissues (Roelofs et al., 2005). Since RNA acts as a TLR7 ligand, it has been demonstrated that B cells are activated by RNA and RNA-associated autoantigens via the synergistic stimulation of B cell antigen receptor and the TLR 7 (Lau et al., 2005).

TLR9 is localized in the endoplasmic reticulum and detects internalized microbial DNA with unmethylated CpG motifs and stimulates B cell activation, proliferation, and the production of IFN- α and IgM. Moreover, TLR9 is expressed by plasmacytoid DCs and B cells. Even though this receptor has not been detected on RASF it has been shown to play a certain role in the pathogenesis of RA. Namely, TLR9 participates in the stimulation of rheumatoid factor (RF) producing B cells since it acts synergistically with the B cell receptor (Chaturvedi et al., 2008). It has also been reported that the stimulation of TLR9 by microbial non-methylated CpGs in combination with the B cell receptor leads to a class switch from IgM to more pathogenic IgG (He et al., 2004). Interestingly, it has been demonstrated that ribosomal DNA (rDNA) containing hypomethylated DNA released from host derived dead cells is present at increased levels in the RA serum (Veiko et al., 2006). This rDNA fragments are thought to stimulate TLR9 signalling. Since Karouzakis et al. in our group could show that the global methylation is reduced in RA synovial tissues and isolated RASF (2007), it is also possible that hypomethylated endogenous DNA derived from apoptotic cells could bind to TLR9 and the innate immune system would recognize hypomethylated host DNA as pathogenic. DNA hypomethylation is known to be essential for apoptotic DNA to

induce systemic lupus erythematosus (SLE)-like autoimmune disease in SLE-non-susceptible mice (Wen et al., 2007). Interestingly, it has been reported that RF⁺ B cells respond to IgG2a immune complexes that contain DNA, but not to monomeric IgG2a antibodies alone (Leadbetter et al., 2002).

The expression of TLR on RASF can also be induced by pro-inflammatory cytokines, heat shock or hypoxia (Seibl et al., 2003). Consequently, activated RASF produce NO which could affect the surrounding tissue (Yao et al., 2005). Activated RASF and macrophages also produce tissue degrading enzymes, like MMP-1, -2, -13 and -14, pro-inflammatory chemokines, like GCP-2, RANTES, MCP-1 and 2, CXCL-16 and cytokines including TNF α , IL-6 and IL-8, that recruit immune cells into the synovium. Moreover, RASF promote invasion of immune cells by expressing adhesion molecules such as VCAM-1 and ICAM-1. Thereby, activated RASF support consistently the survival of immune cells by constituting a favourable milieu for producing pro-inflammatory cytokines, anti-apoptotic proteins and growth factors (Kyburz et al., 2003; Pierer et al., 2004). Taken these results together, it can be concluded that inhibition of TLR signalling could be an attractive approach for therapy in RA. On the other hand, inhibition of TLR signalling may also compromise the immune response against dangerous infections.

2.2. Shared epitopes as part of innate immunity

Recently an interesting article was published presenting a novel concept considering the shared epitope, where it was reported to act as an endogenous ligand stimulating the innate immunity response (Ling et al., 2007). The shared epitope has long been known to be associated with the occurrence of RA (de Vries-Bouwstra et al., 2008). Therefore, it has been speculated that a molecular mimicry between a pathogen and HLA-DR β plays a role in the pathogenesis of RA (Traherne, 2008). Thereby, the T cells were also suggested to recognize a specific self-peptide bound to HLA-DR β and trigger an autoimmune response. However, no single specific peptide has ever been identified. Ling et al. (2007) have now shown that the shared epitope binds to cell surface calreticulin and subsequently triggers the innate immune response such as the production of NO. In individuals positive for HLA-DR β smoking significantly increases the risk of developing RA (Klareskog et al., 2006). On the other hand smoking might induce ligands to trigger TLR signalling (Ospelt and Gay, in press). In conclusion, the latest research proving the involvement of the innate immune system into the pathogenesis of RA contributes to a better understanding of the initiation of the disease as well as might point to possible targets for novel therapies in RA.

3. Epigenetics

The term epigenetics defines modifications in the genome, inheritable over cell generations but not involving the base pair sequence of the DNA. Epigenetic modulations include DNA methylation and histone modifications such as acetylation, methylation (Trenkmann et al., 2008), phosphorylation, ribosylation, ubiquitination and sumoylation (Fig. 1).

So far several epigenetic abnormalities have been described in cancer cells. Recent studies on epigenetics, including DNA methylation and modulations of histone acetylation have also contributed to a better understanding of the pathogenesis of RA. The first report on aberrations in the epigenetic code in RA was published already in 1990 (Richardson et al.). The authors showed that the DNA in T cells of RA patients is hypomethylated. Few years later, we have shown that RASF are intrinsically activated and resemble tumor-like cells in some aspects (Muller-Ladner et al., 1996). Activation of synovial cells leads to an increased production of powerful pro-inflammatory cytokines that in turn attract more inflammatory cells to the joints (Ospelt and Gay, 2008). RASF produce also increased levels matrix degrading enzymes as well as adhesion molecules. Moreover, RASF have intrinsically high levels of small ubiquitin modifier 1 (SUMO-1) which contributes to their resistance to apoptosis (Meinecke et al., 2007). RASF also have increased levels of expression of proto-oncogenes and cell cycle proteins suggesting transformation (Davis, 2003). However, since RASF do not proliferate extensively we prefer to refer to them as cells with activated phenotype (Ospelt and Gay, 2008). It needs to be shown in the future that the epigenetic changes in the SF could lead to this activated phenotype of RASF.

3.1. DNA methylation in RA

DNA methylation in gene promoters containing CpG islands takes place on cytosines of the CpGs, and is connected to a decreased transcription of genes, since transcription factors generally cannot bind to promoters containing methylated CpG islands. In this manner genes in heterochromatin are silenced.

Recent investigations in our group have shown that RASF have decreased levels of global DNA methylation (Karouzakis et al., 2007), which leads to an increased expression of cell activating genes and might stimulate the innate immune response via TLR9. We reported further that the transcription of the retrotransposable element LINE-1 is reactivated in RASF due to hypomethylation of CpG islands in its promoter (Neidhart et al., 2000; Karouzakis et al., 2007). A single unmethylated CpG in a promoter of IL-6 in

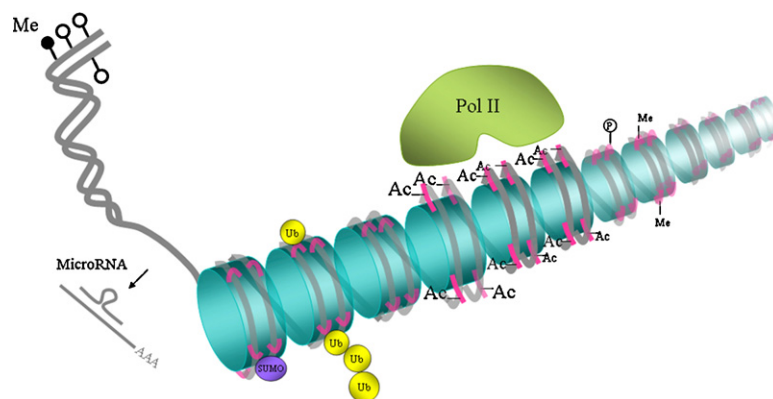


Fig. 1. Epigenetic modulations of the chromatin. The global hypomethylation of the DNA in RA leads to an increase in gene expression. Hyperacetylation of histones in specific promoter sites renders the DNA region accessible for the transcription machinery. MicroRNA encoded by the DNA silences specific genes in a post-transcriptional manner. Other modifications of histones are indicated such as sumoylation, ubiquitination, methylation and phosphorylation. Methylated cytosine in DNA is shown as filled and non-methylated as open circles. The modifications of histone tails are indicated as follows: SUMO, sumoylation; Ub, ubiquitination; Ac, acetylation; P, phosphorylation; Me, methylation.

monocytes was also reported rendering the expression of IL-6 more inducible by LPS stimulation (Nile et al., 2008). On the contrary, the CpG island in the promoter of death receptor 3 (DR3), a member of the apoptosis-inducing Fas gene family, was shown to be specifically methylated in first or second passage RA synovial cells and the expression of DR3 was accordingly downregulated (Takami et al., 2006). The authors concluded that the downregulation of DR-3 could be responsible for the resistance to apoptosis in these cells. This finding resembles certain aspects observed in malignant cells where methylation of specific promoters, for example, in tumor suppressor genes, is observed.

3.2. Histone acetylation in RA

Acetylation of histones H3 and H4 in the nucleosomes of gene promoters is related to the activation of gene expression (Grunstein, 1990; Struhl, 1998). This fact is thought to be due to the binding of an acetyl group to a lysine residue in histones that abolishes the positive charge on the lysine and thus loosens the tight interaction between the positively charged histone octamer and negatively charged DNA. Therefore, the DNA becomes accessible for the transcription machinery. The status of histone acetylation depends on the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Ito and Adcock, 2002). It has been described by Huber et al. (2007) in our laboratory that in RA synovial tissue the balance of HAT/HDAC activity is strongly shifted towards histone acetylation. Specifically, we could show a downregulation of HDAC1 and 2 in the synovium of patients with RA. However, isolated RASF do not have a changed expression of HDAC1 and 2 compared to OASF (Hemmatzad et al., 2008).

In other studies, it has been observed that the HDAC inhibitor FK228 *in vivo* inhibits joint swelling, synovial inflammation and joint destruction in mice through the induction of p16INK4a and the upregulation of p21(WAF1/Cip1) (Nishida et al., 2004). Furthermore, FK228 was recently described to suppress the production of VEGF *in vivo* and to block angiogenesis in synovial tissue in collagen-antibody-induced arthritis (Manabe et al., 2008). Another HDAC inhibitor, Trichostatin A (TSA), was shown to induce cell cycle arrest and apoptosis in RASF and to be particularly effective in combination with ultrasound treatment (Nakamura et al., 2008). TSA was also demonstrated to sensitize RASF to TRAIL-induced apoptosis, however, in this study TSA had no effect on apoptosis when used in the absence of TRAIL (Jungel et al., 2006). In conclusion, on one hand there is a significant downregulation of single HDACs, on the other hand inhibitors of HDACs ameliorate the disease in animal models. These reports suggest that HDACs might be less active in RA and/or misplaced in the subcellular compartments, for example, being localized aberrantly to specific promoter sites, and thereby contributing further to the pathogenesis of RA. However, it needs to be also taken into account that HDACs have in addition non-histone proteins for substrates. Therefore, it might be considered whether inhibition or rather redirection to a proper localization of HDACs is feasible for the treatment of RA (Maciejewska et al., 2008).

3.3. MicroRNA in RA

MicroRNAs (miRNA) are a recently recognized class of modulators of gene expression. MicroRNA are small non-coding single-stranded RNA molecules that downregulate gene expression on the post-transcriptional level. The expression pattern of miRNA has been suggested to be altered in RA. Indeed, expression of miR-155 and miR-146a was found to be higher in RASF than in OASF (Nakasa et al., 2008; Stanczyk et al., 2008b). Moreover, the expression of miR-155 was further enhanced by TNF α , IL-1 β and the ligands of TLR2, 3 and 4 (Stanczyk et al., 2008b). Interestingly, the expression of miR-155 in RASFs had a repressive effect

on the expression of MMP-1 and MMP-3, suggesting an induction of counter regulatory mechanisms in the circle of inflammation and destruction. However, in another study it has been proposed that miR-146a, even though expressed at higher levels in RA, is not able to perform its function as a negative regulator for the expression of TRAF6 and IRAK-1, two molecules involved into the pathway of TNF α synthesis (Pauley et al., 2008). Most interestingly, microparticles, which are small vesicles released from activated or dying cells and present abundantly in the synovial fluid of patients with RA, also contain miRNA (Jungel et al., 2007; Stanczyk et al., 2008a). These observations support the notion that microparticles are intra-cellular mediators of inflammation and entail the activation of synovial cells even more interesting (Distler et al., 2005).

In summary, accumulating data suggest that the intrinsic activation of RASF could be a result of epigenetic modulation (Karouzakis et al., 2006). We hope that the increasing knowledge in the new field of epigenetics has a great potential for developing therapies targeting the aberrant gene expression in RA.

4. Autoimmunity

Even though there has been recently accumulating evidence for the involvement of the innate immune system into the development of synovitis (Ospelt and Gay, *in press*), adaptive immunity has long been known to play a critical role in the pathogenesis of RA. In autoimmune disorders such as RA, the cells of the adaptive immunity recognize self-antigens and propagate a self-directed autoimmune reaction. Numerous studies have shown that self-reactive T cells release pro-inflammatory cytokines and promote the production of immunoglobulins directed to self-antigens by B cells (Halldorsdottir et al., 2000; Kurki et al., 1992; Silman et al., 1992). In this regard, B cell depletion therapy improves RA significantly, whereas targeting the CD4 molecule on T cells did not show to be beneficial in clinical trials (Perosa et al., 2005; Schulze-Koops and Lipsky, 2000). A remarkable specificity of 98% for RA characterizes the antibodies directed to citrullinated peptides (anti-CCP) (Jansen et al., 2002; Mathsson et al., 2008; Schellekens et al., 2000). Among the antigens recognized by anti-CCP antibodies are citrullinated fibrin and vimentin (Mathsson et al., 2008). Anti-CCPs are present very early in the disease, predict radiographic progression and are associated with HLA-DRB1 (de Vries-Bouwstra et al., 2008; Innala et al., 2008; Liao et al., 2008). Although the presence of rheumatoid factors together with anti-CCP show the best predictive value for the development of RA, especially in smokers (Goeb et al., 2008; Klareskog et al., 2006), anti-CCP are not yet included in the ACR criteria. Auto-antibodies reactive with type II collagen (CII), vimentin, decorin, enolase and aldolase A have been described in RA (Kinloch et al., 2005; Mathsson et al., 2008; Polgar et al., 2003; Ukaji et al., 1999). Most recently we have engaged the SEREX technique to identify novel auto-antibodies in RA (Al-Shamisi et al., 2005; Maciejewska et al., 2007). The SEREX technique is a valuable screening method that allows for a fast identification of all cDNA encoded proteins from a tissue that are recognized by autologous sera. The main advantage of the SEREX over classical methods such as Western blot is the possibility for the identification of a broad spectrum of auto-antibodies reactive with proteins expressed extra- and intra-cellularly since it engages screening of a complete gene expression library. Using the SEREX technique we found increased levels of auto-antibodies to serpin E2 in synovial fluids and sera from patients with RA (Maciejewska et al., 2007). Serpin E2 is a natural inhibitor of the plasmin system involved into the activation of more pathogenic enzymes (Mansilla et al., 2008). Interestingly, anti-serpin E2 auto-antibodies interfered with the inhibitory activity of serpin E2. To our knowledge, this is the first reported functionally active auto-antibody in RA that might contribute to the pathogenesis of the disease.

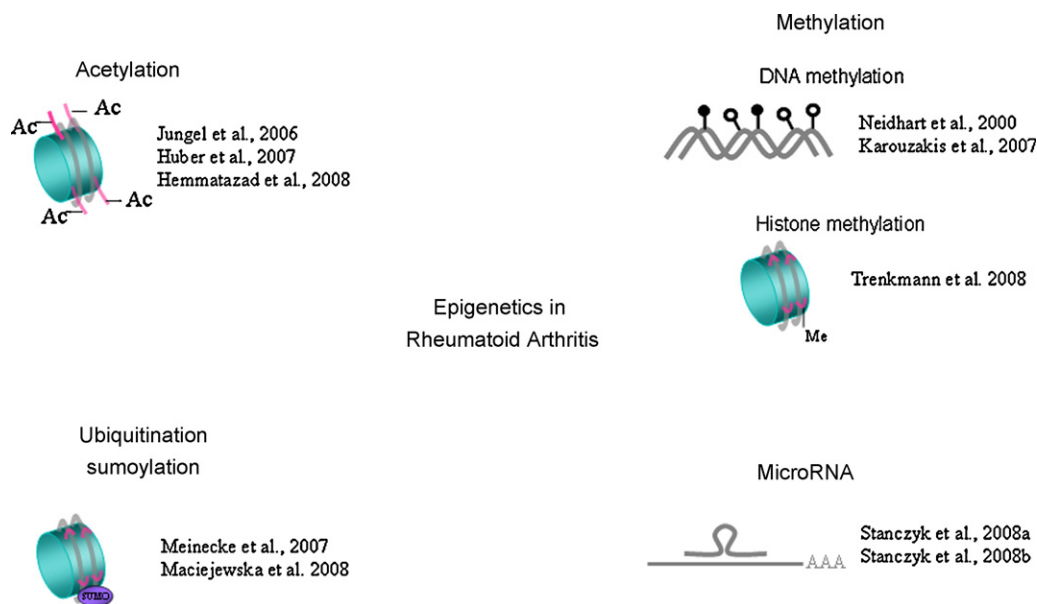


Fig. 2. Epigenetic modifications in RA investigated in the World Health Organization Collaborating Center for Molecular Biology and Novel Therapeutic Strategies for Rheumatic Diseases, University Hospital Zurich.

The data reviewed here suggest a strong interplay between the innate and adaptive immune response in RA. However, it is a great future challenge to study the interactions between the two intertwined immune systems and to elucidate the multiple epigenetic modulations of all cells contributing to innate and adaptive immunity and joint destruction in RA (Fig. 2).

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