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ORIGINAL ARTICLE

Inflammatory response in patients with active and inactive osteoarthritis

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Abstract In the present study, we have investigated comparatively the inflammatory response of patients with active and inactive osteoarthritis. The sera from 31 healthy individuals, 37 patients with active OA, and 19 patients with inactive OA were assayed for TNF-α, IL-6, sRANKL, RANTES, and MRP8 using ELISA in order to evaluate their potential as markers of disease activity. Also, the spontaneous and LSP-induced release of TNF-α and IL-6 by peripheral blood neutrophils was determined. The activation of OA is associated with elevated TNF-α, IL-6, and RANTES serum levels while sRANKL and MRP8 appeared to be increased in both active and inactive OA. The neutrophil spontaneous and up-regulated by LPS cytokine release can contribute to the exacerbation of OA.

Keywords Osteoarthritis · Cytokines · sRANKL · RANTES · MRP8 · Neutrophils

Introduction

Osteoarthritis is a chronic joint disorder characterized by a slow progressive degeneration of articular cartilage, subchondral bone alteration and variable secondary synovial inflammation. The exact etiology of OA is not well understood. The degradation of cartilage matrix components is due to an increased synthesis and activation of extracellular proteinases, mainly matrix metalloproteinases. As a consequence of deficient stimulation by growth factors an insufficient synthesis of new matrix macromolecules occurs. Diagnosis of the disease and the progression of joint damage are mainly based on evaluation of clinical and radiological findings. Molecular markers can serve as promising indicators for OA evaluation because they can provide more direct information about the local inflammation, the alterations in joint tissues and related bone and cartilage turnover. Additionally, these markers may help in the identification of patients at high risk of rapid joint destruction, and the prediction of disease progression [1]. Although OA is defined as a noninflammatory arthropathy, proinflammatory cytokines such as IL-1, TNF-α, and IL-6 have been implicated as important mediators in OA [2]. In the late acute-phase response, these cytokines can provoke many systemic changes, including increased production of acute-phase proteins, C-reactive protein and serum amyloid A, although to a lesser extent than in rheumatoid arthritis (RA). Synovitis has often been observed in experimental OA, and recent data have shown a central role for inflammatory cytokines as biochemical signals which stimulate chondrocytes to release cartilage-degrading proteinases. High levels of IL-1β, IL-10, IL-17, and TNF-α [3, 4] in the serum and synovial fluid were observed in RA and OA arthritis compared to the healthy individuals. In the OA synovium, the inflammatory and destructive responses are driven through a combined action of IL-1 and TNF-α [5–7]. Also, high serum levels of the soluble receptors of TNF-α are associated with lower physical function, increased OA symptoms, and worse knee radiographic scores [8]. Elevated levels of nitric oxide (NO) production are found in

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osteoarthritic joints suggesting that NO is involved in the pathogenesis of OA. NO mediates many of the destructive effects of IL-1 and TNF-α in the cartilage [9]. Elevated serum levels of IL-6 have been detected in patients with OA [10]. However, another authors claim that synovial fluid IL-6 levels may help to classify OA patients, while serum levels are not indicative [11]. In animal model, IL-6 deficiency resulted in a mild transient inflammation whereas wild-type mice developed a chronic, destructive synovitis [12]. TNF-α and IL-1, together with receptor activator of NF-kB ligand (RANKL) are known to promote osteoclast recruitment differentiation, and activation in arthritic disorders [13, 14]. Available data show that synovial and serum RANKL levels are increased in arthritic patients [15, 16] and consequently, RANKL, can powerfully promote osteolysis in RA and OA [17]. Among various chemotactic factors, chemokines constitute a group of important mediators and the major role of RANTES, a C–C family derived chemokine in RA has been established. Synovial fluid obtained from patients with OA and RA show high RANTES level and high serum concentration of the chemokine has been associated with a rapid progression of radiographic changes [18, 19]. Chondrocytes from OA patients produce RANTES and express its receptor [20]. The level of RANTES may be an useful additional marker for disease activity in OA and can be used as an index of prognosis.

Myeloid-related proteins (MRP)-8 and -14 belong to the S-100 family of calcium binding proteins associated with myeloid cell differentiation. They are highly expressed in resting neutrophils, keratinocytes, in infiltrating tissue macrophages and on epithelial cells in active inflammatory disease [21]. Various conditions have shown significant correlation of MRP8/14 and MRP8 levels with disease activity. MRP8/14 and MRP14 are generally associated with acute, and MRP8 with chronic inflammatory conditions [22]. The diagnostic value and advantage of MRPs over other disease markers is that they are released immediately upon activation of the respective cell population. It will be of interest to follow the changes in serum MRPs levels in relation to OA development.

Recent studies have identified an important role for neutrophils in initiating and maintaining inflammatory processes in the joint, since neutrophils may contribute to bone remodeling at inflammatory sites, where they are present in significantly large numbers in human RA and in a murine arthritis models [23]. Neutrophils in the synovial fluid are capable of degrading both proteoglycans and collagens in intact human articular cartilage [24]. Thus, it might be expected that they can contribute to the development and severity of OA.

In the present study, we have investigated comparatively the inflammatory response of patients with active and inactive OA in regard to serum levels of TNF-α, IL-6, sRANKL, RANTES, and MRP8, and to the activation of peripheral blood neutrophils.

Materials and methods

Patients

In this study, informed consent was obtained from all participants. We have included 56 patients, diagnosed as having OA, according to the American College of Rheumathology 1987 revised criteria [25]. They were divided into two subgroups according to the presence of clinical and laboratory data about active inflammatory process. Clinically, the patients expressed pain and limited motion in one or more joints. Pathological lesions of osteoarthritis were demonstrated by conventional radiography of at least one joint. The results showed asymmetric joint space narrowing, subchondral sclerosis, osteophyte formation, and/or joint ankylosis. In OA, the changes in the cartilage proceeded bone erosion, contrary to systemic joint inflammation which begins in the bone and radiography shows bone erosions. The first group with active OA consisted of 37 patients (31 women, 6 men) expressed swelling, local hyperthermia of one or more joints and high ESR. The mean ± SEM duration of disease was 5.9 ± 1.2 years. The second group with inactive OA (12 women, 7 men) enrolled 19 patients with a mean ± SEM disease duration of 3.6 ± 0.9 years and lacked above mentioned symptoms (Table 1). All patients had received a symptomatic therapy with NSAIDs. Thirty-one healthy control subjects (23 women, 8 men) were included.

Isolation of peripheral blood neutrophils

Whole-blood samples were collected in plastic syringes containing heparin (Kabe Labortechnik, Nubrecht-Esenroth, Germany). Erythrocytes were eliminated after 40 min incubation with 6% dextran 500/0.9% NaCl solution (pH 7.4) at room temperature. The purification of isolated human neutrophils was performed after gradient centrifugation on Histopaque 1083 (Sigma-Aldrich, Diesenhofen, Germany) at 1,400×*g* for 40 min at room temperature, followed by the hypotonic lysis of residual erythrocytes with cold distilled water for 30 s. After washing with cold PBS (Cambrex), neutrophils were resuspended in sterile RPMI-1640 (Cambrex) supplemented with 2 mM L-glutamine, 5% fetal calf serum (FCS) (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin. The purity of the cell population containing >95% viable neutrophils was monitored by light microscopy. Neutrophils (2×10^6/ml) were cultured in 24-well plates (Becton Dickinson) in a humidified,

Table 1 Basic characteristics of age- and sex-matched patients and healthy controls

	Healthy controls	Active OA	Inactive OA
No. of subjects	31	37	19
Age, mean ± SEM (range) years	40.0 ± 5.0 (35–45)	58.6 ± 2.1 (43–88)	58.4 ± 2.4 (43–81)
No. of women/no. of men (%women/%men)	23/8	31/6 (83/17)	12/7 (63/37)
Duration of arthritis, mean ± SEM years	NA	5.9 ± 1.2	3.6 ± 0.9
Number of swollen joints, mean ± SEM	NA	2.9 ± 0.3	2.47 ± 0.2

NA not applicable

5% CO2 atmosphere at 37°C and were stimulated with 1 μg/ml of LPS (Sigma-Aldrich) for 24 h. Supernatants were collected and frozen at −70°C.

Determination of cytokine, chemokine, and MPR8 serum concentrations

Sera from RA patients and healthy controls were analyzed using commercial ELISA kits for TNF-α, IL-6, sRANKL and RANTES (Peprotech, UK) and for MRP8 (Bachem, USA) following manufacturers' instructions. All samples were assayed in duplicate and the concentration was calculated from a standard curve of the corresponding recombinant proteins.

Statistics

Data are presented as the mean ± SEM. The 2-tailed Mann–Whitney *U* test was used to compare the levels of cytokines in different groups of patients and healthy individuals. The correlation between different parameters was assessed by linear regression analysis and unpaired *t* test with Welch's correction. *P* values less than 0.05 were considered significant.

Results

Serum levels of cytokines and chemokines in healthy individuals and OA patients

Data in Fig. 1a and b showed that serum levels of TNF-α and IL-6 were significantly elevated in the patients with active OA compared to healthy controls at $P = 0.012$ and $P = 0.004$, respectively. Patients with inactive OA did not exhibit statistically significant difference neither to healthy controls nor to active OA group. In regard to sRANKL we observed elevated levels in patients with active OA ($P = 0.035$) and in inactive OA ($P = 0.0001$) compared to healthy subjects. Also, inactive OA group showed higher

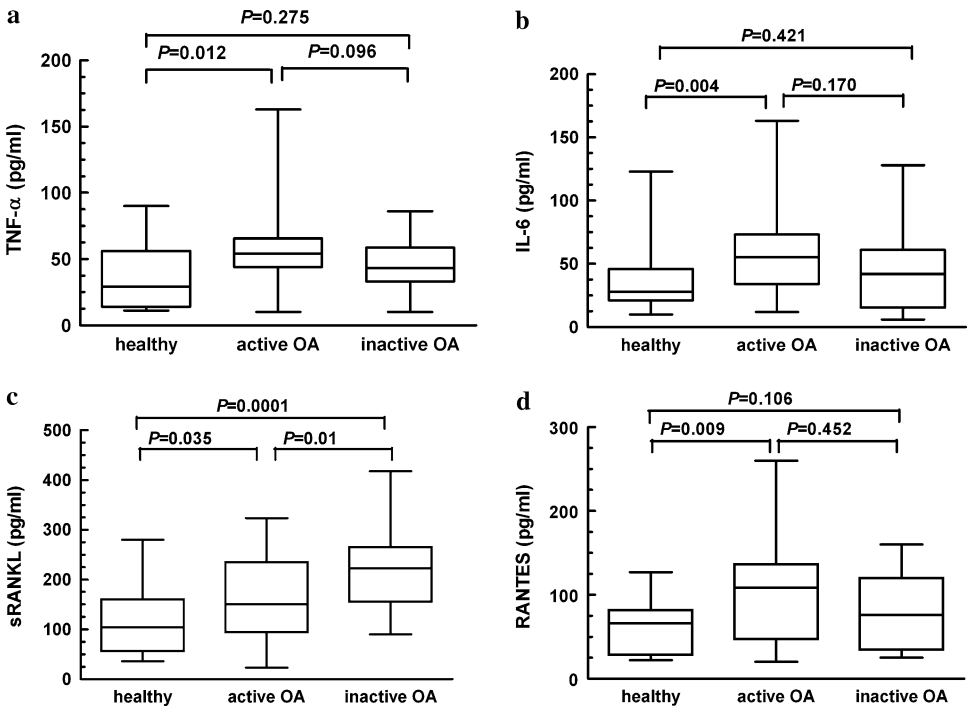

Fig. 1 The levels of TNF-α (**a**), IL-6 (**b**), sRANKL (**c**) and RANTES (**d**) in individual sera of healthy controls ($n = 31$), active OA patients ($n = 37$) and inactive OA patients ($n = 19$). For each group, data are presented as medians in *box plots* ranging from the 25th percentile to the 75th percentile. The *bars* indicate minimum to maximum values. Significance between groups was evaluated by Mann–Whitney test

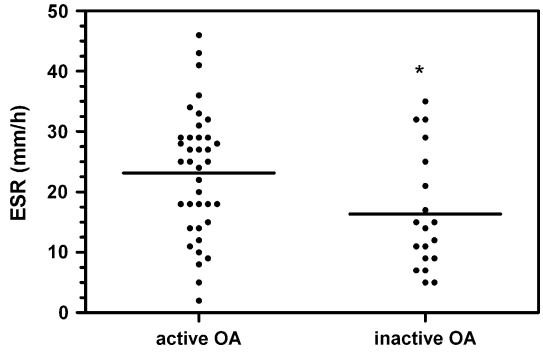

Fig. 2 ESR of active (n = 37) and inactive ($n = 19$) OA patients. *$P < 0.05$, unpaired t test

level of sRANKL than active OA group ($P = 0.01$) (Fig. 1c). Serum level of RANTES was higher in active OA patients while its increase in inactive OA individuals was not statistically significant compared to healthy group (Fig. 1d).

Correlation between cytokine and chemokine serum levels and ESR

ESR is an indicator of the developing inflammatory process, particularly in rheumatic conditions. Patients with active OA showed increased ESR compared to those with inactive OA (Fig. 2). Moreover, we observed a significant correlation between serum TNF-α levels and ESR ($r_s = 0.236$, $P = 0.002$) and between IL-6 levels and ESR ($r_s = 0.116$, $P = 0.035$) in active OA patients (Fig. 3a, b). Also, there was no correlation between sRANKL and RANTES and ESR, neither in active nor in inactive OA patients (data not shown).

Serum MRP8 level

MRP8 is thought to be indicative for the chronic inflammatory diseases, like arthritis. We, therefore, examined the level of this peptide in the circulation of healthy and OA individuals. As shown in Fig. 4 patients with active disease expressed higher level compared to healthy and to inactive OA groups. It should be noted that this difference was due to a relatively small number of patients with extremely high MRP8 level. In the group of active OA they represented 6 from 19 patients (30%), whereas in inactive OA group they represented 3 from 37 patients (8%).

Spontaneous and LPS-induced cytokine secretion by peripheral blood neutrophils

Neutrophils were isolated from healthy and arthritic individuals and in vitro cultivated in the presence or absence of LPS. The concentrations of TNF-α and IL-6 in the supernatants were determined after 24 h. Higher spontaneous production of TNF-α by neutrophils was determined in active OA patients than in healthy individuals (Fig. 5). Twenty-four hours in the presence of LPS increased TNF-α secretion from healthy and inactive OA neutrophil populations. The TNF-α secretion by cells from active OA patients was nonsignificantly stimulated by LPS. The spontaneous IL-6 production was elevated in the group of active OA neutrophils compared to healthy as well as to inactive OA groups. After stimulation all three groups showed enhanced cytokine secretion (Fig. 6).

Discussion

Osteoarthritis is a heterogeneous group of conditions associated with defective integrity of articular cartilage, in addition to related changes in the underlying bone. The chronic inflammatory process is mediated through a complex cytokine network. It is not yet completely understood that all the factors are responsible for initiating the degradation and

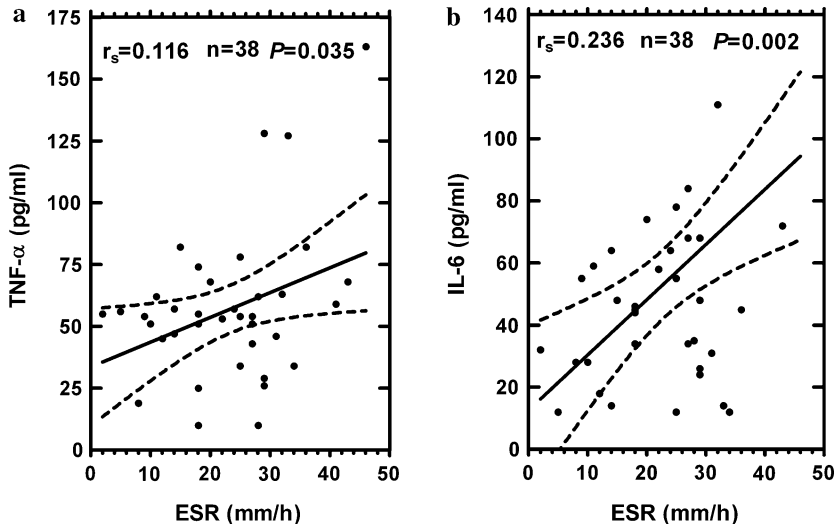

Fig. 3 Correlation between TNF-α and ESR (**a**), and between IL-6 and ESR in active OA patients (**b**). The regression lines and 95% confidence intervals are shown as well as rank correlation coefficient and its P value

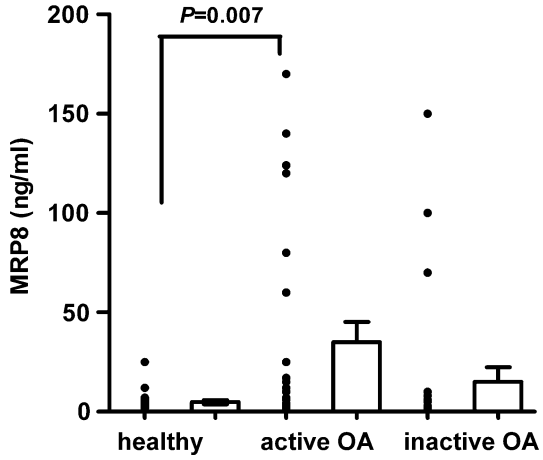

Fig. 4 Serum concentration of MRP8 in healthy controls ($n = 31$), active OA patients ($n = 37$) and inactive OA patients ($n = 19$). *Columns* represent mean ± SEM. *P* values are calculated by unpaired *t* test with Welch's correction

loss of the articular tissues. As cartilage itself has limited capacities of self-renewing, the development of OA is chronic and progressive. The disease is diagnosed in more advanced stages, when clinical and radiographic signs become evident. The early part of the acute-phase response involves the local action and production of cytokines such as IL-1, TNF-α, and IL-6. It is generally accepted that IL-1 is the pivotal cytokine at early and late stages, while TNF-α is involved primarily in the onset of arthritis [26]. In the present study, we have investigated whether serum concentrations of various inflammatory markers are associated with the disease severity. The presence of joint space narrowing, osteophytes, cartilage changes, and the absence of bone erosions suggest OA. In active OA, synovitis appears to be secondary, while in systemic rheumatic diseases synovitis is primary. Two subgroups of OA patients were included according to the presence of active inflammatory process in one or more joints. The age range, duration of OA, and number of affected joints in both groups were very close. In such a way we aimed to minimize their influence on the parameters investigated. We found that plasma TNF-α and IL-6 levels were significantly higher in patients with active OA compared with healthy subjects. Such elevation was not detected in patients with inactive OA. It was observed a great variation in the patterns of cytokine

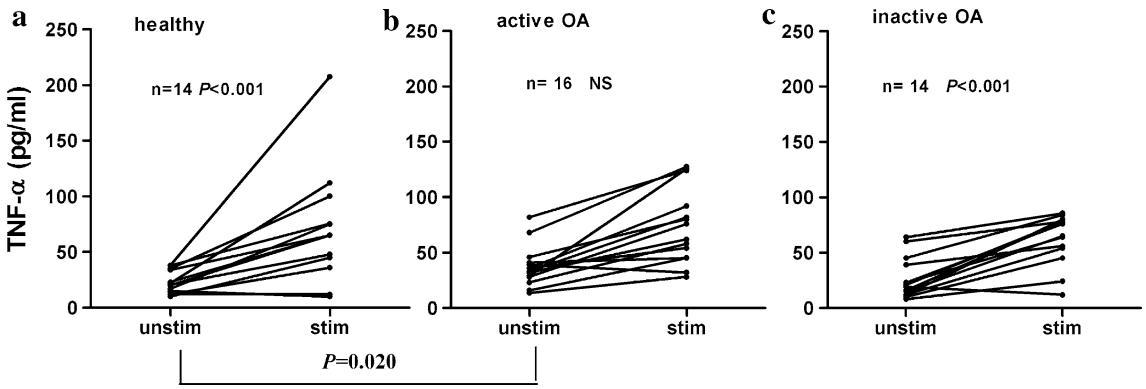

Fig. 5 TNF-α production by unstimulated and stimulated neutrophils. Cells (2×10^6/ml) were cultivated for 24 h at the presence or absence of LPS (1 μg/ml). *P* values are calculated by unpaired *t* test with Welch's correction

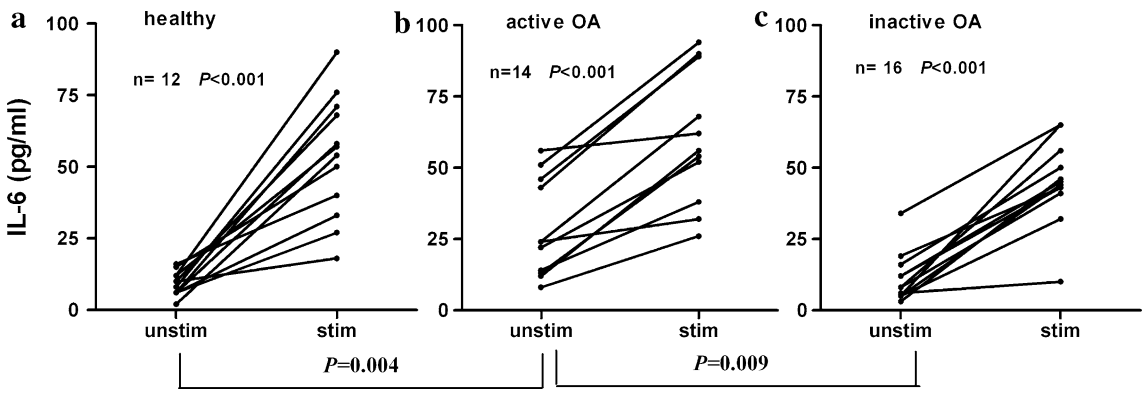

Fig. 6 IL-6 production by unstimulated and stimulated neutrophils. Cells (2×10^6/ml) were cultivated for 24 h at the presence or absence of LPS (1 μg/ml). *P* values are calculated by unpaired *t* test with Welch's correction

concentration between different patients which embarrassed the results' generalization.

Recent studies show higher levels of serum sRANKL in patients with osteoarthritis [27] and in the synovium of active RA and OA patients, which is likely to be an important cause of joint erosions [15]. Our results showed that the increase of serum sRANKL was characteristic for both active and inactive OA. This elevation was less exerted in active OA, pointing on the possibility some mechanisms for sRANKL inhibition to be triggered during the active phase. The chemokine system may play a key role in the cartilage degradation of OA, acting in an autocrine/paracrine manner. Increased serum levels of RANTES have been detected in OA which reflect clinical activity of the disease [19, 28]. We observed that as a result of OA activation the level of RANTES in circulation was increased which further may result in chondrocyte activation and cartilage degradation.

Many inflammatory conditions including arthritis can lead to the increased ESR. It is not unusual to see a patient with OA and an elevated ESR, but we can not attribute this to OA alone. We registered higher ESR values in the group of patients with active OA than in the group of inactive OA patients. The elevation of TNF-α and IL-6 plasma levels well correlated with the enhanced ESR in active OA. Although, sRANKL and RANTES levels were also increased there was no significant correlation with ESR in both active and inactive OA groups.

High levels of myeloid related proteins (MRP) 8 and 14 have been found in the extracellular milieu during inflammatory conditions such as RA. Their concentrations in peripheral blood have been increased in association with the severity of arthritis [29]. The concentrations of MRP8/MRP14 in serum correlated well with those in synovial fluid and after intraarticular therapy, the serum concentrations of the heterodimer decreased significantly in therapy responders, whereas no differences were found in patients who showed no clinical benefit. [30]. The proteins are more enriched in synovial fluid than in blood circulation as a result of infiltration and activation of neutrophils and macrophages in joints of patients with RA [31–33]. Even more scarce data are available about MRP proteins in OA [34]. The present data showed that higher MRP8 plasma level was established in 30% of the patients with active OA and in 8% of the patients with inactive OA. However, we consider that the number of patients was not sufficient to make a definite conclusion that the severity of arthritis correlated with MRP8 plasma level.

The ability of neutrophils to degrade cartilage proteoglycan suggests that the neutrophils that accumulate in the joints of arthritic patients are mediators of tissue damage that results in rapid stimulation of bone erosion during active RA [35]. Neutrophil depletion in animal models of arthritis ameliorated the disease [36]. The regulatory mechanisms which are relevant to the proteoglycan-degrading activity of neutrophils are poorly understood. Present data revealed that neutrophils isolated from patients with active OA spontaneously release TNF-α in contrast to the cells from inactive OA. In the case of IL-6, its release was increased in both active and inactive OA. Thus, it rises the question about the differential role of neutrophils in the active and inactive phases. On subsequent LPS stimulation the cells from healthy as well as from patients with inactive OA were able to up-regulate their TNF-α and IL-6 production. In contrast, neutrophils from patients with active OA expressed low TNF-α production in response to LPS. Probably, being in activated state the cells had reached some threshold making them resistant to stimulation. Simultaneously, they remained responsive in regard to LPS-induced IL-6 production. It might be concluded that during OA peripheral neutrophils can contribute to the elevated levels of proinflammatory TNF-α and IL-6.

In summary, the active phase of OA is associated with an increase of TNF-α, IL-6, and RANTES serum levels. Another markers of joint destruction like sRANKL and MRP8 appeared to be characteristic for both active and inactive OA. These findings may help for better understanding of mechanisms of OA pathogenesis and for elucidation of effective strategies for therapy.

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