Pristane primed rat T cells enhance TLR3 expression of fibroblast-like synoviocytes via TNF-\(\alpha\) initiated p38 MAPK and NF-\(\kappa\)B pathways

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Received 17 July 2014; accepted with revision 25 November 2014
Available online 19 December 2014

Abstract

Based on pristane-induced arthritis (PIA), we found that T cells mediate TLR3 overexpression in fibroblast-like synoviocytes (FLS). The aim of this study is to determine key factors by which T cells induce TLR3 expression. Rat FLS were co-cultured with pristane primed T cell conditioned medium (PPT medium), and TLR3 expression of FLS was significantly induced. TNF-\(\alpha\), IFN-\(\gamma\) and IL-17 were dominantly expressed in PIA T cells. The overexpression of TLR3 and its related genes in FLS co-cultured with PPT medium could be reduced through blocking TNF-\(\alpha\) pathway. CD4\(^+\) T cells from spleen of PIA rats showed increase of TNF-\(\alpha\) secretion. P38 MAPK and NF-\(\kappa\)B were activated in FLS by PPT medium, and their inhibitors decreased TLR3 upregulation significantly. Finally, TNF-\(\alpha\) induced TLR3 expression was confirmed in human synovial cells.

Abbreviations:

DC, dendritic cell; FLS, fibroblast-like synoviocyte; PIA, pristane-induced arthritis; PPT medium, pristane primed T cell conditioned medium; RA, rheumatoid arthritis; RT-qPCR, real-time quantitative PCR; TLR, toll-like receptor.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease which is associated with progressive disability, systemic complications, early death and high socioeconomic costs [1]. The typical pathological manifestations of RA include local synovitis, cartilage and bone erosion and pannus formation in which fibroblast-like synoviocytes (FLS) have been considered proliferated and activated. FLS, the type B synoviocytes, actively drive local inflammation and degradation of the joint by producing inflammatory cytokines, chemokines and matrix-degrading enzymes in the destructive process of RA [2]. Toll-like receptors (TLRs) are proved to mediate the functions of FLS and considered to play a vital role in both RA and experimental arthritis [2,3].

TLRs belonging to the pattern recognition receptor family consist of thirteen members with conserved structure in mammalians, and ten of them have been identified in humans. As stimulated with their specific ligands, pathogen-associated molecular patterns and damage-associated molecular patterns, TLRs activate specific transcriptional factors by MyD88 dependent or independent pathway to regulate the inflammatory gene expression. Accumulating evidence indicates that TLRs, in particular TLR2, TLR3, TLR4, TLR7/8 and TLR9, participate in the pathogenesis of RA and experimental arthritis by affecting the function of key cells including immune cells and FLS [4–10]. It has been found that the overexpressed TLR3 and TLR4 in RA FLS are more responsive to their ligands [5,7]. TLR3 and TLR7 in synovium could trigger the inflammatory cascade of RA via type I interferon [11]. And TLR2 and TLR4 combination engagement upregulates IL-15 synergistically in RA FLS through NF-κB pathway [12].

Among these TLRs, TLR3 recognizes virus dsRNA and endogenous RNA, and subsequently activates IRF family through the MyD88 independent pathway to induce type I interferon, which can modulate the expression of certain inflammation genes [13]. Yaron et al. found that poly(I):C, a synthetic ligand of TLR3, could induce acute joint inflammation in rats in 1979 [14]. Subsequent studies testified that the ligands of TLR3, poly(I):C and dsRNA, did result in arthritis [5,15,16]. Our experiments also showed that poly(I):C synergistically aggravates the pristane-induced arthritis (PIA) [4,5]. Meanwhile, many researches showed that TLR3 expression was increased in some tissues such as synovium and spleen, and especially in some cells such as FLS, macrophages and dendritic cells (DC), in RA patients and arthritis animals [4,5,7,10]. The clinical symptom and pathological manifestation of arthritis were alleviated significantly when TLR3 expression was knocked down in vivo [4]. These results indicate that TLR3 play a crucial role in RA and experimental arthritis.

Antigen presenting cells and FLS are cell types where TLR3 play an important role. It was reported that dsRNA could activate macrophage and trigger arthritis through IL-1R signaling [16]. And the ability of dsRNA triggering arthritis was related to the function of macrophage secreting type I interferon, and also depended on type I interferon signaling [15]. Our previous studies also showed that PIA in rats relied on TLR3 and its downstream cytokines [4,17]. TLR3 ligands were shown to stimulate DC from RA patients to secrete IL-12, MIF [10,18]. Most importantly, FLS are the key cells involved into the local inflammation, which could produce the cytokines such as IFN-γ, IL-6, IL-8 [5,7,19], chemokines such as CCL5, IP-10/CXCL10, and MMP-3, MMP-13 [7,18,19], thymic stromal lymphopoietin [20], VEGF [21], with TLR3 ligand stimulation.

These studies confirmed the phenomenon of the TLR3 overexpression in RA and experimental arthritis, and further disclosed how TLR3 participated in the disease to some extent. However, how TLR3 expression is upregulated remains unclear. Our previous data indicated that the upregulation of TLR3 expression in FLS, which contributed to the activation of joint inflammation in PIA rats, might be caused by the stimulation of T cells [5]. Consequently, in this paper we try to find the possible inflammatory factors from T cells in PIA rats involved into TLR3 overexpression in FLS, and elucidate the further mechanism about the pathways to modulate TLR3 expression.

2. Materials and methods

2.1. Rats

DA rats were bred in a specific pathogen-free animal house of Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Xi’an Jiaotong University Health Science Center. The experiments were approved by the Institutional Animal Ethics Committee of the University.

2.2. Rat FLS co-cultured with T cell conditioned medium

DA rats at age of 8–12 weeks were induced arthritis by a single intradermal injection with 300 μl of pristane (ACROS) at the base of the tail as described previously [5,22]. Four sex-matched rats in both disease and control groups were used in each experiment and three independent repeated experiments were performed to reach significance of the results.

At 14 d after pristane injection, spleens from PIA and control rats were homogenized as single cell suspension and red blood cells were lysed with 0.84% NH₄Cl. Then splenocytes were added into the tissue culture flasks for 1 h incubation and nonadherent cells were recovered for T cell isolation. The pristane primed T cells from PIA rats and control T cells from control rats were isolated using the Nylon Fiber column T (Wako, Japan) as described [5,23]. The purity of the αβ T cells was detected by flow cytometry.
staining with FITC-R73 antibody (a mouse anti-rat TCRαβ monoclonal antibody, IgG1, Serotec), and the proportion of R73 positive cells was more than 90%. All T cells at a density of 5 × 10^5/ml were cultured in RPMI1640 supplemented with 10% FBS and 3 μg/ml Con A (Sigma) for 72 h. Then the supernatant of the culture medium was collected and filtered by a 0.45 μm filter (Millipore).

The FLS from normal rats were isolated by collagenase digestion, cultured as described previously, and used after passage 4 [5,24]. The purity of FLS was detected by flow cytometry staining with rabbit anti-rat vimentin antibody, and the proportion of intracellular vimentin positive cells was more than 95%. FLS were seeded in 6-well plates at a density of 10^5/well and incubated with pristane primed T cell conditioned medium (PPT medium) or control T cell conditioned medium (control medium) respectively for 24 hours. After that FLS were collected for RNA and protein isolation.

2.3. Cytokine blocker treatment

Etanercept (a TNF-α soluble receptor, CPGJ-pharm, China, 25 μg/ml), DB-1 (a mouse anti-rat IFN-γ monoclonal antibody, IgG1, 20 μg/ml) and α-IL-17 antibody (a rabbit anti-rat IL-17 antibody, IgG, Santa Cruz, 20 μg/ml) were administrated to T cell conditioned medium accordingly [25-27]. FLS were seeded in 6-well plates at a density of 10^5/well and incubated with PPT medium or control medium for 24 h with or without the above blocker accordingly, and the isotype-matched IgG control were used as controls. Then FLS were collected to isolate RNA and TLR3, IL-6, MMP3 and MMP13 mRNA expressions in FLS were detected by real-time quantitative PCR (RT-qPCR). And in another experiment, etanercept was applied to the FLS co-cultured model as described above. After 24 h, FLS were collected to isolate protein and TLR3, p-p38, p-p38 or IκB protein levels in FLS were detected by Western blotting.

2.4. Flow cytometry staining

T cells were isolated and purified from spleen of four PIA rats and four control rats respectively at 14 d after pristane injection, and stimulated by Con A as above mentioned. Four hours before flow cytometry staining, Brefeldin A (10 ng/ml, Selleck) was applied to cells. Eight tubes containing 10^6 cells from each rat were incubated with FITC-R73 and CyChrome-OX35 (a mouse anti-rat CD4 monoclonal antibody, IgG2a, Becton Dickinson) or CyChrome-OX8 (a mouse anti-rat CD8 monoclonal antibody, IgG1, Becton Dickinson) antibodies firstly. Then cells were fixed and perforated, and followed by TNF-α intracellular staining by using PE-Hamster anti-rat TNF-α antibody. The proportion and mean fluorescence intensity of TNF-α were detected on R73+OX35+ gated and R73+OX8+ gated cells by a Guava easyCyte Flow Cytometer (Merck Millipore). Data were collected after correction for isotype-matched control.

2.5. The administration of p38 and NF-κB inhibitors to rat FLS

Rat FLS were seeded in 6-well plates at a density of 10^5/well and incubated with PPT medium or control medium, with and without SB203580 (a p38 specific inhibitor, Beyotime, China, 1 μM) or Bay11-7082 (a NF-κB inhibitor, Beyotime, China, 1 μM) for 24 h. In another experiment, TNF-α (PeproTech, 10 ng/ml) was administrated to FLS for 24 h to imitate the effect of PPT medium on FLS, and SB203580 or Bay11-7082 was applied on FLS stimulated with TNF-α. The mRNA expression of TLR3, IL-6, MMP3 and MMP13 of FLS was detected by RT-qPCR, and the protein expression of TLR3 and p38, p-p38 or IκB was detected by Western blotting.

2.6. mRNA quantitation

FLS were collected after various treatments and the mRNA expression of TLRs (TLR2, 3, 4, 7 and 9), IL-6, MMP3, MMP13 and β-actin was determined by RT-qPCR. Total RNA was isolated with TRizol® Reagent (Invitrogen), and cDNA was synthesized by First Strand cDNA Synthesis Kit (Fermentas). Real-time PCR was performed by iQ5 (BIO-RAD) with SYBR® Premix Ex Taq™ II (TaKaRa) for mRNA quantitation. And the relative gene expression normalized by β-actin was calculated with 2^ΔΔCT method. The information of primers, products and annealing temperatures is depicted in Table 1.

2.7. Western blotting

FLS were collected for the protein expression quantitation of TLR3, p38, p-p38, p-ERK1/2, ERK1/2, p-JNK1/2, JNK1/2, IκB and β-actin. Total cell lysates were extracted by using the RIPA solution (Beyotime, China) with a cocktail of protease and phosphatase inhibitors (Roche). The samples were centrifuged at 12,000 x g for 20 min at 4 °C. The supernatants were collected and stored at −80 °C. The final protein concentration of each sample was determined by a BCA kit (Jiankangyuan, Zhuhai, China).

The supernatants (20 μg total protein) were subjected to SDS/PAGE gel according to standard procedures in Bio-Rad system. The specific primary antibody included rabbit anti-TLR3 antibody (Biosen, 1:200), rabbit anti-ERK1/2 monoaotibody (4695, CST, 1:1000), rabbit anti-p-ERK monoantibody (4376, CST, 1:1000), rabbit anti-p-JNK monoantibody (9252, CST, 1:1000), rabbit anti-p-p38 monoantibody (4695, CST, 1:1000), rabbit anti-p-ERK monoantibody (4376, CST, 1:1000), rabbit anti-p-JNK monoantibody (9252, CST, 1:1000), rabbit anti-p-p38 monoantibody (9251, Thr180/Tyr182, CST, 1:1000), rabbit anti-p-p38 monoantibody (9251, Thr183/Tyr185, CST, 1:1000), rabbit anti-iκBα antibody (Biosen, 1:200) and anti-β-actin (CST, 1:1000) overnight. And the signal was further detected by using the secondary antibody of goat anti-rabbit IgG labeled with HRP (Abcam, 1:5000). Signal intensity was determined by Supersignal® West Pico kit (Thermo Scientific).

2.8. ELISA

The concentrations of TNF-α and IFN-γ in the T cell conditioned medium were detected by ELISA. The filtered T cell supernatants were collected, and then TNF-α and IFN-γ were determined by using ELISA development kit (Peprotech, USA). Briefly, 100 μl supernatant was added into TNF-α or IFN-γ antibody coated plate and incubated at 25 °C for 2 h. After adding the biotin-conjugated detecting TNF-α or IFN-γ antibody and incubating for 2 h, streptavidin-HRP was added and TMB was used for development. The OD value was
obtained at the wavelength of 450 nm by multiskan spectrum (Thermo, USA). The complete medium of RPMI1640 + 10%FBS was used as a blank, and the TNF-α or IFN-γ concentration was calculated from the standard curve, which was obtained using the series dilution of recombinant rat TNF-α or IFN-γ.

2.9. TNF-α stimulation of human synovial cells

SW982, a human synovial cell line was cultured by DMEM + 10%FBS, and stimulated by TNF-α (10 ng/ml) for 6 h, 9 h, 12 h and 24 h, respectively. And the protein levels of TLR3, p-p38, total p38 and IκBα were detected by Western blotting. Then SW982 with 10 ng/ml of TNF-α was applied by SB203580 or Bay11-7082 for 24 h, and TLR3 expression in human cells was determined.

2.10. Statistics

Quantitative data were expressed as mean ± standard error of mean (SEM). The statistical analysis of differences between experimental groups was performed by using the Student’s T test. A P value less than 0.05 was considered significant.

3. Results

3.1. Upregulation of TLR3 expression in FLS was induced by inflammatory factors derived from pristane primed T cells

Splenic T cells from PIA and control rats were cultured ex vivo respectively and T cell conditioned medium was collected. FLS were separated from the synovium of DA rats, and then co-cultured with PPT medium or control medium. RT-qPCR results showed that TLR3 mRNA expression but not TLR2, 4, 7 and 9 mRNA expression was significantly increased in FLS co-cultured with PPT medium (Fig. 1A). Meanwhile, TLR3 protein overexpression was also confirmed by Western blotting (Fig. 1B).

The above results suggested that among TLRs, TLR3 should be a specific mediator connecting T cells and FLS in local inflammation, and its upregulation might be induced by proinflammatory factors derived from T cells of PIA rats. PIA is mediated by MHC class II restricted CD4+ T cell [28], and TNF-α, IFN-γ and IL-17, the typical markers of Th1 and Th17, are major cytokines that could be mediating TLR3 expression in FLS. Both TNF-α and IFN-γ were increased in the conditioned medium from pristane primed T cells (Figs. 1C and D). RT-qPCR result showed that IL-17a mRNA expression in pristane primed T cells was also upregulated remarkably (Fig. 1E).

3.2. TNF-α blocker could inhibit the upregulation of TLR3 and its downstream gene expression in FLS

Due to the potential role of TNF-α probably derived from T cells in PIA rats, etanercept, the TNF-α soluble receptor, was added to T cell conditioned medium, which was used to co-culture with FLS. Etanercept significantly inhibited TLR3 mRNA overexpression of FLS co-cultured with PPT medium (Fig. 2A). The mRNA expression of IL-6, a downstream cytokine of TLR3 pathway that has been proved before, was increased in conditioned medium from pristane primed T cells (Figs. 1C and D). RT-qPCR result showed that IL-17a mRNA expression in pristane primed T cells was also upregulated remarkably (Fig. 1E).

### Table 1

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3.3. α-IFN-γ antibody could block the increase of TLR3 and IL-6 expression but not MMPs

DB-1, a monoclonal antibody of IFN-γ, which is also the key cytokine in T cell conditioned medium from PIA rats, was administrated to the FLS co-cultured cell model. DB-1 showed part similarity to the effects of etanercept that TLR3 and IL-6 mRNA overexpression in FLS co-cultured with PPT medium was inhibited (Fig. 2B). However, DB-1 was most likely to have no effect on inhibiting the overexpression of MMP3 and MMP13 mRNA in FLS resulted from PPT medium (Fig. 2B).

3.4. α-IL-17 antibody could not affect TLR3 and downstream gene overexpression in FLS

α-IL-17 antibody was administrated to analyze the potential role of IL-17 in TLR3 mRNA overexpression in FLS induced by T cell conditioned medium from PIA rats. It was clear that α-IL-17 antibody failed to inhibit the TLR3 mRNA overexpression in FLS co-cultured with PPT medium (Fig. 2C). Meanwhile, the administration of α-IL-17 antibody could also not affect TLR3 downstream gene overexpression, including IL-6, MMP3 and MMP13 in FLS co-cultured with PPT medium (Fig. 2C).
3.5. TNF-α which induced TLR3 of FLS was mainly derived from CD4+ T cells of PIA rats

The protein expression of TLR3 in FLS co-cultured with T cell conditioned medium was confirmed by Western blotting, and results showed that with PPT medium stimulation, TLR3 protein was overexpressed (Fig. 3A). T cell induced TLR3 protein expression could be inhibited by etanercept, indicating that TNF-α is involved in induction of TLR3 in FLS (Fig. 3A).

Then, TNF-α expression in CD4+ and CD8+ T cells from both PIA and control rats was checked by using intracellular staining. Flow cytometry staining indicated that in CD4+ T cells of PIA rats, the percentage of TNF-α+ cells showed increased tendency compared with control rats, and the mean fluorescence intensity of TNF-α was significantly upregulated (Fig. 3B). However, TNF-α from CD8+ T cells of PIA rats showed no difference compared with control rats (Fig. 3B). It suggested that increased TNF-α from CD4+ T cells was the main cause of the induction of TLR3 in FLS.

3.6. p38 MAPK was the uniquely activated MAPK pathway in FLS by PPT medium

MAPK pathways, the main downstream pathways after TNF-α activation were then detected in the FLS by Western blotting, including total and phosphorylation levels of p38, ERK1/2 and JNK1/2. The results showed that PPT medium could stimulate and upregulate the protein expression ratio of p-p38/p38 in FLS, whereas it was most likely to have no effect on p-ERK1/ERK1, p-ERK2/ERK2, p-JNK1/JNK1, and p-JNK2/JNK2 (Fig. 4). And the activation level of p38 in FLS co-cultured with PPT medium could further be reduced after inhibiting TNF-α pathway by etanercept (Fig. 4). Combining the above results, it was clear that p38 MAPK pathway in FLS was activated by TNF-α, the inflammatory factor derived from T cells of PIA rats.

3.7. Inhibiting p38 MAPK blocked TLR3 expression upregulation in FLS stimulated by TNF-α derived from T cells of PIA rats

To further confirm the role of p38 on TLR3 expression regulation, SB203580, the inhibitor of p38 signal pathway was combined with T cell conditioned medium to treat with FLS. RT-qPCR results showed that with SB203580 administration, the overexpression of all TLR3 and its related genes, IL-6, MMP3 and MMP13 in FLS co-cultured with PPT medium was inhibited significantly (Fig. 5A). And the upregulation of TLR3 protein level and p38 activation level in FLS co-cultured with PPT medium was also decreased by
SB203580 treatment (Fig. 5C). Thus, inhibiting p38 in FLS could block TLR3 upregulation by PPT medium.

The critical role of TNF-α on TLR3 regulation in FLS had been proved from both mRNA and protein levels. Here, we utilized TNF-α to stimulate FLS directly to mimic the co-cultured cell model, and confirmed the role of p38 on mediating the inflammatory signaling by its specific inhibitor administration. The results showed that as expected, TLR3 mRNA expression in FLS was induced significantly with TNF-α stimulation, and with SB203580 application, TLR3 mRNA overexpression was suppressed (Fig. 5B). And so did its related gene expression. The downstream cytokine, IL-6 and the matrix-degrading molecules, MMP3 and MMP13 mRNA expression all were increased as FLS was stimulated with TNF-α, and the overexpression was inhibited by SB203580 except that MMP3 only had limited effect (Fig. 5B). And Western blotting detection confirmed that TNF-α could stimulate and upregulate TLR3 protein expression and p38 activation in FLS, and both were inhibited after treating with SB203580 (Fig. 5D).

3.8. NF-κB pathway was also activated by TNF-α and contributed to TLR3 upregulation in FLS.

NF-κB is the other critical pathway in the downstream of TNF-α. Its activation in FLS was represented by checking IκBα degradation. Western blotting results showed that IκBα expression was significantly decreased in FLS stimulated by PPT medium, but recovered by etanercept administration (Fig. 6A). It suggested that TNF-α from PPT medium caused the activation of NF-κB pathway.

To further confirm the role of NF-κB, its specific inhibitor, Bay11-7082 was used in combination with PPT medium or TNF-α. Results showed that the administration of this inhibitor could significantly inhibit TLR3 upregulation in FLS (Fig. 6B).

* Figure 3  TNF-α derived from CD4+ T cells caused TLR3 upregulation in FLS. Rat FLS were co-cultured in PPT medium or control medium with or without etanercept for 24 h. TLR3 protein expression (A) was detected by Western blotting, and the intensities of bands were quantified and relative expression was normalized by β-actin. One representative plot and quantitative data from three independent Western blotting are shown. Splenic T cells were isolated and purified from PIA rats and control rats. T cells activated by Con A were stained by FITC-R73 (αβTCR), PE-anti-rat TNF antibody, CyChrome-OX35 (CD4) or CyChrome-OX8 (CD8). The proportion and mean fluorescence intensity of TNF-α (B) were detected on R73′OX35′ gated and R73′OX8′ gated cells by FACS. Data were collected after correction for isotype-matched control. Values are shown as means ± SEM. * represents the comparison between PIA group and control group. Levels of significance were calculated by using Student’s T test (**P < 0.01).

* Figure 4  The determination of MAPK pathways in FLS co-cultured with T cell conditioned medium. Rat FLS were co-cultured in PPT medium or control medium with or without etanercept for 24 h. The protein expression of p-p38, p38, p-ERK1/2, p-JNK1/2 and JNK1/2 in FLS was detected by Western blotting, and each relative expression of phosphorylation form was normalized by the total form. One representative plot and quantitative data from three independent Western blotting are shown.
Bay11-7082 significantly inhibited the upregulation of TLR3 expression induced by PPT medium or TNF-α (Figs. 6B and C), which indicated that besides p38 MAPK pathway, NF-κB pathway was also involved into TLR3 expression modulation in FLS.

3.9. TNF-α could induce TLR3 expression in human synovial cells via p38 MAPK and NF-κB pathways.

It had been confirmed that TNF-α derived from CD4+ T cells of PIA rats resulted in TLR3 upregulation in FLS, and TNF-α activated p38 MAPK and NF-κB pathways might be involved in it. To further confirm our findings in human system, SW982 was cultured and stimulated by TNF-α with different time points (6 h, 9 h, 12 h and 24 h). Western blotting results showed that with the time process, the expression of TLR3 was induced by TNF-α stimulation and showed significantly upregulated at 24 h (Fig. 7A). Meanwhile, the phosphorylation level of p38 was increased in human FLS from 9 h after TNF-α stimulation, and the expression of IκBα was decreased gradually (Fig. 7A). The phenomenon found in rat FLS was successfully confirmed in human system dynamically. Then the role to mediate TLR3 expression modulation was confirmed by using pathway specific inhibitors. Inhibitors of both p38 and NF-κB could block the TNF-α induced TLR3 upregulation, suggesting that both downstream pathways of TNF-α were involved into the regulation of TLR3 (Fig. 7B).

Figure 5  Inhibiting p38 MAPK pathway blocked TLR3 overexpression stimulated by TNF-α. A: Rat FLS were incubated with PPT medium or control medium with or without SB203580 for 24 h. TLR3 mRNA expression and the mRNA expression of IL-6, MMP3 and MMP13 in FLS were detected by RT-qPCR. B: Then rat FLS were stimulated by TNF-α with or without SB203580 for 24 h. The mRNA expression of TLR3 and its related genes in FLS were detected by RT-qPCR. Relative mRNA expression was compared with β-actin. Values are shown as means ± SEM from three independent experiments. * represents the comparison as marked. Levels of significance were calculated by using Student’s T test (* P < 0.05, **P < 0.01, ***P < 0.001). TLR3, p-p38 and p38 protein expressions in FLS with PPT medium (C) or TNF-α (D) stimulation in combination with SB203580 were confirmed by Western blotting. One representative plot and quantitative data from three independent western blotting are shown.
4. Discussion

Accumulating evidence supports a role of TLR3 in RA and in pristane-induced arthritis. However, the induction of TLR3 expression is still ambiguous and we could here show that pristane activated T cells secrete TNF-α that contributes to induction of TLR3 in FLS. Few researches concern on the mechanism of TLR3 overexpression in macrophage, DC, FLS and other cells or tissues from RA patients and arthritis animals. In our previous study, a local inflammation model, co-culturing rat FLS with splenic T cells, was established, and TLR3 mRNA expression in FLS was upregulated when FLS was co-cultured with T cells from PIA rat spleen [5]. Consistent result was observed when FLS were co-cultured only with the PPT medium, suggesting that TLR3 mRNA upregulation might result from soluble inflammatory factors in the T cell conditioned medium. Hence we need to further explore the potential factors involved into TLR3 upregulation in FLS and investigate the possible pathway and mechanism.

The upregulation of both TLR3 mRNA and protein expression in FLS co-cultured with PPT medium was confirmed in the present study (Figs. 1A and B). And an important issue is why the T cell conditioned medium could lead to TLR3 expression upregulation. Maeshima reported that CD4+ T cells separated from synovium could secrete IL-17 and IFN-γ implicated with the inflammation of joint [29]. Our previous work found that there was high amount of TNF-α and IFN-γ in the supernatant of splenocytes restimulated with Con A in vitro [28]. It was known that transferring T cells from PIA DA rats to normal recipient DA rats through the tail vein could lead to the arthritis of recipient rats. Interestingly, when the recipient rats were treated with a recombinant TNF-α soluble receptor (etanercept) or monoantibody of IFN-γ (DB-1) before cell transfer, the adoptively transferred arthritis would be ameliorated both regarding incidence and arthritis severity [28]. This phenomenon indicates that TNF-α and IFN-γ in the conditioned medium which were secreted by spleen T cells play a vital role in the pathogenesis of PIA. Here, we detected the cytokine concentrations in T cell conditioned medium used for co-cultured experiment, and found that both concentrations of TNF-α and IFN-γ in the supernatant of pristane primed T cells were increased (Figs. 1C and D). And the mRNA expression of IL-17a in T cells from PIA rats was also upregulated (Fig. 1E). These results hint that TNF-α, IFN-γ and IL-17 in the T cell conditioned medium might be one reason for overexpression of TLR3.

The previous studies found that TNF-α could stimulate RA FLS to proliferate and produce growth factors, chemokines, proteinases and adhesion molecules, key players in the RA development [30–32]. Hence in the present study we consider there may be a special relation between TNF-α and TLR3 expression in FLS. We applied etanercept to FLS co-cultured with the T cell conditioned medium from PIA. The present result showed that the upregulation of TLR3 and its downstream cytokine and MMP expression resulted from PPT medium could be blocked by etanercept (Figs. 2A and 3A). It suggests that TNF-α in the conditioned medium acts a modulatory role in TLR3 mRNA expression, and also implies that the therapeutic effect of etanercept on RA may be due to its downregulation to TLR3 expression partly. The present results enrich our knowledge about how TNF-α acts in RA pathogenesis.

IFN-γ is another key cytokine concerned by RA researchers. The monoclonal antibody of IFN-γ, DB-1, was also administrated to FLS co-cultured with the T cell conditioned medium from PIA. The increased mRNA expression of TLR3 and IL-6 in FLS co-cultured with PPT medium could be inhibited by DB-1, whereas that of MMPs could not be affected (Fig. 2B). The result verified the function of IFN-γ on TLR3 expression modulation was not as important as that of TNF-α in this cell model. Whatever, our findings might interpret the mechanism of DB-1 therapeutic effect on PIA [28]. Previous research reported that the production of IFN-γ and IL-17 of CD4+ T cell could be suppressed by the JAK inhibitor, which might lead to the decrease of synovitis and cartilage destruction at last [29]. However, many researches showed that IFN-γ could protect articular cartilage and suppressed collagen-induced arthritis [33,34]. The effect of IFN-γ on the local inflammation seems paradoxical and need to be discussed further.

A proverbial fact is that TNF-α and IFN-γ are considered as Th1 cytokines, so our results imply that both of them might participate in RA and PIA known as Th1 related
TNF-α could induce TLR3 overexpression via p38 MAPK and NF-κB pathways in human synovial cells. A: SW982 cells were stimulated by recombinant TNF-α within different time points (0, 6, 9, 12 and 24 h). The protein expressions of TLR3, p-p38, p38 and lκBα in cells were detected by Western blotting. Then SW982 were stimulated by TNF-α with or without SB203580 or Bay11-7082 for 24 h, and TLR3 expression (B) was detected by Western blotting. One representative plot and quantitative data normalized by β-actin from three independent experiments are shown.

Recent studies have shown that Th17 cells also have been found to act in RA and experimental arthritis [35–37], while some studies showed that Th17 did not predominate in the joints of RA patients [38]. Some studies have indicated that there was IL-17 derived from CD4+ T cells in synovium [29]. Thus a question is proposed that if this cytokine belonging to Th17 family has an influence on TLR3 expression in FLS co-cultured with the T cell conditioned medium. A recent study found that IL-17 might increase TLR2, 3, 4 expression in FLS from RA patients [39]. But based on our model, it can be concluded that IL-17, a typical cytokine of Th17 family, might not participate in mediating the PPT medium upregulated TLR3 expression. Although the present evidence on IL-17 seems contradictory, we still could not rule out the role of IL-17 in mediating the local inflammation. Probably, Th17 might participate in the pathogenesis of RA and experimental arthritis through other mechanisms.

The application of etanercept indicated that TNF-α in PPT medium acts in the upregulation of TLR3 expression in FLS, while the administration of DB-1 only partly blocks the upregulation effect. And it is known that etanercept has already been applied to clinical treatment for RA and other diseases, whereas DB-1 could only work well in experimental arthritis. We focus on TNF-α and pay close attention to its downstream signal transduction pathway of TNF-α which results in the increased expression of TLR3 in FLS. As above mentioned, we had found that splenic T cells especially CD4+ αβ T cells could transfer the arthritis in rats. So CD4+ T cells were paid more priority attention to mediating the articular inflammation. The pristane primed T cells are oligoclonal or polyclonal origin as determined by their arthritogenicity after stimulation with several mitogenic anti-TCRVβ antibodies. Even though we considered that CD4+ T cells might play more important role in local inflammation, both CD4+ and CD8+ T cells might be the source of TNF-α which could induce TLR3 expression in FLS. In the present work, we determined the TNF-α expression in CD4+ and CD8+ T cells from both PIA and control rats by using intracellular staining (Fig. 3B). Flow cytometric staining showed that in CD4+ T cells of PIA rats, both the expression of TNF-α and the percentage of TNF-α+ cells were increased, suggesting that TNF-α from CD4+ T cells is the main cause of the induction of TLR3 in FLS which seems in coincidence with our previous presumption.

Previous studies show that MAPK signaling pathway is the key downstream pathway of TNF-α. P-38, ERK and JNK are main members of MAPK family, which have been reported involving in the pathogenesis of RA and experimental arthritis [40]. Hence it is deduced that TNF-α might affect TLR3 expression through p38, ERK or JNK pathway. The activation of MAPK pathways was detected by Western blotting, and the results displayed that only p38 activation was increased in FLS stimulated with T cell conditioned medium from PIA rats. And it also showed that the increase of p-p38/p38 ratio could be abrogate by etanercept treatment, which demonstrates that the p38 activation in FLS resulted from TNF-α stimulation derived from T cells. The above results provide us a clue to discover the role of p38 on TLR3 expression in FLS.

When SB203580, a specific inhibitor of p38 was applied to FLS co-cultured with PPT medium, the overexpression of TLR3 and its downstream molecules were decreased, which confirmed that p38 MAPK was involved into TLR3 modulation in our cell model (Fig. 5). Then TNF-α was treated to FLS directly to mimic the co-cultured cell model, and inhibiting p38 MAPK also blocked the upregulation of TLR3 and its related gene expression (Fig. 5). The result suggested that p38 mediate the modulation of TLR3 upregulation which resulted from TNF-α. The p38 MAPK pathway has been found to play a central role in both the production of and signaling of many proinflammatory molecules in RA and correlates with disease activity [41,42]. The administration of SB203580 to RA synovial membranes could inhibit spontaneous production of proinflammatory cytokines such as TNF-α and IL-1 [43]. However, Dulos found that p38 inhibition enhanced the secretion of chemokines from TNF-α activated RA FLS [44]. In fact, although many p38 inhibitors have entered clinical trials for the treatment of RA, none of these compounds has lived up to the expectations generated from studies in murine models. Whatever, the present results showed that p38 pathway could modulate TLR3 expression in FLS, and our previous study showed that RNAi of TLR3 in vivo could alleviate PIA [4], which give us a hint...
that the blocking of specific downstream molecules of p38 MAPK pathway, or combinational inhibition might be more effective in experimental arthritis. NF-κB pathway is the other critical downstream signal pathway which could be activated by TNF-α. And it has been reported that NF-κB pathway is involved in mediating the local inflammation of arthritis [45]. Many genes including cytokines, chemokines, growth factors, cellular ligands, and adhesion molecules which are part of the pathogenesis of RA, are translated after NF-κB activation. Obviously it should be a potential pathway which participated into TNF-α induced TLR3 expression in FLS. The decreased expression of Il-1β in FLS stimulated by PPT medium and its recovered expression level after etanercept treatment suggested that TNF-α led to the activation of NF-κB pathway. To further confirm the role of NF-κB, its specific inhibitor, Bay11-7082 was used in combination with PPT medium or TNF-α. Results showed that the administration of Bay11-7082 significantly inhibited the upregulation of TLR3 expression, which indicated that the administration of Bay11-7082 significantly inhibited TNF-α, and the blocking of specific downstream molecules of p38 MAPK pathway, or combinational inhibition might be more effective in experimental arthritis.

5. Conclusion

In the present study, we found that TLR3 mRNA and protein expression in rat FLS was induced significantly by PPT medium. And TNF-α derived from CD4+ T cells cause the upregulation of TLR3 expression in FLS via p38 MAPK and NF-κB pathways. Finally the main findings were confirmed in human system by using SW982, a human synovial cell line. And the coincident results in human FLS strengthen our findings significantly. Therefore, the data provide the mechanism of TLR3 upregulation in FLS cell model from certain expect, and discover one way by which p38 and NF-κB might execute their function in experimental arthritis and RA.

References

Pristane primed rat T cells enhance TLR3 expression
