Type I interferons might form the link between Toll-like receptor (TLR) 3/7 and TLR4-mediated synovial inflammation in rheumatoid arthritis (RA)

Roelofs, M F; Wenink, M H; Brentano, F; Abdollahi-Roodsaz, S; Oppers-Walgreen, B; Barrera, P; van Riel, P L C M; Joosten, L A B; Kyburz, D; van den Berg, W B; Radstake, T R D J (2009). Type I interferons might form the link between Toll-like receptor (TLR) 3/7 and TLR4-mediated synovial inflammation in rheumatoid arthritis (RA). Annals of the Rheumatic Diseases, 68(9):1486-1493.

Postprint available at: http://www.zora.uzh.ch

Posted at the Zurich Open Repository and Archive, University of Zurich. http://www.zora.uzh.ch

Type I interferons might form the link between Toll-like receptor (TLR) 3/7 and TLR4-mediated synovial inflammation in rheumatoid arthritis (RA)

Abstract

BACKGROUND: Rheumatoid arthritis (RA) has been associated with an increased risk of infections, but the underlying pathways have not yet been identified. Toll-like receptors (TLR) probably play a role in synovial inflammation and may also contribute to the understanding of the role of infections in RA.

OBJECTIVES: To investigate if the synovial expression of TLR3 and TLR7 in RA correlates with that of inflammatory cytokines, and to assess whether this has functional consequences for local cytokine production and to study potential links between the TLR3/7 axis and TLR4 in RA synovium.

METHODS: Immunohistochemistry was used to study the expression of TLR3, TLR7, interferon alpha (IFNalpha), tumour necrosis factor alpha (TNFalpha) and interleukins IL1beta, IL12, IL17 and IL18 in RA synovium obtained by arthroscopy from 34 patients with RA. Monocytes, monocyte-derived dendritic cells (MoDCs) and RA synovial fibroblasts were stimulated via TLR3 (poly-IC) and TLR7 (loxorubin), after which IL1beta, IL6 and TNFalpha were measured by Luminex bead array technology. Following preincubation with IFNalpha, IL1beta and IL18, TLR3 and TLR7 mRNA expression was assessed using real-time PCR. Cytokine production after preincubation with IFNalpha and subsequent TLR stimulation was measured. RESULTS: Synovial TLR3/7 expression was co-expressed with IFNalpha, IL1beta and IL18, but not with TNFalpha, IL12 and IL17. Stimulation of TLR3/TLR7 on monocytes, MoDCs or synovial fibroblasts led to secretion of type I IFN but no biologically active IL1beta or IL18 could be detected. Type I IFNalpha increased TLR3/7 mRNA expression whereas IL1beta and IL18 did not. In spite of the fact that the mRNA level of TLR4 remained unchanged, IFNalpha enhanced the response to TLR4 agonists, a phenomenon that was clearly more marked in patients with RA.

CONCLUSION: Type I interferons are highly co-expressed with TLR3/TLR7 in RA synovium. They enhance TLR3/TLR7-mediated cytokine production and also TLR4-mediated responses.
Type I interferons might form the link between Toll-like receptor (TLR) 3/7 and TLR4 mediated synovial inflammation in rheumatoid arthritis (RA).

M.F. Roelofs, PhD¹; M.H. Wenink, MD¹; F. Brentano, PhD²; S. Abdollahi-Roodsaz, PhD¹; B. Oppers-Walgreen, MSc¹; P. Barrera, MD, PhD¹; P.L.C.M. van Riel, MD, PhD¹; L.A.B. Joosten, PhD¹; D. Kyburz, MD, PhD²; W.B. van den Berg, PhD¹; T.R.D.J. Radstake, MD, PhD¹.

Affiliations
¹Department of Rheumatology, Radboud University Nijmegen Medical Center, the Netherlands; ²Center of Experimental Rheumatology, University Hospital, Zurich, Switzerland,

T.R. was supported by a VENI and VIDI laureate of the Netherlands organization for scientific research (NWO).

Corresponding Author
T.R.D.J. Radstake, MD, PhD.
Department of Rheumatology
Radboud University Nijmegen Medical Center
Geert Grooteplein 8
6500 HB Nijmegen, The Netherlands

Temporary Address
The Arthritis Center
715 Albany street, MA 02118-2526
Boston, United States of America

Keywords: Toll-like receptors, rheumatoid arthritis, Type I interferons
Abstract

Rationale: Toll-like receptors (TLR) are likely to play a role in synovial inflammation. Rheumatoid arthritis had been associated with infections but the underlying pathways have never been identified. A hypothetical link between various TLR pathways would contribute to our understanding on the role of infections in RA.

Objective: To investigate if the increased synovial expression of Toll-like receptor (TLR) 3 and TLR7 in RA correlates with inflammatory cytokines, whether this has functional consequences for cytokine production and to study a potential link between TLR3/7 axis and TLR4 in RA synovium.

Methods: Immunohistochemistry was used to study the expression of TLR3, TLR7, IFNα, TNFα, IL-1β, IL-12, IL-17 and IL-18 in RA synovium from 34 RA patients obtained via arthroscopy. Monocytes, monocyte-derived dendritic cells and RA synovial fibroblasts were stimulated via TLR3 (Poly-IC) and TLR7 (loxorubicin) after which IL-1β, IL-6 and TNFα was measured by Luminex bead array technology. Upon pre-incubation with IFNα, IL-1β and IL-18 TLR3 and TLR7 mRNA expression upon was assessed using real-time PCR. Cytokine production after pre-incubation with IFNα and subsequent TLR stimulation was measured.

Results: Synovial TLR3/7 expression was co-expressed with IFNα, IL-1β and IL-18, but not with TNFα, IL-12 and IL-17. Stimulation of TLR3/TLR7 on monocytes, MoDC or synovial fibroblasts led to secretion of type I IFN whereas no biologically active IL-1β or IL-18 could be detected. Type I IFN was able to increase TLR3/7 mRNA expression whereas IL-1β and IL-18 was not. Strikingly, despite an unchanged mRNA level of TLR4, IFNα led to an augmented response to TLR4 agonists, a phenomenon that was clearly enhanced in RA patients.

Conclusion: Here we demonstrate that the type I interferons are highly co-expressed with TLR3/TLR7 in RA synovium. Furthermore, we provide clear evidence for the functional role of type I interferons in enhancing not only TLR3/TLR7 mediated cytokine production but also augmenting TLR4 mediated responses.
Introduction
Rheumatoid arthritis (RA) is a systemic autoimmune disease, which is characterized by chronic inflammation of the synovial joints. Although the cause of RA is still unidentified, a role both for genetic and environmental factors has been advocated repeatedly throughout time. As environmental factors, the encounter of bacteria and/or viruses by the host was suggested to trigger autoimmunity. Indeed, evidence for the presence of at least some viruses including Cytomegalovirus (1, 2), Epstein-Barr virus (3) and Parvovirus B19 (4) was demonstrated. In line with these observations, it was demonstrated that dsRNA, which is a common feature of viruses, exerts clear arthrogenic properties (5), further substantiated the potential role of viruses in RA. Although recent studies demonstrated a type I interferon (IFN) signature in the RA synovium the underlying pathways that explains the role for type I IFN remains elusive (6).

Accumulating evidence suggests a role for the Toll-like receptor (TLR) family in the type I IFN mediated response. TLR belong to the family of pattern-recognition receptors (PRR), which were first identified to recognize microbial components, known as pathogen-associated patterns (PAMPs). TLR are constitutively expressed by numerous immune cells and designed to detect and eliminate invading pathogens by activation of both innate as well as adaptive immune responses. TLR3 and 7 serve as receptors for double stranded and single stranded (viral) RNA respectively and TLR9 serves as a receptor for unmethylated CpG motifs in (viral / bacterial) DNA. Therefore they have a key role in antiviral immunity by inducing type I IFN production (7-9). In contrast, TLR2 and TLR4 elicit immune responses upon binding of antigens from bacterial origin and host-derived molecules (so-called endogenous ligands or alarmins) ending up in the production inflammatory mediators including TNF-α and IL-1β (10-13). The increased expression of various TLR subtypes in the synovial tissue of rheumatoid arthritis patients further substantiates the potential role of TLR in RA. More recently, the role of TLR in the inflammatory cascade of arthritis was highlighted in experimental models of arthritis (14, 15). Although TLR and its ligands are abundantly present in the synovial compartment of RA patients, it is hard to conceive that any trigger of a single TLR subtype would be sufficient to convert tolerance to immunity. If such, then autoimmunity should regularly follow from infections. It is therefore more likely that more then one TLR ligand is needed for the
initiation of a chronic and persisting inflammatory response as seen during RA. Accordingly, recent research suggested that simultaneous or sequential triggering of different pathways is perhaps the event that sets off autoimmunity (16). In this light, it is tantalizing to speculate that simultaneous triggering of different TLR pathways might initiate a series of events that forms the basis of the breakthrough of tolerance. Previous evidence from our group demonstrated a synergistic effect with regard to cytokine production when stimulation of two (or more) TLR subtypes was achieved on dendritic cells. Here we sought evidence for a potential link between TLR2/4 and TLR3/7 pathways in RA inflammation.

Here we demonstrate that IL-1β and IL-18, IFNα (the classic type I interferon) are co-expressed with TLR3/7 in RA synovium. In addition, we provide evidence that type I IFN increase the expression of TLR3 and TLR7, but not TLR4, leading to an increased production of cytokines upon TLR3/TLR7 specific triggering. Interestingly, type I IFN also induced a clear augmentation of TLR4 mediated production of pro-inflammatory mediators including IL-6, IL-1β and TNFα, which was clearly more potent in RA. These data illustrate that TLR3/TLR7 stimulation indirectly lowers the threshold for TLR4 mediated immune activation setting the stage for the vicious circle of inflammation as observed in RA. Altogether, these observations underscore the potential role of viruses in RA and provide rationale for interference with TLR signalling in RA.
Methods

Patients / study population

For immunohistochemistry, synovial biopsies from RA patients (n=34) were obtained, using small needle arthroscopy, from the medial and lateral supra-patellar pouch on each occasion. The local Medical Ethics Committee approved the study protocol. For in vitro experiments, heparinized venous blood was collected from 10 RA patients and 11 healthy volunteers. Patients were attending the department of Rheumatology of the Radboud University Nijmegen Medical Centre, fulfilled the American College of Rheumatology criteria for and they all gave their informed consent (17). Patients using high dose prednisolone (>10mg/day) or anti-cytokine therapies (anti-TNFα and/or IL-1Ra) were excluded from this study. The local Medical Ethics Committee approved the study protocol.

Immunohistochemical staining of synovial biopsies.

Tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. Staining of IL-1β, IL-12, IL-17, IL-18 and TNFα was performed as described previously (18). For TLR3, TLR7 and IFNα staining, sections were incubated for 60 minutes with antibodies against human TLR3 (T-17), human TLR7 (V-20) or IFNα (FL-198), which were all obtained from Santa Cruz, California, USA. After this, endogenous peroxidase was blocked with 3% H2O2 in methanol for 15 minutes and subsequently the appropriate biotinylated secondary antibody (mouse anti-goat: Jackson ImmunoResearch, West Grove, PA, USA / swine anti-rabbit: DakoCytomation, Glostrup, Denmark) was incubated for 30 minutes. For TLR3 and TLR7 staining, Vectastain® ABC (Vector Laboratories, Burlingame, CA, USA) reagent was incubated for 30 minutes, developed with DAB (Sigma, St. Louis, MO, USA), and counterstained with hematoxilin for 30 seconds. For IFNα staining, sections were incubated with streptavidin-PO (DakoCytomation, Glostrup, Denmark), developed with DAB (Sigma, St. Louis, MO, USA), and counterstained with hematoxilin for 30 seconds. Staining was semi quantitatively scored on a 5-point scale (scores 0-4) at 200x magnification; a score of 0 represented no or minimal staining, score 1 stands for 10%-20% positive cells, 2 for 30%-40%, 3 for 50%-60%, and a score of 4 represented staining of a more that 60% of the cells.
Isolation and culturing of monocytes and monocyte-derived DC (MoDC).

PBMC were isolated from hepariniz ed venous blood using density gradient centrifugation over Ficoll-Paque (Amersham Biosciences, Roosendaal, The Netherlands). Low-density cells were collected and washed with citrated PBS 5% FCS. For monocytes, CD14+ cell fraction was isolated using MACS® cell separation, according to the manufacturer's instructions. Briefly, PBMC were incubated with anti-human CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 minutes, subsequently cells were washed and CD14+ fraction was separated from the CD14- fraction over a MACS® MS separation column (Miltenyi Biotec, Bergisch Gladbach, Germany). CD14+ cell fraction was eluted from the column, washed again and resuspended in a concentration of 0.5x10^6 cells/ml in RPMI 1640 Dutch Modification (Invitrogen Corporation, Carlsbad, CA, USA), supplemented with 10% FCS, plated in 6-well plates and O/N cultured at 37°C and 5% CO2.

For monocyte-derived dendritic cells, PBMC were allowed to adhere for 1 hour at 37°C in RPMI-1640 Dutch Modification (Invitrogen Corporation, Carlsbad, CA, USA supplemented with 2% human serum (PAA Laboratories, Pasching, Austria) in 25cm² cell culture flasks (Corning Incorporated, NY, USA). Adherent monocytes were cultured in RPMI-1640 Dutch modification supplemented with 10% fetal calf serum (FCS) and antibiotic-antimycotic (Life Technologies) in the presence of IL-4 (500 U/ml, Schering-Plough, Amstelveen, The Netherlands) and GM-CSF (800 U/ml, Schering-Plough, Amstelveen, The Netherlands) for 6 days. Fresh culture medium with the same supplements was added at day 3 where after immature DC were harvested at day 6. Immature DC were resuspended in fresh cytokine-containing culture medium, transferred to 6-well culture plates (Corning Incorporated, NY, USA) in a concentration of 0.5x10^6 cells/ml and cultured for 16 hours at 37°C and 5% CO2.

Isolation and culturing of RA synovial fibroblasts.

Immediately after surgery, the synovial tissue was minced and digested with Dispase at 37°C for 60 minutes. After washing, the cells were grown in Dulbecco's minimum essential medium (Gibco Invitrogen, Basel, Switzerland) supplemented with 10% FCS, 50 IU/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, and 0.2% Fungizone (all from Gibco Invitrogen). Cell cultures were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO2. For the experiments, cultured
synovial fibroblasts, between passages 4 and 8, were grown in 12-well culture plates (6 × 10⁴ RASF/well) and cultured at for 16 hours 37°C and 5% CO₂.

**Stimulation of monocytes, MoDC and RA synovial fibroblasts.**

To study TLR mRNA expression upon cytokine stimulation, monocytes (which express TLR7), monocyte-derived DC and RASF (which both express TLR3) were, after 16 hours resting period, stimulated for 8 hours with TNFα, IL-1β, IFNα, IL-12, IL-17, IL-18 (all R&D systems, Minneapolis, MN, USA). Subsequently, culture supernatants were removed and 1ml TRIzol reagent (Sigma St. Louis, MO, USA) was added to the cells and stored at –20°C until RNA isolation was performed.

To study functional up regulation of TLR, monocytes, monocyte-derived DC and RASF were, after an 16 hours resting period, stimulated IFNα with (R&D systems, Minneapolis, MN, USA) for 24 hours and subsequently stimulated with the TLR3 and TLR7 agonists poly(I:C) and loxoribin respectively (both Invivogen, San Diego, USA) or medium. After another 24 hours, culture supernatants were collected and stored at -20°C until cytokine measurement was performed. In addition, to investigate the role of type I IFN various cell types were pre-incubated with 100 U/ml IFNα for 16 hrs.

**RNA isolation and real-time PCR.**

Total RNA was extracted in 1ml of TRIzol reagent (Sigma St. Louis, MO, USA), an improved single-step RNA isolation method based on the method described by Chomczynski et al. (19). Quantitative real-time PCR was performed using the ABI/Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). PCR conditions were as followed: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, with data collection in the last 30 seconds. All PCR were performed with SYBR Green Master mix (Applied Biosystems), 10 ng cDNA, and primer concentration of 300 nmol/L in a total volume of 25 µl. Quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the gene of interest of each sample with the Ct values of the reference gene GAPDH. Primers sequences for gene expression analysis for hGAPDH, hTLR3, hTLR7 and IL-1β are depicted in Table I.
Measurement of cytokines in culture supernatant

TNFα, IL-1β, IL-6 and IL-10, IL-12p70 levels were measured in the supernatant of the cell cultures, using commercially available kits (Bio-Rad Laboratories, Hercules, USA) according to the manufacturer’s instructions (20). Cytokine levels were measured and analyzed with the Bio-Plex system (Bio-Rad Laboratories). Data analysis was done with Bio-Plex Manager software (Bio-Rad Laboratories).

Statistical analysis

Correlations of the expression of TLR and cytokines in human synovial biopsies were calculated using the Pearson Correlation test. Differences in mRNA expression and cytokine production, upon cell stimulation with cytokines and TLR agonists, were calculated using Mann-Whitney U test. P values were two sided and the level of significance was set at P<0.05.
Results

In RA synovium TLR3/7 are co-expressed with IL-1β and IL-18, and IFNα

Synovial biopsies from 24 active RA patients were used to detect TLR3, TLR7 and IFNα expression (18). TLR3/7 was associated with the expression levels of IFNα, IL-1β and IL-18 but not with IL-12, IL-17 and TNFα. Not unanticipated, IFNα was clearly co-expressed with TLR3 and TLR7 in the lining as well as the sublining from synovial biopsies (Table 2). Moreover, both IL-1β and IL-18 were associated with the expression of TLR in the lining although these correlations were weaker than that observed between TLR and type I IFN. In contrast, nor IL1β or IL-18 was associated with either TLR3 or TLR7 expression in the sublining. TNFα, IL-12, or IL-17 were not correlated with the TLR expression levels in RA synovium.

TLR3/7 mediated stimulation of monocytes, dendritic cells or synovial fibroblasts could not explain its co-expression with IL-1β/IL-18.

Since we found co-expression between TLR3/7 and IFNα, IL-1β and IL-18, we further investigated the functional relation between these mediators and TLR3/7. It is generally accepted that stimulation of TLR3/7 leads to the production of type I IFN. In line with this, we found that TLR3/7 stimulation resulted in clearly enhanced IFNβ mRNA and IFNα protein levels (data not shown). In order to investigate the potential relation between TLR3/7 and IL-1β we stimulated TLR3-expressing monocyte-derived DC and synovial fibroblasts with poly(IC) and TLR7-expressing monocytes with loxoribine. All cell types investigated showed markedly increased expression of IL-1β mRNA upon stimulation of either TLR3 or TLR7, however IL-1β protein could not be detected (data not shown). The stimulation of monocytes, monocyte-derived DC or synovial fibroblasts with TLR3/7 did not lead to the secretion of IL-18 proteins.

TLR3/7 expression is regulated by IL-1β, IL-18 and type I IFN.

Since TLR3/7 stimulation leads to the production of IFNα, but not to IL-1β/IL-18, we next investigated whether the co-expression between TLR3/7 and IL-1β/IL-18 could be explained by IL-1β/IL18-induced up regulation of these receptors. To this aim, we cultured monocytes (TLR7-expressing cells), monocyte-derived dendritic cells (TLR3-expressing cells) and RA synovial fibroblasts (TLR3-expressing cells) in the presence of IFNα, IL-1β and IL-18. IFNα did significantly enhance the TLR3 mRNA expression
on RA synovial fibroblasts and monocyte-derived DC, and TLR7 mRNA expression on monocytes (Figure 1). This increase in TLR expression was equal between RA patients (n=5) and healthy volunteers (n=5) and was specific for TLR3 and TLR7, since the expression of TLR2 as well as TLR4 was not altered (data not shown). In contrast, IL-1β and IL-18 had no effect on the expression of TLR3/7 excluding a direct role in their co-expression with TLR3/7.

**IFNα mediated up regulation of TLR3/7 is functional.**

As we demonstrated that expression of TLR3 and TLR7 was strongly up regulated by IFNα, we sought evidence to whether this enhanced TLR expression was functional, in terms of increased TLR-mediated cytokine production. To this aim, we incubated TLR7-expressing monocytes and TLR3-expressing monocyte-derived DC and RA synovial fibroblasts (n=8) with IFNα or medium and subsequently stimulated TLR3 or TLR7 with poly(IC) and loxoribine as appropriate. As anticipated, TLR7 stimulation on monocytes and TLR3 stimulation on monocyte-derived DC and RASF led to production of IL-6 and TNFα, which was significantly enhanced when the cells were pre-incubated with IFNα, compared to cells that were stimulated with TLR3/7 ligands alone (Figure 2). These data underline that the up regulation of TLR3/7 by type I IFN is functional.

**Type I IFN augments TLR4 mediated cytokine production providing the missing link explaining co-expression between TLR3/7 and IL-1/IL-18.**

Since TLR4 triggering is well recognized as a potent inducer of inflammatory mediators including IL-1β and IL-18, we postulated that stimulation of TLR3/7 leading to the production of type I IFN perhaps augments the TLR4 response ending up in the production of IL-1β and IL-18. To test this, we pre-stimulated monocyte-derived DC with IFNα and next stimulated them with the TLR4 agonist lipopolysaccharide (LPS). Surprisingly, pre-stimulation with IFNα resulted in a marked augmentation (4-fold) of TLR4 mediated secretion of TNFα by DC from healthy controls (3044 pg/ml vs. 800 pg/ml, P = 0.01, n=5) (Figure 3). In addition, pre-incubation with type I IFN augmented the TNF production by the combination of TLR3/4 ligands by almost 3-fold (1880 pg/ml vs. 5010 pg/ml, P = < 0.01). Compared to DC from healthy controls, stimulation of DC from RA patients with TLR4 led to a clear augmented TNF...
production as previously described (22). Notably, DC obtained from RA patients (n=4) produced a 4-fold higher level of TNFα upon stimulation with IFNα/LPS (12344 pg/ml vs. 3044 pg/ml, $P = 0.001$) and IFNα/LPS/Poly-IC (19876 pg/ml vs. 5003 pg/ml), $P = 0.01$) compared to those DC from healthy individuals. Altogether these data implies an augmented TLR4 response that is orchestrated by pre-stimulation with type I IFN, which is even more pronounced in RA patients.
Discussion
In this study we demonstrated that TLR3 and TLR7 in synovial tissue from RA patients was associated with the presence of IL-1β, IL-18 and type I IFN. Since TLR3/7 mediated cell activation did not result in IL-1β/IL-18 secretion and TLR3/7 expression was not regulated by these mediators, we sought evidence for the underlying mechanism that explains the relationship between these mediators as observed in the synovium. Remarkably, we found that pre-incubation of DC with IFNα led to clear augmentation of TLR4 mediated responses. In this light it is tempting to speculate that TLR3/TLR7 triggering leads to the production of type I IFN, which in turn augments TLR4 mediated triggering leading to the production of pro-inflammatory mediators explaining the co-expression between TLR3/7 and IL-1β/IL-18.

IFN type I is the key cytokine that regulates the innate immune response against viruses (21). Type I IFN is released upon transcription of IRF-3 or IRF-7, which can be induced following TLR3- or TLR7-mediated cell activation. TLR3 and TLR7 are highly expressed in synovial tissue from RA patients (22) and TLR3 and TLR7 ligands, such as Cytomegalovirus, Epstein-Barr virus and Parvovirus B19 have been demonstrated in the synovial joints from RA patients also (1, 3, 23). The observation that a type I IFN signature is present in the synovium in a substantial part of RA patients support the notion that TLR3 and/or TLR7 triggering is likely to occur in RA synovium. In this light, it is tempting to speculate that exposure to TLR3/TLR7 agonists might sensitize the synovial milieu for TLR4 ligands via IFNα production acting as a key regulator in maintaining the inflammation processes during RA. For several reasons, IFNβ therapy has been expected to have beneficial effect in RA as comparable to that seen in multiple sclerosis (reviewed in (24)). IFNβ therapy was shown to be effective in animal models of collagen-induced arthritis (CIA). However, several clinical trials of IFNβ in patients with RA have been ineffective so far (25-27). The fact that IFN type I strongly up regulates TLR3/TLR7 expression and tunes TLR4 responses, which in turn are continuously stimulated by ligands present in the synovial joints, might in fact maintain the inflammatory processes and therefore lead to IFN therapy failure.
Identification of endogenous TLR agonists is of great interest in terms of autoimmune disorders. In particular for TLR4 several endogenous agonist have been described thus far. For example hyaluronan fragments, heparan sulfate, fibronectin and (small) heat-shock proteins can all be released in RA joints as a result of inflammation-induced tissue injury and cell stress (11-13, 28). In addition, For instance, Brentano et al. described an endogenous ligand for TLR3, as endogenous RNA, released from necrotic synovial fluid cells, was able to TLR3-mediated activation of RA synovial fibroblast (10). Highly conserved RNA sequences within small nuclear ribonucleoprotein particles are able to activate immune cells via TLR7 and could act as an endogenous auto antigen in systemic lupus erythematosus (29, 30). Thus, triggering of TLR3/7 pathways do not necessarily derive from viruses but might well originate from the host itself. TLR-mediated immune responses in the synovial joints might result in the release of endogenous TLR ligands, originating from cells under stress or tissue damage. Therefore is not unlikely that a self sustaining loop of TLR-activation and generation of new endogenous TLR ligands might lead to a chronic inflammatory process as occurs during RA.

Lately, it has been demonstrated that stimulation of several different TLR at the same time, leads to synergistically induced levels of pro-inflammatory mediators (22, 31). Our data indicates that cytokine levels induced in a synergistic fashion by co-stimulation of TLR3 and TLR4 were even more enhanced when cells were pre-incubated with type I IFN. The exact mechanisms underpinning the augmented TLR4 response after pre-incubation with type I IFN is not yet understood. From our data we can conclude that this phenomenon is not explained by an increased expression of TLR4 itself since pre-incubation with type I IFN did not alter its mRNA expression. Perhaps the explanation for this lies within an up regulation of adaptor molecules or down regulation of intracellular inhibitors that are part of the TLR4 pathways (reviewed in (32)). Since the augmentation of TLR4 responses by type I IFN was significantly more potent in RA patients, further investigation into the causative pathways is highly desirable because it might open novel insights to battle this condition selectively.

Altogether, in this study we showed that the expression of TLR3 and TLR7 was co-expressed with IFNα, IL-1β and IL-18 in RA synovial tissue. Furthermore, we
demonstrated the involvement of IFNα in the regulation of TLR3/TLR7 expression and function but also to an augmented TLR4 mediated response. These observations might, at least partly, explain the role for type I IFN in the inflammatory cascade of RA.
Funding
T.R. was supported by a VENI and VIDI Laureate of the Netherlands organization for scientific research (NWO).

Author’s contributions
Experiments: M.F, F.B.
Interpretation of data: M.F, F.B, L.J, D.K, T.R.
**Table 1:** Oligonucleotide primers for quantitative PCR analysis

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGAPDH</td>
<td>ATC TTC TTT TGC GTC GCC AG</td>
<td>TTC CCC ATG GTG TCT GAG C</td>
</tr>
<tr>
<td></td>
<td>AGA GTT GTC ATC GAA TCA AAT</td>
<td>CAT TGT TCA GAA AGA GGC</td>
</tr>
<tr>
<td></td>
<td>TAA AGA G</td>
<td>CAA AT</td>
</tr>
<tr>
<td>hTLR3</td>
<td>TGC CAT CAA GAA AGT TGA TGC T</td>
<td>GGA ATG TAG AGG TCT GGT</td>
</tr>
<tr>
<td>hTLR7</td>
<td>AAT CTG TAC CTG TCC TGC GTG TT</td>
<td>TGA AGA G</td>
</tr>
<tr>
<td>hIL-1β</td>
<td></td>
<td>TGG GTA ATT TTT GGG ATC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAC ACT CT</td>
</tr>
</tbody>
</table>

Software package Primer Express Version 2.0 (Applied Biosystems) was used to identify appropriate primer sets. All sequences are presented in the 5’→3’ direction.
Table 2. Co-expression of TRL3/7 with Type I IFN, IL-1 and IL-18 in RA synovium.

<table>
<thead>
<tr>
<th>Correlation (r)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Co-expression TLR – Type I IFN</strong></td>
<td></td>
</tr>
<tr>
<td>TLR3 – Type I IFN</td>
<td></td>
</tr>
<tr>
<td>Lining</td>
<td>0.76</td>
</tr>
<tr>
<td>Sub-lining</td>
<td>0.77</td>
</tr>
<tr>
<td>TLR7 – Type I IFN</td>
<td></td>
</tr>
<tr>
<td>Lining</td>
<td>0.70</td>
</tr>
<tr>
<td>Sub-lining</td>
<td>0.84</td>
</tr>
<tr>
<td><strong>Co-expression TLR – Interleukin 1</strong></td>
<td></td>
</tr>
<tr>
<td>TLR3 – IL-1</td>
<td></td>
</tr>
<tr>
<td>Lining</td>
<td>0.54</td>
</tr>
<tr>
<td>Sub-lining</td>
<td>0.22</td>
</tr>
<tr>
<td>TLR7 – IL-1</td>
<td></td>
</tr>
<tr>
<td>Lining</td>
<td>0.32</td>
</tr>
<tr>
<td>Sub-lining</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Co-expression TLR – Interleukin 18</strong></td>
<td></td>
</tr>
<tr>
<td>TLR3 – IL-18</td>
<td></td>
</tr>
<tr>
<td>Lining</td>
<td>0.48</td>
</tr>
<tr>
<td>Sub-lining</td>
<td>0.11</td>
</tr>
<tr>
<td>TLR7 – IL-18</td>
<td></td>
</tr>
<tr>
<td>Lining</td>
<td>0.48</td>
</tr>
<tr>
<td>Sub-lining</td>
<td>0.16</td>
</tr>
</tbody>
</table>

NS; not significant
Legends to the figures

**Figure 1: Effects of type I IFN, IL-1β and IL-18 on TLR3/7 expression.**
IL-1β, IL-18 and IFNα induced TLR3/7 mRNA expression by monocytes (A), monocytes-derived DC (B) and RA synovial fibroblasts (C) from RA patients (n=5) and healthy controls (n=5). Cells were incubated with 20ng IL-1β, IL-18 and 100 IU/ml IFNα for 8 hours and subsequently TLR mRNA expression was determined by real-time PCR. Graphs represent the mean ± SD.

**Figure 2: Functionality of increased TLR3/7 expression by type I IFN.**
Monocytes (panel A), monocyte-derived DC (panel B) and RA synovial fibroblasts (panel C) from RA patients (n=5) were stimulated with TLR3/7 specific ligands after they were incubated with medium only (grey bars) or medium containing IFNα (100 U/ml) (black bars) for 10 hrs. Graphs represent the mean ± SD.

**Figure 3. Pre-incubation with type I IFN augments TLR4 responses.**
TNFα secretion by monocyte-derived DC co-incubated with (24 hrs) agonists for TLR3 (TLR3), TLR4 (purified LPS) or the combination of both. TNFα secretion was compared between DC that were pre-stimulated with 100 U/ml IFNα for 10 hrs and those who were not. DC from healthy individuals (n=5) and RA patients (n=4) were compared. Graphs represent the mean ± SD. * Represents a P-value lower than 0.05.
List of References


