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U1-RNP and TLR receptors in the pathogenesis of mixed connective tissue disease
Part I. The U1-RNP complex and its biological significance in the pathogenesis of mixed connective tissue disease

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Abstract

Mixed connective tissue disease (MCTD) is a rare autoimmune syndrome, signified by complex interactions between disease-related phenomena, including inflammation, proliferative vascular arteriopathy, thrombotic events and humoral autoimmune processes. It is still controversial whether MCTD is a distinct clinical entity among systemic connective tissue diseases, although several authors consider that it is distinct and underline characteristic, distinct clinical, serological and immunogenetic features. The putative target of autoimmunity in MCTD is U1-RNP, which is a complex of U1-RNA and small nuclear RNP. Both the U1-RNA component and the specific proteins, particularly U1-70K, engage immune cells and their receptors in a complex network of interactions that ultimately lead to autoimmunity, inflammation, and tissue injury. U1-RNA is capable of inducing manifestations consistent with TLR activation. Stimulation of innate immunity by native RNA molecules with a double-stranded secondary structure may help explain the high prevalence of autoimmunity to RNA binding proteins.

Key words: mixed connective tissue disease (MCTD), pathogenesis, U1-RNP, TLRs.

Introduction

Autoimmune rheumatic diseases are chronic inflammatory syndromes that begin at a relatively young age, lead to progressive disability and therefore cause social as well as medical problems. The most common among these disorders is rheumatoid arthritis (RA; affecting 1/100,000 people). One of the least frequent is mixed connective tissue disease (MCTD). Mixed connective tissue disease is a relatively rare systematic autoimmune disease that was first described as a new entity with mixed features of several connective tissue disorders, including systemic lupus erythematosus, systemic sclerosis, polymyositis and rheumatoid arthritis. Mixed connective tissue disease is characterized by the presence of vascular abnormalities, chronic inflammation, fibrosis and stimulation of the immune system and B and T cells, with the production of autoantibodies against nuclear and cytoplasmic components [1–3]. When the antigen was characterized as polypeptides on the U1 ribonucleoprotein, an essential component of the spliceosome (U1-RNP), MCTD became the first rheumatic disease syndrome to be defined with a serologic test [4, 5]. Although anti-U1-RNP autoantibodies are a part of the diagnostic criteria for MCTD, this does not imply that they necessarily play any role in the development of the disease. In this disease, the immune system is misdirected against a wide range of autoantigens, and the pathways dependent on the resulting immune effectors lead to some disease-specific damage to the tissues [6]. Moreover, the interaction between the innate and adaptive immune system plays a central role in the development of MCTD. Despite many years of research

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studies, no specific cause of the disease has been discovered so far, although it has been confirmed that pathogenesis of the disease is related to genetic and immunological factors that lead to a breach of immune tolerance. Regardless of genetic factors, the role of immunity-related factors in the pathogenesis of MCTD, which like many rheumatic diseases is not fully understood, has also been confirmed. The clinical symptoms and the presence of autoantibodies suggested that many of the same immunological factors that play a role in well-defined connective tissue diseases (CTDs) may also be involved in MCTD. These factors contribute to immune cell activation via innate signaling through Toll-like receptors (TLRs) and other innate immune receptors, modification of the RNP antigen and its associated RNAs, B cell hyperactivity, abnormal activation of T cells and defects in the clearance of apoptotic cells and immune complexes [7, 8]. The nucleic acid containing immune complex activates the innate response by engaging specific TLRs and promotes the production of autoantibodies [9]. There are many reports indicating that activation of the TLR system and consequently promotion of production of proinflammatory mediators and expression of pathogenic autoantibodies positively correlate with disease activity, suggesting that it may play an important role in pathogenesis of MCTD [1, 8, 9].

U1-snRNP complex structure

U1 small nuclear ribonucleoprotein (snRNP also known as U1-RNP) was discovered as a key component of the spliceosome, which is responsible for removing the vast majority of pre-mRNA introns; the others are U2, U4, U5 and U6 snRNPs and non-snRNP associated splicing factors. All these five uridine-rich (U-rich)-snRNP are compositionally similar but functionally distinct [10–12]. Each snRNP consists of an snRNA (or two in the case of U4/U6) and a variable number of complex-specific proteins. In addition, the U1, U2, U4, and U5 snRNPs all contain seven Sm proteins. In contrast to ribosomal subunits, none of these particles possess a preformed active center and several of the snRNPs are substantially remodeled in the course of the splicing reaction.

Human U1-RNP (248 kDa) consists of a single 165-nucleotide-long RNA molecule and at least 10 proteins. Seven of these, called the Sm proteins (B/B', D, D2, D3, E, F, and G), are common to all the snRNPs, while the proteins 70K, A, and C are contained only in the Ul particle [12–14]. U1-70K and -A proteins are known to bind directly to stem loops of the U1-RNA, whereas the U1-C protein does not bind to naked U1-RNA, but depends on the other U1-RNP protein components for its association.

The **U1-snRNA** molecule is 164 nucleotides long with a well-defined structure consisting of four stem loops (SL1-4) that resemble an asymmetrical X-shape and a single helix H. There are two functionally significant single-stranded stretches in this molecule, without which the particle is incapable of assembly. One is the Sm binding site on U1-RNA, which is located between stem loops 3 and 4, and the second is a strictly conserved sequence at the 5' end that pairs with the 5' splice site within pre-mRNA. Moreover, the U1-RNA is modified during apoptosis by the specific removal of 5/6 nucleotides from the 5' end, which includes the TMG (2,2,7-trimethylguanosine) cap; however it is currently unknown whether this modification affects its antigenicity [12, 14–16].

U1-70kDa (437 nucleotides long) protein is a member of a large family of RNA binding proteins that contains an *N*-terminal domain with proline-rich regions, a RNA binding domain and a *C*-terminus domain that is rich in repeats of serine and arginine residues (RS domain) as well as arginine-asparagine/glutamine residues [11, 12, 14]. The RNA binding domain contains two characteristics RNA recognition motifs (RRMs) that are composed of 80 amino acids and contains elements of a nuclear localization signal (NLS) in loop 5 which presumably interact with the cellular component involved in protein shuttling [17]. Recently studies revealed that at least four isoforms (alternative splice transcripts) of the U1-70K protein exist in humans. Transcripts 1 and 2 differ only by the presence (isoform 1) or absence (isoform 2) of 9 amino acids in the center of the sequence. Furthermore, isoform 3 contains on the *N*-terminal 159 amino acids of transcript 1 and 7 other amino acids in the *C*-terminal domain. In isoform 4 we can observe that the *N*-terminal 159 amino acids of transcript 1 are replaced by 63 other amino acids [16].

U1-A and U1-C are 282 and 159 amino acids in length, respectively. U1-A is composed of two RNA-recognition motifs and an intervening proline-rich domain. This protein contains an RRM motif and has the capacity to directly bind U1-RNA as an individual molecule. In contrast, U1-C protein does not have an RNA binding domain and cannot bind directly to naked U1-RNA, but it contains a zinc-finger domain through which it connects to the U1-70K and Sm proteins [12, 18].

Sm proteins are characterized by conserved structural motifs composed of two short primary sequence segments called Sm1 and Sm2, separated by a variable spacer. None of the Sm proteins alone interact stably with RNA, but the proteins form specific hetero-oligomers involving their Sm motifs, which serve as building blocks during Sm core assembly [11, 12].

U1-RNP function and the role of U1-RNA as a TLR ligand

The conventional function for U1-RNP is in splicing, which is a key process for mRNA maturation, particularly in higher eukaryotes, where most protein-coding transcripts contain multiple introns. Binding of U1-RNP to the 5' splice site of exons is a fundamental step in the formation of the early splicing complex and directs the subsequent assembly of the functional spliceosome. Additionally, when U1 snRNP binds close to a putative polyadenylation site, mostly located in introns, it prevents premature cleavage and polyadenylation and controls the length of most cellular mRNAs. On the other hand, U1 snRNP binding close to the 3' end of some mRNAs inhibits polyadenylation and, therefore, gene expression. Moreover, the absence of effective U1-RNA interaction caused widespread premature cleavage and polyadenylation of pre-mRNA transcripts. The Sm proteins are critical to the assembly, transport and integrity of U1-RNPs. U1-RNP through a combination of interaction with the pre-mRNA transcript and with the transcription initiation machinery plays a central role in early transcriptional events [15, 19].

Both the U1-RNA motif and the specific proteins, especially U1-70K, engage immune cells and their receptors in complex interactions, which lead to widespread autoimmunity, inflammation and tissue damage. Moreover, U1-RNP is a target of autoreactive B and T cells in many rheumatic diseases. This complex contains some structural properties such as a common RNA-binding domain, B and T-cell epitopes and a unique stimulatory RNA molecule that in part explain how it becomes a target of autoreactive immune cells. The RNA-binding domain is central for autoreactivity against U1-RNP. Antigen-presenting cells (APCs) that present epitopes containing the RNA binding domain to T cells would in turn activate B cells to produce autoantibodies against this domain. Monneaux and Muller [20] favor the idea that T cells specific for even one epitope within the RNA-binding domain could stimulate epitope spreading and would be considered as driver T-cell clones. We can also observe some immune mechanisms which may contribute to U1-RNP immunogenicity. Among them we can recognize epitope spreading by B and T-cell interactions, specific modifications during apoptosis and activation of TLRs through stimulation by U1-RNA (Fig. 1).

These results suggest that the U1-RNP complex itself is able to elicit an immune response, autoantibody production directly against select components of the spliceosome, and B and T lymphocyte activation [14, 16, 21]. The modification of the U1-RNP components during apoptosis represents the initial epitopes to which an immune response is generated and may be a trigger for the production of autoantibodies to this complex. Apoptotic modification during cell death, resulting in alteration in the structure of proteins as well as in their antigenic properties, has been characterized for the U1-RNA molecule and for the U1-70K and Sm-F proteins [22, 23]. U1-RNA is modified by the removal of 5–6 nucleotides from the 5' end, the U1-70K is cleaved at the *C*-terminal side at position 341 by caspase-3 while the cleavage of the Sm-F protein generates a 9 kDa apoptotic fragment that remains associated with the U1-RNP complex in apoptotic cells [6, 13, 16, 21]. Cleavage of U1-70K abolishes its function during splicing processes as an enhancer of the interaction between the U1-RNP and the 5'-splice site of an exon. Evidence from animal models of autoimmune disease indicates that disease progression may be due to the activation and recruitment of autoreactive lymphocytes, regardless of the initiating event. These autoreactive lymphocytes are specific for epitopes that are distinct from and non-cross-reactive with the disease-inducing epitope, and result from chronic tissue damage (epitope spreading). Studies by Dai et al. [24] and Tian et al. [25] suggest that autoreactive B cells are key cellular mediators contributing to autoreactive T cell response diversification via their functions that mediate antigen processing and presentation. Furthermore, Peng et al. [26] in a murine model have shown that anti-RNP autoimmunity is T cell dependent. Moreover, T cells through secreted mediators are required for autoantibody production against the U1-RNP complex, which contain both B and T cell epitopes.

The most common B cell epitope overlaps with a T cell epitope recognized on apoptotically modified but not intact U1-70K polypeptide residing within the RNA binding domain of the peptide. These observations coincide with the discovery of a series of pathogen-associated pattern recognition receptors such as TLRs, which play a role in host defense through their recognition of bacterial and viral cell products [27–29]. Hoffman et al. [30] using U1-RNA and TLR mutant endometrial cell lines were the first to demonstrate that the U1-RNA molecule (which has a substantial double-stranded secondary structure) was able to stimulate the TLR3 at similar efficiency as known TLR-3 agonist polyriboinosinic: polyribocytidylic acid [poly(IC)]. U1-RNA may also induce innate immunity through other cellular RNA sensors, including TLR7 and 8 and protein kinase R. The study by Greidinger et al. [31] suggested that U1-RNA through the TLR7 system leads to activation of the proinflammatory cascade and end-organ damage. Stimulation of innate immunity by native RNA molecules with a double-stranded secondary structure may help explain the high prevalence of autoimmunity to RNA binding proteins. Moreover, the

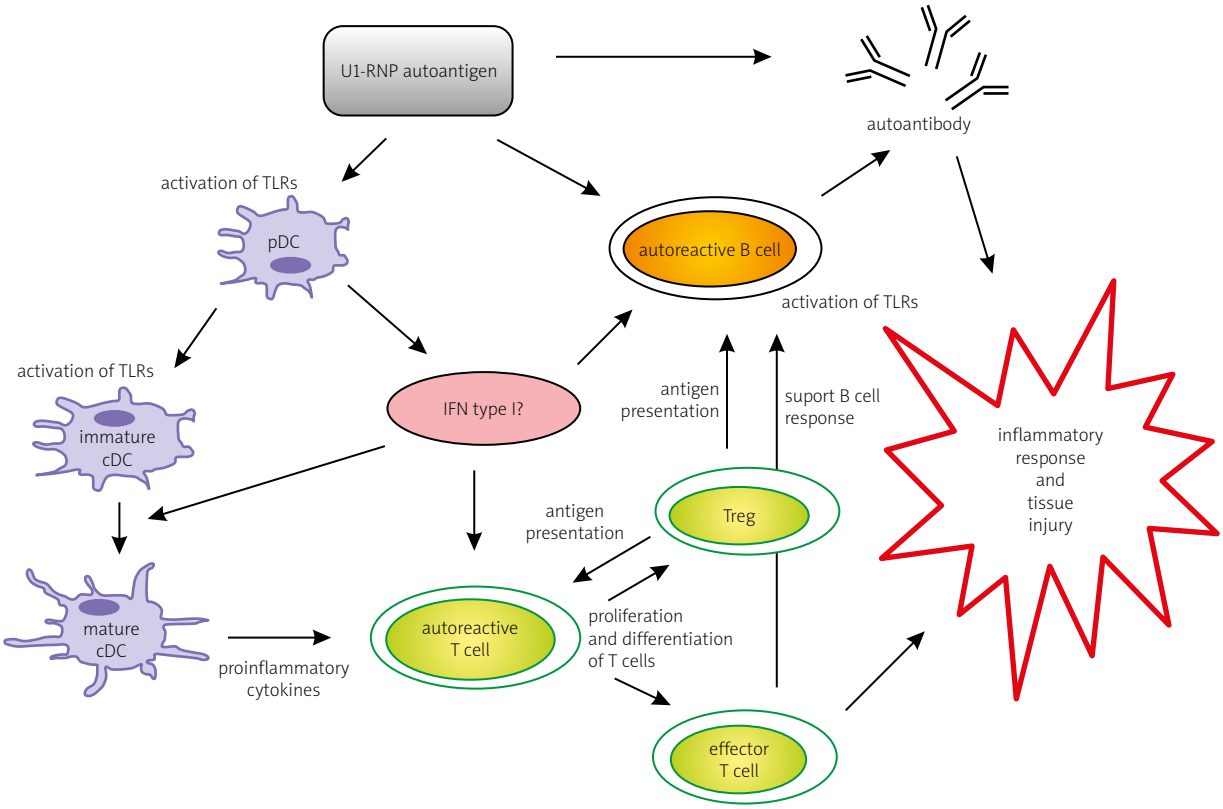

Fig. 1. Probably role of U1-RNP autoantigen and endosomal TLRs in the pathogenesis of MCTD. Self-antigen (U1-RNP) triggers immune system and breaks immune tolerance. Plasmacytoid dendritid cells (pDCs) via TLR7 induce production of type I IFN. In turn IFN I promotes autoreactive T cells activation and autoantibody production by autoreactive B cells. Activated autoreactive T cells differentiate into effector T cells and regulatory T cells (Treg); Self-antigen also activates classical DCs (cDCs) and they promote the release of inflammatory cytokines and the priming of T cells that are specific for self-antigens in a process, which is also facilitated by IFN I. Role of IFN I in the pathogenesis of MCTD is only speculated.

ability of U1-RNA to act as a TLR ligand may serve as an important link between innate immunity and the development of the anti-U1-RNP immune responses [32].

Clinical aspect, epidemiology and etiology of mixed connective tissue disease

The concept of MCTD as a separate immune-mediated connective tissue disorder was first introduced by Sharp and co-workers in 1972 [4]. They describe the disease as a connective tissue inflammatory syndrome with overlapping features of systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), polymyositis/dermatomyositis (PM/DM) and systemic sclerosis (SSc), associated with antibodies against RNase-sensitive extractable nuclear antigen. Clinical features include a high frequency of arthritis, swollen fingers and hands, skin and vascular changes, Raynaud's phenomenon, arthralgia, myositis, esophageal dysmotility, and gastrointestinal and pulmonary involvement. Clinical course and outcomes might vary from mild forms with good clinical prognosis to severe rapidly progressive life-threatening diseases. The main causes of death are pulmonary arterial hypertension, infections and cardiac and renal involvement [33].

Mixed connective tissue disease is classified among systemic autoimmune disorders because of the presence of anti-U1-RNP and other autoantibodies such as anti-cyclic citrullinated peptides (anti-CCP), anti-phospholipids, anti-Ro/SS-A, anti-ssDNA, anti-Sm, anti-dsDNA as well as rheumatoid factor (RF) [18, 34]. Cases with high titers of anti-U1-RNP autoantibodies without any criteria of MCTD or other defined CTD usually evolved into MCTD over 2 years. Antibodies to U1-RNP interact with endothelial and mononuclear cells, which provide multiple putative pathways for tissue injury in MCTD.

The general prevalence of MCTD is still uncertain; in Caucasians it has been estimated to be 3.8 per 100,000 adults [34–37]. MCTD affects women much more often than men (9 : 1), and the mean age of onset is 28–37 years.

Mixed connective tissue disease is one of the rheumatic autoimmune syndromes where environmental, genetic and immune factors contribute to development and progression of its inflammatory manifestation. Although some studies [38–40] have shown that certain HLA-DR4, -DR1, and, to a lesser extent, -DR2 alleles play a role in genetically determined susceptibility to MCTD, their presence is not sufficient to induce the disease, and the total genetic background of MCTD remains unexplained. In contrast, SLE turned out to be associated mainly with HLA-DR2, PM/DM with HLA-DR3, and SSc shows an association with HLA-DR5. The HLA evidence is in favor of MCTD as a disease distinct at least from SSc or PM/DM, and as a T-cell-dependent disease, given the HLA class II association.

In this disease, the immune system is misdirected against a wide range of autoantigens, and the pathways dependent on the resulting immune effectors lead to some disease-specific damage to the tissues. Moreover, the interaction between the innate and adaptive immune system plays a central role in the development of MCTD. Clinical symptoms and presence of autoantibodies suggest that many of the immunological factors which play a role in other CTDs may also be involved in MCTD. One of these factors is production of various cytokines by activated antigen-specific T-cells, which may trigger an inflammatory response and stimulate autoantibody production by B cells. Cytokines also play a key role in regulation of the type and magnitude of immune responses, and the polymorphic nature of the cytokine genes may confer flexibility on immune and inflammatory responses towards different serological and clinical phenotypes [41–43].

Anti-U1-RNP autoantibodies in mixed connective tissue disease

Mixed connective tissue disease is a chronic inflammatory disease in which the autoimmune response is initiated by the activation of antigen-specific T cells, which appear to play a central role in the pathogenesis of this syndrome. T cells react with various snRNP polypeptides, including U1-A and U1-70K polypeptides, and their associated U1-RNA. T cells reacting against U1-70K possess a typical phenotype of helper cells associated with production of Th1- (e.g. IL-12, IFN-γ), Th2- (e.g. IL-10) and Th17- (e.g. IL-17A, IL-17F) derived cytokines, which may trigger inflammatory responses [44–48].

Originally, autoreactivity in patient sera against the U1-RNP complex was named anti-U1-RNP. Anti-U1-RNP autoantibodies are detected in nearly all MCTD patients, as anti-RNP reactivity is a criterion for the diagnosis of MCTD. Antibodies specifically directed against U1-70K are found in 75–90% of MCTD patients and represent the most commonly detected U1-RNP component. Autoantibodies to the U1-70K and Sm-B/B' proteins more frequently appear early in the anti-RNP response, next there appear autoantibodies directed against U1-A and -C, and finally autoantibodies targeting Sm-D are detected [16]. Furthermore, the U1-RNA component and specific proteins, especially U1-70K, engage immune cells and their receptors in a complex network of interactions that ultimately lead to autoimmunity, inflammation and tissue injury.

Cellular events, such as apoptosis or oxidation, may lead to the development of modified autoantigens and distinct antibodies to modified forms of U1-70K. Apoptotically modified 70K is antigenically distinct from intact 70K, which may have clinical implications in breaking immune tolerance to the autoantigen. Antibodies to apoptotic U1-70K are associated with lupus skin diseases, but the antibodies to oxidatively modified U1-70K are associated with Raynaud's phenomenon [32]. Moreover, several patients have antibodies to the apoptotic fully modified form of the U1-70K protein at high titer, before substantial titers of antibodies to the naïve form of U1-70K are found. In contrast, among patients with MCTD there are also identified cases with autoantibodies to the naïve form of U1-70K but without autoantibodies to the antiapoptotic 70K autoantibodies. This suggested that the apoptotically modified 70K may be the antigen toward which U1-RNP tolerance is first broken in many MCTD patients [49].

Anti-U1-RNP antibodies may induce endothelial cell activation and damage, causing not only intimal hyperplasia, obliterated vasculopathy and pulmonary arterial hypertension, but also upregulated expression of intracellular adhesion molecule-1, endothelial leukocyte adhesion molecule-1 and MHC class II molecules. Moreover, it has been shown that production of anti-U1-RNP antibodies in MCTD patients is associated with HLA-DR4. The frequency of the DR4 allele was higher in patients with anti-U1-RNP antibodies compared to control groups. Moreover, in some patients with MCTD, antibody titers against the U1-RNP complex are correlated with disease activity and could even possess prognostic value. Patients with a high titer of anti-U1-RNP rarely develop severe CNS or renal manifestations such as psychosis, seizures or diffuse proliferative glomerulonephritis [50]. Finally, the disappearance of anti-U1-RNP antibodies, and especially antibodies against U1-70K, -A, -C and Sm-B/B', is associated with prolonged remission in MCTD patients [14, 32].

Summary

Mixed connective tissue disease is a condition characterized by a simplified clinical and serological profile and the absence of severe organ involvement. In the last

decade, several advances in knowledge of the disease course and pathogenesis of MCTD have been made. Moreover, recent data have shown that anti-U1-RNP autoantibodies may play an important role in the pathogenesis of MCTD. Both U1-RNA and autoantibodies to U1-RNP have multiple effects on the innate and adaptive immune responses, implicating them in the autoimmunity, inflammation and tissue damage. Understanding of the structure and dynamics of the molecular network between an immunogenic autoantigen and an individual's immune system is extremely important for better understanding of this disease, as their further analysis will not only provide support in research on MCTD but also optimize antigen-specific therapies that could effectively target autoimmune responses.

The author declares no conflict of interest.

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