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How Important are Innate Immunity Cells in Osteoarthritis Pathology

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1. Introduction

Osteoarthritis (OA) is a chronic degenerative bone disorder leading to cartilage loss, frequently associated with the aging process. It is widely spread in society and often causes disability. Joint swelling which attends OA is due to osteophyte formation or to synovial fluid accumulation. Pathologically, focal damage of cartilage in load-bearing areas are observed together with the formation of new bone at the joint margins, as well as with changes in subchondral bone, and synovitis. Current diagnosis of OA is based on the clinical history and on the radiographical data which occur late at the disease and are very irreversible. Ideally, we wish to detect osteoarthritis at an early stage by following the changes in expression of particular molecular markers, assuming that these markers are sufficiently sensitive, specific, and quantitative for the disease. OA is no longer considered an exclusively degenerative joint disorder because it is related to changes in the synovial membrane as a result of more or less exerted inflammation (Hedbom & Hauselmann, 2002). Trauma or some mechanical problem might be the primary reason for OA initiation. The initiation and progression of OA is sometimes associated with synovial inflammation and the production of proinflammatory and destructive mediators from the synovium causing the invasion of chondrocytes into the cartilage. In early OA the influx of mononuclear cells is enhanced simultaneously with overexpression of inflammatory molecules compared with late OA (Benito et al., 2005). A fundamental question is which cell populations in OA contribute to and maintain synovial inflammation and cartilage destruction.

2. Neutrophils as the main participants in the development of OA

2.1 Phenotype of OA neutrophils

Neutrophils are an essential part of the innate immune system, triggering the initial inflammatory response and the development of host defense mechanisms. During inflammation they leave the circulation and enter the tissues in which they are under the influence of various local factors such as cytokines, endogenous growth factors, microbial products etc. In a result, neutrophils adopt effector functions of great importance for initiation and maintaining of many chronic inflammatory diseases (Duan et al., 2001; Edwards & Hallett, 1997; Mitsuyama et al., 1994).

Neutrophils develop from progenitor cells in bone marrow. They have short lifespans of several hours and then die via apoptosis. Constitutive apoptosis of neutrophils is

regulated by two transcription factors: hypoxia-inducible factor 1 (HIF1) and forkhead box O3A. When certain stimuli such as granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF- α , IL-8 and IFN- γ are provided, the lifespan of blood neutrophils is significantly prolonged (Brach et al., 1992; Kilpatrick et al., 2006). Increased neutrophil survival is related with an enhanced expression of anti-apoptotic genes (Marshall et al., 2007) and by death-inducing receptors belonging to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor super-family, such as Fas, TNF-related apoptosis-inducing ligand (TNFSF10) receptors, TNFRSF9 (CD137), and the type I TNF receptor (Simon et al., 2003).

It has been shown that RA synovial fluid counteracts neutrophil apoptosis and leads to prolonged survival (Ottonello et al., 2002). Such inhibited apoptosis is characteristic for the earliest phase of RA in contrast to other early arthritides (Raza et al., 2006), suggesting that the suppression of apoptosis in RA patients at high risk is a possible therapeutic approach. RA neutrophils are also functionally different from healthy neutrophils as it has been demonstrated by up-regulated expression of complement receptors CR1, CR3 and CR4 (Felzmann et al., 1991). They show an increased chemotaxis to synovium (Pronai et al., 1991) promoted by TNF- α , IL-17, IL-20 and IL-24 (Kragstrup et al., 2008; Shen et al., 2005). Reports about apoptosis of neutrophils in OA are few and controversial. Bell et al. showed that synovial fluid from OA patients contains factors inhibiting neutrophil survival (Bell et al., 1995). There is also a hypothesis that pyrophosphate dihydrate (CPPD) and basic calcium phosphate (BCP) crystals present in the OA joint fluid and tissue can activate Ca²⁺ signal in neutrophils thereby prolonging their survival and reducing their apoptosis (Rosenthal, 2011). Chakravarti et al. isolated a subset of blood neutrophils which represents 8–17% of the total neutrophil population and persists beyond 72 h after an exposure to GM-CSF, TNF- α and IL-4. These neutrophils secrete IL-1, IL-1Ra and IL-8, and interact strongly with resident stromal cells (Chakravarti et al., 2009). The phenotype of “long-lived” neutrophils also differs from that of the circulating neutrophils. Although they express the common neutrophil cell surface markers CD32, CD18 and CD11b, they acquire new surface markers, such as HLA-DR and the co-stimulation molecule CD80 (Cross et al., 2003).

Neutrophils participate actively in joint inflammation as proven by their depletion in an experimental model of arthritis (Santos et al., 1997). They affect chemotaxis of macrophages and dendritic cells by cleaving prochemerin to chemerin. Neutrophils produce TNF- α and other cytokines like IL-1, IL-6 that drive the differentiation and activation of dendritic cells and macrophages. The destructive potential of neutrophils is related with their ability to release reactive oxygen species and granules with myeloperoxidase, defensins and MMP-8, MMP-9, MMP-25. MMPs are secreted in response to IL-8 and through ERK1/2 and Src-family kinase pathways (Chakrabarti & Patel, 2005).

2.2 RANKL expression on OA neutrophils

The uncoordinated bone remodeling events in OA results from the impaired balance between bone resorption mediated by mature osteoclasts and bone formation mediated by osteoblasts. The receptor activator of nuclear factor- κ B ligand (RANKL) and its receptor RANK are actively involved in osteoclast formation (Yasuda et al., 1998). Immature osteoblasts express RANKL which binds to RANK on osteoclasts, initiating the recruitment of osteoclast precursors in bone marrow and promoting their differentiation.

Intracellularly, RANK interacts with TNF receptor-associated factor 6 (TRAF6), which unlocks signaling through NF- κ B, p38 kinase, and c-Jun N-terminal kinase (Teitelbaum, 2000). OPG is released by osteoblasts and stromal cells and is expressed by macrophages in synovial lining layer. Bone resorption is controlled by the balance between RANKL, RANK and osteoprotegerin (Crotti et al., 2002). The inhibition of RANKL in serum transfer model (Ji et al., 2002), in TNF- α -induced model (Keffer et al., 1991) and in autoimmune type II collagen-induced arthritis (Kamijo et al., 2006) resulted in amelioration of bone destruction.

Recently, Poubelle and co-workers reported that RA neutrophils express RANKL and are activated through RANK/RANKL interaction (Poubelle et al., 2007). Despite the studies in RA, there are no investigations showing the involvement of RANKL positive neutrophils in the pathogenesis of OA. A little is known about the expression of RANKL in active or inactive stages of OA. We have conducted a study on RANKL expression by neutrophils in OA patients and in mouse models. OA patients have been divided into two groups depending on the presence of active inflammatory process. The first group, with active OA had swelling, local hyperthermia of one or more joints and high erythrocyte sedimentation rate (ESR). The second group with inactive OA lacked above mentioned painful swelling and local hyperthermia. A number of healthy controls have also been included (Table 1).

	healthy controls	active OA	inactive OA
No. of subjects (women/men)	10 (4/6)	12 (5/7)	14 (6/8)
Duration (years)	not assessed	4.5 \pm 1.2	2.5 \pm 1.2
ESR (mm/h)	<20	24.8 \pm 6.2	14.0 \pm 3.6
RF	not assessed	<20	<20
CRP	<0.01	58.9 \pm 30.4	<0.01

CRP - C reactive protein; ESR-erythrocyte sedimentation rate; OA-osteoarthritis; RF-rheumatoid factor; Data are expressed as mean \pm standard error of the mean

Table 1. Basic characteristics of healthy donors and OA patients

The intensification of OA symptoms at established phase of the disease can be due to calcium-containing crystals. The basic calcium phosphate (BCP) and hydroxyapatite (HA) crystals are often found in joint fluid and tissues of OA patients. The reason for their accumulation is not clear but their contribution to the aggravation of inflammation is obvious (Mebarek et al., 2011; Rosenthal et al., 2011). The elevated ESR might reflect their action on innate immunity cells, inducing inflammatory signals and amplification of already generated. Moreover, the increase of BCP concentration correlates with the severity of the disease (Yavorsky et al., 2008). To provoke an inhibition of crystal deposition or their degradation represents a novel and tempting approach for application in chronic phase which can limit joint damage.

In our study low percentage of RANKL positive blood neutrophils was detected in healthy donors ($0.8 \pm 0.4\%$). After their *in vitro* stimulation with TLR2 agonist, zymosan, they responded with an increased RANKL expression ($8.22 \pm 0.07\%$). Significantly higher percentage of RANKL positive blood neutrophils were observed in patients with active OA ($14.75 \pm 5.07\%$; $p < 0.001$ vs healthy) and inactive OA ($9.77 \pm 2.16\%$; $p < 0.01$ vs healthy) but only inactive group responded to zymosan stimulation (Fig. 1).

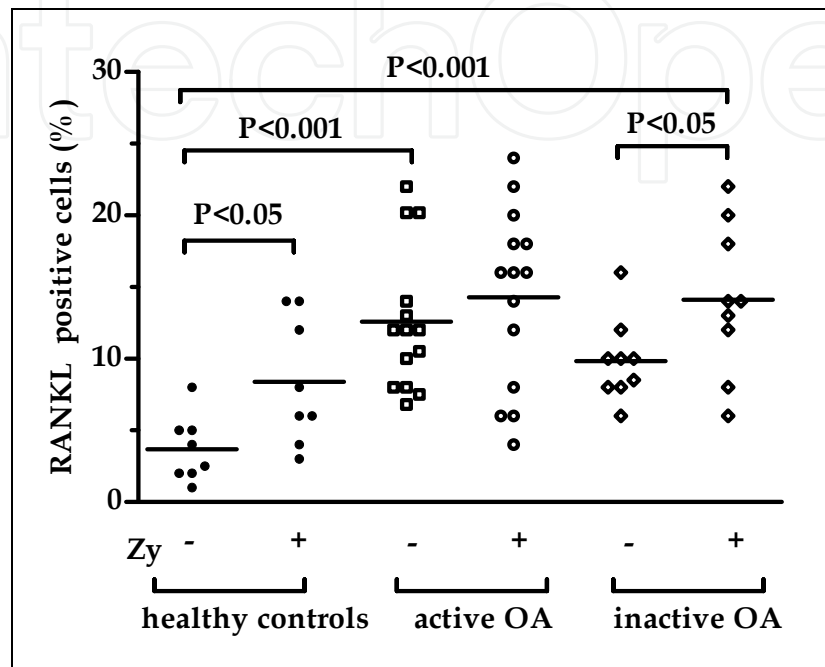


Fig. 1. RANKL expression on neutrophils isolated from healthy controls and patients with active and inactive osteoarthritis (OA). Cells ($1 \times 10^6/\text{ml}$) were incubated (37°C , 2 h) in the absence or presence of $10 \mu\text{g}/\text{ml}$ of zymosan (Zy). After washing neutrophils were stained with rat antibody against human RANKL and isotype control, followed by secondary FITC-conjugated anti-rat antibody. Data represent percentage of RANKL positive cells with the median value in the group (see lines in each group).

Experimental osteoarthritis was induced according to a method described by Blom et al. (Blom, et al. 2007). Mice received two intra-articular injections of collagenase (2 times 1×10^6 collagenase) at day 0 and 2. Arthritis onset occurs within 7 days. ZIA was induced by intra-articular injection of $180 \mu\text{g}$ ($10 \mu\text{l}$) of zymosan. In both models BALB/c mice 10-12 week old with weight 20-22 g were used. Whole-blood samples were collected in heparin and neutrophils were isolated by dextran sedimentation followed by gradient centrifugation, resuspended at $1 \times 10^6/\text{ml}$ and stimulated for 2 h with zymosan ($10 \mu\text{g}/\text{ml}$). After washing neutrophils were stained with rat antibody against mouse RANKL and isotype control, followed by secondary FITC-conjugated anti-rat antibody. Data from one representative experiment shows the mean fluorescence intensity and the percentage of RANKL positive cells.

Further, we have investigated RANKL expression on neutrophils in two mouse models: collagenase-induced osteoarthritis and zymosan-induced arthritis. We found low frequencies of RANKL positive neutrophils in blood of CIOA mice and a weak response to *in vitro* zymosan stimulation (Fig. 2). Blood neutrophils from ZIA mice expressed RANKL and responded to TLR2 engagement with increased RANKL expression (Fig. 2).

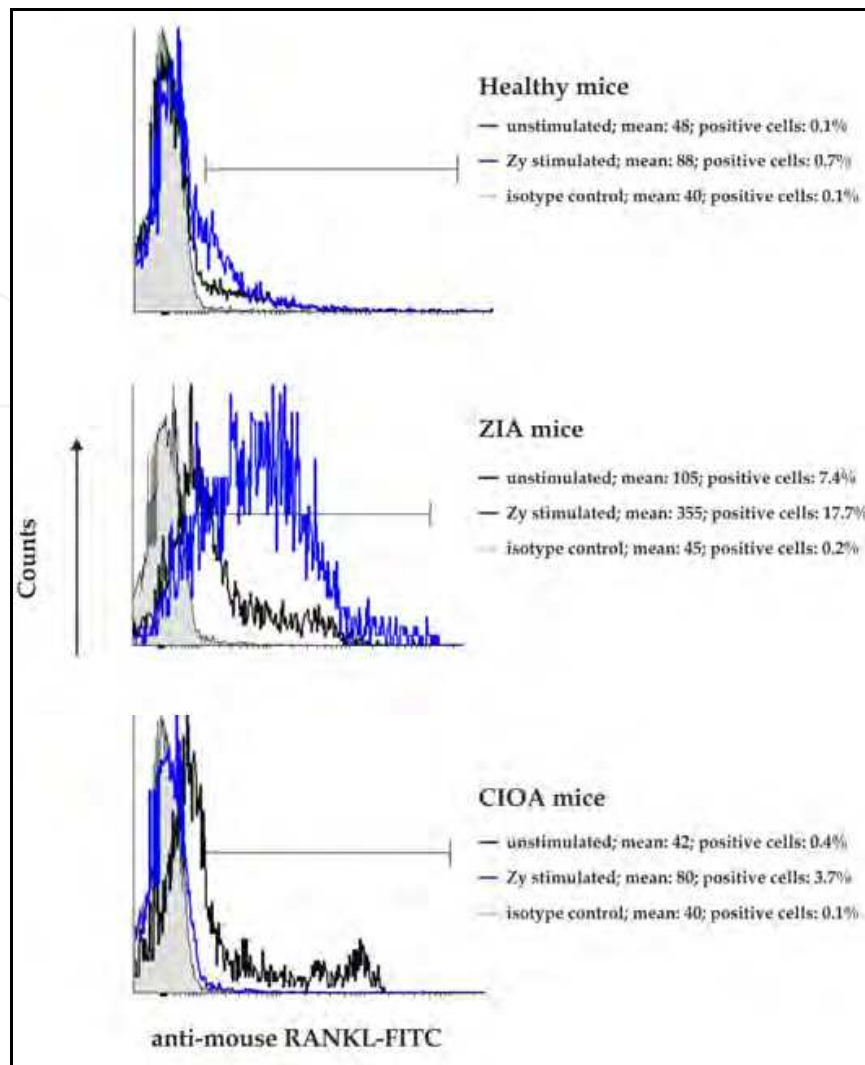


Fig. 2. RANKL expression by blood neutrophils isolated from mice with zymosan-induced arthritis (ZIA) and collagenase-induced osteoarthritis (CIOA) at day 30 of disease.

2.3 MRP8/MRP14 as a potential OA marker related to neutrophil recruitment

The rate of neutrophil and macrophage infiltration at the site of inflammation is associated with myeloid-related proteins (MRP)-8 and -14, belonging to S-100 family of calcium binding proteins. MRP8 and MRP14 are secreted by human monocytes after activation of protein kinase C (PKC) (Rammes et al., 1997). In inflammation they are expressed by infiltrating neutrophils, keratinocytes and monocytes in contrast to resting-tissue macrophages and lymphocytes (Frosch et al., 2000; Kerkhoff et al., 1998). Increased expression of these molecules has been established in various inflammatory disorders including RA (Youssef et al., 1999), psoriasis (Kunz et al., 1992), inflammatory bowel disease (Rugtveit et al., 1994), PsA (Kane et al., 2003). MRP8/MRP14 expression is very low in normal tissues and in RA patients in clinical remission, but high in patients with active disease (Brun et al., 1994). Similar levels of MRP8/MRP14 are found in synovial fluid from patients with psoriatic arthritis, RA and spondyloarthritis without a correlation with disease duration and clinical expression of arthritis activity (Bhardwaj et al., 1992). The proteins enriched more the synovial fluid than the blood circulation as a result of infiltration and

activation of neutrophils and macrophages in the joints of RA patients. Very scarce data are available about MRP proteins in OA. It is supposed that the contact of phagocytes with activated endothelium leads to release of MRP8/MRP14, which induces the switch from selectin-mediated reversible adhesion to integrin-dependent tight contact (Frosch et al., 2000).

However, MRP8/MRP14 levels might be reliable prognostic marker for disease activity and for the effectiveness of immunosuppressive therapy. Methotrexate (MTX) treatment resulted in reduced MRP8/MRP14 serum levels (Kane et al., 2003; Ryckman et al., 2003). It is considered that in acute inflammation MRP8/14 and MRP14 are well represented, while MRP8 is associated with chronic inflammatory conditions (Roth et al., 2003). In our study we have included OA patients with or without an active inflammatory process in one or more joints (Toncheva et al., 2009). We observed high MRP8 plasma level in 30% of the patients with active OA (number of patients =37) and in 8% of the patients with inactive OA (number of patients =19). In healthy donors MRP8 level was very low (number of donors =31). Although our data suggest that the severity of OA might correlate with MRP8 plasma level it will be important to continue such investigations in larger groups of patients. The diagnostic value and advantage of MRPs over other disease markers is that they are released immediately upon activation of the particular cell population.

2.4 TLR2 expression by OA neutrophils

The progression of arthritic processes might be supported by the recognition of microbial or host-derived ligands found in arthritic joints. It has been shown that TLR2 and TLR4, but not TLR9, play distinct roles in disease pathogenesis (Abdollahi-Roodsaz et al., 2008). Knockout (IL1rn/-) mice spontaneously develop T cell-mediated arthritis dependent on TLR activation since germ-free mice fail to develop arthritis. Activation of TLRs on macrophages and dendritic cells leads to the production of proinflammatory cytokines, including TNF- α and IL-1 β . The role of the innate immune system in RA and in experimental models of RA has been the object of recent investigations. An increased expression of TLR2 and TLR4 on peripheral blood monocytes and in the synovial tissues from patients with RA has been observed (De Rycke et al., 2005; Iwahashi et al., 2004). Limited data exist on the quantitative TLR2 and TLR4 expression by synovial macrophages, but it is known that their expression was increased in RA compared with osteoarthritis or normal synovial tissue (Radstake et al., 2004). TLRs through NF- κ B activation provoke the production of innate immunity mediators, like IL-1, IL-6, IL-8, and TNF- α (Takeuchi et al., 2000; Wang et al., 2001). Consequently, these molecules up-regulate TLR expression, e.g. TNF- α stimulates TLR2 gene expression in contrast to TLR4 (Matsuguchi et al., 2000). The stimulation of cultured RA synovial cells with IL-1 β and TNF- α leads to an increase of TLR2 mRNA expression but such increase of TLR4 and TLR9 expression is not detected in the absence or in the presence of stimuli (Seibl et al., 2003). These results are not specific for RA, because cells derived from joints of patients with OA responded similarly to TNF- α stimulation.

Neutrophils express all known human TLRs except TLR3 (Hayashi et al., 2003). TNF- α and IL-6 production in neutrophils can be triggered upon the engagement of TLR2, TLR4, TLR9. In neutrophils the ligation of TLRs results in activation of MAPK and NF- κ B but not always involves the adaptor protein MyD88. In respect to TLR4, recently it has been reported that

LPS can induce different response in adherent and in suspended neutrophils. In adherent cells TLR4 ligation triggers Jun activation and the release of the chemokine monocyte chemoattractant protein-1, an activated protein-1-dependent gene product that is important for monocyte recruitment. Adherent neutrophils interact with matrix proteins through shedded CD43. CD43 (leukosialin) is a heavily sialylated molecule that is cleaved by neutrophil elastase near the plasma membrane. In blood CD43 binds albumin that protects it from the elastase action. In inflammatory conditions neutrophils secrete elastase enhancing the spread and rolling of neutrophils. In RA the destruction of cartilage and bone might be associated with the activation of synovial cells through TLR2. The ligands of TLR2 include lipopeptides and peptidoglycan (Aliprantis et al., 1999; Schwandner et al., 1999), and zymosan acting in collaboration with TLR6 and CD14 (Ozinsky et al., 2000). While TLR4 is weakly represented on the surface of human neutrophils, TLR2 and CD14 are well expressed (Kurt-Jones et al., 2002).

To investigate the involvement of TLR2 in OA we followed its constitutive and Zy-induced expression in blood neutrophils. Data in Fig. 3 show that the percentage of TLR2 positive neutrophils from OA patients was approximately 4 fold higher compared to healthy donors. Zymosan stimulation resulted in 2 fold enhancement of TLR2 expression on neutrophils from all groups.

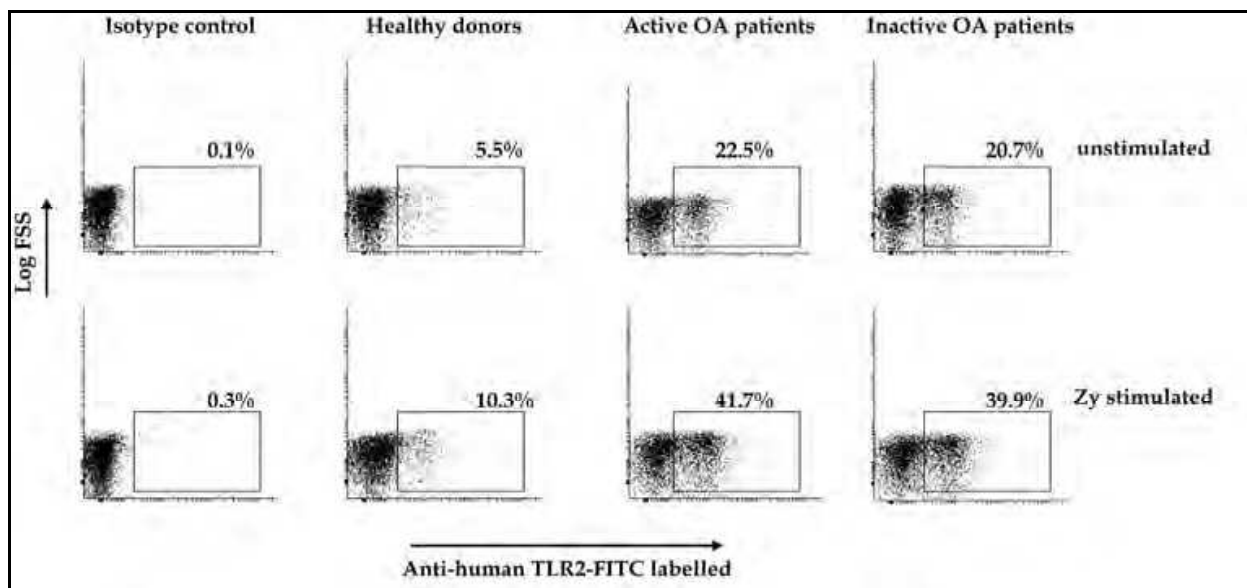


Fig. 3. TLR2 positive neutrophils from healthy donors and patients with active and inactive osteoarthritis (OA). Cells (1×10^6 /ml) were incubated (37°C , 2 h) in the absence or presence of $10 \mu\text{g}/\text{ml}$ of zymosan (Zy). Cells were collected, washed and stained with rat antibodies against human TLR2 ($3 \mu\text{g}/\text{ml}$), followed by secondary FITC-conjugated anti-rat antibody.

Neutrophils isolated from healthy and OA donors were *in vitro* simulated with zymosan and the secretion of $\text{TNF-}\alpha$ was determined. Neutrophils from patients with active OA spontaneously release the cytokine in contrast to healthy and inactive OA groups. Zymosan significantly enhanced $\text{TNF-}\alpha$ secretion of healthy donors and inactive OA (Fig. 4A). The spontaneous release of $\text{TNF-}\alpha$ was higher in ZIA and CIOA groups compared to healthy group. Neutrophils from arthritic mice did not respond significantly to zymosan stimulation, while healthy group showed increased $\text{TNF-}\alpha$ production (Figure 4B).

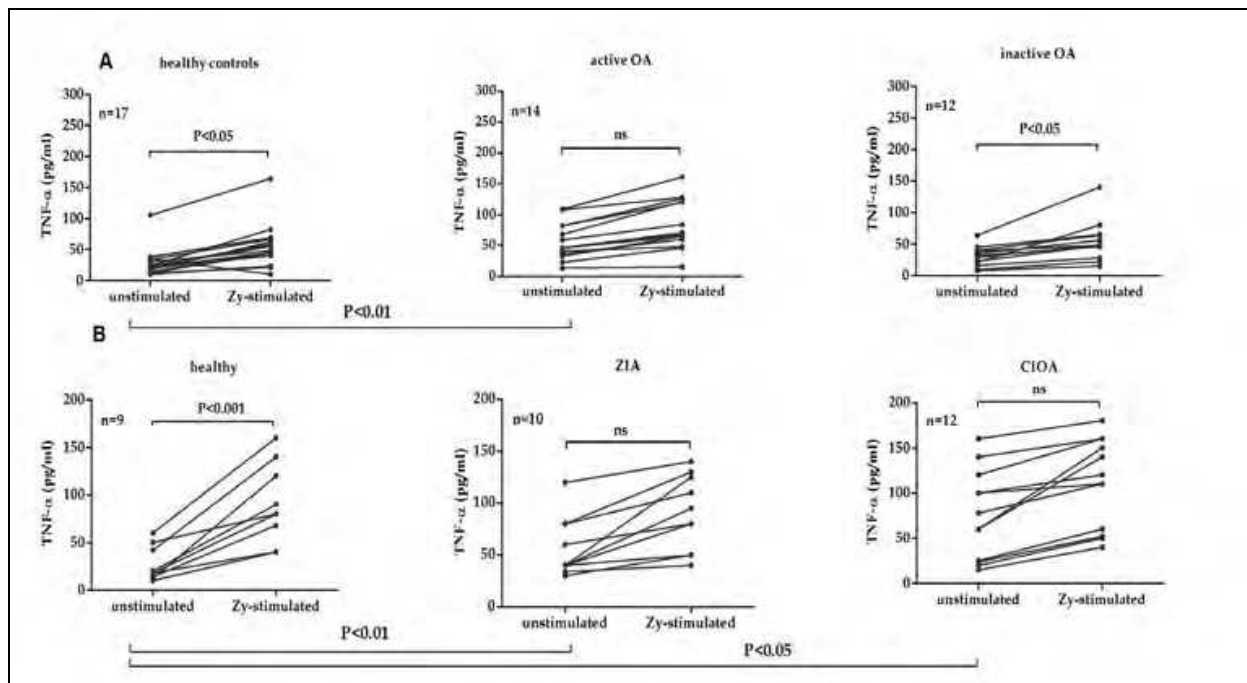


Fig. 4. Spontaneous and zymosan-induced TNF- α production by blood neutrophils. (A) Cells isolated from healthy donors and patients with active and inactive osteoarthritis (1×10^6 /ml) were incubated (37°C , 24 h) in the absence or presence of $10 \mu\text{g}/\text{ml}$ of zymosan. (B) Cells isolated from healthy mice and mice with zymosan-induced arthritis (ZIA,) and collagenase-induced osteoarthritis (CIOA) at day 30 of arthritis (1×10^6 /ml) were incubated (37°C , 24 h) in the absence or presence of $10 \mu\text{g}/\text{ml}$ of zymosan. TNF- α concentration in the supernatants was determined by ELISA.

The up-regulation of TLR2 corresponds either to a response to an exposure to microbial compounds or is secondary to the inflammatory milieu present in the rheumatoid joints. TNF- α is a key mediator in inflammatory joint diseases. We observed the activation state of neutrophils at least in active OA, which was witnessed by their spontaneous *ex vivo* TNF- α release. Our previous results showed that TLR4 ligand LPS is able to trigger *in vitro* TNF- α release by neutrophils from patients with inactive OA (Toncheva et al., 2009). Zymosan also enhanced TNF- α production of neutrophils from inactive OA patients. Probably in active OA, being in activated state cells has reached a threshold after which they become unresponsive. The results from ZIA and CIOA point on such possibility if we accept that ZIA is relevant to more severe inflammatory condition than CIOA. In synovial explant cultures it has been proved that a monoclonal antibody against TLR2 can inhibit the spontaneous release of TNF- α , IFN- γ , IL- 1β and IL-8. Such data are a good base for future investigations on the use of TLR2 antagonists by clinicians, because the effect of anti-TLR2 antibodies is comparable with that of the TNF inhibitor adalimumab (Nic An Ultaigh et al., 2011).

2.5 Activation of monocytes and macrophages during OA

Monocytes and macrophages play an important role in various inflammatory conditions, depending on their stage of activation (Burmester et al., 1997; Tak et al., 1997). Monocytes are subdivided into two different populations with distinct phenotype and functional activity. While classical monocytes are CD14^{hi}CD16⁻ in humans and GR1⁺ in mouse, non-classical monocytes are CD14^{low}CD16⁺ in man and GR1⁻ in mouse. Classical monocytes highly express the CC-chemokine receptor 2 (CCR2), CD62 ligand (CD62L) (Tacke et al., 2007) and vascular cell adhesion molecule 1 (VCAM1 or CD106), and produce low levels of proinflammatory cytokines like TNF- α and IL-1. The latter mediators activate synovial endothelial cells and other leukocytes and initiate inflammatory process. Non-classical monocytes or “resident” monocytes express high level of CX₃C-chemokine receptor 1 (CX₃CR1) and are potent antigen-presenting cells. It has been shown that monocytes from arthritic patients with active disease have reduced HLA-DR expression and decreased capacity to stimulate T cells *in vitro* than healthy cells (Muller et al., 2009). Moreover, TNF- α inhibits HLA-DR synthesis in arthritic myeloid cells via the expression of class II transactivator (CIITA). In comparison to RA, OA patients showed lower density of HLA-DR expression on peripheral monocytes (Koller et al., 1999) suggesting their intrinsic functional abnormality to act as antigen-presenting cells.

An accumulation of macrophages is found in the synovium of patients with early OA (Benito et al., 2005). These resident tissue cells are CD68 positive. CD68⁺ macrophages in OA synovium were restricted to the lining layer while in RA patients they were found in the sublining layer and the areas around newly formed micro-vessels. In the study of Bloom et al. macrophages are depleted prior the development of early OA by injection of clodronate liposomes (Blom et al., 2004). The lack of macrophages reduced the size of osteophytes and lining thickness, and inhibits chondrocyte ossification and fibrosis. Macrophage depletion also down-regulates the expression of bone morphogenetic proteins, BMP2 and BMP4 in synovium (van Lent et al., 2004). Both molecules are important regulators of bone remodeling process and their inhibition has good therapeutic potential in OA as we have observed in a model of CIOA (Ivanovska & Dimitrova, 2011).

Under OA conditions, CD68⁺ macrophages in synovial lining layer are persistently activated via NF- κ B, STAT and Pi3K signaling pathways. They are the source of inducible nitric oxide and of proinflammatory cytokines like TNF- α and IL-1 β (Benito et al., 2005) driving inflammatory process in OA and causing synovitis and bone erosion. Apart of this role, macrophages can participate directly in cartilage degradation. They are capable to produce enzymes degrading extracellular matrix macromolecules like disintegrin-metalloproteinases with thrombospondin motifs and MMPs like MMP-2, MMP-3 and MMP-9 (Smeets et al., 2003). While MMP-2 activates the expression of other MMPs in chondrocytes, MMP-3 is directly involved in cartilage destruction. Serum level of MMP-3 is associated with joint space narrowing in OA patients (Lohmander et al., 2005). MMP-3-deficient mice show significantly decreased cartilage damage (Blom et al., 2007). In rabbit model of experimental arthritis MMP-3 is initially up-regulated in the synovium contributing to the appearance of cartilage lesions while MMP-3 derived from chondrocyte exacerbate cartilage loss at late phases of OA (Mehraban et al., 1998). Blom et al. showed that at early experimental OA synovial macrophages are responsible for the initial MMP-3 production (Blom et al., 2007). These data are based on the observation that MMP-3 expression in the synovium is strongly reduced in the absence of synovial macrophages.

Secreted by macrophages MMPs are proteases that cleave not only collagen in bone matrix but also can modify other molecules present in the OA synovium. For example MMP-9 cleaves chemokines CXCL1 and CXCL8 increasing their potency to attract neutrophils and to amplify inflammation and bone erosion (Van den Steen et al., 2000).

Synovial macrophages can secrete pro-inflammatory mediators that increase MMP expression by synovial cells or chondrocytes. Macrophages isolated from synovial lining layer of OA patients produce spontaneously IL-1 β and TNF- α . In synovial cell co-cultures macrophages stimulate the synovial fibroblasts to produce MMPs and cytokines like IL-6 and IL-8 via a synergistic action of IL-1 β and TNF- α (Bondeson et al., 2006). IL-1 β synthesis in OA is independent of TNF- α and correlates with OA severity. The biological activity of IL-1 β is regulated by the balance between the expression of the active receptor IL-1RI and the inactive or decoy receptor IL-1RII. While the IL-1RI is highly expressed the decoy receptor IL-1RII is little or missing in OA. This receptor imbalance in turn decreases the ability of IL-1RII to neutralize completely and eliminate active IL-1. IL-1 together with TNF- α promotes osteoclast differentiation and bone resorption. It has been shown that IL-1 alone can induce osteoclastogenesis but only in osteoclast precursors over-expressing IL-1RI (Kim et al., 2009). Several *in vitro* studies show that IL-1 inhibition by natural inhibitors such as IL-1 receptor antagonist or soluble receptors decreases MMP expression and cartilage destruction in OA (Jacques et al., 2006).

Macrophages can produce pro-inflammatory cytokine IL-18. The administration of IL-18 at the initial and late phase of arthritis accelerates the development of disease. Despite that IL-18 is detected at low amounts in OA synovium (Gracie et al., 1999) it can stimulate the expression of MMP-3, MMP-13, aggrecanase-2, TIMP-1 in chondrocytes promoting bone destructive process. Recently, it has been shown that IL-21 is a proinflammatory cytokine that increases CXCL8 production by monocyte-derived macrophages. The receptor for IL-21 was detected on monocytes, monocyte-derived macrophages and on synovial macrophages from RA patients (Jungel et al., 2004). IL-21R has limited expression in OA synovium but it is expressed in the areas with enhanced catabolic processes and might participate in destructive process.

Macrophages also release factors that are important for tissue repair and suppression of inflammatory response. Among these factors are vascular endothelial growth factor CD106 (Haywood et al., 2003), prostaglandin E₂, IL-10 and TGF- β . TGF- β expression from macrophages can be triggered by lipoxin A₄ produced by activated neutrophils. TGF- β inhibits T cell proliferation and inflammatory responses. The anti-inflammatory cytokine IL-10 regulates the synthesis of IL-4, and inhibits IFN- γ production in T cells and TNF- α and IL-1 β production in macrophages. The important role of anti-inflammatory cytokines IL-10 and IL-4 in OA has been well described in studies on experimental OA where these cytokine are administrated. The combined treatment with low dosages of IL-4 and IL-10 has potent anti-inflammatory effects and markedly protected against OA cartilage destruction. Improved anti-inflammatory effect was achieved with IL-4/prednisolone treatment (Joosten et al., 1999).

The progression of OA might result in an inappropriate differentiation of resident tissue macrophages, and expression of functionally distinct phenotype (Mantovani et al., 2007; Xu et al., 2005). Macrophage functions are tightly regulated by reversible histone acetylation with acetylases (HAT enzymes) and deacetylation with deacetylases (HDAC enzymes) (Grabiec et al., 2010).

Resident macrophages in different tissues such as lung, liver and synovium express Z39Ig (CRIg) protein (Helmy et al., 2006). The Z39Ig is a receptor for complement fragments C3b and iC3b and is a type 1 transmembrane protein of the immunoglobulin superfamily member. Significant Z39Ig staining is detected in macrophage-enriched areas in the lining and sublining areas of RA synovium (Lee et al., 2006). In OA synovial tissue the number of cells expressing Z39Ig was lower and the positive staining was restricted to the lining-layer macrophages. A significant number of Z39Ig+CD11c+cells observed in some cases of OA and PsA points that Z39Ig+CD11c+cells deserve extended investigations to clarify their functional activity in OA (Tanaka et al., 2008).

It has been shown that prostaglandin E2 secreted by macrophages contributes to pain hypersensitivity by promoting sensory neurons hyperexcitability. Tissue-resident macrophages constitutively express receptor P2X4 and the stimulation via P2X4R triggers calcium influx and p38 MAPK phosphorylation and COX-dependent release of PGE2 (Ulmann et al., 2010). These data suggest that synovial lining macrophages might be important effectors that control pain relieve in OA patients.

Osteoclasts and monocytes are not only derived from a common myeloid progenitor but their activity might be influenced by common mediators (Ross, 2000). Activation of CD40 signaling in monocytes/macrophages results in up-regulation of nitric oxide generation (Tian et al., 1995), and induction of metalloproteinase production (Malik et al., 1996). Recently, it was found that except CD40L, the known activator of monocytes/macrophages, also OPGL can express such function (Andersson et al., 2002). Mice deficient in CD40L expression display a deficiency in T cell-dependent macrophage-mediated immune responses (Stout et al., 1996). The development of osteoclasts is strongly dependent on the interactions between two members of TNF superfamily, OPGL and its receptor RANK (Lacey et al., 1998; Yasuda et al., 1998). One of the pathways for triggering inflammatory processes by OPGL is through p38 MAPK and p42/44 ERK and inducing cytokine and chemokine secretion (Suttles et al., 1996). Results from *in vivo* application of RANK-Fc in a model of antibody-mediated arthritis showed that it blocked OPGL activity and ameliorated arthritis development (Seshasayee et al., 2004).

3. TLR9 expression in OA

Toll-like receptors are involved not only in pathogen recognition but they can participate in triggering the inflammatory and joint destructive process in arthritis or they can enhance the progression of already established synovitis. TLR-mediated inflammatory response may induce further tissue damage and can provoke a self-sustaining inflammatory loop responsible for chronic progression of arthritis processes. The expression of TLR9 in the joints was assessed in chronic phase (day 30) of ZIA and CIOA. In healthy mice no detectable expression of TLR9 was found in the synovium in contrast to CIOA and ZIA, where synovial lining was extensively stained (Fig. 5A).

We observed stronger TLR9 positive staining in the bone and bone marrow of ZIA than of CIOA, in comparison to healthy mice showing only single positive cells (Fig. 5B). Most intensive accumulation of TLR9 positive cells was established in ZIA mice, especially well exerted in the sites of osteophyte formation (Fig. 5C)

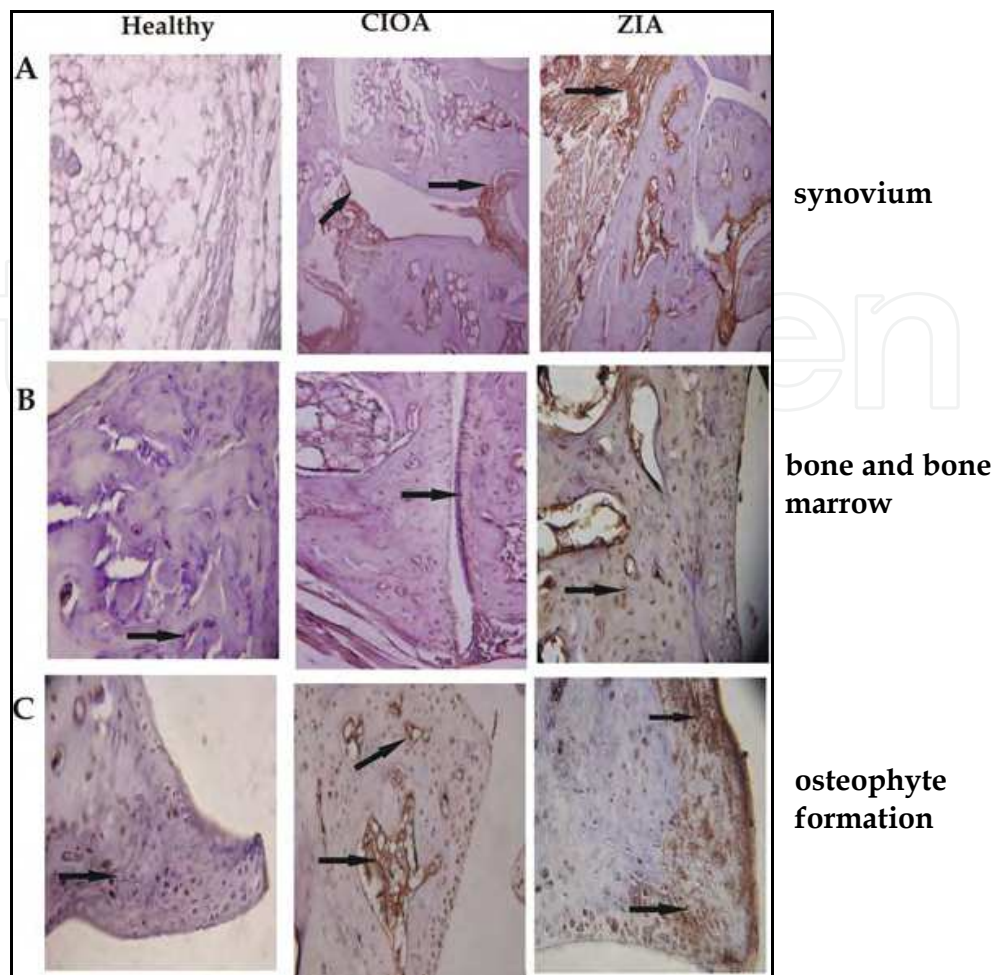


Fig. 5. Histological analyses of TLR9 expression in the joint. Dissected ankle joints were fixed in 10% paraformaldehyde/PBS, decalcified in 5% nitric acid for 1 week, dehydrated and embedded in paraffin. Sections (6 μm thickness) were blocked with 5% bovine serum albumin/PBS for 1 h and the endogenous peroxidase was blocked with 0.3% H_2O_2 in 60% methanol for 10 min. After washing, the sections were incubated for 40 min at room temperature with antibodies against mTLR9 (10 $\mu\text{g}/\text{ml}$). Isotype anti-mouse IgG was used as a background staining control. Then, the joint sections were incubated for 10 min with biotinylated anti-mouse IgGs and streptavidin-peroxidase was added for 10 min. The sections were washed and incubated with DAB solution kit (3',3'-diaminobenzididne kit, Abcam) for 10 min and counterstained with Gill's hematoxylin. Arrows show positive TLR9 staining (magnification 40x).

The different TLR9 expression might be due to the difference in both models. Zymosan-induced arthritis is an example for proliferative arthritis, which is restricted to the joint injected with zymosan (Bernotiene et al., 2004). Using this model we found that histologically, joint sections showed cell infiltration into cartilage, capsule, osteoid and surrounding soft tissue and synovial hyperplasia without aggressive pannus formation. Proteoglycan depletion in cartilage, detected by loss of safranin O staining intensity, and changes in joint architecture were observed at late stage of arthritis. Interestingly, we observed the immunoreactivity for TNF- α R and C5aR in cartilage, along with C5aR positive inflammatory cells in the areas of bone and synovium. At the onset of ZIA, TNF- α plays a

dominant role in inflammation. In the synovial extracts were found increased levels of IL-6 and C5a that can regulate the expression of C5aR on infiltrating neutrophils (Dimitrova et al., 2010; Dimitrova et al., 2011). CIA was provoked by intraarticular injections of collagenase, leading to acute ligament instability and prolonged inflammatory cartilage erosion over a period of six weeks. The model is relevant to human osteoarthritis pathology. In order to look for correlation between elevated TLR expression and the severity of arthritis we investigated the changes of TLR9 expression by macrophages from different origin in established ZIA. Data showed high presence of TLR9 positive cells in peritoneal exudates and PLNs in arthritic animals, while such elevation was not established for spleens (Fig. 6). These results deserve further experiments on the role of macrophages in the maintenance of inflammation in different organs. The elevation in lymph node population might be due to the fact that PLN is located most closely to the site of zymosan injection in inflamed joint.

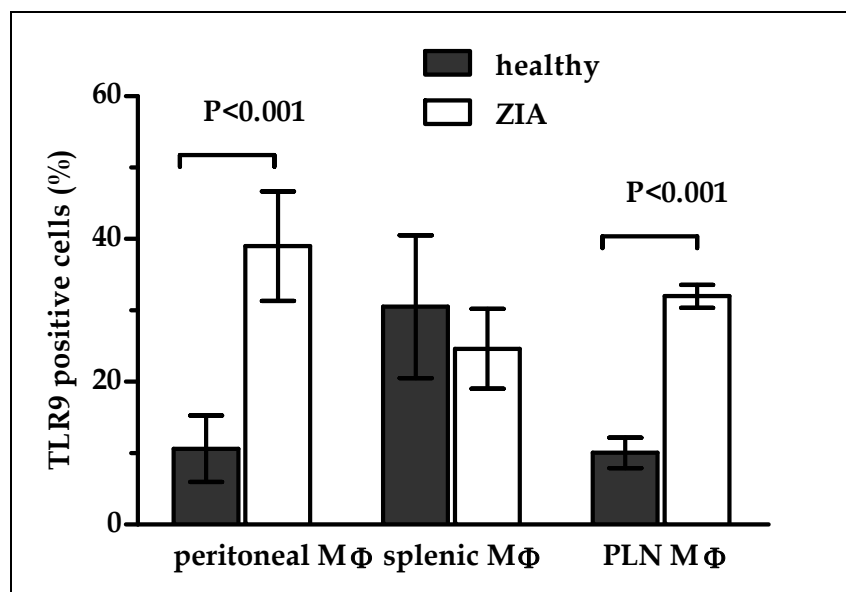


Fig. 6. TLR9 expression in macrophages isolated from different compartments at day 30 of ZIA. Differentiated macrophages isolated from peritoneal exudates (peritoneal Mφ), spleens (splenic Mφ) and popliteal lymph nodes (PLN Mφ) of healthy and ZIA mice (1×10^6 /ml) were stained with antibodies against mouse TLR9 ($1 \mu\text{g}/\text{ml}$), followed by secondary FITC-conjugated anti-mouse antibody and subjected to FACS analysis.

4. Natural killer cells

Natural killer cells are firstly described by their capacity to limit the growth of malignant cells and to eliminate virus-infected cells. They are innate immune effectors that produce immunoregulatory cytokines, such as interferon (IFN)- γ and granulocyte macrophage-colony-stimulating factor GM-CSF (Bancroft, 1993; Feng et al., 2006). Later, it became evident that NK cells can play a critical role in various autoimmune diseases, including rheumatoid arthritis by improving or exacerbating immune responses. They can play dual role in autoimmune diseases, either support or suppress pathogenic processes. In animal models it is established that NK cells are responsible for the induction and progression of K/BxN serum transfer model, being engaged through activation of their Fc γ III receptors (Kim et al., 2006) and also being involved in the acceleration and exacerbation of CIA (Chu

et al., 2007). The induction of CIA in NK-depleted mice reduces the severity of arthritis and almost completely prevents bone erosion (Soderstrom et al., 2010). In these experiments, also significantly reduced inflammation, pannus formation, and synovitis have been observed. NK cells are enriched within the joints of RA patients but how they contribute to disease pathology is currently not fully elucidated. In RA patients NK cells comprise 20 % of the synovial fluid cells at the early phase of disease (de Matos et al., 2007; Tak et al., 1994). These cells express CD56 and CD94/NKG2A phenotype, but failed to express CD16 similarly to peripheral RA NK cells.

Two distinct subsets of mature NK cells have been recognized, CD56^{bright} and CD56^{dim}. CD56^{bright} subset is the source of IFN- γ , TNF- β , IL-10, IL-13, and GM-CSF, whereas the CD56^{dim} NK cell subset produces significantly less of these cytokines in vitro (Cooper et al., 2001). The CD56^{bright} NK cells express a chemokine receptor pattern similar to that of monocytes including high-affinity receptors for IL-15 (Carson et al., 1994). This subset has been identified in the joints of patients with early synovitis in RA. Several studies have shown that IL-15 is a critical factor for the development of human and murine NK cells (Kennedy et al., 2000; Mrozek et al., 1996). This might be due to its stimulation of M-CSF and RANKL expression by NK cells. IL-15 alone did not change the ability of monocytes to enhance osteoclast formation, while this process was dramatically supported in the presence of both NK cells and IL-15, indicating that the effect of IL-15 is mediated through NK cells (Soderstrom et al., 2010).

NK cells may be implicated in the initiation, the maintenance or the progression of autoimmune diseases directly or through their interaction with dendritic cells, macrophages or T lymphocytes. Whether neutrophils are capable to regulate and influence the activity of NK cells is not well defined. Generally, data on neutrophils and NK cells concern RA and are focused mainly on cytokines. There are no available investigations in OA in regard to remodeling events and the participation of these cells in OA has been underestimated. The investigations of NK-cell functions in patients with OA will improve our capacity to monitor these cells as possible markers for disease activity and will provide new prospects for NK-cell-directed therapies.

5. Mast cells

Nearly two decades ago available data show that when compared with healthy individuals, patients with OA had elevated numbers of intact and degranulated mast cells in the synovium and synovial fluid of diseased joints. Histological studies confirmed significant numbers of mast cells in both RA and OA synovium (Dean et al., 1993; Kopicky-Burd et al., 1988). Moreover, the numbers of mast cells in OA are comparable with those in RA when clinically active arthritis is envisaged. Prednisone used as anti-rheumatic drug lowered synovial mast cell number (Bridges et al., 1991). Mast cells containing triptase (MCT) expanded in the SF of OA patients (Buckley et al., 1998), while cells containing triptase and chimase (MCTC) expand in RA but not in OA (Gotis-Graham et al., 1998). When mast cell numbers in RA and OA patients are compared without respect to mast cell distribution in the subsynovial layer or the stratum fibrosum, no statistical differences between the diseases could be observed (Fritz et al., 1984). Synovial fluid collected from patients with hand OA expressed elevated number of mast cells in correlation with high content of histamine and elevated levels of tryptase and NO (Renoux et al., 1996). Such data support the hypothesis that in OA the increase of mast cells may participate in the pathological process, at least they can contribute in concert with other inflammatory cells.

6. Adipokines as potential participants in OA

Although leptin has been discovered fifteen years ago, the investigations on adipokines as potential participants in arthritic diseases are now at its beginning. Innate immunity cells appeared to be a source of adipokines as well as an object of their action. Leptin realizes its action as a proinflammatory cytokine through modulation of monocytes, macrophages, neutrophils, basophils, eosinophils, natural killer and dendritic cells (Otero et al., 2005). It seems to play a role in auto immune diseases such as RA and OA, by expressing harmful as well protective action on joint structures in RA (Lago et al., 2007). Leptin has been detected in SF obtained from patients with OA, and it was strongly overexpressed in human OA cartilage and in osteophytes (Dumond et al., 2003). The administration of exogenous leptin in rats increases IGF1 and TGF β 1 production suggesting that high circulating leptin levels might protect cartilage from osteoarthritic erosion but it also can induce osteophyte formation. Although adiponectin was discovered almost at the same time as leptin, its role in obesity-related disorders has now begun to be investigated. In joint disorders adiponectin might play proinflammatory role being involved in matrix degradation. The pathogenic role of adiponectin is largely unknown in concern to RA and OA. Recent data proved that in chronic RA patients adiponectin plasma levels are higher compared to healthy controls, but lower plasma levels than in OA (Laurberg et al., 2009). Another member of this group is resistin (FIZZ3) which is found in adipocytes, macrophages and other cell types. It has been determined in the plasma and the synovial fluid of RA patients. The injection of resistin into mice joints induces an arthritis-like condition, with typical leukocyte infiltration in the synovium and tissue hypertrophy (Bokarewa et al., 2005). There are not enough data to make firm conclusions about the exact role of adipokines in arthritic processes. Their physiological role in RA or OA and their use as disease markers deserve to be a subject of further studies.

7. Conclusions

In the hope of defining novel therapeutic targets in OA much attention has been paid on degenerative processes of cartilage and secondary bone damage. But in recent years, the synovium, and in particular the participation of synovial cells and their mediators are under intensive study. Macrophages, neutrophils and lymphocytes act together with resident fibroblasts in the destructive phases of arthritis through liberation of proinflammatory molecules. Activated macrophages produced chemoattractants such as chemoattractant protein 1, RANTES, MIP-2 α and epithelial neutrophil activator (Choy & Panayi, 2001). Many studies have been devoted to investigating various signaling pathways involved in proinflammatory cytokine production from OA synovial macrophages. The promising results of anticytokine therapies in RA prompted that such approach might be used in OA (Bondeson, 2010). Major population of infiltrated cells in synovium are neutrophils. This is valid for the early phase of inflammation as well as for its maintenance. The injection of anti-neutrophil antibodies ameliorated the established disease in collagen-LPS-induced model and K/BxN model (Nandakumar et al., 2003; Tanaka et al., 2006). Instead of macrophage/monocyte or neutrophil depletion which can impair host resistance, neutralizing antibodies blocking their chemotaxis might be used. Promising results in animal models have been obtained with ant-MIP-1 α antibodies (Kagari et al., 2003) and pertussis toxin blocking of signals from G protein-coupled receptors (GPCRs) (Becker et al., 1985; Painter et al., 1987; Spangrude et al., 1985).

There is a large unmet need for reliable biochemical markers that will predict the subset of patients who are at risk of the disease progression and will be used to find molecular targets in future therapies. Elevated RANKL levels in synovium and circulation, and particularly increased RANKL expression by neutrophils in OA, makes it a suitable candidate for disease prognosis.

The ability of known TLR agonists to activate neutrophil functions supports the notion that TLRs are an important pattern recognition receptors in the function of these cells. Consequently, while producing chemokines they trigger migration of other immune cells, such as more neutrophils, monocytes, macrophages, NK cells, and immature dendritic cells. These results prompt a possible correlation between the increase of TLR2 positive blood cells, including neutrophils and existing OA condition and strongly support the idea that OA inflammation might be influenced through TLR2. Also, elevated TLR9 expression is detected in the joints of arthritic mice. Whether TLRs involvement is similar for both inactive and active OA is a question to be resolved. TLR-dependent mechanisms may contribute to the activation of synovial cells, possibly leading to the destruction of cartilage and bone in the pathogenesis of RA and OA.

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9. Abbreviations

CIOA - collagenase-induced osteoarthritis; ERK - extracellular signal-regulated kinase; FITC - fluorescein isothiocyanate; GM-CSF - granulocyte-macrophage colony-stimulating factor; IFN- γ - interferon gamma; IL - interleukine; MAPK-mitogen-activated protein kinase; MIP-2 - macrophage inflammatory protein; MMP - matrix metalloproteinase; MRPs - myeloid-related proteins; NF- κ B - nuclear factor-kappa B; NK cells - natural killer cells; OA - osteoarthritis; OPG - osteoprotegerin ; Pi3K - phosphatidylinositol 3-kinases; RA - rheumatoid arthritis; RANK - receptor activator of nuclear factor-kappa B; RANKL - receptor activator of nuclear factor-kappa B ligand; RANTES - Regulated upon Activation, Normal T-cell Expressed, and Secreted; STAT - Signal Transducer and Activator of Transcription; TGF- β - transforming growth factor beta; TIMP-1 - TIMP metalloproteinase inhibitor 1; TLR - Toll-like receptor; TNF - tumor necrosis factor; Z39Ig - Immunoglobulin superfamily protein Z39IG; ZIA - zymosan-induced arthritis

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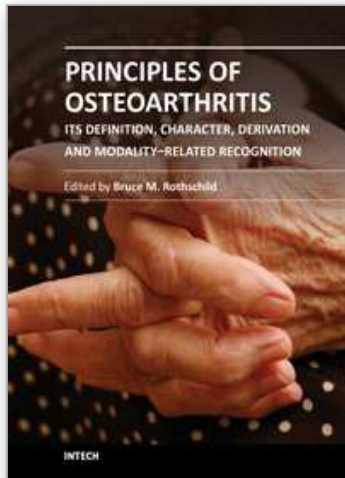
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This volume addresses the nature of the most common form of arthritis in humans. If osteoarthritis is inevitable (only premature death prevents all of us from being afflicted), it seems essential to facilitate its recognition, prevention, options, and indications for treatment. Progress in understanding this disease has occurred with recognition that it is not simply a degenerative joint disease. Causative factors, such as joint malalignment, ligamentous abnormalities, overuse, and biomechanical and metabolic factors have been recognized as amenable to intervention; genetic factors, less so; with metabolic diseases, intermediate. Its diagnosis is based on recognition of overgrowth of bone at joint margins. This contrasts with overgrowth of bone at vertebral margins, which is not a symptomatic phenomenon and has been renamed spondylosis deformans. Osteoarthritis describes an abnormality of joints, but the severity does not necessarily produce pain. The patient and his/her symptoms need to be treated, not the x-ray.

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