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Exposure to nuclear antigens contributes to the induction of humoral autoimmunity during TNF alpha blockade

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Abstract

Objective—type I interferons and apoptotic particles contribute to anti-nuclear autoimmunity in experimental models. We assessed if similar mechanisms contribute to break peripheral B cell tolerance in humans by studying the induction of anti-nuclear antibodies by TNF blockade in spondyloarthritis (SpA)

Methods—We studied 40 SpA patients treated with infliximab or etanercept and 20 renal cell carcinoma patients treated with sorafenib. Serum anti-nucleosome IgM and nucleosomes were measured by ELISA. Type I IFN serum activity was measured using a functional reporter cell assay. Synovial apoptosis was assessed by TUNEL assay and anti-active caspase-3 immunostaining. Complement was measured by nephelometry.

Results—Despite similar clinical improvement and reduction of synovial inflammation, anti-nucleosome IgM were induced by infliximab but not etanercept. This induction did not correlate with type I IFN activity, which was transiently downmodulated by infliximab but persistently upregulated by etanercept. In contrast, anti-nucleosome IgM levels did correlate with serum nucleosome levels, which were significantly upregulated by infliximab but not by etanercept treatment. This increase in serum nucleosome levels was not directly related to massive cell death, but rather to a decrease of complement 3 and 4 serum levels during infliximab treatment.

Conclusion—Infliximab and etanercept have a differential effect on both type I IFN activity and nucleosome levels. However, only elevated serum nucleosomes relate to the induction of anti-nucleosome antibodies after infliximab treatment.

Keywords

Spondyloarthritis; TNF blockade; humoral autoimmunity; nucleosomes; type I interferon

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Introduction

Random joining of immunoglobulin gene segments during V(D)J recombination generates multiple poly- and autoreactive specificities in the early stages of human B cell development (1). In normal individuals, discrete checkpoints in the bone marrow remove most of the self-reactive cells from the repertoire by deletion and/or receptor editing (2). Nevertheless, up to 20% of the antibodies expressed by mature naïve B cells in healthy individuals appear to be autoreactive, indicating that these mechanisms of central tolerance are partially leaky (1). An additional peripheral checkpoint at the transition between the mature naïve B cell pool and antigen-experienced, IgM memory B cells removes these cells from the repertoire and thereby avoids overt autoimmunity (3).

In humoral autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), the increased self-reactivity in the mature naïve B cell pool even before the onset of an active immune response indicates that the central tolerance mechanisms are impaired (4-6). This increase of autoreactivity in the naïve pool is not expected to lead to overt autoimmunity as long as the peripheral tolerance mechanisms are intact. Therefore, additional mechanisms contribute to break peripheral tolerance in these diseases in order to allow the appearance of autoreactivity in the memory pool. Data from experimental models suggest a potential contribution of an overload of nuclear antigens stimulating the B cell receptor and Toll-like receptors (7-10), as well as of B cell receptor-independent stimuli such as type I interferons (IFN). In how far similar mechanisms contribute to break peripheral B cell tolerance in humans remains to be investigated.

In contrast to SLE and RA, spondyloarthritis (SpA) is a chronic inflammatory arthritis in which the absence of known autoantibodies suggests intact B cell tolerance checkpoints. We recently described that even in this condition TNF blockade leads to the induction of anti-nuclear antibodies (ANA) (11;12). Such novel autoreactivities can either result from a break in peripheral tolerance or be generated de novo by somatic hypermutation as demonstrated in healthy, non-autoimmune prone individuals (13). The fact that the TNF blockade-induced anti-nuclear antibodies were largely restricted to the IgM isotype without corresponding IgG reactivities argues against the latter possibility. As such, TNF blockade in SpA provides us with a unique human model to study the regulation of peripheral B cell tolerance in humans. Taking advantage of the differential effect of the monoclonal anti-TNF antibody, infliximab, and the soluble TNF receptor, etanercept on the autoantibody induction (12), we investigated which of the mechanisms described in experimental SLE models could contribute to the induction of IgM anti-nuclear antibodies in SpA. Based on the published data on the induction of apoptosis in lymphocytes and monocytes by infliximab but not etanercept (14-16), the hypothesis investigated here is that the differential induction of ANA by TNF blockers could relate to the fact that infliximab treatment can induce cell death and a subsequent release of nucleosomes. As it has been shown that type I interferons are central players in the response to these autoantigens (17-19), we additionally investigated involvement of this cytokine in the autoantibody induction.

Materials and methods

Patients

We included 40 SpA patients who fulfilled the European Spondyloarthropathy Study Group criteria (20) and had active disease defined as the presence of at least one swollen joint and/or inflammatory spinal pain. Twenty of these patients were treated with infliximab 5 mg/kg intravenously at weeks 0, 2, and 6 followed by 10 mg/kg every 14 weeks. The other group of 20 patients was treated with etanercept 25 mg subcutaneously twice a week. None of the patients received disease-modifying anti-rheumatic drugs and/or systemic or local

corticosteroids. The median age was 49 year (38-66) and the median disease duration was 18.5 years (1-43) in the infliximab cohort. In the etanercept group, the median age was 38 years (20-71) and the median disease duration 10 years (1-41). Additionally, we included 20 patients with renal cell carcinoma who were treated with sorafenib at weeks 0, 8, and 16. The median age of these patients was 60 years (56-67). These patients responded to therapy; the time to regression was 181 days (116-309) compared to patients who did not receive therapy (75 days (21)). All patients gave written informed consent to participate in the study as approved by the local Medical Ethics Committee.

Samples

Serum samples were obtained at week 0, 2, 6, 12, and 34 in the infliximab-treated patients, at week 0, 4, 8, 12, and 32 in the etanercept-treated patients, and at week 0, 2, 8, and 16 in the renal cell carcinoma patients. Synovial biopsies were obtained from a clinically swollen knee joint by needle arthroscopy before and after 12 weeks of TNF blockade in all SpA patients. Additional biopsies were also obtained after 1 week of treatment (n=12 infliximab-treated patients). Eight biopsies per patient were snap frozen for immunohistochemistry (22-24).

Synovial histopathology

Immunohistochemistry was performed with the following antibodies: anti-CD4 (Clone MT310, DakoCytomation, Glostrup, Danmark), anti-CD8 (Clone DK25, DakoCytomation), anti-CD20 (Clone L26, DakoCytomation), and anti-CD68 (Clone EBM11, DakoCytomation). Concentration and isotype matched negative controls were included. Cells were visualized using the LSAB+ kit and AEC substrate (DakoCytomation). Sections were blinded and scored on a semi-quantitative four-point scale by two independent observers (25).

Serum measurements

Serum anti-nucleosome antibodies were detected by Anti-Nucleo ELISA (GA Generic Assays, Dahlewitz, Germany). The supplied anti-human IgG conjugate was replaced by a specific anti-human IgM (rabbit) HRP (Abcam, Cambridge, UK). We previously validated the specificity of this secondary antibody (12). Serum nucleosomes were detected with the Cell Death Detection ELISA^{plus} (Roche Diagnostics, Penzberg, Germany) (26;27). Serum levels of complement C3 and C4 were assessed by nephelometry. Rheumatoid factor may interfere with these assays, but all patients were rheumatoid factor negative.

Type I IFN serum activity

Type I IFN serum activity was measured as previously described (28;29). Briefly, WISH epithelial cell line cells were cultured in the presence of 50% serum for 6 hours. RNA was extracted using the RNeasy Mini kit (Qiagen, Chatsworth, CA) and reverse-transcribed to cDNA (Superscript III RNase H⁻ reverse transcriptase, Invitrogen, Carlsbad, CA). Real-time quantitative PCR reaction was performed for following interferon inducible genes: IFIT-1, PRKR, and MX-1. HPRT-1 was used as a housekeeping gene control.

Detection of synovial apoptosis

Apoptotic cells were detected by both Terminal Transferase nick-end-labeling (TUNEL) and anti-active caspase 3 staining (14;30;31). The in situ cell death detection kit (Roche Applied Science, Indianapolis, IN) was used to visualize DNA strand breaks. Positive and negative controls were included. Active caspase 3 was detected using a polyclonal rabbit antibody (R&D Systems, Abingdon, UK). Concentration and isotype matched negative control was included. Staining was visualized using the LSAB+ kit and AEC substrate (DakoCytomation).

Statistical analysis

All data were non-parametric, they are represented as medians (interquartile range). The Mann-Whitney U test and the Wilcoxon signed rank test were performed. Correlations were calculated with the Spearman's rho correlation coefficient. P-values ≤ 0.05 were considered significant.

Results

Infliximab, but not etanercept, induces anti-nucleosome IgM

The global efficacy of infliximab and etanercept treatment in SpA has been described previously (22;23;32). Both TNF blockers lead to clinical improvement (Table 1) and induced a decrease of the global inflammatory infiltration as well as the number of T lymphocytes and macrophages in the synovial sublining (Table 1). Titters of anti-nucleosome IgM antibodies were not different between healthy controls and SpA patients at baseline (Figure 1a). Infliximab treatment induced a median increase of the anti-nucleosome IgM levels of 94% (-22% to 648%) at week 12 ($P<0.001$) and 123.5% (7% to 453%) at week 34 ($P<0.001$) (Figure 1b). In contrast, there was no increase of the anti-nucleosome IgM levels in the etanercept-treated patients, with median changes compared to baseline of 6% (-25% to 280%) at week 12 and -1% (-29% to 218%) at week 32 (Figure 1c). As previously reported, the induction of anti-nucleosome antibodies corresponded well with the ANA and anti-dsDNA reactivity. However, anti-histone antibodies or other anti-nuclear reactivities were not detected (11). The induction of IgM anti-nucleosome antibodies by infliximab but not etanercept in patients without humoral autoimmunity at baseline provides a unique human model to investigate what factors contribute to break peripheral B cell tolerance to nucleosomes.

The differential effect of infliximab and etanercept on type I IFN serum activity does not correlate with the induction of anti-nucleosome IgM

Type I IFN are a group of cytokines promoting humoral immunity. They play a crucial role in autoimmune pathology such as SLE and Sjögren's syndrome (17;33;34). As TNF alpha suppresses IFN alpha production by plasmacytoid dendritic cells in vitro (35) and blockade of TNF alpha in vivo leads to an increased activation of the type I IFN pathways in juvenile chronic arthritis and Sjögren's syndrome (29;35), we investigated if type I IFN contributes to the induction of anti-nucleosome IgM during TNF blockade in SpA. At baseline, the type I IFN serum activity in SpA patients was not different from healthy controls (1.81 (1.45-2.15) versus 1.85 (1.25-2.21) (29)). During infliximab treatment, type I IFN serum activity was downmodulated at week 2 ($P=0.005$) but returned to baseline at week 12 (Figure 2a). On the contrary, etanercept induced a persistent upregulation of type I IFN serum activity ($P<0.03$ from week 4 to 12) (Figure 2b). The induction of anti-nucleosome IgM by infliximab but not etanercept can thus not be explained by increased type I IFN activity during treatment.

Serum nucleosome levels increase during infliximab but not etanercept treatment and correlate with the induction of anti-nucleosome IgM

As humoral autoimmunity in experimental SLE models can result from excessive exposure of the immune system to nuclear antigens (7-10), we investigated if serum levels of nucleosomes were modulated by TNF blockade. Compared to baseline, infliximab treatment induced a median increase in nucleosome levels of 88.5% (-51% to 483%) at week 2 ($P=0.002$) and 26% (-88% to 438%) at week 6 ($P=0.014$) (Figure 2c). The nucleosome levels returned to normal at week 12 (-4%, from -90% to 133%). In contrast, etanercept treatment did not increase the nucleosome levels from baseline to week 4 (-5.5%, from -83% to 233%) or to week 8 (-11%, from -75% to 108%). At week 12 of etanercept treatment, there was even a significant decrease of the nucleosome levels (-45%, from -85% to 107%; $P=0.021$) (Figure 2d). Thus, infliximab, but not etanercept, induced an early and transient rise of serum nucleosome levels in SpA

patients. Moreover, this increase in serum nucleosome levels was significantly correlated with the rise in anti-nucleosome IgM antibodies at week 12 ($r=0.383$; $P=0.008$) and at week 34 ($r=0.453$; $P=0.014$) (Figure 2e and f). A similar trend towards correlation was found when analyzing the infliximab-treated cohort separately ($r=0.329$; $P=0.078$), indicating that those patients with an early and pronounced increase in serum nucleosome levels were more likely to develop anti-nucleosome antibodies.

Neither infliximab nor etanercept increase synovial apoptosis

Considering the suggested induction of apoptosis of activated lymphocytes and monocytes by infliximab but not etanercept in vitro and in Crohn's disease in vivo (15;16;32;36-41), we investigated if a similar mechanism contributes to the rise of serum nucleosome levels. At baseline, TUNEL staining of synovial biopsies showed the presence of distinct cells exhibiting DNA damage in both the intimal synovial lining layer and the synovial sublining (Figure 3a and c). However, neither infliximab nor etanercept significantly increased the number of TUNEL positive cells at week 12 compared to baseline (Figure 3b and d and Table 1). Similar results were obtained analyzing anti-active caspase-3 staining (Table 1). Additional analysis of SpA biopsies obtained after one week of treatment with infliximab also failed to show increased apoptosis (data not shown). Whereas these data show that DNA fragmentation is observed in inflamed synovium, they do not provide evidence for apoptosis induction after infliximab or etanercept treatment in vivo.

Induction of massive cell death in vivo is not sufficient to increase serum nucleosome levels

As this synovial analysis in SpA cannot exclude the possibility of apoptosis induction at sites other than the inflamed joint, we used a different type of targeted treatment to investigate the relationship between the induction of cell death and the elevation of serum nucleosomes and anti-nucleosome antibodies. Sorafenib, a tyrosine-kinase inhibitor used in renal cell carcinoma, is known to induce acute and massive renal cell death (42). In our cohort of 20 renal cell carcinoma patients, sorafenib treatment at week 12 induced marked necrosis of the tumour (Figure 4e and f). However, the serum levels of nucleosomes did not increase during treatment up to week 16 (data not shown), indicating that induction of massive cells death is not sufficient to increase serum nucleosome levels. Accordingly, there was no induction of anti-nucleosome IgM in these patients (data not shown).

Infliximab but not etanercept decreases serum complement levels

As the increase of serum nucleosome levels could not be explained solely by enhanced release during massive cell death, we investigated if TNF blockade with infliximab affected clearance of apoptotic material. CRP is an important acute phase protein implicated in clearing mechanisms. We observed a similar decrease of CRP in both infliximab and etanercept treated patients (Table 1). In accordance, we did not observe any correlation between CRP levels or changes in CRP levels and the anti-nucleosome levels. Beside CRP, the complement factors C3 and C4 have also been reported to play an important role in the clearance of apoptotic cells (43-45). Baseline levels of serum C3 and C4 were comparable to healthy controls (data not shown). C3 levels dropped significantly during infliximab treatment (from 1.3 g/L (1.2-1.4) to 1.0 g/L (0.9-1.2); $P<0.001$) (Figure 4A). In contrast, C3 levels remained stable during etanercept treatment over 12 weeks (from 1.3 g/L (1.1-1.5) to 1.2 g/L (1.0-1.3)) (Figure 4B). Similarly, C4 remained stable during 12 week of treatment with etanercept, but dropped significantly during infliximab treatment (from 0.26 g/L (0.23-0.32) to 0.20 g/L (0.14-0.22); $P=0.001$). Taken together, these data indicate that infliximab but not etanercept treatment is associated with a significant drop in serum C3 and C4 levels.

Discussion

Whereas the absence of known autoantibodies suggests normal, functional central and peripheral tolerance B cell checkpoints in SpA, we previously reported the induction of IgM anti-nuclear antibodies by TNF blockade in these patients (11). As there is a differential induction by the monoclonal anti-TNF antibody, infliximab, and the soluble TNF receptor, etanercept (12), we investigated which of the mechanisms described in experimental SLE models could contribute to break peripheral B cell tolerance in this unique human model of anti-nuclear antigen autoimmunity.

We observed a clear modulation of type I IFN serum activity. Type I IFN are a group of cytokines reported to play a crucial role in autoimmune pathology such as SLE and Sjögren's syndrome (17;33;34). They are central players in a positive feedback loop generating more nuclear material and anti-nuclear antibodies (17-19). TNF blockade sustains type I IFN production in vitro (35). In agreement with these experimental data, we reported previously that etanercept treatment induces a persistent upregulation of type I IFN in Sjögren's syndrome (29). We confirmed this observation in SpA patients treated with etanercept. As to infliximab, a cross-sectional analysis of 5 patients with active systemic onset juvenile chronic arthritis despite anti-TNF treatment for variable periods showed an increase in both IFN type I regulated genes in PBMC's and anti-dsDNA antibody levels (35). In contrast, our prospective, longitudinal data in 20 SpA patients with a good clinical response to infliximab showed that type I IFN serum activity was transiently downmodulated before returning to normal levels. Neither baseline levels nor changes of type I IFN activity correlated with the induction of anti-nucleosome IgM in SpA. This is consistent with the observation that type I IFN changes during etanercept treatment in Sjögren's syndrome did not affect specific autoantibody levels (29). Although the reasons for the differential effect of infliximab and etanercept on type I IFN serum activity remain speculative, these data emphasize the complexity of the cross-regulation between TNF and type I IFN in vivo and indicate that changes in type I IFN are not directly related to the break of B cell tolerance in our SpA model.

Beside factors affecting autoreactive B cells, the antigen itself can also determine the outcome of the B cell response. Apoptotic material represents an important source of autoantigens as intracellular and nuclear antigens are modified during the apoptotic process and are translocated to the cell membrane (7-10). Under normal physiologic conditions, apoptotic cell blebs are rapidly cleared in a non-immunogenic process. However, overload with apoptotic bodies due to increased production or impaired clearance can induce the production of anti-nuclear antibodies (8;10). A link between infliximab treatment and an increase of serum nucleosomes was reported in a previous study in 11 RA patients (26), although it is probably not a very early event as we did not observe it within the first 24 hours of infliximab treatment (46). In the present study, we confirm a significant increase of serum nucleosome levels upon 2 weeks of infliximab treatment. We demonstrate that this is specific for infliximab and not for etanercept and that this phenomenon relates directly to the subsequent appearance of anti-nucleosome IgM antibodies. As such, these data identify the elevated levels of serum nucleosomes as an important factor contributing to the break of B cell tolerance during TNF blockade.

The appearance of elevated levels of serum nucleosomes can be due to high levels of release during cell death and/or disruption of the normal pathways of clearance of the cell debris (27). In mice, increased in vivo cell death induced by LPS or anti-Fas resulted in a rise of plasma nucleosomes in a dose-dependent fashion (47). As infliximab has been suggested to induce apoptosis of activated leucocytes in vitro (15;37;39), this mechanism could contribute to the subsequent release of nucleosomes in the circulation in vivo. However, there is at present no evidence for specific induction of cell death by infliximab but not etanercept in human

arthritis. Several studies in RA and PsA demonstrated that infliximab does not induce apoptosis in the inflamed synovium despite marked clinical and histologic response to treatment (30; 31). Confirming the former studies (30;31), we could not detect any increase of synovial apoptosis in SpA treated with any of these TNF blockers. As the discrepancy between the increase in serum nucleosomes and the absence of synovial apoptosis may be due to infliximab-induced cell death at other sites, we turned to another model to assess the potential relationship between massive cell death and a rise in serum nucleosomes and, eventually, anti-nucleosome antibodies. In renal cell carcinoma, treatment with the tyrosine-kinase inhibitor, sorafenib, leads to massive cell necrosis (42). Necrosis, as opposed to apoptosis, is not a strictly regulated physiological process: the release of inflammatory mediators and modified antigens evokes a strong inflammatory response and is therefore more likely to induce humoral autoimmunity against nuclear antigens. However, the massive cell death in this model did not lead to a rise in serum nucleosome and, subsequently, anti-nucleosome antibodies. Taken together, these data consistently indicate that the rise in serum nucleosomes can not be merely explained by infliximab-induced cell death.

The serum nucleosome levels are not only regulated by release but also by adequate clearance. Factors such as mannose-binding lectin, serum amyloid P, CRP, and complement prevent nuclear antigens from appearing in the circulation under normal conditions. Mice with deficiencies in some of these factors exhibit a delayed clearance of apoptotic cells, which is directly linked to the production of autoantibodies and the development of autoimmune disease (48-50). In our SpA patients, we observed a similar decrease of CRP during infliximab and etanercept treatment, but only the former lead to a highly significant decrease in C3 as well as C4 after 12 weeks of treatment. As both nucleosomes and anti-nucleosome antibodies are induced, it can be expected that they form immune-complexes which lead to complement consumption. On the other hand, infliximab does not induce complement activation (46;51) suggesting that the decrease in C3 and C4 serum levels might be a direct effect rather than due to the consumption, but this hypothesis needs to be formally confirmed. In this context, it is also noteworthy that the increase of nucleosomes was transient and disappeared after 12 weeks despite the low complement levels. On the other hand, the anti-nuclear antibodies themselves persist over more than 2 years, but disappear after interruption of infliximab treatment (12). This suggests that anti-nucleosome IgM antibodies contribute to clear these nucleosomes and disappear when the triggering mechanism has been relieved. Although clearly different from preimmune natural IgM antibodies, the anti-nuclear antibodies induced by infliximab treatment are also restricted to the IgM isotype and could have a similar function in contributing to clear rapidly potentially deleterious antigens (52;53).

In conclusion, in our human model of anti-nuclear antibody induction we could demonstrate that the break in peripheral B cell tolerance is not due to elevated levels of type I IFN, but can be attributed to an increase in the availability of the antigen. The increase in serum nucleosome levels is most probably due to defects in clearance mechanisms rather than an increased release of nuclear antigens.

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Figure 1.

Anti-nucleosome antibody levels at baseline were not different between healthy donors (HD) (n=15) and spondyloarthritis patients (SpA) (A). Changes in anti-nucleosome IgM levels in SpA patients treated with either infliximab (n=20) (B) or etanercept (n=20) (C). The bars represent the median. * P<0.05; ** P <0.01.

Figure 2.

Infliximab and etanercept had a differential effect on type I IFN serum activity (A and B) and serum nucleosome levels (C and D). The data are represented as median and interquartile range compared to baseline values (* $P < 0.05$ ** $P < 0.01$). The early changes in serum nucleosome levels (between baseline and week 2 for infliximab treated patients and week 4 for etanercept treated patients) correlated with the changes in serum anti-nucleosome IgM antibodies between baseline and week 12 ($r = 0.383$; $P = 0.008$) (E) and between baseline and week 34 ($r = 0.453$; $P = 0.014$) (F).

Figure 3.

Representative pictures of TUNEL (Terminal Transferase nick-end-labeling) staining in spondyloarthritis (SpA) synovium before and after 12 weeks of TNF blockade with either infliximab or etanercept (A-D) (magnification $\times 320$). Figure E and F depict representative CT scans of the kidneys of a patient treated with sorafenib, at baseline (E) and after 8 weeks of treatment (F) showing massive necrosis of the renal cells.

Figure 4.

Serum levels of complement C3 were not different at baseline between both groups of spondyloarthritis (SpA) patients (A and B, left data set). Infliximab (A) but not etanercept (B) decreased the serum complement C3 levels after 12 weeks of treatment. ** $P < 0.01$.

Response to treatment in infliximab-treated and etanercept-treated spondyloarthritis (SpA) patients. The patients were evaluated at baseline and after 12 weeks for clinical and histological response. The patient's assessment of pain and the patient's and physician's assessment of global disease activity were scored on a 100 mm Visual Analogue Scale (VAS). Histological parameters were scored on a 4 point semi-quantitative scale. Data are represented as median (range).

Table 1

		Infliximab		Etanercept	
		baseline	week 12	baseline	week 12
Clinical parameters	Patient Pain (VAS)	69 (14-100)	16 (1-86)**	69 (17-99)	12 (0-80)**
	Patient Global (VAS)	69 (17-100)	15 (0-73)**	73 (18-100)	14 (0-69)**
	Physician Global (VAS)	64 (35-89)	15 (8-75)**	54 (36-89)	14.5 (1-59)**
	Duration of morning stiffness (min)	133 (0-300)	10 (1-90)**	75 (18-100)	12 (0-74)**
	C-reactive protein (mg/L)	17 (10-29)	3 (0-79)*	10 (0-150)	3 (0-34)*
	Erythrocyte sedimentation rate (mm/h)	28 (11-101)	7 (1-59)**	16 (1-86)	8 (1-39)*
	Swollen joint count (number)	7 (0-24)	1 (0-7)**	3 (1-19)	0.5 (0-10)**
	Tender joint count (number)	10 (1-20)	0 (0-6)**	5 (1-23)	1 (0-13)**
	Inflammatory infiltration	1.8 (0-3.0)	1.0 (0-2.5)*	2.0 (0-3.0)	0.5 (0-2.5)*
	CD4+ T lymphocytes	1.0 (0-3.0)	0.5 (0-2.0)*	1.0 (0-2.0)	0 (0-2.0)*
Synovial histology	CD8+ T lymphocytes	1.0 (0-3.0)	1.0 (0-3.0)*	1.0 (0-3.0)	0 (0-2.5)*
	CD20+ B lymphocytes	1.0 (0-3.0)	1.0 (0-3.0)	0.5 (0-2.5)	0 (0-3.0)
	CD68+ macrophages	1.5 (0-3.0)	0.5 (0-2.0)*	1.5 (0-2.5)	0.5 (0-2.5)
	Tunel	2.0 (0-3.0)	1.8 (0-3.0)	2.0 (0-3.0)	2.0 (0-3.0)
	Anti-active caspase 3	0 (0-2.0)	0 (0-1.5)	0 (0-1.5)	0 (0-2.5)
		0.5 (0-2.5)	0 (0-3.0)	0 (0-2.5)	0 (0-2.0)

* P < 0.05;

** P < 0.001.