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Functional complement activity is decisive for the development of chronic synovitis, osteophyte formation and processes of cell senescence in zymosan-induced arthritis

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ABSTRACT

Synovial inflammation plays a critical role in the symptoms and structural progression of arthritis which leads to irreversible damage of the adjacent cartilage and bone. Activation of complement system is strongly implicated as a factor in the pathogenesis of chronic synovitis in human rheumatoid arthritis (RA). In this study, we show that the depletion of functional complement activity at the time of the initiation of zymosan-induced arthritis, significantly reduced the expression of TGF-beta1/3, BMP2 and pSmad2 and decreased the number of Sudan Black B positive cells in the synovium. Also, the excessive synthesis of proteoglycans and glycosaminoglycans was diminished. The appearance of apoptotic and senescent cells among the adherent bone marrow cells cultivated *in vitro* was not observed in complement depleted mice. Therefore, the lack of functional complement prevented the development of chronic synovitis, osteophyte formation and the generation of pathologic senescent arthritic cells.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease manifested by cell infiltration into the synovium, followed by hyperplasia of the synovial lining and resulting in progressive cartilage and bone destruction [1,2]. The crosstalk between the joint tissues such as synovium and cartilage at the cellular levels within an innate immune inflammatory network, can promote synovitis and cartilage degradation. Activation of innate immune signaling pathways and complement can lead to generation of pro-inflammatory mediators, produced by a various type of cells such as synovial fibroblasts, macrophages, and chondrocytes [3]. The transforming growth factor (TGF)-beta superfamily consists of two functional groups, the TGF-beta-like group and the bone morphogenetic protein (BMP)-like group [4]. TGF-beta-like proteins induce their intracellular signals *via* phosphorylation of R-Smad2/3, while BMPs phosphorylate and activate R-Smad1/5/8 [5]. TGF-beta1 is one of the major factors keeping the balance between the dynamic processes of bone resorption and bone formation. TGF-beta1 can increase osteoprotegerin (OPG) secretion by osteoblasts thus preventing the maturation of osteoclasts, but the cytokine also, can enhance bone resorption through accumulation of osteoclast precursors at the bone and triggering osteoclast differentiation by enhanced expression of receptor activator of nuclear factor κB (RANK) on the surface of monocytic/preosteoclastic cells [6,7]. In fact, TGF-beta and BMP might play quite different roles in cartilage homeostasis. IL-1beta is one of the 11 representatives of the IL-1 family that acts as a negative regulator for chondrocyte proliferation and induces dedifferentiation in chondrocytes [8]. It is considered one of the major cytokines involved in the pathogenesis of arthritis either independently or being combined with other mediators to damage articular cartilage and other elements of joints [9]. IL-1beta was shown to increase BMP2 expression and to anabolically activate articular chondrocytes [10].

During RA, many pathological changes are observed in the affected joints, including loss of articular cartilage and, new formation of ectopic cartilage and bone (osteophytes) in addition, to the activated synovium. Osteophytes develop from mesenchymal cells at the junction of periosteum and synovium and although it is considered as a repair mechanism to help stabilize joints, excessive osteophytes also cause negative effects, such as pain and loss of movement. Both, TGF-beta and BMP2 have been implicated in osteophyte formation however, little is

Abbreviations: ALP, alkaline phosphatase; AP, alternative pathway; BMP, bone morphogenetic protein; CVF, cobra venom factor; GAGs, glycosaminoglycans; IL, interleukine; M-CSF, macrophage colony-stimulating factor; PGs, proteoglycans; RANKL, receptor activator of nuclear factor κB ligand; RA, rheumatoid arthritis; TRAP, tartrate-resistant acid phosphatase; TGF, transforming growth factor; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; ZIA, zymosan-induced arthritis

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known about their endogenous *in vivo* role in models for RA [11,12] TGF-beta-induced Smad family member protein (Smad2) signaling has been shown as a crucial signaling in cartilage, since it prevents the deleterious terminal differentiation of cartilage cells [13,14]. Smad proteins bind to membrane bound serine/threonine receptors, and are activated by the kinase activity of the receptors [15].

Activation of the complement system is a major event that underlies various pathologic responses in a number of diseases [16]. The activation can be realized through each of three distinct pathways; the classical (CP), alternative (AP), and lectin (LP) pathways. Complement plays pathogenic role in RA through generation of potent anaphylatoxins such as C3a or C5a [17]. It was found an increased in situ expression of mRNA for C3 and factor B in RA synovium that point on their local production. The expression pattern of C3 and factor B was different from that for C3aR and C5aR [18]. LP and the CP are initiated by the recognition of target molecules, the activation of the AP can also be started when properdin promotes initial C3b deposition [19]. The important role played by the complement system in tissue damage of RA patients is proved by the elevated levels of complement fragments and their pro-inflammatory receptors in the rheumatoid joints [20]. The role of complement is also studied in different models of arthritis [21,22]. The alternative pathway is required for the induction of arthritis following injection of anti-collagen antibodies related to enhanced C3 activation [22,23]. Cobra venom factor (CVF) is structurally identical to C3b fragment of complement component C3, able to form a stable complex with Factor B thus acting as C3–C5 convertase and in the presence of Factor D and Mg^{2+} leading to exhaustion of complement activity. Data in animals depleted with CVF showed decreased susceptibility to collagen-induced arthritis and to *Yersinia*-induced reactive arthritis, thus demonstrating that functional complement has an important role in joint inflammation [24,25].

Previously, by the use of a model of zymosan-induced arthritis in properdin-deficient mice, we have shown that the alternative pathway is definitely involved in the development and progression of chronic joint inflammation [26]. Moreover, our results have revealed that complement depletion provoked by pre-treatment with CVF, ameliorates the course of chronic synovitis, concerning macrophage infiltration, C3aR and C5aR expression in the joints, and the elevated levels of C5a and soluble RANKL (sRANKL) in the synovial fluid [27]. The aim of the present study was to estimate the role of functional complement system in the induction of arthritis and development of chronic synovitis, osteophyte formation and cell differentiation of adherent bone marrow cells in zymosan-induced model of arthritis.

2. Materials and methods

2.1. Animals

Female ICR outbred mice (7 to 8 weeks old; local breeder, Bulgaria) were used in the experiments because of their relatively high serum complement activity, compared to inbred strains (BALB/c and C57Bl/6). They were maintained on a 12:12 h light/dark cycle and received tap water and pelleted chow. All experiments were carried out in accordance with the Bulgarian Food Safety Agency Guidelines no. 352 06.01.2012 and the international laws and policies (EEC Directive of 1986; 86/609/EEC and the recommendation 2007/526/EC from European Community) and the protocols were approved by the Animal Care Committee at the Institute of Microbiology, Sofia.

2.2. Induction of arthritis

Mice received an intra-articular (i.a.) injection of 180 μg zymosan A from *Saccharomyces cerevisiae* (Sigma-Aldrich, Germany) under brief anaesthesia (sodium pentobarbital 50 mg/kg, i.p.) (day 0). Control animals received an i.a. injection of an equal volume of sterile phosphate buffered saline (PBS).

2.3. Cobra venom factor treatment

Mice were injected intraperitoneally (i.p.) with 10 ng/g body weight of CVF (*Naja naja* CVF, cobra venom anti-complement protein, Sigma-Aldrich, St. Louis, MO) 72 and 48 h before zymosan (CVF group). To evaluate the efficiency of the decomplementing effect of CVF, 5–7 ICR mice in each experiment were injected with CVF at the same time points, as the experimental groups. Blood was collected by retro-orbital puncture and the total AP complement activity in sera was determined by the method of Klerx et al. [28]. The pretreatment value was set to 100%. CVF treatment resulted in a remarkable suppression of total AP and C3 complement activities which persisted for 5 days.

2.4. Histologic examination

Tissue samples were dewaxed with xylene and dehydrated until 70% ethanol. For Sudan Black B (SBB) staining the slides were mounted in saturated 70% ethanol solution for 8 min and then embedded into 50% ethanol, transferred and washed in distilled water, then counterstained with 0.1% Nuclear Fast Red for 10 min. Lipofuscin staining was considered positive when perinuclear and cytoplasmic aggregates of blue-black granules were evident inside the cells. For detection of glycosaminoglycans joint sections were stained with 1% toluidine blue or stained with iron hematoxylin, followed by 0.001% fast green and 1% safranin O for detection of proteoglycans.

2.5. Immunohistochemistry

The hind paws were removed, fixed in 10% buffered paraformaldehyde (PFA) for 48 h and decalcified by incubation in 10% EDTA/0.2% PFA in PBS at 4 °C, with the decalcifying solution changed twice weekly, for three weeks. The paws were embedded in paraffin, serial sections (5–7 μm) were cut and mounted on glass slides. After deparaffinization and rehydration, slides were permeabilized with 0.05% Triton for 10 min and then for 10 min with 3% H_2O_2 to block endogenous peroxidase, followed by washing procedure with PBS and immediately blocked with 5% BSA at room temperature for 10 min. The sections were then incubated for 30 min with anti-mouse TGF-beta1 antibody (10 μg/ml), TGF-beta3 (10 μg/ml), BMP2 (0.1 μg/ml) or pSmad2 (20 μg/ml) (all from BioLegend, San Diego, USA), washed with PBS and HRP-streptavidin (1:100 diluted; Sigma-Aldrich, Germany) was added for 10 min. Isotype matched antibodies with rat or rabbit origin (BioLegend, San Diego, USA) were used as a background staining control. The sections were washed and incubated with DAB solution kit (3′,3′ diaminobenzididne kit, Sigma-Aldrich, Germany) for 10 min and counterstained with Gill's hematoxylin for 3 min. For the different antigens, the number of positive cells in the synovium was determined by a blinded observer. For each antigen, a threshold was set in such a manner that only the cells that were found to be positive (brown stained cell) were selected. The computer program determined the number of positive cells in at least three tissue sections. To correct for differences in cell number between the groups, the average number of cells was determined in sections stained with hematoxylin only, based on a similar selection procedure with the exception that selection of cells was based on the blue staining from heamatoxylin instead of brown staining.

2.6. Cultivation of bone marrow cells

Bone marrow was isolated aseptically from tibiae and femora of 8–10 weeks healthy mice or from mice at day 30 of ZIA and mice with ZIA preatreated with CVF. The suspension was gently aspirated to disrupt cell aggregates and after centrifugation cells were resuspended at a concentration of 2×10^6/ml in minimum essential medium (α-MEM, Sigma Aldrich, Germany), supplemented with 10% FBS and 1% penicillin–streptomycin, (Sigma-Aldrich, Germany), and cultured