Targeting TNF and Its Family Members in Autoimmune/Inflammatory Disease
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Targeting TNF and Its Family Members in Autoimmune/Inflammatory Disease

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Tumor necrosis factor (TNF), a pleiotropic cytokine mainly produced by activated macrophages, modulates a wide range of biological functions in multiple tissues and organs. Besides its effects on tumor cell death, TNF is a key mediator of both acute and chronic inflammation. Since the description of TNF in the 1970s and through consideration of structural homologies, a total of 19 TNF-related cytokines have now been regrouped into a large family called the TNF ligand superfamily (TNFSF), whose members interact with TNF receptor superfamily (TNFRSF) members. More than 150,000 scientific publications (!) concerning TNF and its family members are available, demonstrating the strong interest of the scientific community in this molecule. Numerous studies implicating TNF family members in the pathophysiology of human autoimmune/inflammatory diseases have supported the emergence of TNF blocking agents developed for treatment of human disease, particularly over the last decade. These biotherapies, in the form of (I) chimeric, humanized, or human anti-TNF monoclonal antibodies or (II) fusion proteins involving a soluble TNF receptor, have been very successful in ameliorating disease signs and symptoms, especially in patients suffering from rheumatoid arthritis (RA) and Crohn's disease. Nonetheless, several aspects of these beneficial effects remain enigmatic. Moreover, the modulatory factors influencing TNF production by macrophages are not all known. Nevertheless, it is expected that over the next few years we will witness an increasing number of diseases for which TNF-blockade therapy is indicated.

In this special issue, eleven papers including research articles, review articles, and clinical studies provide new information and interesting discussion regarding current questions related to this hot topic.

In the first group of articles, interesting data is presented about TNF-blocking therapies and modulation of TNF generation by macrophages. Y. Lv et al. studied the nonneuronal cholinergic system existing in macrophages and show in a murine monocyte/macrophage cell line that bacterial lipopolysaccharide (LPS) exposure enhances autocrine acetylcholine production associated with an attenuation of TNF release. In this same cell line, K. Borzęcka et al. determined that, during high dose LPS stimulation, CD14 together with scavenger receptors is required for the binding of LPS but has a limited and dispensable contribution to TNF production. R. Cascão et al. recently identified gambogic acid as a simultaneous blocker of IL-1β and TNF secretion and described here a beneficial anti-inflammatory effect of gambogic acid in rat antigen-induced arthritis. Interestingly, F. R. Spinelli et al. show that blocking TNF biological effects during RA is associated with an increase of circulating endothelial progenitor cells, which might positively affect the endothelial function and may help in correcting the endothelial dysfunction observed in the disease.
In the second group of articles, possible off-label uses of anti-TNF therapy in various disorders are discussed. Due to their low prevalence, discussion of the use of anti-TNF modalities in Behçet’s disease, sarcoidosis, and noninfectious uveitis in the review by D. Sánchez-Cano et al. was mainly based on case reports and case series. The clinical study of C. García-De-Vicuña et al. supports the usefulness of adalimumab in the treatment of refractory uveitis associated with juvenile idiopathic arthritis. P. A. Jarrot and G. Kaplanski review the use of anti-TNF therapy in vasculitis and conclude that, except for Behçet’s disease, this treatment has not shown significant efficacy. In their review article, A. Kumar et al. described how HIV infection is modulated by TNF and TNFR superfamily pathways and then discuss the emerging therapeutic options based on the modulation of TNF activity.

The third group of articles tackle the topic of the management of anti-TNF therapy for its well-established indications. B. Mörck et al. envision being able to reduce the drug costs in active HLA-B27 positive ankylosing spondylitis over time by reducing the infliximab dose and by extending the interval between infliximab doses. On the other hand, R. Altwegg and T. Vincent discuss the usefulness of monitoring serum trough levels and anti-drug antibodies in the optimization of anti-TNF therapies during inflammatory bowel disease.

Lastly, Y. Aiba and M. Nakamura open a debate concerning a putative therapeutic role of TL1A/DR3 inhibition. They suggest that the modulation of this particular TNF/TNFR superfamily member interaction may be a potential therapeutic target in several autoimmune diseases including inflammatory bowel disease, RA, ankylosing spondylitis, and primary biliary cirrhosis.

We hope that readers of the journal will find in this special issue not only accurate data and updated reviews on the targeting of TNF and its family members in autoimmune/inflammatory disease treatment, but also relevant questions that remain to be resolved including the extension of current therapeutic indications and the optimization of the anti-TNF therapies to find a better balance between cost and effectiveness.

Sophie Desplat-Jégo
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Review Article

TNF Blocking Therapies and Immunomonitoring in Patients with Inflammatory Bowel Disease

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Since their appearance in the armamentarium for inflammatory bowel disease (IBD) more than a decade ago, antitumor necrosis factor (TNF) inhibitors have demonstrated beneficial activity in induction and maintenance of clinical remission, mucosal healing, improvement in quality of life, and reduction in surgeries and hospitalizations. However, more than one-third of patients present primary resistance, and another one-third become resistant over time. One of the main factors associated with loss of response is the immunogenicity of anti-TNF biologics leading to the production of antidrug antibodies (ADAbs) accelerating their clearance.

In this review we present the current state of the literature on the place of TNF and its blockage in the treatment of patients with IBD and discuss the usefulness of serum trough levels and ADAAb monitoring in the optimization of anti-TNF therapies.

1. Introduction

Antitumor necrosis factor (TNF) biologics appeared over a decade ago in the armamentarium for inflammatory bowel disease (IBD). Originally evaluated in Crohn’s disease (CD) and thereafter in ulcerative colitis (UC), their efficacy was demonstrated in both diseases and has deeply modified the management of patients with IBD [1]. Although they are potentially able to change the natural course of IBD and to decrease the need for surgery, absence or loss of response is frequent and only one-third of patients remain in clinical remission at 1 year [2]. Clinical response, steroid-free remission, and mucosal healing have been correlated with drug trough levels [3, 4]. However, anti-TNF pharmacokinetic is characterized by a considerable interindividual variability and antidrug antibodies (ADAbs) have been identified as one of the major factors impacting their clearance [5]. Thus, serum trough levels and ADAb measurement have been proposed for the monitoring of anti-TNF drugs and algorithms were defined for the management of patients with IBD [6].

2. Role of TNF in IBD Pathophysiology

While the etiology of IBD is still unknown, it is thought to involve complex interactions between genetic disposition, environmental conditions, life style, and microbial and immune factors resulting in a deregulated and excessive immune response directed against components of the normal microflora. CD and UC have been associated with exaggerated T helper (Th) type 1 and Th2 responses, respectively. More recent studies demonstrated that tissue damages result from mucosal inflammation mainly mediated by proinflammatory Th1 and Th17 lymphocyte subpopulations and their respective proinflammatory effector cytokines. In the gut of CD patients, activated Th1 and Th17 cells produce IFNγ and IL17 (A and F), respectively, which stimulate macrophages and induce the production of other inflammatory cytokines such as IL-1β and TNFα that subsequently promote matrix metalloproteinases (MMPs) production by stroma cells and mucosal damage [7]. Thus, it is now widely accepted that TNFα plays a strategic role in IBD pathophysiology, at the cross talk of the different inflammatory pathways involved.
in gut mucosal inflammation [8]. Accordingly, most of the efficient biologic therapies developed so far in IBD aimed at neutralizing the proinflammatory activity of the TNF pathway. The effects of TNFα are known to be mediated by TNF receptor I (TNF-RI) or TNF-RII. Ligation of TNF-RI, which is expressed on a wide range of immune and nonimmune cells, results in NF-κB activation, cytotoxicity, and induction of proinflammatory cytokines and chemokines as well as antiapoptotic peptides [9, 10]. The effects on T lymphocytes are mainly mediated by interaction of TNFα with TNF-RII inducing a costimulatory signal to TCR-mediated T cell activation, thereby increasing T cell proliferation, expression of T cell activation markers (CD25, human leukocyte antigen-DR, and TNF-RII), and secretion of inflammatory cytokines including IFNγ and TNFα [11]. Accordingly, anti-TNF are able to inhibit T cell activation resulting in a decrease of proliferation and cytokine secretion (IFN-γ, IL-13, IL-17A, and TNFα) of both CD4+ and CD8+ T cell populations derived from UC patients [12]. On the other hand, TNFα and TNF-RII are also able to activate and expand protective CD4(+)FoxP3(+) regulatory T cells (Tregs) and seem critical for the stabilization of their phenotype and function in the inflammatory environment of the lamina propria in a mouse model of colitis [13]. These contrasting effects of TNFα on effector versus regulatory T cells may explain unexpected and disappointing results obtained with anti-TNF in some autoimmune diseases such as multiple sclerosis [14]. Altogether, these data underline the complexity of TNFα function via TNF-RI or TNF-RII on the course of intestinal inflammation, due to different susceptibility of epithelial cells and effector or regulatory immune cells. As an illustration, in dextran sulfate sodium- (DSS-) induced acute colitis in BALB/c mice, TNF-RI ablation led to exacerbation of the disease with increased cytokine secretion and intestinal damage, while TNF-RII deficiency had opposite effects [15]. Nonetheless, studies in patients with IBD have extensively demonstrated the efficiency of anti-TNF therapies which directly inhibit activation of effector T cells and sensitize them to Treg-mediated inhibition with final restoration of immune homeostasis, resolution of inflammation, and mucosal healing. Further studies are now required to better understand the respective protective and deleterious effects mediated by TNFα on immune and nonimmune cells through TNF-RI and TNF-RII in order to develop more specific inhibitors with potentially an increased efficacy and/or safety.

3. Anti-TNF Therapies in Patients with IBD

TNFα is the major target molecule of biologic treatments in CD and UC. Numerous randomized clinical trials and meta-analyses have demonstrated the efficacy of monoclonal antibodies against TNFα for both induction and maintenance of remission in both CD and UC [16–18]. Infliximab (IFX), a chimeric monoclonal antibody composed of human constant and murine variable regions, and adalimumab, a fully human monoclonal IgG1 anti-TNF antibody, demonstrated their efficacy for the control of disease activity and the induction of clinical remission and mucosal healing in luminal CD and UC both in children and adult patients [1, 19–25]. Several randomized clinical trials showed a better efficacy in inducing steroid-free clinical remission for a combination therapy with immunomodulators than anti-TNF monotherapy in CD and UC [26]. Moreover several studies established the use of infliximab and adalimumab in active fistulizing CD in adult patients [27, 28]. Certolizumab, a polyethylene-glycolated Fab′ fragment of anti-TNF Ab, also produced significant clinical benefit and mucosal healing in adult patients with CD [29]. Recently, golimumab, a fully human monoclonal antibody to TNFα, was shown to induce and maintain clinical response in patients with active moderate-to-severe UC [30, 31].

However, although 60 to 80 percent of patients exhibit a good initial response to anti-TNF treatments (defined as a Crohn’s Disease Activity Index (CDAI) decrease from baseline >70 points for CD and a decrease in the Mayo score of at least 3 points and at least 30 percent for UC), only one-third of patients are in clinical remission without steroids at one year (defined as a CDAI <150 for CD and a total Mayo score of 2 points or lower, with no individual subscore exceeding 1 point for UC) [18]. Consequently, 20 to 30 percent of patients require dose intensification or interval adjustment in order to maintain long-term clinical benefit and an average of 10 to 20 percent per year lose response [32–36].

4. Drug Monitoring of Anti-TNF Biologics

Despite the high effectiveness of anti-TNF in patients with IBD, more than one-third of patients present primary resistance, and another one-third become resistant over time [37]. Optimal clinical response required the maintenance of clinically effective drug concentrations, but the pharmacokinetic of anti-TNF is highly variable among patients and could be influenced by numerous factors including gender, body weight, associated treatments (immunosuppressants are known to increase anti-TNF trough levels), route of administration, serum albumin concentration, and systemic inflammation with a markedly decreased half-life in patients with severe disease [38–40]. However, the main factor impacting anti-TNF pharmacokinetic and efficacy over time is immunogenicity whereby antidrug antibodies (ADAbs) accelerate anti-TNF monoclonal Abs clearance and shorten their half-life [41, 42]. Although humanized (e.g., certolizumab) and fully human Abs (e.g., adalimumab and golimumab) are logically less immunogenic as compared with chimeric ones (e.g., IFX), they can all induce ADAbs targeting murine and/or variable domains of the monoclonal Ab. Other factors may promote immunogenicity such as genotype in a minority of patients and drug agitation or freeze-thaw cycles that can induce immunogenic protein aggregates (for review [43]). Contrastingly, prescription of maintenance therapy with concomitant immunomodulators and achievement of suitable trough drug levels have been shown to reduce the risk of ADAbs [44].

Several studies assessed IFX trough levels after induction treatment or during maintenance therapy as predictors of

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sustained clinical response and showed a significant correlation between low IFX trough levels and decreased clinical response in CD and UC adult patients [3, 4, 34, 45–47] and in children with UC [48]. In a recent prospective study of IBD patients who have developed secondary failure to IFX, Paul et al. have shown that the only factor associated with mucosal healing after IFX optimization was a significant increase in IFX trough levels [49]. Antibodies to IFX (ATIs) were described in up to 60% when IFX was used on an ad hoc basis in practice and in 10 to 20% of patients in randomized controlled trials of maintenance therapy [43]. ATIs were associated with loss of clinical response, deterioration of endoscopic activity, infusion reactions, and low serum IFX concentrations [5, 41, 44, 46, 50–52]. However, some studies did not observe significant correlation between trough levels of IFX and CD activity or between positivity of ATIs and loss of clinical response or deterioration of endoscopic activity [3, 4, 53–55]. These discrepancies could be explained by different methods of measurements for ATIs and IFX concentrations, by the short follow-up time in some studies, and by the lack of consensual optimal levels of IFX for prediction of efficacy.

There are fewer data for adalimumab, but some studies also described a positive association between serum adalimumab concentration and clinical remission in CD [56–58]. Furthermore, while fully human, antiadalimumab antibodies were described in 2.6 to 17 percent of patients treated for CD or rheumatoid arthritis and significantly associated with low serum adalimumab trough levels and decreased clinical response [56, 59–61]. The relationship between pharmacokinetic data and efficacy is less clear for adalimumab than IFX with considerable variability and overlap in serum concentrations between patients with and without remission [57]. However, in an observational study evaluating the efficacy of adalimumab in 168 active CD patients who failed to respond to IFX, long-term clinical benefit was significantly associated with higher serum trough concentrations and absence of ADAbs [56]. A recent study using adalimumab maintenance therapy in 40 adult patients with CD or UC showed a significant association of high trough levels of adalimumab with clinical remission and mucosal healing. Antiadalimumab antibodies were associated with low trough levels of adalimumab and lack of mucosal healing [58].

There is so far no data concerning trough levels and antidrug antibodies for adalimumab in children and in all patients for certolizumab and golimumab.

Serum trough levels measurement to detect subtherapeutic drug concentrations and identification of ADAbs (therapeutic drug monitoring or TDM) are the most relevant and useful parameters for the monitoring of anti-TNF drugs to facilitate informed decision making in IBD patients with secondary loss of response to TNF antagonists. The clinical utility of the immunomonitoring was evaluated in a retrospective study conducted on 155 patients with IBD and loss of response to IFX [6]. They showed that measuring IFX and ADAbs concentrations may impact treatment decision in 73%. When ADAbs were detected, the switch to another anti-TNF molecule allowed a partial or complete response in 92% versus 17% for dose escalation whereas drug escalation was the most efficient strategy in patients with subtherapeutic IFX concentration (86% versus 33% of partial or complete response, resp.). They concluded that increasing anti-TNF doses is ineffective in patients with ADAbs but appropriate in case of subtherapeutic drug concentration and proposed an algorithm for optimization of therapeutic strategy in IBD patients with loss of response to IFX based on ADAbs and trough drug measurement [6].

Interestingly, in a prospective study examining the course of ADAbs formation and the clinical relevance of its assessment in the followup of patients with rheumatoid arthritis, Bartelds et al. showed that, among patients positive for antiadalimumab Abs, 67% developed ADAbs during the first 28 weeks and almost one-third during the first month of treatment [62]. However and despite a poor clinical response, patients with ADAbs discontinued treatment only after 52 weeks of therapy indicating an important delay between ADAbs appearance and treatment adjustment. Furthermore, early trough level measurement after induction might also have a prognostic value with IFX trough levels above 3.5 μg/mL at 14 weeks being associated with a sustained therapeutic response [63].

On the other hand, supratherapeutic anti-TNF trough levels might also be associated with paradoxical inflammatory side effects such as psoriasiform eczema or arthralgia [64]. In such patients, lowering doses could be beneficial in terms of not only safety but also decrease of the cost for the healthcare payer.

Altogether, these data plead for the clinical and economical utility of early therapeutic drug monitoring in the management of patients receiving TNF inhibitors. In case of a loss of response with low trough level without ADAbs, an intensified therapy with the same drug should be recommended by increasing doses and/or decreasing intervals and eventually adding an immunosuppressant. When low trough level is related to the presence of ADAbs, therapy should be switched within the anti-TNF class and if necessary to a drug with another mode of action [63]. The addition of an immunomodulator might also be able to induce a decrease in ADAbs level and to restore clinical response [65]. Of note, clinical response can occur despite the presence of ADAbs as described recently in a retrospective study [66]. Continued maintenance therapy with IFX induced ADAbs disappearance in two-thirds of these patients after a median of 4 infusions suggesting that continued anti-TNF treatment could be considered in patients with clinical response and first ADAbs detection. Indeed, recent studies investigating the kinetics of ATI formation confirmed that ADAb secretion may be transient and disappeared over time in almost one-third of patients [67, 68]. Compared to nontransient ATI that appeared usually within the first 12 months of therapy, transient ATI was detected throughout the duration of IFX therapy [68]. Patients with sustained ATI were more likely to discontinue IFX treatment compared with patients with transient ATI [67].

In a very recent study using a decision analytic model that simulated 2 cohorts of patients with CD who become resistant to anti-TNF inhibitors, Velayos et al. compared the effectiveness of empiric dose escalation versus testing-based strategy over a 1-year time period [69]. Although both
strategies yielded similar rates of remission (66% versus 63%, resp.) and quality-adjusted life year (0.800 versus 0.801), the testing-based strategy was less expensive than empiric dose escalation ($31,870 versus $34,266, resp.). Similarly, Steenholdt et al. showed in a randomized controlled trial that a testing-based strategy using an algorithm designed to identify the mechanism leading to secondary loss of response to IFX is more cost effective than empiric dose escalation in patients with CD [70]. In the monitored arm, patients with low serum IFX and ATIs were switched to adalimumab, patients with low serum IFX without ATIs underwent dose intensification, and patients with high IFX trough levels with or without ATIs were switched to an out-of-class therapy or screened for an alternate cause of their symptoms. Compared to the current dose intensification strategy, individualized therapy substantially reduced average treatment costs per patient with similar clinical response rates.

Large prospective and randomized studies are still required to validate all these approaches in patients with IBD and clear dose toxicity/efficacy relationships have yet to be established for anti-TNF inhibitors.

Finally, we have to keep in mind that, in the absence of standardization, the numerous assays developed for serum trough levels and ADAb measurement (Table 1) exhibit variable performances that could explain discrepancies between studies and difficulties in establishing clear cutoff values. There are currently no defined gold standard assays for quantification of anti-TNF drugs and ADAbs. A recent study compared three in house or commercially available assays (ELISA, bridging ELISA, and RIA) developed for the analysis of IFX levels and ATIs [71]. There was a good correlation between IFX and ATI levels measured with all 3 tests. The sensitivity of the three assays to detect ATIs was comparable with a slight advantage for the RIA test which is less sensitive than ELISA to drug interference caused by the presence of IFX in the serum impeding the detection of low ATI concentrations. Nevertheless, discrepancies between the three assays were not rare and conclusions of the study were highly debated highlighting the high need for standardization [72, 73].

## 5. Conclusion

Since the advent of anti-TNF biologics more than a decade ago, they have demonstrated beneficial activity in induction and maintenance of clinical responses, mucosal healing, improvement in quality of life, reduction in surgeries and hospitalizations, and the treatment of extraintestinal manifestations of IBD. However, despite good overall initial effectiveness, a significant proportion of patients lose response over time mainly because of ADAb production and accelerated drug clearance. Although optimal treatment strategies remain controversial, therapeutic algorithms were proposed based on serum trough levels and ADAb monitoring in order to rationalize drug adjustment. For the future, a better understanding of the ambivalent protective and deleterious effects mediated by TNFα and its receptors on immune and nonimmune cells during IBD might be crucial for the development of more efficient and safe biological inhibitors.

### Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Research Article

Upregulating Nonneuronal Cholinergic Activity Decreases TNF Release from Lipopolysaccharide-Stimulated RAW264.7 Cells

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Nonneuronal cholinergic system plays a primary role in maintaining homeostasis. It has been proved that endogenous neuronal acetylcholine (ACh) could play an anti-inflammatory role, and exogenous cholinergic agonists could weaken macrophages inflammatory response to lipopolysaccharide (LPS) stimulation through activation of α7 subunit-containing nicotinic acetylcholine receptor (α7nAChR). We assumed that nonneuronal cholinergic system existing in macrophages could modulate inflammation through autocrine ACh and expressed α7nAChR on the cells. Therefore, we explored whether LPS continuous stimulation could upregulate the nonneuronal cholinergic activity in macrophages and whether increasing autocrine ACh could decrease TNF release from the macrophages. The results showed that, in RAW264.7 cells incubated with LPS for 20 hours, the secretion of ACh was significantly decreased at 4 h and then gradually increased, accompanied with the enhancement of α7nAChR expression level. The release of TNF was greatly increased from RAW264.7 cells at 4 h and 8 h exposure to LPS; however, it was suppressed at 20 h. Upregulating choline acetyltransferase (ChAT) expression through ChAT gene transfection could enhance ACh secretion and reduce TNF release from the infected RAW264.7 cells. The results indicated that LPS stimulation could modulate the activity of nonneuronal cholinergic system of RAW264.7 cells. Enhancing autocrine ACh production could attenuate TNF release from RAW264.7 cells.

1. Introduction

Acetylcholine (ACh), traditionally regarded solely as a neurotransmitter, is synthesized in cholinergic neurons and released via vesicular machinery in response to physiological and pharmacological stimulation. ACh acts through nicotinic and muscarinic receptors in nerves and peripheral tissues. In 1966, Morris reported that ACh was synthesized in the placenta and made the initial description of nonneuronal ACh synthesis [1]. Subsequently, growing evidence indicates that, besides neuronal ACh, a broad variety of nonneuronal cell types throughout the body (such as lymphocytes, macrophages, dendritic cells, adipocytes, keratinocytes, endothelial cells, and epithelial cells) also produce and release ACh and express choline acetyltransferase (ChAT), acetylcholinesterase (AChE), and acetylcholine receptors (AChRs) [2–9]. In 1998, Wessler et al. proposed the concept of “nonneuronal acetylcholine” [10]. A wide distribution of nonneuronal acetylcholine on multiple tissues or cells suggested that ACh and its receptors played a regulating role in maintaining the homeostasis of these tissues or cells by autocrine/paracrine signaling pathway, or as a participant in the progression of severe pathologies of some diseases [10–13].

Borovikova et al. [14] found that vagus nerve stimulation attenuated the systemic inflammatory response to endotoxin (lipopolysaccharide (LPS)) and developed the concept of “cholinergic anti-inflammatory pathway.” It was observed that vagotomy followed by LPS stimulation resulted in a higher level of tumor necrosis factor (TNF) alpha in serum compared with control animals receiving LPS alone or sham vagotomy [15]. It suggested that, under normal conditions (i.e., in the absence of nicotine), the vagus nerve release of acetylcholine at sites of peripheral tissue innervations
provides the source of agonist for the nAChR. The nAChR responsible for this effect was pharmacologically identified to be the α7 subunit-containing nicotinic acetylcholine receptor (α7nAChR) [16], which was later supported by studies in mice whose α7nAChR subunit was genetically eliminated. Wang et al. first reported that acetylcholine or nicotine pretreatment of human peripheral blood mononuclear cells acted through a posttranscriptional mechanism to reduce the amount of TNF present in the media 2 hours following challenge. Whether enhancing autocrine ACh in RAW264.7 cells could induce macrophages hyporesponsiveness to continuous or nonneuronal cholinergic system, which were involved in inducing hyporesponsiveness to exogenous antigenic stimulation.

Prior exposure of macrophages to minute amounts of LPS causes them to become refractory to subsequent LPS challenge, a phenomenon called “endotoxin tolerance” [18, 19]. The molecular mechanisms underlying endotoxin tolerance remain elusive. Because the inhibition of TNF release in endotoxin tolerant macrophages [20, 21] was similar to that in macrophages treated with cholinergic agonists [14–16, 22], accordingly we hypothesized that macrophages stimulated by minute LPS may modulate the synthesis and release of ACh as well as α7nAChR expression via their innate nonneuronal cholinergic system, which were involved in inducing macrophages hyporesponsiveness to continuous or subsequent LPS challenge.

Therefore, in this study we investigated whether LPS challenge affects nonneuronal cholinergic components in RAW264.7 cells (mice peritoneal macrophage line) and whether enhancing autocrine ACh in RAW264.7 cells could ameliorate the TNF release and benumb the response to LPS challenge.

2. Materials and Methods

2.1. Cell Line. RAW264.7 cells were purchased from the American Type Culture Collection (ATCC TIB-71, Manassas, VA, USA) and maintained in RPMI1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine and 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Carlsbad, CA, USA). Cell cultures were maintained at 37 °C, 5% CO2 atmosphere.

2.2. Cell Culture and Treatment. RAW264.7 cells were seeded in 12-well tissue culture plates at 10⁴ cells per well and were cultured overnight in RPMI1640 with 10% FBS. Prior to adding LPS or PBS, the cells were washed and replaced with fresh RPMI1640 with 10% FBS. Cells were exposed to lipopolysaccharide (LPS; endotoxin) (Escherichia coli, L4130 0111:B4; Sigma, St. Louis, MO, USA) (100 ng/mL) for 20 h or PBS (volume equaled with the added LPS) for 4 h. Cell culture media were collected at the times indicated in the figure legends (PBS, LPS 4 h, LPS 8 h, and LPS 20 h) and centrifuged at 1500 rpm for 5 min to sediment cell debris. The centrifuged media were aliquoted and were frozen at −80°C until ELISA analysis was performed.

2.3. Determinations of TNF and ACh. TNF and ACh secreted into the media by RAW264.7 cells were determined by enzyme-linked immunosorbent assay (ELISA) (Cusabio Biotech, China) according to the manufacturer’s instructions.

2.4. Determination of AChE Activity in Culture Medium. AChE activity of culture medium was determined by the method reported by Ellman et al. [23], which is based on the formation of the yellow 5-thio-2-nitrobenzoate anion produced in the reaction between 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) and thiocholine, after the AChE-mediated hydrolysis of ACh. The reaction was followed spectrophotometrically by the increase of absorbance at 412 nm. AChE activity of the medium was expressed as μmole per mL. The assay was performed according to the corresponding instruction of the assay kit supplied by Jiancheng Biological Institute (Nanjing, China).

2.5. Preparation of Cell Lysates and ChAT Content Analysis. RAW264.7 cells (10⁵/mL) were seeded in 6-well tissue culture dishes (2 x 10⁶ cells per well) and cultured overnight and replaced the medium. Then the cells were incubated with LPS or PBS for different periods of time at 37°C and 5% CO2 atmosphere. The cells were rinsed twice with PBS and disassociated with nonenzyme cell detach solution. The cells were collected and centrifuged 5 min at 1500 rpm at 4°C. The cells were washed twice with ice-cold PBS and centrifuged as above. The supernatants were discarded and 1 mL nonadenated cell lysis buffer was added to each sample to lyse the cells. The suspensions were vortexed for 10 s and kept on ice bath for 20 min. The lysates were centrifuged at 12000 rpm for 15 min at 4°C to remove the cell debris, and the supernatants were used to assay ChAT and TNF content with ELISA. The protein concentration of the lysates was determined by bicinchoninic acid (BCA). The reagents used in preparation of cell lysates were purchased from Applygen Technologies Inc. in China.

2.6. Flow Cytometric Analysis for α7nAChR Expression. Change in the expression of α7nAChR on RAW264.7 cells was detected by flow cytometry (FACS). RAW264.7 cells were stimulated with 100 ng/mL LPS for 4 h, 8 h, and 20 h, respectively, and the cells incubated with PBS (volume equaled with the added LPS) for 4 h were used as the control. At the corresponding time points, the cells were rinsed with PBS, disassociated with nonenzyme cell detach solution, and transferred to tubes. After being centrifuged 5 min at 1500 rpm at 4°C and washed with PBS, the cells were fixed in 4% polyformaldehyde/PBS 30 min. Then the cells were washed with PBS for three times and suspended in 0.4 mL PBS. A mouse monoclonal antibody against α7nAChR (sc-374284, Santa Cruz Biotechnology Inc., USA) was added in the suspension (1:100), incubated overnight at 4°C, followed by washing with PBS, and stained with FITC-conjugated goat anti-mouse IgG (H + L) (EarthOx, USA) for 20 min at room temperature. The cells were washed with 2 mL PBS and resuspended in 0.4 mL PBS. All FACS data were analyzed on 10⁶ cells in FACSCalibur 4-color flow cytometer.
2.7. RNA Extraction, Reverse Transcription, and Quantitative Real-Time Polymerase Chain Reaction. To determine TNF, ChAT, and AChE mRNA expression level, total RNA was isolated from PBS and LPS-treated RAW264.7 cells using TRIzol reagent (Gibco BRL, USA) according to the manufacturer’s instructions. The extracted RNA quality and quantity were determined by measuring absorption at 260/280 nm by spectrophotometer. Reverse transcription was carried out with 2 μg total RNA using the SuperScript II reverse transcription system (Invitrogen, USA) according to the manufacturer’s recommendations. Semiquantification of mRNA expression level was performed by real-time PCR using Fast SYBR Green Master Mix (ABI, USA) and a StepOne 7500 Fast (ABI, USA). PCR primers of the different target genes and their products’ length are indicated in Table 1. PCR program: 1 cycle of 95°C for 20 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. β-Actin was used as a housekeeping gene to normalize the amplification signals of target genes. The relative amounts of PCR product were determined using the standard ΔΔCT method. The dissociation stages, melting curves, and quantitative analyses of the data were performed using ABI instrument software SDS2.1.

2.8. RAW264.7 Cells Transfections. RAW264.7 cells were seeded in 12-well tissue culture plates at 5 x 10^4 cells per well and grown overnight in RPMI1640 supplemented with 10% fetal bovine serum. In the next day, the cells were infected with either empty vector or ChAT expression vector of constructed lentivirus with MOI20 according to the manufacturer’s protocol (GeneChem Co., China) for 12 hours. The media were discarded, and the cells were washed twice and replaced with fresh RPMI1640 supplemented with 10% FBS. After being cultured for 72 h, the transfected cells were disassociated and seeded in 24-well tissue plate at 6 x 10^5 cells per well and cultured for 20 h. The media were collected for determining ACh and TNF concentrations with ELISA. Cells lysates, RNA isolation, and RT-PCR were performed as described above. The transfection experiment was performed for three independent times.

2.9. ChAT Overexpressed RAW Cells Were Treated with LPS. The transfected RAW264.7 cells were seeded in 24-well tissue plate at 5 x 10^5 cells per well and cultured overnight. The cells were washed and replaced with fresh RPMI1640 with 10% FBS and then treated with LPS (100 ng/mL) or PBS for 4 hours. The media were collected, centrifuged, and aliquoted for determining TNF and ACh secretion. The cells lysates and total RNA extraction were performed as mentioned above. The TNF content in lysates was assayed using ELISA, and TNF mRNA expression level in the cells was quantified by real-time PCR as described in Section 2.6.

2.10. Statistical Analysis. The data, expressed as mean ± standard deviation (SD), were analyzed from one of two or three independent experiments. Significant differences were assessed by using one way analysis of variance (ANOVA) followed by least-significant-difference (LSD) test. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. LPS Stimulation Induces the Enhancement of Both TNF Release and mRNA Expression of RAW264.7 Cells. RAW264.7 cells, originating from mice macrophages, could respond to endotoxin and showed overproduction of inflammatory cytokines such as TNF, IL-1, IL-6, and IL-12. When stimulated with LPS (100 ng/mL), RAW264.7 cells released a large number of TNF (Figure 1(a)) and upregulated TNF mRNA expression level (Figure 1(b)). However, the increase of TNF release did not synchronize with that of its mRNA expression level: the amount of TNF release arrived at the peak following LPS stimulation for 8 hours, and subsequently it was decreased; TNF mRNA expression level was gradually enhanced with time-dependent manner during 20 h LPS incubation.

3.2. LPS Stimulation Modulated ACh Production and Upregulated α7nAChR Expression Level of RAW264.7 Cells. It has been known that prior exposure of macrophages to minute amount of endotoxin for 18–20 h could make them become refractory to subsequent endotoxin challenge, a phenomenon called “endotoxin tolerance.” We supposed that the innate nonneuronal cholinergic members in macrophages may be involved in inducing their tolerance to LPS overstimulation. So, we analyzed the change in ACh production and its ligand α7nAChR expression of RAW cells stimulated by LPS for 20 h. We observed that, in RAW264.7 cells, ACh secretion was significantly dropped following 4 h LPS exposure and then gradually increased by time-dependent fashion (Figure 2(a)). α7nAChR expression level of RAW264.7 cells was greatly enhanced following LPS stimulation and had been kept at high level during a period of 20 h LPS incubation (Figure 2(b)). It was shown that the increase of both ACh secretion and α7nAChR expression went with decrease of TNF release when RAW264.7 cells were incubated with LPS for 20 h but without relation to TNF mRNA expression fashion.

3.3. Effect of LPS Stimulation on ChAT Content in Lysates, AChE Activity in Culture Supernatants, and Their mRNA Expression Levels in RAW264.7 Cells. ACh, ChAT (an enzyme catalyzing ACh synthesis), AChE (an enzyme catalyzing ACh degradation), and AChRs constitute an integrated nonneuronal cholinergic system in RAW264.7 cells. Based on altered ACh production and upregulated α7nAChR expression in LPS treated RAW cells, we further analyzed changes in ChAT contents and AChE activity of LPS stimulating RAW cells. Generally speaking, the increase of ACh concentration is accompanied with the augmentation of ChAT content or the weakness of AChE activity. Curiously, we observed an unexpected phenomenon: ChAT content in RAW cells was greatly enhanced at LPS challenge 4h,
**Table 1: Sequences of primers used for real-time quantitative PCR analysis.**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primer sequences</th>
<th>PCR product length (bp)</th>
</tr>
</thead>
</table>
| TNFα        | F: ATGGGAAGGGAATGAATTCCACC  
              R: GTCCACATCCTGTAGGGCGTCT | 281 |
| ChAT        | F: GTCTCTGAATACTGGCTGATG  
              R: TGGTGCTTGGGAAGGTGCTG | 106 |
| AChE        | F: ACTACCGAGTGGGAACTTTGGC  
              R: CCTGTGGAAGAGGCTCCTGCTG | 224 |
| β-Actin     | F: GCCGTGACATCAAAGAGAAGC  
              R: AGCAGTGTTGGCATAGAG | 270 |

F: forward, R: reverse.

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**Figure 1:** Kinetics of TNF protein and mRNA expression in RAW264.7 cells stimulated with 100 ng/mL LPS. (a) TNF content in the supernatant was determined by ELISA; data are shown as OD450; (b) TNF mRNA expression level was determined by using real-time PCR. β-Actin was used as a control gene. The data shown are representative of three independent experiments. All values are expressed as mean ± SD (n = 3). *P < 0.05 compared with PBS, #P < 0.05 compared with LPS 4 h, and &P < 0.05 compared with LPS 8 h.

---

**Figure 2:** LPS stimulation reduced ACh release and upregulated α7nAChR expression of RAW264.7 cells. (a) ACh secretion in the supernatant of RAW264.7 cells incubated with LPS (100 ng/mL). ACh secretion decreased from RAW264.7 cells challenged with LPS for 4 h and then gradually recovered along with the LPS incubated time. (b) The rate of α7nAChR positive cells was increased in LPS stimulated RAW264.7 cells, which was maintained at a high level during 20 h LPS exposure. The data are expressed as fold increases compared to unstimulated cells (PBS group). All values are expressed as mean ± SD (n = 3), *P < 0.05 compared with PBS, #P < 0.05 compared with LPS 4 h, and &P < 0.05 compared with LPS 8 h. The data shown are representative of two individual experiments.
followed by a gradual decrease, which showed a negative change with autocrine ACh production. ChAT mRNA expression level was upregulated in RAW264.7 cells during 20h LPS stimulation. AChE activity in culture medium and mRNA expression level of RAW264.7 cells were not altered significantly during 8h LPS incubation. But at 20h following LPS stimulation, AChE activity in the supernatant was lowered obviously and its mRNA expression level was elevated significantly (Figure 3).

3.4. Upregulated Expression of ChAT by Transfecting Lentiviral Carried ChAT Gene Could Promote Autocrine ACh Synthesis and Suppress TNF Release of RAW264.7 Cells. To explore whether autocrine ACh could ameliorate TNF release from RAW264.7 cells, we employed the method of transfecting lentiviral carried ChAT gene into RAW264.7 cells to make the infected cells overexpressing ChAT and overproducing ACh. The data in Figure 4 showed that the cells transfected with ChAT expression vector (ChAT) could overexpress ChAT mRNA and produce more ACh than the control cells transfected with empty vector (EV) (Figures 4(c) and 4(d)). ACh production in the transfected cells (in the lysate) was greatly elevated; however, there was no significant difference in the amount of ACh secretion in culture supernatants between overexpressed ChAT cells and the control cells.

3.5. Increasing Autocrine ACh of RAW264.7 Cells Suppresses TNF Release but Upregulates TNF mRNA Expression. We next observed the effect of increasing autocrine ACh production on TNF release of RAW264.7 cells. Figure 5 shows TNF content in the culture supernatant and lysate, as well as TNF mRNA expression level of both ChAT vector and empty vector transfected RAW264.7 cells. In the presence or absence of LPS, the quantity of TNF in the supernatant was less in ChAT transfection RAW264.7 cells than in the control cells. However, it is puzzling why TNF content in lysate and TNF mRNA expression abundance were enhanced when ACh production was increased in ChAT overexpression RAW264.7 cells (Figure 5(c)). The results suggested that the increase of autocrine ACh production may inhibit TNF release rather than reduce TNF mRNA expression and protein synthesis.

4. Discussion

The present study suggests that continuous LPS stimulation could modulate the nonneuronal cholinergic autocrine activity of RAW cells. It is evidenced by changes of ACh secretion and upregulated expression of cholinergic ligand α7nAChR in LPS stimulated murine macrophages (RAW264.7 cells). Moreover, increasing autocrine ACh production via upregulating ChAT gene expression in RAW264.7 cells could suppress TNF release in presence or absence of LPS. The results indicated that macrophages could establish the adaptation to LPS stimulation partly via their innate nonneuronal cholinergic autocrine loop.

Animals pretreated with a low dose of endotoxin have a markedly reduced mortality when rechallenged with a normally “lethal” dose of endotoxin [24, 25], a phenomenon termed “endotoxin tolerance.” In humans, endotoxin tolerance was developed during five consecutive LPS administrations as demonstrated by the attenuated release of proinflammatory cytokines on the fifth day and was associated with less leukocyte and endothelial activation [26]. This refractoriness to LPS is considered to be an adaptation to prevent overstimulation from the continuous exposure to LPS [27, 28]. Nevertheless, it is now evident that the early proinflammatory phase of the sepsis is immediately followed by an anti-inflammatory response that rapidly results in an immunosuppressive state. Immunosuppression is believed to be the critical factor for the patients’ secondary infection and to increase the risk of mortality [29–31]. The development of immunosuppression is associated with negative feedback regulation and the so-called endotoxin or microbial tolerance. Various mechanisms have been proposed for endotoxin tolerance. Among them are the downregulation of the LPS-receptor TLR4 [32], loss of tyrosine phosphorylation of TLR4 [33], and decreased recruitment of the adaptor protein MyD88 to TLR4 or suppressed interaction between MyD88 and the kinase IRAK-1 [34, 35]. Furthermore, a shift from transcriptionally competent NF-κB heterodimers (p50/p65) to inactive homodimers (p50/p50) was associated with the tolerance state [36]. The upregulation of inhibitory proteins (such as IRAK-M, SOCS-1, and SHIP1) [37–39] and anti-inflammatory cytokatoes (IL-10 and TGF) [40] induced by low dose microbial stimulation also is involved in the generation of the hyporesponsive state. Recently, microRNAs in LPS-induced gene reprogramming urge a reevaluation of endotoxin tolerance [41]. Tracey’s group proposed that exacerbated release of TNF and other proinflammatory cytokines could be suppressed by the efferent vagus nerve based cholinergic anti-inflammatory pathway [14, 17] and further demonstrated that α7nAChR is an important component underlying the anti-inflammatory efficacy [16]. The discovery of the cholinergic anti-inflammatory pathway provides not only a new strategy for targeting excessive inflammatory diseases but also a novel clue for exploring the mechanisms inducing macrophages tolerance to LPS stimulation.

In this study, we observed that there are ACh, α7nAChR, ChAT, and AChE in RAW264.7 cells, which constitute a complete nonneuronal cholinergic system. ACh secreted in the supernatant dropped sharply when incubation of RAW264.7 cells with LPS for 4h subsequently increased in a time-dependent fashion during the period of 4h–20h LPS exposure. In contrast, α7nAChR expression on RAW264.7 cells, one of the cholinergic members, was upregulated significantly and maintained at a high level during 4h–20h LPS incubation. The relative increased autocrine ACh and upregulated expression of α7nAChR could strengthen the cholinergic activity in LPS challenged RAW264.7 cells through promoting the interaction between ACh and α7nAChR. It has been identified that prior exposure of macrophages to minute LPS for 12h–29h could lead to hyposresponsiveness to subsequent LPS challenge [20, 42]; meanwhile endogenous ACh and exogenous cholinergic agonists could decrease inflammatory cytokines release from LPS stimulated macrophages [14, 15, 22]. We infer that sustained LPS stimulation could upregulate the nonneuronal cholinergic activity in the challenged
RAW264.7 cells, which was likely to play a role in limiting the excessive inflammation of primed RAW264.7 cells. This kind of negative feedback regulation would contribute to the restoration of homeostasis of the primed cells. Nevertheless, the continuing negative feedback effect in vivo is likely to be one of the mechanisms mediating immunosuppression in sepsis.

Physiologically, the balance between synthesis and degradation of ACh depends on the coordination of ChAT and AChE expression. We assume that change of ACh secretion induced by LPS should associate with upregulation of ChAT expression or downregulation of AChE activity. However, we accidentally found that ChAT content was changed negatively with autocrine ACh production during 20 h LPS incubation and AChE activity was not changed obviously during 8 h LPS stimulation. Interestingly, at 20 h after LPS stimulation, there was cooperativity among ACh secretion, ChAT content, and AChE activity, which was presented as increased ACh release and reduced AChE activity in the supernatant and augmented ChAT content in the lysate of RAW264.7 cells. The correlation between AChE and ChAT levels was observed by Kaufer et al. [43]. They found that the ACh increase due to forced swimming stress or inhibitors of the acetylcholine-hydrolysing enzyme AChE could result in markedly upregulated expression of AChE mRNA, as well as downregulated expression of ChAT mRNA in cortex or sagittal corticohippocampal slices of mice. Their results indicate that acute cholinergic stimulation promotes selective bidirectional changes in the expression of genes regulating acetylcholine metabolism. In the present study, the coordinated regulation of AChE and ChAT in sustained exposure to LPS could support the balance between ACh synthesis and degradation in RAW264.7 cells and therefore make the cells adapt to LPS challenge. The regulation of AChE expression was complex and multiple.
Waiskopf et al. reported that fluoxetine as an antidepressant could intercept the LPS-induced decreases in intracellular AChE [44]. LPS exposure induced overexpression of the AChE-targeted microRNA-132 [45, 46] and AChE antisense ODN mEN101 [47] also have been found to suppress AChE expression. The induced expression of AChE possesses dual effects. On the one hand, the stressful induced AChE directly hydrolyzes Ach to promote inflammation. On the other hand, the intracellular AChE could interact with the nuclear factor kappa B-activating intracellular receptor for activated C kinase 1 to take part in anti-inflammation [44]. In addition, the induced AChE could selectively contribute to cellular apoptosis through its noncatalytic properties [48, 49]. The therapeutic agents can thus be targeted to the AChE protein, its encoding mRNA transcripts, or the regulator, opening new venues for therapeutic interference with immune system diseases.

TNF is one of potent proinflammatory cytokines induced by LPS challenge [50], as well as most probably the best marker of endotoxin tolerance as assessed by its dramatically reduced production following an LPS challenge in tolerant animals and macrophages [51, 52]. We examined the kinetics of TNF production and mRNA expression level in LPS treated RAW264.7 cells. It was found that, in the duration of 20 h exposure to LPS, TNF mRNA expression level was gradually increased in a time-dependent manner. However, the amount of TNF release was raised greatly at 4 h and arrived at the maximum at 8 h, followed by decrease at 20 h following LPS stimulation. The increase of TNF mRNA expression did not synchronize with that of TNF release. Since cholinergic anti-inflammatory pathway inhibits TNF expression at the posttranscriptional level [14, 52], we infer that the weakened TNF release at 20 h of LPS stimulation was associated with the increases of autocrine ACh release and α7nAChR expression. Nahid et al. have also reported that, in supernatant from cultured LPS-stimulated THP-1 cells, TNF started to appear within 2 h and reached a maximal level at 4 h of stimulation followed by gradually decreased starting at 8 h. They found that TNF increased up to 4 h and then decreased gradually implicating a negative correlation with miR-146a progression [41]. Hamano et al. found that nicotine suppressed the expression of CD14, toll-like receptor 4, intercellular adhesion molecule 1, B7.1, and CD40 on monocytes and the production of TNF via...
α7nAChR [53]. These data indicated that multiple regulating pathways, including nonneuronal cholinergic system, may take part in ameliorating the RAW264.7 cells’ response to LPS overstimulation. It is likely that autocrine ACh mediates macrophages’ hyporesponsiveness to continuous LPS stimulation through downregulating LPS signaling molecules.

In order to explore the effect of autocrine ACh on TNF release from RAW264.7 cells, ChAT expression vector of lentivirus was constructed and transfected into RAW264.7 cells. The infected RAW264.7 cells could upregulate ChAT expression, which resulted in increasing ACh production and decreasing TNF release from both LPS-stimulated and unstimulated RAW264.7 cells. Unexpectedly, both protein content and mRNA level of TNF were higher in ChAT expression vector infected cells than in empty vector infected cells. This means that the increase of ACh is able to suppress TNF release but unable to inhibit TNF mRNA expression and its protein synthesis. These data agree with Borovikova et al. finding that the anti-inflammation of cholinergic agonists is operated at the posttranscriptional level [14].

In summary, we described here for the first time that continuous LPS stimulation could upregulate autocrine ACh production and α7nAChR expression of RAW264.7 cells, which may play a primary role in inducing the hyporesponsiveness of macrophages to LPS stimulation. Increasing autocrine ACh could effectively ameliorate TNF release from RAW264.7 cells. These findings establish the autoregulatory effect of nonneuronal cholinergic system on RAW 264.7 cells.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Yi Lv and Jiayang Lu researched, analyzed, and interpreted the data; Yi Lv and Sen Hu wrote the paper; Ning Dong, Qian
Liu, Minghua Du, and Huiping Zhang assisted with research and analysis.

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References


Review Article

Anti-TNF-Alpha Therapy and Systemic Vasculitis

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TNF-α is a pleiotropic cytokine, which plays a major role in the pathogenesis of numerous autoimmune and/or inflammatory systemic diseases. Systemic vasculitis constitutes a group of rare diseases, characterized by inflammation of the arterial or venous vessel wall, causing stenosis and thrombosis. Treatment of the different type of vasculitis mainly relies on steroids and immunosuppressive drugs. In case of refractory or relapsing diseases, however, a second line of treatment may be required. Anti-TNF-α drugs have been used in this setting during the last 15 years with inconsistent results. We reviewed herein the use of anti-TNF-α therapy in different kind of vasculitis and concluded that, except for Behcet’s disease, this therapeutic option has not demonstrated significant improvement in the treatment of vasculitis.

1. Introduction

Tumour necrosis factor alpha (TNF-α) is a pleiotropic cytokine known to play a major role in host defense mechanisms, initiating a beneficial local inflammation which in excess, however, may cause tissue damage [1]. Since 1999, anti-TNF-α therapy has been used with success in the treatment of patients suffering from rheumatoid arthritis (RA), inflammatory enterocolitis (Crohn’s disease and ulcerative colitis), spondyloarthropathies, or psoriasis [2–6]. Randomized international studies have shown the efficacy of five currently commercially available anti-TNF-α molecules. These molecules have also been tested in other autoimmune and inflammatory systemic diseases such as severe vasculitis refractory to conventional treatment but, to date, vasculitis are not included in the list of therapeutic indications of anti-TNF-α agents.

Systemic vasculitis is a group of rare diseases characterized by inflammation of the arterial or venous vessel wall, causing stenosis or thrombosis [7]. Initially classified by the size of the vessel involved, primitive vasculitis has been recently reclassified with the introduction of immunological markers in the new Chapel Hill Consensus classification [8]. One can distinguish between large vessels vasculitis (giant cell arteritis (GCA) and Takayasu arteritis (TA)), medium vessels vasculitis (periarteritis nodosa (PAN)), and small vessels vasculitis with immune complex deposits (mixed cryoglobulinemia (MC)) or associated with anti-neutrophil cytoplasmic antibodies (ANCA) (granulomatosis with polyangitis (GPA) formerly Wegener granulomatosis, eosinophilic granulomatosis with polyangitis (EGPA) formerly Churg Strauss disease, and micropolyangitis (MPA)). In addition, some diseases may affect vessels of variable size (Behçet disease (BD)) [8]. We reviewed the published experience related to the use of anti-TNF-α therapy in these diseases, pointing to the fact that data are relatively rare and often contradictory.

2. Rationale for the Use of Anti-TNF-α in Vasculitis?

Two forms of TNF-α are synthesized by activated macrophages and dendritic cells: a transmembrane precursor form (26 Kda) which is proteolytically cleaved in a soluble form (17 kda) by a TNF-α converting enzyme (TACE) [9]. These two forms bind to two ubiquitous cell surface receptors (TNFR1 and TNFR2) on target cells to initiate proinflammatory genes transcription via the activation of
Nuclear Factor Kappa B (NFκB) and Mitogen Activated Protein (MAP) Kinase pathways, as well as proapoptotic genes transcription by the induction of death signal pathways [1, 10]. TNF-α induces leukoendothelial adhesion via increased expression of various adhesion molecules, such as E-selectin, Intercellular Adhesion Molecule 1 (ICAM-1), and Vascular Adhesion Molecule 1 (VCAM-1), and mediates tissue leukocyte infiltration through chemokine synthesis [11]. TNF-α induces metalloproteinase production and may also participate in endothelial cell death directly via apoptosis or indirectly via neutrophil activation [10].

In addition, TNF-α may play a role in neutrophil “priming” inducing membrane expression of proteinase-3 or myeloperoxidase, which are subsequently recognized by ANCA in ANCA-associated vasculitis (AAV) [12]. This cytokine may thus be involved in the pathogenesis of different kind of vasculitis. In addition, binding of anti-TNF-α to membrane-associated TNF-α can have an agonistic action, initiating reverse signaling and processes such as apoptosis, cytokine suppression, and cell activation, which could constitute an interesting target in the treatment of vasculitis [11, 13]. To date, 5 different anti-TNF-α drugs have been developed and are commercially available, 3 consisting in monoclonal antibodies (infliximab (IFX), adalimumab (ADA), and golimumab). IFX is usually used intravenously at 3 to 5 mg/kg every 8 weeks, and ADA and golimumab are used subcutaneously, 40 mg every 2 weeks for the former and 50 mg once a month for the latter. The fourth available drug is a dimer of a chimeric protein genetically engineered by fusing the extracellular ligand binding domain of human tumour necrosis factor receptor 2 (TNFR2/p75) to the Fc domain of human IgG-1 (etanercept (ETN)) and is used subcutaneously at 25 mg twice a week. The last is a humanised Fab fragment conjugated to polyethylene glycol (certolizumab pegol) but has never been used in vasculitis. Monoclonal antibodies and certolizumab are active on the two molecular forms of TNFα, whereas etanercept neutralizes soluble TNF-α only.

3. Large Vessels Vasculitis

3.1. Giant Cell Arteritis (GCA). The pathogenesis of GCA seems due to an abnormal cell-mediated immune response taking place in the vessel wall, leading to macrophage activation, giant cell formation, and excess production of interferon gamma [14]. Other proinflammatory cytokines such as IL-1, IL-6, and IL-17 may be involved in GCA pathogenesis, whereas experimental data showing the role of TNF-α in this disease are sparse [15]. GCA mostly affects people older than 50 years. Long-term corticosteroids remain the main treatment which is unfortunately commonly complicated by many side effects [16]. Immunosuppressive drugs such as methotrexate (MTX) or azatioprine (AZT) have been used in order to have a steroid sparing effect and in some corticoidependent/resistant patients. MTX was tested in 3 prospective studies with contradictory effects, and AZT gave disappointing results in a controlled study enrolling 31 patients [17–20]. Thus, after a few cases showing successful anti-TNF-α treatment in corticoidependent GCA patients have been reported, a comparative double blind study was conducted using IFX but was subsequently stopped due to the lack of efficacy on the prevention of relapse [21]. Regarding ETN, a randomized controlled study against placebo was conducted on 17 patients and demonstrated a significant corticosteroid sparing effect after one year, but not for a longer period which was nevertheless the primary end point [22]. Finally, ADA showed no benefit in a prospective study including 70 patients with a primary end point of steroid sparing at week 26 [23]. In view of these different studies, anti-TNF-α therapy is not recommended in GCA (Table 1).

3.2. Takayasu Arteritis (TA). TA is characterized by inflammation of large vessels, primarily the aorta and its main branches, resulting in anerysm formation, vascular stenosis, or occlusion, affecting mainly young women. TA is a chronic idiopathic granulomatous panarteritis, resulting from infiltration of the three layers of the vessel wall by macrophages, T lymphocytes, and natural killer cells [14, 24]. First line treatment consists in high doses of corticosteroids [24]. However, almost 50% of TA patients demonstrate glucocorticoid resistance or relapsing disease, requiring the addition of immunosuppressive agents such as AZT, cyclophosphamide (CYC), or mycophenolate mofetil (MMF) with inconsistent efficiency [25]. To date, approximately 120 TA patients have been treated with anti-TNF-α, mostly with IFX, as a second/third line immunosuppressive therapy. A first open label prospective study involving 15 patients with active or relapsing disease (8 treated by infliximab and 7 by etanercept) suggested some therapeutic efficacy which was confirmed by other studies (90% rate of remission and 60% for sustained remission) [26–28] (Table 2). Relapses, however, seem to be common after the drug is stopped following remission [27]. In summary, despite the fact that no prospective controlled study has been conducted to date, anti-TNF-α may constitute an interesting therapeutic option in TA patients who have been unable to achieve or maintain remission with steroids alone or common immunosuppressive agents.

4. Medium Size Vessels Vasculitis

4.1. Periarteritis Nodosa (PAN). PAN is a rare necrotizing vasculitis complicating hepatitis B virus chronic carriage affecting medium size vessels, whose incidence has declined since the introduction of hepatitis B vaccination and antiviral treatments [29]. The treatment of PAN consists in steroids or immunosuppressive drugs in association with antiviral therapy, according to the gravity of the disease [29, 30]. In a few case reports, IFX has been used in refractory forms of the disease or because of intolerance of conventional drugs and seems to be effective [31].

5. Small Size Vessels Vasculitis

5.1. Mixed Cryoglobulinemia (MC). MC is a small vessel vasculitis involving skin, joints, peripheral nerves, and the kidney, which is mainly associated with hepatitis C, Sjögren’s syndrome, or lymphoma [32]. MC is a model of immune-complex-mediated inflammation of blood vessels and may involve TNF-α [33]. Before the area of anti-CD-20-targeted
5.2. ANCA-Associated Vasculitis (AAV). AAV is a group of multisystemic diseases characterized by a pauci-immune small vessel vasculitis which includes three different entities: two recently renamed granulomatosis with polyangiitis (GPA), eosinophilic GPA (EGPA), and microscopic polyangiitis (MPA). AAV pathogenesis is consistent with a primary role for neutrophils, which are both the effector cells responsible for endothelial damage via oxygen radical synthesis and enzyme degranulation and the targets of ANCA [37]. Current AAV treatment is based on a six-month induction phase associating high-dose steroids with immunosuppressive drugs such as CYC or rituximab, followed by an 18-month maintenance therapy with AZT [38]. However, using these standard treatment protocols, relapses are very common, occurring in 49% and 35% of patients with GPA and MPA, respectively [29, 39]. Moreover, some patients remain refractory to conventional treatments, raising the need for new therapeutic options.

Etanercept was tested initially in an open label trial including 20 relapsing or incompletely controlled GPA and seemed efficient with a 3-point reduction of the Birmingham Vasculitis Activating (BVAS) score at 6 months [40]. These results were not confirmed in a larger controlled prospective study including 180 GPA patients [41]. However, it should be noted that this latter study had some design limitations since the two groups were not homogeneous at the baseline, and some patients had localized forms of the disease. Finally, although ETN is known to be of little usefulness in granulomatous disease, only the WGET study provides data confirming that the addition of ETN to usual treatments is ineffective in the maintenance regimen of GPA [41]. Anti-TNF-α monoclonal antibodies have also been tested in refractory AAV patients. The efficiency of infliximab has been observed in prospective observational studies [42, 43]. One multicentric prospective randomized control trial involving 17 patients compared the efficacy of infliximab (n = 9) or rituximab (n = 8) in association with steroids and immunosuppressive drugs in refractory GPA for a median follow-up of 30.6 months (+/-15.4). Efficacy of infliximab and/or rituximab to obtain remission at one year was observed, with an advantage for rituximab. During long-term follow-up, rituximab was also more effective at obtaining and maintaining remission [44]. Recently, 33 patients with active AAV (BVAS > 10) were enrolled in an open prospective trial to study infliximab adjunction to standard therapy in order to obtain remission for a median follow-up of 12 months and demonstrated no benefit with anti-TNF-α. However, this was a noncontrolled study and groups lacked homogeneity [45]. The last open label prospective study was conducted with adalimumab associated with standard therapy in refractory AAV with renal involvement. Although no significant gain in response rate was observed, a significant steroid sparing effect was noted [46].

In conclusion, it seems that a short course anti-TNF-α therapy using infliximab may be useful in complement to
6. Variable Size Vessels Vasculitis

6.1. Behcet’s Disease (BD). BD is a chronic and relapsing systemic inflammatory disorder characterized by recurrent orogenital ulcerations with possible mucocutaneous, ocular, digestive, vascular, and/or central nervous system involvements. BD pathogenesis is still unclear, but an association between genetic intrinsic factors (HLA-B5) and triggering extrinsic factors is suspected [49, 50]. First line treatments are adapted to each organ involvement, such as colchicine for mucocutaneous symptoms or combination of steroids and immunosuppressive drugs in case of severe visceral involvement [51]. Some patients however may develop severe and even life-threatening complications despite these standard treatments regimens. The pathogenic role of TNF-α in mediating tissue injury during BD seems to be major, and increased levels of TNF-α and soluble TNF receptors have been found in the peripheral blood of patients with active BD [52, 53].

In 2001, anti-TNF-α treatments were first tested for severe eye involvement showing promising results [54]. A meta-analysis collecting 158 patients included in 14 prospective studies testing infliximab in BD with ocular lesions refractory to immunosuppressive drugs was realized in 2011. A complete or partial remission was achieved in 65% and 24% of patients receiving infliximab, allowing glucocorticoids and immunosuppressive release in about 40% [55]. A recent open label prospective study enrolling 63 patients receiving infliximab showed similar results after one year of treatment [56]. Interestingly, the improvement was rapidly obtained following the initiation of infliximab [57].

Five open prospective studies demonstrated a beneficial effect of long-term infliximab treatment on the prevention of relapse, maintenance of visual acuity, and immunosuppressive drugs tapering [57–61] (Table 4). Intravitreous injection of infliximab was also tested in refractory uveitis in 15 patients and demonstrated a positive effect [62]. Regarding the use of adalimumab, etanercept, and golimumab, only case reports are currently available but showed beneficial effects in refractory uveitis, [63–65].

Anti-TNF-α treatments were tested in severe refractory cutaneous manifestations, especially etanercept which revealed a significant efficacy compared to placebo on oral ulcers, and nodular lesions [66].

Infliximab in monotherapy was also tested in refractory entero-Behcet during a prospective open trial and showed 100% improvement on clinical, CT-scan, and colonoscopy [67]. Adalimumab may also be efficient in this kind of patients [68].

In addition, infliximab has been used in BD affecting the central nervous system (CNS) in open prospective

<table>
<thead>
<tr>
<th>References</th>
<th>Design/anti-TNF-α therapy</th>
<th>Number of patients</th>
<th>Main objectives</th>
<th>Follow-up</th>
<th>Main results</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stone et al. [40]</td>
<td>Open label trial ETN</td>
<td>20 GPA</td>
<td>BVAS at 6 months adverse events during 6 months</td>
<td>6 months</td>
<td>3 points Decrease of BVAS (P &lt; 0,05)</td>
<td>Injection site reaction in 25% of patients still taking ETN</td>
</tr>
<tr>
<td>WGET research group [41]</td>
<td>Randomized controlled trial ETN versus placebo</td>
<td>180 GPA (89 ETN, 91 placebo)</td>
<td>Sustained remission at 27 months (BVAS = 0)</td>
<td>27 months</td>
<td>69,7% for ETN versus 75,3% for placebo (NS)</td>
<td>56,2% for ETN versus 57,1% for placebo had a life threatening event (NS)</td>
</tr>
<tr>
<td>Morgan et al. [45]</td>
<td>Open label trial IFX</td>
<td>33 (22 GPA, 11 MPA) (16 IFX, 17 standard treatment)</td>
<td>Time to clinical remission (BVAS ≤ 1)</td>
<td>12 months</td>
<td>No difference between the two groups</td>
<td>Infections in 8 patients for IFX and 7 for standard treatment (NS)</td>
</tr>
<tr>
<td>De Menthon et al. [44]</td>
<td>Randomized controlled trial IFX versus rituximab</td>
<td>17 GPA (9 IFX, 8 RTX)</td>
<td>CR/PR at month 12</td>
<td>12 months</td>
<td>IFX: 2 CR, 1 PR RTX: 4 CR, 1 PR</td>
<td>One death in both groups (invasive Aspergillosis for IFX and sudden death for RTX)</td>
</tr>
<tr>
<td>Laurino et al. [46]</td>
<td>Phase 2 open label trial ADA</td>
<td>14 (9 GPA, 5 MPA)</td>
<td>(i) Induction of remission within the first 14 weeks, (ii) time to remission</td>
<td>17 months</td>
<td>(i) 78,5% achieved remission (ii) Time to remission 12 weeks</td>
<td>Infections in 3 patients (1 mild and 2 severe including 1 death)</td>
</tr>
</tbody>
</table>

NS: Nonsignificant; RTX: rituximab; CP: complete remission; PR: partial remission.
Table 4: Open label and randomized controlled trials performed in Behcet’s uveitis.

<table>
<thead>
<tr>
<th>References</th>
<th>Design/anti-TNF-α therapy</th>
<th>Number of patients</th>
<th>Main objectives</th>
<th>Follow-up</th>
<th>Main results</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Okada et al. [56]</td>
<td>Open label trial IFX</td>
<td>63</td>
<td>Efficacy of IFX in the first year of treatment</td>
<td>12 months</td>
<td>Improvement in 69% Improvement somewhat in 23% Unchanged in 8%</td>
<td>46% of side effects including 3 infusion reactions No serious side effects</td>
</tr>
<tr>
<td>Sfikakis et al. [57]</td>
<td>Open label trial IFX</td>
<td>25</td>
<td>Remission at day 28</td>
<td>28 days</td>
<td>89% of complete remission</td>
<td>No serious side effects</td>
</tr>
<tr>
<td>Ohno et al. [58]</td>
<td>Open label trial IFX</td>
<td>12</td>
<td>Frequency of ocular attacks</td>
<td>14 weeks</td>
<td>Reduction in the number of relapses for IFX (5 mg/kg and 10 mg/kg) One case of tuberculosis (IFX 10 mg/kg)</td>
<td></td>
</tr>
<tr>
<td>Accorinti et al. [59]</td>
<td>Open label trial IFX</td>
<td>12</td>
<td>Frequency of ocular attacks</td>
<td>15 months</td>
<td>91% of reduction in the number of relapses</td>
<td>33% of side effects including one tuberculosis and one herpetic keratitis</td>
</tr>
<tr>
<td>Tognon et al. [60]</td>
<td>Open label trial IFX</td>
<td>7</td>
<td>Frequency of ocular attacks</td>
<td>23 months</td>
<td>21 to 6 ocular attacks observed in the equivalent period of time before treatment One infusion reaction</td>
<td></td>
</tr>
<tr>
<td>Tugal-Tutkun et al. [61]</td>
<td>Open label trial IFX</td>
<td>13</td>
<td>Absence of ocular attacks</td>
<td>6 years</td>
<td>31% remained attack-free 32 to 13 ocular attacks observed in the equivalent period of time before treatment No serious side effects (7 respiratory tract infection and one infusion reaction)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Open label and randomized controlled trials performed in BD with cutaneous, intestinal, and central nervous system involvements.

<table>
<thead>
<tr>
<th>References</th>
<th>Type of involvement/design/anti-TNF-α therapy</th>
<th>Number of patients</th>
<th>Main objectives</th>
<th>Follow-up</th>
<th>Main results</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melikoglu et al.  [66]</td>
<td>Cutaneous/entero randomized controlled trial/ETN versus placebo</td>
<td>40 (20 ETN/20 placebo)</td>
<td>(i) Pathergy response and monourate sodium status (ii) frequencies of mucocutaneous manifestations</td>
<td>4 weeks</td>
<td>(i) No differences between the two groups (ii) decrease of frequency of mucocutaneous manifestations</td>
<td>No serious side effects</td>
</tr>
<tr>
<td>Iwata et al. [67]</td>
<td>Entero-Behcet/open label trial/IFX</td>
<td>10</td>
<td>Clinical manifestations CT-scan</td>
<td>12 months</td>
<td>Rapid and dramatic improvement for all the patients</td>
<td>No serious side effects</td>
</tr>
<tr>
<td>Kikuchi et al. [69]</td>
<td>Neuro-Behcet/open label trial/IFX</td>
<td>5</td>
<td>Clinical manifestations brain magnetic resonance imaging</td>
<td>24 weeks</td>
<td>Improvement in 3 patients</td>
<td>One pneumocystis pneumonia</td>
</tr>
<tr>
<td>Giardina et al. [70]</td>
<td>Neuro-Behcet/open label trial/IFX</td>
<td>21</td>
<td>Clinical manifestations (CR/PR)</td>
<td>54 weeks</td>
<td>85% of CR 9% of PR</td>
<td>No serious side effects</td>
</tr>
</tbody>
</table>

CR: complete remission; PR: partial remission.

studies and was almost always successful [69, 70]. These patients were refractory to high-dose steroids combined with various immunosuppressive drugs and demonstrated major improvement or stabilization of their symptoms. Long-term remission (6–18 months) after discontinuation of infliximab therapy was noted in 75% of patients [69, 70] (Table 5). Adalimumab and etanercept have also been tried in case reports of BD with CNS involvement with a favorable outcome [71, 72]. In case of a first anti-TNF-α failure in refractory BD, a switch of molecule was made in up to 25% of cases with a 70% improvement [73].

In summary, infliximab seems effective in induction treatment and relapse prevention in severe BD refractory to glucocorticoids or immunosuppressive drugs, especially
in case of eye involvement. It could also be an alternative therapy to immunosuppressive drugs in case of central nervous system or gastrointestinal manifestations. Nevertheless, the main limitation of the present analysis is that most information originated from limited cases or noncontrolled studies, strongly raising the need for properly randomized controlled clinical trials.

7. Safety and Tolerance of TNF-Alpha Blockade

In vasculitis, anti-TNF-α are often prescribed as second/third line treatments in patients already immunocompromised by long-term use of glucocorticoids and immunosuppressive drugs. Despite this fact, side effects have been reported up to 46% and are mostly moderate. The literature review suggests that these drugs are rather safe, in agreement with what is known in RA and spondyloarthropathy [56, 74].

Patients treated with anti-TNF-α are prone to develop soon after initiation bacterial and viral infections mostly affecting respiratory or urinary tracts and cutaneous or soft tissues. Furthermore, reactivation of latent tuberculosis or extrapulmonary forms of this infection is another well-known threat [75–81].

The role of anti-TNF-α therapy in carcinogenesis and tumor progression remains a matter of controversy. A large study assessed the risk of cancer in a RA cohort treated with anti-TNF-α and showed a relative risk (RR) of 1.00 (95% CI: 0.86–1.15) compared to the biotherapy naïve RA cohort. RR did not increase with longer exposure or with the cumulative duration of active anti-TNF-α therapy during a 6-year follow-up period [82]. Regarding the risk of hemopathy, infliximab and etanercept were not associated with the occurrence of lymphoma in a study involving 19,000 patients with RA [83]. One observational study, however, showed a positive association between anti-TNF-α therapy in RA and nonmelanoma skin cancers with a follow-up period of 3 years [84]. In vasculitis, especially AAV, an unusually high frequency of solid cancer was reported in a randomized controlled trial that evaluated etanercept for maintenance of remission in 180 patients with GPA. But all the patients (n = 6) who developed cancer also received standard therapy associating MTX or CYC, which are known to be involved in cancerogenesis [44]. Without large specific epidemiological studies, caution is however advised.

Besides infection and malignancy, anti-TNF-α treatments can induce acute infusion reaction, which may lead to discontinuation of the treatment. Anti-TNF therapy may also favor antinuclear antibodies appearance which are, however, weakly associated with clinical symptoms [85, 86]. Other extending complications are allocated to anti-TNF-α treatments, such as the occurrence of demyelinating and sarcoid-like granulomatous diseases [87].

Nevertheless, anti-TNF-α should be used in the treatment of BD refractory to immunosuppressive drugs, especially in case of ocular, CNS, or digestive tract involvement. Some interesting data are also available for the use of anti-TNF-α treatments in refractory AAV, but the recent reports on rituximab efficacy in these diseases and the relative innocuity of this drug may limit the use of anti-TNF-α in these diseases, in the future. In both BD and AAV, an anti-TNF monoclonal antibody especially infliximab should be preferred to etanercept. In addition, physicians must be aware of the risk of infection using these drugs, especially in patients already immunocompromised by previous treatments. Regarding other vasculitis, published data are not in favor of efficiency of anti-TNF-α which, therefore, should not be used.

8. Conclusion

Anti-TNF-α treatments in vasculitis did not demonstrate the same efficacy as in other inflammatory diseases such as RA.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References


Potent Anti-Inflammatory and Antiproliferative Effects of Gambogic Acid in a Rat Model of Antigen-Induced Arthritis

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Background. We have previously reported a continuous activation of caspase-1 and increased interleukin (IL)-1β levels in early rheumatoid arthritis (RA). These observations raised the hypothesis that drugs targeting the IL-1β pathway, in addition to tumour necrosis factor (TNF), may be particularly effective for early RA treatment. We have recently identified gambogic acid as a promising therapeutic candidate to simultaneously block IL-1β and TNF secretion. Our main goal here was to investigate whether gambogic acid administration was able to attenuate inflammation in antigen-induced arthritis (AIA) rats.

Methods. Gambogic acid was administered to AIA rats in the early and late phases of arthritis. The inflammatory score, ankle perimeter, and body weight were evaluated during the period of treatment. Rats were sacrificed after 19 days of disease progression and paw samples were collected for histological and immunohistochemical evaluation.

Results. We found that inflammation in joints was significantly suppressed following gambogic acid administration. Histological and immunohistochemical evaluation of treated rats revealed normal joint structures with complete abrogation of the inflammatory infiltrate and cellular proliferation.

Conclusions. Our results suggest that gambogic acid has significant anti-inflammatory properties and can possibly constitute a prototype anti-inflammatory drug with therapeutic efficacy in the treatment of inflammatory diseases such as RA.

1. Introduction

Rheumatoid arthritis (RA) is the most common disease of the inflammatory joint diseases, affecting about 1% of the world population. The disease can have a very aggressive course and poor outcome as inferred by the analysis of its social impact (after 10 years of disease duration, more than 50% of RA patients are unable to perform professional activities) [1] and life expectancy diminishes 10 years due to disease activity and associated comorbidities [2]. RA is a chronic systemic inflammatory disease characterized by synovial hyperplasia caused by a large proliferative cellular infiltrate of leukocytes, high expression levels of proinflammatory cytokines, and consequent erosion of joint cartilage and bone. The therapeutic approach of RA has been revolutionized in the last decade with the discovery of specific targeted treatments. However, despite all available therapeutic options, RA remains a progressive, destructive, and debilitating disease with only 20% of patients reaching remission [3]. Anakinra, an antagonist of interleukin (IL)-1, was approved for RA treatment in the last decade. However, the real impact on disease activity has been shown in practice to be lower than what was anticipated from clinical trial results, casting doubts on the role of IL-1β as a therapeutic target [4]. Nonetheless, we have previously reported increased levels of IL-1β in very recent onset arthritis and in the synovial fluid of established RA patients [5]. This observation could be explained by the activation of caspase-1 that we also have observed both in early and established RA patients [6]. Therefore, it is possible that IL-1β plays an important role in early rather
than late stages of the disease and that pathways regulating this cytokine, such as caspase-1 and NF-κB activation, can potentially constitute promising therapeutic targets for specific drugs. The effect might be further boosted if an inhibitory effect on tumour necrosis factor (TNF) can also be achieved. Based on the results of a recent drug screen for compounds that simultaneously inhibit IL-1β and TNF secretion, we chose gambogic acid as a promising therapeutic candidate for the treatment of arthritis. Gambogic acid is a polypropylated xanthone abundant in resin derived from *Garcinia hanburyi* and *G. Morella* and is used in Southeast Asia complementary and alternative medicine [7]. Recent studies showed that gambogic acid could inhibit the growth of a wide range of tumour cells [8]. Our aim in this study was to investigate whether gambogic acid administration was able to attenuate inflammation in a rat model of antigen-induced arthritis (AIA).

2. Materials and Methods

2.1. Ethics Statement. All experiments were approved by the Animal User and Ethical Committees at the Instituto de Medicina Molecular, according to the Portuguese law and the European recommendations.

2.2. Compounds. Gambogic acid was purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

2.3. IL-1β and TNF Quantification. THP-1 cells were stimulated for 6 hours with 4% PFA-fixed DH5 *Escherichia coli* (*E. coli*) at a multiplicity of infection (MOI) of 20 bacterial cells per THP-1 cell, 1 hour after incubation with gambogic acid. Cell supernatants were collected and IL-1β and TNF cytokines were quantified by enzyme-linked immunosorbent assay (ELISA) (R&D systems, USA) according to the provider’s instructions.

2.4. AIA Rat Model and Assessment of Arthritis. Female Wistar AIA rats were purchased from Charles River Laboratories International (MA, USA) and maintained under specific pathogen-free (SPF) conditions. Animals were inoculated under isoflurane anesthesia by subcutaneous injection of complete Freund’s adjuvant (CFA) containing *Mycobacterium butyricum* in the rat right pad which leads to a profound systemic inflammation resulting in severe joint swelling and destruction. Gambogic acid was administrated at a dose of 4 μg/g body weight every day [9]. Drugs and vehicle control (dimethyl sulfoxide, DMSO) were dissolved in normal saline solution and injected intraperitoneally to AIA rats after 4 days (early treatment group, *N* = 10) and after 11 days (late treatment group, *N* = 5) of disease induction, when arthritis was already present. Healthy nonarthritic (*N* = 10) and vehicle-injected (*N* = 10) animals were used as controls for comparison. The inflammatory score, ankle perimeter, and body weight were measured during the time of treatment. Inflammatory signs were evaluated through the counting of the score of each joint in a scale of 0–3 (0: absence, 1: erythema, 2: erythema and swelling, and 3: deformities and functional impairment). The total score of each animal was defined as the sum of the partial scores of each affected joint [10]. Rats were sacrificed after 19 days of disease evolution and paw samples were collected for histological and immunohistochemical evaluation.

2.5. Histology and Immunohistochemistry. For histopathological observation, paws, lungs, livers, kidneys, spleens, and pancreas samples were collected at the time of sacrifice. Samples were fixed immediately in 10% neutral buffered formalin solution and then dehydrated using increased ethanol concentrations (70%, 96%, and 100%). Paw samples, after being fixed, were also decalcified in 10% formic acid. Samples were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin for morphological examination. Paws were also used for immunohistochemical staining with Ki67 antibody, a cellular proliferation marker. Tissue sections were incubated with primary antibody against rat polyclonal Ki67 (Abcam, UK) and with EnVision + (Dako, Denmark). Colour was developed in solution containing diaminobenzidine-tetrahydrochloride (Sigma, USA), 0.5% H2O2 in phosphate-buffered saline buffer (pH 7.6). Slides were counterstained with hematoxylin and mounted. All images were acquired using a Leica DM 2500 (Leica microsystems, Germany) microscope equipped with a colour camera. Data regarding the degree of proliferation of synovial cells was scored from 0–3 (0: fewer than three layers, 1: three to four layers, 2: five to six layers, and 3: more than six layers). Lymphoid cell infiltration was scored from 0–3 (0: none to diffuse infiltration, 1: lymphoid cell aggregate, 2: lymphoid follicles, and 3: lymphoid follicles with germinal center formation) [11].

2.6. Caspase-1 and NF-κB Assay. THP1 (ATCC TIB-202) macrophage-like cell line and THP1/NF-κB reporter cell line were cultured in R10-RPMI media 1640 supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin, 1% (v/v) pyruvate, 1% (v/v) L-glutamine, 1% (v/v) nonessential amino acids, 1% (v/v) hepes buffer, and 2-mercaptoethanol to a final concentration of 0.05 M, as recommended by the American Tissue Culture Collection (ATCC). Cells were cultured at 250,000 cells/mL, incubated with 10 μM of gambogic acid for 1 h at 37°C 5% CO2, and then stimulated with PFA-fixed *E. coli* (20 *E. coli* per cell) for 8 h and 24 h at 37°C 5% CO2. Simultaneously, nonstimulated negative control cells were also cultured at the same density as the stimulated population for comparison. Caspase-1 activity was measured in THP1 macrophage-like cell line using the Carboxyfluorescein FLICA Detection kit for Caspase Assay (Immunochemistry Technologies, LLC, USA) following the reagent instructions. Briefly, cells from the different assays were protected from light exposure while incubated for 1 hour at 37°C with 30X FLICA solution at a 1:30 ratio. NF-κB activity was measured in THP1/NF-κB reporter cell line. Lentiviral particles carrying a NF-κB-responsive GFP-expressing reporter gene (Cignal Lenti Reporters, SABiosciences, USA) were used to infect THP-1 cells and to establish a stable cell line. All samples were analyzed by flow
cytometry using a FACS Calibur (BD biosciences, USA). The data collected were further analyzed using FlowJo software (Tree Star Inc., USA).

2.7. Statistical Analysis. Statistical differences were determined with nonparametric Kruskal-Wallis and Mann-Whitney tests using GraphPad Prism (GraphPad, USA). Differences were considered statistically significant for \( P < 0.05 \).

3. Results

3.1. Gambogic Acid Reduces IL-1β and TNF Production. To study the effect of this drug on the inhibition of IL-1β and TNF secretion, we treated the human THP-1 macrophage-like cell line with growing concentrations of gambogic acid for 1 hour before challenging them with PFA-fixed E. coli for 6 hours. The conditioned media was then probed for the secretion of either IL-1β or TNF using ELISA. Gambogic acid significantly inhibited the secretion of both cytokines over a wide range of concentrations (Figure 1), confirming the previously reported effect of gambogic acid in blocking the secretion of these cytokines [12] and validating our earlier findings.

3.2. Gambogic Acid Inhibits the Activation of NF-κB and Caspase-1. Pro-IL-1β and TNF both depend on NF-κB activation for the transcription of their respective mRNAs. Pro-IL-1β processing is further dependent on the activation of caspase-1. We therefore tested the effect of gambogic acid on these key pathways. To investigate the effect of this drug in the activation of NF-κB, we used an NF-κB reporter cell line created by stably infecting THP-1 cells with a commercial lentiviral GFP reporter under the control of a minimal CMV promoter and tandem repeats of the NF-κB transcriptional response element (TRE). Gambogic acid was able to suppress NFκ-B reporter activation upon E. coli stimulation in comparison with cells that were also stimulated but did not receive treatment (Figure 2(a)). To test the effect of this drug on caspase-1 processing and activation, we used a caspase-1 fluorescent substrate and measured relative active caspase-1 levels using FACS. Also in this setting, gambogic acid significantly decreased the activation of caspase-1 (Figure 2(b)).

3.3. Gambogic Acid Is Able to Suppress Inflammation in Wistar Rat Antigen-Induced Arthritis. To study the anti-inflammatory properties of gambogic acid in vivo, AIA rats were treated daily with 4 µg/g body weight of gambogic acid intraperitoneally after the disease had already become symptomatic. We started the treatment after 4 days of disease induction (early treatment group) and after 11 days of disease induction (late treatment group). The inflammatory score and ankle perimeter were evaluated during the period of treatment. As shown in Figure 3, by the 4th day, all induced animals already presented with arthritis. All induced animals received either vehicle or gambogic acid at that time point. After 6 days of treatment, the vehicle-injected group increased sharply the inflammatory manifestations, whereas, in gambogic acid-treated rats, there was minimal inflammatory activity or even complete abrogation of arthritis manifestations. In the late treatment group, drug administration was started after 11 days of disease evolution, when animals presented a mean inflammatory score of 6. Also in this group, by the second day of treatment with gambogic acid, the inflammatory manifestations started to significantly decrease over time. This result shows that this drug has anti-inflammatory effects even when administrated in a later phase of arthritis. After 15 (early treatment group) and 8 (late treatment group) days of treatment, gambogic acid showed significant anti-inflammatory effects, as assessed by the evaluation of the inflammatory score (Figure 4) and ankle perimeter (\( P = 0.0126 \) in early group and \( P = 0.0126 \) in late group versus untreated animals). We have also tested 2 µg/g body weight subcutaneously every day. With this regimen, in the early treatment group, the anti-inflammatory effects were significant (\( P = 0.0048 \)) but not as dramatic as the 4 µg/g body weight intraperitoneally, and, in the late treatment group, the drug had no efficacy. In addition, we have observed that a subset of animals kept a low inflammatory score, even after stopping the administration of the drug at day 13. We were able to observe this anti-inflammatory effect in the absence of drug until day 21 (\( \text{data not shown} \)).

Of note, in some of the intraperitoneally treated animals, we have observed decreased body weight and ascites, in accordance with previous literature reports. During autopsy, we, together with a veterinary, have observed macroscopically internal organs that showed no alterations when comparing gambogic acid-treated, vehicle-treated, and healthy
Figure 2: (a) **NF-κB reporter activation is suppressed by gambogic acid treatment.** NF-κB expression was measured by flow cytometry in a THP-1/NF-κB reporter cell line incubated with gambogic acid and then stimulated for 24 h with *E. coli*. Each thin line in the histogram corresponds to untreated but *E. coli* stimulated cells, the shaded area corresponds to drug-treated and *E. coli* stimulated cells, and the thick line corresponds to untreated non-stimulated cells as a control. (b) **Caspase-1 activation is decreased with gambogic acid treatment.** Caspase-1 activation was measured using flow cytometry in a THP-1 cell line incubated with gambogic acid and then stimulated for 8 h with *E. coli*. Each thin line in the histogram corresponds to untreated but *E. coli* stimulated cells used as control and the thick line corresponds to drug-treated and *E. coli* stimulated cells.

Figure 3: **Gambogic acid is able to suppress inflammation throughout time.** After 6 days of treatment, the vehicle-injected group increased inflammatory manifestations, whereas, in gambogic acid-treated rats, there was a significant reduction in the inflammatory activity. Arrows indicate the beginning of treatment after 4 and 11 days of disease induction. Differences were considered statistically significant for *P* values < 0.05.

Figure 4: **Gambogic acid possesses anti-inflammatory properties.** Inflammatory score in gambogic acid-treated AIA rats is significantly diminished in comparison with vehicle-treated rats after treatment. Differences were considered statistically significant for *P* values < 0.05.

nonarthritic rats. Additionally, we have observed sections of internal organs collected at the time of sacrifice by histology, which have also shown no alterations comparing all experimental groups of animals. Specifically, in the case of the spleen, which in some RA patients and in animal models of arthritis present splenomegaly, we have observed that gambogic acid-treated and vehicle-treated rats showed spleen hyperplasia, with increased cellularity, compared with healthy nonarthritic rats (see Supplemental Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/195327). This result might be explained by the fact that gambogic acid is not modulating the lymphocyte development but is instead targeting downstream caspase-1 and NF-κB activation.
3.4. Gambogic Acid Prevents Joint Inflammatory Infiltration and Proliferation. To evaluate the infiltration of immune cells within joints in AIA rats, joint tissue sections were stained with hematoxylin and eosin. The histological evaluation shown in Figure 5 revealed that rats treated with gambogic acid had a normal joint structure with complete abrogation of the inflammatory infiltrate ($P < 0.0001$ in early treatment group versus untreated animals). In contrast, vehicle-treated rats exhibited infiltration of inflammatory cells, bone invasion and erosions (Figure 5). We also studied the levels of proliferation of immune cells by staining joint tissue sections with Ki67. The immunohistochemical results revealed that rats treated with gambogic acid presented reduced proliferation of immune cells within joints ($P = 0.0098$ versus untreated animals). Moreover, treatment with gambogic acid prevented cartilage and bone damage (Figure 5).

4. Discussion

Our results demonstrated that treatment with gambogic acid protected Wistar AIA rats from arthritis development with a complete abrogation of joint immune cellular infiltration and proliferation, preventing cartilage and bone damage.

Our laboratory has used a THP-1 macrophage-like cell line to screen 2320 drugs for those that simultaneously inhibit IL-1β and TNF secretion [13]. We have selected gambogic acid as a promising therapeutic candidate for the inhibition of both IL-1β and TNF secretion, due to the reduction in caspase-1 and NF-κB activation, and consequently for the treatment of arthritis. Previous reports have demonstrated that gambogic acid can inhibit the growth of a wide variety of tumour cell lines, possibly due to its ability to induce apoptosis [14] via the transferrin receptor (TfR1) [15]. Additionally, recent data have shown that this drug can inhibit NF-κB signalling pathway in human leukemia cancer cells [8] and in a noncancerigenous macrophagic cell line [16] also via TfR1. Therefore, the anti-inflammatory effects of gambogic acid appear to be mediated by the inhibition of NF-κB activation pathway which in turn leads to the silencing of most of the inflammatory genes. In fact, the inhibition of NF-κB in animal models has shown the ability to inhibit inflammatory arthritis, demonstrating that NF-κB may be an important therapeutic target in RA [17–19]. Indeed, NF-κB participates in the transcription of the genes encoding many proinflammatory cytokines and chemokines, in the regulation of the different immune cells, and in the regulation of the expression of adhesion molecules and matrix MMPs [20–22]. As recently reviewed, the IκB kinase IKKβ is essential for the inflammatory cytokine-induced activation of NF-κB [23]. Importantly, it has already been reported that gambogic acid is able to inhibit IKKβ activity [16]. In our study, we demonstrated that the anti-inflammatory properties of this drug in AIA rats might not only be related with its ability to suppress the activation of NF-κB but also to its effect on inhibiting caspase-1 activation.

Gambogic acid has a dual effect on the downregulation of TNF and IL-1β production. Interestingly, Joosten et al. showed uncoupled activities of IL-1β and TNF in joint swelling and ongoing cartilage destruction. Also, it has been
5. Conclusion

In conclusion, we have found that Wistar AIA rats can be effectively treated by gambogic acid. The effects of this drug probably rely on the inhibition of IL-1β and TNF secretion, possibly explained by its ability to downregulate caspase-1 and NF-kB activation and by blocking synovial hyperplasia due to its significant antiproliferative properties. These results support our initial hypothesis that a double inhibition of IL-1β and TNF could be effective in the treatment of inflammatory diseases, such as RA, and further indicate that the antiproliferative properties of gambogic acid may prove essential for an effective clinical control and to induce early remission in RA patients. Based on our observations indicating possible toxicity in some of the regimens used, we suggest that gambogic acid might not be suitable to directly enter phase I clinical trials, but it can certainly serve as a prototype drug to search for derivative compounds with similar effects on inflammatory mediators and cell proliferation and a more favourable safety profile.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


Clinical Study

Usefulness of Adalimumab in the Treatment of Refractory Uveitis Associated with Juvenile Idiopathic Arthritis

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Purpose. To assess the efficacy and safety of adalimumab in patients with juvenile idiopathic arthritis (JIA) and associated refractory uveitis. Design. Multicenter, prospective case series. Methods. Thirty-nine patients (mean [SD] age of 11.5 [7.9] years) with JIA-associated uveitis who were either not responsive to standard immunosuppressive therapy or intolerant to it were enrolled. Patients aged 13–17 years were treated with 40 mg of adalimumab every other week for 6 months and those aged 4–12 years received 24 mg/m² body surface. Results. Inflammation of the anterior chamber (2.02 [1.16] versus 0.42 [0.62]) and of the posterior segment (2.38 [2.97] versus 0.35 [0.71]) decreased significantly between baseline and the final visit (P < 0.001). The mean (SD) macular thickness at baseline was 304.54 (125.03) μm and at the end of follow-up was 230.87 (31.12) μm (P < 0.014). Baseline immunosuppression load was 8.10 (3.99) as compared with 5.08 (3.76) at the final visit (P < 0.001). The mean dose of corticosteroids also decreased from 0.25 (0.43) to 0 (0.02) mg (P < 0.001). No significant side effects requiring discontinuation of therapy were observed. Conclusion. Adalimumab seems to be an effective and safe treatment for JIA-associated refractory uveitis and may reduce steroid requirement.

1. Introduction

Uveitis is a well-known extra-articular manifestation of spondyloarthritides, which may lead to severe functional impairment [1]. Childhood uveitis is relatively rare and may be secondary to a variety of causes. The majority of children with uveitis have idiopathic uveitis, with uveitis secondary to juvenile idiopathic arthritis (JIA) being the second most
common diagnosis [2, 3]. Uveitis occurs in around 10–15% of the patients with JIA, although most reports are retrospective and describe referral centers’ experiences rather than population-based studies [4]. It has been largely recognized that uveitis is a serious manifestation of JIA. Complications increase with the duration of the disease, with a potential for cataract, glaucoma, band keratopathy, synchiae, macular edema, and significant ocular damage with impaired vision and even blindness [5, 6]. Antinuclear antibody (ANA)—positive girls younger than 7 years of age with oligoarticular JIA are at the greatest risk of developing eye disease [7]. Presence of complications at first visit and uveitis manifestation before arthritis have been identified as predictors for complications [8, 9].

JIA-associated refractory chronic uveitis is a challenge for treatment. Topical and systemic corticosteroids are the first-line standard therapy, often reinforced by conventional disease-modifying antirheumatic drugs (DMARDs) including a variety of immunomodulatory agents, but methotrexate remains the most commonly used drug [10–12]. During the last few years, tumor necrosis factor-alpha (TNF-α) blocking agents (infliximab, etanercept, and adalimumab) have been used to treat chronic refractory uveitis in childhood [12–15] and, particularly, uveitis associated with JIA in children who have failed topical and second-line DMARD therapy [16, 17]. Adalimumab, a TNF-α antagonist, has shown remarkable efficacy in rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, Crohn’s disease, and plaque-type psoriasis. Currently, there is a large experience of the beneficial effects of adalimumab in the treatment of these conditions in daily practice. Since 2008, adalimumab has been approved for the treatment of active juvenile idiopathic arthritis (JIA).

Adalimumab has shown promising results in controlling intraocular inflammation, even though this has been used primarily as a rescue therapy for refractory uveitis [18]. Open-label evaluations have demonstrated the efficacy of adalimumab therapy for childhood uveitis [19, 20]. In a recent comparative cohort study on anti-TNF-α treatment for sight-threatening childhood uveitis, adalimumab was more efficacious than infliximab in maintaining remission of chronic childhood uveitis for over 3 years [21]. In recent data of a large retrospective cohort of children with JIA and refractory chronic uveitis, treatment with adalimumab for a mean of 2 years was associated with an overall improvement of disease activity in 57% of the cases [22]. In recent evidence-based interdisciplinary guidelines for anti-inflammatory treatment of uveitis associated with JIA, adalimumab is recommended as the preferred TNF-α inhibitor [23].

A prospective multicenter study was design to assess the efficacy and safety of adalimumab therapy in a cohort of patients with JIA and associated uveitis who were treated with adalimumab in daily practice.

2. Materials and Methods

A prospective open-label, noncomparative, and multicenter study was conducted in the outpatient clinics of the services of ophthalmology or uveitis units of 10 centers throughout Spain and 1 center in Latin America (Mexico, DF) in daily practice conditions. The objective of the study was to assess the efficacy and tolerability of adalimumab for treating children and adolescents with uveitis in conjunction with JIA. The diagnosis of JIA was based on the 2001 revised International League Against Rheumatism (ILAR) classification criteria [24]. All diagnoses of JIA were confirmed by a pediatric rheumatologist. To be considered eligible for this study, patients were required to have disease onset prior to 16 years, bilateral or unilateral, chronic, and noninfectious uveitis that was refractory to standard immunosuppressive therapy for uveitis, with systemic corticosteroids and at least one other immunosuppressive medication, or to be intolerant to such therapy. Refractory was considered as persistent active uveitis for at least 3 months despite systemic steroids and immunosuppressive treatment. Uveitis was classified anatomically according to the International Uveitis Study Group (IUSG) classification [25]. The study protocol was approved by the ethics committee of each participating center, and written informed consent was obtained from the parents of legal guardians as well as from the patients older than 16 years of age.

All patients underwent a protein-purified derivative (PPD) skin test and chest radiography before enrollment because of the risk of tuberculosis reactivation associated with TNF-α inhibition. Patients diagnosed with latent tuberculosis, defined as a PPD skin conversion consisting of an induration of 5 mm or larger without radiographic or clinical evidence of disseminated or pulmonary disease, received antituberculosis prophylaxis at least 3 weeks prior to the first dose of adalimumab.

In all patients, treatment with adalimumab was initiated because of active refractory uveitis. Children and adolescents between 13 and 17 years of age were treated with 40 mg of adalimumab (Humira, Abbott Laboratories, Madrid, Spain), subcutaneously (s.c.) every other week for 6 months. For children aged between 4 and 12 years, doses were administered as indicated in the product label (i.e., 24 mg/m² body surface area up to a maximum single dose of 40 mg s.c. every other week). The volume of injection is selected according to the weight and height of the patient. Patients (or parents in case of young children) were instructed by a specialized nurse in self-administration of adalimumab.

Outcome variables included intraocular inflammation, visual acuity, immunosuppression load, and macular thickness. Inflammatory activity was graded according to the SUN Working Group grading schemes [26] for the anterior (cells and flare) and posterior (vitreous cells and haze) chambers, from grade 0 to 4. Worsening was defined as a two-step increase in the level of inflammation or as an increase from 3+ to 4+ (this was also the adopted definition for relapse during follow-up). Improvement was defined as a two-step decrease in the level of inflammation or decrease to grade 0.

The best-corrected visual acuity (BCVA) was measured according to the ETDRS protocol adapted by the Age Related Eye Disease Study [27]. Three lines (±0.3 logMAR; ±15 letters) of change were chosen as the standard for worsening or improvement in visual acuity, because as reported
in previous uveitis clinical trials, this was the minimum necessary number to reflect a clinical significant change.

The immunosuppression load was assessed with a semi-quantitative scale for each medication as described by Nussbaurnall et al. [28]. The grading scheme provides a combined, single numeric score for the total immunosuppression load per unit of body weight per day. Grades for each agent (prednisone, cyclosporine, azathioprine, methotrexate, and chlorambucil) ranged on a scale from 0 to 9. For patients receiving multiple medications, the sum of the grading scores for each drug was used to calculate the total immunosuppression score at baseline visit and at the end of follow-up on a scale from 0 to 15. Topical or periocular corticosteroid therapy was excluded from the calculation of the immunosuppression load. A ≥ 50% reduction in the immunosuppression load was considered a significant reduction in the associated immunosuppressant therapy.

Macular thickness was measured by optical coherence tomography (OCT) (Stratus OTC, Carl Zeiss Meditec, Dublin, CA, USA). Changes in the mean 1 mm central foveal retinal thickness served to evaluate the reduction in the cystoid macular edema (CME).

**Statistical Analysis.** Categorical data are expressed as absolute number and percentages and continuous data as mean and standard deviation (SD). Continuous variables were analyzed with the paired Student’s t-test (e., immunosuppression load and macular thickness) and qualitative variables (degree of intraocular inflammation) with the Wilcoxon signed-rank test. Statistical significance was set at $P < 0.05$. The analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 12.0 for Windows.

### 3. Results

A total of 39 patients, 11 boys and 28 girls, with a mean (SD) age of 11.5 (7.9) years were included in the study. All patients presented initially with arthritis. The age at diagnosis of arthritis ranged between 18 months and 6 years, with a median of 4.4 years. Manifestations of uveitis developed approximately 8 months after the diagnosis of JIA.

All patients were followed for at least 6 months. Twenty-nine (74.4%) patients had chronic anterior uveitis, 1 (2.6%) intermediate and anterior uveitis and 9 (23.1%) panuveitis (anterior with secondary posterior uveitis). The right eye was involved in 2 (5.1%) patients, the left eye in 5 (12.8%), and both eyes in the remaining 32 (82.1%) patients.

Changes of outcome variables associated with adalimumab therapy are shown in Table 1. A marked decrease of intraocular inflammation with adalimumab at the end of follow-up was observed. The baseline anterior chamber degree of inflammation was 2.02 (1.16) and the posterior chamber degree of inflammation was 2.38 (2.97) on the standardized scale, and at the end of follow-up they were 0.42 (0.62) and 0.35 (0.71), respectively. There was a statistically significant difference in the mean degree of intraocular inflammation of the anterior chamber between the initial visit and at the end of follow-up ($P < 0.001$). Differences in the mean degree of intraocular inflammation of the posterior chamber were also statistically significant ($P < 0.005$).

The visual acuity improved by $-0.3 \text{logMAR} (+15 \text{letters})$ in 4 (12.5%) eyes out of 32 eyes, remained stable in 26 (81.2%) eyes, and worsened by $+0.3 \text{logMAR} (-15 \text{letters})$ in 2 (6.25%) eyes at the end of follow-up. The baseline visual acuity was $+0.30 \pm 0.32$ (mean ± SD) logMAR and $+0.24 \pm 0.35$ logMAR at the end of follow-up. There was no statistically significant difference between the mean baseline visual acuity and the mean visual acuity at the end of follow-up ($P = 0.226$).

The OCT macular examination revealed that 3 eyes (7.7%) had CME at baseline, and at the end of follow-up, there was complete resolution of CME in all cases (Figure 1). The mean (SD) macular thickness at baseline was 304.54 (125.03) μ and at the end of follow-up was 230.87 (31.12) μ. There was a statistically significant difference between pretreatment and final macular thickness ($P < 0.014$) (Figure 2).

Baseline immunosuppression load was 8.10 (3.99) as compared with 5.08 (3.76) at the final visit. There was a significant difference in the mean immunosuppression load between baseline and end of follow-up ($P < 0.001$).

Treatment with adalimumab was also associated with a decrease in the mean dose of corticosteroids from 0.25 (0.43) at the pretreatment visit to 0 (0.02) mg at the end of follow-up ($P < 0.001$).

Adalimumab was well tolerated in all patients during the 6-month follow-up period except for the case of a 9-year-old girl that at the fifth month of administration of adalimumab had a severe uveitis reactivation that needed a switch to infliximab therapy to control the episode. Minor side effects

## Table 1: Comparison of outcome variables before treatment with adalimumab and at the final visit.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Week 0 (baseline)</th>
<th>Month 6 (final visit)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior chamber inflammation</td>
<td>2.02 (1.16)</td>
<td>0.42 (0.62)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Posterior chamber inflammation</td>
<td>2.38 (2.97)</td>
<td>0.35 (0.71)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Macular thickness, μ</td>
<td>304.54 (125.03)</td>
<td>230.87 (31.12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Immunosuppression load</td>
<td>8.10 (3.99)</td>
<td>5.08 (3.76)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data as mean (standard deviation).
at the site of injection, such as pain, erythema, localized rash, or minor hemorrhage were the most common side effects observed. During the follow-up period, 3 (7.8%) patients experienced reactivation of uveitis, which was severe in only 1 patient (3.1%), who discontinued the adalimumab therapy and improved with infliximab treatment. The remaining 2 patients did not discontinue the adalimumab therapy because the inflammation was controlled with periocular steroid injection of the affected eye.

4. Discussion

This prospective study carried out in a clinical series of 39 children and adolescents with JIA-associated uveitis confirms the value of adalimumab as a treatment option in patients who were poorly responsive to conventional therapy for uveitis. The effectiveness of adalimumab was demonstrated by a statistically significant decrease of anterior chamber and vitreous cavity degree of inflammation, reduction of macular thickness, and decrease of the immunosuppression load.

Most reported treatment studies in pediatric uveitis are retrospective case series, which may be explained by the relative rarity of uveitis associated with JIA, urgency of treatment to prevent complications, difficulty in examining younger children, and the lack of controlled clinical trials [4]. The course of uveitis is chronic with waxing and waning activity, and follow-up times should be longer enough to assess the efficacy of treatment [4]. TNF-α appears to play a role in the pathogenesis of uveitis [29–31] and therefore TNF-α blockade is a rational therapy for uveitis refractory to standard treatment [16].

Adalimumab is currently considered the most efficacious TNF-α blocker for childhood uveitis and the preferred biologic drug for the treatment of uveitis associated with JIA [23]. In this respect, data of the present study collected in a prospective series of patients with JIA and refractory uveitis add evidence to the preferential role of adalimumab in the therapeutic armamentarium of this condition. The beneficial effects of adalimumab in reducing ocular inflammation together with the good tolerability profile of this agent are consistent with data previously published in the literature. Previous studies, however, are generally retrospective or small series of case studies, including 8 patients reported by Sen et al. [32], 6 patients described by Katsicas and Russo [33], 3 patients reported by Foeldvari et al. [12] among 47 patients with JIA-related uveitis treated with anti-TNF-α collected in a multinational survey, and 9 patients reported by Vazquez-Cobian et al. [17]. Other clinical studies with a larger number of patients include a group of 17 patients reported by Biester et al. [20] and a retrospective observational study of 20 patients with JIA and chronic uveitis reported by Tynjälä et al. [34]. Findings of these studies in children with JIA who were poorly responsive to conventional therapy for uveitis are consistent with a decrease in ocular inflammation, sustained response, and decrease or discontinuation of other immunosuppressive agents. In a recent retrospective cohort of 54 patients with JIA treated with adalimumab for active associated uveitis, improvement of ocular inflammation was recorded in 28% of the cases [21]. In this study, in which treatment with adalimumab was given for a mean of 2 years, at the end of the follow-up, only 4 patients remained on adalimumab monotherapy and the rest were on combined therapy with prednisone, methotrexate, cyclosporine A, or other antimetabolite drugs.

The three TNF-α antagonists (etanercept, infliximab, and adalimumab) appear to have similar efficacy in rheumatoid arthritis (JIA), but this does not appear to be the case with uveitis where infliximab seems to be more effective than etanercept, and adalimumab more effective than infliximab. In an open-label prospective comparison of infliximab and adalimumab in 33 children with refractory noninfectious uveitis, 22 of them with associated JIA, at 40 months of follow-up, 60% of children on adalimumab compared to 18.8% of children on infliximab were still in remission on therapy [21].

In summary, treatment with adalimumab for at least 6 months was associated with improvement of symptoms and decrease in inflammatory activity in 39 patients with refractory uveitis associated with JIA. It also allowed a significant reduction of concomitant immunosuppressive therapies.

Conflict of Interests

The authors declare that they have no financial conflict of interests.

Authors’ Contribution

All the authors contributed to the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, and approval of the paper.

Acknowledgments

The study protocol was approved by the ethics committee of each participating center. The authors thank Marta Pulido, M.D., for editing the paper and editorial assistance.
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Research Article

CD14 Mediates Binding of High Doses of LPS but Is Dispensable for TNF-α Production

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Activation of macrophages with lipopolysaccharide (LPS) involves a sequential engagement of serum LPS-binding protein (LBP), plasma membrane CD14, and TLR4/MD-2 signaling complex. We analyzed participation of CD14 in TNF-α production stimulated with 1–1000 ng/mL of smooth or rough LPS (sLPS or rLPS) and in LPS binding to RAW264 and J744 cells. CD14 was indispensable for TNF-α generation induced by a low concentration, 1 ng/mL, of sLPS and rLPS. At higher doses of both LPS forms (100–1000 ng/mL), TNF-α release required CD14 to much lower extent. Among the two forms of LPS, rLPS-induced TNF-α production was less CD14-dependent and could proceed in the absence of serum as an LBP source. On the other hand, the involvement of CD14 was crucial for the binding of 1000 ng/mL of sLPS judging from an inhibitory effect of the anti-CD14 antibody. The binding of sLPS was also strongly inhibited by dextran sulfate, a competitive ligand of scavenger receptors (SR). In the presence of dextran sulfate, sLPS-induced production of TNF-α was upregulated about 1.6-fold. The data indicate that CD14 together with SR participates in the binding of high doses of sLPS. However, CD14 contribution to TNF-α production induced by high concentrations of sLPS and rLPS can be limited.

1. Introduction

Mechanisms of the innate immunity assure a rapid response directed against microbes which have successfully overcome physical barriers protecting the body. These reactions are triggered upon recognition of evolutionarily conserved constituents of microorganisms named pathogen-associated molecular patterns (PAMPs) by distinct cellular receptors among which Toll-like receptors (TLR) are of great importance [1]. The prototypical PAMP is lipopolysaccharide (LPS, endotoxin), a major constituent of the outer membrane of Gram-negative bacteria. LPS activates TLR4 of leukocytes and initiates signalling cascades leading to production of proinflammatory mediators exemplified by tumor necrosis factor-α (TNF-α), chemokines like MIP-2 and RANTES, and type I interferons [2, 3]. The presence of high LPS concentrations in the blood and the following exaggerated production of TNF-α and other pro-inflammatory mediators can lead to a systemic inflammatory reaction, termed sepsis [4, 5].

LPS molecules consist of three components: the polysaccharide chain named the O-antigen, the core oligosaccharide and lipid A with the latter determining the proinflammatory activity of endotoxin. The greatest variability in LPS structures is observed within the O-specific chain and concerns the chemical nature and the number of sugar residues assembling the polysaccharide, as well as the position and stereochemistry of the O-glycosidic linkages [6, 7]. In certain species or mutants of Gram-negative bacteria, or in distinct growth conditions, the O-specific chain may be absent giving rise to a so-called rLPS (from a "rough" phenotype of bacterial
colony) in contrast to the typical phenotype of “smooth” colonies synthesizing sLPS with the O-antigen. The lack of the O-specific chain modulates the process of LPS recognition by cells of the immune system which can lead eventually to differences in the magnitude of the cytokine production, as it was found for LPS originating from Salmonella sp., Brucella sp., and Escherichia coli [8–10].

An optimal response of macrophages to LPS requires a cooperation of a number of extracellular and plasma membrane proteins, including serum LPS-binding protein (LBP) which monomerizes LPS and transfers LPS molecules to the plasma membrane-anchored CD14 [11]. CD14 is 56 kDa protein which forms homodimers and binds lipid portion of LPS in its NH$_2$-terminal hydrophobic pocket [12, 13]. The protein is incorporated in the outer leaflet of the plasma membrane via a glycosylphosphatidylinositol anchor and contains no transmembrane or cytoplasmic domains. It was the reason why CD14 together with LBP was assumed to play merely a role of sensors efficiently capturing LPS molecules and transferring them to a signalling complex composed of MD-2 protein associated with TLR4. Dimerization of TLR4/MD-2 complexes induced upon LPS binding triggers two signalling pathways depending on the association of TLR4 with either MyD88/TIRAP or TRIF/TRAM adaptor proteins, respectively [14–17]. Recent studies indicate that CD14 is important for the initiation of proinflammatory signalling triggered by sLPS rather than rLPS [10]. However, CD14 may fulfill also other functions in the process of cell stimulation than simple LPS recognition. In macrophages isolated from mice with mutant CD14, the TRIF-dependent signalling pathway of TLR4 was nullified [9]. This disabled pathway was linked to CD14-dependent endocytosis of LPS-activated TLR4 [18]. CD14 participates also in LPS internalization in a pathway which leads to an intracellular detoxification of LPS. This LPS uptake is attributed mainly to the activity of scavenger receptors (SR) and cooperation of SR with CD14 was indicated [19–22].

On the other hand, CD14 is not the only one coreceptor of TLR4 in LPS-stimulated cells. Measurements of the resonance energy transfer between fluorescently labelled membrane proteins in LPS-stimulated monocytes revealed that activated TLR4 co-clustered with CD14 and also with heat-shock proteins 70 and 90, CD55, CD11/CD18, and chemokine receptor 4 (CXCR4) [23, 24]. These proteins can participate in LPS-induced production of TNF-α by functioning as LPS-binding molecules similarly to CD14; however, signalling properties of CXCR4 were also indicated [25, 26].

The complexity of the TLR4-accompanying plasma membrane receptors potentially involved in LPS recognition prompted us to analyze the participation of CD14 in TNF-α production stimulated by sLPS and rLPS of E. coli and in sLPS binding. We found that CD14 moderately affected TNF-α production induced by high doses of sLPS and rLPS. On the other hand, CD14 together with SR participated in the binding of high doses of sLPS. The data suggest that the involvement of CD14 is important for recognition and binding of sLPS. However, CD14 contribution to TNF-α production induced by high doses of sLPS and rLPS can be limited.

2. Materials and Methods

2.1. Cell Culture and Stimulation. RAW264 and J774A.1 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 5% CO$_2$. Cells were stimulated with ultrapure smooth LPS (sLPS) from E. coli 0111:B4 (List Biological Laboratories) or rough LPS (rLPS) from E. coli, serotype 515, Re mutant (Enzo), or S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-lysyl3-lysine (Pam$_3$CSK$_4$), or N-palmitoyl-S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-lysyl3-lysine (Pam$_2$CSK$_4$), or polyinosinic-polycytidylic acid (polyI:C) (all from InvivoGen). When indicated, cells were exposed to sLPS labeled with Alexa Fluor 488 hydrazide (Molecular Probes) or hydrazide-LC-biotin (Thermo Scientific) according to [27]. Concentration of LPS after labeling was estimated using Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific). To determine the AF488 content in LPS-AF488 samples, their absorbance at 492 nm was measured. A 5:1 labeling ratio of AF488-to-LPS was obtained. Labeling of LPS with biotin was confirmed by dot-blot analysis of the binding of streptavidin-peroxidase (Sigma) to LPS-biotin applied onto a nitrocellulose in a 5–50 ng/mL range. Labeling of sLPS either with AF488 or biotin did not diminish endotoxin activity, as indicated by its ability to induce TNF-α production in comparison to sLPS prior to the labeling.

2.2. Silencing of TLR4 Gene. RAW264 cells ($1.5 \times 10^5$) were suspended in 1 mL of RPMI medium supplemented with 5% FBS, mixed with 1 mL of serum-free RPMI containing 20 µL of TrueFect-Lipo (United BioSystems) and 200 pmol of either TLR4 small interfering RNA (siRNA) or control scrambled bodies and chondroitin sulfate or dextransulfate. Subsequent stimulation of cells with either LPS or other above-mentioned ligands was conducted in the presence of appropriate drugs and antibodies. Concentrations of sulfates and antibodies were reduced by half after LPS addition. Levels of TNF-α and MIP-2 in culture supernatants were determined after 4 h while RANTES after 6 h of stimulation, with an application of murine ELISA kits (R&D Systems, Biolegends, Peprotech). The product absorbance was measured using a Sunrise plate reader (Tecan Group).
2.4. LigandTracer Binding Assay. J774 cells (1 x 10⁶ in 3 mL of DMEM/10% FBS) were seeded in a local area of tilted 8.7 cm cell dish according to [28] and cultured at 5% CO₂, 37°C. After 6 h, the dish was supplemented with 7 mL of DMEM/10% FBS and cells were grown overnight. Prior to experiments, cells were washed once with DMEM/10% FBS supplemented with 20 mM Hepes, pH 7.4, layered with 2 mL of the medium containing the mixture of 10 µg/mL anti-CD14 rat IgG2b (clone 4C1) and 50 µg/mL dextran sulfate or 50 µg/mL chondroitin sulfate A and incubated for 15 min at 37°C. The dish was placed on a tilted, rotating support of the instrument LigandTracer Green (Ridgeview Instruments AB, Uppsala, Sweden) in an incubator at 37°C/5% CO₂ for the baseline setting. After 30 min, 40 µL of sLPS-AF488 was added to the medium to final concentration of 3 µg/mL of LPS and real-time LPS-cell association was monitored for 1 h. In this time, repeated measurements of the fluorescence from dish areas covered with and devoid of cells were performed generating an output signal defined as a difference between the fluorescence of the cell-containing area and the fluorescence of the surrounding reference area. After removal of the medium and washing, the dish was filled with 2 mL of DMEM/10%FBS/20 mM Hepes, pH 7.4, with appropriate drugs, and the measurements were carried out for another 1 h as above mentioned to follow retention of LPS in cells. After adding 100 µg/mL of concanavalin A-FITC to the medium, the measurements were continued for 30−40 min to assess the association of the lectin with cells.

2.5. Binding and Internalization of LPS-Biotin. Cells were plated at 4 x 10⁴/well in 96-well plates in DMEM/10% FBS. After 18 h, cells were incubated in the presence of 10 µg/mL anti-CD14 antibody or 10 µg/mL control rat IgG2b, or 50 µg/mL chondroitin sulfate or 50 µg/mL dextran sulfate or a combination of those compounds in DMEM/10% FBS (30 min, 37°C). Subsequently, the cultures were supplemented with sLPS-biotin at 1 µg/mL, reducing concentration of antibodies and sulfates by half. After 1 h (37°C), cell were washed twice with PD buffer (125 mM NaCl, 4 mM KCl, 10 mM NaHCO₃, 1 mM KH₂PO₄, 10 mM glucose, 20 mM Hepes, pH 7.4) and to facilitate uptake of sLPS-biotin they were incubated in DMEM/10% FBS for another 1 h in the presence of appropriate drugs or antibodies. After final wash with PD buffer, cells were exposed to 150 µL of a hypotonic solution of 2 mM EGTA, 2 mM EDTA, 20 mM Hepes, pH 7.4 (10 min, 4°C) and sonicated on ice for 5 min at 0.25 cycle, amplitude 25% using an UP200S Hielscher sonifier (Germany). The homogenates were transferred into eppendorf tubes and centrifuged (10 min, 10 000 g, 4°C); supernatants were diluted twice with TBS buffer and applied in 100 µL quantities onto nitrocellulose membranes. After blocking with 3% bovine serum albumin in TBS buffer containing 0.05% Tween 20, blots were incubated with streptavidin-peroxidase and immunoreactive dots were visualized by chemiluminescence, using SuperSignal West Pico substrate (Pierce).

To assess the binding of sLPS-biotin but to prevent its internalization, cells were preincubated for 30 min at 37°C with 0.05% NaN₃ prior to adding 5–15 µg/mL of the anti-CD14 antibody, or 10 µg/mL of control rat IgG2b or 50 µg/mL of chondroitin sulfate or 50 µg/mL of dextran sulfate or the mixture of 5 µg/mL anti-CD14 antibody and 50 µg/mL dextran sulfate. After 30 min (37°C), cultures were supplemented with 1 µg/mL of sLPS-biotin for 1 h (37°C) in the presence of 0.05% NaN₃. Cells were washed twice with PD buffer and processed for dot-blot analysis as mentioned above. Blots were analyzed densitometrically using the ImageJ software. For normalization, dot intensity values found in nonstimulated cells and reflecting a nonspecific binding of streptavidin-peroxidase to cell homogenates were subtracted from those found in LPS-treated cells. The data are expressed in relation to LPS level found in cells exposed to cIgG and arbitrarily equalized to 100.

2.6. Immunoblotting. Proteins of whole cell lysates were separated by 10% SDS-PAGE, transferred onto nitrocellulose, and probed with rabbit anti-TLR4 (Santa Cruz Biotechnology) and mouse anti-actin antibodies (BD Biosciences) followed by goat anti-rabbit or anti-mouse IgG conjugated with peroxidase. Immune-reactive bands were visualized and analyzed densitometrically as above to assess TLR4 level normalized against actin content in samples.

2.7. Data Analysis. The significance of differences between groups was calculated using Student’s t-test. P values ≤ 0.05 were considered to be statistically significant.

3. Results

3.1. CD14 Moderately Affects TNF-α Production Triggered by High Doses of LPS. To assess the involvement of CD14 in LPS-induced signaling, we examined effects of neutralizing of CD14 on the production of TNF-α and RANTES. TNF-α is synthesized in MyD88-dependent while RANTES in TRIF-dependent signaling pathways of TLR4 [3]. RAW264 cells were stimulated with 1–1000 ng/mL of sLPS or rLPS of E. coli in the presence of 4C1 antibody which excluded LPS binding to CD14 [29]. We found that this blocking of function of CD14 nearly abolished TNF-α and RANTES production induced by 1 ng/mL of LPS. In cells stimulated with 10 ng/mL of sLPS the antibody significantly, by 51% and 88%, reduced production of TNF-α and RANTES, respectively (Figures 1(a) and 1(b)). In contrast, there was a clear difference in the CD14 involvement in TNF-α and RANTES generation induced by higher, 100 and 1000 ng/mL, doses of sLPS. In these conditions, the blocking of the LPS/CD14 interaction by the 4C1 antibody inhibited the production of TNF-α by 25–27% only (Figure 1(a)) while RANTES generation was reduced by 58–76% (Figure 1(b)). For comparison, when stimulation of cells was conducted in a medium devoid of FBS as an LBP source, production of both TNF-α and RANTES induced by 1–1000 ng/mL sLPS was greatly inhibited approaching the level found in cells prior to the stimulation. When cells were deprived of FBS and additionally exposed to the anti-CD14 function blocking antibody, the cytokine release remained very low (Figures 1(a) and 1(b)).
As signaling properties of LPS can be modulated by the O-antigen polysaccharide chain [9, 10], we next examined the involvement of CD14 in rLPS-induced cytokine production. In cells stimulated with rLPS, TNF-α production was less dependent on CD14 than in cells exposed to sLPS. Neutralizing CD14 with the 4C1 antibody inhibited by 65% the production of TNF-α induced by 1 ng/mL rLPS. However, in cells stimulated with 10–1000 ng/mL of rLPS, the production of TNF-α was reduced by 5–30% only (Figure 1(c)). When used at higher concentrations, despite the presence of the CD14-neutralizing antibody, 100 or 1000 ng/mL of rLPS was able to induce as much as 77–82% of the RANTES production found in control cells (Figure 1(d)). In further contrast to sLPS, even in the absence of FBS, rLPS at 100 or 1000 ng/mL induced the production of TNF-α and RANTES which approached 47–66% of control levels (Figures 1(c) and 1(d)). The absence of FBS combined with the neutralizing of CD14 diminished strongly the production of TNF-α and RANTES induced by 1–100 ng/mL of rLPS. However, in these conditions rLPS at 1000 ng/mL was still able to induce synthesis of TNF-α and RANTES reaching about 51% and 58% of control values, respectively (Figures 1(c) and 1(d)). Taken together, the data indicate the following: (i) CD14 is dispensable for TNF-α production induced by higher (100–1000 ng/mL) concentrations of both rLPS and sLPS; (ii) rLPS, when used at higher doses, can bypass not only CD14 but also LBP involvement to activate TLR4-dependent release of TNF-α and RANTES.

We then examined whether cytokine production induced by sLPS and rLPS in those lines of experiments was indeed attributed to TLR4 activation. The silencing of expression of TLR4-encoding gene in RAW264 cells suppressed TNF-α production induced by sLPS or rLPS by 47–59% regardless of LPS concentration (Figures 2(a) and 2(c)). Notably, downregulation of TLR4 inhibited RANTES release by 55–61% resembling the inhibitory effect exerted on TNF-α production (Figures 2(b) and 2(d)). This fairly even inhibition of cytokine production corresponded to 50–55% reduction of TLR4 level.

**Figure 1:** Interference with LPS/CD14 interaction moderately affects TNF-α production induced by high concentrations of sLPS or rLPS. RAW264 cells were pretreated with 10 μg/mL of the anti-CD14 antibody, clone 4C1, or isotype-matched control rat IgG2b (30 min, 37°C) and subsequently stimulated with 1–1000 ng/mL sLPS (a, b) or 1–1000 ng/mL rLPS (c, d) in the presence or absence of 10% FBS, as indicated. Concentrations of TNF-α (a, c) and RANTES (b, d) were measured by ELISA tests in supernatants of the cells. Data shown are mean ± SEM from three or four experiments each run in triplicate. * Significantly different from cells stimulated with a corresponding LPS concentration in the presence of control IgG and FBS.
Figure 2: Silencing of TLR4 gene expression significantly downregulates production of TNF-α and RANTES in cells stimulated with sLPS or rLPS. RAW264 cells were transfected with TLR4-specific siRNA or scrambled siRNA and, after 50 h, stimulated with 10–1000 ng/mL sLPS (a, b) or 10–1000 ng/mL rLPS (c, d). Production of TNF-α (a, c) and RANTES (b, d) by the cells was estimated by ELISA tests. Data shown are mean ± SEM from three experiments each run in triplicate. * Significantly different from cells treated with scrambled siRNA and stimulated with a corresponding LPS concentration. (e) Immunoblotting analysis of TLR4 protein level versus actin level in cells transfected with TLR4-specific or scrambled (sc) siRNA. nt: not transfected cells. On the left, a molecular weight marker (prestained phosphorylase b, 101 kDa) is indicated. Data from two independent experiments are shown.

in cells transfected by specific siRNA in comparison to cells treated with scrambled siRNA (Figure 2(e)) pointing to TLR4 as mediating inflammatory responses to sLPS and rLPS in these studies.

We also analyzed whether the neutralizing of CD14 can affect production of TNF-α and RANTES induced by ligands of other TLRs in RAW264 cells. The anti-CD14 antibody induced partial inhibition of TNF-α production in a response to synthetic lipopeptides, Pam2CSK4 and Pam3CSK4, ligands of TL2/TLR6 and TL2/TLR1, respectively. No inhibition of TNF-α release was found in cells exposed to 100 ng/mL of Pam3CSK4 or Pam3CSK4. However, at lower doses, 10–50 ng/mL, the lipopeptides the production of TNF-α was suppressed by about 50% in cells stimulated with Pam3CSK4 and by 15–24% when Pam2CSK4 was used (Figures 3(a) and 3(b)). These results are in agreement with suggestions that CD14 serves as a sensor of amphipathic molecules [30–32], although data arguing against the involvement of mouse CD14 in TLR2/TLR1 signaling should be noted [9]. On the other hand, production of RANTES in cells stimulated with 5–20 μg/mL of poly(I:C), a ligand of endosomal TLR3, was not changed by the anti-CD14 antibody (Figure 3(c)). The data indicate that CD14 is not involved in endocytosis of TLR3 ligands which can be delivered to the receptor by scavenger receptor A (SR-A) [33].

3.2. CD14 and Scavenger Receptors Participate in the Binding of High Doses of LPS. The moderate effect exerted by the CD14 neutralizing antibody on TNF-α production induced by 100 or 1000 ng/mL of LPS can indicate that CD14 is not
crucial for the binding of high doses of endotoxin. To test this assumption, we measured the binding and internalization of 1000 ng/mL of sLPS conjugated with biotin in RAW264 cells (Figure 4). To block sLPS internalization, the binding of the endotoxin was performed in the presence of 0.05% NaN₃ (1h), after which cells were homogenized and amounts of sLPS-biotin bound to the cell surface were measured by a dot-blot analysis. In these conditions, treatment of cells with 5–15 μg/mL of the anti-CD14 antibody strongly inhibited the binding of 1000 ng/mL of sLPS. The amounts of bound sLPS were reduced by about 72% at 5 μg/mL and by about 86–88% at 10–15 μg/mL of the anti-CD14 antibody in comparison to cells treated with the isotype-matched control antibody (Figures 4(a) and 4(b)). We established that the effect of neutralizing CD14 exerted on sLPS binding in RAW264 cells was comparable to that of 50 μg/mL of dextran sulfate, a competitive ligand of class A SR [34] which mediated uptake and detoxification of large quantities of LPS. Dextran sulfate at 50 μg/mL inhibited sLPS-biotin binding by about 80%, whereas simultaneous action of 5 μg/mL of the anti-CD14 antibody and 50 μg/mL of dextran sulfate abolished the binding (Figures 4(a) and 4(b)). Both the anti-CD14 antibody and dextran sulfate also inhibited to a similar extent, by over 60%, the internalization of 1000 ng/mL of sLPS-biotin. A joint influence of these two agents very strongly diminished sLPS uptake, suggesting partial separation of CD14- and SR-dependent internalization routes of LPS (Figures 4(c) and 4(d)). Taken together, the data suggest that CD14 and SR participate in the binding and internalization of large quantities of sLPS while the CD14-mediated sLPS binding contributes only partially to TLR4 signaling that leads to TNF-α generation.

The data were reinforced by an analysis of participation of CD14 in sLPS binding and TNF-α production in J774 cells which express higher amounts of CD14 on the surface than RAW264 cells (not shown). The application of J774 cells allowed us to assess the amounts of sLPS-biotin bound to the surface of NaN₃-treated cells after 1h of incubation (Figures 5(a) and 5(b)). In addition, we were also able to perform a real-time analysis of the binding and internalization of sLPS-AF488 in the LigandTracer Green instrument which requires cells well adhering to the substratum (Figure 5(c)). The dot-blot analysis of the binding of 1000 ng/mL of sLPS-biotin revealed a dose-dependent inhibition of the binding by the anti-CD14 antibody. The binding was unaffected by 5 μg/mL of the neutralizing antibody; however, it was reduced by about 58% at 10 μg/mL and by about 77% at 15 μg/mL of the antibody (Figures 5(a) and 5(b)). For comparison, 50 μg/mL dextran sulfate inhibited the sLPS binding by J774 cells by about 40% but joint action of 5 μg/mL of the anti-CD14 and 50 μg/mL of dextran sulfate reduced the binding further by 61% (Figures 5(a) and 5(b)) pointing to the involvement of both CD14 and SR in the binding of high amounts of sLPS to cells. A control polyanion chondroitin sulfate at 50 μg/mL did not affect the binding or internalization of sLPS in RAW264 and J774 cells (Figures 4, 5(a), and 5(b)).

These data on CD14 and SR engagement in sLPS association with cells were confirmed by an analysis of a real-time binding and internalization of sLPS-AF488 to living J774 cells performed in the LigandTracer instrument. For this analysis, J774 cells were pretreated with 10 μg/mL of the anti-CD14 antibody and 50 μg/mL of dextran sulfate or with 50 μg/mL of chondroitin sulfate in a control. Subsequently, the cells were placed into the LigandTracer at 37°C and exposed to 3 μg/mL of sLPS-AF488. In these conditions, an association of sLPS-AF488 with cells (including LPS binding and internalization) was monitored for 1h, after which the excess of sLPS-AF488 was washed out and the retention of endotoxin in cells was assessed for another 1h. As can be seen in Figure 5(c), simultaneous exposition of cells to dextran sulfate and the CD14-neutralizing antibody suppressed both the association and retention of 3 μg/mL sLPS-AF488 in cells, apparently abolishing its accumulation in cells. The lack of retention of sLPS-AF488 was not caused...

**Figure 3:** Effect of neutralizing CD14 on cytokine production during activation of TLR2/TLR6, TLR2/TLR1, and TLR3. RAW264 cells were pretreated with 10 μg/mL of the anti-CD14 antibody or isotype-matched control rat IgG2b (30 min, 37°C) and exposed to indicated concentrations of Pam3CSK4 (a) or Pam3CSK4 (b) or poly(I:C) (c) in the presence of 10% FBS. Amounts of TNF-α (a, b) and RANTES (c) were measured by ELISA tests in supernatants of the cells. Data shown are mean ± SEM form two or three experiments each run in triplicate.

*Significantly different from cells stimulated with a corresponding ligand concentration in the presence of control IgG.
Figure 4: Binding of high doses of sLPS to the surface of RAW264 cells is mediated by CD14 and SR. (a, b) Binding of sLPS-biotin to the surface of RAW264 cells. Cells were preincubated with 0.05% NaN₃ (30 min, 37°C) and either left untreated (ns) or exposed to 5 or 10 μg/mL of the anti-CD14 antibody or 10 μg/mL isotype-matched control IgG (clgG) or 50 μg/mL dextran sulfate (DS), or 50 μg/mL chondroitin sulfate (CS), or the mixture of 5 μg/mL anti-CD14 antibody and 50 μg/mL dextran sulfate. After 30 min (37°C), cells were stimulated with 1 μg/mL sLPS-biotin (1 h, 37°C) in the constant presence of 0.05% NaN₃. The amount of sLPS-biotin bound to the cells surface was assessed by dot-blot analysis of cell homogenates using streptavidin-peroxidase (a). (c, d) Internalization of sLPS-biotin. Cells were preincubated with 10 μg/mL anti-CD14 antibody or 10 μg/mL isotype-matched control IgG or 50 μg/mL dextran sulfate or 50 μg/mL chondroitin sulfate or a mixture of the anti-CD14 and dextran sulfate for 30 min at 37°C. Subsequently, the samples were supplemented with 1 μg/mL of sLPS-biotin for 1 h and washed and incubated for another 1 h at 37°C prior to homogenization and dot-blot analysis (c). (b, d) Quantification of cell surface-bound (b) and internalized (d) sLPS-biotin based on densitometric analysis of dot-blots. Data are mean ± SEM from three experiments. *Significantly different from cells exposed to control IgG.

Taken together, the data indicate that CD14 and SR mediate the binding and internalization of large quantities of LPS. Despite the participation of CD14 in the binding of high amounts of sLPS, neutralizing of CD14 with 10 μg/mL of the 4C1 antibody in J774 cells inhibited TNF-α production induced by 100 or 1000 ng/mL of sLPS by 27–35% only. The function blocking anti-CD14 antibody exerted, however, a detachment of cells from the substratum, since they were still able to bind FITC-labeled concanavalin A (Figure 5(c)). Taken together, the data suggest that CD14 and SR mediate the binding and internalization of large quantities of LPS. Despite the participation of CD14 in the binding of high amounts of sLPS, neutralizing of CD14 with 10 μg/mL of the 4C1 antibody in J774 cells inhibited TNF-α production induced by 100 or 1000 ng/mL of sLPS by 27–35% only. The function blocking anti-CD14 antibody exerted, however, a strong inhibitory effect on TNF-α released by J774 cells at 10 ng/mL of sLPS, consistent with the results obtained in RAW264 cells (Figure 5(d) compared with Figure 1(a)). RANTES production in J774 cells induced by sLPS was markedly inhibited by the anti-CD14 antibody (Figure 5(e)). Taken together, the data suggest that the interference with the binding of high amounts of sLPS to CD14 has more pronounced inhibitory effect on the association of LPS with cells than on TNF-α production.
Figure 5: Disparate requirements for CD14 participation in the binding of high doses of LPS and production of TNF-α in J774 cells. (a, b) Binding of sLPS-biotin to the surface J774 cells. Cells were preincubated with 0.05% NaN₃ (30 min, 37°C) and either left untreated (ns) or supplemented with 5, 10, or 15 μg/mL of the anti-CD14 antibody or 10 μg/mL isotype matched control IgG (cIgG) or 50 μg/mL dextran sulfate (DS) or 50 μg/mL chondroitin sulfate (CS) or a mixture of 5 μg/mL anti-CD14 antibody and 50 μg/mL dextran sulfate for 30 min (37°C). Subsequently, 1 μg/mL of sLPS-biotin was added to the cultures for 1 h in the presence of 0.05% NaN₃. (a) Dot-blot analysis of sLPS-biotin in cell homogenates. (b) Densitometric analysis of dot-blot exemplified in (a). Data are mean ± SEM from three experiments. (c) LigandTracer real-time analysis of the binding and internalization of sLPS-AF488. Cells were preincubated for 15 min at 37°C with a mixture of 10 μg/mL anti-CD14 antibody and 50 μg/mL dextran sulfate (DS) or with 50 μg/mL chondroitin sulfate (CS) and transferred into the LigandTracer. After 30 min of the baseline setting (37°C), cells were exposed to 3 μg/mL of sLPS-AF488 for 1 h (association phase), washed to remove the unbound LPS, and monitored for another 1 h to measure retention of sLPS-AF488 in cells. Traces with open symbols and closed symbols reflect sLPS-AF488 binding and internalization in CS-treated and anti-CD14/DS-treated cells, respectively. Concanavalin A-FITC (100 μg/mL; ConA) was added to the anti-CD14/DS-treated culture for 40 min to ensure that the cells were still adherent. (d, e) Production of TNF-α (d) and RANTES (e) in cells pretreated with 10 μg/mL of the anti-CD14 antibody or isotype-matched control rat IgG2b and stimulated with 10–1000 ng/mL of sLPS. Data are mean ± SEM from three experiments. *Significantly different from cells exposed to control IgG.

3.3. Modulation of TNF-α Production by SR. The interference with the LPS/CD14 interaction by the 4C1 antibody only partially reduced TNF-α production induced by high doses of LPS (100–1000 ng/mL) which suggested the involvement of other LPS acceptors, like SR, in this process. To test this assumption we measured TNF-α production in RAW264 cells stimulated with 10–1000 ng/mL of sLPS in the presence of dextran sulfate. An attenuation of the cytokine release in these conditions would indicate the importance of LPS/SR interaction for TNF-α production. However, blocking of sLPS binding to SR by dextran sulfate was found to upregulate rather than inhibit TNF-α production induced by 100 or 1000 ng/mL of sLPS. In these conditions, 50 μg/mL dextran sulfate either alone or in combination with a control antibody enhanced TNF-α release by 20–70% (Figure 6(a)). Similar enhancement was found for MIP-2, another cytokine produced mainly in MyD88-dependent manner (Figure 6(b)), and for RANTES generated in MyD88-independent manner (Figure 6(c)). The presence of dextran sulfate did not affect significantly production of TNF-α and MIP-2 induced by 10 ng/mL of sLPS while RANTES production was moderately inhibited in these conditions (Figures 6(a)–6(c)). Of note, the stimulatory effect exerted on TNF-α, MIP-2, and RANTES production by dextran sulfate at 100–1000 ng/mL LPS was reversed by a simultaneous interference with the LPS/CD14 interaction by the 4C1 antibody (Figures 6(a)–6(c)). In these conditions, the production of TNF-α reached the level found in cells exposed to the anti-CD14 antibody alone...
Figure 6: Occupation of SR by dextran sulfate upregulates sLPS-induced TNF-α production with CD14 participation. RAW264 cells were pretreated with 50 μg/mL dextran sulfate or 50 μg/mL chondroitin sulfate or 10 μg/mL anti-CD14 or 10 μg/mL isotype-matched rat IgG2b (clgG) or combination of the agents, as indicated, and subsequently were exposed to 10–1000 ng/mL of sLPS (37°C). Generation of TNF-α (a), MIP-2 (b), and RANTES (c) was estimated in culture supernatants by ELISA tests. Arrows directed upwards point to the enhancement of cytokine production stimulated by 100 or 1000 ng/mL of sLPS in the presence of dextran sulfate while arrows directed downwards indicate the inhibition of cytokine generation by the anti-CD14 in comparison to cells exposed to sLPS accompanied by dextran sulfate. Data shown are mean ± SEM from three or four experiments each run in triplicates. *Significantly different from cells exposed to chondroitin sulfate and sLPS; # significantly different from cells exposed to dextran sulfate and sLPS.

(Figure 6(a)). These data suggest that the binding of LPS to CD14 is required to support the enhanced TNF-α production stimulated in the presence of dextran sulfate.

4. Discussion

TNF-α is a major pro-inflammatory cytokine produced by mammals during infection with Gram-negative bacteria. This cytokine is generated mainly by macrophages which reside in many tissues and trigger an array of innate immune responses upon encounter of invading pathogens [35]. Monocyte-to-macrophage differentiation is accompanied by upregulation of CD14 expression [36]. Therefore, macrophages are prone to recognize LPS monomers (provided by LBP) by the binding of the lipid A part of LPS molecule to the hydrophobic pocket of CD14 from which endotoxin can be transferred to the TLR4/MD-2 [11, 12, 37]. A line of studies performed on living cells indicates that LPS induces formation of multimolecular
complexes in the plasma membrane composed of TLR4/MD-2, CD14, and several other proteins potentially involved in LPS recognition [23–25]. To assess participation of CD14 in signaling pathways leading to LPS-induced TNF-α production we conducted studies on RAW264 and J774 macrophage-like cells. Our preliminary data indicated that these cells differed greatly in the level of expression of CD14 on their surface; cytometric analysis revealed that the fluorescent signal attributed to CD14 was 2.6-fold stronger in J774 cells than in RAW264 cells (not shown). In both cell lines CD14 was found indispensable for TNF-α production induced by 1 ng/mL of sLPS or rLPS. However, TNF-α production induced by higher concentrations of sLPS and rLPS (100 or 1000 ng/mL) required CD14 to a much lower extent. The inhibition of TNF-α generation exerted by the CD14 neutralizing antibody reached 25–27% at 1000 ng/mL of sLPS in RAW264 and J774 cells. These data indicate that at higher LPS concentrations participation of CD14 in TNF-α production can be partially omitted. On the other hand, a reduction of TLR4 level in cells by half correlated with 50–60% inhibition of TNF-α and RANTES production induced by 1–1000 ng/mL of sLPS and rLPS. This indicates that the cytokines were generated in TLR4-dependent manner, although further confirmation of these results will require studies on TLR4-/− macrophages. Our data are in line with results obtained on human monocytes exposed to the function blocking anti-CD14 antibody MY4 and bone marrow-derived dendritic cells or macrophages of CD14-/− mice stimulated with rLPS or sLPS, respectively [18, 25]. It seems likely that, at high LPS doses, endotoxin molecules can bind either directly to TLR4/MD-2 complex or be transferred to the signaling complex by other LPS-binding proteins localized in the plasma membrane proteins [24]. It was reported recently that albumin also forms complexes with LPS monomers and can transfer endotoxin directly to MD-2 protein [38].

The interference with LPS/CD14 interaction by the anti-CD14 antibody inhibited rLPS-induced TNF-α production relatively weaker in comparison to TNF-α generation induced by sLPS (Figure 1; compare (a) and (c)). In addition, both LPS forms displayed a striking difference in the FBS requirement for the initiation of TNF-α production, and lack of which nullified sLPS activity. These data indicate the differences between sLPS and rLPS modes of action which are just being appreciated. In 2005 Beutler’s group showed that rLPS activity is less CD14-dependent compared to sLPS to induce TNF-α production in murine peritoneal macrophages, and recently these observations were reinforced by studies of sLPS- and rLPS-induced TNF-α production in murine dendritic cells [9, 39]. Disparate requirements for participation of CD14 and FBS (as LBP source) for sLPS- or rLPS-induced IL-6 production were found also in murine mast cell lacking CD14 expression [10] and more recently for TNF-α production in human macrophages [40]. These different requirements of sLPS and rLPS for accessory proteins to mediate cell activation can be attributed to differences in physicochemical properties between these two forms of LPS. It was suggested that highly hydrophobic rLPS can incorporate directly into the plasma membrane to get an access to TLR4 [10]. Furthermore, it was shown that rLPS aggregates rather than monomers are the biologically active form of endotoxin [41] which could explain why rLPS can act without participation of LPS monomer-binding proteins. It is tempting to speculate that aggregates of rLPS (100–1000 ng/mL) can be also responsible for the CD14- and FBS-independent production of RANTES in RAW264 cells (Figure 1(d)). Recently, Watanabe et al. demonstrated that LPS-liposomes can induce RANTES generation without CD14 participation [42]. Otherwise, CD14 is required for sLPS-induced endocytosis of activated TLR4 followed by TRIF-dependent synthesis of cytokines like RANTES ([18]; see also Figure 1(c) in this paper).

In contrast to the conditional involvement of CD14 in sLPS-induced cytokine production, CD14 seems to be crucial for the binding of high amounts of sLPS to the surface of RAW264 and J774 cells. After the blocking of the function of CD14 with 10 µg/mL of the anti-CD14 antibody in RAW264 cells, the binding and internalization of 1000 ng/mL sLPS-biotin were reduced by about 90% and 64%, respectively, while TNF-α production induced by 1000 ng/mL of sLPS was diminished by 25% only (compare Figures 4(b), 4(d), and I(a)). Similar discrepancy in the magnitude of inhibition of sLPS binding and TNF-α production was found in J774 cells exposed to 10 µg/mL of the anti-CD14 antibody (Figures 5(b) and 5(d)). More pronounced sensitivity of sLPS binding to the inhibition by the anti-CD14 antibody in RAW264 cells than in J774 can be attributed to the differences in CD14 surface amounts between these cells. Taken together, the data indicate that in macrophages CD14 participates in the binding and internalization of large quantities of sLPS, similarly as indicated for monocytes [21, 43]. However, CD14-mediated binding and internalization of sLPS are required only to a certain extent for the signaling of TLR4 and TNF-α production. The internalized sLPS serves possibly as an activator of TRIF-dependent signaling pathway of TLR4 in endosomes or is directed for detoxification. The participation of CD14 in the internalization of high doses of sLPS was reflected by sustained reduction of the cell surface level of CD14, reaching 21% after 1h and 38% after 2h of J774 cell stimulation with 1000 ng/mL of sLPS (not shown).

Significant portion of sLPS can be bound and internalized also with the participation of SR judging from the attenuation of those processes by dextran sulfate, a competitive ligand of SR. Among scavenger receptors, SR-A was indicated earlier as mediating uptake of large quantities of LPS in macrophages having a protective function against excessive pro-inflammatory responses to LPS [19, 20, 44]. Participation of SR in removal of the excess of sLPS can explain why TNF-α, MIP-2, and RANTES production increased in conditions when SR/LPS binding was inhibited by dextran sulfate (Figure 6). Recent data indicate, however, more complex scenario of SR-A involvement in LPS-induced cytokine production [22, 45]. As we reported earlier, activation of SR-A by dextran sulfate can upregulate CD14 and TLR4 expression on the cell surface and contribute this way to the high TNF-α production when both LPS and dextran sulfate are present [22]. The function blocking anti-CD14 antibody reversed
the enhancement of TNF-α generation induced by 100–1000 ng/mL sLPS accompanied by dextran sulfate (Figure 6) indicating that participation of CD14 is a limiting factor in this SR-related activity. It should be noted that both CD14 and SR mediate the binding of high doses of sLPS since this process was strongly inhibited by the anti-CD14 antibody and dextran sulfate (Figures 4 and 5). Although an additive effect of these two agents suggests a partial separation of CD14- and SR-dependent binding of sLPS, a cooperation between these two receptors is possible. Taking into account that CD14 is a plasma membrane raft protein [46] and SR associates with caveolae rafts [47] these regions of the plasma membrane can serve as platforms for the putative interaction between CD14 and SR-A. This suggestion is supported by recent report demonstrating that activation of SR-A enhanced the interaction of SR-A, caveolin, and major vault protein (MVP) located in rafts which led to the increase of TNF-α production in RAW264 cells [48].

5. Conclusion

Our data suggest that CD14 participates in the binding and internalization of large quantities of sLPS but these events only to some extent are required for signaling leading to TNF-α production. The generation of TNF-α induced by sLPS is even less dependent on CD14. Besides CD14, SR are involved in the binding of large quantities of sLPS and the involvement of SR modulates sLPS-induced TNF-α production.

Conflict of Interests

Hanna Björkland is employed by Ridgeview Instruments AB. Other authors declare that they have no conflict of interests.

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References


Review Article
The Role of TL1A and DR3 in Autoimmune and Inflammatory Diseases

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TNF-like ligand 1A (TL1A), which binds its cognate receptor DR3 and the decoy receptor DcR3, is an identified member of the TNF superfamily. TL1A exerts pleiotropic effects on cell proliferation, activation, and differentiation of immune cells, including helper T cells and regulatory T cells. TL1A and its two receptors expression is increased in both serum and inflamed tissues in autoimmune diseases such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and ankylosing spondylitis (AS). Polymorphisms of the TNFSF15 gene that encodes TL1A are associated with the pathogenesis of irritable bowel syndrome, leprosy, and autoimmune diseases, including IBD, AS, and primary biliary cirrhosis (PBC). In mice, blocking of TL1A-DR3 interaction by either antagonistic antibodies or deletion of the DR3 gene attenuates the severity of multiple autoimmune diseases, whereas sustained TL1A expression on T cells or dendritic cells induces IL-13-dependent small intestinal inflammation. This suggests that modulation of TL1A-DR3 interaction may be a potential therapeutic target in several autoimmune diseases, including IBD, RA, AS, and PBC.

1. Characteristics of TL1A and DR3

1.1. TL1A. TL1A, also referred to as vascular endothelial growth inhibitor (VEGI)-251, is a member of the tumor necrosis factor superfamily (TNFSF) of ligands, which was identified by Migone et al. in 2002 [1]. Although TL1A was identified as a longer variant of TL1/VEGI, the fourth exon of TL1A encodes the majority of TL1/VEGI, and it has been presumed that the original TL1/VEGI was a cloning artifact. TL1A exhibits approximately 20–30% homology to other TNFSF members [1]. Human TL1A consists of 251 amino acids: 35 in the cytoplasmic domain, 24 in the transmembrane region, and 192 in the extracellular domain. There are two potential N-linked glycosylation sites in the TL1A amino acid sequence, specifically Asn residues at amino acids 133 and 229 [1]. TL1A is a type II transmembrane protein. TL1A is initially expressed as a membrane-bound protein and is subsequently released as a soluble protein via ectodomain shedding by a metalloproteinase such as TNF-α converting enzyme (TACE) [2, 3]. TL1A expression is detected on human umbilical vein endothelial cells and synovial fibroblast-like cells and is upregulated by stimulation with proinflammatory cytokines such as TNF-α, IL-1, and PMA, a phorbol ester known to be a potent activator of protein kinase C [1, 4]. TL1A expression has also been confirmed on antigen-presenting cells and lymphocytes that are activated by Toll-like receptor (TLR) ligands, enteric bacteria, and Fcγ receptor (FcγR) crosslinking [5–7].

1.2. DR3. DR3, also known as APO-3, TRAMP, LARD, and WSL-1, is a member of the tumor necrosis factor receptor superfamily (TNFRSF) with a typical death domain that consists of an approximately 60-amino-acid globular bundle of 6 conserved α helices found in the cytoplasmic region. Although DR3 is most homologous to TNFR1, which is
widely expressed, its expression is mostly restricted to lymphocytes such as NK cells and T cells, in particular NKT cells and is enhanced upon their activation [8–10]. DR3 is more highly expressed on Th17 cells than on Th1 and Th2 cells, and is also expressed on naturally occurring and TGF-β-induced Treg cells (n-Treg and i-Treg, resp.) [11–13]. It was recently shown that DR3 expression on B cells was induced by anti-IgM stimulation, although its expression was not detectable on resting B cells [14]. There are several expression differences between human and mouse [9]. DR3 splicing variants of 13 in human [8, 15, 16] and 3 in mice [17] have been identified. Pappu et al. showed that DR3 splicing variants are differentially expressed on T-cell subsets in mice [13].

2. Role of TL1A-DR3 Signaling in Cell Fate Determination

TL1A-DR3 signaling induces both NF-κB activation and apoptosis in vitro [1, 18, 19]. TL1A-DR3 interaction induces the formation of signaling complexes containing TRADD, TRAF2, and RIP and activates the NF-κB and MAPK pathways (ERK, p38, and JNK). The activation of NF-κB induces c-IAPs, which protect against apoptosis [20]. On the other hand, DR3 overexpression in embryonic cells also induces FADD- and caspase-8-dependent apoptosis [21, 22]. Blocking of TL1A-DR3 signaling by adding NF-κB inhibitors or protein synthesis inhibitors induces apoptosis [20], suggesting that NF-κB activation by TL1A-DR3 interaction is responsible for resistance to apoptosis. Analysis of DR3-deficient mice has shown that DR3 is required for negative selection in the thymus [23]. Inhibition of TL1A-DR3 interaction has shown that TL1A-DR3 signaling is required for effective T-cell immune responses in the target organs of T-cell-mediated autoimmune diseases and inflammatory diseases [24]. Thus, TL1A-DR3 signaling may be involved in lymphocyte homeostasis by modulating either cell death or lymphocyte activation.

3. Role of TL1A and DR3 Signaling in the Immune System

3.1. Th1. Under TCR stimulation, TL1A induces cell proliferation and the secretion of proinflammatory cytokines (including IFN-γ, GM-CSF, and TNF-α) in T cells, in particular memory CD4+ T cells [1, 25]. Sustained TL1A expression on T cells or dendritic cells leads to an increase in the number of activated CD4+ T cells and memory CD4+ T cells, whereas sustained TL1A expression on dendritic cells does not stimulate conventional T cells in the absence of TCR stimulation in vivo, suggesting that TL1A may act as a costimulator for T cells to regulate inflammatory cytokines and cell proliferation [26, 27]. TL1A synergizes with IL-12/IL-18 to promote IFN-γ production in T cells in an antigen-independent manner [25, 28]. TL1A itself cannot directly induce Th1 differentiation of native CD4+ T cells, while TL1A-deficient mice show the decrease of IFN-γ-producing CD4+ T cells [13]. Collectively, it is speculated that TL1A indirectly or synergistically with other cytokines enhances Th1 responses of activated and memory CD4+ T cells. The differential T-cell responsiveness for TL1A between naïve and activated/memory T cells might be explained by the regulation of DR3 splicing variants, in particular full-length transmembrane variant, which encodes complete transmembrane DR3 protein. The expression of full-length DR3 mRNA and protein is low level or not detected in naïve and resting T cells [8], whereas it is upregulated in activated T cells [9, 25, 29]. In mice with chronic intestinal inflammation, the transmembrane DR3 expression is upregulated [25].

3.2. Th17. Although exogenous TL1A induces IL-2 secretion and responsiveness in T cells [1, 24], IL-2 is a negative regulator for Th17 cells [30]. Therefore, when IL-2 is blocked, exogenous TL1A induces the differentiation of Th17 cells from naïve CD4+ T cells stimulated with TCR under Th17 polarization condition in vitro. TL1A also induces the proliferation of in vitro-differentiated Th17 effector cells but neither Th1 nor Treg cells even in the absence of TCR stimulation [11, 13]. This differential TL1A responsiveness in T-cell subset might be explained by DR3 expression. DR3 expression is upregulated at a later stage but not early stage of Th17 differentiation. Total DR3 expression is increased in Th17 cells as compared with Th1 and Th2 cells, and full-length transmembrane DR3 expression is increased in Th17 cells as compared with Treg cells [13]. Thus, TL1A-DR3 interaction might preferentially act on Th17 cells and differentially affect the differentiation and maturation of Th17 cells. On the other hand, TL1A inhibits Th17 cell differentiation even in the presence of anti-IL-2 neutralizing antibody in vitro [11]. Activation of STAT1 signaling is induced by inflammatory cytokines such as IL-27, IFN-γ, and type I IFN and inhibits Th17 differentiation in vitro [31]. However, the inhibitory mechanism of Th17 differentiation by TL1A was independent of activation of STAT1 signaling as well as IL-2 signaling [11]. Further, DR3 is dispensable for Th1, Th2, and Th17 differentiation from naïve CD4+ T cells in vitro [24]. Thus, the role of TL1A in Th17 differentiation is still controversial in vitro. However, TL1A transgenic mice [26, 27], and Th17-mediated autoimmune disease model mice, experimental autoimmune encephalomyelitis [13] and dextran-sulfate-induced chronic colitis [32], show that TL1A-DR3 interaction could positively regulate Th17 cell function in vivo. Further research will be required to elucidate the regulatory mechanism of TL1A-DR3 interaction for Th17 cell function in vitro and in vivo.

3.3. Th2. TL1A-DR3 interaction is involved in Th2- as well as Th1- and Th17-mediated immune responses. Transgenic mice that constitutively express TL1A specifically in T cells or dendritic cells develop Th2 cytokine IL-13-dependent small intestinal inflammation [26, 27]. Intranasal immunization with ovalbumin (OVA) together with TL1A in mice induces Th2-mediated immune responses, including OVA-specific IgG1 antibody production in serum, IgA antibody production in mucosal tissues, and the production of Th2 cytokines IL-4 and IL-5 from OVA-restimulated splenocytes in vitro [33]. Studies have shown that in DR3-deficient mice, or following blockade of TL1A-DR3 interaction by TL1A neutralization antibodies, OVA-induced lung inflammation is attenuated
and Th2 cytokines IL-4, -5, and -13 production is reduced in a mouse model of asthma [10, 24]. In mice with small intestinal inflammation or OVA-induced lung inflammation, NKT cells, activated and memory CD4+ T cells, or eosinophils are likely to be a main source of the Th2 cytokines that are induced by the TL1A-DR3 signaling pathway [10, 24, 26, 27], suggesting that TL1A-DR3 signaling in these cells might be a therapeutic target in asthma and ulcerative colitis.

3.4. Treg Cells. TL1A transgenic mice show the proliferation and activation of Treg cells in the secondary lymphoid organs and the small intestinal lamina propria [26, 27, 34]. Although exogenous TL1A itself does not affect either n-Treg or i-Treg proliferation in vitro, it promotes Treg cell proliferation in the presence of antigen presenting cells with TCR stimulation both in vitro and in vivo [13, 35], suggesting that TCR signaling is required for costimulation of Treg cells as well as conventional T cells by TL1A. Agonistic anti-DR3 antibodies expand the proliferation of preexisting Treg cells in a manner dependent on TCR and IL-2 signaling in vivo, and the expanded Treg cells inhibit OVA-induced lung inflammation [12]. Although Treg cells derived from TL1A-treated mice have highly suppressive activity ex vivo, both exogenous TL1A and agonistic anti-DR3 antibodies directly inhibit the suppressive activity of Treg cells in vitro [12, 35]. Treg cells derived from mice that constitutively express TL1A under the CD2 promoter maintain their suppressive ability [27]. These results suggest that the effect of TL1A-DR3 interaction on T cells might be highly dependent on experimental conditions in vitro or the context of the immune response that is being modulated in vivo.

3.5. NK and NKT Cells. TL1A and agonistic anti-DR3 antibodies synergize with IL-12 and IL-18 to augment IFN-γ production in NK cells and NKT cells as well as T cells [28]. The fold-induction of IFN-γ production by the addition of TL1A is significantly lower in NK cells and NKT cells than in CD4+ T cells and CD8+ T cells. The combination of IL-12 and IL-18 drastically increased the DR3 expression in NK cells but minimally in T cells. These data suggest that the augmentation of IL-12/IL-18-induced IFN-γ in response to TL1A is differentially induced in T cells and NK cells. TL1A also enhances IL-12/IL-18-induced NK cell cytolytic activity, which is independent of IFN-γ production [36], suggesting that TL1A might be an attractive molecule for tumor therapy. Agonistic anti-DR3 antibodies costimulate the proliferation and IL-13 production of NKT cells stimulated with α-galactosceramide or anti-CD3 antibodies [10]. NKT-deficient mice, which are resistant to OVA-induced allergic lung inflammation, restore the lung inflammation upon adoptive transfer of wild-type NKT cells, but not after transfer of dominant negative DR3 transgenic NKT cells, suggesting that DR3 signals in NKT cells play an important role for triggering lung inflammation [10].

3.6. B Cells. In contrast to T cells, there have been few reports on the significance of TL1A-DR3 interaction in B cells. Membrane-bound TL1A expression on resting B cells was found to be at very low levels in mice [27]. TL1A expression is not induced in B cells either during resting or activated conditions. In vitro, TL1A directly reduces B-cell proliferation induced by anti-IgM antibodies and IL-2, whereas it does not affect B-cell proliferation induced by a combination of anti-IgM antibodies and other B-cell-specific stimulators, namely, CpG oligodeoxynucleotide and CD40 ligand [14].

Collectively, these reports indicate that TL1A-DR3 interaction exerts pleiotropic effects on adaptive immune cells, including their activation, proliferation, differentiation, cytokine production, and maintenance.

4. Association of TNFSF15 Gene Polymorphisms with Autoimmune and Inflammatory Diseases

To examine the association of TNFSF15 gene polymorphisms with autoimmune diseases, Yamazaki et al. performed a genomewide case-control study and found that TNFSF15 gene polymorphisms are associated with the susceptibility to CD in a Japanese population as well as IBD in a European population [37]. Subsequent replication studies and genomewide association studies have revealed that TNFSF15 is only one gene that is associated with CD or IBD in both Asian and Caucasian population [38–45]. TNFSF15 gene polymorphisms are also associated with the severity of CD and IBD in Japanese and Caucasian population, respectively [46–48]. TNFSF15 haplotypes A and B (which consist of five polymorphisms: rs3810936, rs6478108, rs6478109, rs7848647, and rs7869487) are risk and protective factors, respectively, for susceptibility in both Asian CD and Caucasian IBD patients [37, 39, 49], and haplotype B is a risk factor for severity and antibody status for E. coli outer membrane porin C in Jewish CD patients [49, 50]. A polymorphism of TNFSF15 haplotype A increases promoter activity in stimulated T cells [51], whereas TNFSF15 haplotype B is associated with increased soluble and membrane TL1A expression in some Jewish CD patients [50]. In addition to IBD, TNFSF15 gene polymorphism rs4263839 is associated with susceptibility to irritable bowel syndrome and ankylosing spondylitis in Caucasians [52, 53], and we recently found that TNFSF15 gene polymorphism rs4979462 is associated with susceptibility to PBC in a Japanese population [54]. These findings suggest that TNFSF15 gene polymorphisms contribute to altered TL1A production, leading to the pathogenesis of autoimmune and inflammatory diseases. In addition to TNFSF15, polymorphisms of IL-23R and IL-12A/IL-12RB2 are associated with susceptibility to IBD [55, 56] and PBC [57], respectively. TL1A and IL-23 or IL-12 synergistically induce Th1- and Th17-effector cells, implicating the TL1A-IL12/IL-23 pathway in the pathogenesis of both IBD and PBC. Zhang et al. reported that TNFSF15, NOD, and IL-23R are susceptibility genes for leprosy in a Chinese population [58, 59]. These genes are also susceptibility genes in CD, suggesting that CD and leprosy...
may share a common disease pathway, in particular innate immunity and inflammatory responses.

5. Role of TL1A and DR3 in Autoimmune and Inflammatory Diseases

5.1. RA. TL1A expression is elevated in the serum, synovial fluid, and synovial tissues of RA patients, in particular patients who are positive for rheumatoid factor (RF). Its expression is correlated with the severity of RA [6, 60]. DR3 gene duplication is more prevalent in RA patients as compared to healthy subjects [61]. Immunohistochemical staining found that TL1A-positive cells in the synovial tissue of RA patients, in particular RF-positive patients, are positive for CD14 and CD68, which are surface markers of macrophages and monocytes [6]. TL1A is induced in human synovial fibroblasts stimulated with TNF-α and IL-1β [4] and in monocytes stimulated with insoluble immune complexes derived from RA patients [6]. TL1A induces T cells to secrete TNF-α and IL-17 under TCR stimulation or Th17 polarization conditions, respectively [4], and it synergizes with IFN-γ and augments the production of CXCL8 and matrix metalloproteinase 9 in the human monocytic cell line THP-1 [62]. These inflammatory cytokines and chemokines are associated with RA pathology, and therefore it is possible that TL1A and these inflammatory cytokines form a vicious loop that aggravates RA pathogenesis. Indeed, administration of TL1A and these inflammatory cytokines form a vicious loop associated with RA pathology, and therefore it is possible that serum TL1A levels in RA patients [60]. In a mouse model, RA [4]. DR3 knockout mice show resistance to development of adverse bone pathology in experimental antigen-induced arthritis (AIA), and TL1A promotes osteoclastogenesis in a DR3-dependent manner in AIA model mice [63], suggesting that DR3 is involved in the generation of osteoclasts at site of bone pathology. Collectively, TL1A-DR3 interaction forms a part of the inflammatory cytokine network and contributes to RA pathogenesis by promoting osteoclastogenesis and the production of inflammatory cytokines and autoantibodies. Furthermore, administration of neutralizing antibodies against TL1A ameliorated both AIA and CIA [63], suggesting that the TL1A-DR3 pathway may be a potential therapeutic target in RA patients.

There has been growing evidence that RA patients are at elevated risk for cardiovascular disease [64]. It was reported that TL1A and its receptors are correlated with atherosclerosis [65]. TL1A regulates the expression of genes implicated in the uptake (scavenger receptors such as SR-A, SR-B1, and CD36) and efflux of cholesterol (ABCG-1, ABCA-1, and ApoE), leading to promotion of foam cell formation in human macrophage [65]. This TL1A-induced macrophage foam cell formation is dependent on DR3. DR3 itself also promotes macrophage foam cell formation by regulating the expression of genes implicated in the uptake and efflux of cholesterol [65]. In addition, it was recently reported that elevated serum TL1A at baseline positively correlates with the progression of atheromatic plaque height in RA patients [66]. A combination of low TL1A and undetectable DcR3 levels in serum at the baseline correlates with decrease of new atheromatic plaques in carotid arteries and/or femoral arteries of RA patients over a follow-up period of 3.5 years, suggesting that serum TL1A and DcR3 levels might predict a preserved atherosclerosis profile in carotid and/or femoral arteries. The expression of D3R mRNA but neither TL1A nor DcR3 shows a trend to evaluation in atheromatic plaques of arterial tissue. Collectively, these data highlights that TL1A-DR3/DcR3 signaling is involved in chronic inflammation and atherosclerosis.

5.2. Human IBD. In IBD patients, TL1A expression is increased in both serum and intestinal tissues and is correlated with the disease activity [67–69]. DR3 expression is also increased in lymphocytes, in particular T cells, in the intestinal lamina propria in these patients [68]. Interestingly, TL1A-expressing cells in the lamina propria are macrophages and CD4+ or CD8+ T cells in CD patients, whereas they are mainly plasma cells in UC patients [68]. The diversity of TL1A expression might reflect differences in the pathogenesis of CD and UC. TL1A expression is increased only in lamina propria CD4+ macrophages (but not peripheral monocytes or monocyte-derived macrophages) in CD patients but not in UC patients or healthy subjects. The membrane-bound expression of TL1A is induced in lamina propria CD4+ macrophages stimulated by commensal bacteria such as E. coli [70]. In UC, IgG-producing plasma cells were found to infiltrate areas of mucosal inflammation, and IgG immune complex stimulation increased TL1A expression in macrophages in the lamina propria [71]. These findings indicate that mononuclear phagocytes are likely to be a major source of TL1A in inflamed loci in the intestines of IBD patients. In CD patients, exogenous TL1A and agonistic anti-DR3 antibodies augment IFN-γ production in lamina propria mononuclear cells as well as peripheral blood mononuclear cells [68, 69]. TL1A synergizes with IL-12/IL-18 and induces IFN-γ production in lamina propria CCR9+ T cells derived from CD patients, which is thought to be implicated in the pathogenesis of CD [72]. Exogenous TL1A and IL-23 synergistically induce IFN-γ and IL-17 secretion in lamina propria CD4+ T cells. TL1A enhances the differentiation of IL-17- and IFN-γ/IL-17-producing Th17 cells when naïve CD4+ T cells are stimulated with lamina propria macrophages derived from CD patients [70]. These reports demonstrate that TL1A-DR3 interaction may contribute to Th1- and Th17-mediated responses that are characteristic of CD.

5.3. IBD Mouse Model. TL1A is increased in the intestines of IBD model mice, including dextran-sodium-sulfate (DSS)-induced chronic colitis mice and TNFαARE chronic ileitis mice. Its major source is likely to be dendritic cells in mesenteric lymph nodes and small intestinal lamina propria mononuclear cells [25, 32]. Mice with constitutive TL1A expression in antigen-presenting cells and T cells show intestinal inflammation and colonic fibrosis with a high percentage of T cells that are positive for CCR9 and CCR10,
both of which are gut-homing chemokines in T cells [34]. As with TL1A, DR3 expression is increased in DSS-induced chronic colitis mice, and its transmembrane splicing variant is increased in correlation with inflammation in chronic ileitis mice, TNA^ARE^ mice, and SAMPI/Yit Fc mice [25, 32]. Administration of blocking anti-TL1A monoclonal antibodies inhibits DSS-induced colonic inflammation in mice [32], and DR3-deficient mice are protected from intestinal inflammation even after colitis induction [27]. In mice with 2,4,6-trinitrobenzenesulfonic acid- (TNBS-) induced colitis, colonic inflammation is inhibited by administration of anti-TL1A or anti-DR3 antibodies [27]. These reports demonstrate that TL1A-DR3 interaction plays an important role in the pathogenesis of these chronic intestinal inflammatory conditions in mice. TL1A synergizes with IL-12 or IL-23 and induces both IFN-γ and IL-17 secretion in CD4^+^ T cells derived from gut-associated lymphoid tissue (GALT) of DSS-induced chronic colitis mice [32]. IL-23 itself or IL-23 in combination with TL1A induces IFN-γ/IL-17 double-positive CD4^+^ T cells that are known to be colitogenic [32]. Sustained TL1A expression in T cells or dendritic cells promotes goblet cell and Paneth cell hyperplasia in the small intestine in mice [26, 27, 34]. Hyperplasia of goblet cells and Paneth cells is associated with elevated Th2 cytokine production [73, 74]. In mice with sustained TL1A expression, IL-13 and IL-17 expression is increased in mesenteric lymph node cells, ileum, and CD4^+^ T cells isolated from the lamina propria. Administration of antagonistic anti-IL13 antibodies but not anti-IL17 antibodies attenuates intestinal inflammation in mice constitutively expressing TL1A [27], suggesting that IL-13 plays an important role in the small intestinal inflammation induced by sustained TL1A expression on T cells or dendritic cells. Although agonistic anti-DR3 antibodies and glycosphingolipid enhance the production of Th2 cytokines IL-13 and IL-4 in NKT cells [10], mice with sustained TL1A expression and small intestinal inflammation show decreased NKT cells and increased activated and memory CD4^+^ T cells, suggesting that in these mice the major source of IL-13 is likely to be activated and memory T cells [26, 27]. In human, CD is associated with the Th1/Th17 cytokines IL-12 and IL-23, and UC with the Th2 cytokine IL-13 [75]. Taken together, these findings in IBD model mice provide evidence that TL1A-DR3 interaction may contribute to both Th1/Th17 and Th2 signaling pathways in human IBD.

5.4. Psoriasis. Bamias et al. reported an association of TL1A and its two receptors with psoriasis [76]. TL1A is mainly expressed in keratinocyte, basal cells, vascular cells, and infiltrating inflammatory cells of psoriatic skin but is rarely expressed in those of normal skin. DR3 and DcR3 are expressed in normal skin and are upregulated in psoriatic skin. The expression levels of TL1A and its two receptors are upregulated in lesional skin as compared to nonlesional skin in psoriasis patients, suggesting that TL1A and its two receptors may be involved in the pathogenesis of psoriasis. Immunohistochemical staining shows that TL1A localizes at nuclear region in inflammatory and fibroblast-like cells in psoriasis patients, although previous studies reported that TL1A localizes at cytoplasmic region in inflamed tissues of several autoimmune diseases [6, 68]. In this paper, the unique nuclear location is also confirmed in synoviocytes and inflammatory cells in RA patients. Further study is needed to show the significance of nuclear TL1A localization. TL1A transgenic mice develop chronic intestinal inflammation and a minority of the mice also develop ulcerative skin lesions and arthritis. Concurrent rate among IBD, RA, and psoriasis is high in human. These reports might provide the evidence that TL1A is one of common denominators of gut, joint, and skin inflammation.

5.5. PBC. We recently found that TL1A expression is increased in both serum and liver tissues of PBC patients. In the liver, TL1A expression is positive for infiltrating mononuclear cells, endothelial cells, Kupffer cells, and biliary epithelial cells. Serum TL1A levels are decreased in early-stage but not late-stage patients being treated with ursodeoxycholic acid, the only therapeutic drug for PBC approved by the Food and Drug Administration, suggesting that TL1A has a potential to be a new serum marker and therapeutic target for PBC [77].

6. The Association of DcR3 with Autoimmune and Inflammatory Diseases

DcR3, also known as TR6, and M68, is a member of the TNFRSF. DcR3 consists of 300 amino acids lacking a transmembrane domain of TNFRSF and be released as secreted protein. DcR3 functions as a decoy receptor for TL1A as well as FasL and LIGHT and inhibits these ligands mediated apoptosis and lymphokine secretion [78–81]. DcR3 is induced in human antigen-presenting cells such as monocytes and myeloid dendritic cells and intestinal epithelial cells lines by lipopolysaccharide or lipoteichoic acid and is also induced in human dermal microvascular endothelial cells by TNF-α and IL-1β [82, 83]. DcR3 modulates the differentiation and maturation of monocyte, macrophage, and dendritic cells, polarization of naïve T cells into Th-2 immune response [84], and the negative regulation for activation of B cells by TL1 ligands [85]. DcR3 is rarely detectable in serum of healthy subjects, whereas its expression is increased in that of various autoimmune and inflammatory diseases such as IBD [67, 86], SLE [78], RA [60], PBC [77], silicosis [87], viral infections [82], renal failure [88], and atopic dermatitis [89] as well as cancer [90]. DcR3 is not found in mouse genome, suggesting that additional complexity of TL1A-DR3 pathway in human as compared to mouse. DcR3 protects the development of autoimmune diabetes [91, 92], IgA nephropathy [93], and crescent glomerulonephritis [94] model mice, while DcR3-transgenic mice develop SLE-like syndrome [95]. These reports suggest that DcR3 also plays an important role in the pathogenesis of autoimmune and inflammatory diseases.

7. Conclusion

TL1A expression is transiently induced by inflammatory cytokines, TLR ligands, enteric bacteria, and FcyR
crosslinking in antigen-presenting cells or non-immune cells. Although TL1A was initially characterized as a costimulator for inducing cell proliferation and cytokine secretion in T cells, there is growing evidence that TL1A has pleiotropic effects such as cell death, differentiation, and maintenance of lymphocytes, as well as osteoclastogenesis and atherosclerosis. Increased TL1A expression and/or TL1A (TNFSF15) gene polymorphisms are associated with the pathogenesis of various autoimmune and inflammatory diseases. Analysis of murine models of autoimmune diseases and TL1A or DR3 transgenic mice suggests that TL1A-DR3 interaction plays an important role in local inflammation of T-cell-dependent autoimmune diseases. Thus, TL1A connects innate immune responses to adaptive immune responses and is critically involved in the induction of autoimmune and inflammatory diseases (Figure 1), suggesting that inhibition of TL1A-DR3 interaction could be an effective therapeutic strategy for ameliorating local inflammation in target organs of individuals with autoimmune diseases.

Conflict of Interests
The authors declare that they have no conflict of interests.

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Review Article

TNF and TNF Receptor Superfamily Members in HIV infection: New Cellular Targets for Therapy?

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Tumor necrosis factor (TNF) and TNF receptors (TNFR) superfamily members are engaged in diverse cellular phenomena such as cellular proliferation, morphogenesis, apoptosis, inflammation, and immune regulation. Their role in regulating viral infections has been well documented. Viruses have evolved with numerous strategies to interfere with TNF-mediated signaling indicating the importance of TNF and TNFR superfamily in viral pathogenesis. Recent research reports suggest that TNF and TNFRs play an important role in the pathogenesis of HIV. TNFR signaling modulates HIV replication and HIV proteins interfere with TNF/TNFR pathways. Since immune activation and inflammation are the hallmark of HIV infection, the use of TNF inhibitors can have significant impact on HIV disease progression. In this review, we will describe how HIV infection is modulated by signaling mediated through members of TNF and TNFR superfamily and in turn how these latter could be targeted by HIV proteins. Finally, we will discuss the emerging therapeutics options based on modulation of TNF activity that could ultimately lead to the cure of HIV-infected patients.

1. Introduction

The term tumor necrosis factor (TNF) came into existence in 1975 with the work of Carswell and colleagues while studying hemorrhagic necrosis by endotoxin [1]. It was described as a host factor, a glycoprotein induced in response to endotoxin that has the capacity to kill the tumor. As the time progressed, TNF was realized to be rather a member of a superfamily that governs by binding to their receptors. TNF and TNF receptors (TNFR) are growing members of ligand and receptor superfamily that regulate several complex signaling pathways leading to apoptosis, inflammation, cellular differentiation, and antiviral state. The first member of TNF superfamily discovered is TNF-alpha (old name cachectin), a pleiotropic proinflammatory cytokine that plays pivotal role in several pathological conditions due to inflammation and infection [2]. Role of TNF in malignancies and inflammation conditions like arthritis have been reviewed extensively elsewhere [3–5].

Till date TNF superfamily comprises of 19 ligands and 29 receptors [4]. All members are proinflammatory in nature playing diverse roles [4]. Most of the members act like dual edge sword, both beneficial and in adverse role [4, 6, 7]. First two members of TNF ligand (TNFL) superfamily were TNF-alpha and TNF-beta, recognized first at protein level followed by identification of their respective cDNAs, while rest of the members were discovered based on cDNA sequence homology [4, 8, 9]. All members of TNF superfamily and their receptors have been comprehensively reviewed recently [4]. Besides TNF-alpha and TNF-beta, TNFL superfamily include CD40L, CD30L, FasL, TNF-related apoptosis-inducing ligand (TRAIL), lymphotoxin-beta (LT-beta), LIGHT, receptor activator of NF-kappaB ligand (RANKL), 4-1BBL, CD27L, OX40L, TNF-related weak inducer of apoptosis (TWEAK), a proliferation-inducing ligand (APRIL), B-cell activating factor (BAFF), vascular endothelial cell growth inhibitor (VEGI), ectodysplasin A (EDA)-A1, EDA-A2, and GITRL.
2. TNF-Alpha-Mediated Cell Signaling: An Overview

Most extensively studied member of TNF superfamily is TNF-alpha. TNF-alpha is produced in response to pathological conditions like inflammation and infection mainly by activated macrophages and T lymphocytes [4, 7], but also by several cell types including natural killer (NK) cells, mast cells, and fibroblasts. TNF-alpha is synthesized as pro-TNF, a 25 kDa plasma membrane bound protein that is further processed by metalloproteinase called TNF-alpha converting enzyme into a 17 kDa soluble form [12]. Both forms are functional in their trimeric forms via binding to their receptors. Data suggest that plasma membrane associated 25 kDa TNF-alpha form binds to the TNFR2 with high affinity whereas soluble 17 kDa form interacts with TNFR1 with high specificity [13, 14].

TNF-alpha triggers several signaling cascades which include apoptotic pathways, NF-kappaB stimulation, and activation of p38 MAPK, ERK, and JNK [4, 7] (Figure 1). Binding of the ligand TNF-alpha to its receptor TNFR1 leads to the recruitment of a 34 kDa adapter protein called TNFR-associated death domain (TRADD). Latter interacts with the cytopathic death domain of TNFR1 through its own death associated death domain (TRADD). TNF-alpha can activate transcription factor called activator protein-1 (AP-1) by binding to TNFR1 followed by sequential contribution of TRADD, TRAF2-RIP, MEKK1, M KK7 and JNK [4, 7, 20] (Figure 1).

Cell signaling associated with TNFR2 is poorly understood. TNFR2 lacks death domain, despite of that it interacts with TRAF2 through which it can activate transcription factors NF-kappaB and AP-1 (Figure 1). There are several reports where TNFR2 has been reported to be involved in cellular proliferation, apoptosis, and the induction of granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion [21–23].

Significance of TNF-alpha can be evaluated by this fact that several human pathogens have evolved mechanisms to combat TNF-alpha-mediated response against infection [24]. 2013 has been marked as 30 years of discovery of HIV. In this review, we will focus on TNF and TNFR and their family members in context to HIV infection and potentially how to modulate them by TNF inhibitor therapy.

3. Role of TNF and TNFR Superfamily Members in HIV Pathogenesis

3.1. TNFRI and TNFR2

3.1.1. TNF and HIV Entry. The first and foremost part of any virus life cycle is its entry into permissive cells. TNF-alpha is known to target HIV entry step specifically in macrophages but not in peripheral blood lymphocytes [25]. Notably, in cell culture, TNF-alpha is released by primary macrophages infected with HIV type 1 (HIV-1) or treated with HIV envelope protein gp120 [26]. One of the plausible strategies of inhibition of HIV entry by TNF-alpha may be by downregulating the expression of HIV receptor and coreceptor on cell surface (CD4 and CCR5) that may explain inhibition of HIV entry into permissive cells [27]. In addition, GM-CSF secretion is stimulated by TNF-alpha that in turn can downregulate CCR5 and may inhibit the entry of CCR5-dependent viruses into macrophages [28]. Moreover, it has been reported by Herbein and colleagues that pretreatment of tissue culture-differentiated macrophages (TCDM) with human recombinant TNF-alpha (hrTNF-alpha) resulted in remarkable delay in detection of HIV DNA long terminal repeat (LTR) as a result of strong inhibition of virus entry into these cells. Furthermore, using TNF-R1 and TNF-R2 mutants, they demonstrated that this inhibition was mediated through TNF-R2 not TNF-R1 [25–27].

3.1.2. TNF and HIV Postentry Stages. TNF-alpha can activate HIV-1 in chronically infected T cell lines and promonocytic cell lines through translocation of NF-kappaB to the nucleus followed by activation of HIV LTR [29–33]. However, contradictory findings have been also reported where TNF-alpha has shown to inhibit HIV-1 replication in several cell types including freshly infected peripheral blood monocytes, alveolar macrophages, and TCDM [25, 34, 35]. These findings
Mediators of Inflammation

NF-κb mediated gene expression

Apoptosis

Caspase 8

Mitochondria

ROS, cyto C, bax

Caspase 3

Caspase 9

JNK

IKK

NF-αB

Figure 1: TNF and TNFR superfAMILY-mediated cell signaling. Binding of TNF-alpha to TNFRs results in activation of its receptors followed by recruitment of adaptor proteins (TRADD, FADD, TRAF, and RIP) in sequential manner that activates several signaling cascades leading to the activation of transcription factors NF-kappaB, AP-1, and/or caspase cascades. Most of the members of TNF superfamily activate also NF-kappaB.

indicate that TNF-alpha may have contrasting impact on HIV-1 replication in chronically infected cells and cells coming in contact with the virus for the first time [35]. In addition, TNF-alpha induces several HIV suppressive factors such as RANTES in lymphoid cells [36, 37] and alveolar macrophages [35], MIP-1alpha, and MIP-1beta in human fetal microglia cells [38, 39] that may explain the negative role of TNF-alpha in HIV replication.

3.1.3. TNF and HIV-Induced Apoptosis and Transformation. CD8+ T-cell apoptosis that occurs in HIV pathogenesis could result from the interaction between macrophage-membrane bound TNF-alpha with TNFR2 present on CD8+ T cells [40]. Additionally, HIV-1 Tat is known to induce the expression of TNF-beta in a human B-lymphoblastoid cell line (Raji cells). There is a possibility that HIV-1 Tat protein induces the growth of Kaposi’s sarcoma cells via TNF-beta induction [41–43].

Interestingly, increased levels of sCD40L in the cerebrospinal fluid and plasma of HIV-infected patients with cognitive impairment have been documented [46, 48]. In vitro experiments with recombinant CD40L (rCD40L) and HIV-1 Tat show that they act synergistically to enhance the yield of TNF-alpha by microglia and monocytes [46]. This enhancement may be contributed by ability of Tat to increase CD40 expression via NF-kappaB activation [46, 49]. Furthermore sCD40L interacts with CD40 leading to CD40-mediated signal cascade resulting in activation of NF-kappaB in microglia and monocytes [46]. This results in production of high amounts of inflammation mediators such as TNF-alpha that may explain HIV-associated dementia [46].

3.2. CD40. CD40 a member of TNF superfamily, is a 45–50 kDa integral membrane glycoprotein found on B-cells, monocytes, dendritic cells, endothelial cells, and epithelial cells [44, 45]. Ligand for CD40 is CD40L (CD154), a 33 kDa transmembrane glycoprotein that is mainly expressed by activated B-cells, T-cells, and platelets [45, 46] (Figure 1). CD40-CD40L mediated signaling plays indispensable role in the development of cellular and humoral responses [46]. Membrane dissociated truncated soluble CD40L (sCD40L) is released by activated cells and binds to the CD40 molecule expressed on the target cells to activate it [46, 47].

Thus there is fair possibility that Tat-stimulated CD40L in platelet may contribute to HIV-related thrombocytopenia [52]. In addition, Tat is known to enhance the expression of TNF ligand superfAMILY members FasL and TRAIL in macrophages and T cells. As a consequence apoptosis can be induced in the bystander cells [53–55].

Worth mentioning, CD40L can be embedded on the surface of HIV-1 virion generated by peripheral blood and through budding from stimulated CD4+ T cells in cell culture as well as in HIV-1 infected patients [56–58]. CD40L-associated virions can induce strong activation of B-cells
and modulate genes including members of TNF superfamily (FAS, A20, TNFIP1, CD40, lymphotoxin alpha, and lymphotoxin beta), cytokines, and transcription factors [57, 58]. Additionally, macrophages expressing Nef or activated by CD40L-CD40 receptor interaction release factors (CD23 and soluble ICAM) which makes T cells present in their vicinity susceptible to HIV infection, thus expanding the HIV cellular reservoir [59].

3.3. Lymphotoxin-Beta Receptor (LT-betaR). Another important member of growing TNFR superfamily is lymphotoxin-beta receptor (LT-betaR) that governs the signaling pathways involved in the organogenesis of lymphoid tissue and function of follicular dendritic cells in a manner distinct from TNFR signaling pathways through activation of NF-kappaB [60–65] (Figure 1). LT-betaR stimulation favors HIV-1 replication in monocytes [62]. Additionally, when TNF receptors and LT-betaR are activated by their respective ligands (TNF-alpha and LT-alpha/beta), an additive effect on HIV-1 replication is observed in U1 cells [62].

3.4. CD27. CD27, a TNFR superfamily member, is a 55 kDa type 1 transmembrane protein that exists in a homodimeric form [66]. Ligand for CD27 is CD27L (CD70), a member of TNF ligand superfamily. CD27 plays an important role in the activation of T cells and infection of T cells by HIV-1 [67, 68]. One critical feature of HIV-1 life cycle is its integration into the host genome using virus-encoded integrase. There are reports describing preference of HIV integration into transcriptionally active genes [68, 69]. A recent study shows that HIV-1 integrates into the coding region of CD27 gene in CD4+ T cells which may disturb CD27 open reading frame and hence can hamper the help response of CD4+ T cells by cytotoxic CD8+ T cells [68].

3.5. CD30. TNFR superfamily member CD30 is present on activated T cells, B-cells, several other transformed lymphocytes, and NK cells [70–72]. CD30 plays role in triggering developmental process in B-cells via CD30-CD30 ligand interaction [72, 73]. Ligation of CD30 with an anti-CD30 monoclonal antibody (functionally equivalent of CD30L) in chronically HIV-1-infected human T cell line ACH-2 has been reported to enhance the HIV gene expression via binding of activated NF-kappaB to the HIV-1 LTR [74, 75]. Investigation of molecular mechanism responsible for the induction of NF-kappaB revealed that NF-kappaB translocation into nucleus was mediated by TRAF2, independent of TNF-alpha/beta (Figure 1).

3.6. Fas. The Fas also known as Apo-1, CD95 and TNFSF6, is a TNFR superfamily member that governs apoptosis when activated by its ligand FasL (Figure 1). Role of Fas-FasL signaling cascade in HIV pathogenesis has been extensively reviewed [55, 76]. Production of Fas and FasL is increased in CD4+ T cells isolated from HIV infected individuals [77, 78]. Increased expression of Fas is observed in B-cells, CD4+, and CD8+ T cells whereas FasL increased expression is associated with macrophages, NK cells, and monocytes [78, 79]. In vitro studies reveal that HIV-infected macrophages can induce apoptosis in Jurkat T cells and in peripheral blood T lymphocytes via FasL that could be one factor responsible for the depletion of lymphocytes during HIV pathogenesis [78, 80].

3.7. 4-1BB (CD137). 4-1BB (CD137) is a TNFR expressed predominately on T cells, NK cells, mast cells, and neutrophils [4]. Ligation of 4-1BB with agonistic monoclonal antibodies has been shown to effectively increase the HIV-1 replication in CD4+ T cells isolated from HIV-infected patients. There is a possibility that 4-1BB receptor may be involved in the activation of HIV from latency in CD4+ T cells [81].

3.8. OX40 (CD134). OX40 (CD134), a member of the TNFR superfamily, is expressed on activated CD4+ T cells and neutrophils. It is crucial for the survival of antigen specific CD4+ T cells [4, 82]. Natural ligand for this receptor is OX40L (also called CD252 and gp34), a member of TNFL superfamily. OX40-gp34 interaction, in HIV-1 (both acutely and chronically) infected T cell lines result in increase in HIV-1 replication, independent of TNF-alpha or TNF-beta production. The increase in viral replication has been shown to mediate by activation of NF-kappaB following stimulation of HIV-1 LTR [33]. Recent study suggests that OX40 activation suppresses the CCR5-tropic (R5) HIV-1 infection in PBMCs by generating anti-HIV beta-chemokines [83].

3.9. DR 4 and DR 5. Death receptor (DR), DR4 (also called TRAILR1, TNFRSF10A, and Apo2) and DR5 (also called TRAILR2 and TNFRSF10B) are the fourth and fifth members of TNFR superfamily, respectively. They are expressed in most of the normal as well as transformed cells [4]. They govern their activity by binding to their ligand TRAIL (tumor-necrosis-factor related apoptosis inducing ligand or Apo2L) leading to receptor oligomerization, assembly of death inducing signaling complexes that ultimately govern the activation of caspase pathways [4, 78]. TRAIL is a TNFL superfamily member expressed in NK, T cells, and dendritic cells. In HIV-infected CD4+ T cells, TRAIL is involved in the apoptosis of infected cells by binding with DR4 and DR5 [4, 78]. Plasma level of TRAIL has been found to be high in HIV-1 infected individuals, whereas HIV-1 infected patients undergoing antiretroviral therapy demonstrate decrease in TRAIL levels in plasma and also decreased viral load suggesting crucial role of TRAIL in HIV-1 pathogenesis [84]. In vitro testing of recombinant TRAIL (rTRAIL) against HIV-infected peripheral blood lymphocytes and monocyte-derived macrophages isolated from HIV-infected patients results in apoptosis of the target cells. However, rTRAIL shows no effect against target cells isolated from uninfected patients [84]. This raises the possibility of using TRAIL as anti-HIV agent.

4. HIV Proteins Mimicking TNF/TNFR Signaling

Several HIV proteins especially Vpr, Tat, and Nef exhibit molecular mimicry with respect to TNF signaling in HIV-infected cells particularly in macrophages (Figure 2).
Viral protein R (Vpr) is a small (14 kDa) multifunctional virion-associated accessory protein that participates in import of viral preintegration complexes to the nucleus. In addition, Vpr exerts antiapoptotic effect in HIV infected cells on the other hand, it induces apoptosis in the surrounding cells [85]. Besides these functions, Vpr also triggers mitochondrial dysfunction [86, 87]. Although Vpr is dispensable for HIV replication in T cells, it is critical for HIV replication in nondividing cells, for example, macrophages [10] (Figure 2). There are several reports where Vpr has been shown to stimulate HIV-1 growth using serum derived or synthetic Vpr [88, 89]. Virion derived (HIV-1) Vpr activates NF-kappaB in primary T cells, macrophages as well as in promonocytic cell line U937 [90]. Similarly, synthetic Vpr has been shown to activate NF-kappaB, AP-1, and JNK in primary macrophages and U937 cells resulting in increase in viral replication [10, 89] (Figure 2). In addition, recombinant Vpr stimulates HIV production by utilizing toll-like receptor 4 (TLR4) and IL-6 secretion [87]. Synergistic effect of Vpr and Tat leads to enhanced stimulation of HIV-1 LTR [91]. Even Tat alone mimics TNF-alpha. Like TNF-alpha, Tat triggers translocation of NF-kappaB into nucleus and activation of AP-1/cJun by MAPK activation JNK, p38, and ERK1/2 [10, 92] (Figure 2).

In contrast, there are several reports describing the suppression of NF-kappaB activity by Vpr. It has been shown that glucocorticoid receptor and Vpr act in harmony to inhibit NF-kappaB mediated gene expression via a pathway involving the suppression of poly (ADP-ribose) polymerase (PARP)-1 nuclear trafficking in response to TNF-alpha [85, 93].

HIV Nef, the most abundantly expressed HIV accessory protein, is a multifunctional 27 kDa myristoylated cytoplasmic protein expressed in early phase of viral life cycle [94]. Nef helps in the establishment of HIV persistence in infected cells [95] and interferes with several signaling events [10]. Recombinant Nef (rNef) has been shown to induce expression and release of several cytokines mediated by NF-kappaB activation in culture monocyte-derived macrophages (MDMs) [96, 97]. In U937 cells and MDMs, exogenously added rNef triggers NF-kappaB activation resulting in HIV LTR activation [98] (Figure 2). In addition, rNef induces transcription of several inflammatory genes in response to addition of rNef to MDMs. Convincible, analysis of rNef treated MDMs supernatants revealed induction and release of TNF-alpha and other macrophage inflammatory proteins (MIP-1alpha and MIP-1beta) and IL6 [96]. Moreover, in chronically infected promonocytic cells U1, addition of rNef leads to increase in HIV-1 replication [98]. Furthermore, rNef is able to rapidly and transiently induce phosphorylation of several key-signaling molecules including alpha/beta subunits of Ikappa B kinase, ERK1/2, JNK, and p38 in MDMs [97]. Signaling scenario observed in post-rNef treatment is more or less similar to what is observed in post TNF-alpha treatment [10] suggesting their similar impact on HIV infection at least in mononuclear macrophages (Figure 2).

5. Targeting Members of TNF and TNFR Superfamily in HIV-1 Infection

In this section, we will discuss current status and future potential therapeutic use of TNF based therapies for HIV-1 and HIV-1 related diseases and their pros and cons.

The targeting of TNF signaling has proven the most successful and clinical efficacious therapy at reducing the inflammation in several diseases. Several TNF related inflammatory cytokines and their cognate receptors are now in preclinical or clinical phase of development as a possible target for treating various diseases such as cancer, autoimmune, and inflammatory disorders. HIV infection is characterized by immune activation and inflammation [99]. Therefore, TNF blocking agents and/or TNF inhibitor therapy could be useful to modulate HIV disease.

There are several types of drugs targeting TNF and TNFR superfamily that are being used for therapeutic application [100]. Five different antibodies or receptors based drugs targeting TNF and LTalpha are approved for treating various inflammatory diseases. The chimeric antibody infliximab, TNF targeted drug, was approved in 1998 followed by etanercept [101]. The fully human antibodies adalimumab and golimumab were approved in 2002 and 2009, respectively. Cetolizumab pegol, an another therapeutic monoclonal antibody with pegylated Fab fragment was approved in 2008. As polyethylene glycol does not cross the placenta, this drug can be administrated to pregnant women who have autoimmune diseases and are in need of anti-TNF-alpha therapy [102]. These drugs neutralize the biological activity of TNF-alpha by binding with high affinity to the soluble as well as transmembrane form of TNF-alpha. Thus they prevent the binding of TNF-alpha to their natural receptors. Worth mentioning, drugs adalimumab and infliximab have the potential to lyse the cells involved in inflammation. Besides the availability and affordability, these drugs fall in the class of immune suppressors which may have serious complications such as blood disorders, infections, liver injury, skin lesion, and reactivation of tuberculosis [100].

5.1. Anti-TNF Therapy and HIV-1 Infection. HIV-1 infection induces TNF expression, and high amount of TNF is present in all stages of HIV-1 infection [29, 103, 104]. This elevated level of serum TNF has been associated with increased viral replication and depletion of CD4+ T cells [105, 106]. The treatment of HIV-1 infected patients with thalidomide (a weak TNF inhibitor) reduces serum TNF level that results in lower viral load [107, 108]. Furthermore, it has been shown that LMP-420, a small inhibitor of TNF, suppresses the transcription and biosynthesis of TNF, which ultimately inhibits the replication of HIV-1 [109]. Multiple studies have reported that use of anti-TNF therapy in patients with HIV-1 does not appear to increase the mortality rates [110]. As there is a theoretical risk that immunosuppressive drugs increase the risk of opportunistic infection and progression to HIV-1 disease, several studies have shown that anti-TNF therapy may improve the HIV-1 associated symptoms [111]. Further, etanercept has been used as anti-TNF therapy in HIV-1 infected patients for the treatment of rheumatic disease. In
most of the cases, the therapy was well tolerated. In addition, no opportunistic infection was observed in HIV-1 infected rheumatic patients unless they had uncontrolled HIV [112, 113]. Similar results were obtained by using other anti-TNF agents such as infliximab and adalimumab in HIV-1 infected patients. These drugs were safe and effective with normal CD4+ T cell counts [114, 115]. Therefore, in the patients whose HIV disease is under control of HAART, the anti-TNF therapy may be helpful for the treatment of autoimmune disease without enhancing plasma viremia [111, 116]. Worth mentioning, above clinical studies are based on a little number of patients; therefore, results must be analyzed based on large cohorts to assert a safe and real benefit to the community.

5.2. Costimulatory TNFRs and HIV-1-Specific T Cell Response. Several studies have been carried out to compare the efficacy of different costimulatory TNFR family members for the activation of HIV-1-specific T cells in vitro. These costimulatory signaling pathways could be used to activate CD8 T cell responses to HIV in vivo (Figure 3) [117]. The OX40L signaling pathway plays an important costimulatory role in DC/T cell interactions. OX40 binding to CD4+ T cells by human OX40L-IgG1 enhances ex vivo expansion of HIV-1 specific CTL from HIV-1 infected individuals [118]. This mechanism of CTL expansion was independent of induction of cytokines such as IL-2 or any inhibitory effect on CD4+ T helper cells, but it was associated with a direct effect on proliferation of CD4+ T cells. This mechanism of action of OX40 represents a potentially novel immunotherapeutic strategy that could prevent the persistent HIV-1 infection [118]. Like OX40, the 4-1BB is transiently expressed following TCR ligation [119, 120]. Furthermore, the ligation of 4-1BB/4-1BBL enhances CTL expansion that has both antiviral and antitumor activity [121]. In addition, dual costimulation by OX40L in combination with 4-1BBL resulted in improved expansion and effector function of CTL over costimulation with individual costimulatory molecules [122]. Furthermore, urelumab is a monoclonal antibody that specifically binds to and activates 4-1BB expressing immune cells and stimulates the CTL response against tumor cells. Although 4-1BB ligand (4-1BBL) could be an important costimulatory molecule for exhausted CD8+ T cells from chronically infected patients, anti-4-1BB therapy is associated with several immunological side effects such as splenomegaly, hepatitis, and several immunological disorders [123, 124]. Additionally, it has been reported that stimulation of 4-1BB in T cells enhances HIV-1 replication [81]. To be an effective therapy, the 4-1BB agonist should induce HIV-1 specific CD8+ T cell response and also should not induce viral replication. This issue may be solved by incorporation of 4-1BBL along with CD8+ T cell epitopes into vaccine vectors. The incorporation of 4-1BBL into a fowlpox vector along with HIV-1 Gag enhances Gag-specific CD8+ T cell responses, suggesting this could be a useful approach in a therapeutic vaccine [125].

5.3. TNF and TNFR Superfamily and HIV-1 Reservoirs. The persistence of HIV-1 reservoirs is a major challenge for complete viral eradication in HIV-1 infected patients [126–128]. To date, resting or memory CD4+ T cells are the most well-characterized HIV-1 reservoirs [128]. During the development of memory CD4+ T cells, the NF-kappaB signaling ensures T cell survival during the initial differentiation of effector cells into memory T cells. OX40 along with CD30 induces NF-kappaB-dependent expression of antiapoptotic genes such as Bcl-2 and Bcl-xL that in turn play an important role in the survival of memory CD4+ T cells.
Figure 3: 4-1BB and OX40 signaling enhance anti-HIV immunity, leading to therapeutic effects. Agonists 4-1BB or OX40 specific antibodies can induce enhanced anti-HIV T cell (CD4+ and CD8+ T cell) response in HIV-1 infected patients. The ligation of OX40/4-1BB antibodies activates OX40/4-1BB signaling that results in enhanced CD4+ T cell response through increased cytokine production and increased survival of memory T cells. TRAFs and NF-κB signaling has been shown to be important for the generation of memory T cells. Potential antiviral mechanisms of OX40/4-1BB include an increase in CD8+ T cell cytotoxicity through perforins and granzymes and an increase in Fas/FasL mediated HIV-infected cell killing.

within the small intestine lamina propria [129]. Low levels of NF-κB activity by control of the TNF and TNFR superfamily members may contribute to the establishment and maintenance of latent HIV-1 reservoirs in memory CD4+ T cells. Activation induced purging of HIV-1 in patients receiving HAART has long been a proposed mechanism to eradicate the latent HIV pool [130]. TNF-alpha has been used to reactivate HIV-1 from latently infected cells, but it is not the optimal reactivation treatment [131]. In Jurkat based HIV-1 latency model, TNF-alpha consistently activates latent HIV-1 provirus. However, in primary CD4 T cell model, TNF-alpha does not appear too effective to purge HIV-1 from latent reservoirs [132, 133]. Moreover, there are some concerns about the toxicity associated with TNF treatment [78]. Since TNF-alpha is only a weak enhancer of HIV-1 reactivation to purge cellular reservoirs, new approaches have been used to target the epigenetic regulation of HIV gene expression (Figure 4). Many studies have reported that the combination of HDACIs (histone deacetylase inhibitors) with TNF-alpha synergistically reactivates HIV-1 from latency [134, 135]. The HDACIs such as trichostatin A (TSA), trapoxin (TPX), valproic acid (VPA), and sodium butyrate (NaBut) activate HIV-1 transcription by remodeling nucleosome of HIV-1 promoter [136, 137]. Thus, an ideal anti-AIDS therapy would consist in eliminating the pool of latently cells by inducing forced HIV-1 gene expression by HDACIs and TNF-alpha, while maintaining an effective HAART regimen [131, 136, 138]. Furthermore, HDACIs potentiate TNF-alpha mediated NF-κB activation and also delay IκBα cytoplasmic reappearance [139]. Thus, the use of TNF-alpha and HDACIs in the presence of HAART not only could purge HIV-1 for latent reservoirs but also could suppress plasma viremia and formation of further new viral reservoirs. In addition to HDACI, inhibitors of histone methyltransferase (HMTI) have also been recently shown to reactivate HIV-1 from latency in resting CD4+ T cells from HIV-infected HAART-treated patients [140]. Furthermore, JNK inhibitors, such as AS601245, prevent HIV-1 reactivation from latency despite potent NF-κB activity [141]. Since stimulation of CD40 and 4-1BB induces JNK activation [142], the use of agonist of 4-1BB or CD40 could lead to the reactivation of the HIV-1 from latency. Combining TNF-alpha treatment
with HDACIs/HMTI could have significant impact on the clearance of HIV-1 from cellular reservoirs and ultimately could lead to the cure of HIV-infected patients [143].

6. Conclusion

TNF ligands and TNF receptors superfamily are the integral part of our immune system. TNF signaling exerts significant impact on HIV life cycle. In turn, HIV encoded proteins also modulate TNF signaling pathways resulting in the survival of HIV-infected cells and killing of bystander cells. Anti-TNF therapy has been successfully used in several inflammatory diseases. Combinatorial therapy involving HAART, anti-TNF therapy, along with use of HDACIs/HMTIs might be a viable option for the treatment of HIV infection to reach the ultimate goal, the clearance of HIV-1 from cellular reservoirs.

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Research Article

Effect of Therapeutic Inhibition of TNF on Circulating Endothelial Progenitor Cells in Patients with Rheumatoid Arthritis

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Endothelial dysfunction has been detected in RA patients and seems to be reversed by control of inflammation. Low circulating endothelial progenitor cells (EPCs) have been described in many conditions associated with increased cardiovascular risk, including RA. The aim of this study was to investigate the effect of inhibition of TNF on EPCs in RA patients. Seventeen patients with moderate-severe RA and 12 sex and age-matched controls were evaluated. Endothelial biomarkers were tested at baseline and after 3 months. EPCs were identified from peripheral blood mononuclear cells by cytofluorimetry using anti-CD34 and anti-vascular endothelial growth factor-receptor 2. Asymmetric dimethylarginine (ADMA) was tested by ELISA and flow-mediated dilatation (FMD) by ultrasonography. Circulating EPCs were significantly lower in RA patients than in controls (P = 0.001). After 3 months EPCs increased significantly (P = 0.0006) while ADMA levels significantly decreased (P = 0.001). An inverse correlation between mean increase in EPCs number and mean decrease of DAS28 after treatment was observed (r = −0.56, P = 0.04). EPCs inversely correlated with ADMA (r = −0.41, P = 0.022). No improvement of FMD was detected. Short-term treatment with anti-TNF was able to increase circulating EPCs concurrently with a proportional decrease of disease activity suggesting that therapeutic intervention aimed at suppressing the inflammatory process might positively affect the endothelial function.

1. Introduction

Rheumatoid Arthritis (RA), as other autoimmune systemic diseases, is associated with increased cardiovascular morbidity and mortality [1], mostly attributable to accelerated atherosclerotic process [2]. Data in the literature demonstrated that inflammatory nature of RA contributes to the excess of atherosclerosis observed in this disease [3]. Rheumatoid synovia and atherosclerotic plaque share a common inflammatory cellular and molecular milieu characterized by an activated endothelial phenotype, expression of the same pattern of adhesion molecules, cytokines, and infiltrating leucocytes [4]. Impairment of endothelial function represents the earliest and reversible stage of atherosclerotic plaque formation, originating from the loss of protective antioxidant and anti-inflammatory systems [5]. Integrity of vascular endothelium is essential for arterial wall functions and homeostasis, and its dysfunction represents the key event which subsequently leads to vascular wall disorders. Less than twenty years ago, Asahara and coll firstly identified endothelial progenitor cells (EPCs) as precursors circulating in peripheral blood, mobilized form bone marrow, and able to differentiate in situ into endothelial cells; such cells contribute to the recovery of injured endothelium, thus, limiting atherosclerotic plaque formation [6–8]. Mobilization and differentiation of the EPCs
is known to be regulated by nitric oxide (NO) produced through the activation of the endothelial NO synthase (eNOS) [9].

The number and functional activity of EPCs seem to influence cardiovascular risk. An inverse correlation between the number of EPCs and the Framingham risk factor score has been demonstrated [7], and defective number and function of these cells have been found in different clinical conditions associated with an increased cardiovascular risk [10].

Endothelial dysfunction has been documented in both long-standing [11] and early RA patients [12, 13] with Doppler ultrasound assessment of brachial artery flow-mediated dilatation (FMD) or evaluation of artery wall stiffness [14]. An improvement of endothelial function after treatment has been demonstrated by several authors [15–19]. Patients with RA also show a reduced number of circulating EPCs, which inversely correlates with disease activity and seems to be responsive to glucocorticoids [20]. Moreover, an association between the endogenous eNOS inhibitor asymmetric dimethyl arginine (ADMA) and the number of circulating EPCs has been detected in RA patients who have no other cardiovascular risk factors [21].

To date, anti-TNF agents represent a milestone of RA treatment. Given the evident role of TNF in atherosclerosis, a beneficial effect of TNF inhibition has been postulated; however, observational studies and data form registries did not always demonstrate a decrease in cardiovascular events [1]. Long-term controlled studies, directly evaluating the effect of this class of drugs on atherosclerotic process progression, are needed.

The aim of our study was to investigate the effect of short-term subcutaneous administration of anti-TNF drugs on EPCs number in patients with active RA.

2. Materials and Methods

2.1. Patients and Controls. Consecutive patients affected by RA according to 1987 criteria [22], designated to start subcutaneous anti-TNF drugs, were recruited from the "biological drugs-dedicated outpatient clinic" of the Rheumatology Unit of Sapienza University of Rome. All patients were prospectively followed up for at least 3 months. As control, 12 age and sex-matched healthy subjects were studied. All patients signed an informed consent before entering the study. At recruitment, demographic and clinical data, and comorbidities were recorded. Patients and controls were excluded in case of a diagnosis of cardiovascular diseases, chronic kidney failure, dyslipidemia, and/or diabetes. Before starting anti-TNF, patients were screened for latent tuberculosis and hepatitis virus B and C.

2.2. Disease Activity Assessment. RA disease activity was evaluated at baseline, and after 3 months of anti-TNF treatment, by 28-joint disease activity score (DAS28).

2.3. Blood Samples. Blood samples were collected from each patient at baseline and 3 months later. Heparinized vials were used to test EPCs on the same day of the blood draw. The remaining samples were centrifuged at 3000 × g for 10 minutes at room temperature and serum collected and frozen at −80°C until analyzed.

As for control group, blood samples from healthy subjects were collected on the same day of baseline patients’ visit.

2.4. Circulating Endothelial Progenitor Cell Analysis. Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation (Lymphocyte-H; Cedarlane Laboratories, Hornby, Ontario, Canada), and phenotypic characterization was performed as previously described by Vasa et al. [23]. In brief, after incubation with FcR-blocking reagent (Miltenyi Biotec, Bergisch-Gladbach, Germany), cells were incubated for 30 min on ice with phycocerythrin (PE)-labeled mAb anti-CD34 (BD Immunocytometry Systems, San Jose, CA) and allopheycocyanin (APC)-labeled mAb anti-VEGF R2/KDR (R&D Systems, Minneapolis, MN). Appropriate isotype controls were used. Acquisition was performed on a FACS Calibur (BD Immunocytometry Systems) and included 100.000 to 400.000 events per sample. Data were analyzed using the CellQuest Pro software (BD Immunocytometry Systems). EPCs were defined as to CD34/KDR double-positive cells, and their number was expressed as a percentage of cells within the lymphocyte gate [21, 23]. A representative dotplot is shown in Figure 1.

2.5. ADMA. ADMA serum levels were detected by a commercial human enzyme linked immunosorbent assay (ELISA) kit (Vinci Biochem, Florence, Italy), according to manufacturer’s instructions. Sera were tested in triple and result expressed as mean value ± standard deviation.

2.6. Assessment of Flow Mediated Dilatation. Flow mediated dilation in response to reactive hyperemia (endothelium dependent vasodilatation) was evaluated on brachial artery by employing a high-resolution B-mode Doppler (ATL HDI 5000 with a 7.4 MHz linear-array transducer) and following the guidelines published by the International Brachial Arterial Reactivity Task Force [24].

All subjects were evaluated fasting between 8 and 11 AM, in a quiet and stable temperature environment. A straight, nonbranching segment of the brachial artery 5–15 cm above the antecubital fossa was identified by a B-mode longitudinal scan. Vessel diameter was recorded in a segment with clear anterior and posterior intimal interfaces between the lumen and vessel wall at rest and during reactive hyperemia. Brachial artery diameter was measured offline by an automatic edge-detection system. A blood pressure cuff was then inflated around the forearm to a supra-systolic pressure (at least 50 mm Hg above the systolic pressure to occlude arterial inflow) for the standardized length of 5 minutes. Measurement of the maximal diameter of the artery was taken 45 to 60 seconds after cuff release. Absolute FMD was expressed as: (postdeflation diameter–resting diameter); FMD relative values were also calculated as percent change from the baseline diameter as follows: 100% × ((postdeflation diameter – resting diameter)/resting diameter).
Two cardiologists (FC and LA), blinded to participants’ clinical data, interpreted the ultrasound results using an offline method. The intra- and interobserver variability of the FMD were calculated within the study population by plotting the patients’ FMD estimates from each measurement against the estimates by two independent measurements. The estimate’s standard error was calculated using this plot. The intra- and interobserver variability were 4.2% and 5.1%, respectively.

In order to evaluate the readers’ ability to identify positive results, 15 hypertensive patients with known coronary artery disease were also evaluated.

2.7. Statistical Analysis. The study was designed to investigate the effect of short-term subcutaneous anti-TNF therapy on the amount of circulating EPC in RA patients. The threshold for significance was set at 0.05. Data were expressed as mean ± standard deviation. Data for matched pairs were analyzed with Wilcoxon signed-rank test. Correlations were evaluated with the Spearman rank correlation test. Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad software Inc. La Jolla, CA - USA).

The study protocol was approved by the Institutional Review Board of Policlinico Umberto I - Sapienza University of Rome.

3. Results

We recruited 17 RA patients (14F:3M, mean age 50.4 ± 14.4 years, range 26–68 years) with long-standing disease (mean disease duration 103 ± 104.4 months, range 24–360) who were designed to start a subcutaneous anti-TNF drug. Fourteen were treated with etanercept 50 mg/week/subcutaneously and 3 with adalimumab 40 mg/every other week/subcutaneously.

At the time of enrollment, all patients were taking glucocorticoids; none of the patients increased steroid dose during the followup. Clinical characteristics of RA patients at baseline and after 3 months of anti-TNF treatment are reported in Table 1. After 3 months a significant decrease in DAS28 (versus baseline values) was recorded ($P = 0.001$).

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>F/M</th>
<th>Mean ± SD (range)</th>
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<tbody>
<tr>
<td>Age—yrs</td>
<td>14/3</td>
<td>50.4 ± 14.4 (26–68)</td>
</tr>
<tr>
<td>Disease duration—months</td>
<td></td>
<td>103.0 ± 104.4 (24–360)</td>
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<tr>
<td>DAS28 baseline</td>
<td></td>
<td>5.2 ± 1.1 (4.27–7.78)</td>
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<tr>
<td>DAS28 followup</td>
<td></td>
<td>3.3 ± 1.3 (0.56–4.03)*</td>
</tr>
<tr>
<td>Glucocorticoid dose (mg) baseline</td>
<td></td>
<td>7.5 ± 5 (5–20)**</td>
</tr>
<tr>
<td>Glucocorticoid dose (mg) followup</td>
<td></td>
<td>5.2 ± 0.6 (5–7.5)**</td>
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<tr>
<td>Methotrexate</td>
<td>10/17</td>
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<tr>
<td>Leflunomide</td>
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<td>Sulfasalazine</td>
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<td>Etanercept</td>
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DAS28: disease activity score 28. *$P = 0.001$ versus baseline value, **prednisone-equivalent.

3.1. Circulating EPCs. At baseline, the percentage of circulating EPCs was significantly lower in active RA patients than in
4. Discussion

The results of our study demonstrate that short-term treatment of RA with TNF inhibitors is associated to an increase in circulating EPCs concurrently to a proportional decrease of disease activity; these findings suggest that therapeutic intervention aimed at suppressing the inflammatory process might also positively affect the “health” of endothelial barrier. In RA patients, traditional risk factors, genetic predisposition, and inflammatory mechanisms are now recognized to act synergistically in determining the increased risk of subclinical atherosclerosis and consequent CV events [25, 26].

3.3. Doppler Ultrasound Assessment of Flow Mediated Dilatation. Mean FMD at baseline was 8.25 ± 0.09% in RA patients, and 4.3 ± 0.7% in positive controls (P = 0.001). At 3 months followup, a not significant increase of FMD was observed in RA patients (8.70 ± 0.06%, P = 0.49 versus baseline). However, even after anti-TNF administration, mean FMD was below the normal value of 10%.

3.2. ADMA Serum Levels. At baseline, mean ADMA levels were 0.64 ± 0.12 μmol/L. After 3 months of anti-TNF, ADMA serum levels significantly decreased below the values detected before treatment, (0.47 ± 0.04 versus 0.64 ± 0.12 μmol/L, P = 0.001).

healthy subjects (0.01±0.02% versus 0.05±0.03%, P = 0.001). At 3 months followup, the number of EPCs was significantly higher compared to basal values (from 0.01 ± 0.02% to 0.05 ± 0.04%, P = 0.0006 versus baseline; P = n.s. versus healthy subjects) (Figure 2). No significant correlation between EPCs and DAS28 values was detected (P = 0.056); However, an inverse correlation between mean increase in EPCs number and mean decrease of DAS28 after 3 months of anti-TNF therapy was observed (r = −0.56, P = 0.04) (Figure 3). Moreover, EPCs number inversely correlated with ADMA serum levels (r = −0.41, P = 0.022) (Figure 4). No other correlations between EPCs and clinical characteristic nor FMD values were detected.
Systemic inflammation is responsible for a proatherogenic profile characterized by oxidative stress, lipid abnormalities, insulin resistance, hypercoagulable state, and upregulation of proatherogenic inflammatory leukocytes [25] each contributing to endothelial injury. Healthy endothelium represents the main regulator of vascular tone, inflammation, and remodeling; consequently, a loss of its function initiates the atherosclerotic process which ultimately leads to the development of the plaque. Different cardiovascular risk factors act on endothelial cells inducing senescence and apoptosis, thus, determining endothelial dysfunction [27]. Growing evidence suggests that EPCs circulating in peripheral blood play a crucial role in endothelium repair. In RA patients, deficiency of circulating EPCs number and functions has been proven [20, 21, 28, 29]. An in vitro study demonstrated that endothelial progenitor cells obtained from RA patients showed impaired migratory response to vascular endothelial growth-factor (VEGF) and adhesive properties to mature endothelial cells after stimulation with TNF, when compared to cultured cells from healthy subjects [29]. Other in vitro data demonstrated that TNF-alpha negatively affected proliferative, migratory, and adhesive capacity of human EPCs [30]. After TNF-inhibitor administration, a significant increase in adhesion property of EPCs was detected [28].

Concerning the number of circulating EPCs, a reduction of peripheral blood EPCs was described in RA. In their paper, Herbrig et al. aimed at evaluating, ex vivo and in vitro, number and function of endothelial progenitors in 13 patients with impaired endothelial function; all patients were treated with methotrexate, and 6 out of 13 were also taking anti-TNF drugs [29]. The authors suggested two hypotheses explaining the alteration in EPCs number and function: the inflammatory disease itself and the effect of methotrexate administration [29]. When comparing the frequency of circulating EPCs in patients with high or low disease activity, Grisar et al. [31] observed a significant difference between subjects with active disease and those with low disease activity or in remission who showed EPCs levels comparable to healthy subjects. Differently from the population studied by Herbrig et al. [29], we enrolled long-standing RA patients with moderate-high disease activity (DAS28 ≥ 3.2) nonresponders to standard Disease Modifying Anti-Rheumatic Drugs (DMARDs) and eligible for anti-TNF therapy; in this population we detected a reduced number of circulating EPCs compared to healthy subjects not correlating with disease activity. The homogeneity of our population did not allow any stratification based on disease activity status. In another population of moderately active RA patients, no correlation between circulating EPCs and disease activity was found [20].

Contrary to most published data on endothelial precursors in RA, few studies demonstrated a higher or similar number of circulating EPCs in RA patients compared to patients with other systemic autoimmune diseases or healthy subjects [32–34]. However, different surface markers and techniques were used to characterise endothelial precursor cells. Endothelial precursors represent an extremely rare population among peripheral blood mononuclear cells, and this scarcity contributes to the difficulty in cell isolation and definition [35, 36]. An additional way to define EPCs is to quantify their ability to proliferate by colony forming unit (CFU) assay. With this method, a depletion of peripheral endothelial progenitors was confirmed [20, 31, 34].

Interestingly, besides a decrease in circulating number, rheumatoid synovia seems to be enriched with EPCs suggesting a role for these precursors in local vasculogenesis [37]. Migration of endothelial precursor cells recruited from the peripheral blood through α4β1 integrin/vascular cell adhesion molecule (VCAM)-1 [38] might explain the depletion in peripheral blood which compromises the endothelial renewal, thus, beginning the atherosclerotic process.

Serum levels of proinflammatory cytokines, such as IL6, showed an inverse correlation with the number of circulating EPCs in RA patients [29]. Recently, in other condition characterized by endothelial impairment, even TNF showed an inverse correlation with the number of circulating EPCs [39]. Given the pivotal role of TNF in the pathogenesis of both RA and atherosclerosis [40], we aimed at investigating the effect of TNF inhibition on markers of endothelial function. A first
observation of TNF effect on endothelial precursors in RA patients comes from an in vitro study in which the cytokine was demonstrated to impair the CFU formation activity of EPCs, while the addition of TNF-inhibitor infliximab to cultured cells reversed this effect [20]. Moreover, other in vitro data showed that TNF was able to stimulate expression of fractalkine on EPC surface, which determines progenitor cell killing by natural killer cells [41]. Further evidence of TNF-mediated effect on the number of circulating EPCs was provided by Ablin et al. [28] who demonstrated ex vivo a positive influence of infliximab administration; the authors investigated the effect of a single dose of the anti-TNF drug in 14 RA patients who were already treated with infliximab and methotrexate and observed a significant increase of EPC number and adhesive function 14 days after drug infusion. The improvement of endothelial precursors after treatment was related to a statistically, even if not clinically, significant decrease in DAS28 score (from 5.1 ± 1.4 to 4.2 ± 1.1) [28]. A drug-mediated effect on EPCs number or an indirect effect, working through a reduction of disease activity can be hypothesized; however, it should be considered that patients evaluated in the study were still moderately active after a single infusion of infliximab [28]. One week treatment with intermediate doses of glucocorticoids was also associated to significant reduction of TNF levels and increase of EPC numbers [20].

To the best of our knowledge, the present study is the first one specifically designed to investigate short-term effect of repeated subcutaneous administration of TNF-inhibitor on EPCs. Differently from a previous study investigating the potential effect of TNF inhibition, in our work we enrolled only RA patients who were naive to any biological drugs. We decided to select patients at their first course of anti-TNF drug in order to minimize potential confounding effect of circulating drugs. As expected, in our RA patients, 3 months treatment with TNF blockers significantly decreased disease activity. As previously reported by others [28], parallel to a significant reduction in DAS28 score our patients showed a significant increase in percentage of circulating EPCs which was inversely correlated with the extent of disease activity reduction. Whether the effect of anti-TNF on EPCs increase is related to drug itself rather than indirectly mediated by the reduction of disease activity can be arguable. Normal number of progenitors previously detected in patients with low disease activity [31] is in line with indications of reduced cardiovascular risk among RA patients effectively treated with methotrexate [42]. However, Methotrexate has demonstrated a proapoptotic effect on cultured endothelial precursors which could at least partially contribute to the decrease of circulating EPCs seen in RA patients irrespective of disease activity status [29]. Contrary to the study by Ablin et al. at the time of first evaluation, all our patients were anti-TNF naïve; however, similarly to this previous study, 10/17 (58,8%) were already treated with MTX so we cannot definitively exclude a contribution of this drug to the decrease of baseline EPCs.

In 2007 an inverse correlation between EPCs and ADMA serum has been demonstrated [21], ADMA is an endogenous inhibitor of NO synthase [43] coming into the limelight as a biomarker of endothelial function. Elevated ADMA serum levels have been described in many conditions associated to increased cardiovascular risk, including long-standing and early RA [21, 44, 45]. Moreover, after effective RA treatment both with standard DAMRDs and biological drugs, decreased ADMA serum levels were observed [44, 45]. In the present study, we confirmed a significant reduction in ADMA levels after 3 months of treatment with TNF-inhibitors. This is in line with previous observations that disclose that anti-TNF blockade led to a decrease of the levels of endothelial cell activation biomarkers in patients undergoing anti-TNF-α therapy because of severe disease refractory to conventional therapy [46]. This result, together with the evidence of increased EPCs, further suggests the ability of anti-TNF to reverse the effect of chronic low-grade inflammation on endothelial biomarkers in RA patients. FMD is considered as a noninvasive, standardized method to investigate endothelial function. However, this ultrasonographic technique is limited by operator dependence, and it is related to the environmental conditions in which it is performed [27]. This might explain the reason for nonsignificant improvement of FMD recorded in our patients even if we observed a significant improvement of biomarkers of endothelial function. Even if we detected a slight increase in FMD, the relative small cohort size may partially account for not reaching a statistically significant value.

This small prospective study was designed to evaluate the effect of anti-TNF treatment of EPCs in RA patients. The major shortcoming of this pilot study is the small size of the cohort evaluated and the relatively short followup. Further assessment of endothelial biomarkers on a wider and heterogeneous RA population and longer followup would confirm the result of our study and allow stratifying patients for cardiovascular risk.

In conclusion, our results enhance the current knowledge on the impairment of endothelial biomarkers in RA, as evaluated by EPCs and ADMA. An effective treatment with anti-TNF agents, aimed at reducing disease activity, seems to contribute to the improvement of endothelial barrier function. Such observation suggests a possible role of these drugs in reducing atherosclerotic damage by controlling inflammation.

References


Clinical Study

Infliximab Dose Reduction Sustains the Clinical Treatment Effect in Active HLAB27 Positive Ankylosing Spondylitis: A Two-Year Pilot Study

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The rationale of the study was to evaluate the efficacy of infliximab (IFX) treatment in patients with ankylosing spondylitis (AS) and to determine whether IFX dose reduction and interval extension sustains the treatment effect. Nineteen patients were included and treated with IFX 5 mg/kg every 6 weeks for 56 weeks. All patients concomitantly received MTX with median dose 7.5 mg/weekly. During the second year, the IFX dose was reduced to 3 mg/kg every 8 weeks. Eighteen patients completed the 1-year and 15 patients the 2-year trial. The ≥50% improvement at week 16 from baseline of BASDAI was achieved in 16/19 (84%) patients. Significant reductions in BASDAI, BASFI, and BASMI scores, decrease in ESR and CRP, and improvement in SF-36 were observed at weeks 16 and 56. The MRI-defined inflammatory changes in the sacroiliac joints disappeared in 10/15 patients (67%) already at 16 weeks. IFX treatment effect was sustained throughout the second year after IFX dose reduction and interval extension. We conclude that IFX treatment is effective in well-established active AS and a dose reduction sustains the treatment effect. These observations are of clinical importance and open the opportunity to reduce the drug costs. This trial is registered with ClinicalTrials.gov NCT01850121.

1. Introduction

Ankylosing spondylitis (AS) is a chronic, progressive inflammatory disease that primarily affects the spine and sacroiliac joints. The disease has a prevalence of about 0.55% of the European population [1, 2] and is closely associated with HLA-B27 positivity. The disease affects mostly young individuals in the third and fourth decade of their life and therefore may have a major impact on their work ability, which is associated with increased costs to the patient and the healthcare system [3].

Disease modifying antirheumatic drugs (DMARDs), including methotrexate and sulfasalazine, have not shown efficacy in treating the axial manifestations of AS but may be beneficial in treating peripheral joint disease [4]. NSAIDs along with patient educational programs, regular physiotherapy, and exercises have been recommended as the standard therapy for axial AS. TNF-alpha antagonists have made it possible to notably improve the health status in AS patients. The efficacy of TNF antagonists has been demonstrated in several short-term clinical studies [5] as well as in long-term studies [6–8]. Although compelling data is increasing indicating that infliximab is effective for treatment of AS, most randomized, placebo-controlled studies have evaluated a treatment dose of 5 mg/kg every 6 weeks. A few reports have been published showing that infliximab in a low-dose regimen (3 mg/kg) is also effective in suppressing signs and symptoms of active AS [9–15]. However, the need for dose escalation up to 5 mg/kg due to partial treatment effect has been reported highly varying in different study cohorts [13–15]. Dose escalation was necessary in 15% and 18% of patients as reported by Maksymowych et al. [10] and Jois et al. [14], respectively. In contrast, in two other studies it was found that 61%–63% of patients required dose escalation [12, 15].
However, it is currently unknown whether the treatment effect achieved with a dose of 5 mg/kg of infliximab every 6 weeks is maintained after dose reduction to 3 mg/kg every 8 weeks.

The initial objectives of the current study were to evaluate the efficacy of infliximab (5 mg/kg) treatment on the clinical disease activity, MRI assessed inflammatory changes in the sacroiliac joints and quality of life in patients with HLA-B27 positive active AS at 16 and at 56 weeks. Additional objective of importance was to determine whether infliximab dose reduction to 3 mg/kg every 8 weeks during second year would retain the treatment effect.

2. Materials and Methods

2.1. Patients and Study Protocol. Twenty-three consecutive patients with active AS identified at the Department of Rheumatology Outpatient Clinic, Sahlgrenska University Hospital, Gothenburg, during the period of June 2003 to November 2006, were invited to participate in the study. The diagnosis in each patient had been made prior to the study by the treating rheumatologist (Boel Mörck). None of the patients had received previous treatment with biological agents. The patients had to fulfill the following four inclusion criteria: (I) age between 18 and 60 years, (II) proven diagnosis according to the modified New York criteria [16] for definitive AS, (III) active disease with Bath AS Disease Activity Index (BASDAI) score ≥4, and (IV) current or previous treatment with conventional nonsteroidal anti-inflammatory drugs (NSAID) in adequate doses without sufficient effect.

Exclusion criteria were as follows: current signs or symptoms of severe, progressive, or uncontrolled hepatic, hematological, pulmonary, cardiac, neurological, or cerebral disease; ongoing or past serious infection (including HIV and past or current tuberculosis); pregnancy or breast feeding; current malignancy or history of malignancy within the past five years; congestive heart failure; any contraindication to MRI.

This study was approved by the Regional Ethics Committee in Gothenburg and an acceptance was obtained from the Medical Product Agency since infliximab was not approved for treatment of AS at the commencement of the study. The study was performed in accordance with the Declaration of Helsinki and informed consent was obtained from all patients.

2.2. Treatment Protocol. The patients fulfilling the inclusion criteria were treated with intravenous infusion of infliximab (5 mg/kg) at week 0, week 2, and week 6 of the study and thereafter every 6 weeks for a total of 56 weeks. All patients were concomitantly treated with methotrexate. DMARDs other than methotrexate were not allowed and were discontinued at least 4 weeks prior to inclusion. Treatment with NSAIDs and methotrexate (median dose 7.5 mg/weekly) remained unchanged or a dose reduction was allowed during the study period. Initiation of oral corticosteroids was not permitted during the study. After having completed the first year, the dosage of infliximab was reduced to 3 mg/kg and median infusion interval extended to every 8 weeks during the second year. Patients were thereafter followed up on a regular basis. Inflammatory parameters and BASDAI were recorded at two-year followup.

2.3. Study Assessments. The efficacy of therapy was determined by evaluating changes in MRI of the sacroiliac joints and by judging alterations in clinical and functional assessments. The Spondyloarthritis Research Consortium of Canada (SPARCC) MRI Spinal Inflammation Index was calculated [17]. Peripheral involvement was assessed by counting the number of swollen/tender joints out of a total of 28 joints; disease activity score 28 (DAS 28) [18] and Health Assessment Questionnaire (HAQ) [19] were recorded. The markers of inflammation (C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and hemoglobin) were recorded.

The initial study endpoints were (1) to determine the proportion of responders at week 16 defined as ≥50% and/or 2 cm improvement from baseline of Bath AS disease activity score (BASDAI) [20] and (2) to determine the improvement at weeks 16 and 56 from baseline in the following parameters: BASDAI [21], spinal movement (Bath AS Metrology Index; BASMI) [22], spinal function (Bath AS Functional Index; BASFI) [23], patient’s global assessments (BASG) [24], inflammation in the sacroiliac joints as measured by MRI- and health-related quality of life (HRQoL) assessed using the Short Form- (SF-) 36 questionnaire [25, 26]. The second endpoint of the study was to evaluate the treatment effect maintenance following infliximab dose reduction during the second year by assessing the laboratory inflammatory parameters and AS disease activity (BASDAI).

2.4. Health Related Quality of Life. HRQoL was assessed with the Swedish version of the Medical Outcomes Study (MOS) 36-Item Short Form Survey (SF-36) at baseline and at weeks 16 and 56. The questionnaire consists of 36 questions on different dimensions of quality of life and global health, including physical function (PF), role-functioning (RP), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), role-emotional (RE), and mental health (MH). The first four variables are summarized into a physical component score (PCS) and the last four into a mental component score (MCS). The scores of the different dimensions range between 0 and 100, with 100 corresponding to the best possible health and zero to the worst conceivable self-perceived health. The PCS and the MCS are standardized to a mean (SD) value of 50 [25–27]. A randomly chosen sex and age-matched reference group of healthy controls (n = 528) from the Swedish SF-36 national normative database (n = 8930) was used for comparison [28].

2.5. Statistical Analysis. Non-parametric statistical methods were employed due to small sample size. The data is presented as medians (25th–75th percentiles). The Mann-Whitney U test was used to calculate changes between patient variables and controls in the SF-36 test. Fisher’s exact test was employed to calculate changes at different time points with respect
patients who had completed the study at 16 and 56 weeks, respectively, were used. For the third endpoint, data was available for 15 patients. Analyses were performed using Stat View version 5.0.1 for Microsoft Windows. A $P$ value $<0.05$ was considered statistically significant.

### 3. Results

#### 3.1. Patient Demographics and Baseline Characteristics

Out of 23 patients invited to participate, 3 were screening failures (Figure 1). One patient dropped out shortly after inclusion due to depression demanding hospitalization and was therefore excluded from the study and analysis. Nineteen patients were included in the study and analysis. Fifteen (79%) completed the study after 2 years (Figure 1).

All patients were HLA-B27 positive and fulfilled the modified New York criteria [16] for AS with radiological confirmation. Seven patients (37%) had a history of uveitis and eight patients (42%) had peripheral joint involvement. The patients’ characteristics are shown in Table 1.

At inclusion, 18 patients received medication with NSAIDs and one patient was treated with 5 mg prednisolone that was gradually tapered out within the first weeks. At the time of infliximab treatment initiation, all patients were also receiving a low dose methotrexate (median dose 7.5 mg/week). The purpose of adding MTX to the treatment regimen was mainly to improve the treatment effect trying to prevent the antidrug antibody formation against infliximab.
Comparisons between continuous measures at different time points as compared to baseline values were calculated employing the Wilcoxon signed rank test for paired samples and comparisons between the groups regarding percentage were calculated using Fisher’s exact test. The level of significance is expressed as follows: \( * P < 0.05 \), \( ** P \leq 0.005 \), \( *** P \leq 0.0005 \), ns: not significant.

### 3.2. Clinical Treatment Response during the First Year

The proportion of responders in the study defined as \( \geq 50\% \) or \( 2 \) cm improvement at week 16 from baseline of BASDAI was 16 (84\%) patients. Significant reduction in disease activity as assessed by BASDAI index (\( P = 0.0002 \)) was observed both at weeks 16 and 56 in comparison with baseline (Table 1, Figure 3), as well as significant increase in spinal functional activity as assessed by BASFI score (\( P < 0.0004 \)) along with significant decrease in spinal metrological measures (BASMI, \( P < 0.007 \)). CRP was elevated in 74\% of patients at study start and decreased significantly along with ESR, whereas increase in hemoglobin levels was observed (Table 1). At 16-week followup, 10 patients (53\%) required regular concomitant use of NSAIDs as compared to 18 (95\%) at baseline (\( P = 0.0078 \)). DAS28 decreased significantly already 16 weeks after treatment initiation (\( P = 0.0037 \)) and none of patients displayed peripheral arthritis at week 56.

### 3.3. The Effect of Infliximab Dose Reduction on Disease Activity

During the second treatment year the dosage of infliximab was reduced to 3 mg/kg and the infusion interval extended to every 8 weeks. Laboratory inflammatory parameters and BASDAI were recorded. After a significant reduction between baseline and weeks 16 and 56, the clinical signs and symptoms did not significantly change during the second year of treatment. No significant increase in BASDAI (median 2.1 (IQR 0.6–3.6) versus 3.2 (0.4–4.1), ns) was observed. The increased ESR and CRP at baseline normalized following treatment with infliximab after 16 weeks and were sustained throughout the study period of 56 weeks and 2 years. Importantly, patients did not have any greater need for NSAIDs or analgesics after the infliximab dose reduction (Table 1).

### 3.4. Radiologic Findings

All patients had NY criteria unilateral grade 3 sacroiliitis or worse on preinclusion radiological studies. Four had bilateral ankylosis and one had unilateral ankylosis with contralateral grade 3 sacroiliitis. Eleven patients had bilateral grade 3 sacroiliitis; three patients had grade 3 on one side and grade 2 sacroiliitis on the other. All four patients with radiological bilateral ankylosis had bilateral

<table>
<thead>
<tr>
<th>Characteristics</th>
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<th>56 weeks</th>
<th>2 years</th>
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<td>Number of men/women</td>
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<td>14/5</td>
<td>14/4</td>
<td>13/2</td>
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<td>Age (mean years ± SD)</td>
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<td></td>
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<td>AS symptom duration (mean years ± SD)</td>
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<td>AS diagnosis duration (mean years ± SD)</td>
<td>6.9 ± 7.0</td>
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<td></td>
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<tr>
<td>Peripheral arthritis, number (%) of patients</td>
<td>8 (42%)</td>
<td>2 (10%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<tr>
<td>Uveitis, present or in history, number (%) of patients</td>
<td>7 (37%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>MTX dose mg/week; median (IQR)</td>
<td>7.5 (7.5–9.4)</td>
<td>7.5 (7.5–7.5)</td>
<td>7.5 (7.5–7.5)</td>
<td>7.5 (7.5–7.5)</td>
</tr>
<tr>
<td>Concomitant NSAID use, number (%) of patients</td>
<td>18 (95%)</td>
<td>10 (53%)*</td>
<td>9 (50%)*</td>
<td>7 (47%)*</td>
</tr>
</tbody>
</table>

**Table 1: Clinical and laboratory characteristics of patients at baseline and following treatment with infliximab 5mg/kg every 6 weeks (time points at 16 weeks and 56 weeks) and after continuing infliximab treatment 3mg/kg every 8 weeks (time point at 2 years).**

BASDAI: Bath AS Disease Activity Index; BASFI: Bath AS Function Index; BASMI: Bath AS Metrology Index; BASG1: Bath AS Patients Global score (last 6 months); CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; HAQ: Health Assessment Questionnaire Disability Index; DAS28: Disease Activity Score; NA: not analyzed.

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bone marrow oedema at inclusion. MRI of the sacroiliac joints showed signs of inflammation at baseline in 15 patients (75%), with median SPARCC score 3.75 (IQR 2.25–9). The MRI-defined inflammatory changes in the sacroiliac joints decreased significantly ($P = 0.0012$) after initiation of infliximab therapy in these 15 patients (median decrease in SPARCC score $-2.25$, IQR $-1.5–6$) and disappeared in 10 patients (67%) at 16 weeks (Figures 4(a) and 4(b)). At 56 weeks, two patients still had MRI-verified inflammatory changes. The change in SPARCC scores over time in the whole study group is shown in Figure 2.

3.5. Health Related Quality of Life. At baseline, the patients scored their quality of life significantly worse ($P < 0.0001$) than the age-matched reference population on all SF-36 subscales and their component summary scores were significantly lower ($P < 0.0001$) as compared to controls (Table 1 and Figure 5). A significant increase in patients’ health parameters was observed at week 16 as compared with baseline values regarding physical function (PF; $P = 0.01$), bodily pain (BP; $P = 0.001$), general health (GH; $P = 0.02$), vitality (VT; $P = 0.026$), and social functioning (SF; $P = 0.046$). By week 56 after induction of infliximab treatment, the patients’ quality of life had significantly improved also regarding their functioning role (RP; $P = 0.005$) and mental health (MH; $P = 0.025$). Importantly, at week 16 the AS patients had reached an MH status comparable with age-matched reference population and by week 56 no differences could be observed between the groups regarding their MH or emotional role (RE) (Figure 5).

4. Discussion

In the present pilot study, we evaluated the clinical effect of infliximab treatment (5 mg/kg) on disease activity, patients’ quality of life, and MRI-defined inflammatory changes in the sacroiliac joints in patients with HLA-B27 positive active AS. In addition, we explored whether the clinical treatment effect achieved with a standard dose of 5 mg/kg of infliximab every 6 weeks was maintained after a dose reduction to 3 mg/kg every 8 weeks.

It is currently recommended that infliximab 5 mg/kg body weight should be administered every 6 weeks to treat AS and most randomized, placebo-controlled studies have evaluated this treatment regime [29]. Recently, a few reports have been published indicating that infliximab, when initiated in a lower dose as commonly used for treatment of rheumatoid arthritis (3 mg/kg at 8 weekly intervals), was also effective in decreasing inflammatory symptoms of active AS, although increased doses are needed in 15%–62% of patients according
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Figure 4: (a) A representative MRI study at inclusion (top row) with bilateral inflammatory changes (arrows). There is a 2 × 3 cm high signal intensity lesion on the sacral side of the left sacroiliac joint on the STIR images (coronal and axial) with corresponding low signal intensity on the coronal T1-weighted image. At the right sacroiliac joint, there are smaller and more subtle lesions on both sides of the joint. There are sclerotic changes along the left joint (arrowheads). At 16 weeks (bottom row), no oedematous lesions can be seen. (b) An MRI study showing bilateral sacroiliitis in an AS patient at inclusion and at 16 weeks. At inclusion, there are bilateral inflammatory changes on the MRI images (top row, arrows). At the right sacroiliac joint, there is a 1 × 1 cm high signal intensity lesion in the sacrum on the STIR images (coronal and axial) with corresponding low signal intensity on the coronal T1-weighted image. On the left, there are smaller and more subtle lesions on both sides of the joint. There are sclerotic changes along the right sacroiliac joint. At 16 weeks (bottom row), no oedematous lesions can be seen.

Although our study population consisted of HLA-B27 positive AS patients with long disease duration, the initial endpoint of the study at 16 weeks was fulfilled in 84% of patients, the treatment response being surprisingly high. After 12 weeks of treatment with infliximab, 53% of patients achieved >50% of reduction in BASDAI score as first reported by Braun et al. [30]. In the ASSERT trial, the respective improvement in BASDAI score from baseline to results obtained from different studies [10–15]. To date, there is no published data regarding whether infliximab treatment effect is also maintained after dose decline and extension of infusion frequency. We observed in our pilot study that infliximab dose reduction from 5 mg/kg to 3 mg/kg along with longer infusion interval during the second year maintained the treatment effect and no significant increases in inflammatory parameters and/or BASDAI were seen.
At week 56, TNF antibody of IgG subtype and has the potential to cause alterations in apoptosis and thereby reveal nuclear mediated cytotoxicity reaction, infliximab has been suggested to cause alterations in apoptosis and thereby reveal nuclear antigens to the immune system [36]. Infliximab is, due to its chimeric nature, immunogenic, and concomitant treatment with methotrexate might therefore possibly enhance the efficacy of infliximab in AS, by preventing anti-infliximab antibody formation. Of note, since the patients were tested for antibodies at the study end, the data regarding anti-infliximab antibodies in drop-out patients is unavailable. Infliximab treatment decreases disease activity and inflammatory parameters from the very beginning of treatment and our results are consistent with previously published studies [37–39]. In accordance with the clinical results in the current study, treatment with infliximab resulted in a significant reduction of inflammatory changes of the sacroiliac joints as depicted by MRI using the SPARCC scoring system [17, 40]. We also observed that the efficacy of infliximab for improving the clinical signs and symptoms of AS along with reduced sacroiliac joint inflammation is associated with significant improvement in patients’ health related quality of life. As shown by SF-36, substantial and sustained improvements of several physical health domains were seen already after 16 weeks of infliximab treatment. Interestingly, in the current trial mental domains improved as well and no significant differences were observed regarding mental health as compared to healthy control population at week 16 or regarding mental health and role emotional at 56 weeks. However, in ASSERT trial and in subsequent 2-year followup of ASSERT cohort, the results regarding SF-36 mental health component in infliximab treated AS patients did not show significant improvement as compared to patients treated with placebo [5, 39].

There are limitations of this study that should be taken into consideration. The main shortcoming of this study is the relatively small number of included patients and that it is not a blinded randomised controlled study. However, to our knowledge this is the first pilot study describing maintained effect of infliximab dose reduction in treating patient with established active HLA-B27 positive AS with long disease duration in the clinical praxis. These observations are of clinical importance and open the opportunity to reduce the drug costs and improve safety profile since infliximab is expensive and carries higher risk for side effects. However, there is a need for a prospective randomised controlled trial with larger sample size to confirm our results.

In conclusion, the current study has shown that treatment with infliximab along with methotrexate in patients with active HLAB27 positive AS with long disease duration resulted in significant and rapid improvement, which was sustained during the course of the study despite reduced dose and extended infusion intervals.

**Disclosure**

Rille Pullerits, Mats Geijer, Tomas Bremell, and Helena Forsblad-d’Elia have no disclosures to declare.

**Authors’ Contribution**

Boel Mörck and Rille Pullerits contributed equally to this work.
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References


Mediators of Inflammation


Review Article

Off-Label Uses of Anti-TNF Therapy in Three Frequent Disorders: Behçet’s Disease, Sarcoidosis, and Noninfectious Uveitis

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1. Introduction

Tumor necrosis factor- (TNF-) α is a pleiotropic cytokine which plays a major role in the development, homeostasis, and adaptive responses of the immune system. In fact, it is central to the initiation and maintenance of inflammation in multiple autoimmune and nonautoimmune disorders. TNF-α, which is released by macrophages, monocytes, and T lymphocytes, as well as other types of cells, can be found both in its soluble form and bound to the cellular membrane [1–3].

The better understanding of the pathogenesis of autoimmune disorders has led to the search of new targets for its treatment. In this way, biological therapies, which are synthesized from living organisms, can be designed to specifically act on certain inflammatory mediators, among them, TNF-α. Thus, reinforcement or even substitution of usual immunosuppressive therapies has now been made possible [1, 3].

Currently, five anti-TNF agents are commercially available: infliximab, etanercept, adalimumab, certolizumab pegol, and golimumab (Table 1).

(a) Infliximab (INX) is an IgG1 chimeric monoclonal antibody with a constant human region and a variable murine one. This agent binds both the soluble and the cell-bound TNF-α but not TNF-β. It is administered intravenously on an outpatient basis, and even though serious infusion reactions are rare, development of antibodies against INX has been reported (incidence varying between 15% and 50%). The latter has been associated with a lower efficacy and a higher rate of infusion reactions. In order to avoid the formation of these antibodies, low-dose methotrexate (usually 7.5 mg weekly) is frequently added to INX [1, 3].

(b) Etanercept (ETP) is a soluble receptor of human TNF. It is obtained by means of recombinant DNA technology, by fusion of the extracellular region of two type II TNF receptors and the Fc region of human immunoglobulin G1 [1, 3]. ETP binds both soluble TNF-α and TNF-β, leaving them biologically inactive. It is administered by subcutaneous route, once or twice a week. Skin reactions at the site of injection have been reported in up to 40% of cases, and
although antibodies against ETP are present in less than 10%, a lower efficacy has not been observed [1, 3]. (c) Adalimumab (ADB) is a fully humanized IgG1 monoclonal antibody, specifically directed against TNF-α, which binds both its soluble and cell-bound forms. It is administered once every two weeks by subcutaneous injection. Due to its more recent release, data regarding safety, as well as the incidence of antibodies directed against ADB and their possible effect on its efficacy, are insufficient [3]. (d) Certolizumab pegol (CZP) is a pegylated humanized antibody Fab' fragment of a monoclonal antibody specifically directed against TNF-α, which binds both its soluble and the cell-bound forms. It is administered subcutaneously every other week [4]. (e) Golimumum (GLB) is a fully humanized IgG1 monoclonal antibody, specifically directed against TNF-α, which binds both its soluble and cell-bound forms. It is administered subcutaneously once a month [4, 5]. It should be administered in conjunction with methotrexate in rheumatoid arthritis (RA) [6].

These five TNF-blocking agents are currently licensed for the treatment of a variety of disorders, namely, RA (INX, ETP, ADB, CZP, and GLB), juvenile idiopathic arthritis (JIA) (ETP and ADB), ankylosing spondylitis (AS) (INX, ETP, ADB, and GLB), psoriasis (INX, ETP, and ADB), psoriatic arthritis (PsA) (INX, ETP, ADB, and GLB), Crohn’s disease (CD) (INX, ADB, and CZP) and ulcerative colitis (UC) (INX and ADB) [3–5, 7–9]. Nonetheless, the TNF-blocking agents are being used in an increasing number of autoimmune disorders, in those cases which are severe and resistant, or intolerant, to standard immunosuppressive therapies. The aim of this paper is to discuss the available data regarding the off-label uses of the anti-TNF agents in three specific frequent disorders: Behçet’s disease, sarcoidosis, and noninfectious uveitis. At present, there is no published literature with regard to the use of CZP in these three conditions, while that concerning the use of GLB is limited to a few cases of uveitis or retinal vasculitis with dissimilar results [5]. Therefore, we will focus on INX, ETP, and ADB.

2. Behçet’s Disease

Behçet’s disease (BD) is a systemic vasculitis involving arteries and veins of any size, with a chronic-relapsing course, of unknown cause and with HLA-B51 as an admitted predisposing factor. The main manifestation of BD is recurrent oral ulcers, to which genital ulcers and systemic manifestations may be associated. Thus, BD may also involve the eyes, skin, nervous system, joints, kidneys, and arteries and veins of all sizes. Since sensitive or specific laboratory tests or specific pathologic findings are currently absent, the diagnosis is based on clinical criteria. Topical treatment is initially used for oral ulcer and mild ocular involvement. In the rest of cases, other therapies have been employed, such as colchicine, steroids, dapsone, thalidomide, methotrexate, azathioprine, ciclosporine A, cyclophosphamide, and mycophenolate. Notably, the main issue is the lack of controlled evidence regarding therapeutic options, especially in cases of neurological, vascular, and gastrointestinal manifestations [10]. TNF-α is believed to be a central inflammation mediator in BD, and, consequently, the TNF-blocking agents have been used in this disorder with different results.

As it will be detailed later on, existing evidence on anti-TNF therapy in BD suggests that INX seems to be effective in ocular inflammation (mainly in posterior uveitis with serious risk of view loss), as well as extracutaneous manifestations. On the contrary, ETP has apparently shown better results in mucocutaneous lesions, even though enough data regarding its efficacy in ocular and articular involvement are insufficient, chiefly based on a few single case reports [11–13]. In fact, Melikoglu et al., in a 4-week randomized, double-blind, placebo-controlled trial, observed that patients treated with ETP achieved sustained remission for oral ulcers and nodular lesions, whereas no significant differences could be found regarding genital ulcers and papulopustular lesions. Patients receiving ETP showed a lower number of arthritis episodes, although the difference was not significant [14]. Conversely, two patients with neuro-BD have been reported to respond to ETP [15, 16].

A panel expert meeting on BD held in May 2006 [17] recommended considering the use of INX in patients with two or more relapses of posterior uveitis or panuveitis per year, loss of visual acuity secondary to chronic macular edema, refractory parenchymal central nervous system disease, selected patients with intestinal inflammation, or in those with articular or mucocutaneous involvement which

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**Table 1: Characteristics of the different anti-TNF agents.**

<table>
<thead>
<tr>
<th>Type</th>
<th>Infliximab</th>
<th>Etanercept</th>
<th>Adalimumab</th>
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<td>Binding to soluble TNF-α and TNF-β</td>
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<td>Dose</td>
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<td>25–50 mg once or twice a week</td>
<td>40 mg every other week</td>
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significantly affects their quality of life (in whom ETP might also be considered). In cases of bilateral posterior uveitis with serious risk of view loss, a single dose of INX could be administered in order to prevent irreversible damage of the retina with permanent visual acuity loss. After that, the usual immunosuppressive therapy would follow (cyclosporine A or azathioprine, or even interferon-alpha, combined with low-dose corticosteroid). Subsequent to the publication of these recommendations, several case series have been published regarding eye, central nervous system, and bowel involvement, which further support the role of INX in the treatment of BD, usually being well tolerated and with hardly any side effects (except for one case of cytomegalovirus colitis) [17–23]. INX has subsequently been approved in Japan for the use of BD-related uveoretinitis not responding to conventional treatments [17]. Before and after the aforementioned recommendations, several prospective studies on the therapeutic use of INX for posterior uveitis reported a sustained response, with improvement of visual acuity and reduction of eye inflammation, either complete (65%) or partial (24%). This ocular remission was better maintained if combination with immunosuppressive agents was employed (azathioprine, cyclosporine A, and/or methotrexate), although it was only statistically significant for combination with cyclosporine A [12]. In another prospective study, the authors found that the effects of INX on reducing ocular inflammation were significantly faster than those of intravenous methylprednisolone or intravitreal triamcinolone acetonide, while the effect on visual acuity did not differ among them [24]. Besides, the efficacy of INX on BD uveitis seems to be maintained in the long term [25]. On the other hand, the intravitreal use of a single dose of IFX has been tested on a 15-patient pilot study with BD-associated relapsing posterior uveitis at the beginning of a unilateral attack. The outcomes of this study were significantly positive, while no side effects were noticed, either ocular or extraocular [26]. However, despite the good results reported in all these studies, uveitis may recur. In fact, Yamada et al., in a retrospective study, observed that 13 of 23 patients presented with recurring uveitis following treatment with INX, with a variable timing. The authors gave three possible explanations: (1) it might be a rebound effect due to a quick discontinuation of the patients’ previous immunosuppressants; (2) it might be the result of too long interval between infusions after the fourth one; (3) it might be the consequence of neutralization of INX by antibodies directed against it [27]. Furthermore, small prospective studies and some case series and isolated patients have shown excellent response to INX in gastrointestinal involvement, central nervous system manifestations, pulmonary aneurysms, and other vascular involvements but not in hepatic vein thrombosis [12, 28–30]. For example, in the prospective study by Iwata et al., 10 patients with severe gastrointestinal involvement and who were irresponsible or intolerant to corticosteroids responded rapidly and dramatically to INX monotherapy. Moreover, improvement of abdominal computed tomography and colonoscopy in the long-term evaluation was noted [31]. In another study, five patients with Behçet’s disease and resistant to methotrexate and steroids received INX as add-on therapy.

They all showed clinical amelioration, along with regression of parenchymal lesions in magnetic resonance imaging and decrease of IL-6 levels in cerebrospinal fluid [32]. However, not only has the clinical response of INX in BD been analyzed but also its effects on health-related and vision-related quality of life, with significant improvement of the scores [33].

As far as ADB is concerned, the available data were initially very limited. Nonetheless, since it shares a similar mechanism of action with INX, the results were therefore expected to be comparable to those of the latter. In fact, a good number of the cases reported in the literature refer to patients who were switched from INX to ADB due to intolerance or failure to treatment compliance in cases of eye involvement, with no loss of efficacy [34, 35]. A subsequent observational study reported either sustained remission or good response in 17 BD patients (including mucocutaneous and neurological manifestations, as well as retinal vasculitis), who had to be switched to ADB due to lack or loss of efficacy, or else, infusion reactions [36]. A more recent case series observed a clinical improvement following treatment with ADB in 17 out of 19 patients, mainly with ocular, gastrointestinal, mucocutaneous, and peripheral nervous system involvement. Five of them had previously received INX and 2 ETP, both of which had proved ineffective. Furthermore, the number and dose of previous immunosuppressants could be reduced. Only 1 patient had it stopped due to the development of urticaria-angioedema [37]. Further case reports or small case series have shown good results with ADB in patients with different manifestations (gastrointestinal, mucocutaneous, neurological, articular, ocular, and vascular), either in anti-TNF naïve patients or following failure of INX [38–40].

3. Sarcoidosis
Sarcoidosis is a chronic, multisystemic disorder of unknown etiology, whose main characteristic is the development of noncaseating granulomas. These lesions may involve any part of the body, mostly the lungs and the thoracic lymphatic nodes. Granulomas can also affect the nodes of other parts of the body, skin, and eyes. The diagnosis is based on both clinical and histopathological findings. Systemic steroids remain the mainstay of treatment. Other options include hydroxychloroquine, azathioprine, methotrexate, cyclophosphamide, and mycophenolate.

The fact of TNF-α being one of the participating cytokines in the formation of the sarcoid granuloma, along with the successful use of immunomodulators inhibiting the TNF-α such as pentoxiphylline and thalidomide, has led to the utilization of anti-TNF agents in numerous patients. Most of them have received treatment with INX, and while skin involvement seems to show better results, those regarding pulmonary involvement tend to be less positive. Likewise, a good response to INX in other locations, such as ocular, neurologic, articular, cardiac, hepatic, renal, vertebral, and parotid gland, has also been reported [3, 38, 41–59]. A multicentre, double-blind, randomized, placebo-controlled trial on the efficacy of INX in 138 patients affected with extrapulmonary sarcoidosis was recently published. Severity of extrapulmonary sarcoidosis decreased in over 40% of patients
compared to placebo after 24 weeks of treatment, even though this difference disappeared once INX was discontinued [60]. In addition, a retrospective study involving 54 patients with lupus pernio found that INX seemed to be superior to systemic steroids in achieving resolution or near resolution of lesions, with or without other additional medications [61]. A subsequent double-blind, placebo-controlled trial with 134 patients with chronic pulmonary sarcoidosis found a modest though significant increase of forced vital capacity following INX therapy. The clinical response was stronger in those patients with an important baseline systemic inflammatory profile (which included different chemokines, neutrophil-associated proteins, acute-phase proteins, and metabolism-associated proteins), and it correlated with the decrease of inflammatory serum proteins, namely, MIP-1β and TNF-RII [60]. Also, in a small case series, 9 patients with pulmonary involvement experienced an improvement in lung functions tests after treatment with INX, even though only one patient normalized chest radiograph [62]. Finally, two small, retrospective studies have assessed the long-term efficacy of INX in sarcoidosis with pulmonary and extrapulmonary involvement. In one of them, with 16 patients, 88% of patients maintained the good initial response on follow-up. Only one patient had INX discontinued due to adverse events [63]. In the other one, with 26 patients, a sustained remission or improvement in almost 59% of the organs evaluated was achieved. INX had to be withdrawn because of adverse events in 3 patients, namely, severe pneumonia, positive purified protein derivative tuberculosis skin test, and recurrent sinusitis [64].

On the contrary, results with ETP are discouraging, similarly to other granulomatous disorders [3, 42, 43]. In two small studies, one with patients with pulmonary sarcoidosis and another with patients with ocular sarcoidosis, ETP failed to provide any amelioration of the disease. In addition, the first trial had to be terminated due to excessive treatment failures [65, 66]. This lack of efficacy of ETP in granulomatous conditions has been attributed to its different mechanism of action, compared to that of INX and ADB [56].

Experience with ADB, on the contrary, is more limited. Several case reports have shown ADB to be efficacious in patients with different involvements (systemic, cutaneous, lymphadenopathies, inner ear, neurological, pulmonary, ocular, vertebral and bone marrow), in one of them after ETP failure [56, 59, 67–73]. More recently, a double-blind, placebo-controlled trial assessing the effect of ADB in 16 patients with cutaneous sarcoidosis was published. ADB proved to be effective in improving both clinical lesions (Physician Global Assessment, target lesion area and target lesion volume) and Dermatology Life Quality Index score, even though this effectiveness partially wore off after an 8-week period of ADB withdrawal [74]. Another recent prospective study with 26 patients with refractory posterior uveitis revealed improvement or stabilization of intraocular inflammatory signs in 85% and 15% of patients, respectively. Other indicator of disease activity (pulmonary lung tests, laboratory tests) also improved [75].

Finally, the report of the onset of new sarcoid granulomas following treatment with anti-TNF agents for another reason (RA, AS, PsA, and SAPHO syndromes) is but paradoxical. The locations affected vary (lungs, central nervous system, bone marrow, skin, eyes, lymph nodes, liver, kidneys, joints, and parotid gland), and all the three TNF-blocking agents have been associated with this unexpected adverse event. The sarcoid granulomas usually resolved upon cessation of TNF-α blockade and/or increase or initiation of oral steroids [76–89]. Even more shocking are the cases reported in which sarcoid granulomas disappeared after switching from ETP to ADB (since the initial patient’s conditions so required) or did not relapse after reintroducing the same agent [90, 91]. The mechanism by which anti-TNF agents could induce sarcoidosis remains unclear. On the one hand, the role of TNF-α in sarcoidosis seems to be partial and to change as the disease evolves [84]. On the other hand, there is evidence that TNF-α and interferon-α (IFN-α) show cross-regulation in vitro [92] and in vivo [93]. Thus, suppression of TNF-α levels would lead to an increase in those of IFN-α. And IFN-α has been reported to induce sarcoidosis and other autoimmune diseases [94].

4. Noninfectious Uveitis

The results from experimental models in animals as well as studies in humans suggest that TNF may play a central role in promoting ocular inflammation. Thus, intravitreal injection of TNF in rabbits and Lewis rats has shown to induce the development of uveitis [95–97]. Likewise, increased levels of TNF have been detected in serum and/or aqueous humor of uveitis patients when compared with controls. Based on these observations, therapy with TNF blockade has been utilized in ocular inflammatory disorders, either isolated or associated with systemic conditions. Even if limited, the existing evidence implies that INX seems to be more effective than ETP in the treatment of ocular inflammation [42].

The reported cases and case-series regarding INX suggest its efficacy in treating noninfectious uveitis and other ocular inflammatory disorders. As far as uveitis associated with other disorders, such as JIA, AS, CD, psoriasis and Takayasu arteritis, is concerned, INX has revealed equally effective [7, 42, 55, 98–107]. Braun et al. [108] reviewed the outcomes of different open studies and placebo-controlled trials with AS patients treated with either INX or ETP. They found that attacks of anterior uveitis (AU) had become less frequent (15.6 per 100 patient-years in the placebo group versus 6.8 per 100 patient-years in the patients treated with anti-TNF agents \( P = 0.01 \)). This reduction in frequency was more marked, though not significant, in the patients receiving INX than in those treated with ETP (3.4 per 100 patient-years and 7.9 per 100 patient-years, resp.). More recently, Cantini et al. observed that INX was effective and safe in the long term in 14 patients with idiopathic posterior uveitis [25]. Furthermore, Farvardin et al. treated 10 eyes of 7 patients with chronic persistent noninfectious uveitis with a single intravitreal injection of IFX, resulting in a significant improvement of visual acuity and reduction of central macular thickness [109]. Data regarding BD and sarcoidosis-associated uveitis have already been reviewed before.
As to ADB, it was initially mainly used in infantile non-infectious uveitis. Thus, Vazquez-Cobian et al. [110] treated 14 children with uveitis, 5 idiopathic, and 9 JIA associated. They obtained a reduction of inflammation in almost 81% of eyes. Additionally, ADB was found to be effective in 16 out of 18 children with uveitis treated by Biester et al. [111]. Since ADB possesses a similar inflammation decrease in 50% of the 10 eyes of the 5 patients receiving ADB [112]. Since ADB possesses a similar mechanism of action to that of INX, comparable results to those of the latter could be assumed. In fact, a retrospective study with 43 spondyloarthropathies treated with anti-TNF agents, noted that those receiving INX or ADB presented a lower rate of uveitis relapses than those treated with ETP (number of uveitis flares/100 patient-years before and during ETP treatment: 54.6 versus 58.5 \( P = 0.92 \), number of uveitis flares/100 patient-years before and during INX or ADB treatment 50.6 versus 6.8 \( P = 0.001 \)) [113]. A European multinational, open-label, nonplacebo-controlled trial with 1250 AS patients treated with ADB was recently published. In this study, it was observed that the AU attack rate was reduced by 51% in all patients, by 58% in the 274 patients with a previous history of AU, by 68% in the 106 patients with a recent history of AU, and by a 50% in the 28 patients with active AU at the beginning of the trial, and by 45% in the 43 patients with chronic uveitis. Furthermore, AU attacks during ADB treatment were generally mild [114]. In another study by Díaz-Llopis et al., 19 patients with refractory autoimmune uveitis received treatment with ADB. After one year of follow-up, visual acuity had improved in 31% of eyes, control of intraocular inflammation had been achieved in 63% of patients, the cystoid macular edema present in 86% of eyes at baseline had disappeared in 55% of eyes, and all patients had been able to have their dosage of baseline immunosuppressants reduced at the end of follow-up in at least a 50%. Uveitis relapsed in 42%, but it was easily controlled with a single intraocular injection of steroids [115].

Further increasing evidence in recent years supports the benefits of ADB in the treatment of uveitis. Thus, a case interventional study with 17 children with chronic uveitis (9 of whom had previously received another anti-TNF blocker) found that ADB improved visual acuity. Ocular inflammation also improved or stabilized. However, it did not completely manage to avoid the need for steroid treatment. One patient had ADB discontinued due to the development of varicella zoster infection [116]. In a larger case series study (131 adults with idiopathic or secondary refractory uveitis), the authors observed a significant improvement of visual acuity as well as intraocular inflammation parameters after a 6-month period of treatment with ADB. Additionally, patients could significantly reduce their baseline immunosuppression load (8.81 [5.05] versus 5.40 [4.43]; \( P = 0.001 \)). In fact, 85% of patients had been able to reduce at least 50% of their baseline immunosuppression load at the end of the study. Only 9 patients presented severe relapses during follow-up [117]. Three further case series with 31 (prospective), 60 (retrospective), and 21 patients (prospective), respectively, have shown equally positive outcomes when treating refractory uveitis with ADB [118–120].

Finally, two studies have compared ADB with INX in the treatment of uveitis. In one of them, 48 pediatric patients with JIA-associated AU from the National Italian Registry were treated with IFX, while 43 received ADB for the same reason. After at least one year of follow-up, remission rate was significantly higher with ADB (67%) than with IFX (43%) [121]. In the other one, an open-label prospective study with 33 children with chronic uveitis from different etiologies (16 received ADA and 17 INX), authors observed a significantly higher probability of remission with ADB than with INFX after 40 months of follow-up [122].

In contrast to these results, there exist multiple cases in the literature reporting the occurrence of uveitis following anti-TNF therapy. In this regard, a study based on a registry of uveitis cases developed under treatment with IFX, ADB, or ETP in the USA found a significantly higher number of cases related to the use of ETP than to the other two agents (43 with ETP, 14 with INX, and 2 with ADB), even when the patients whose underlying disorder was associated with uveitis had been excluded. The authors concluded that these results suggest a relationship with the development of agent-specific uveitis rather than with the anti-TNF blockers on the whole. Nonetheless, they admitted the lack of enough evidence to discourage the use of ETP in the treatment of uveitis [53]. In another subsequent study based on a French survey, 31 cases of new onset of uveitis during treatment with anti-TNF blockers were reported. Again, ETP was the agent most frequently associated with this event, 23 cases as opposed to 5 with INX and 3 with ADB. In addition, the author performed a review of the English literature, which produced comparable results: 121 cases (including those of the aforementioned study), 103 of which were on ETP at the time of apparition of the uveitis [123].

5. Safety Issues

No specific adverse effects of anti-TNF therapy when treating BD, sarcoidosis, and noninfectious uveitis have been reported, other than those already known to these agents. These adverse effects include infections (especially tuberculosis), demyelinating diseases, malignancies (lymphomas), allergic reactions, development of autoimmunity, hepatitis, and new onset or worsening of existing congestive heart failure. As to safety during pregnancy, anti-TNF agents are classified in Category B [60].

6. Conclusions

TNF blockade has widely been used off-label, even though there is not any trial-based evidence to support it, except for the experience provided by cases and case series. This experience, which is continuously increasing, has yielded encouraging results, especially regarding ocular, cutaneous, and articular involvement, both in the disorders for which this therapy is licensed and in those for which they are not, such as BD. As far as individual agents are concerned, the largest available experience and the best outcomes on the whole are with INX, particularly in ocular, neurological, and gastrointestinal involvement in BD, skin lesions in
sarcoidosis, and noninfectious uveitis, associated or not to another disorder. Instead, ETP has not proved effective in granulomatous diseases, as it had already been observed in conditions for which the TNF blockade is approved, such as CD, which could be the consequence of a different composition and mechanism of action. As to ADB, the experience was initially very limited. Nowadays, however, the growing evidence suggests that ADB may be more effective than INX in certain cases, such as noninfectious uveitis. Besides, its subcutaneous administration, which allows the patient to self-administer it at home, along with a similar mechanism of action to that of INX, makes the future of ADB most promising. Finally, the safety profile of these agents needs to be more specifically established. Nonetheless, it seems clear from the published literature that incidence of opportunistic infections (mostly tuberculosis) is increased and so does the development of autoimmunity. On the contrary, aspects like the association with demyelinating diseases and the occurrence of lymphomas need additional clarification. In sum, further randomized, controlled trials are required to adequately assess the actual benefits and safety profile in the long term of anti-TNF agents in BD, sarcoidosis, and noninfectious uveitis.

References


