Neutrophil-derived lactoferrin induces the inflammatory responses of rheumatoid arthritis synovial fibroblasts via Toll-like receptor 4

K. Umekita, S. Miyauchi, H. Nomura, K. Umeki, A. Okayama

Department of Rheumatology, Infectious Diseases, and Laboratory Medicine, Faculty of Medicine, University of Miyazaki, Japan.

Abstract

Objective

Damage-associated molecular patterns (DAMPs) are proposed to drive aberrant stimulation of Toll-like receptors (TLRs) in rheumatoid arthritis (RA) inflamed joints. In the current study we investigated the role of the neutrophil-derived lactoferrin (LTF), as an endogenous ligand for TLR4 in the inflammatory response of RA synovial fibroblasts (RASFs).

Methods

RASFs were stimulated with LTF, and the expressions of inflammatory cytokines in RASFs were measured. To clarify the TLR4 signalling pathway associated with LTF stimulation, a small molecular inhibitor of TLR4 (TAK242) and NF-κB inhibitor were used. The role of nuclear factor of activated T cells 5 (NFAT5) was identified using small interfering RNA. To reveal the interaction between NF-κB and NFAT5, cerulenin, which disrupts their interaction, was used.

Results

Stimulation of RASFs with LTF significantly increased the expressions of inflammatory cytokines and chemokines, such as IL-6, CCL20 and IL-8, in RASFs. LTF enhanced the mRNA expressions of these cytokines in RASFs stimulated by TNF-α. TAK242 almost completely inhibited the expressions of inflammatory cytokines and chemokines in RASFs stimulated by LTF. The NF-κB inhibitor partially repressed the expressions of IL-6 and IL-8 mRNAs induced by LTF, but not CCL20 mRNA expression. On the other hand, NFAT5 silencing decreased the expressions of CCL20 and IL-8 mRNAs induced by LTF, but not IL-6 mRNA expression. Cerulenin repressed the expressions of IL-6, CCL20 and IL-8 in RASFs stimulated by LTF.

Conclusion

Neutrophil-derived LTF may play a role as an endogenous ligand for TLR4 expressed on RASFs. NFAT5-NF-κB enhanceosome might regulate the expressions of LTF-TLR4-responsive genes in RASFs.

Key words

rheumatoid arthritis, innate immunity, Toll-like receptor, enhanceosome

Kunihiko Umekita, MD, PhD
Shunichi Miyauchi, MD, PhD
Hajime Nomura, PhD
Kazumi Umeki, PhD
Akihiko Okayama, MD, PhD

Please address correspondence to:
Dr Kunihiko Umekita,
Department of Rheumatology, Infectious Diseases, and Laboratory Medicine,
Faculty of Medicine,
University of Miyazaki,
Kihara 5200, Kiyotake,
889-1692 Miyazaki, Japan.
E-mail:
kunihiko_umekita@med.miyazaki-u.ac.jp

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Introduction

A previous study has shown that rheumatoid arthritis synovial fibroblasts (RASFs) can be strongly stimulated by Toll-like receptor (TLR) pathways (1). TLRs belong to a family of pattern-recognition receptors, and they play a crucial role in the activation of the innate immune system in response to the invasion of microorganisms. According to their corresponding ligands, TLRs can be categorised into TLRs that are receptors for lipid-based pathogen-associated molecular patterns (PAMPs) (TLRs 1, 2, 4 and 6) and TLRs that are receptors for nucleic acid-based PAMPs (TLRs 3, 7, 8 and 9). Among expressed TLRs, TLR3 and TLR4 have been shown to be abundantly expressed in synovial fibroblasts (2). Exogenous and endogenous TLR ligands have been identified in the joints of RA patients (1, 3). It was found that activation of innate immunity via TLRs is connected to activation of the adaptive immune system, in addition to the identification of endogenous ligands for certain TLRs, and this strengthened the hypothesis that early dysfunction of TLR pathways might result in autoimmune inflammation in joints (4).

Lactoferrin (LTF) is an iron-binding protein that is released from activated neutrophils at sites of inflammation. It has anti-microbial as well as anti-inflammatory properties in bacterial infections. The LTF level in RA synovial fluid is higher compared to that in osteoarthritis (OA) (5). LTF contributes to extended neutrophil survival in inflamed joints in the established phase of RA (6, 7). Indeed, the LTF expression in neutrophils in RA synovial fluid was higher than that in OA (8). Recent evidence has indicated that LTF activates NF-κB through the TLR4 pathway in mouse embryonic fibroblasts (9). However, the role of LTF as an endogenous ligand for TLR4 in the inflammatory response of RASFs is still unclear.

Transcription factors belonging to the family of nuclear factors of activated T cells (NFAT) play an essential role in diverse biological processes, such as inflammation, immune response and cell proliferation (10) NFAT transcription factors are expressed in the synovial tissue of RA patients. NFAT5 is predominantly expressed in the synovial lining of RA joints, including the pannus, but is not detected in the normal synovium (11, 12). Recently, pro-inflammatory cytokines, such as TNF-α and IL-1β, were found to induce the expression of NFAT5 in RASFs (13). Known NFAT5 target genes are involved in the pathogenesis of RA, including inflammation promotion and joint destruction. In a previous study, mice that were haploinsufficient for NFAT5 presented with strongly reduced synovial proliferation and angiogenesis in a collagen antibody-induced arthritis model (13). Additionally, lipopolysaccharide (LPS), which is one of the TLR4 ligands, was shown to trigger the activity of NFAT5 in macrophages through the IKK- NF-κB pathway (14). These findings suggested that NFAT5 might play an important role in not only innate immune but also autoimmune rheumatic disorders. However, it is still not clear whether the TLR4 pathway induces NFAT5 activation in RASFs. Additionally, no study has assessed the role of LTF in regulation of NFAT5 activity via TLR4.

The present study aimed to investigate the function of LTF as an endogenous ligand for TLR4 in the inflammatory responses of RASFs.

Methods

Cell culture

Synovial fibroblasts from patients with RA were purchased from Articular Engineering (IL, USA). These patients fulfilled the American College of Rheumatology 1987 criteria for RA (15). RASF cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (PSN). RASF of passage 4–8 were used in the experiments. RASF were serum-starved (0.5% FCS) for stimulation experiments.

Reagents and stimulation assays

Cultured RASFs were grown in 6-well culture plates (1×10^5 RASFs/well). RASFs were cultured with 50μg/ml recombinant neutrophil-derived LTF (Sigma-Aldrich Japan, Tokyo, Japan) for 24 and 48 h in screening of cytokine production. In dose-response experi-

ment, RASFs were stimulated with 0, 10, 50, and 100μg/ml LTF for 24 h or 48 h. The mRNA expressions of cytokines and chemokines in RASFs were evaluated at 24 h of different levels of LTF stimulation. The amounts of cytokines and chemokines proteins in culture medium of RASFs were measured at 48 h of different levels of LTF stimulation. To investigate the synergistic effect, RASFs were cultured with 50μg/ml LTF with/without 10 ng/ml of TNF-α (R&D Systems, Minneapolis, MN, USA) for 24 h. The culture supernatants were collected and maintained at -80°C for a multi-cytokine assay. RASFs were treated with 1.0 μM TAK242 (Cosmobio, Tokyo, Japan) to inhibit TLR4 signalling or with DMSO (control) for 1 h before addition of LTF. Additionally, RASFs were treated with 10 μM BMS-345541 (Sigma-Aldrich Japan) to inhibit NF-κB signalling or with DMSO (control) for 1 h before addition of LTF. Moreover, RASFs were treated with 10 μM cerulenin (Sigma-Aldrich Japan) to inhibit the activity of NF-κB -NFAT5 enhanceosome or with ethanol for 1 h before addition of 50μg/ml LTF or 100ng/ml lipopolysaccharide (LPS) purified from *Pseudomonas aeruginosa* (Sigma-Aldrich Japan, Tokyo, Japan). Then, RASFs were stimulated with LTF or LPS for 24h. All reagents were tested for endotoxins, and the endotoxin levels did not exceed 0.1 endotoxin units/ml (detection limit) in the tested samples.

Multi-cytokine assay

To measure the levels of inflammatory cytokines in cell culture supernatants, the multi-cytokine assay system MAGPIX (Merck Millipore Co., Darmstadt, Germany) was used, according to the manufacturer's instructions. Twenty-five cytokines related to differentiation and activation of Th1, Th2 and Th17 cells were measured in this assay. In addition, the concentrations of IL-6, CCL20 and IL-8 in culture supernatant of RASF stimulated with LTF were also measured using MAGPIX.

Western blotting

Cultured RASFs were lysed in sample buffer (62.5 mM TrisHCl, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 5 mM β-mercaptoethanol) after collecting the culture medium. Whole cell lysates were separated on 10% SDS polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, UK). Membranes were blocked for 1 h in 5% (w/v) non-fat milk in TBST (20 mM Tris base, 137 mM sodium chloride, 0.1% Tween 20, pH 7.6). After blocking, the membranes were probed with antibodies against NFAT5 (Santa Cruz Biotechnology, TX, USA) or α-tubulin (Abcam, Cambridge, UK) at 4°C overnight. HRP-conjugated goat anti-rabbit or goat anti-mouse antibodies (Cell Signaling Technology Japan, Tokyo, Japan) were used as secondary antibodies. Signals were detected using ECL Western blotting detection reagents (GE Healthcare) and LAS3000 (Fujifilm, Tokyo, Japan). Protein expression was analysed through pixel quantification of electronic images using Image J software (National Institutes of Health, Bethesda, MD, USA).

Reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR)

Total RNA from RASFs was isolated using TRIZOL Reagent (Invitrogen, CA, USA). RNA (100 ng) was reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen). TaqMan real-time PCR was performed using Light Cycler 2.0 (Roche Diagnostics, Mannheim, Germany). The primer and probe sequences were as follows: NFAT5 primer: forward 5'-GGAGGCACAATGAACCAACTG-3' and reverse 5'-CAGAGGTTAAAAGCTGACTACAGTTA-3', NFAT5 probe: 5'-ATTCTCCTGGCTCATCTCAGCAGACATCAG-3'; IL-6 primer: forward 5'-AGTCCTGATCCAGTTCCTGCA-3' and reverse 5'-CTTCGTCAGCAGGCTGGC-3', IL-6 probe: 5'-ATGCAATAACCACCCCTGACCCAACCAC-3'; CCL20 primer: forward 5'-TGTGACATCAATGCTATCATCTTTC-3' and reverse 5'-GACGCACAATATATTTCACCCAAG-3', CCL20 probe: 5'-AGTTGTCTGTGTGCGCAAATCCAAAACA-3'; IL-8 primer: forward 5'-CACTGTGTGTAAACATGACTTCCAA-3' and reverse 5'-TGGCAAAACTGCACCTTCACA-3', IL-8 probe: 5'-CGTGGCTCTCTTGGCAGCCTTCCTG-3'.

The data were analysed using the comparative C_t method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was measured as an endogenous control.

Transfection of small interfering RNA (siRNA)

RASFs were transfected with 40 nM small interfering RNA (siRNA) targeting NFAT5 or AllStars Negative Control siRNA as a scrambled control (Qiagen, Hilden, Germany) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cell culture medium was replaced after transfection of siRNA for 24h. Then, siRNA-transfected RASFs were treated with or without LTF for 24h. The expressions of NFAT5, IL-6, CCL20 and IL-8 mRNAs in RASFs were analysed using RT-qPCR and/or Western blot analysis.

Statistical analysis

For the statistical analysis, GraphPad Prism 6 v. 6.05 (GraphPad Prism Software, San Diego, CA, USA) was used. Each experiment was performed at least 5 times independently. Differences between various groups were evaluated using the paired *t*-test. The data are presented as mean ± SEM. A p-value <0.05 was considered statistically significant.

Results

LTF induced the production of inflammatory cytokines in RASFs

To investigate whether LTF induced the inflammatory responses of RASFs, a multi-cytokine assay of 25 inflammatory cytokines was performed (Fig. 1A). The amount of IL-6 and CCL20 in the culture medium of RASFs increased on treatment with LTF (Fig. 1B-C). The mRNA expressions of inflammatory cytokines, such as IL-6, CCL20 and IL-8, increased in RASFs at 24 h of LTF stimulation in a dose-dependent manner (Fig. 2A-C). In addition, the amount of IL-6, CCL20, and IL-8 in culture medium of RASFs also increased at 48h of LTF stimulation in a dose-dependent manner (Fig. 2D-F). Both mRNA expressions and protein of inflammatory cytokines in RASFs in-

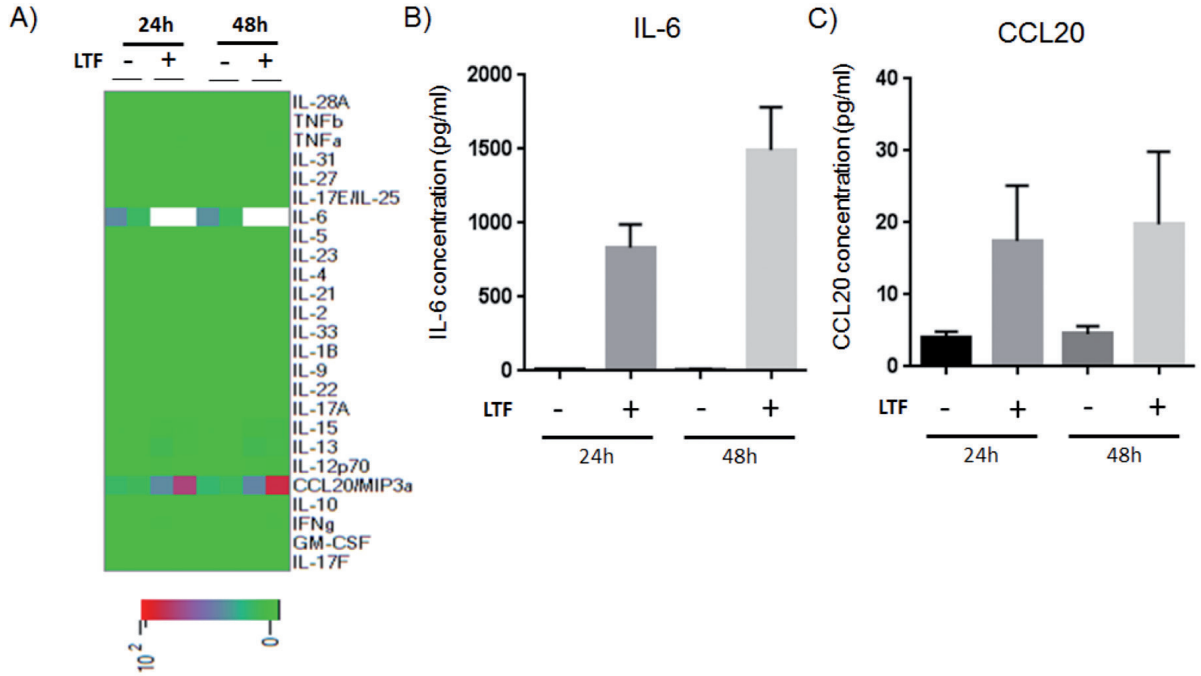

Fig. 1. Cytokine production induced by lactoferrin (LTF) stimulation.
A: Rheumatoid arthritis synovial fibroblasts (RASFs) treated with 50 μg/ml LTF for 24 and 48 h. Twenty-five inflammatory cytokines and chemokines associated with Th1, Th2 and Th17 differentiation in culture supernatants of RASFs treated with LTF were measured using a multi-cytokine assay system. The heat-map indicates the levels of cytokines and chemokines. Green indicates an undetectable protein level. Red and white indicate high levels of proteins.
B-C: Levels of IL-6 and CCL20 in the culture medium of RASFs treated with LTF for 24 h and 48 h (n=2, independent experiments).

creased after LTF stimulation in a dose-dependent manner.

LTF enhanced the expressions of inflammatory cytokines in RASFs stimulated by TNF-α

High local levels of inflammatory cytokines, such as TNF-α and IL-6, have been reported in RA synovial fluid. These cytokines induce the secretion of chemokines, including CCL20 and IL-8, in RASFs (16). To reproduce the inflammatory situation in the RA synovium, we investigated whether LTF enhances the expressions of inflammatory cytokines in RASFs in the presence of TNF-α. RASFs were pre-treated with increasing concentrations of TNF-α (10 ng/ml) with or without LTF. TNF-α increased the expressions of IL-6, CCL20 and IL-8 mRNAs in RASFs. LTF enhanced the expressions of IL-6, CCL20 and IL-8 mRNAs in RASFs stimulated by TNF-α (Fig. 3A-C).

The expressions of IL-6, CCL20 and IL-8 mRNAs in RASFs via the LTF-TLR4 signalling pathway

LTF is considered to be one of the TLR4 ligands (9). We investigated whether TAK242, a specific TLR4 inhibitor, could repress the mRNA expressions of inflammatory cytokines in RASFs induced by LTF stimulation. RASFs were pre-incubated with 1.0 μM TAK242 for 1h. Then, RASFs were treated with 50 μg/ml LTF for 24h. TAK242 markedly inhibited the expressions of IL-6, CCL20 and IL-8 mRNAs in RASFs stimulated by LTF (Fig. 4A-C). There was no cytotoxic effect of 1.0 μM TAK242 on RASFs in this experiment (data not shown). LTF may play a role as endogenous ligand for TLR4 expressed on RASFs. In addition, to clarify the signalling pathway of TLR4 associated with the expressions of inflammatory cytokines, we examined whether activation of NF-κB was functionally linked to LTF-induced expressions of IL-6, CCL20 and IL-8 in RASFs. RASFs were treated with 10 μM BMS345541, a chemical inhibitor of IkB kinase, which acts up-stream of NF-κB nuclear translocation. As shown in Figure 4, the NF-κB inhibitor BMS345541 partially reduced the expressions of IL-6 and IL-8 induced by LTF (Fig. 4A, C). On the other hand, NF-κB inhibitor did not repress the increasing expression of CCL20 mRNA in RASFs stimulated by LTF (Fig. 4B).

NFAT5-p65 complex enhanceosome played a role in inflammatory responses of RASFs via LTF-TLR4 signalling

TLR4 signalling pathways play an important role in innate immune responses. LPS stimulates macrophages via the activated transcription factor NFAT5 (14). Therefore, we focused on the role of NFAT5 in inflammatory responses of RASFs via the LTF-TLR4 signalling pathway (Fig. 5). Silencing of NFAT5 decreased the expressions of NFAT5 mRNA and protein in RASFs (Fig. 5A-B). The optical density of immune blotting bands of the NFAT5 protein significantly decreased ($p<0.01$, Fig. 5C). Silencing of NFAT5 repressed the expressions of CCL20 and IL-8, but not IL-6 mRNA in RASFs stimulated by LTF (Fig. 5D-F). Some reports indicated that there is an interaction between NF-κB p65 and NFAT5 for the induction of the expression of the target genes of these transcription factors. Cerulenin is an inhibitor of fatty acid synthase, which is isolated from *Cephalosporium caeru-*

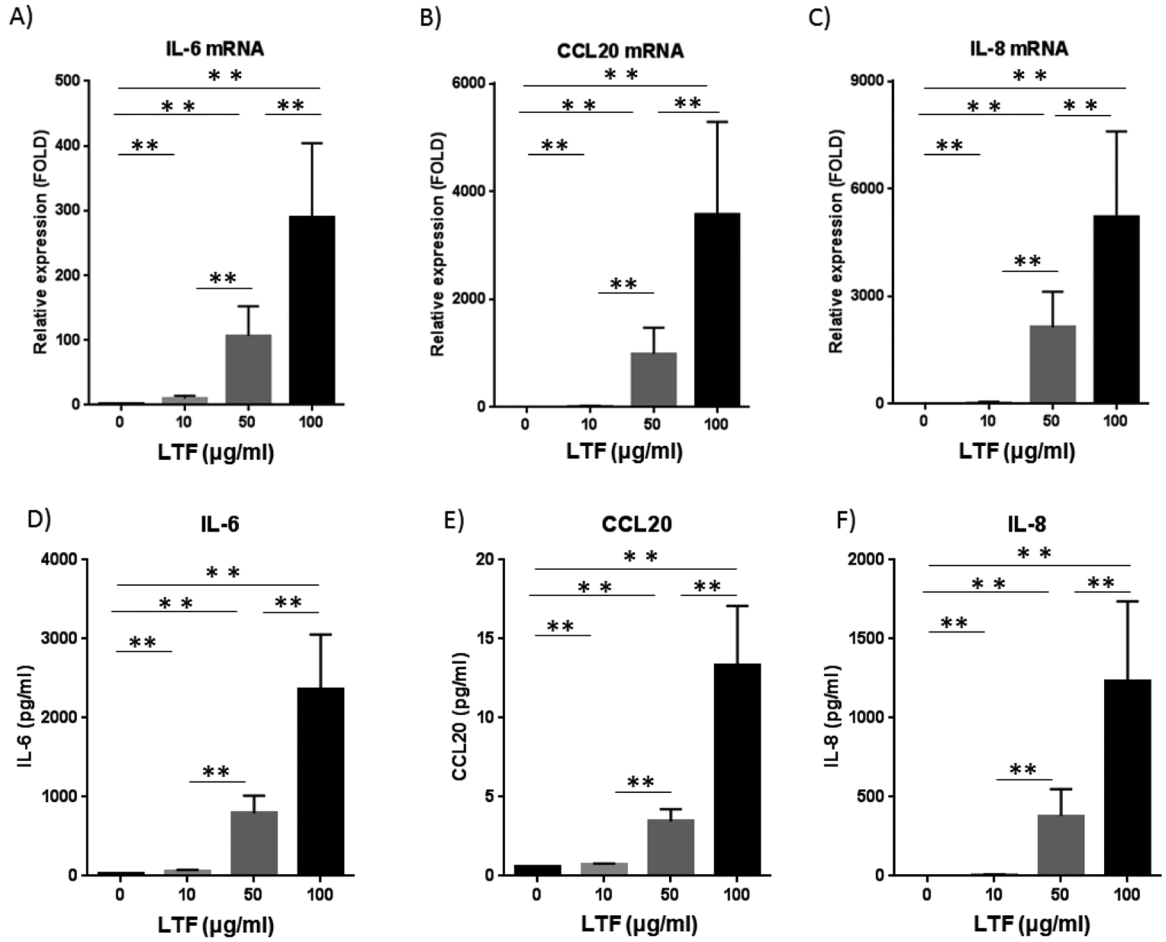

Fig. 2. Expressions of inflammatory cytokine and chemokine in rheumatoid arthritis synovial fibroblasts (RASFs) treated with different levels of lactoferrin (LTF). **A-C:** RASFs were stimulated with different levels of LTF (10, 50 and 100 μg/ml) for 24 h. LTF increases the expressions of IL-6, CCL20 and IL-8 mRNAs in RASFs, in a dose-dependent manner (n=8, independent experiments). **D-F:** RASFs were stimulated with different levels of LTF (10, 50 and 100 μg/ml) for 48 h. LTF increases the amounts of IL-6, CCL20 and IL-8 protein in culture mediums of RASFs, in a dose-dependent manner (n=10, independent experiments). Results are shown as mean ± SEM. **p<0.01, significantly different.

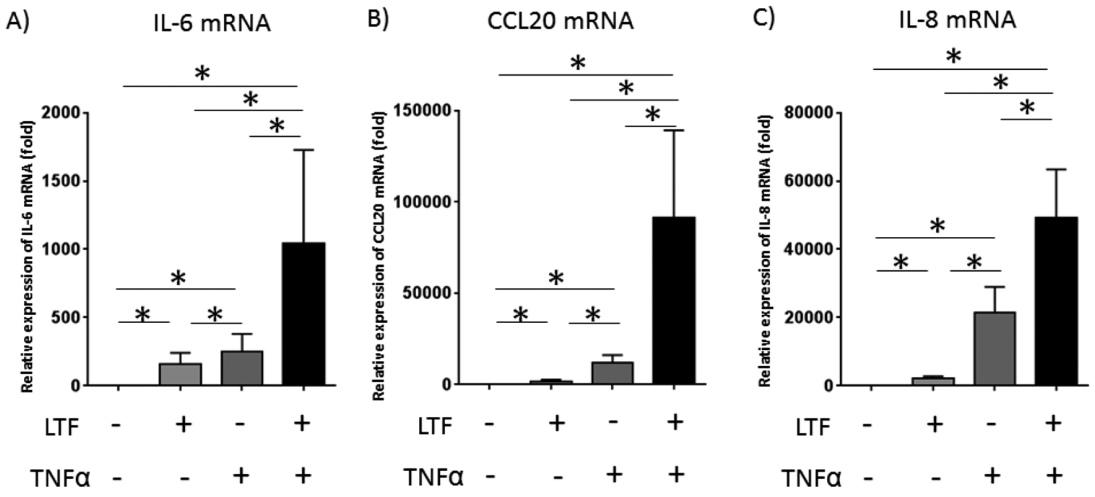

Fig 3. Expressions of inflammatory cytokine and chemokine mRNAs in rheumatoid arthritis synovial fibroblasts (RASFs) treated with lactoferrin (LTF) with or without TNF-α stimulation. **A-C:** Expressions of IL-6, CCL20 and IL-8 mRNAs in RASFs treated with 50 μg/ml LTF with or without 10 ng/ml TNF-α for 24 h (n=6, independent experiments). Results are shown as mean ± SEM. *p<0.05, significantly different.

lens as an antibiotic agent (17). A recent study demonstrated that cerulenin disrupted the p65-NFAT5-p300 interaction and inhibited the activity of NF-κB (18). Therefore, we characterised cerulenin action in LTF-TLR4 signalling of activated RASFs in this experiment. Expectedly, cerulenin repressed the expressions of IL-6, CCL20 and IL-8 mRNAs in RASFs treated with LTF or LPS after 24h (Fig. 5G-I).

Discussion

In the present study, we demonstrated for the first time that neutrophil-derived LTF

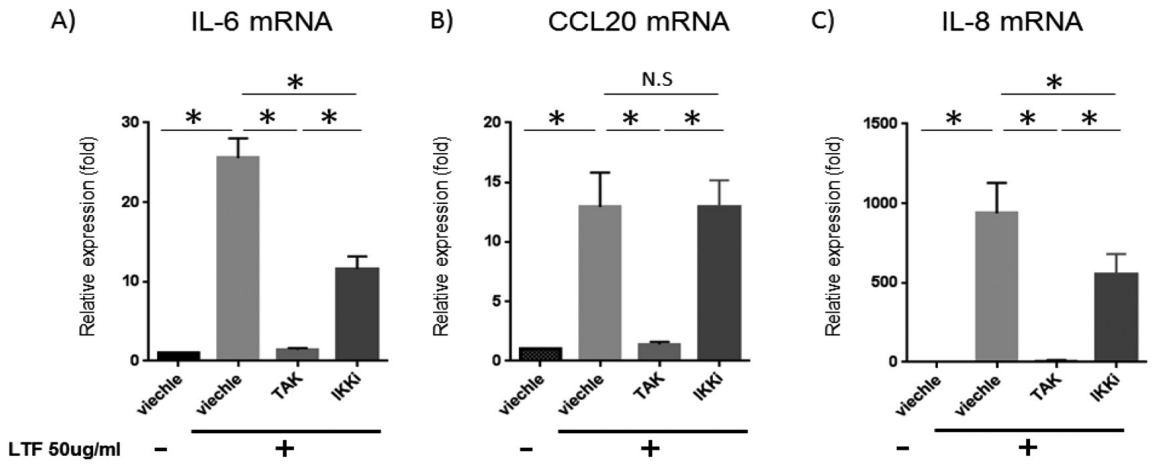

Fig. 4. Effects of a Toll-like receptor (TLR) 4 inhibitor (TAK242) and NF-κB inhibitor (BMS345541) on the expressions of cytokines and chemokines in rheumatoid arthritis synovial fibroblasts (RASFs) stimulated by lactoferrin (LTF). **A-C:** Expressions of IL-6, CCL20 and IL-8 mRNAs in RASFs stimulated (+) or unstimulated (-) with 50 μg/ml LTF for 24 h after 1 h of pre-treatment with a specific inhibitor (n=5, independent experiments). The Toll-like receptor-4 inhibitor TAK242 (TAK) and IKK-NF-B inhibitor BMS-345541 (IKKi) were used in the experiments. DMSO was used as a vehicle. Results are shown as mean ± SEM. *p<0.01, significantly different. NS: not significant.

acts as a ligand for TLR4 expressed on RASFs. Numerous activated neutrophils recruited into RA inflamed joints release pro-inflammatory mediators (19). LTF is present in the secondary granules of neutrophils. Therefore, activated neutrophils release not only inflammatory mediators but also LTF in RA joints. Some reports demonstrated that the level of LTF is higher in the synovial fluid of RA than in that of OA (7, 8). LTF exerts several biological effects, including antimicrobial, immunoregulatory and anti-inflammatory effects (20). In the present study, we demonstrated that neutrophil-derived LTF induced the expression of inflammatory cytokines and chemokines in RASFs. The level of LTF has been reported to be well correlated with the levels of IL-6, IL-8 and TNF in the inflammatory synovial fluid of RA (6). LTF synergistically enhanced the expressions of these cytokines in RASFs stimulated by TNF-α in this study. TNF-α has been shown to induce the expression of LTF in activated neutrophils in RA inflamed joints (8). Our results suggested that neutrophil-derived LTF contributes to the worsening inflammatory responses of RASFs, instead of the malignant loop of inflammation in RA. In other words, LTF may play a role as an inflammatory mediator in the malignant loop of inflammation in RA.

The activities of IL-8 include chemotactic attraction and activation of neutrophils. IL-8 contributes to the recruitment of activated neutrophils into RA inflamed joints. The newly characterised IL-17-secreting helper T cell, named Th17, plays a pathogenic role in autoimmune diseases, including RA (21). Strong enhanced and specific expression of CCR6 in human Th17 cells has been demonstrated (22). Its ligand CCL20 is expressed in the rheumatoid synovium (23). IL-6 also plays an important role in the differentiation of Th17 from naive T cells. Increased production of cytokines and chemokines from RASFs by LTF may drive the recruitment of white blood cells, such as neutrophils and Th17 cells, in RA inflamed joints. Therefore, our data suggested that neutrophil-derived LTF contributes to the creation of an inflammatory environment favouring neutrophil and lymphocyte migration and sequestration to perpetuate rheumatoid inflammation.

The recent identification of endogenous ligands for several different TLRs has generated great interest because of their potential importance for autoimmunity (24). Several DAMPs have been already identified as endogenous TLR4 activators in RA synovial fluid (25). TAK242, a small molecular inhibitor for TLR4 signalling, markedly repressed the inflammatory responses of RASFs stimulated by LTF in the present study. Therefore, neutrophil-derived LTF was shown to act as an endogenous ligand for TLR4 expressed on RASFs.

The present study demonstrated that different mechanisms regulate the expressions of IL-6, IL-8 and CCL20 in RASFs stimulated by LTF. TAK242 binds selectively to TLR4 and subsequently disrupts the interaction of TLR4 with adaptor molecules, such as Toll/interleukin-1 receptor domain-containing adaptor protein (TIRAP) and Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon-β-related adaptor molecule (TRAM) (26). The activation of these adaptor molecules activates downstream NF-B signalling pathways and subsequently triggers inflammatory mediator production (27, 28). NF-κB is also known as a master regulator down-stream of TLR4 signalling in RASFs. TLR4 activators induce both the phosphorylation and shuttling of NF-κB in RASFs (29). Therefore, we expected involvement of TIRAP- and TRAM-mediated activation of NF-κB in the expression of inflammatory cytokines via LTF-TLR4 signalling in RASFs. The NF-κB inhibitor decreased the expressions of IL-6 and IL8, but not completely. However, the NF-κB inhibitor could not repress the expression of CCL20 mRNA induced by LTF stimulation. These results suggested that the expressions of IL-6, IL-8 and CCL20 mRNAs via the LTF-TLR4 signalling pathway might not be influenced by NF-κB in RASFs. Therefore, we focused on the novel transcription factor NFAT5 in the pre-

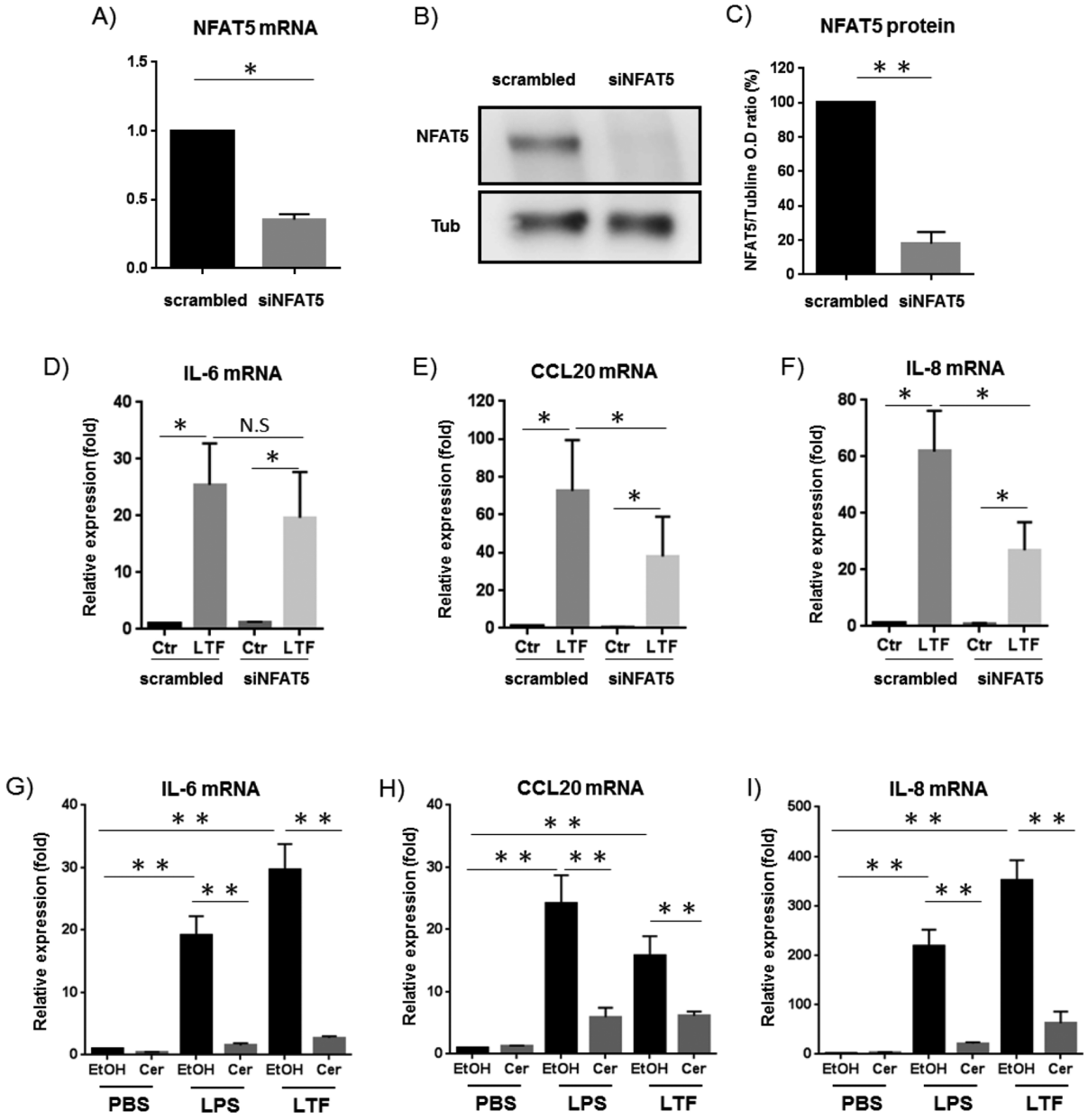

Fig. 5. Role of NFAT5 in the expressions of cytokines and chemokines in rheumatoid arthritis synovial fibroblasts (RASFs) stimulated by lactoferrin (LTF). **A-C:** Expressions of NFAT5 mRNA and protein in RASFs after silencing of NFAT5 (siNFAT5) (n=6). Optical density (OD) ratio (NFAT5/Tubline) was measured using Image J software. **D-F:** Expressions of IL-6, CCL20 and IL-8 mRNAs in RASFs treated with 50 μg/ml LTF with or without silencing of NFAT5 (n=6, independent experiments). **G-I:** Expressions of IL-6, CCL20 and IL-8 mRNAs in RASFs pre-treated with or without 10 μM cerulenin (Cer) induced by lipopolysaccharide (LPS) or LTF after 24 h (n=4, independent experiments). Ethanol (EtOH) was used as a vehicle.
Results are shown as mean ± SEM. *p<0.05 and **p<0.01, significantly different. NS: not significant.

sent study. NFAT5 was originally identified as a tonicity-regulated transcription factor involved in the protection of cells from hypertonic stress (30, 31). The expressions of IL-6 and Nos2 were shown to be regulated by NFAT5 in macrophages in response to LPS (14). Furthermore, pro-inflammatory stimuli can activate NFAT5 in an osmotic stress-independent manner, concomitantly triggering several other downstream signalling pathways, including NF-κB (13, 14). These findings suggested that NFAT5 is one of the critical regulators of RASFs in inflammatory responses. The expressions of CCL20 and IL-8, but not IL-6 mRNA induced by LTF were repressed by silencing of NFAT5 in RASFs. On the other hand, our findings demonstrated that the NF-κB inhibitor partially repressed the expressions of IL-6 and IL-8 in RASFs induced by LTF-TLR4 signalling. The NF-κB inhibitor did not repress the expression of CCL20 in RASFs stimulated by LTF. Thus, the expression of CCL20 appears to be dependent on the activity of NFAT5 in the LTF-TLR4 pathway in RASFs. These results suggested that NF-κB and NFAT5 may partially regulate the expression of these cytokines and chemokines. NFAT5 and NF-κB transcription factors have similar DNA binding mechanisms and share enhancer elements in response to different cellular signals (33). It has been shown

that NF-κB activity is enhanced after binding of NF-κB-NFAT5 complexes to the κB elements of NF-κB -responsive genes (34). A recent report demonstrated that the LPS-induced NF-κB enhanceosome requires NFAT5 without DNA binding. NFAT5 can interact with NF-κB and co-activator p300 (18). The shuttling of NFAT5 to the nucleus of a RASF after LTF stimulation may therefore amplify the activation of NF-κB. In other words, NFAT5 enhances the gene expression cooperatively with the activity of NF-κB p65. The present study demonstrated that cerulenin, which disrupts the NF-κB -NFAT5 enhanceosome, inhibited the gene expressions of IL-6, CCL20 and IL-8 in RASFs stimulated by LTF. These results suggested that NFAT5-NF-κB enhanceosome regulates the expression of LTF-TLR4 responsive genes in RASFs. Inflammatory gene expression induced by LTF-TLR4 signalling in RASFs requires the transcription factor NFAT5.

In conclusion, we demonstrated that neutrophil-derived LTF acts as an endogenous ligand for TLR4 expressed on RASFs. In the TLR4 signalling pathways of RASFs, NFAT5 plays an important role in the expressions of pro-inflammatory cytokines and chemokines. The interaction between NFAT5 and the p65 enhanceosome plays a critical role in the inflammatory responses of RASFs via the LTF-TLR4 signalling pathway. Thus, insights into LTF-TLR4 signalling and regulation of the enhanceosome in RASFs are of particular interest for understanding the pathogenesis and treatment of RA.

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