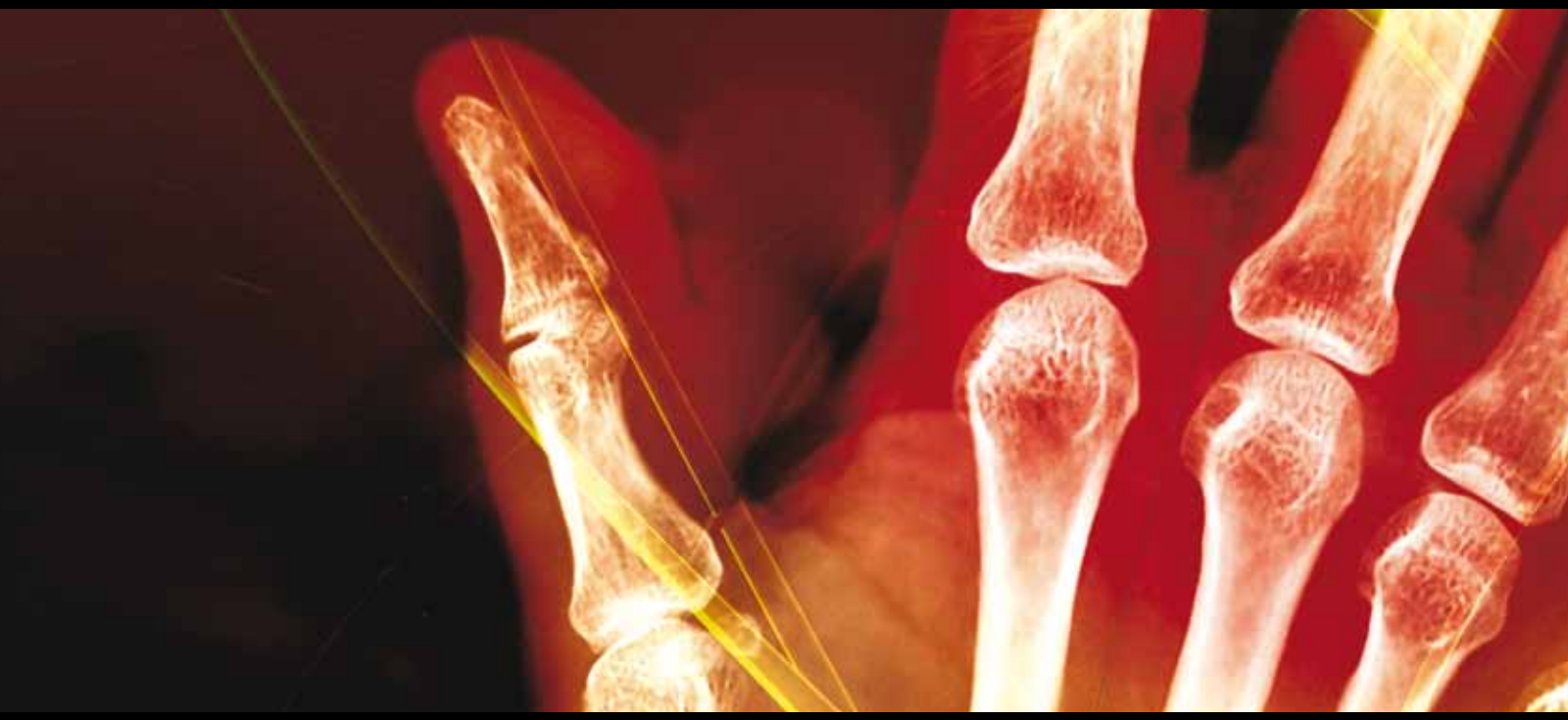
International Journal of Rheumatology

Biologicals

Guest Editors: Jozélio Freire de Carvalho, Simone Appenzeller, and Yehuda Shoenfeld



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Editorial Board

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Editorial
Biologicals

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Biological agents are currently a very important option for refractory autoimmune diseases [1]. From about ten years to now, several drugs are available to the clinical practice, mainly, in the rheumatology one. The main biological agents are antitumor necrosis factor (anti-TNF) medications, however there are other important drugs such as rituximab, which blocks lymphocyte B CD20, tocilizumab that inhibits anti-interleukin-6 receptor [2, 3]. In this special issue on biologicals from *Autoimmune Diseases* journal, we have invited several papers that address this modern issue.

The paper entitled "*Biological therapy systemic lupus erythematosus*" of this issue addresses the rational for the use of biological agents in patients with systemic lupus erythematosus. Several aspects including anti-CD20 and anti-CD22 antibodies, B-cell tolerogens, costimulatory blockers, anti-TNF, anti-interferon, anti-interleukins 1, 10, and 18, and also complement inhibitors. The paper entitled "Use of biological agents in ocular manifestations of rheumatic disease" reviews the different studies published in the literature regarding biological agents use on ocular disorders.

The paper entitled "*Tocilizumab for the treatment of rheumatoid arthritis and other systemic autoimmune diseases: current perspective and future directions*" is not only an elegant review of the use of tocilizumab in rheumatoid arthritis, but also in other autoimmune diseases such as lupus, systemic sclerosis, polymyositis and large vessel vasculitis.

Complications related to biologicals are discussed in this paper entitled "*Risk of orthopedic site surgical infections in patients with rheumatoid arthritis treated with anti-tumor necrosis factor alfa therapy*." The authors performed a literature review of infections associated to orthopedic surgery in rheumatoid arthritis patients treated with anti-TNF and found inconclusive data in this field.

An experimental study was presented on the paper entitled "*TNF-alpha in the locomotor system beyond joints: high degree of involvement in myositis in a rabbit model*." In this research paper, the authors have evaluated the role of TNF in an experimental model of myositis and found interesting results.

The paper entitled "*Immunosuppresive exosome: a new approaching for treating arthritis*" brings a new methodology for treating arthritis. The rational consists that some vesicles derived from immunosuppressive dendritc cell may have immune suppressive properties and the authors reviewed several studies developed by themselves and others that used these vesicles (exosomes) as in experimental animals as in humans with the objective of treatment of arthritis.

Jozélio Freire de Carvalho
Simone Appenzeller
Yehuda Shoenfeld

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Research Article

TNF-Alpha in the Locomotor System beyond Joints: High Degree of Involvement in Myositis in a Rabbit Model

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The importance of TNF-alpha in arthritis is well documented. It may be that TNF-alpha is also markedly involved in muscle inflammation (myositis). An animal model where this can be investigated is needed. A newly developed rabbit myositis model involving pronounced muscle overuse and local injections of substances having proinflammatory effects was therefore used in the present study. The aim was to investigate the patterns of TNF-alpha expression in the developing myositis and to evaluate the usefulness of this myositis model for further TNF-alpha research. Human rheumatoid arthritis (RA) synovial tissue was examined as a reference. TNF-alpha immunoexpression and TNF-alpha mRNA, visualized via in situ hybridization, were detected in cells in the inflammatory infiltrates of the affected muscle (soleus muscle). Coexistence of TNF-alpha and CD68 immunoreactions was noted, suggesting that the TNF-alpha reactive cells are macrophages. Expression of TNF-alpha mRNA was also noted in muscle fibers and blood vessel walls in areas with inflammation. These findings demonstrate that TNF-alpha is highly involved in the myositis process. The model can be used in further studies evaluating the importance of TNF-alpha in developing myositis.

1. Introduction

Tumour necrosis factor alpha (TNF-alpha) is one of the most frequently studied pro-inflammatory cytokines. It drives the activation and recruitment of inflammatory cells, amplifies the production of other pro-inflammatory cytokines, and activates nuclear transcription factors, thereby promoting and maintaining the inflammatory response [1]. TNF-alpha is likely to be a key cytokine in several autoimmune diseases such as rheumatoid arthritis (RA), inflammatory bowel disease, systemic sclerosis, and systemic lupus erythematosus [1–3].

A very large number of studies have been performed investigating the importance of TNF-alpha in arthritis, especially RA. TNF-alpha has also attracted interest in recent years for its possible role in skeletal muscle damage. Increased protein degradation, as well as decreased body weight and food consumption, was demonstrated when TNF-alpha was administered to rats via a catheter into the external jugular vein [4]. Crush injury in mice leads to elevated TNF-alpha levels in skeletal muscle tissue [5] and there is an increase in TNF-alpha serum levels in response to repetitive strain injuries [6]. However, the experiments that have been performed have sometimes yielded apparently conflicting results. For example, an experiment in which TNF-alpha was administered to mice via an osmotic pump led to accumulation of inflammatory cells in skeletal muscles but no signs of atrophy or injury [7]. Furthermore, the results of studies on TNF receptor knockout and TNF-alpha antibody-neutralized mice indicate that TNF-alpha can actually be involved in the recovery of muscle function after traumatic muscle injury [8]. Therefore, it might be that the role of TNF-alpha in muscle injury varies with the type, severity, and stage of the injury [9]. In humans, TNF-alpha is known to be intimately involved in cachexia [10], a complex condition characterised by progressive muscle loss that affects up to 13% of patients with RA [11]. Nevertheless, a recent study showed acute elevation of TNF-alpha not to affect markers of systemic or skeletal muscle turnover in healthy humans [12].

Remarkably little data is available on the role of TNF-alpha in situations where there is a pronounced infiltration

Table 1: Summary of the five subgroups of animals analyzed. The number of animals for which soleus specimens were analysed is shown. Subgroups 3–5 had been given pro-inflammatory substances/endopeptidase inhibitors in combination with exercise. The subgroups 1 and 2 are collectively referred to as comprising the "nonmyositis group" and those of subgroups 3–5 as comprising the "myositis group" in the text.

Subgroup	Exercise	Injection	Number of animals
1	No	—	6
2	Yes	NaCl	5
3	Yes	Substance PCaptoprilDL-Thiorphan	5
4	Yes	CaptoprilDL-Thiorphan	6
5	Yes	Captopril	6

of inflammatory cells in the muscle tissue, that is, myositis. From studies in tissue other than muscle, it is known that macrophages and other immunoactive cells such as monocytes, mast cells, and neutrophils are responsible for TNF-alpha production [13–16]. Data addressing a possible TNF-alpha production by inflammatory cells in myositis comes almost entirely from studies of patients affected by a group of diseases known as "idiopathic inflammatory myopathies" (inflammatory myopathies) [17, 18]. These autoimmune diseases include mainly the subgroups inflammatory myopathic polymyositis, dermatomyositis, and inclusion body myositis [19]. In these conditions, inflammatory cell-related TNF-alpha expression is localised predominantly to macrophages [18]. TNF-alpha is also expressed in the inflammatory cells in crush-injured and transplanted muscle autografts in mice [7]. Finally, blockade of TNF-alpha in the dystrophic (mdx) mouse, which is the most frequently used model of Duchenne's muscular dystrophy, reduces TNF-mediated adverse responses to exercise-induced muscle damage [20, 21]. However, without further information, it is difficult to reach conclusions on the importance of TNF-alpha and the possible usefulness of TNF-blocking in muscle disorders, including in myositis [22]. Furthermore, it should be stressed that the majority of information on the TNF system for skeletal muscle tissue has come from studies of cultured myoblasts (e.g., [23]). Animal models are needed to advance our understanding of the disease mechanisms of TNF-alpha that are involved in myositis.

Our laboratory has developed a rabbit model of marked muscle (m. triceps surae) and tendon overuse that, when combined with injections of substances eliciting pro-inflammatory effects, results in significant myositis [24]. This model causes myositis that morphologically resembles that seen in inflammatory myopathies [17, 18] but without having an apparent autoimmune origin. The types of white blood cells involved in the inflammatory infiltrates were defined [24]. The model leads to a muscle pathology that to some extent resembles the morphology seen in overuse musculoskeletal disorders (see [25], for a review, see [26]). In studies using this model, we noted evidence of local glutamate signaling in the cells of the inflammatory infiltrates within the muscle tissue [24]. We have taken advantage of this model in order to examine the TNF-system during myositis development. Thus, the aim of this study was to examine the pattern of TNF-alpha expression in one segment of the triceps sursae muscle (the soleus muscle) affected by myositis using immunohistochemistry and in situ hybridization in order to get an insight into the possible usefulness of this model for further studies on the importance of TNF-alpha in myositis.

2. Material and Methods

2.1. Animals and Experimental Procedures

2.1.1. Animals. Twenty-eight adult female New Zealand white rabbits were used for the studies. The animals had an average weight of 4 kg and ranged in age from 6 to 9 months. The animals were kept in ordinary cages allowing good freedom of movement.

Six of the animals corresponded to control nonexercised animals (subgroup 1) and 22 were assigned to an exercise protocol leading to marked overuse of the triceps surae muscle (subgroups 2–5). In order to increase the muscle affection, including the degree of inflammation, the muscle overuse was combined with paratendinous injection treatment (cf. the following). As a control for this, five of the exercised animals (subgroup 2) were given control substance (NaCl) just outside the tendon of the triceps surae muscle (i.e., the Achilles tendon). In essence, these five animals did not develop myositis (Song et al., unpublished observations). For the purpose of achieving muscle inflammation, 17 of the exercised animals (subgroups 3–5) were in parallel to being subjected to marked overuse, given local injections of pro-inflammatory substance (substance P and/or endopeptidase inhibitors; Captopril, DL-Thiorphan) outside the Achilles tendon. Substance P was given as this neuropeptide has well-known pro-inflammatory effects [27] and the injections of the endopeptidase inhibitors were given in order to diminish endopeptidase activities and thereby lead to more pronounced effects of substance P.

For clarification of all the various animal groups, see Table 1.

2.1.2. Exercise Procedure. The animals were exposed to an exercise procedure designed to cause marked overuse of the triceps surae muscle and the associated tendon (the Achilles tendon). The procedure is performed according to previously described procedures [28], with some modifications [29]. Throughout the experiment, the rabbits were kept under anaesthesia, induced by intramuscular (i.m.) injections of diazepam (5 mg/mL; 0.2 mL/kg) and fentanylfluanison (0.2-0.3 mL/kg). Fentanylfluanison (0.1 mL/kg) was injected each

30–45 min during the experiment in order to maintain the anaesthesia. Each experimental session lasted for 2 hours. For analgesia, buprenorphine (0.03 mg/kg) was given subcutaneously (s.c.) after each experiment session. The experiment was repeated every second day for one week (4 exercise sessions in total).

An apparatus (kicking machine) was used to achieve passive repetitive flexions and extensions of the right ankle joint; a pneumatic piston attached to the right foot produced the movements. During the plantar flexion, an active contraction was furthermore induced by electrical stimulation via surface electrodes (Pediatric electrode 40 426 A, Hewlett Packard, Andover, MA, USA) placed 2 cm apart over the triceps surae muscle of the right leg. The stimulation was synchronized with the plantar flexion movement of the piston by a microswitch, which trigged the stimulator unit (Disa stimulator Type 14E10, Disa Elektronik A/S, Herlev, Denmark). An impulse of 0.2 ms duration was delivered 85 ms after the initiation of the plantar flexion at an amplitude of 35–50 V. The movement frequency was 150 repetitions per minute. The left leg was not attached to the kicking machine. The pelvis was strapped down and there were no ankle movements on the left side. One day after the final exercise session, the animals were sacrificed by an overdose of Pentobarbital. For further details about the apparatus and the exercise protocol, see [24, 28–30].

2.1.3. Injection Treatments. Injections were given into the loose connective tissue around the Achilles tendon, that is, in the paratenon region. The injections were given directly after each of the 2-hour exercise periods. The substances injected were (a) NaCl (0.91% w/v, volume: 1 mL) (subgroup 2; $n = 5$), (b) Substance P (10^{-8} μmol/mL) and Captopril (Sigma) (c4042, 30 μmol/kg) both in distilled water (volume: 1 mL) and Dl-Thiorphan (N-[(RS)-2-Benzyl-3-mercaptopropanoyl]-glycine) (Sigma) (500 μg/mL; 0.02 mL) (subgroup 3; $n = 5$), (c) Captopril (Sigma) (c4042, 30 μmol/kg, dissolved in distilled water, volume 1 mL) + DL-Thiorphan (Sigma) (500 μg/mL, 0.02 mL) (subgroup 4; $n = 6$), and (d) Captopril (Sigma) alone (c4042, 30 μmol/kg, dissolved in distilled water, volume 1 mL) (subgroup 5; $n = 6$). For further details, see Table 1.

2.1.4. Grouping of the Animals. Based on recent observations in our group (Song et al., unpublished observations) and as recently reported [24], it has become obvious that subgroups 3–5 develop myositis. Therefore, these subgroups were grouped together and further on referred to as the "myositis group". As there are minimal or no signs of myositis in the NaCl-treated subgroup (subgroup 2), the animals in this group were grouped together with the nonexercised animals (subgroup 1), comprising the "non-myositis group".

2.1.5. Collection of Muscle Samples: Sectioning

Muscle Samples. After the animals were sacrificed, the right triceps surae muscle was dissected out and immediately transported on ice to the laboratory. Samples conforming to the soleus muscle part (5–8 × 10 mm) were dissected out and fixed by immersion overnight at 4°C in an ice-cold solution of 4% formaldehyde in 0.1 M phosphate buffer (pH 7.0). The samples were thereafter thoroughly washed in Tyrode's solution containing 10% sucrose at 4°C overnight, mounted on thin cardboard in OCT embedding medium (Miles Laboratories, Naperville, Ill, USA), frozen in propane chilled with liquid nitrogen, and stored at −80°C. Series of 5 μm thick sections were cut using a cryostat. The sections were mounted on slides precoated with chrome-alum gelatine and were then processed for immunohistochemistry. Other sections were processed for morphology or in situ hybridization.

2.1.6. Human RA Tissue Studied in Parallel. As a reference, human RA synovial tissue was analyzed. The tissue was fixed and further processed in the same way as were the rabbit specimens (cf. the following).

2.2. Processing for Immunohistochemistry and Morphology

2.2.1. Staining for Demonstration of Morphology. One section from all specimens was stained in Harris Haematoxylin solution for 2 min. These sections were then rinsed in distilled water, dipped in 0.1% acetic acid for a few seconds, and then washed in running water. Counterstaining was achieved by immersion in eosin for 1 min. The sections were dehydrated in ethanol and mounted in Permount.

2.2.2. Immunohistochemistry. Sections of all specimens were processed for immunohistochemistry. The sections were pretreated with acid potassium for 2 min, a procedure found to enhance specific immunofluorescence reactions [31]. Thereafter followed incubation for 20 min in a 1% solution of Triton X-100 (Kebo lab, Stockholm) in 0.01 M phosphate buffer saline (PBS), pH 7.2, containing 0.1% sodium azide as preservative, and three 5 min washes in PBS. The sections were then incubated for 15 min in 5% normal donkey serum (code no: 017-000-121, Jackson Immune Research Lab. Inc.) in PBS. Next, incubation with the primary antibody, diluted in PBS (pH 7.4), occurred in a humid environment for 60 min at 37°C. After incubation with specific antiserum, and three 5 min washes in PBS, another 15 min incubation in normal donkey serum followed. Next, the sections were incubated with either of these donkey antigoat IgGs for 30 min at 37°: FITC-(fluorescein isothiocyanate-) conjugated AffiniPure donkey antigoat IgG (Jackson ImmunoResearch Lab Inc, dilution 1 : 100) or Alexa FluorO 488 donkey antigoat (Invitrogen, dilution 1 : 300). The sections were thereafter washed in PBS and then mounted in Vectashield Mounting Medium (H-1000) (Vector Laboratories, Burlingame, CA, USA). Examination was carried out in a Zeiss Axioscope 2 plus microscope equipped with epifluorescence optics and an Olympus DP70 digital camera.

2.2.3. Double Stainings. To clarify the TNF-alpha immunoreaction pattern in relation to that of white blood cells,

double stainings were made. As it is frequently emphasized that macrophages [13, 16] show TNF-alpha expression, double stainings for TNF-alpha/macrophage marker (CD68) were performed. Double stainings for TNF-alpha/T-cell-neutrophil marker were also performed.

Alexa FluorO donkey antigoat was used as secondary antibody for TNF-alpha immunolabelling, and TRITC (tetramethylrhodamine isothiocyanate-) conjugated rabbit antimouse antibody was used for stainings for CD68 and T-cell/neutrophil marker. For detailed information about the staining procedures for TNF-alpha, see above. When doing double stainings for CD68 and T-cell/neutrophil marker, 5% normal rabbit serum (code no: X0902, DAKO Cytomation, Glostrup, Denmark), diluted in 0.1% BSA (bovine serum albumin) in PBS, was used as normal serum, and TRITC-conjugated rabbit antimouse antibody (R0276, DAKO Cytomation), diluted 1 : 40 in 0.1% BSA in PBS, as the secondary antibody.

2.2.4. TNF-Alpha Antibody and Control Stainings. An antibody against TNF-alpha produced in goats was used (AF-210-NA; R&D Systems). Various dilutions were trialled to achieve the optimal fluorescence to background ratio, with a dilution of 1 : 50 found to be optimal. The supplier reports that this antibody is directed against *E. coli*-derived recombinant human TNF-alpha. The TNF-alpha-specific IgG was purified by human TNF-alpha affinity chromatography. It is described to be specific via having the ability to neutralize the biological activity of recombinant human TNF-alpha. Of note, the TNF-alpha amino acid sequence homology between species is reported to be highly conserved and TNF-alpha DNA sequence comparison shows an overall high sequence homology between various species (including rabbit) [32].

In control stainings, preabsorption of the primary antibody with TNF-alpha antigen (T6674; Sigma; 20 μg/mL antiserum) was performed overnight at 4°C. Control staining also included staining when the primary antibody was omitted.

2.2.5. Antibodies against CD68 and T-Cell/Neutrophil Marker and Reference Concerning Double Stainings. A macrophage (CD68) antibody (M0814) from DAKO Cytomation (Glostrup, Denmark) was used. It is an affinity purified mouse monoclonal antibody and was used at a dilution of 1 : 100 in 0.1% BSA in PBS. The antigen for this antibody is glycosylated transmembrane glycoprotein, which is mainly located in lysosomes. A mouse antirabbit T-cell and neutrophil antibody (MCA805G) from AbD Serotec (Oxford, UK) was furthermore used. It is an affinity purified mouse monoclonal antibody against a cell surface antigen, which is expressed by a subset of T-cells, thymocytes, neutrophils, and platelets in rabbits. The dilution was 1 : 100 in 0.1% BSA in PBS.

Stainings performed in a parallel project on rabbit soleus muscle [24] were used as a reference (control) for the current double stainings. In that project, double stainings were performed using the same CD68 and T-cell/neutrophil marker antibodies as in the current double stainings. In this previous study, double stainings were made against an antibody produced in goats (against VGluT2; Santa Cruz), that is, being of the same type as the TNF-alpha antibody used in the current study. In these reference stainings, the same types of secondary antibodies as described previously were utilized.

3. Processing for In Situ Hybridization

In situ hybridization was used as a complementary method to detect the expression of TNF-alpha, namely, at the mRNA level. A digoxigenin-(DIG) hyperlabeled oligonucleotide probe (ssDNA) for detection of rabbit TNF-alpha mRNA was used on sections from myositis (4 specimens) and nonmyositis (1 specimen) groups (GD1001-DS custom designed; GeneDetect, New Zealand). The antisense sequence of the probe was CGGCGAAGCGGCTGACAGTG-TGAGTGAGGAGCACGTAGGAGCGGCAGC. The procedures were performed according to an established protocol [33], using an alkaline phosphatase-labeled anti-DIG antibody for detection [34]. The probe for TNF-alpha mRNA was used at 50 ng in 15 μL of hybridization solution.

The tissue specimens were cut into 10 μm thick fresh cryosections using a cryostat (with a knife washed in 70% EtOH in DEPC [diethylpyrocarbonate]-H_2O) and mounted onto Super Frost Plus slides (nr.041200, Menzel Gläser). The protocol that thereafter followed was that previously used in our laboratory for detection of mRNA for other substances (e.g., [34–36]).

An alkaline phosphatase-(AP) labelled anti-DIG antibody (Roche, Germany, 11 093 274 910) was used for detection. The sections were finally mounted in Pertex mounting medium.

The corresponding sense DIG-hyperlabeled ssDNA probe was used as a negative control. As a positive control probe, a β-actin antisense probe (GD5000-OP) was used, comparisons being made with sense β-actin probe (GeneDetect, New Zealand).

Ethics. The study protocol was approved by the local ethical committee at Umeå University.

4. Results

4.1. Morphology. Myositis was observed in subgroups 3–5, and these are now collectively being referred to as the "myositis group" (cf. above). The most noteworthy feature was the presence of an inflammatory infiltrate (Figure 1), although muscle fiber changes were also observed, including muscle fiber necrosis (cf. [24]). Variations in the levels of myositis were observed between the different subgroups, as well as between different animals within the subgroups. The inflammatory infiltrates were seen in some parts of the specimens. In the other groups (subgroups 1 and 2), there were no or very marginal changes seen and these are now collectively referred to as the "nonmyositis group".

4.2. Reference Studies Concerning the TNF-Alpha Antiserum Used. It was considered relevant to examine a reference tissue regarding the demonstration of TNF-alpha. Human RA

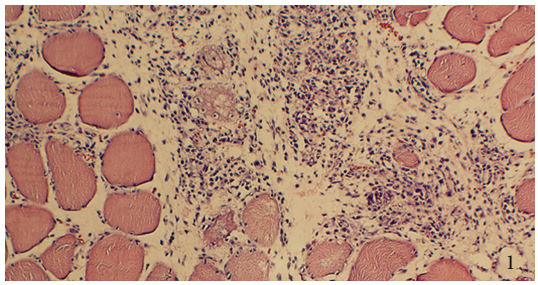


Figure 1: Soleus muscle of a specimen of the myositis group in a section stained with htx eosin. There is a marked inflammatory infiltrate (middle part): myofibers to the left and to the right.

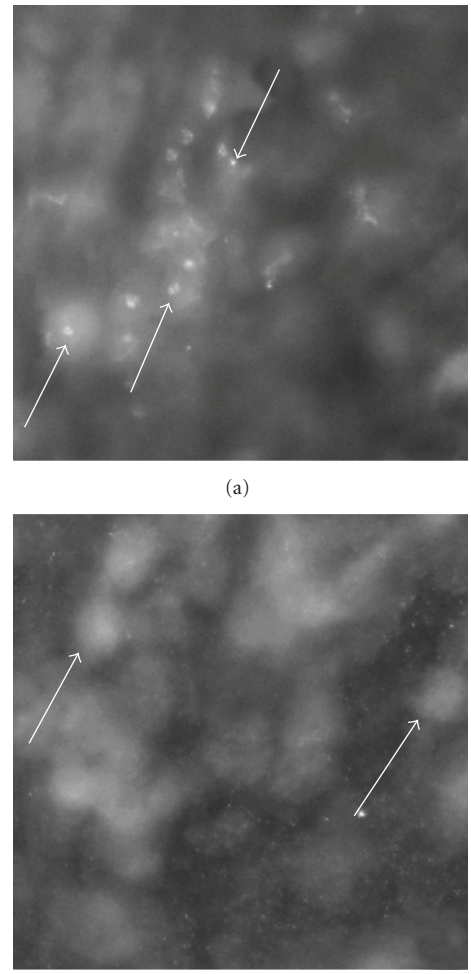
synovial tissue was therefore analysed to provide reference information for the particular TNF-alpha antibody used, as it a well-known fact that there is a marked TNF-alpha expression in the inflammatory infiltrates in the synovial tissue of patients with RA [37].

We observed that mononuclear-like cells of the human synovial tissue exhibited immunoreactions when incubated with the TNF-alpha antiserum (Figures 2 and 3). The reactions were in high magnification seen in the form of intracellular granular reactions (cf. Figures 2 and 3). The specificity of the reactions was confirmed via preabsorption with synthetic antigen (Figure 2). The cells occurred as parts of immune cell aggregates or as isolated cells in the synovial tissue.

4.3. TNF Alpha Immunoreactions in the Rabbit Soleus Muscle. Pronounced TNF-alpha immunoreactions were observed in cells of the inflammatory infiltrates (Figures 4 and 5) in all subgroups in the myositis group. It was noteworthy that the immunoreaction patterns seen in the cells resembled those observed for the mononuclear-like cells of the human RA synovial tissue. Thus, the reactions showed a granular pattern in high magnification (Figures 4 and 5). In lower magnification, the reactions were of a more diffuse type (Figure 6(a)). The specificity of the reactions was verified via preabsorptions (Figure 4).

Fibroblasts in the connective tissue did also to some extent display TNF-alpha immunoreactions, however the reactions were very faint. No specific TNF-alpha reactions were noted for blood vessel walls, the nerve fascicles, muscle spindles, and the muscle fibers (not shown).

4.4. Results of Double Stainings. In order to clarify the patterns of cellular reactions for TNF-alpha in the inflammatory infiltrates, double stainings for TNF-alpha/CD-68 and TNF-alpha/T cells and neutrophil marker were performed. It was found that CD68 coexisted with TNFalpha in cells in the inflammatory infiltrates (Figure 6). On the other hand, colocalization between TNF-alpha and T cells and neutrophil marker was not observed (not shown). In the reference studies (cf. Section 2) using the same secondary antisera and the same white blood cell markers but a primary goat antibody not directed against TNF-alpha, completely different colocalization patterns were noted [24].



(a)

(b)

Figure 2: (a, b) Sections of synovial tissue of a patient with rheumatoid arthritis. The sections were stained for demonstration of TNF-alpha (a) and for TNF-alpha after preabsorption with TNF-alpha antigen (b). Inflammatory cells show specific immunoreaction in (a) (arrows). There are no specific immunoreactions in these in (b) (arrows). The inflammatory cells had in parallel sections been identified via staining for routine morphology (htx eosin).

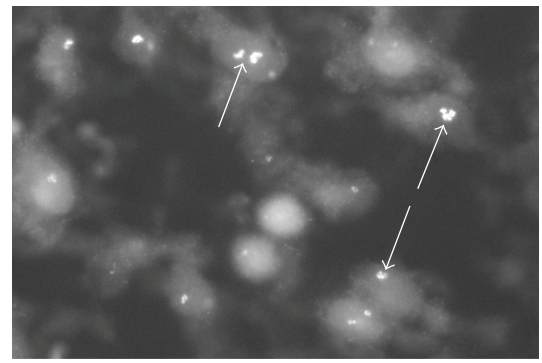
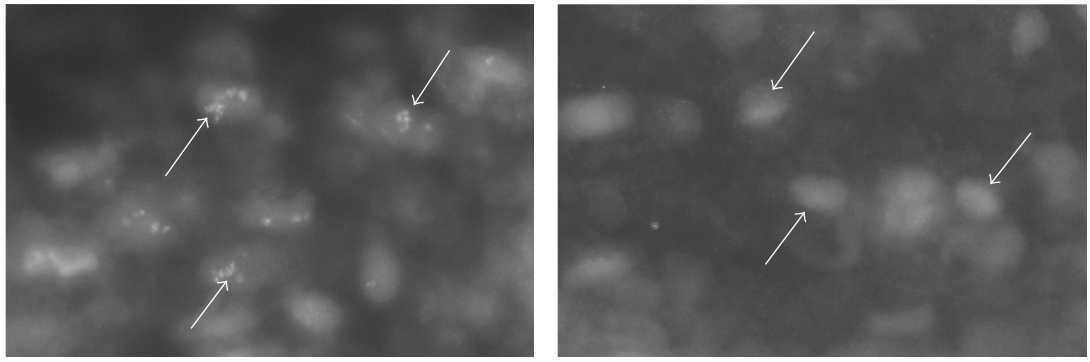
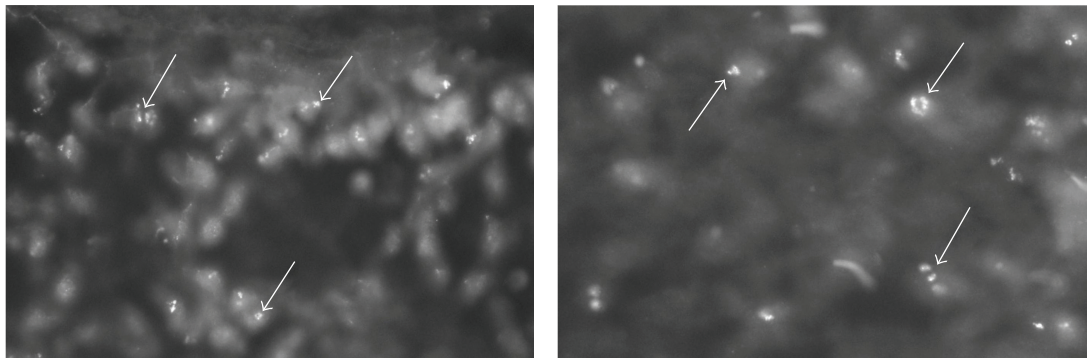


Figure 3: Staining for demonstration of TNF-alpha in a synovial tissue specimen of a rheumatoid arthritis patient. There are specific reactions in inflammatory cells (arrows). The reactions show a granular appearance.



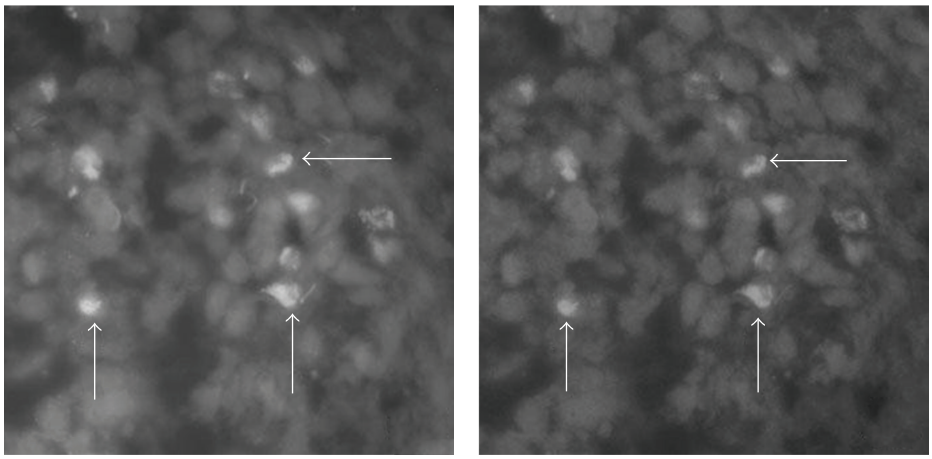
(a) (b)

Figure 4: Sections of a myositis specimen stained for TNF-alpha (a) and for TNF-alpha after preabsorption with TNF-alpha antigen (b). The cells of an inflammatory infiltrate show immunoreactions for TNF-alpha (a) (arrows). There are no specific reactions in (b) (arrows at some of the cells).



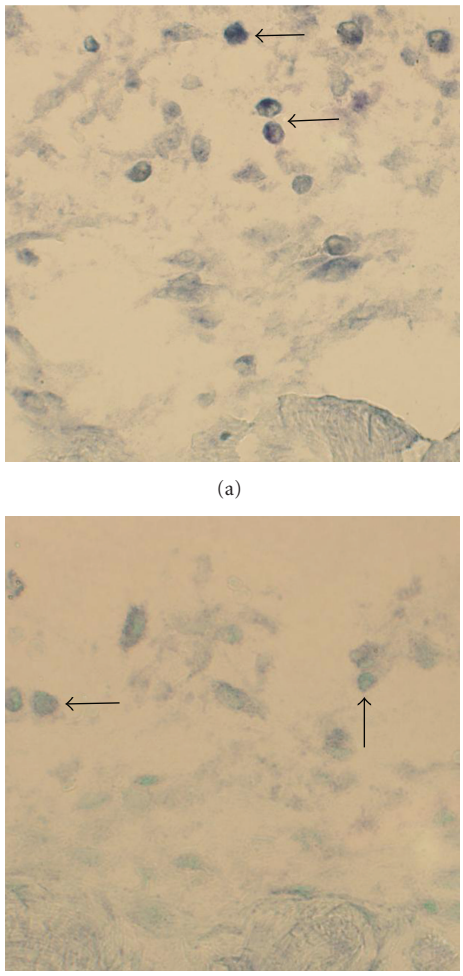
(a) (b)

Figure 5: (a, b) Sections of myositis specimens after processing for TNF-alpha. Cells show specific immunoreactions (arrows). The reactions show a granular appearance.



(a) (b)

Figure 6: (a, b) Inflammatory infiltrate in a soleus muscle (myositis specimen). Double-staining for demonstration of TNF-alpha (a) and CD68 (b). Immunoreactions are seen in the same cells (arrows).



(a)

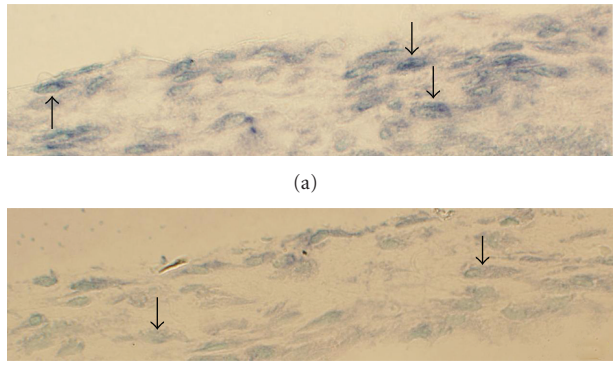
(b)

Figure 7: In situ hybridization for the demonstration of TNF-alpha mRNA in inflammatory cells. Adjacent sections are shown: antisense staining (a), and sense staining (b). There are reactions in (a) but not in (b). The arrows point at inflammatory cells.

4.5. In Situ Hybridization. Reactions for TNF-alpha mRNA were revealed for white blood cells of the inflammatory infiltrates in the myositis specimens (Figure 7). Reactions were also seen for fibroblasts (Figure 8) and sometimes for blood vessel walls (Figure 9) and muscle fibers (Figure 10). The muscle fibers and blood vessels for which reactions were seen were located in the regions with inflammatory infiltrates. It was noted that the muscle fibers with reactions for TNF-alpha mRNA were often infiltrated by inflammatory cells. The majority of the muscle fibers and blood vessels in the tissue of the myositis samples had no demonstrable reaction. There were no reactions at all in the musculature and the blood vessel walls in the nonmyositis samples. No reactions were noted for nerve fascicles and muscle spindles in any of the specimens from the myositis or non-myositis groups.

5. Discussion

It is well known that TNF-alpha is highly involved in arthritis, notably in RA. Accordingly, in our reference studies



(a)

(b)

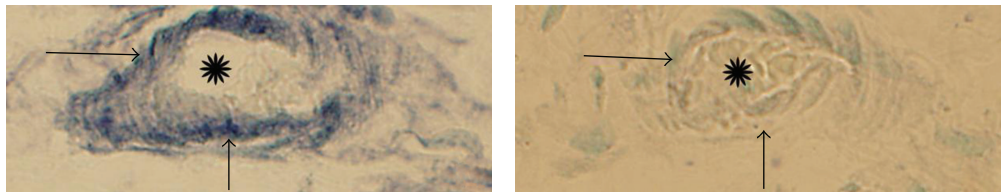
Figure 8: In situ hybridization for the demonstration of TNF-alpha mRNA in fibroblasts. Adjacent sections are shown: antisense staining (a), and sense staining (b). There are reactions in (a) but not in (b). The arrows point at fibroblasts.

in the present investigation we found that TNF-alpha was expressed in mononuclear-like cells in the RA synovial tissue. Detection of TNF-alpha reactions was thus clarified from the methodological point of view, and verifications were obtained via preabsorption stainings. With this as a basis, studies on TNF-alpha in myositis were performed.

A unique model for the production of myositis in rabbit musculature (the soleus muscle) was utilized. The main finding was that cells in the inflammatory infiltrates in the myositis muscles were found to express TNF-alpha at both at the mRNA and protein levels. Colocalization between TNF-alpha and CD68 was noted for these cells. Expression of TNF-alpha in macrophages has previously been noted in other situations (e.g., [13, 16]), including inflammatory myopathies [17]. In contrast, in our recent studies using the current myositis model, expression of the vesicular glutamate transporter VGluT2 was noted in white blood cells in the inflammatory infiltrates other than macrophages [24]. A further main finding was that the muscle fibers and blood vessel walls in areas showing inflammatory infiltration exhibited TNF-alpha mRNA and that fibroblasts also were seen to exhibit TNF-alpha mRNA.

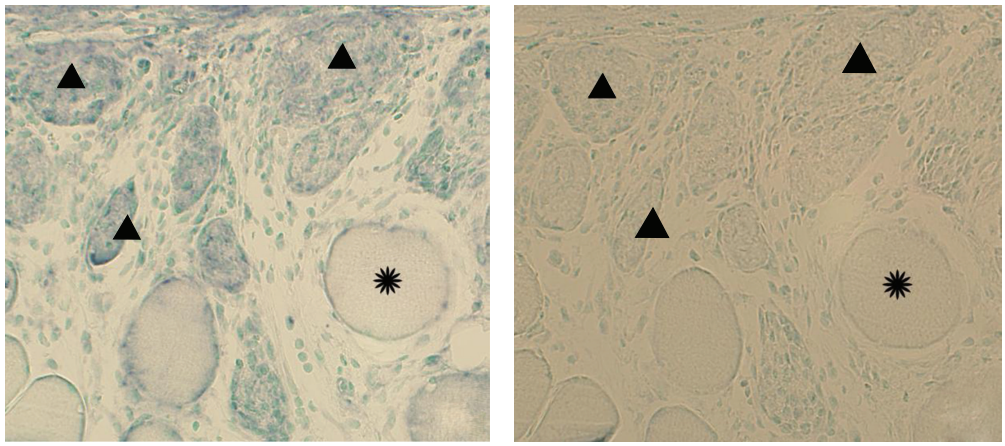
From a methodological point of view, it was clear that muscle fibers, blood vessel walls, and fibroblasts exhibited TNF-alpha mRNA but that no reactivity (muscle fibers, blood vessel walls) or very weak reactivity (fibroblasts) was noted at the protein level. The production level in these locations is therefore likely to be low, which precluded clear detection with our immunohistochemical methods. It is also possible that our in situ hybridization method detects very small quantities of TNF-alpha mRNA. Nevertheless, it has previously been shown that TNF-alpha can be expressed not only in inflammatory cells but also in injured muscle fibers and fibroblasts in response to muscle injury (crush-injury) [7] as well as in muscle fibers and cells in the connective tissue in inflammatory myopathies [17, 18].

The patterns of morphologic appearances of the inflammatory infiltrates and other morphologic changes seen resembled the appearances that can be seen in the muscle



(a) (b)

Figure 9: In situ hybridization for the demonstration of TNF-alpha mRNA in a small blood vessel: antisense staining (a), and sense staining (b). The vessel was located in the proximity of an inflammatory infiltrate. Arrows point at the wall. There are reactions in the wall in (a) but not in (b). Asterisks are in the lumen.



(a) (b)

Figure 10: In situ hybridization for the demonstration of TNF-alpha mRNA in the muscle tissue. Adjacent sections are shown, one processed with antisense probe (a), the other with sense probe (b). There are reactions in muscle fibers in (a) but not in (b). Arrowheads indicate corresponding muscle fibers in (a) and (b). As verified via examinations of parallel sections processed for morphology, the reactive muscle fibers are infiltrated by inflammatory cells. Certain muscle fibers appear to be unaffected (asterisk).

tissue in inflammatory myopathies [38]. Nevertheless, preliminary analysis using ELISA detecting anti-Jo-1 antibodies, which are known to correlate with disease activity for patients with inflammatory myopathy [39], does not lend proof to the theory that the myositis in our model is autoimmune in origin (unpublished observations). However, further studies on this aspect are warranted.

As noted previously, TNF-alpha immunoreactions in inflammatory cells invading muscles affected by myositis have previously only been documented in biopsies from patients with inflammatory myopathies [17, 18] and in muscle of mice in response to crush-injury [7]. Thus, in combination with this previous work, our results imply that TNF-alpha is intimately involved in the inflammatory process in myositis. Indeed, it has been suggested that TNF-alpha may have a role in the pathogenesis of the myositis in the inflammatory myopathies [40, 41] and that a marked inflammatory response involving TNF-alpha may be directly responsible for damaging muscle fibres in myopathic conditions [42].

Whether or not the TNF-alpha produced by the cells of the inflammatory infiltrates is entirely responsible for pro-inflammatory and damaging effects remains open to speculation. It is well known that TNF-alpha administration can have pro-inflammatory and detrimental effects, for example, leading to various catabolic changes as seen in studies on cultured skeletal muscle cells [4, 43, 44]. However, there is a marked discrepancy in the literature regarding the effect of TNF-alpha on the musculature. Some studies on myoblast cell culture show that TNF-alpha administration does not have catabolic effects (e.g., [45]), and other studies documenting accumulations of inflammatory cells in skeletal muscle in response to TNF-alpha administration [4] show no decrease in skeletal muscle proteins and no signs of muscle atrophy or injury. Perhaps these discrepancies reflect a dual role of TNF-alpha where in some circumstances inflammatory cell derived TNF-alpha can play a protective role [46] and also be involved in the recovery of muscle function after traumatic injury [9] and in muscle regeneration [47]. The discrepancies may also reflect the fact that different methods have been used in the studies that have been performed. The results in preliminary studies on inflammatory myopathies suggest that TNF blocking might be useful [48], but it is also emphasized that further studies are needed in order to clarify if this type of treatment is indeed useful [22].

Results of in vitro studies suggest that targeting TNF-alpha might be worthwhile in myositis [49, 50] and studies on dystrophic mdx mice subjected to wheel exercise indicate that TNF blockade can reduce myofiber necrosis [20, 21]. The use of anti-TNF treatment in studies on a rat model of repetitive reaching and grasping leads to an improvement in grip strength and attenuated task-induced increases in inflammatory cytokines, including TNF-alpha [51].

Although the use of other animal models have shown inflammation in muscle tissue in response to various forms of exercise [50, 52], the myositis model used in the current experiment is clearly distinguishable from these models. Thus, in contrast to these models, it leads to a marked presence of inflammatory infiltrates in the muscle tissue, that is, a morphology resembling that seen in inflammatory myopathies. In fact, no experimental myositis model exists which resembles the one used here and in which a marked presence of inflammatory infiltrates becomes present in the muscle tissue. Those for which such an infiltration has been demonstrated are the model of crush-injury described above [7] and models designed to help understand the mechanisms of inflammatory myopathies that occur in man. In these latter cases, myositis is induced by various infectious agents [53, 54], immunization with muscle components, for example, myosin [55, 56], and intraperitoneal injections with lipopolysaccharide [57]. The TNF system has not been examined in any of these myositis models replicating the inflammatory myopathies seen in man. Interestingly, there is evidence indicating a relationship between the inflammatory myopathies and another condition, muscular dystrophy, in the form of complex interactions between immunological and nonimmunological features of the diseases [58].

A noteworthy aspect with the currently used model is that marked overuse is applied in the procedures. Nevertheless, a limitation of the present study is that the relative contributions of the exercise protocol and the injections of the proinflammatory substances to the observed myositis are unclear. Forthcoming studies will clarify this issue. In any case, the model will, as it is currently used, provide the opportunity to evaluate the effects of interference with TNF-alpha actions in myositis development.

6. Conclusions

An animal model in which the importance of TNF-alpha for myositis development can be followed has previously been lacking. Using a newly established rabbit model of myositis development, a marked TNF-alpha expression has here been shown for the cells of the inflammatory infiltrates within damaged muscle. There was thus a clear evidence of local TNF-alpha production via infiltrated inflammatory cells, presumably leading to secondary inflammation-modifying effects. Using in situ hybridization, it was also seen that TNF-alpha mRNA was detected for muscle fibers and blood vessel walls in regions of inflammatory infiltrates. The current model can be used for further studies on the importance of TNF-alpha in the development of myositis and to document the expression patterns of other cytokines and signal substances in this condition.

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Review Article

Immunosuppressive Exosomes: A New Approach for Treating Arthritis

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Rheumatoid arthritis (RA) is a chronic autoimmune disease and one of the leading causes of disability in the USA. Although certain biological therapies, including protein and antibodies targeting inflammatory factors such as the tumor necrosis factor, are effective in reducing symptoms of RA, these treatments do not reverse disease. Also, although novel gene therapy approaches have shown promise in preclinical and clinical studies to treat RA, it is still unclear whether gene therapy can be readily and safely applied to treat the large number of RA patients. Recently, nanosized, endocytic-derived membrane vesicles "exosomes" were demonstrated to function in cell-to-cell communication and to possess potent immunoregulatory properties. In particular, immunosuppressive DC-derived exosomes and blood plasma- or serum-derived exosomes have shown potent therapeutic effects in animal models of inflammatory and autoimmune disease including RA. This paper discusses the current knowledge on the production, efficacy, mechanism of action, and potential therapeutic use of immunosuppressive exosomes for arthritis therapy.

1. Introduction

Arthritis refers to joint inflammation resulting from an autoimmune disease, joint wear and tear, or bacterial/viral infection. Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disorder in which endogenous synovial joints and other connective tissues are attacked by the immune cells. The pathological infiltration of inflammatory cells and synovial hyperplasia usually leads to the progressive destruction of articular cartilage and ankylosis of the joints. Although different types of treatment are available to alleviate symptoms and/or improve disease pathologies, no known therapy has been effective in reversing disease progression.

Conventional therapies for RA include nonsteroidal anti-inflammatory drugs, steroids, so-called "disease-modifying medications" (DMARDs), and surgery. These therapies are mostly palliative, can cause significant side effects, and offer no cure for the disease. In the early 1990s, biological therapies were demonstrated to alleviate symptoms of disease, but not to necessarily cure disease. Currently several different biologics, in particular inhibitors of tumor necrosis factor-α (TNF-α), are the leading drugs for treating RA. However, the need for constant infusion of drugs, either intravenously or subcutaneously, as well as the fact that not all patients response to anti-TNF therapy, necessitates the development of new RA therapies.

Gene therapy approaches also have been developed to treat arthritis and related joint disorders, demonstrated to be highly therapeutic in many animal models and safe in several clinical trials [1–3]. Gene transfer can be used to deliver genes encoding factors that inhibit proinflammatory cytokines (e.g., IL-1 receptor antagonist (IL-1Ra) for IL-1β inhibition, TNF receptor for TNF-α inhibition, and IL-18 neutralizer), Th2-polarizing and anti-inflammatory cytokines (e.g., IL-4, IL-10 and TGF-β), apoptosis-inducing factor (e.g., FasL), NF-κB inhibitors or decoy oligodeoxynucleotides, or cartilage destruction inhibitor (e.g., Ribozymes and MMP-1 antisense construct), either locally to the inflamed joints or systemically [4–7]. The types of vectors for gene delivery include nonviral (e.g., plasmid DNA) and viral (e.g., retrovirus, adenovirus, adenoassociated virus, lentivirus and herpes simplex virus) vectors. The potential problems with *in vivo* gene transfer using viral vectors include possible toxicity and immunogenicity [8, 9]. Among

these vectors, adenoassociated virus vector, which has limited immunogenicity and toxicity, has been used safely in several Phase I and II gene therapy trials for gene transfer of the TNF soluble receptor locally to joints.

Ex vivo gene transfer is an alternative gene delivery strategy where cells are genetically modified *in vitro* followed by local or systemic injection. The advantages of *ex vivo* gene transfer include better safety by controlling the type of cells transduced and reduced immunogenicity following injection. Synovial fibroblasts (SFs) or fibroblast-like synoviocytes (FLS) have been a major target for *ex vivo* gene transfer in RA therapy. Intra-articular injection of virally transduced SFs expressing IL-1Ra was therapeutic in animal models of arthritis [10–12] and a clinical study using IL-1Ra-transduced autologous RASFs for injection into knuckle joints of RA patients showed evidence of therapeutic effects without any adverse events [13]. Still, RASFs have the problems of low transduction efficiency and low proliferation rate which hamper their application on a large scale [14]. In addition to RASFs, dendritic cells (DCs, discussed below) as well as antigen-specific T cells [15] have also been used as vehicles to deliver immunosuppressive cytokines for the treatment of collagen-induced arthritis (CIA) in mouse models.

DCs are antigen-presenting cells (APCs) derived from $CD34^+$ stem cells that can regulate immune reactivity. Although DC were initially considered as instigators of immune responses, including organ graft rejection and autoimmune disorders, more recent data have implicated DC in the induction and even maintenance of tolerance to allo- or autoantigens in experimental models. The ability of DC to either stimulate or suppress immune responses is mediated by various factors; the most important being their stage of differentiation/maturation/activation and their hematopoietic lineage affiliation. Immature DCs, characterized by low levels of MHC and costimulatory molecules (e.g., CD80, CD86, CD40, ICAM-1, and ICOSL), are able to suppress antigen-specific T-cell responses. These "tolerogenic" DCs produce reduced levels of type 1 cytokines (e.g., IL-12 family members) and increased level of immunosuppressive cytokines (e.g., IL-10, TGF-β, and VEGF). They can also express high levels of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO), which degrades free tryptophan and "starves" responder T cells of the essential amino acid, resulting in increased T-cell apoptosis [16]. The mechanisms through which tolerogenic DCs exert regulatory functions can include the induction of antigen-specific T-cell anergy or deletion [17]; induction of regulatory T cells [18–20]; polarization of T cells away from a Th1 or Th17-type response and toward a Th2-type response [21]. The immunosuppressive/tolerogenic properties of DCs can be enhanced and stabilized by genetic modification. Genetically modified DCs have been shown to successfully ameliorate symptoms and control disease progression in animal autoimmune disease models including RA and type 1 diabetes [22–26]. In fact, DCs transduced with viral vectors expressing immunosuppressive agents were found to be more effective in treating murine CIA than similarly modified fibroblasts or T cells [22, 27].

More recently, exosomes derived from immunosuppressive DCs have been found to confer potent and lasting immunosuppressive effects, similar to their parental DC. Exosomes are a type of secreted membrane vesicles produced by most cell types. They are characterized by a size of 30–100 nm in diameter and an endocytic origin, formed by the reverse budding of the multivesicular bodies and released upon their fusion with the plasma membrane [28–30] (Figure 1(a)). Their protein content largely reflects that of the parental cells and is enriched in certain molecules including adhesion molecules, membrane trafficking molecules, cytoskeleton molecules, heat-shock proteins, cytoplasmic enzymes, signal transduction proteins, and cell-specific antigens [28, 31, 32]. APC-derived exosomes are enriched in MHC classes I and II as well as co-stimulatory molecules. Exosomes also contain functional mRNA and microRNAs molecules [33–35] (Figure 1(b)). Most hematopoietic cells, including DCs, produce copious amount of exosomes. Certain types of exosomes have been shown to confer immunosuppressive effects in different disease models including RA. Thus it is likely that exosomes represent a novel effective and safe therapeutic approach for treating arthritis. Indeed, exosomes derived from immunosuppressive DCs and from peripheral blood (Figure 2) have shown the ability to suppress inflammation.

2. Immunosuppressive DC-Derived Exosomes for Arthritis Treatment

2.1. DC/IL-10 Exosomes. DCs transduced with adenovirus expressing the IL-10 gene (Ad.IL-10) or treated with recombinant murine IL-10 (rmIL-10) were demonstrated to be anti-inflammatory and suppress mouse CIA [36, 38]. Interestingly, exosomes secreted by those DCs were also found immunosuppressive. Exosomes derived from Ad.IL-10 DCs were capable of decreasing T-cell proliferation in a mixed lymphocytes reaction. In mice immunized with keyhole limpet hemocyanin (KLH) antigen, local injection of DCs (ad.vIL-10 or rmIL-10 treated) or their exosomes both resulted in significant suppression of KLH-induced delayed-type hypersensitivity (DTH) response. Moreover, a single dose of these exosomes systemically delivered after the onset of CIA effectively ameliorated disease progression, in contrast to the ineffectiveness of direct injection of rmIL-10 [36]. Similar efficacy was observed using DC/IL-10 exosomes to suppress DTH response and CIA compared with DC/IL-10 cell treatment, making DC exosomes an even attractive therapy than DCs.

Although not fully understood yet, the suppressive effect of DC/IL-10 exosomes is not simply due to the delivery of the suppressive cytokine IL-10. Instead, the therapeutic effect of exosomes requires the integrity of the exosome membrane, as repeated freeze and thaw cycles that disrupt the exosome structure abrogated the effect. The effect is also MHC class II dependent since exosomes deficient in MHC class II did not suppress DTH [36]. In addition, the presence of B7-1/2 (CD80 and CD86) on IL-10-treated BMDC-derived exosomes is required for their suppressive

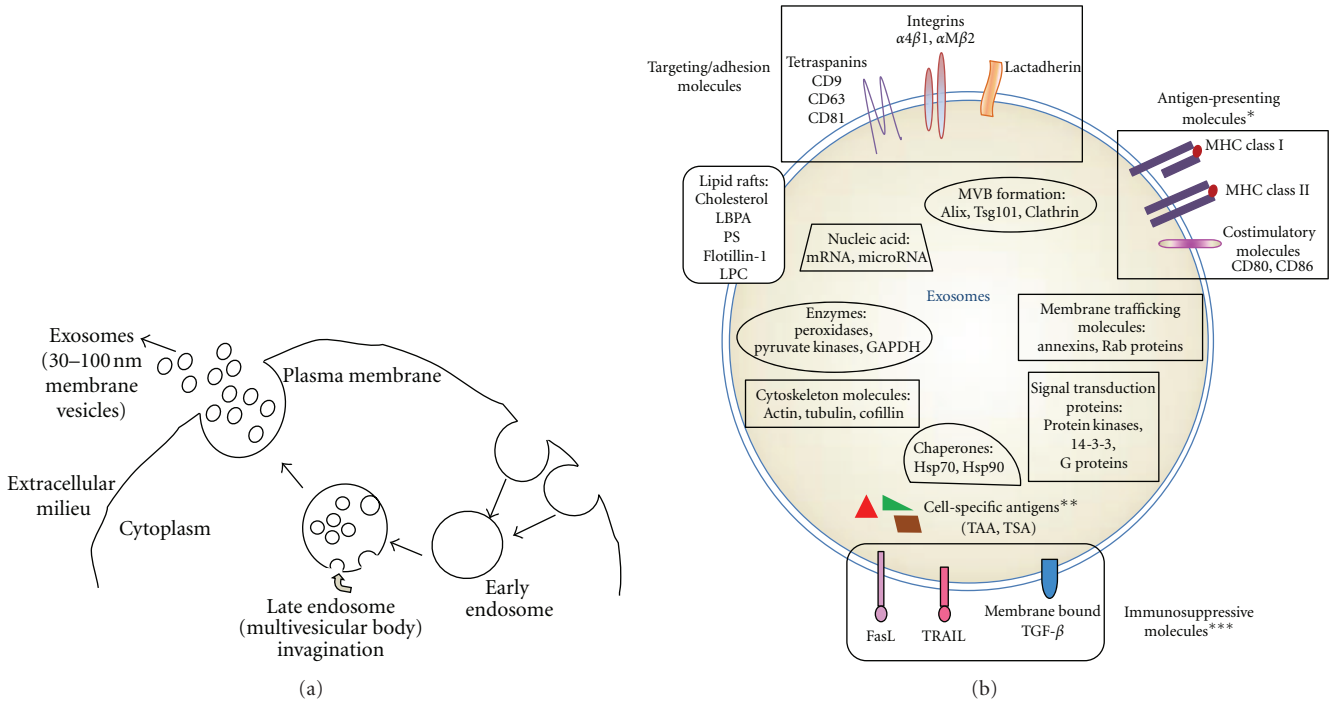


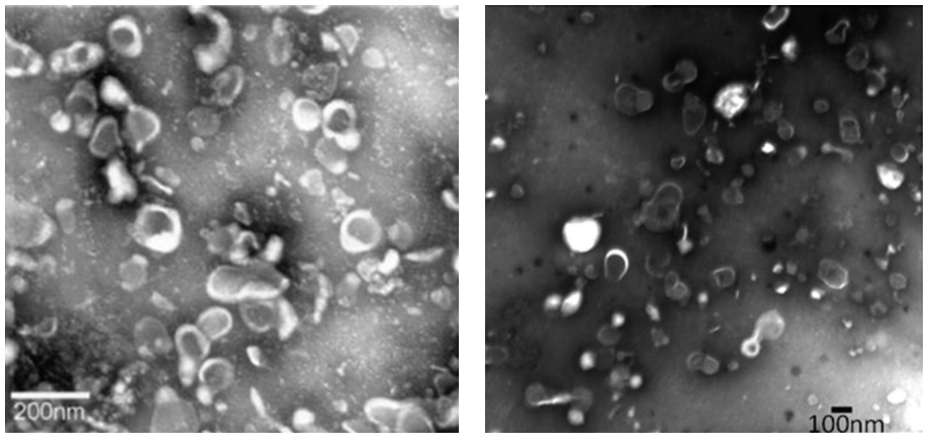
Figure 1: Exosome biogenesis and molecular composition. (a) Exosomes are small membrane vesicles formed by invagination of the multivesicular bodies (MVBs) in the late endocytic compartment. They are released upon the fusion of MVBs with the plasma membrane. (b) Exosomes are typically enriched in certain molecules including targeting/adhesion molecules, membrane trafficking molecules, cytoskeleton molecules, proteins involved in MVB formation, chaperones, cytoplasmic enzymes, signal transduction proteins, and functional mRNA and microRNA populations. *APC-derived exosomes contain antigen-presenting molecules including MHC class I, MHC class II, and co-stimulatory molecules. **Exosomes also contain cell-specific antigens (e.g., tumor antigens in tumor-derived exosomes). ***Immunosuppressive molecules such as FasL, TRAIL, or TGF-β are present on certain APC or tumor-derived exosomes.

effects [39]. Thus it is possible that IL-10 treatment results in DC-derived exosomes with a different composition that makes the vesicles more immunosuppressive.

2.2. DC/IL-4 Exosomes. We and others have demonstrated that DCs genetically engineered to express the Th2 cytokine IL-4 were an effective treatment for murine CIA [22, 23]. Specifically, we found that a single i.v. injection of immature BMDCs infected with adenoviral vector expressing IL-4 into mice with established CIA achieved almost complete suppression of the disease lasting at least 4 wk posttreatment. The therapeutic effect of the IL-4 expressing DC (DC/IL-4) was significantly better than repeated injection of recombinant IL-4 or direct injection of adenoviral IL-4 [23]. DCs retrovirally transduced with the IL-4 gene also reduced the incidence and severity of CIA after a single i.p. injection [22]. In both studies, DC/IL-4 reduced the disease-associated humoral responses and conditioned splenic cells towards a Th2-polarized response upon antigen stimulation.

Similar to the significant immunosuppressive effects of DC/IL-4, exosomes derived from those DCs were shown to reduce the severity and the incidence of established CIA when delivered systemically (i.v.), and suppressed DTH response when injected locally [40]. Suppression of the DTH response is MHC restricted in that only syngeneic DC exosomes, but not allogeneic exosomes, were effective in conferring immunosuppression. Furthermore, systemically injected DC/IL-4 exosomes were found to migrate to spleen and liver and interact with CD11c+ DCs and F4/80+ macrophages. Adoptive transfer of CD11c+ or CD3+ splenic cells isolated from antigen-immunized mice that have been systemically treated with exosomes into the footpad of recipient mice significantly reduced footpad swelling in the DTH model, suggesting that exosomes from DC/IL-4 can directly or indirectly modify the function of endogenous APCs and T cells, either by inducing a regulatory subset and/or depleting antigen-reactive Th1 cells [40].

2.3. DC/Death Ligand Exosomes. Selective inducing apoptosis of antigen-specific T cells by APCs genetically modified to express death ligand (e.g., FasL and TRAIL) is an alternative way to downregulate antigen-specific T-cell responses. DCs genetically modified to express FasL can induce donor-specific T-cell hyporesponsiveness to alloantigen and facilitate allograft survival [41]. FasL-expressing DCs are also able to suppress collagen-reactive T cells and inhibit the progression of murine CIA after systemic injection [26]. Exosomes derived from FasL-expressing DCs showed an anti-inflammatory effect in a murine DTH model upon local administration [42]. The therapeutic effect was abolished



(a) (b)

Figure 2: Transmission electronic micrograph of (a) exosomes isolated from murine BMDC culture [36]. *Copyright 2005. The American Association of Immunologists, Inc.* and (b) exosomes isolated from murine blood plasma [37]. *Copyright 2007. The American Association of Immunologists, Inc.*

when *lpr* (Fas-deficient) mice were used as recipients or when exosomes were derived from the DCs of *gld* (FasL-deficient) mice. However, the immunosuppressive effect of FasL-deficient DC exosomes could be restored by gene transfer of FasL to DCs. The ability of DC/FasL exosomes to suppress DTH response was also antigen specific as optimal suppressive effects were achieved when the DCs were prepulsed with the same antigen used for mice immunization. Using DC and DC-derived exosomes from different knockout mice, the suppressive effect was shown to be MHC class II dependent, but MHC class I independent. Systemic injection of DC/FasL exosomes was also effective in treating established murine CIA [42].

It was shown that infiltrating T cells present in the synovial fluid of RA patients were more susceptible to apoptosis induced by APO2L/TRAIL, a TNF superfamily member capable of inducing cell apoptosis. Bioactive APO2L/TRAIL associated with exosomes was detected in the synovial fluid of RA patients compared with synovial fluid of traumatic arthritis patients [43]. Interestingly, bioactive APO2L/TRAIL conjugated to the membrane of liposomes, artificial lipid vesicles resembling exosomes, was demonstrated to substantially reduce inflammation after intra-articular injection in a rabbit model of RA, more effectively than soluble, unconjugated APO2L/TRAIL. The increased bioactivity is possibly due to the enhanced receptor cross-linking as a result of increased local concentration of the protein upon liposome delivery [44].

2.4. DC/IDO Exosomes. Immunoregulatory DCs expressing the tryptophan catabolic enzyme IDO can inhibit T-cell activation and suppress T-cell responses to auto- and alloantigens by tryptophan starvation and/or production of toxic metabolites [45, 46]. The immunosuppressive potency of DCs genetically modified to express IDO and their exosomes was also investigated in RA models. BMDCs adenovirally transduced to express IDO and the resulting DC/IDO exosomes both showed anti-inflammatory effect in murine DTH and CIA models. In addition, transduction of DCs with the IDO inducer CTLA4-Ig resulted in induction of IDO and the derived exosomes were also able to reduce inflammation [47]. The suppressive effect of DC/CTLA4-Ig exosomes was reduced when DCs were pre-treated with the competitive IDO inhibitor 1-MT or excessive L-tryptophan, suggesting that the effect was dependent on the IDO activity in DCs and IDO-mediated tryptophan deprivation. Similar to exosomes derived from DC/IL-10, the immunosuppressive effect of DC/IDO exosomes was partially dependent on B7-1/2 molecules, as exosomes of DCs isolated from B7-1/2 knockout mice had an attenuated anti-inflammatory effect when transduced with IDO [47].

2.5. Mechanism(s) of Exosome Function. Although the exact functional mechanism of immunosuppressive DC-derived exosomes remains to be determined, exosomes are believed to function more than vehicles that simply deliver immunosuppressive factors derived from their parental DCs. This is evidenced by the facts that most of the suppressive effects observed have antigen specificity and are dependent on the presence of certain molecules on exosomes as well as in recipient animals, in particular MHC class II molecules and B7-1/2. Furthermore, similar to direct gene transfer or DC cell therapy [38, 48–51], distal therapeutic effects (contralateral effects) were observed when exosomes were delivered locally. However, trafficking analysis suggested that there is only limited cross-trafficking of exosomes to the contralateral lymph node [42]. Therefore, it is likely that immunosuppressive DC exosomes are able to modify the behavior of endogenous immune cells, such as APCs, which then are responsible for conferring a systemic suppressive/anti-inflammatory effect. The interactions between exosomes and APCs could be at the membrane level or, in some cases, involve the internalization

of these vesicles where vesicle-contained proteins and RNAs could be functionally transferred.

3. Immunosuppressive Exosomes in Body Fluids

In addition to exosomes derived from immunosuppressive DCs, other sources of suppressive exosomes may also have the potential to treat inflammatory arthritis diseases. Many types of tissue- or body-fluid-derived exosomes have been found to be immunoregulatory or tolerogenic. For instance, placenta-derived exosomes and exosomes isolated from the maternal peripheral circulation are able to induce T-cell signaling defects, possibly attenuating immune responses against the fetus [52, 53]. Exosomes isolated from serum shortly after antigen feeding are able to induce antigen-specific tolerance in naïve recipient animals [54]. In addition, exosomes isolated from the bronchoalveolar fluid of mice respiratory exposed to pollen allergen can prevent antigen-specific allergic reaction [55]. Also, exosomes isolated from human breast milk and colostrum can increase the number of T regulatory cells and inhibit effector T-cell activation *in vitro* [56]. Interestingly, exosomes derived from certain body fluids, in particular conditioned blood plasma or serum, have been found to reduce arthritic inflammation.

3.1. Plasma-Derived Exosomes. We have demonstrated that exosome-like vesicles can be isolated from the blood plasma of both naïve mice and antigen-immunized mice, with certain surface protein markers including MHC class I, MHC class II, CD11b, CD71, FasL, and CD86. Plasma-derived exosomes isolated from mice immunized with KLH antigen showed potent suppressive effect on the KLH-induced DTH response after local administration [37]. The effect was antigen specific since plasma-derived exosomes isolated from mice immunized with an irrelevant antigen did not induce effective suppression. MHC class II+ exosomes were responsible for conferring the suppressive effect as depletion of MHC class II+ exosomes from plasma-derived exosomes abrogated this effect. The anti-inflammatory effect also required Fas/FasL signaling. Additionally, the effect was time dependent since the optimal immunosuppressive activity was obtained with exosomes isolated 14 days after immunization. This result suggests the presence of exosomes with antigen-specific immunosuppressive activity in the circulation of individuals that are hyperreactive to certain antigens. It also suggests the possibility of utilizing autologous plasma-derived exosomes therapeutically for the suppression of antigen-specific inflammation.

3.2. Clinical Studies with Serum-Derived, Anti-Inflammatory Exosomes. An effective method for stimulating *de novo* production of anti-inflammatory cytokines in blood was developed by incubating whole blood with CrSO4-treated glass beads for a short period of time [57]. Such treatment resulted in the robust induction of IL-1Ra and an increase in the levels of IL-4 and IL-10 as well as certain growth factors such as insulin-like growth factor-1 (IGF-1). This approach for the preparation of autologous conditioned serum (ACS) with enhanced anti-inflammatory cytokines has been used for the clinical treatment of patients with RA, osteoarthritis (OA), and spinal disorders, with efficacy and safety both observed [58, 59]. Exosomes with anti-inflammatory properties were isolated from ACS and have been tested clinically for the treatment of RA. Intra-articular injection of these exosomes appears to be therapeutic for RA patients who do not respond well to conventional therapy, with reduced pain in multiple joints and decreased inflammatory markers in the blood [60]. The therapeutic use of these ACS-derived exosomes also appears to be safe, supporting the development of similar exosome treatments for other inflammatory and autoimmune diseases. The immunosuppressive exosomes shown to be effective in treating murine and human RA are summarized in Table 1.

4. Pathogenic Exosomes

We have discussed the potential usage of immunosuppressive exosomes for arthritis treatment. However, additional studies have also suggested that exosomes of certain sources could contribute to disease progression. For example, exosomes produced by the synovial fibroblasts obtained from RA patients were found to contain a membrane form of TNF-α, which could play a role in tissue destruction and autoimmune inflammation. These TNF-α positive exosomes rendered activated T cells resistant to apoptosis, favoring the pathogenesis of RA [61]. In addition, citrullinated proteins, known to be autoantigens in RA, were detected in exosomes purified from the synovial fluids of RA patients [62]. Similarly, the autoantigen nuclear protein DEK, which contributes directly to joint inflammation in juvenile arthritis (JA), is secreted in both a free form and an exosome-associated form in the synovial fluids of JA patients [63, 64]. Exosomes containing annexins, which promote pathological mineral formation and articular chondrocytes destruction, were found more in the articular cartilage in OA patients [65]. These observations demonstrate the presence of disease-contributing exosomes, which could be useful inflammation markers of arthritis diseases. In theory, selective elimination of these exosomes would be beneficial to arthritis therapy.

5. Conclusion

Immature DCs with enhanced immunosuppressive/tolerogenic properties can be produced by genetic modification or by exposure to cytokines or cytokine inhibitors. Exosomes derived from immunosuppressive DCs have shown therapeutic effects comparable to or better than their parental DCs in treating animal DTH and CIA and thus have the potential to be used clinically for RA treatment. While DCs manipulated *ex vivo* still have the risk of maturation in inflammatory environment, DC-derived exosomes are more stable following isolation and thus are safer than autologous cells for *in vivo* administration. The unique biological composition of exosomes also gives them a half-life that

Table 1: Immunosuppressive exosomes for the treatment of arthritis.

Exosome source	Cell modification/treatment	Model	Application and effect	Reference
BMDC	BMDCs transduced with adenoviral IL-10 or treated with rmIL-10	Mouse	Footpad injection suppressed DTH response; systemic delivery (i.v.) ameliorated CIA progression. The effect requires exosome integrity and MHC class II and B7-1/2 molecules.	[36, 39]
BMDC	BMDCs transduced with adenoviral IL-4 or retroviral IL-4	Mouse	Systemic injection (i.v.) reduced the incidence and severity of established CIA; local injection suppressed DTH response. Exosomes interacted with DCs and macrophages in spleen and liver and were able to modify the function of endogenous APCs and T cells.	[40]
BMDC	BMDCs transduced with adenoviral FasL	Mouse	Local administration suppressed DTH response. The effect was dependent on Fas-FasL interaction and MHC class II molecules. Systemic injection was also effective in treating established CIA.	[42]
BMDC	BMDCs transduced with adenoviral IDO or CTLA4-Ig	Mouse	DC/IDO exosomes were anti-inflammatory in both DTH and CIA models. DC/CTLA4-Ig exosomes reduced DTH response. The effect was dependent on the IDO activity in DCs and partially dependent on B7-1/2 molecules.	[47]
Blood plasma	Exosomes were isolated from the plasma of antigen-immunized mice	Mouse	Local administration of plasma-derived exosomes suppressed DTH response in an antigen-specific manner. The effect was dependent on MHC class II+ exosomes.	[37]
Serum	Exosomes were isolated from physicochemically conditioned autologous patient serum	Human clinical trial	ACS exosomes exhibited anti-inflammatory properties. Local injection of these exosomes was safe and beneficial to RA patients that do not respond to conventional therapy. Reduced joint pain and decreased blood inflammatory markers were observed.	[58–60]

appears longer than many cell types after injection. However, the paucity of clinical trials using immunosuppressive DC exosomes for arthritis treatment still prevents a comprehensive evaluation of their effects on human patients.

The fact that exosomes isolated directly from ACS appears to improve disease in RA patients strongly supports the further clinical development of immunosuppressive exosomes. However, it is important to note that while the clinical results are encouraging in terms of feasibility, safety, and efficacy, the blood plasma- or serum-derived exosomes have heterogeneous cellular origins and poorly defined composition. Further investigation is needed to determine the functional components of these therapeutic exosomes. Taken together, there is considerable evidence supporting the ability of immunosuppressive exosomes to help control the overreactive immune system. Compared with gene and cell therapies, exosome-based therapy could provide a new and safe therapeutic approach for arthritis.

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Review Article

Risk of Orthopedic Surgical Site Infections in Patients with Rheumatoid Arthritis Treated with Antitumor Necrosis Factor Alfa Therapy

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Introduction. International guidelines recommend interruption of anti-TNF medications in the perioperative period, but there are no randomized trials to support such recommendation. *Objectives*. To study literature evidence assessing the risk of surgical site infections in orthopedic surgery patients with RA using anti-TNF drugs, compared to untreated patients or those using conventional DMARD. *Methods*. Systematic review of cohort studies is concerning surgical site infections in orthopedic procedures in patients with RA. *Results*. Three studies were selected. Only one was considered of high-quality, albeit with low statistical power. The review resulted in inconclusive data, since the best quality study showed no significant differences between groups, while others showed increased risk of infections in patients using anti-TNF medications. *Conclusion*. It is unclear whether patients with RA using anti-TNF medications are at increased risk of surgical site infections. Randomized controlled trials or new high quality observational studies are needed to clarify the issue.

1. Introduction

Rheumatoid arthritis (RA) affects between 0.2 and 1% of brazilian population [1]. Twenty-five percent of RA patients undergo some surgery for the treatment of orthopedic sequelae after 22 years of followup [2]. Currently, most patients with RA are in use of conventional modifying disease activity drugs (DMARD), and some of them are on antagonists of tumor necrosis factor (anti-TNF) medications [3, 4].

Anti-TNF drugs have been used to treat patients with RA who do not get to remission with one or more conventional DMARD. Systematic reviews have shown no increased risk of bacterial infections after treatment with such drugs [5, 6].

There is no consensus in the literature on the use of immunosuppressive drugs in the perioperative period in orthopedic surgery since there are few studies on the topic. A randomized clinical trial showed no increased perioperative infections in patients on methotrexate [7]. There are no clinical trials assessing treatment with anti-TNF medications in this context so far. The guidelines of the American College of Rheumatology (ACR), British Society of Rheumatology, and Japan College of Rheumatology recommend the suspension of anti-TNF medications in the perioperative period [8–10], but this might lead to worsening of disease activity, which could affect the postoperative rehabilitation.

In observational studies of patients undergoing hip and knee arthroplasties, several independent risk factors for surgical site infection were found, including RA itself, male gender, age greater than or equal to 75 years, secondary osteoarthritis, type of prosthesis, no cement prosthesis, comorbidity index, joint injury by trauma, American Society of Anesthesiology physical status classification greater than

or equal to 3, wound hematoma, days of wound drainage, and surgical time, which is the most consistently described risk factor [11–15].

The objective of this study was to perform a systematic review of observational studies on the risk of surgical site infections (SSI) in orthopedic surgery in patients with RA, treated with anti-TNF drugs, compared to untreated patients or those using conventional DMARD.

2. Methods

2.1. Types of Studies. Prospective or retrospective cohort studies that assessed the risk of SSI in orthopedic surgery in patients with RA, treated with anti-TNF drugs, compared to untreated patients or those using conventional DMARD were eligible. Studies could evaluate patients undergoing any type of orthopedic surgery, including arthroplasty. The minimum followup should be one year, so that all prosthetic infections were accounted [16].

2.2. Types of Patients. RA patients are classified according to ACR 1987 criteria [17].

2.3. Outcomes. Superficial or deep incisional infections or prosthesis infections, defined by objective criteria.

2.4. Search Strategy. We used the following keywords: "anti-tumor necrosis factor," "DMARD," "rheumatoid arthritis", "orthopedic surgery," and "infection," all simultaneously and in combinations between themselves. Search was performed in the Cochrane Collaboration, MEDLINE, EMBASE, CINAHL, and LILACS databases. We included only those studies in English, Portuguese, and Spanish.

2.5. Data Collection and Analysis of Studies. After the search results, the abstracts were initially assessed. After selecting articles that met inclusion criteria, we performed a general reading of the articles, followed by methodological analysis. Data were collected in a systematic manner on a standardized form. Initially, it was noted whether the study was prospective or retrospective and whether there was sample size calculation. Then, we applied Newcastle-Ottawa Cohort Quality Assessment Scale [18], which has good applicability for the purposes of this review. These criteria split the analysis into three areas: selection, comparability, and outcome. In each of these areas, we applied a number of questions and, according to the answer, a "asterisk" is attributed. In the fields "selection" and "outcome," it is possible to assign one "asterisk" for each question, while it is possible to assign two "asterisks" to the question "comparability." We performed an adaptation to questions so that they could apply to the scenario of this review. The assessment details are shown in Table 1. Studies were considered of high quality if they had at least one "asterisk" in each area, and the sum of the "asterisks" were equal to or greater than five.Due to heterogeneity of the studies, no qualitative data analysis was performed.

Table 1: Newcastle-Ottawa Cohort Quality Assessment Scale, adapted for review purposes, as description from authors. Acronym: RA: rheumatoid arthritis.

Selection
(1) Representativeness of the exposed cohort
(a) truly representative of the average RA patient in the community*
(b) somewhat representative of the average RA patient in the community*
(c) selected group of users e.g., nurses, volunteers
(d) no description of the derivation of the cohort
(2) Selection of the nonexposed cohort
(a) drawn from the same community as the exposed cohort*
(b) drawn from a different source
(c) no description of the derivation of the nonexposed cohort
(3) Ascertainment of exposure
(a) secure record (e.g., surgical records)*
(b) structured interview *
(c) written self-report
(d) no description
(4) Demonstration that outcome of interest was not present at start of study
(a) yes*
(b) no
Comparability
(1) Comparability of cohorts on the basis of the design or analysis
(a) study controls for surgical time*
(b) study controls for any additional factor *
Outcome
(1) Assessment of outcome
(a) independent blind assessment*
(b) record linkage*
(c) self-report
(d) no description
(2) Was followup long enough for outcomes to occur?
(a) yes (1 year)
(b) no
(3) Adequacy of followup of cohorts
(a) complete follow up—all subjects accounted for*
(b) subjects lost to followup unlikely to introduce bias—small number lost—>80% follow up, or description provided of those lost*
(c) followup rate < 80% and no description of those lost
(d) no statement

* Studies were considered of high quality if they had at least one asterisk in each area, and the sum of the asterisks were equal to or greater than five.

3. Results

3.1. Search. Initially 283 abstracts were found in MEDLINE. Search in other databases did not add additional abstracts. Six abstracts were selected according to inclusion criteria

and, after general manuscript reading, three studies [19–21] were included in the review. Details of articles selection are shown in Figure 1. Since we selected few articles, it was possible to describe each one separately.

3.2. Description of Studies. Studies included 1767 procedures. The study by Momohara et al. was not clear about the number of of patients, so was not possible to express their exact number.

The study by den Broeder et al. [19] is a retrospective cohort that included 1219 patients in 768 procedures from two centers in the Netherlands. Its population consisted of patients who underwent various types of orthopedic surgery between 2001 and 2004. Procedures were excluded if the time from the last procedure was less than three months. Patients were divided into two groups: those who were on anti-TNF therapy (cohort 2) versus those who had never used these medications (cohort 1), but it was not clear which DMARD they were in use. The first group was then divided into two groups: patients who were in use of anti-TNF therapy in the perioperative period (cohort 2b) versus those who had discontinued the drug at least four half-lives before the surgery (cohort 2a). In cohort 2, patients were being treated with infliximab in 80 procedures, etanercept in 79, and adalimumab in 37 procedures. In addition to the primary outcome, this study also evaluated the incidence of wound dehiscence, bleeding or hematoma, subluxation, reoperation, and death. The rate of SSI in cohorts 1, 2ł, and 2b was 4.0%, 5.8% and 8.7%, respectively. No increased risk of SSI was found in cohort 2, compared to cohort 1. In this comparison, odds ratio (OR) values and confidence intervals (CI) were not provided and were calculated by the main author of this review (OR 1.84, 95% CI 0.98 to 3.44). The comparison between the cohorts 2a and 2b showed similar numbers of SSI between groups (OR 1.56, 95% CI 0.52 to 4.66). Regarding the "wound dehiscence" outcome, there was an increased incidence in patients who continued using the anti-TNF compared with those who had stopped (OR 11.2, 95% CI 1.4 to 90). Comparsion between patients on anti-TNF therapy who discontinued the drug and patients who were anti-TNF naive showed a reduced incidence in the former group (OR 2.4, 95% CI 1.1 to 5.0). There were no data on disease activity.

The study by Kawakami et al. [20] is a retrospective cohort that included 128 procedures in 112 patients from a single center in Japan. The population consisted of patients undergoing joint surgery between 2004 and 2009, in which most of them were arthroplasties. A 1 : 1 matching was performed among patients receiving anti-TNF versus conventional DMARD. In the group on anti-TNF therapy, patients were taking infliximab in 35 surgeries and etanercept in 29 surgeries. Patients on conventional DMARD were using methotrexate in 48 cases, sulfasalazine in 18 cases, bucillamine (an immunomodulator drug developed in Japan, similar to D-penicillamine) in 6 cases, and D-penicillamine in 4 cases, either alone or in combination. In addition to the primary outcome, the presence of arthralgia and deep vein thrombosis (DVT) diagnosed with ultrasonography was assessed, but without specifying whether tests were performed in all patients. Anti-TNF medications were discontinued 2–4 weeks before surgery and it is unclear if conventional DMARD were also discontinued. OR and CI were not provided and were calculated by the main author based on information collected in the study. The SSI rate was higher among patients on anti-TNF therapy than in patients receiving conventional DMARD (12.5% and 1.6% resp.) (OR 9.0, 95% CI 1.1–74.22). The incidence of DVT was higher among patients on anti-TNF therapy than in patients receiving conventional DMARD (OR 2.9, 95% CI 1.2 to 6.9). Regarding the outcome "arthralgia," comparisons were made only within the group of patients treated with anti-TNF medications.The study of Momohara et al. [21] is a retrospective analysis of a prospective cohort that included 420 procedures performed by the same group of Kawakami et al. The population consisted of patients undergoing hip (81 cases) and knee (339 cases) arthroplasties between 2005 and 2009. Initially, the authors divided patients into two groups: individuals on anti-TNF therapy versus those using conventional DMARD. However, when reporting the results, the authors have chosen to make comparisons according to the outcome, setting a nested case-control design. As the study provided the data for each group, it was possible to calculate OR and CI for the comparison according to the risk factor. In the group of patients using anti-TNF medications, 19 patients were treated with infliximab, 23 with etanercept, and 2 with adalimumab. In the group of patients using conventional DMARDs, 279 patients were treated with methotrexate, 93 with sulfasalazine, 52 with bucillamine, 7 with minocycline, 4 with leflunomide, 31 with tacrolimus, 15 with mizoribine (a drug with immunomodulatory mechanism of action similar to mycophenolate mofetil), 3 with cyclophosphamide, 9 with actarit (an immunomodulator drug developed in Japan, a nitric oxide inhibitor), 4 with auranofin, 1 patient with aurothiomalate, and 16 patients with D-penicillamine, alone or in combination. Conventional DMARD were kept during

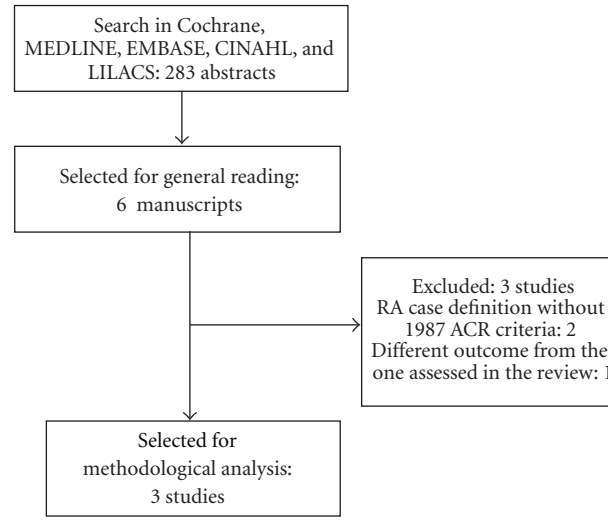

Figure 1: Fluxogram of studies selection. Acronyms: RA: rheumatoid arthritis; ACR: American College of Rheumatology.

Table 2: Quality of studies according to Newcastle-Ottawa Cohort Quality Assessment Scale.

Studies	Selection	Comparability	Outcome	Total	Quality
den Broeder AA e cols.	****	**	***	9	high
Kawakami K e cols.	****			4	low
Momohara S e cols.	****			4	low

* Studies were considered of high quality if they had at least one asterisk in each area, and the sum of the asterisks were equal to or greater than five.

the perioperative period, and anti-TNF medications were discontinued 2–4 weeks before surgery. The SSI rate was higher among patients using anti-TNF than in patients receiving conventional DMARD (20.8% and 4.0% resp.) (OR 6.3, 95% CI 2.6 to 14 9). Most infections were superficial, and there was no data on disease activity.

3.3. Quality Rating. The only study considered of high quality by our assessment was AA den Broeder et al. (Table 2). It was also the only study that included patients receiving adalimumab and assessed allocation bias. However, the study did not achieve sufficient statistical power to detect small differences. A *post hoc* calculation of statistical power of this study was 49.4%. The other two studies showed several methodological flaws and heterogeneous methodologies, which hampered the statistical analysis between groups with and without the use of anti-TNF medications, including logistic regression, so it is likely to have occurred association bias. On the other hand, in these studies, anti-TNF medications were discontinued 2–4 weeks before surgery, which may have diminished the risk of infections with such drugs. As followup was not informed, it is not clear whether all infections were recorded. There was no information about the frequency of progression to deep infections.

4. Discussion

Currently, there are no randomized trials that have assessed safety of anti-TNF medications in the orthopedic surgery perioperative period. The available body of evidence is based on observational studies and expert opinion. Although some international guidelines recommend discontinuation of medications before surgery, according to drug half-life [6–8], the results of this review indicate that there is insufficient evidence to support these recommendations. Two Japanese studies, performed by the same group, have shown significantly increased risk of surgical site infections in patients on anti-TNF therapy when compared to patients using conventional DMARD, but it is not clear if both studies included some patients in common. In contrast, the single high quality study (den Broeder AA et al.), performed in another ethnic group, showed no increased risk of infections. Moreover, it was the only study that compared anti-TNF naïve patients to the ones in current treatment with anti-TNF medications and to others who discontinued the anti-TNF drugs before surgery, including patients receiving adalimumab. It was also the only study to describe follow-up time. Unfortunately, this study had a low statistical power, probably because the estimated number of infections was less than expected. In the Japanese studies, anti-TNF drugs were discontinued in the perioperative period, not allowing the observation of outcomes in the presence of full serum levels of the analyzed drugs.

On the other hand, none of the studies properly assessed disease activity. Discontinuation of immunomodulatory treatment may allow the reactivation of joint inflammatory activity in the perioperative period, what can lead to difficulties in the rehabilitation process.

In conclusion, it is not clear, according to the body of evidence currently available, whether patients with RA using anti-TNF medications are at increased risk of surgical site infections, compared to patients receiving conventional DMARD. Multicenter randomized controlled trials or new prospective high quality observational studies are needed to make it possible to reach a firm conclusion, including patients from distinct ethnicities, with sufficiently numerous population and assessing adalimumab use. For now, we recommend that discontinuation of anti-TNF drugs should occur after a case-based discussion between clinicians and surgeons, considering the risks and benefits, taking into account patient characteristics, the intended procedure and the institution where the surgery will be performed.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Biological Therapy in Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is a prototypic inflammatory autoimmune disorder characterized by multisystem involvement and fluctuating disease activity. Symptoms range from rather mild manifestations such as rash or arthritis to life-threatening end-organ manifestations. Despite new and improved therapy having positively impacted the prognosis of SLE, a subgroup of patients do not respond to conventional therapy. Moreover, the risk of fatal outcomes and the damaging side effects of immunosuppressive therapies in SLE call for an improvement in the current therapeutic management. New therapeutic approaches are focused on B-cell targets, T-cell downregulation and costimulatory blockade, cytokine inhibition, and the modulation of complement. Several biological agents have been developed, but this encouraging news is associated with several disappointments in trials and provide a timely moment to reflect on biologic therapy in SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune, multisystemic, relapsing, and remitting disease that is characterized by the production of antibodies against nuclear antigens. The pathogenesis includes genetic, environmental, and hormonal factors, but the cause of SLE remains unclear. A broad array of clinical manifestations ranging from mucocutaneous and arthritis to severe organ- and life-threatening disease are observed in SLE patients [1, 2].

The current treatment options include the use of corticosteroids, hydroxychloroquine, and other immunosuppressive medications (e.g., azathioprine, mycophenolate, and cyclophosphamide) [2]. More recently, belimumab was approved by the FDA for SLE treatment [3].

Due to earlier diagnosis and better treatment options of both disease and complications, the prognosis has markedly improved in the last decades. The 5-year survival of patients with SLE has exceeded 90% in most centers [4, 5]. However, morbidity, especially renal failure, and mortality from cardiovascular events after long-term followup are still an important issue [5].

In the last decade new treatment strategies have been developed. Advanced knowledge of the pathogenesis of SLE has led to new therapeutic approaches targeting specific molecules [4]. Beside autoantibody production, B-cells are the key for the activation of the immune system, particularly through cytokines and as antigen-presenting cells. An important part of B-cells is activated in a T-cell-dependant manner.

This paper will review the rational of biologic therapies in SLE and discuss potential therapeutic options.

2. B-Cell Targets

B cells have been largely implicated in the pathogenesis of SLE as sources of autoantibody, as antigen-presenting cells, and as initiators and regulators of inflammation through cytokine secretion [6–8]. B-cell-targeted therapies, including anti-CD20 monoclonal antibody (Rituximab) and anti-B lymphocyte stimulator (BLyS), are at forefront of new SLE therapies [8, 9] (Table 1).

2.1. Anti-CD20 Antibody. The first B-cell depleting antibody used in SLE was rituximab, a chimeric murine/human monoclonal antibody against CD20 (Figure 1). CD20 is expressed early in the development of B lymphocytes. Rituximab administration results in rapid depletion of CD20-positive B lymphocytes [7, 38, 39]. After rituximab treatment some patients reconstitute with naive B cells and enter remission.

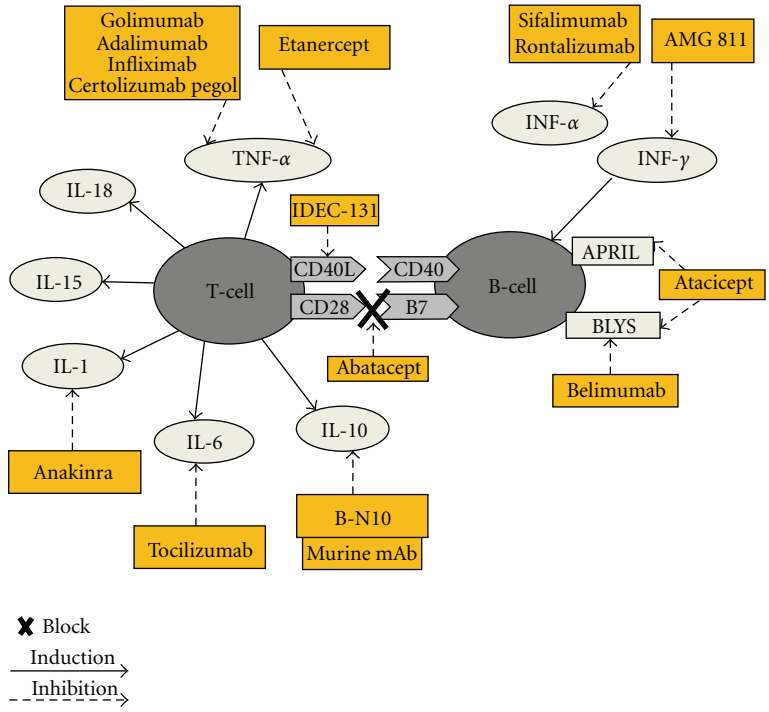

Figure 1: Potential targets and relevant drugs in connection with B and T cells in the management of SLE.

Others, however, do not deplete B cells completely and they reconstitute with memory B cells and might therefore benefit from rituximab retreatment [10]. Two recent open-label studies confirmed that repeated cycles of rituximab are effective in treating refractory SLE, may produce a sustained clinical response and have a favorable safety profile [10, 11].

Rituximab has been used in open trials and improvements in disease activity has been observed [12, 13]. In addition it has been shown to be safe and well tolerated [13, 40, 41]. Two large multicenter randomized placebo-controlled trials with rituximab in moderately to severely active SLE (EXPLORER) [14] and in proliferative lupus nephritis patients (LUNAR) [15] could not demonstrate a significant benefit of rituximab when compared to placebo. The inclusion of milder forms of SLE, the ethnic background of patients, the concomitant use of steroids and other immunosuppressive drugs, and the short followup (52 weeks) could explain in part why no benefit could be demonstrated for rituximab in these studies [14, 15, 42]. Despite the lack of evidence in randomized trials, rituximab has been used in refractory patients and improvement in up to 89% of the patients has been observed [43–47].

Adverse events associated with the use of rituximab are most often mild, but infusion reactions (30–35%), neutropenia (8%), and human antichimeric antibodies (9%) production have been observed [10]. In addition, two cases of fatal progressive multifocal leukoencephalopathy (lethal encephalitis caused by the polyomavirus JC) in SLE patients after rituximab treatment have been reported [11].

New monoclonal anti-CD20 antibodies have been developed. Ocrelizumab, a recombinant humanized monoclonal anti-CD20 antibody has been studied in Phase III trials in extrarenal SLE (BEGIN study) [16] and lupus nephritis (BELONG study) [17]. However, treatment with ocrelizumab has been suspended in SLE trials, following the negative outcome of a similar study design with the anti-CD20 antibody and also due to an increase in the treatment group [16, 17, 48].

2.2. Anti-CD22 Antibodies. Epratuzumab is a fully humanized antibody against CD22. CD22 is 128a 135-kD B-lymphocyte restricted type I transmembrane sialoglycoprotein of the Ig superfamily and modulates B-cell function without B-cell depletion [9, 49]. Epratuzumab was evaluated in randomized controlled trials in patients with moderate-to-severe SLE flares [18]. An improvement in BILAG scores and reduction in corticosteroid doses with a good safety profile was observed; however the trial was interrupted due to problems in the biologic supply [18]. Two studies are currently evaluating the efficacy of epratuzumab in a subset of serologically active SLE, and results have yet not been presented [19, 20].

2.3. B-Lymphocyte Tolerogens. Abetimus (LJP-394) is a B-cell tolerogen. It consists of four double-stranded DNA (dsDNA) epitopes on a polyethylene glycol platform [50]. It cross-links anti-dsDNA surface immunoglobulin receptors on B-cells, leading to anergy or apoptosis. It also reduces titers of anti-dsDNA antibodies [21]. Abetimus was the first B-cell tolerogen developed for SLE and was studied in human trials for the treatment of nonrenal lupus and lupus nephritis [21]. Initial trials suggested a reduction in renal flares in patients who have high-affinity antibodies to the DNA epitope contained within the abetimus molecule [4, 21]. After an analysis

Table 1: Biological therapies proposed for SLE treatment.

Biologic drug	Main results
B-cell targets	
Anti-CD20 antibody	
Rituximab	Effective in treating refractory SLE [10, 11] Improvements in disease activity [12, 13] No benefit in proliferative lupus nephritis [14, 15]
Ocrelizumab	No benefit in lupus nephritis [16, 17]
Anti-CD22 antibody	
Epratuzumab	Improvement in BILAG scores [18] Reduction in corticosteroid doses with a good safety profile [19, 20]
B-lymphocyte tolerogens	
Abetimus	No long-term benefit in patients with lupus nephritis [21]
Edratide	No results released [22]
BLyS blockers	
Belimumab	Reduction in activity and new flares [23]
Atacicept	Significant decrease in IgM and IgG levels [24]
T-cell target and costimulatory blockers	
Abatacept	Improvements in non-life- threatening SLE manifestations [25, 26]
IDEC-131	No clinically effective in human SLE [27]
Efalizumab	Reduction in cutaneous SLE manifestations [28]
AMG557	No results released [29]
Sirolimus	Safe and effective for refractory SLE [30]
Cytokine inhibition	
Anti-TNF-α	
Infliximab	Long-term efficacy for lupus nephritis [31]
Anti-IFN-α/-γ	
Sifalimumab	No results released [32]
Rontalizumab	No results released [33]
AMG 811	No results released [34]
Anti-IL-1	
Anakinra	Improvements in SLE arthritis [35]
Anti-IL-6	
Tocilizumab	Improvements in clinical and serologic responses [36]
Anti-IL-10	
B-N10[a]	Improvements in disease activity [37]

[a] Murine Lupus; BILAG: The British Isles Lupus Assessment Group; BLyS: B cell survival molecule B lymphocyte stimulator; Ig: immunoglobulin; TNF: tumor necrosis factor; INF: interferon; IL:interleukin.

of a phase III Abetimus Sodium in patients with a history of lupus nephritis (ASPEN) trial, the trial was terminated when interim efficacy analysis indicated no benefit to continue [51].

Another tolerogen, TV-4710 (Edratide) a peptide composed of 19 amino acids based on the complementarily determining regions (CDR1) of a human anti-dsDNA antibody, was tested in a phase II trial [22]. This study has been concluded but there are yet no results released [22].

2.4. BLyS Blockers. The B-cell survival molecule B-lymphocyte stimulator (BLyS) also known as B-cell activation factor of the TNF family (BAFF) plays a key role in the activation and differentiation of B cells [4]. BLyS represents, therefore, an excellent target for interventions in SLE. High serum levels of soluble BLyS, and its homolog APRIL (a proliferation inducing ligand), are found in SLE patients and in murine lupus. Selective blockade of BLyS reduces transitional type 2 follicular and marginal-zone B cells and significantly attenuates immune activation [4, 9].

Belimumab is a fully human monoclonal antibody that binds to BLyS and inhibits its biological activity (Figure 1). Efficacy, tolerability, and safety of three different doses of belimumab in SLE were evaluated in a multicenter phase II study [23]. After 52 weeks of analysis, belimumab was associated with a reduction in activity and new flares. Two phase III trials (BLISS-52 and BLISS-76) showed that belimumab plus standard care achieved a significant improvement in patient response rate and increased time to-first-flare compared with placebo plus standard care [23, 52]. Based on these results, FDA recently approved Belimumab for the treatment of SLE [3].

An alternative blocker to BlyS is atacicept (also known as TACI-Ig). It is a soluble transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) receptor, which binds both BAFF and APRIL (Figure 1). In a phase I trial in SLE patients, atacicept was well tolerated [24].

Atacicept is of interest in SLE because of its profound effects on plasma cells, but its use leads to significant decrease in IgM and IgG immunoglobulin levels [53, 54]. A phase II study of atacicept plus mycophenolate in SLE nephritis was terminated because of an increased number of infections [53]. The increased number of infection could be explained by the fact that plasma cells require APRIL and so serum Ig was reduced. A phase II/III trial of atacicept for generalized SLE (April SLE) is still ongoing [55].

3. T-Cell Target and Costimulatory Blockers

Costimulatory molecules provide the necessary second signal for T-cell activation by antigen-presenting cells. The inhibition of this mechanism has been demonstrated to be effective in murine lupus models [56, 57]. The most important antigen-independent signal for T-cell activation is the CD28:B7 costimulatory interaction [4]. CD28 is expressed on T cells, whereas the ligands B7-1 and B7-2 (CD80 and CD86) are found on antigen-presenting cells [4]. CTLA4 inhibits T-cell activation by binding to B7-1 and B7-2 (CD80 and CD86) expressed on antigen-presenting cells. Therefore CTLA4 interacts with B7 but inhibits T-cell activation, by preventing the costimulatory signal CD28-B7 interaction necessary for T-cell activation [4] (Figure 1).

Abatacept is a soluble receptor or fusion protein encoded by fusion of CTLA-4 with the Fc portion of IgG1. Abatacept blocks CD28-B7 interaction and subsequent T-cell-dependent B-cell function [58, 59] (Figure 1). In murine model, abatacept prevents initiation but not evolution of antiphospholipid syndrome in NZW/BXSB mice [59]. In SLE patients, abatacept has been tested in phase I to III trials [25, 26].

CD40-CD40 ligand (CD40L) is another important costimulatory pair that induces T-cell-dependent B-cell proliferation and antibody production. CD40 is expressed on B cells, endothelial cells, and antigen-presenting cells and binds to CD40L (or CD154) on CD4+ T helper cells [4] (Figure 1). In lupusprone mice with nephritis treated with anti-CD40L antibodies reduction in anti-dsDNA antibody, milder renal disease and increased survival was observed [60]. Unfortunately, anti-CD40L monoclonal antibody (mAb) (IDEC-131) did not prove to be clinically effective in human SLE compared with placebo [27]. Another study (BG9588) was terminated prematurely after a few patients demonstrated life-threatening prothrombotic events despite improvement in serologic activity [61].

Efalizumab is a monoclonal antibody directed against CD11a, the alpha-subunit of the leukocyte-functioning antigen-1. It plays an important role in T-cell activation, re-activation, extravasation, and trafficking from the circulation into the skin, through its binding to intercellular adhesion molecules (Figure 1). Efalizumab seems to reduce cutaneous manifestations in SLE patients [28]. The majority of patients with difficult lupus discoid had an important response to treatment with the mean time to response being 5.5 week [28]. However, this study evaluated only a small number of patients. There is a need for more prospective studies with long-term followup to better define the efficacy and safety of efalizumab in SLE.

The inducible costimulator (ICOS) is a T-cell-specific molecule structurally and functionally related to CD28. ICOS regulates T-cell activation and T-helper cell differentiation and is mainly involved in humoral immune responses and, thus, autoantibody production. A fully humanized anti-B7RP1 antibody (AMG557) is currently being investigated and may represent a further target for SLE therapy [29].

Mammalian target of rapamycin (mTOR) has multiple regulatory functions in T- and B-cell intracellular signaling [62]. It controls the expression of T-cell receptor-associated signaling proteins through increased expression of the endosome recycling regulator genes and enhances intracellular calcium flux [63]. Rapamycin (Sirolimus) interacts with mTOR by influencing gene transcription and multiple cellular metabolic pathways. This interaction has been proven to be beneficial in murine lupus [64]. Rapamycin appeared to be a safe and effective therapy for refractory SLE in a small pilot study [30].

4. Cytokine Inhibition

As cytokine dysregulation can be demonstrated in murine and in SLE patients, an anticytokine approach seems promising in this autoimmune disease [65]. Cytokines such as tumor necrosis factor alpha (TNF-α), interferon alpha and gamma (IFN-α/-γ) and interleukins (IL) 1, 6, 10, 15, and 18 are upregulated in SLE and play important roles in the inflammatory processes that leads to tissue and organ damage [65]. These cytokines have been considered potential targets for the reduction of chronic inflammation in SLE (Figure 1).

4.1. Anti-TNF-α. TNF-α is a pleiotropic cytokine that exerts several functions in the immune system and can either promote or reduce autoimmunity. In SLE, its role is controversial. TNF-α promotes apoptosis and significantly affects the activity of B and T cells and dendritic cells (DCs). In different strains of lupus mice, the expression of TNF-α is often variable, and beneficial effects on the disease can be observed either after administration of TNF-α or upon TNF-α blockade [31, 66–68]. TNF-α blockers are associated with the development of autoantibodies, such as antinuclear, anti-dsDNA, and anticardiolipin, as well as with rare cases of drug-induced lupuslike syndromes, all of which disappear after therapy is discontinued [65].

There are several TNF-α inhibitors available for clinical use such as infliximab, adalimumab, golimumab, and certolizumab pegol and a fusion protein that acts as a "decoy receptor" for TNF-α (etanercept) [31, 69] (Figure 1). TNF-α inhibitors are usually well tolerated; however their use may increase the overall risk of opportunistic infections, in particular the reactivation of latent tuberculosis [70, 71]. The appearance of neutralizing antibodies has been described in patients treated with infliximab, which is a chimeric human/mouse mAb, as well as in those treated with adalimumab, in spite of its fully human sequence [71]. The concomitant use of an immunosuppressive drug like methotrexate has been shown to prevent the development of neutralizing antibodies [72].

4.2. Anti-IFN-α/-γ. IFN-α plays a significant role in the pathogenesis of SLE. IFN-γ is elevated in (New Zealand Black [NZB] $\times$ New Zealand White [NZW]) F1 (NZB/W) lupus mice, and a correlation with disease activity has been observed [73, 74]. In addition, administration of IFN-γ accelerates murine lupus, while anti-IFN-γ antibody (or soluble IFN-γ receptor or IFN-γ receptor-immunoglobin) delays the disease [75–77]. Finally, it has been demonstrated that late treatment with IFN-γ in MRL/lpr mice accelerates SLE, while early treatment protects disease progression [78]. IFN-α levels are increased in SLE patients and correlate with disease activity and kidney involvement [79]. In addition an increased expression of interferon-regulated inflammatory genes in the peripheral blood mononuclear cells of the SLE patients (known as "interferon signature") has been observed [80, 81].

Sifalimumab (MEDI-545) is a monoclonal human antibody that blocks multiple IFN-α subtypes. It is currently being tested in phase I/II clinical trials to evaluate safety and tolerability of multiple intravenous and subcutaneous doses in SLE [32] (Figure 1).

Rontalizumab, a humanized mAb against IFN-α (rhuMAb IFN-α) is in a phase II, randomized, double-blind,

placebo-controlled trial that evaluates the efficacy and safety in patients with moderately to severely active SLE [33] (Figure 1).

AMG 811, a human mAb to IFN-γ, is under investigation in a phase Ib, randomized, multicenter study in SLE patients with and without glomerulonephritis [34].

4.3. Anti-IL-1. IL-1 levels are increased by serum TNF levels and by anti-dsDNA antibody. The increase in serum IL-1 level is associated with lupus disease activity and a low level of IL-1 receptor antagonist is seen in patients with lupus nephritis [82, 83]. Anakinra, a nonglycolysated version of the human IL-1Ra (IL-1 receptor antagonist), neutralizes the biological activity of IL-1 (Figure 1). It has been used as an alternative in individual patients with lupus arthritis not responding to conventional treatments [35]. Anakinra has shown both safety and efficacy in improving arthritis in an open trial on four SLE patients, however short-lasting therapeutic effects were observed in two patients [35].

4.4. Anti-IL-6. IL-6 induces B-cell differentiation to plasma cells, hyperactivity, and secretion of antibodies and also promotes T-cell proliferation, cytotoxic T-cell differentiation, and local inflammation [65]. IL-6 is highly expressed in patients with lupus nephritis. IL-6 is induced in DCs by nucleic acid containing immune complexes as well as by multiple cytokines, including TNF, IL-1, and IFN-γ. In NZB/W mice IL-6 promotes disease, and anti-IL-6 therapy delays lupus nephritis, suggesting that IL-6 blockade might also be beneficial in SLE patients [84].

Tocilizumab is a humanized IgG1 antibody directed to human IL-6 receptor that inhibits IL-6 signaling [85] (Figure 1). An open-label, dose escalating phase I study of tocilizumab in SLE patients has recently been published [36]. Although neutropenia may limit the maximum dosage of tocilizumab in SLE patients, the observed clinical and serologic responses are promising and warrant further studies to establish the optimal dosing regimen and efficacy [36].

4.5. Anti-IL-10. IL-10 is produced by Th2 cells and considered an inhibitory cytokine for T cells and contrasts the activity of other proinflammatory cytokines such as TNF-α and IFN-γ. In SLE patients, IL-10 levels are increased in sera and are associated with disease activity [50]. NZB/W mice treated with anti-IL-10 mAb have reduced anti-dsDNA antibody titers and a delay in the onset of proteinuria and glomerulonephritis [86].

In the absence of a humanized mAb to IL-10, the murine anti-IL-10 mAb (B-N10) was used to inhibit the activity of IL-10 in a small uncontrolled, open-label study in SLE patients with relatively mild disease [37] (Figure 1). Disease activity improved and inactivity was observed in SLE patients up to 6 months after treatment. However, all patients developed antibodies against the murine mAb [37].

4.6. Anti-IL-15. IL-15 is mainly produced by the macrophage/monocyte cell line [87]. High serum levels of IL-15 are found in 40% of SLE patients; however its levels are not directly associated with disease activity [87]. IL-15 might be responsible for some immune abnormalities of the disease, such as stimulating lymphocytic expression of B-cell lymphoma 2 (Bcl-2) and CD25 (in both B and T-cells) [87]. Therapeutic agents against IL-15 are currently being tested in other autoimmune diseases.

4.7. Anti-IL-18. IL-18 is a proinflammatory cytokine closely related to IL-1. Several groups have observed increased serum levels of IL-18 in SLE patients, which appear to be associated with TNF levels [88–90]. IL-18 is overexpressed in the nephritic kidneys of MRL/lpr mice. Moreover, MRL/lpr mice benefit from targeting IL-18 [91]. Until now, IL-18 blockade has not been used in human SLE (Figure 1).

5. Complement Inhibition

The complement system consists of 3 pathways and more than 30 proteins, including those with biological activity that directly or indirectly mediate complement effects, plus a set of regulatory proteins necessary to prevent inadvisable complement activation [9]. The complement system appears to have a protective effect in SLE, since homozygous deficiencies of classic pathway components are associated with an increased risk for SLE. The deposition of immune complexes, however, observed in human and animal models, leads to an activation of the complement system, amplifying the inflammatory response. Pathologic evidence of immune complex-mediated activation of complement in affected tissues is clearly evident in both experimental and human SLE [92].

Two complement inhibitors, soluble complement receptor 1 (TP10) and a monoclonal anti-C5 antibody (Eculizumab) have been shown to inhibit complement safely and now are being investigated in a variety of clinical conditions [9]. Eculizumab has shown to reduce hemolysis and has been approved by the FDA in paroxysmal nocturnal hemoglobinuria [93]. Although still no clinical trial has been performed in SLE, they hold promise to be used therapeutically in SLE [93].

6. Conclusion

In recent years advances in our understanding of the mechanisms of SLE has offered better drug targets for treatment. Over the next years, we will test the efficacy of many new therapeutic agents. The knowledge on how to divide patients into subsets according to genetic susceptibility, pathogenetic mechanisms, and phases of the disease will maximize the therapeutic effect of each agent and minimize its toxicity.

Abbreviations

TNF-α: Tumor necrosis factor alpha
IFN-α/γ: Interferon alpha and interferon gamma
IL: Interleukin
mAb: Monocloral antibodies
BLyS: B lymphocyte stimulator
APRIL: Proliferation inducing ligand
CTLA-4: Cytotoxic T lymphocyte-associated antigen 4.

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Review Article

Tocilizumab for the Treatment of Rheumatoid Arthritis and Other Systemic Autoimmune Diseases: Current Perspectives and Future Directions

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Interleukin (IL)-6 is a cytokine featuring redundancy and pleiotropic activity. While IL-6, when transiently produced, contributes to host defense against acute environmental stress, continuous dysregulated IL-6 production plays a significant pathological role in several systemic autoimmune diseases. In response to the expectation that IL-6 blockade would constitute a novel therapeutic strategy for the treatment of these diseases, tocilizumab, a humanized anti-IL-6 receptor antibody, was developed. Clinical trials have verified the efficacy and the safety of tocilizumab for patients with rheumatoid arthritis, resulting in approval of this innovative biologic for the treatment of rheumatoid arthritis in more than 90 countries worldwide. Pathological analyses of the effect of IL-6 on the development of autoimmune diseases and a considerable number of case reports and pilot studies have also indicated the beneficial effects of this antibody on other systemic autoimmune diseases, including systemic lupus erythematosus, systemic sclerosis, polymyositis, and large-vessel vasculitis.

1. Introduction

Interleukin (IL)-6 is a cytokine featuring redundancy and pleiotropic activity. It was successfully cloned in 1996 as a B-cell differentiation factor, which promotes B-cell differentiation into antibody-producing cells [1]. Subsequent in vitro studies and analysis of IL-6 transgenic mice have shown that IL-6 acts not only on B cells but also on T cells, hepatocytes, hematopoietic progenitor cells, and various other cells [2–4]. One of the important functions of IL-6 is the differentiation of $CD4^{positive}$ naïve T cells into effector cells. IL-6 in the presence of TGF-β promotes naïve T-cell differentiation into Th17 cells, while IL-6 inhibits TGF-β-induced regulatory T-cell (Treg) differentiation [5], causing imbalance between Th17 and Treg, which is a primary pathogenic factor in several autoimmune diseases [6].

IL-6 transmits its signal through its binding to transmembrane receptors or the soluble IL-6 receptor (IL-6R) [7, 8]. After binding of IL-6 to IL-6R, the resultant IL-6/IL-6R complex associates with gp130 and induces homodimerization of gp130, which triggers signal transduction system [9].

The pathological significance of IL-6 for diseases was first demonstrated in a case of cardiac myxoma [10]. The culture fluid obtained from the myxoma tissues of a patient who presented with fever, arthritis with positivity for antinuclear factor, increased C-reactive protein (CRP) levels and hypergammaglobulinemia and was diagnosed with undifferentiated connective tissue disease, contained a large quantity of IL-6, which suggested that IL-6 might contribute pathologically to chronic inflammation and autoimmunity. Subsequent studies have shown that dysregulation of IL-6 production is implicated in the pathogenesis of Castleman's disease [11], rheumatoid arthritis (RA) [12], and various other autoimmune, inflammatory, and malignant diseases [2–4].

Because of the biological activities of IL-6 and its pathological role in diseases, it was anticipated that IL-6 blockage would constitute a novel treatment strategy for autoimmune and inflammatory diseases [4, 13–15]. To this

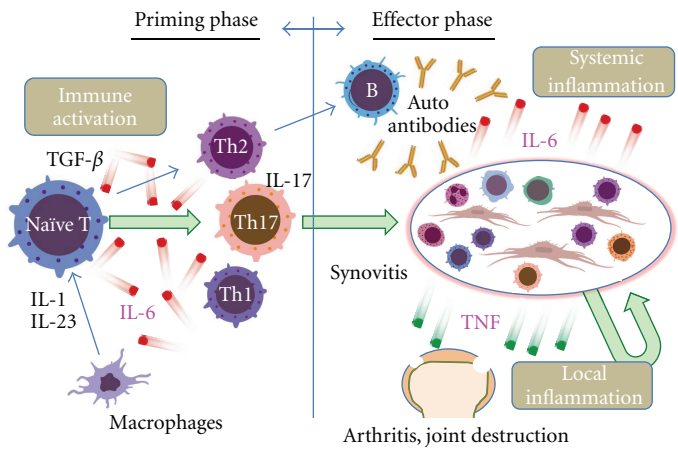

Figure 1: Pathological role of IL-6 in rheumatoid arthritis. IL-6 is important for development of Th17 and induction of autoantibodies such as rheumatoid factor. Activated Th17 cells and autoantibodies generate pannus in combination with activated fibroblastic synoviocytes, macrophages, and lymphocytes. Inflamed synovitis such as pannus is a major source of inflammatory cytokines including IL-6, and systemic inflammation (resulting in production of acute phase protein, anemia, and fatigue) is mainly mediated by IL-6. Tumor necrosis factor (TNF) plays a major role in the progression of local types of inflammation (arthritis) such as arthralgia, swelling, and joint destruction but plays a minor role during the priming phase.

end, tocilizumab was developed, which is a humanized anti-IL-6R monoclonal antibody (Ab) of the IgG1 class that was generated by grafting the complementarity determining regions of a mouse anti-human IL-6R Ab onto human IgG1. Tocilizumab blocks IL-6-mediated signal transduction by inhibiting IL-6 binding to transmembrane and soluble IL-6R.

2. Approval of Tocilizumab for the Treatment of Rheumatoid Arthritis

2.1. Pathological Role of IL-6 in Rheumatoid Arthritis. RA is a chronic, progressive inflammatory disease of the joints and surrounding tissues accompanied by intense pain, irreversible joint destruction, and systemic complications such as fatigue, anemia, and fever [16]. At the local level, inflammatory cells invade the otherwise relatively acellular synovium leading to neovascularization, synoviocyte hyperplasia, and formation of pannus tissue, which in turn causes destruction of cartilage, erosion of the adjacent bone, and, ultimately, loss of function of the affected joint. The biological activities of IL-6 such as proinflammatory activity, augmentation of synovial fibroblast proliferation, osteoclast differentiation, matrix metalloproteinase (MMP), and vascular endothelial growth factor (VEGF) production, as well as lymphocyte differentiation and its elevation in both serum and synovial fluids of patients with RA [17–22] indicate that IL-6 is one of the key cytokines involved in the development of RA.

It has been demonstrated in animal model of RA, that are type II collagen-induced arthritis (CIA), and antigen-induced arthritis, IL-6 performs a major role in the development and progression of joint destruction, while IL-6 deficiency generated by gene knockout or IL-6 blockade by means of anti-IL-6R Ab reduces the incidence and severity of arthritis in these models [23–28]. In the CIA model, immunization with type II collagen predominantly increased the frequency of Th17 cells and treatment of mice with anti-IL-6R Ab during priming markedly suppressed the induction of Th17 cells and arthritis development, while treatment with anti-IL-6R Ab on day 14 failed to suppress both Th17 differentiation and arthritis [29]. Similarly, in a glucose-6-phosphate-isomerase- (GPI-)induced arthritis model, administration of anti-IL-6R Ab on day 0 or 3 suppressed Th17 differentiation and protected against arthritis induction, while injection of anti-IL-6R Ab on day 14, at the peak of arthritis, did not bring about any improvement in arthritis [30]. Arthritis of anti-type II collagen antibody-induced arthritis (CAIA) is another arthritis model, but, in this model, the priming phase of T cell dependent antibody generation is skipped. Although IL-6 is also elevated in this model, CAIA was profoundly suppressed in $TNF^{-/-}$ mice but not in $IL\text{-}6^{-/-}$ mice [31], indicating that TNF may play a more significant role in the development of CAIA than IL-6. These observations suggest that in the priming phase IL-6 is a required factor for the activation of T cell response and production of antibodies specific for joint components and that in the effector phase TNF is the main generator of arthritis [32]. We found that tocilizumab was not effective for clinical improvement in the condition of two patients with psoriatic arthritis, for whose development immune activation does not appear to be required [33]. The clinical antiarthritic effect of tocilizumab is slower than that of TNF inhibitors, which may be due to the different pathological roles of IL-6 and TNF in the development of RA (Figure 1).

2.2. Efficacy of Tocilizumab in Randomized Controlled Trials. As shown in Table 1, seven phase III clinical trials of tocilizumab subsequent to phase I and II studies demonstrated its efficacy either as monotherapy or in combination with disease-modifying antirheumatic drugs (DMARDs) for adult

Table 1: Phase III randomized controlled trials of tocilizumab for RA patients. Summary of the results of seven phase III randomized controlled trials of tocilizumab. DMARDs: disease modifying antirheumatic drugs, IR: inadequate response, TCZ: tocilizumab, anti-TNF: anti-tumor necrosis factor inhibitor, MTX: methotrexate.

Study	Reported year	Population	Week at evaluation	Treatment arms	Patient (n)	Response rates (%)			Remission rate (%)	Radiological progression		
						ACR20	ACR50	ACR70	DAS28< 2.6	TSS: Total Sharp score	ES: Erosion score	JSNS: Joint space narrowing score
SAMURAI	2007	DMARDs IR	52 W	TCZ (8)	157	78	64	44	59	2.3	0.9	1.5
				DMARDs	145	34	13	6	3	6.1	3.2	2.9
TOWARD	2008	DMARDs IR	24 W	TCZ (8) + DMARDs	803	61	38	21	30			
				DMARDs	413	25	9	3	3			
RADIATE	2008	Anti-TNF IR	24 W	TCZ (4) + MTX	161	30	17	5	8			
				TCZ (8) + MTX	170	50	29	12	30			
				placebo + MTX	158	10	4	1	2			
OPTION	2008	MTX IR	24 W	TCZ (4) + MTX	186	48	31	12	13			
				TCZ (8) + MTX	191	59	44	22	27			
				placebo + MTX	189	26	11	2	1			
SATORI	2009	MTX IR	24 W	TCZ (8)	61	80	49	30	43			
				MTX	64	25	11	6	2			
AMBITION	2010	MTX, anti-TNF naïve	24 W	TCZ (8)	286	70	44	28	34			
				MTX	284	53	34	15	12			
LITHE	2011	MTX IR	52 W	TCZ (4) + MTX	394	47	29	16	30	0.34	0.21	0.13
				TCZ (8) + MTX	398	56	30	20	47	0.29	0.17	0.12
				MTX	393	25	10	4	8	1.13	0.71	0.42

Table 2: Reevaluation of antirheumatic effects of tocilizumab in actual medical practice. Summary of the contents of the three actual medical practice of tocilizumab for rheumatoid arthritis.

Study	Country	Patient number	Registry	Evaluation
TAMARA	Germany	286	Sep. 2008~Sep. 2009	Disease activity EULAR response ACR response Adverse events 2011 ACR/EULAR remission
DAMBIO	Denmark	178	~April 2010	Disease activity EULAR response Drug survival
REACTION	Japan	229	April 2008~March 2009	Disease activity EULAR response Adverse events Drug survival

patients with moderate to severe RA [34–40]. A Cochrane database systematic review concluded that tocilizumab-treated patients taking concomitant methotrexate were four times more likely to achieve American College of Rheumatology (ACR) 50 improvement (absolute %, 38.8% versus 9.6%) and 11 times more likely to achieve Disease Activity Score (DAS) remission (30.5% versus 2.7%) than patients taking a placebo [41]. Furthermore, the SAMURAI [34] and LITHE studies [40] proved that radiological damage of joints was significantly inhibited by the treatment. The findings of the RADIATE trial showed that, among RA patients who had previously discontinued TNF inhibitors 50% achieved ACR20, 28.8% ACR50, and 12.4% ACR70 responses [36]. The ACR improvement and DAS remission criteria include an acute-phase reactant component, so that there was concern that the effect of tocilizumab evaluated with these criteria might be overestimated. However, it was found that, even when criteria such as the Simplified Disease Activity Index (SDAI) and Clinical Disease Activity Index (CDAI) were used, remission rates for patients treated with tocilizumab were in the same range as those for patients treated with TNF inhibitors [42, 43].

2.3. Efficacy of Tocilizumab in Actual Medical Practice. On the basis of the excellent results obtained for the efficacy of tocilizumab, it was approved in April 2008 for the treatment of RA in Japan. The recommended posology of tocilizumab (proprietary name, Actemra) is 8 mg/kg, every 4 weeks. Subsequently, the European Medicines Agency approved tocilizumab (proprietary name, RoACTEMRA) for RA in January 2009 at a recommended dose of 8 mg/kg. In the United States, it was approved for RA in January 2010, but the recommended starting dose is 4 mg/kg administered once every 4 weeks followed by an increase to 8 mg/kg depending on clinical response. While the dosage differs among countries, tocilizumab has now been approved for the treatment of RA in more than 90 countries worldwide [14].

In addition to clinical trials, the efficacy of tocilizumab was reconfirmed in actual medical practice. The finding by the three recent studies, the German phase IIIb real-life study (TAMARA study) [44, 45], the Danish nationwide cohorts of RA patients (DANBIO registry) study [46], and the multicenter retrospective real-life study (REACTION study) [47, 48] are shown in Table 2. In the TAMARA study, 286 patients were registered for an analysis of the effectiveness and safety [44, 45], 41.6% of whom had previously been treated with TNF inhibitors. ACR50 and ACR70 response rates at week 24 were 50.7% and 33.9%, respectively, while 47.6% of the patients achieved DAS remission and 54.9% the European League Against Rheumatism (EULAR) good response. Remission rates with the new ACR/EULAR Boolean-based criteria for clinical studies were 15.0% after 12 weeks and 20.3% after 24 weeks, and CDAI and SDAI remission rates were 24.1% and 25.2%, respectively. For the DANBIO registry in Denmark, 178 patients with RA treated with tocilizumab were identified [46]. The disease activity decreased at all-time points, with remission rates for tocilizumab treatment of 39% after 24 weeks and 58% after 48 weeks. EULAR good or moderate response rates were 88% and 84%, respectively. These response rates were comparable to those found for patients switching to their second TNF inhibitors and to the response rates previously observed in phase III clinical trials. In Japan, 229 patients were registered in the REACTION study for an analysis of the effectiveness of tocilizumab [47, 48]. Clinical remission at week 52 was observed in 43.7% of the patients, radiographic non-progression in 62.8%, and functional remission in 26.4%. The retention rates at 24 and 52 weeks were 79.5% and 71.1%, respectively, and were the same for those with or without previous anti-TNF treatment. These results indeed show the efficacy of tocilizumab for the treatment of RA in actual medical practice.

2.4. Safety Profile of Tocilizumab. The safety and tolerability profiles of tocilizumab monotherapy for Japanese RA patients obtained from six initial trials and five long-term extensions have been published [49]. For these studies, 601 patients with a total exposure to tocilizumab of 2,188 patient-years (pt-yr) were enrolled. The medial treatment duration was 3.8 years. The incidence of adverse events

(AEs), including abnormal laboratory test findings, was calculated as 465/100 pt-yr, with infections being the most common serious AEs (6.2/100 pt-yr). Of the patients treated more than 5 years, 59.7% met the DAS28 remission criteria at 5 years, which demonstrates the excellent tolerability and high efficacy of tocilizumab. In addition, a systemic literature review to assess the risk of AEs for RA patients treated with tocilizumab reported that pooled odds ratios (ORs) indicated statistical significance for an increased risk of AEs for patients treated with 8 mg/kg of tocilizumab plus methotrexate compared with controls (OR = 1.53; 95%CI = 1.26–1.86), as well as a heightened risk of infection (OR = 1.30; 95%CI = 1.07–1.58) [50]. However, no increases in the incidence of malignancy or hepatitis were detected.

The results of an interim analysis of a postmarketing surveillance of all patients treated with tocilizumab in Japan were recently reported [51]. This analysis comprised 3,881 patients who received 8 mg/kg of tocilizumab every 4 weeks, and was observed for 28 weeks. Occurrence of a total of 3,004 AEs in 1,641 patients (167/100 pt-yr) and 490 serious AEs in 361 patients (27/100 pt-yr) was reported. The most frequent AE and serious AE were infection at 31/100 pt-yr and 9/100 pt-yr, respectively, with the majority of infections being pneumonia and cellulitis. Cardiovascular events were observed in 0.9% (myocardial infarction in 4 patients or 0.1%). Abnormalities in laboratory test findings, such as increases in lipid and liver function parameters were common, and total and serious AEs associated with laboratory test abnormalities were 35/100 pt-yr and 2/100 pt-yr, respectively. The increased lipid level resulting from tocilizumab administration is perhaps mediated by its effecting on lipoprotein receptor expression, since it was recently shown that overproduction of IL-6 reduces blood lipid levels via upregulation of very-low-density lipoprotein receptors [52]. In contrast, we and others observed that HbA1c levels and insulin sensitivity improved as a result of tocilizumab treatment [53, 54]. While white blood cell and neutrophil counts usually decreased just after tocilizumab injection, this was not related to the incidence of infection. Twenty-five patients died for a standardized mortality ratio of 1.66, which was similar to the results reported for a Japanese cohort study of RA. The results of this analysis thus demonstrated that tocilizumab is acceptable in the actual clinical setting.

Seven cases of gastrointestinal (GI) perforation in six patients were reported in this postmarketing surveillance. In the worldwide Roche clinical trials, 26 (0.65%) cases of GI perforation were found among patients with RA treated with tocilizumab for a rate of 1.9/1,000 pt-yr and most cases appeared to be complications of diverticulitis [55]. This rate is intermediate between the rates of GI perforations of 3.9/1,000 pt-yr for corticosteroids and 1.3/1,000 pt-yr for anti-TNFα agents reported in the United Health Care database.

The reactivation of tuberculosis is a major concern during anti-TNF treatment [56], but there is no medical consensus regarding the effect of IL-6 blockade on tuberculosis. Okada et al. examined the effects of IL-6 and TNFα blockade on the development of tuberculosis infection in mice and observed that there was less tuberculosis infection for anti-IL-6R Ab than for anti-TNFα Ab [57]. In addition, we showed that tuberculosis antigens-induced interferon (IFN)-γ production was suppressed by the addition of TNF inhibitors (infliximab and etanercept) but not of tocilizumab [58]. Although it seems likely that the incidence of reactivation of tuberculosis is lower during tocilizumab treatment than that during anti-TNF treatment, further detailed studies will be needed to clarify this point.

2.5. The Place of Tocilizumab in Rheumatoid Arthritis Treatment. A number of biologics are available for the treatment of RA. These include anti-TNF blockers (infliximab, etanercept, adalimumab, golimumab, and certolizumab), an IL-1 antagonist (anakinra), a B-cell depletor (rituximab), an IL-6 receptor inhibitor (tocilizumab), and a T-cell activation blocker (abatacept). These biological modifiers target different molecules and B cells, leading to different clinical effects and causing different adverse effects. Since no head-to-head comparative studies have been made of the efficacy of these various agents, it has not yet been determined which of these biologics should be selected for a given patient. Currently, one of the anti-TNF drugs is chosen as a first-line biologic, but between 14 and 38% of patients show no or little response to anti-TNF treatment, with as many as 40% of patients discontinuing these drugs within a year and 50% within 2 years. The findings of the RADIATE trial showed that RA patients who had previously discontinued TNF inhibitors, mainly due to their inefficacy, achieved ACR20/50/70 responses of 50%, 28.8%, and 12.4%, respectively, when tocilizumab was administered at 8 mg/kg every four weeks [36]. At present, tocilizumab is likely to be prescribed as a second-line biologic therapy but will have to overcome significant competition from established anti-TNF therapies.

It is anticipated that tocilizumab will be selected as a first-line biologic for moderately to severely active RA patients with certain complications. AA amyloidosis is a serious complication of RA, and amyloid fibril deposition causes progressive deterioration in various organs [59, 60]. Since the gene activation of serum amyloid A, a precursor protein of amyloid A fibril, depends primarily on IL-6 [61, 62], tocilizumab administration was found to promptly reduce serum concentrations of SAA, just as in the case of CRP [60]. Three case reports showed the clinical ameliorative effect of tocilizumab on gastrointestinal symptoms due to intestinal amyloidosis [63–65], and amyloid A fibril deposits were found to have disappeared in two cases after three injections of tocilizumab [63, 65]. This suggests that tocilizumab may be suitable as a first-line drug for RA patients who are complicated with or are at high risk of developing AA amyloidosis.

2.6. Drug-Free Remission Rate. Remission induction is the current goal for RA, and with the development of biological modifiers, a growing number of RA patients has been able to achieve this goal [66]. The long-term efficacy after cessation of tocilizumab followed by DAS28 remission was demonstrated in the DREAM (drug-free remission after cessation of actemra monotherapy) study [67]. The continuous rate

of tocilizumab-free efficacy was 35.1% at 24 weeks and 13.4% at 52 weeks. Serum levels of IL-6 and MMP-3 are useful markers for identifying patients who may be able to discontinue tocilizumab without risk of recurrence. In addition, the RESTORE study (retreatment efficacy and safety to tocilizumab in patients with rheumatoid arthritis at recurrence) demonstrated that retreatment of all relapsed patients with tocilizumab resulted in re-remission [68].

3. Therapeutic Implications of Tocilizumab for Other Systemic Autoimmune Diseases

3.1. Systemic Lupus Erythematosus. Systemic lupus erythematosus (SLE) is a multisystem autoimmune disorder with a broad spectrum of clinical presentations of unknown etiology that mainly affects young women [69]. The pathogenesis of SLE remains unclear, but the concept of apoptosis goes some way towards explaining how the immune system may recognize mainly intracellular antigens. Defects in the clearance of apoptotic cells have been recognized in SLE patients, leading to aberrant uptake by macrophages, which then present intracellular antigens to T and B cells, thus driving the autoimmune process [70]. Cytokine dysregulation is pervasive, and its expression profiles may serve as a marker of disease activity and severity. Recent findings have highlighted type I interferon pathway [71] or Th17 cell activation [72] in the pathogenesis of SLE.

Levels of CRP have been shown to rise in acute illness but not in SLE flares, indicating that IL-6, a major regulator of CRP production, has a minor role in SLE development. However, recent findings suggest that CRP dysregulation also plays a part in the pathogenesis of SLE [73] and SLE may well be a potential target for IL-6 blockade [74]. Serum IL-6 levels of SLE patients were elevated [75–77]. Urinary excretion and renal expression of IL-6 was elevated in SLE patients with active proliferating lupus nephritis [76, 78–81], as were IL-6 levels in the cerebrospinal fluid of SLE patients with central nervous system involvement [82]. Compared to healthy controls, SLE patients had significantly more IL-6 secreting peripheral blood mononuclear cells [83, 84]. Lymphoblastoid cells isolated from SLE patients produced higher levels of IL-6 and blocking of IL-6 inhibited anti-double-stranded DNA (dsDNA) Ab production in vitro [85, 86], indicating that IL-6 is involved in autoantibody production. In murine SLE models, age-associated increases in serum IL-6, soluble IL-6R, and abnormal expression of IL-6R have been detected in MRL/lpr mice [87–89]. In old NZB/W mice, anti-IL-6 Ab reduced and exogenous IL-6 increased production of IgG dsDNA Ab by B cells [90, 91]. Furthermore, IL-6 administration exacerbated glomerulonephritis [92, 93], while IL-6 blockade by means of anti-IL-6R or anti-IL-6 Ab prevented the onset and progression of the disease [94, 95]. Mice with epidermal loss of JunB reportedly developed an SLE phenotype linked to increased epidermal IL-6 secretion, and facial skin biopsies of SLE patients displayed low levels of JunB protein expression, high IL-6, and activated STAT3 levels within lupus lesions [96]. These findings led to an open-label phase I dosage-escalation study of tocilizumab (2 mg/kg, 4 mg/kg or 8 mg/kg, every 2 weeks for 12 weeks) with an enrollment of 16 SLE patients with mild-to-moderate disease activity [97]. Significant improvement in the modified Safety of Estrogens in Lupus Erythematosus National Assessment version of the Systemic Lupus Erythematosus Disease Activity Index score was observed in 8 of the 15 evaluable patients, accompanied by a median reduction in anti-dsDNA Ab levels of 47%. The percentage of $CD38^{high}CD19^{low}IgD^{negative}$ plasma cells in the peripheral blood, which was higher for SLE patients than for normal controls (mean 5.3% versus 1.2%), was significantly reduced to 3.1% at 6 weeks. These results indicate that tocilizumab represents a promising therapeutic biologic for SLE.

3.2. Systemic Sclerosis. Systemic sclerosis (SSc) is a connective tissue disease, characterized by fibrosis of the skin and internal organs, vasculopathy, and immune abnormalities [98]. IL-6 is a definite therapeutic target in SSc [99]. IL-6 in the serum of SSc patients was reportedly elevated and the level correlated with the skin severity score [100–104]. Moreover, the culture supernatants of peripheral blood mononuclear cells and skin tissues from SSc patients contained higher concentrations of IL-6 than those from controls [105–109]. In vitro studies demonstrated that IL-6 may contribute to fibrosis by inducing collagen production [110] and induce α-smooth muscle actin (α-SMA) expression by dermal fibroblasts [111], leading to their differentiation into myofibroblasts. On the other hand, anti-IL-6 Ab suppressed procollagen type 1 production in fibroblasts derived from SSc patients in vitro [112]. SSc serum mediated largely by IL-6 was found to induce endothelial cell activation and apoptosis in endothelial cell-neutrophil cocultures [113]. IL-6 is also associated with humoral and cellular immunological abnormalities in SSc [98, 99]. IL-6 is thus thought to play a significant role in producing the characteristics of SSc. Moreover, in a SSc model mouse, induced by immunization with topoisomerase I and complete Freund's adjuvant, loss of IL-6 expression could ameliorate skin and lung fibrosis [114]. We also examined the clinical effect of tocilizumab on two diffuse SSc patients who had been resistant to conventional treatment regimens [115]. Six months after the treatment, both patients showed softening of the skin with reductions of 50.7% and 55.7% for the total z-score determined with the Vesmeter, a novel device for measuring the physical properties of the skin [116], and of 51.9% and 23% for the modified Rodnan total skin score. Histological examination showed thinning of the collagen fiber bundles and reduction of the number of α-SMA positive cells in the dermis. Since there are few therapeutic drugs for SSc at the present time [117], these improvements suggest that tocilizumab appears to be a promising biologic for the treatment of SSc.

3.3. Polymyositis. The inflammatory myopathies encompass a group of heterogenous muscle diseases which share the common clinical features of slowly progressive symmetrical muscle weakness, decreased muscle endurance, and

fatigue [118]. They include polymyositis (PM), dermatomyositis, and inclusion body myositis, but are generally considered to be distinct diseases with different pathophysiological mechanisms. Muscles produce IL-6 [119], and IL-6 has been also shown to play a regulatory role in muscle wasting [120]. Among these inflammatory myopathies, PM appears to be another suitable target disease for tocilizumab. Excessive IL-6 expression has been found in the sera and infiltrating mononuclear cells in the muscles of PM patients [121–123]. Infiltrating cytotoxic T cells are thought to be involved in muscle fiber damage, and IL-6 functions as a helper factor in the induction of cytotoxic T cells [124]. Moreover, in a model of myosin-induced experimental myositis it was shown that control mice developed clinically manifest muscle damage, whereas IL-6-deficient mice showed no clinical or histological signs of muscle damage [125]. In another model of PM, known as C-protein-induced myositis, intraperitoneal administration of anti-IL-6R Ab suppressed the severity of myositis preventatively as well as therapeutically [126]. We tested the efficacy of tocilizumab in two PM patients who had been refractory to corticosteroids and immunosuppressive drugs [127]. Creatine phosphokinase levels of both patients normalized and MR images showed the disappearance of high-intensity zones in the thigh muscles. These findings suggest that tocilizumab may also be effective as a novel drug for refractory PM.

Dermatomyositis is a complement-mediated microangiopathy associated with destruction of capillaries, hypoperfusion, and inflammatory stress on the perifascicular regions, so that the pathology is different from that of PM [118]. Production of IL-6 and type I interferon signature genes was recently proposed as a biomarker for disease activity in childhood dermatomyositis [128], which thus may be another disorder suitable for tocilizumab targeting.

3.4. Takayasu's Arteritis and Giant Cell Arteritis. Vasculitis refers to inflammation where blood vessels are the primary site of inflammation. The pathological consequence of such inflammation is destruction of the vessel wall, which is histologically detected as fibrinoid necrosis. Takayasu's arteritis (TA) and giant cell arteritis (GCA) belong to an entity designated vasculitis syndrome, and involve both large and medium-sized arteries [129, 130]. The pathogenesis of TA and GCA remains unclear, but it is clear that IL-6 is involved in their development [129–133]. Tocilizumab treatment for a 20-year-old woman with refractory active TA improved the clinical manifestations and abnormal laboratory findings [134], and subsequent studies reported that tocilizumab treatment induced a rapid remission in 2 patients with TA and 5 patients with GCA [135]. Surprisingly, two of the patients with GCA went into remission without concomitant use of corticosteroids. Moreover, tocilizumab was also shown to be effective as rescue treatment for three GCA patients for whom the prednisone dose could not be tapered to less than 30 mg/day [136]. Positron emission tomography/CT scans revealed that in two patients generalized large-vessel vasculitis was detected during the active phase, which completely resolved upon a 6-month course of tocilizumab therapy. These reports strongly imply that IL-6 inhibition may serve as an innovative strategy for the treatment of both TA and GCA. However, several studies have suggested that GCA patients with a lesser inflammatory response without an increase in IL-6 expression were at a higher risk of developing ischemic manifestations than were other patients [137], since the angiogenic activity of IL-6 offers protection against ischemia in such GCA patients [138]. These findings indicate that further clinical studies are required to evaluate the efficacy and safety of tocilizumab for GCA and TA.

It is worthy of note that IL-6 has been also implicated in the development of other types of vasculitis syndrome such as polyarteritis nodosa (PAN) and antineutrophil-cytoplasmic-antibody- (ANCA) associated vasculitis [139–142]. However, so far there have been no reports about off-label use of tocilizumab for PAN or ANCA-associated vasculitis.

4. Therapeutic Implications for Other Autoimmune and Inflammatory Diseases

On the basis of excellent results of the efficacy of tocilizumab for Castleman's disease [143, 144] and systemic juvenile idiopathic arthritis [145–147], it has been approved and used as the first-line biologic in Japan. Pilot studies and case reports with off-label use of tocilizumab also indicate the potential indications of this biologic for various other organ-specific autoimmune and chronic inflammatory diseases. These include relapsing polychondritis [148], acquired hemophilia A [149], autoimmune hemolytic anemia [150], adult-onset Still's disease [151–165], Crohn's disease [166], Bechet's disease with posterior uveitis [167], polymyalgia rheumatica [135, 168], remitting seronegative, symmetrical synovitis with pitting edema [169], spondyloarthritides [170–175], graft-versus-host disease [176, 177], TNF-receptor-associated periodic syndrome [178], and pulmonary arterial hypertension complicated with Castleman's disease or mixed connective tissue disease [179–181]. Further clinical trials are essential, however, to evaluate the efficacy and safety of tocilizumab for these diseases.

5. Conclusion

Acute IL-6 synthesis provides a warning signal and protects the host from environmental stress, while its prolonged production causes the onset and progression of various autoimmune diseases. Several clinical trials have verified the efficacy and safety of tocilizumab for RA, systemic juvenile idiopathic arthritis and Castleman's disease, resulting in approval of this innovative biologic for the treatment of these diseases. Case reports of off-label use or pilot studies have also raised the possibility that tocilizumab could become the biological drug of choice for other systemic autoimmune diseases including SLE, systemic sclerosis, polymyositis and large vessel vasculitis. At present, the mechanisms through which tocilizumab exerts its clinical ameliorative effects on phenotypically different autoimmune diseases are not completely understood. IL-6 blockade may suppress autoantibody production or correct the imbalance of autoantigen-specific Th17 and/or Th1 versus Treg. Thus, clarification of

the mechanisms as well as further clinical trials to evaluate the efficacy and safety of tocilizumab for these diseases are important issues.

Conflict of Interests

Toshio Tanaka declares no conflict of interests.

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Review Article

Use of Biologic Agents in Ocular Manifestations of Rheumatic Disease

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Biologic agents have dramatically shifted the treatment paradigm for rheumatic disease. Use of these agents can decrease disease burden, allow the patient to be weaned from corticosteroids, and reduce the likelihood of relapse. Eye disease associated with rheumatic conditions may present with a wide range of signs and symptoms. This coexisting pathology should not be overlooked and should be considered a reason for initiation or continuation of biologic therapy. Additionally, many of the ocular manifestations of rheumatic disease respond preferentially to specific targeting molecules. This paper summarizes the available studies on the use, efficacy, and safety of biologic agents in the treatment of ocular manifestations of rheumatic disease.

1. Introduction

Eye disease associated with rheumatic conditions may present with a wide range of signs and symptoms. The treating physician must be careful not to overlook ocular manifestations, as they can be quite subtle. Dry eye syndrome, acute and chronic anterior uveitis, panuveitis or vitritis, scleritis, keratitis, retinal vasculitis, and ischemic optic neuropathy represent ocular disorders frequently associated with rheumatic diseases. In patients presenting with nonspecific signs and symptoms, ocular findings may be the only clue to the final diagnosis. Alternatively, in patients with long-standing rheumatic disease, ophthalmic flares may suggest further deterioration or relapse.

The mainstay of management of inflammatory ocular conditions has been topical agents with occasional oral corticosteroid use. However, for some conditions these therapies are often inadequate. Biologic therapies (Table 1) have demonstrated efficacy in the control of many of the primary manifestations of rheumatic disease. Their targeted use in the ocular manifestations of rheumatic disease has not been as extensively studied. This paper seeks to compile the available reports on the use, efficacy, and safety of biologic agents in the treatment of ocular symptoms of rheumatic disease.

2. Adamantiades-Behçet's Disease

Ocular symptoms occur in 43–72% of Adamantiades-Behçet's disease (ABD) patients and affect males more commonly than females. The classic appearance is that of an anterior uveitis with a sterile hypopyon; however, presentations more often feature a posterior or diffuse uveitis with accompanying retinal vasculitis [1].

ABD is an aggressive, sight-threatening disease that requires immunosuppressive therapy to avoid vision loss. TNF-α antagonists are the preferred first line agent for treatment of ABD. They have proven successful in controlling symptoms, reducing ocular relapses, and significantly decreasing the daily dose of corticosteroids [2, 3]. Infliximab has emerged as the foremost agent with several prospective studies demonstrating remission of anterior and posterior segment inflammation, resolution of macular edema, and successful control of uveitis refractory to previous therapy [4, 5]. Among the anti-TNF-α agents, infliximab has been

Table 1: Biologic agents.

Biologic agent	Trade name	Mechanism of action
	TNF-α blockers	
Infliximab	*Remicade*	Chimeric monoclonal antibody against TNF-α
Etanercept	*Enbrel*	TNF receptor-IgG fusion protein
Adalimumab	*Humira*	Human monoclonal antibody against TNF-α
Certolizumab pegol	*Cimzia*	PEGylated Fab of a humanized TNF inhibitor monoclonal antibody
Golimumab	*Simponi*	Humanized monoclonal antibody against TNF-α
	Lymphocyte inhibitors	
Rituximab	*Rituxan*	Chimeric monoclonal antibody against CD20
Abatacept	*Orencia*	Selective inhibitor of T-cell costimulation
	Anti-interleukin antibodies	
Anakinra	*Kineret*	IL-1 receptor antagonist
Daclizumab	*Zenapax*	Humanized monoclonal antibody to IL-2 receptor
Tocilizumab	*Actemra*	Humanized monoclonal antibody to IL-6R
Basiliximab	*Simulect*	Chimeric monoclonal antibody to the CD25
	Specific receptor antibodies	
Efalizumab	*Raptiva*	CD11a, a pan-leukocyte surface marker, inhibitor
Alefacept	*Amevive*	CD2 inhibitor
Alemtuzumab	*Campath-1H*	CD52, a pan-lymphocyte antigen, antagonist
	Anti-VEGF-A antibodies	
Ranibizumab	*Lucentis*	Monoclonal antibody fragment (Fab) targeting VEGF-A
Bevacizumab	*Avastin*	Monoclonal antibody targeting VEGF-A

shown to achieve the best control of ocular signs and symptoms [1, 6].

While not as extensively studied, several reports have suggested etanercept and adalimumab are effective in controlling ABD disease severity [7, 8]. Treatment with etanercept achieves a greater response in ocular manifestations of ABD over other complications such as oral ulcers, arthritis, and skin lesions [9]. Adalimumab may induce and maintain sustained remission of refractory ocular inflammation in about 90% of patients [10].

Among the other biologic agents, rituximab has shown success in retinal vasculitis associated with ABD [11]. A case report describing the effective management of ABD with anakinra did not address ocular symptoms [12]. The ability of therapy targeting interleukin activity to reduce disease burden suggests IL-1β is a mediator of inflammation in ABD and may effectively treat its ocular manifestations.

3. Rheumatoid Arthritis

Rheumatoid arthritis (RA) gives rise to significant eye disease in 15–30% of affected patients. Characteristic presentations include keratoconjunctivitis sicca (KCS), stromal keratitis, sclerosing keratitis, scleritis, and episcleritis. KCS is by far the most common ocular manifestation (11.6%), followed by episcleritis and scleritis [13].

Dry eyes can be significantly disabling and difficult to treat. Aggressive lubrication, punctal plugs, autologous serum drops, prednisolone drops, and topical cyclosporine make up the ophthalmologist's armamentarium. This localized approach satisfactorily controls most patients; however, systemic steroids and increased methotrexate are occasionally necessary. Although not sight threatening, symptoms can exert an increasing burden as the disease progresses or increases in severity. KCS patients have various degrees

of health-related quality of life impairment [14]. Although unconventional, initiation of infliximab in otherwise quiescent RA has successfully controlled KCS symptoms [15]. Evidence suggests that the indirect costs of KCS actually may outweigh the expense of biologic treatment [16].

Reinforcing the importance of TNF-α in the control of corneal inflammation, the TNF-α antagonists, infliximab, adalimumab, and etanercept have been shown to be effective therapies for RA-associated keratitis. They have shown differing levels of efficacy [17–19]. Infliximab has been shown to be the most effective agent to control RA-associated keratitis [20, 21]. Rituximab has been used successfully in the treatment of severe peripheral ulcerative keratitis (PUK) demonstrating prior resistance to anti-TNF agents [22]. However, a case of bilateral PUK following treatment with rituximab has been reported. Causation was not established [23].

Necrotizing scleritis is the most destructive form of scleritis and has considerable ocular morbidity. In patients with RA, it is associated with a high mortality, especially when not treated with immunosuppressants. Anti-TNF-α agents have the most evidence supporting their use and efficacy in scleritis. Certolizumab pegol has been shown to control scleritis in a patient with RA who had failed other TNF-α antagonists [24].

Inflammation control in the eyes of RA patients remains a challenge. Head to head comparison trials of the many biologics have not been completed. A large review of several individual studies indicated that TNF-α antagonists (certolizumab, adalimumab, infliximab, etanercept), the B-cell inhibitor (rituximab), and the IL-6 blocker (tocilizumab) are superior to T-cell costimulation inhibitor (abatacept) and the IL-1 blocker (anakinra). However, none of the comparisons between these biologics reached statistical significance [25].

4. Juvenile Idiopathic Arthritis

Uveitis occurs in 10%–15% of patients with juvenile idiopathic arthritis (JIA) and represents the primary cause of uveitis in childhood. Contrary to the red painful eye seen in adults with uveitis; the inflammation seen in children is often asymptomatic and bilateral, with an indolent chronic course. Severe vision loss, even blindness, occurs in an unacceptably high percentage of patients with one-quarter of children with JIA becoming blind in one eye [26].

Topical steroids are the first line of therapy, but only around 40% of patients respond to such treatment. The associated risks of increased intraocular pressure and cataract are particularly unappealing in a pediatric population. An investigation into the effectiveness of the three most popular anti-TNF-α agents, etanercept, infliximab, and adalimumab, found infliximab to be more effective than etanercept. No statistically significant conclusion was drawn regarding adalimumab [27]. Infliximab, however, was found to have a high rate of side effects in a prospective study [28]. The preferred anti-TNF-α agent is adalimumab, which is more effective against uveitis than etanercept and better tolerated by children [29].

Use of biologic agents not targeting TNF-α has only recently been published. Rituximab has reported efficacy in patients refractory to treatment with TNF-α antagonists [30]. Preliminary studies of high-dose intravenous administration of IL-2 antagonist, daclizumab demonstrate a reduction in active inflammation in JIA-associated anterior uveitis [31]. In a prospective study of abatacept in cases refractory to anti-TNF-α treatment, all seven children exhibited decreased anterior segment inflammation. However, only one demonstrated complete resolution with this treatment [32]. A head-to-head comparison of these newer agents has not been performed.

5. Sjogren's Syndrome

KCS, or severe dry eye, is the hallmark of Sjogren's syndrome (SS) and indicates an autoimmune attack on the lacrimal gland. Therapeutic options for the debilitating xerophthalmia are currently limited to symptomatic relief with aggressive artificial lubrication, autologous serum eye drops, topical cyclosporine, topical corticosteroids, and punctal occlusion.

Understanding the inflammatory cascade involved in SS suggests elevated levels of proinflammatory cytokines (e.g., IL-1α, IL-1β, IL-6, TNFα) and immunoactivators (e.g., ICAM-1, CD40, CD40 ligand) play a role the ocular symptoms [33]. Newer treatment strategies are targeting these pathways as therapeutic options. Preliminary studies of the anti-TNF-α agents were promising; however, a large randomized trial failed to demonstrate a difference in response between placebo and an infliximab-treated group [34]. Similarly, etanercept was also no more effective than placebo in a 12-week study [35].

While a recent double-blind, randomized, placebo-controlled trial indicated that rituximab is effective and safe in the treatment of patients with SS, ocular signs and symptoms were not among the measures demonstrating improvement with treatment [36]. A case report did report improvement in subjective and objective measures of xerophthalmia in 2 patients treated with rituximab [37].

6. Seronegative Spondyloarthropathy

The seronegative spondyloarthropathies, ankylosing spondylitis (AS), psoriatic arthritis (PsA), inflammatory bowel disease (IBD), and reactive arthritis (ReA) represent a group of diseases that share clinical, genetic, and pathological characteristics. They share an association with HLA-B27, an absence of positive rheumatoid factor (negative serostatus), and extra-articular features, such as involvement of eyes, skin, and genitourinary tract.

Anterior uveitis reportedly occurs in up to 30% of patients with AS. There is now accumulating evidence that targeted anti-TNF-α therapy is highly effective in spondyloarthritis [38]. Patients taking anti-TNF-α agents exhibited significantly reduced rates of recurrence of anterior uveitis in the major trials, with stronger protection afforded by infliximab and adalimumab [39, 40]. Other biologics have not shown as much promise; abatacept failed to show improvement in any outcome measures in one prospective study [41].

The classic triad of ReA includes arthritis, nongonococcal urethritis, and conjunctivitis. However, ocular manifestations may also include acute anterior uveitis. Current opinion is that because TNF-α drives the pathogenesis of reactive arthritis and suggests TNF-α antagonists will be efficacious therapeutic tools. Like AS, definitive studies are lacking but isolated case reports support the use of anti-TNF-α agents [42, 43].

Biologic treatment of the uveitis associated with PsA, like the other seronegative spondyloarthropathies, centers on the anti-TNF-α therapies [44]. Evidence suggests infliximab provides the greatest response among the anti-TNF-α agents [45, 46].

Inflammatory bowel disease (IBD), like the other spondyloarthropathies, can be associated with an acute anterior uveitis. It also can present with a keratitis or scleritis. Successful control of the ocular inflammation has been seen in IBD patients using TNF-α antagonists [47]. Of the TNF-α inhibiting therapies, etanercept is ineffective in controlling both the systemic symptoms of IBD and the associated uveitis [48].

7. Relapsing Polychondritis

Fifty-nine percent of patients with relapsing polychondritis (RP) have ocular components of their disease. The most common manifestation is scleritis, seen in 41% of patients; uveitis is seen in one-quarter of patients; conjunctivitis, episcleritis, keratitis, and retinal vasculitis are seen less frequently [49]. Immunosuppressive chemotherapy is usually required to successfully treat the ocular manifestations of RP, especially nodular and necrotizing scleritis [50]. Infliximab was shown to diminish ocular manifestations [51].

8. Systemic Vasculitic Disease

Giant cell arteritis (GCA) can cause an incredibly rapid total or near total loss of vision. Without prompt recognition and initiation of high-dose intravenous steroids, bilateral vision loss may result in up to 50% of individuals. Maintenance therapy with biologic agents is an attractive theory, as it would allow avoidance of chronic steroids and the associated morbidity. Studies of TNF-α antagonists have mixed results. Infliximab was not shown to be effective in a prospective randomized trial [52]. However, a case of reported steroid-resistant GCA was treated successfully with adalimumab [53]. Rituximab was also effective in reducing inflammatory markers in a patient with GCA refractory to treatment with corticosteroids [54].

Ocular manifestations are very common in Wegener's granulomatosis (WG), affecting approximately half of patients. Scleritis, keratitis, orbital disease, and less commonly retinovasculitis or uveitis are all potential manifestations of WG [55]. The other ANCA-associated vasculitides, microscopic polyangiitis, and Churg-Strauss present much less frequently with ophthalmic complications. Reversal of vision loss was seen in a case of severe posterior scleritis in WG treated with infliximab [56]. Rituximab has emerged as an effective tool in treating ocular manifestations of WG [57, 58]. Recent evidence suggests it is equivalent to cyclophosphamide for the induction of remission, with particular efficacy at inducing remission in patients with relapsing disease [59]. It was shown to be superior to infliximab by a small prospective study [60].

9. Anterior Uveitis Induced by Anti-TNF Agents

As discussed above, extensive evidence supports the efficacy of TNF-α antagonists in the treatment of uveitis associated with rheumatologic disease. Paradoxically, use of these agents has been implicated to cause uveitis. Several anecdotal case reports suggested an association between use of these agents and development of uveitis [61]. Subsequently, a review of medication adverse event registries not only confirmed this observation but also suggested etanercept caused a greater number of reported uveitis cases compared to infliximab and adalimumab [62]. A retrospective review reported a frequency of 1 case per 100 patient-year for patients treated with a TNF-α antagonist for seronegative spondylopathy [63].

10. Conclusion

The last decade has seen a dramatic increase in the number and nature of biologic agents. We continue to expand our knowledge of rheumatologic disease and the role of the inflammatory cascade in the ocular manifestations of those diseases. Systemically, administered small molecular antibodies and antagonists have become valuable tools in the treatment of refractory ophthalmic symptoms of rheumatic disease. In certain cases, these agents can even be considered primary therapeutic options.

Recently, two humanized monoclonal antibodies targeting vascular endothelial growth factor-A (VEGF-A), ranibizumab (Lucentis) and bevacizumab (Avastin) have revolutionized the treatment of eye diseases such as age-related macular degeneration. These agents are delivered via an intraocular injection and are effective and well tolerated [64]. Preliminary safety studies to evaluate toxicity of intravitreal injection of TNFα inhibitors have been performed in rabbits with experimental uveitis, with promising results [65, 66]. Future treatment of ocular manifestations of rheumatic disease will certainly build upon the documented efficacy of biologic agents and the ability to locally inject these antibodies and antagonists.

Disclosure

The authors have no financial interests or conflict of interests to disclose.

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