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Humoral autoimmune response against specific collagen type II epitopes in Bulgarian patients with rheumatoid arthritis

ABSTRACT

Collagen type II (CII) is a strong candidate autoantigen for rheumatoid arthritis (RA) pathogenesis. CII is the main structural protein of synovial cartilage and it is attacked by both antibodies and T-cells during RA disease course. Experiments with mouse models have identified an immunodominant T-cell epitope from CII as well as several epitopes that are recognized by the majority of CII-specific autoantibodies. It has been shown that some epitope-specific anti-CII antibodies are arthritogenic and are associated with development of chronic arthritis. In addition, the immunodominant CII epitopes could be posttranslationally modified and these modified epitopes might be involved in induction and/or perpetuation of autoimmune humoral response and arthritic pathology. The aim of the present study was to evaluate the CII epitope-specific humoral response in a subgroup of Bulgarian patients with rheumatoid arthritis. Our results demonstrate that RA patients have significantly increased levels of anti-CII antibodies compared to healthy individuals and patients with other type of autoimmune disease. The majority of anti-CII antibodies in Bulgarian patients are directed against the U1 and J1 conserved epitopes. We show that D8 epitope-specific antibodies react to the triple-helical structure of the epitope and thus recognize both the native and the posttranslationally citrullinated D8. This is the first article presenting an evaluation of CII-specific humoral autoimmune response in Bulgarian patients with rheumatoid arthritis..

Key words: rheumatoid arthritis, autoimmune response, collagen type II, epitope-specific antibodies

Introduction

Rheumatoid arthritis (RA) is a common autoimmune disorder that affects approximately 0.5-1% of the population worldwide. It is characterized by inflammation of synovial membranes of peripheral joints leading to joint deformation and destruction of cartilage, followed by bone erosion (Bartok & Firestein, 2010). RA is a systemic autoimmune disease because the inflammatory process could also affect the heart, blood vessels, lungs, eyes, kidneys and other organs (Hart, 1969; Turesson et al., 2003). A significant part of RA patients is rheumatoid factor (RF)-positive, i.e. the majority of RA patients have a high serum level of autoantibodies that bind to the Fc-fragment of immunoglobulins (Song & Kang, 2010). RFs are secreted by plasma cells in the inflamed synovial tissue and their titer correlate with disease activity in RA patients (Magalhaes et al., 2002). Nevertheless, the specificity of these antibodies is low (approximately 75%) – they are present in the sera of 3 to 5% of healthy young people and in 10-30% of healthy elderly people, as well as in patients with other autoimmune disease (Sjögren syndrome) or chronic infections (like hepatitis C infection or tuberculosis) (Newkirk, 2002; van Boekel et al., 2002). Other types of autoantibodies that are detected in the blood serum of most RA patients are antibodies specific to citrullinated proteins and cyclic citrullinated peptides. Anti-citrullinated protein antibodies (ACPA) show greater specificity than RFs: they are detected in only 2% of healthy individuals and in less than 10 % of patients with other autoimmune disease (Sjögren syndrome, systemic lupus erythematosus). ACPA antibodies are present

in patients' sera even before the clinical onset of RA: a fact that highlights the significance of ACPA antibodies as a prognostic marker for RA (Kokkonen et al., 2011; Rantapaa-Dahlqvist et al., 2003). ACPA recognize different citrullinated proteins (vimentin, fibrinogen, type II collagen, α-enolase) and some of them are enriched in joints of RA patients (Snir et al., 2010). However, the contribution of citrullinated proteins to RA pathogenesis is still unclear.

Type II collagen is a major candidate autoantigen in RA pathogenesis. CII is the principal component of hyaline cartilage and hence a target of autoimmune response in inflamed joints (Dobritzsch et al., 2011; Lindh et al., 2014). Anti-CII antibodies are present in the blood serum and synovial fluid of many RA patients (Kim et al., 2000; Nandakumar, 2010). Moreover, the titer of IgG anti-CII antibodies correlates with inflammation progression in RA (Kim et al., 2000). Thus, it is suggested that anti-CII antibodies play an important role in disease development (Nandakumar, 2010). Experiments with mouse models of RA led to identification of several conserved epitopes from CII that are recognized by the majority of CII-specific antibodies, i.e. D8, J1 and U1 epitope (Burkhardt et al., 2002; Kraetsch et al., 2001; Schulte et al., 1998). It has been shown that antibody clones specific to these epitopes are arthritogenic and their serum concentration correlates with development of chronic arthritis in mice (Bajtner et al., 2005; Nandakumar & Holmdahl, 2005). On the other hand, it has been demonstrated that a portion of ACPA reactivity in many RA patients is directed toward citrullinated CII (Burkhardt et al., 2005; Snir et al., 2010; Snir et al., 2009). Consequently, analysis of anti-citrullinated CII antibody titers could contribute to RA diagnosis, whereas accumulation of immune complexes containing anti-citrullinated CII antibodies in affected joints could contribute to disease pathogenesis (Yoshida et al., 2006).

To date, there aren't reports on CII-specific autoantibody responses in Bulgarian patients with RA. Therefore, the aim of our study was to analyze the levels of anti-human CII antibodies as well as the levels of antibodies specific to native and citrullinated immunodominant CII epitopes in a group of ACPA-positive RA patients. In this report we show for the first time significantly elevated levels of anti-human CII antibodies in Bulgarian RA patients that are ACPA-positive. Our results demonstrate that the majority or CII-specific antibodies are directed against the J1 and U1 epitopes. Anti-D8 epitope-specific antibodies in the examined patients group recognize both the native and the posttranslationally citrullinated epitope suggesting a reactivity to the common triple helical structure of D8.

Materials and Methods

Patient samples

All experiments were performed in concordance to the WMA Declaration of Helsinki concerning the ethical principles for medical research involving human subjects. Samples from RA patients, healthy individuals and patients with other autoimmune disease were obtained following a written informed consent.

Twenty ACPA-positive RA patients were included in the study. All patients were women between age of 40 and 70 years with established disease (disease duration more than 1 year) and had already received treatment (disease modifying antirheumatic drug therapy or anti-tumor necrosis factor therapy) at the time of sampling. Data for rheumatoid factor status were not available for 7 patients. Control samples were obtained from 43 healthy women (age range 35-70 years) with no reported inflammatory joint disease. 12 samples from women (age range 40-70 years) diagnosed with other autoimmune disease (Hashimoto's thyroiditis or systemic lupus erythematosus) were also included in the analyses.

Serum samples from routine laboratory analyses were used. All samples were coded and assayed in a blinded way. The authors did not have direct contact with the patients and control healthy subjects. Only data concerning history of disease, gender and age were collected.

Antigens

The following antigens were used for detection of CII-specific antibodies: *human CII protein* obtained from individuals with hip trauma (without manifestations of rheumatoid arthritis or osteoarthritis) undergoing surgery; *synthetic single-chain peptides* containing the D8 (C1^{III}) epitope (CII 359-369) – *D8* peptide (GARGLTGRPGDA), citrullinated peptide *0309* (GP-HYP-GA(**Cit**)LTG(**Cit**)-HYP-GDAGP-HYP-amid), *T+B peptide* containing both T-cell and B-cell epitope (GARGLTGRPGDAGIA GFKGEQGPKGET); *synthetic triple-helical CII peptides* – *tr. D8* (GP-HYP-GP-HYP-GP-HYP-GP-HYP-GP-HYP-GA**R**GLTG**R**-HYP-GDAGP-HYP-GP-HYP-G-eACA) 3-triple), *tr. J1* (GP-HYP-GP-HYP-GP-HYP-GP-HYP-GP-HYP-GE**R**GAAG-HYP-IAGPKGP-HYP-GP-HYP-G-eACA) 3-triple), *tr. U1* (GP-HYP-GP-HYP-GP-HYP-GP-HYP-GP-HYP-GLVGP**R**G-HYP-E**R**GFGP-HYP-GP-HYP-

G-eACA) 3-triple), *citrullinated triple-helical peptide 0123* (GP-HYP-GP-HYP-GP-HYP-GP-HYP-GP-HYP-GA-**Cit**-GLTG-**Cit**-HYP-GDAGP-HYP-GP-HYP-G-eACA) 3-triple). All peptides were synthesized by Schafer-N, Denmark. Human CII protein (huCII) was purified as previously described (Dzhambazov et al., 2005). All synthetic peptides and huCII proteins were dissolved in 0.1M acetic acid and stored at 4ºC.

ELISA for anti-CII-specific antibodies

Costar ELISA plates were coated with 10 μg/ml huCII or 5 μg/ml CII peptides for 2 h at room temperature (RT). Unspecific binding was blocked using 5% fetal calf serum (Sigma-Aldrich, Germany) incubated on the plate for 1h at RT. Then the plates were washed three times with ELISA buffer (PBS supplemented with 0.05% Tween 20) and serum samples in serial dilutions (1:10, 1:100, 1:1000, 1:10000) were added to the plate and incubated for 2h at RT. After that, the plates were washed four times with ELISA buffer, 1 μg/ml goat anti-human IgA/IgG/IgM-[HRP] (Novus Biologicals, USA) was pipetted and incubated for 1h at RT. At the end of the incubation period the plates were washed five times with ELISA buffer and peroxidase substrate solution was added to each well (ABTS, Roche Diagnostics, Switzerland). As a result color reaction developed in 5 to 15 min and 405 nm wavelength absorption was detected using Synergy-2 reader (BioTek Instruments, USA).

Statistics

StatView software (SAS Institute) was used for data analyses and graph generation. Statistics was calculated using Mann-Whitney U test. *P* values less than 0.05 were considered statistically significant.

Results

To determine the total antibody response against CII in RA patients and healthy controls we performed ELISA for anti-collagen type II IgM/IgG/IgA antibodies. The results from this analysis are shown on Figure 1. They demonstrate significantly higher levels of anti-CII protein antibody levels in RA patients compared to healthy controls. These data suggest a role for anti-CII antibodies in RA pathogenesis. The CII-specific antibody levels were elevated in all RA patients although there were variations between the samples (data not shown). We assume that these variations are due to differences in disease stage and progression in individual patients.
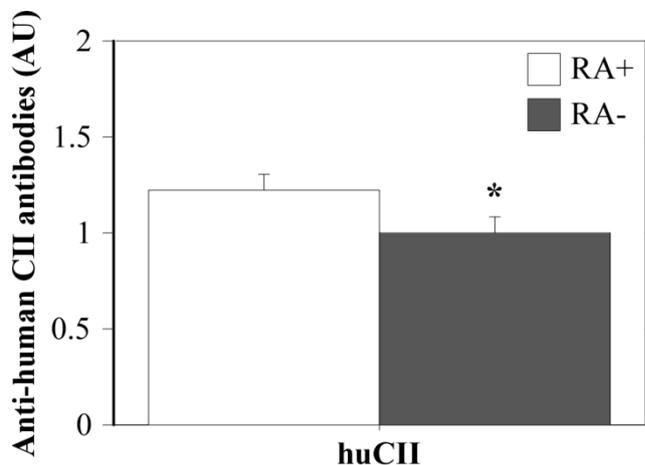

Figure 1. *Anti-collagen type II antibody levels in RA patients and healthy individuals. Data are present as ±SEM (Standard Error of the Mean). RA+: patients with rheumatoid arthritis (n=20); RA-: healthy controls (n=43); * $p < 0.05$.*

In order to characterize the fine specificity of anti-CII humoral immune response we analyzed the antibody levels against the three major CII immunodominant B-cell epitopes in RA – D8, J1, and U1. Figure 2 represents the results from this experiment. Significant titers of antibodies reactive to all three epitopes were detected in RA patients. Triple-helical U1, J1 and D8 peptides were used for detection of CII epitope-specific antibodies. Anti-D8 humoral reactivity was additionally evaluated using single-chain peptides. Thus, we determined that anti-D8 antibodies recognize the epitope in both single-chain and triple-helical structure. Comparing the levels of different epitope-specific antibodies it is evident that anti-CII humoral autoimmune response is directed mainly against the U1 and the J1 epitope.

The J1 epitope contains one arginine residue whereas D8 and U1 epitopes contain two arginine residues. These residues could be posttranslationally modified to citrulline. To investigate the humoral autoimmune response to modified by citrullination CII epitopes we used the synthetic peptides 0309 and 0123. Peptide 0123 is triple-helical – its structure mimics the typical triple-helical structure of the CII protein. The amino acid sequence of this peptide as well as 0309 peptide corresponds to the D8 epitope except the arginine residues that are converted to citrulline. Peptide 0309 has a single-chain structure and two citrulline instead of arginine residues.
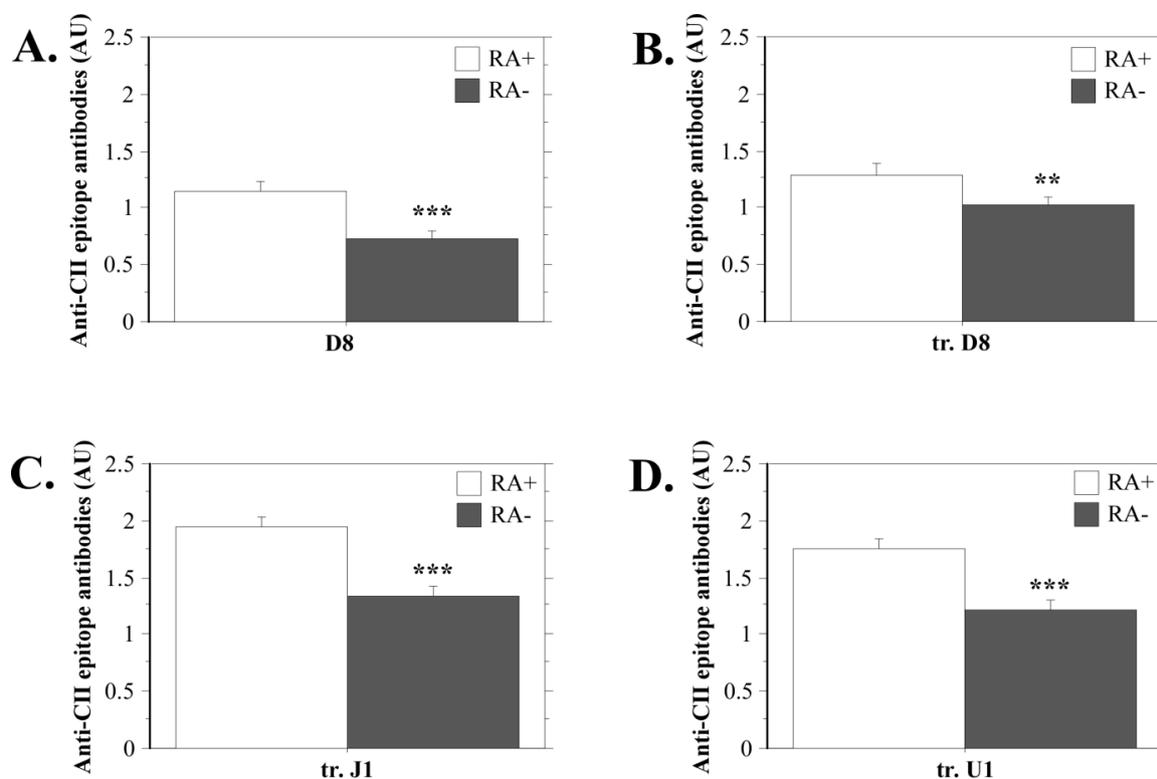
Figure 2. *CII epitope-specific humoral autoimmune response in RA patients and healthy controls. Data are present as ±SEM (Standard Error of the Mean). RA+: patients with rheumatoid arthritis (n=20); RA-: healthy controls (n=43); (A) D8 peptide; (B) tr. D8 – triple-helical D8 peptide, (C) tr. J1 – triple-helical J1 peptide, (D) tr. U1 – triple-helical U1 peptide; *** $p<0.001$, ** $p<0.01$.*
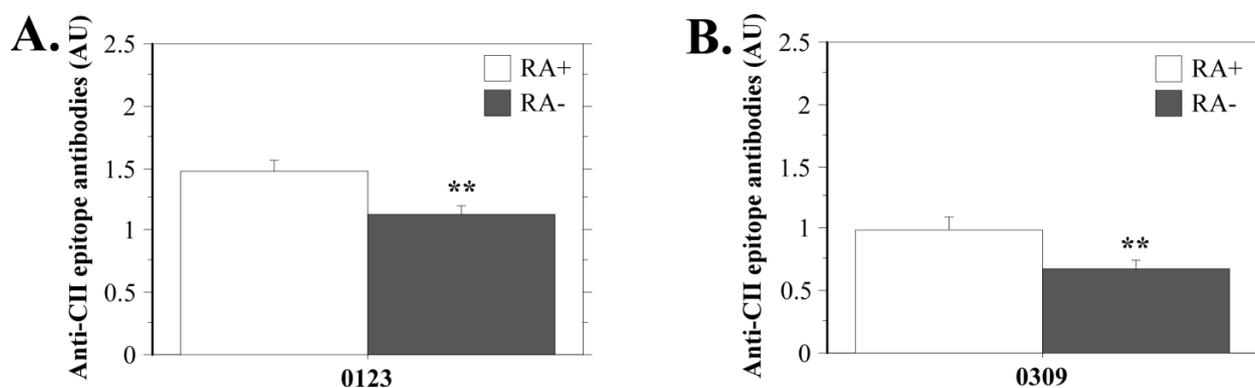

Figure 3. *Anti-citrullinated CII epitope antibody levels in RA patients and healthy individuals. Data are present as ±SEM (Standard Error of the Mean). RA+: patients with rheumatoid arthritis (n=20); RA-: healthy controls (n=43); (A) Peptide 0123, (B) Peptide 0309. ** $p<0.01$.*

The results presented on Figure 3 demonstrate a significant level of antibody response to the citrullinated peptides suggesting that anti-D8 antibodies exhibit cross-reactivity and recognize the epitope in both native and posttranslationally citrullinated form. Anti-D8 antibodies show similar reactivity to the single-chain and the triple-helical structure of the epitope.

In addition to the levels of antibodies specific to the main immunodominant B-cell CII epitopes, we evaluated the humoral response to a chimeric peptide containing the amino acid sequence of both the D8 epitope and the major T-cell CII epitope (CII259-273). Again, RA patients demonstrated significantly higher titer of antibodies reactive to the chimeric CII epitope compared to healthy controls (Figure 4). This result could be explained with cross-reactivity of anti-CII epitope-specific antibodies, as well as with presence of antibodies recognizing the T-cell epitope.
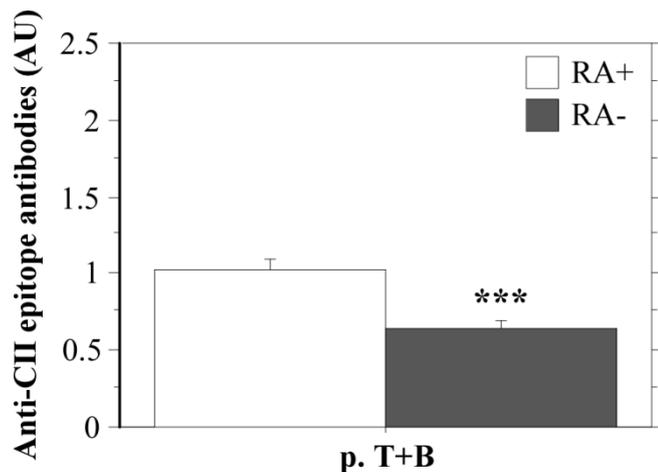

Figure 4. *Humoral immune response against chimeric CII epitope. Data are present as ±SEM (Standard Error of the Mean).p. T+B – peptide containing the amino acid sequence of D8 and T-cell CII259-273 epitope. RA+: patients with rheumatoid arthritis (n=20); RA-: healthy controls (n=43); *** p < 0.001.*

Approximately half of the RA patients in the examined group were RF-positive. Thus, we were interested to compare the CII-specific antibody levels between RF-negative and RF-positive samples. As shown on Figure 5 there was a clear tendency for higher anti-CII protein response in RF-positive patients (Figure 5). On the contrary, we did not find a difference in the levels of CII epitope-specific antibodies (Figure 6). These results support the hypothesis that RF-positive RA patients produce antibodies specific for other epitopes on the CII molecule.

The levels of CII-specific antibodies in RA patients were also compared to patients with other autoimmune diseases (Hashimoto's thyroiditis and systemic lupus erythematosus). The results from this analysis are presented on Figure 7. They clearly demonstrate higher levels of CII-specific antibodies in the group of RA patients. We could not see significant difference only in the levels of anti-0309 peptide antibodies. A possible reason for this is the presence of cross-reactive autoantibodies in the patients with Hashimoto's thyroiditis or/and systemic lupus erythematosus. It is also likely that the structure of the 0309 peptide shows similarity with antigenic epitope that is characteristic for another autoimmune disease.
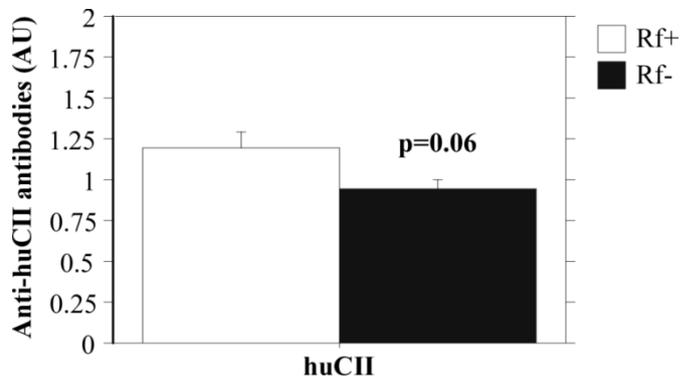

Figure 5. *Anti-CII total antibody levels in RF-positive and RF-negative RA patients. Data are present as ±SEM (Standard Error of the Mean). Rf+: RF-positive RA patients (n=9); Rf-: RF-negative RA patients (n=4).*
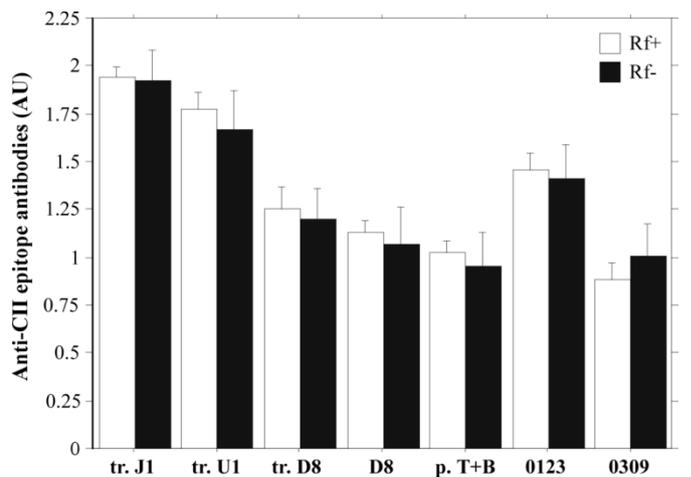

Figure 6. *CII epitope-specific antibody levels in RF-positive and RF-negative patients. Data are present as ±SEM (Standard Error of the Mean). Rf+: RF-positive RA patients (n=9); Rf-: RF-negative RA patients (n=4); tr. D8 – triple-helical D8 peptide, tr. J1 – triple-helical J1 peptide, tr. U1 – triple-helical U1 peptide.*
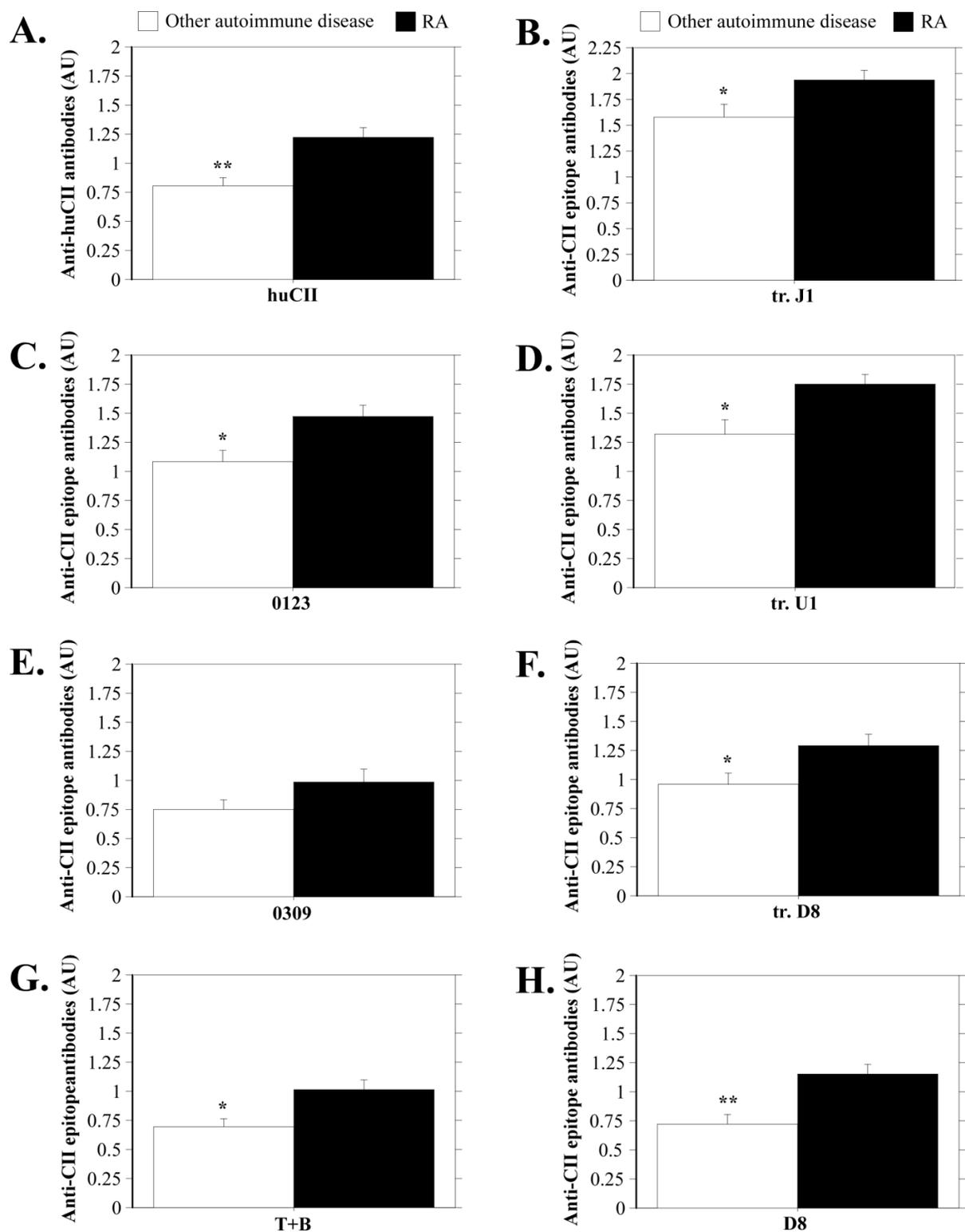
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Figure 7. *Comparison of CII-specific antibody levels between RA patients and patients with other autoimmune diseases. Data are present as ±SEM (Standard Error of the Mean). * p<0.05, ** p<0.01; RA patients (n=20); Patients with other autoimmune diseases (n=12).*

Discussion

Anti-collagen type II autoimmunity has not been investigated so far in Bulgarian RA patients. Here, we show for the first time analysis of CII-specific humoral response in an ACPA-positive subgroup of RA patients from Bulgaria. We detected significantly higher anti-CII antibody levels in RA patients compared to both healthy individuals and patients with other autoimmune disease. These results are in concordance with the data for RA patients from other countries published by different research groups (Cook et al., 1996; Kim et al., 2000; Lindh et al., 2014) and suggest a role for CII-specific immunity in disease pathogenesis. Mullazehi et al. (2012) assume that anti-CII antibodies might stimulate production of metalloproteinases by fibroblasts and macrophages within the synovial tissue leading to cartilage and bone destruction (Mullazehi et al., 2012).

Comparing the data for individual RA patients we noticed certain variations in anti-CII antibody titers. This is a common tendency for tests with patient samples and it's possibly due to differences in disease stage and progression in individual patients. It has been shown that high anti-CII antibody titers are associated with elevated degree of joint destruction at the time of diagnosis (Mullazehi et al., 2012). Thus, the lower anti-CII response in some of the tested RA patients could be linked to earlier RA disease stage and initial joint destruction.

In animal models for RA (collagen-induced arthritis) as well as in RA anti-CII antibodies recognize and bind to specific conserved immunodominant epitopes (Burkhardt et al., 2002; Schulte et al., 1998). The majority of CII-specific antibodies react against the C1 (D8), J1 and U1 epitopes. Experiments with mouse models for RA have shown that antibodies specific to the three immunodominant B-cell epitopes from CII are arthritogenic and their levels correlate with development of chronic arthritis (Bajtner et al., 2005; Nandakumar & Holmdahl, 2005). Our tests showed significantly higher levels of antibodies specific to the D8, J1 and U1 CII epitopes in RA patients. The highest titers detected were for J1- and U1-specific antibodies. These results support the recent data from Lindh et al. who reported an evaluation of CII-specific humoral autoimmune response in a selected group of Scandinavian RA patients and in experimental models (mice and primates) with collagen-induced arthritis (Lindh et al., 2014). The same report showed highly conserved anti-CII antibody specificity in RA patients, primates and rodents – a fact that supports the pathogenic role of antibodies reacting to the main CII epitopes. Lindh et al. also found high titers of CII epitope-specific antibodies in synovial fluid samples which suggest that production of these antibodies takes place in the inflamed joints (Lindh et al., 2014).

To determine anti-D8 antibodies fine specificity we preformed ELISAs using single-chain D8 native and citrullinated peptides, as well as triple-helical D8 native and citrullinated peptides. Interestingly, we found that D8-specific antibodies react to the single-chain and triple-helical structure of the epitope irrespective of posttranslational citrullination of its arginine residues. These antibodies cross-react to the native and modified D8 epitope, which is a common phenomenon for B-cell epitopes. On the contrary, the immune response to T-cell epitopes is highly specific for each posttranslational modification (Backlund et al., 2002; Corthay et al., 1998).

In conclusion, the present study clearly demonstrates the presence of CII-specific humoral autoimmune response in a group of ACPA-positive Bulgarian patients with rheumatoid arthritis. We determined that anti-CII antibodies react against the major B-cell CII epitopes – U1, J1 and D8. Anti-J1 and anti-U1 antibodies show the highest levels among all tested epitope-specific antibodies. Anti-D8 antibodies exhibit cross-reactivity and bind both the native and citrullinated D8 epitope.

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