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The Toll-Like Receptor Adaptor Proteins MyD88 and Mal/TIRAP Contribute to the Inflammatory and Destructive Processes in a Human Model of Rheumatoid Arthritis

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The widespread distribution of Toll-like receptors (TLRs) and their ligands raises the question whether they contribute to the production of inflammatory and tissue destructive molecules in rheumatoid arthritis (RA). We examined the expression and function of TLR2 and TLR4 and their downstream signaling adaptors MyD88 and Mal/TIRAP in synovial membrane cultures from RA tissue. Both TLR2 and TLR4 were detected by flow cytometry, and stimulation with TLR2 and TLR4 ligands augmented the spontaneous production of tumor necrosis factor-α, interleukin (IL)-6, and IL-8, indicating that TLR2 and TLR4 are functional in these cultures. In addition, overexpression of dominant-negative forms of MyD88 and Mal/TIRAP significantly down-regulated the spontaneous production of cytokines tumor necrosis factor-α, IL-6, and vascular endothelial growth factor, and enzymes MMP-1, MMP-2, MMP-3, and MMP-13 in RA synovial membrane cell cultures. Because TLR2 and TLR4 require both MyD88 and Mal/TIRAP for signaling, this study suggests that TLR function may regulate the expression of these factors in the RA synovium. Conditioned media from synovial membrane cell cultures stimulated human macrophages in a MyD88- and Mal-dependent manner, suggesting the release of a TLR ligand(s) from these cells. Thus, TLRs not only protect against infection but may also promote the inflammatory and destructive process in RA. ***(Am J Pathol 2007, 170:518–525; DOI: 10.2353/ajpath.2007.060657)***

Rheumatoid arthritis (RA) is an autoimmune disease primarily characterized by synovial inflammation and destruction of cartilage and bone. Cytokines and matrix metalloproteinases (MMPs) play important roles in these processes, a fact highlighted by the clinical effectiveness of anti-cytokine biologicals (antibodies or soluble receptors) targeting tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 receptor.[1,2] However, it is still unclear what regulates cytokine production or triggers and prolongs the expression of inflammatory and tissue-destructive mediators in RA.

Toll-like receptors (TLRs) recognize microbial products termed pathogen-associated molecular patterns in the response to infection. In humans, there are at least 10 TLRs that have different pathogen-associated molecular pattern specificities, eg, TLR4 for lipopolysaccharide (LPS), TLR2 for lipoproteins and TLR3, -7, and -8 for single- or double-stranded RNA. These ligands are potent inducers of inflammatory cytokines. The TLR signal transduction pathway that activates nuclear factor (NF)-κB shares many components with IL-1R signaling mechanisms, due to the common use of the signaling adaptor molecule MyD88 that binds to both TLRs and IL-1R. However, unlike the IL-1R family, some TLRs also require other TIR adaptors such as MAL/TIRAP (TLR2 and 4), TRIF (TLR3 and 4), and TRAM (TLR4) to function.[3]

TLRs have also been reported to recognize a number of endogenous ligands, (eg, fibronectin fragments,[4] hyaluronan fragments,[5] self-mRNA,[6] HMGB1[7]). These potential danger signals would indicate tissue damage, are likely to be abundant in chronically inflamed tissue,[8,9] and could potentially initiate or sustain an inflammatory

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response. There is considerable evidence from rodent models that activation of the TLRs can induce or exacerbate inflammatory arthritis.[10] However, its relevance to human disease is limited because all of these studies used microbial products such as LPS and mycobacterial DNA to induce arthritis. So far, data on any role for TLRs in RA have been circumstantial. In humans, infection of the joints induces strong immune responses that often lead to a destructive septic arthritis. In addition, activation of fibroblast-like synoviocytes with TLR ligands results in NF-κB activation and increased expression of inflammatory cytokines, chemokines, adhesion molecules, and MMPs.[11,12] Interestingly, peptidoglycans and bacterial DNA derived from gut-colonizing bacteria have been detected in RA joints, but the relevance is unclear because they are also found in osteoarthritic joints.[13] Immunohistological staining has detected TLR2 and TLR4 in the RA joint synovial tissue although, curiously, the Asp299Gly polymorphism that inactivates TLR4 function has been associated with RA susceptibility but not severity.[14]

This study investigates whether there is a role for the TLRs in chronic inflammatory processes of RA. Using a human disease model of RA, total synovial tissue cultures,[15,16] we show that TLR2 and TLR4 are present and responsive to exogenous ligands. More importantly, we show that signaling mediated by the pan-TLR adaptor MyD88 and by Mal/TIRAP, which is used by TLR2 and TLR4, is involved in the spontaneous production of cytokines and MMPs in RA synovial membranes and that the RA membrane cell cultures release a factor(s) that can stimulate macrophages in a MyD88- and Mal-dependent manner. These data provide evidence, for the first time to our knowledge, that the TLR signaling system is involved in the pathogenesis of a human chronic inflammatory disease.

Materials and Methods

Reagents

Phenol-chloroform-purified *Escherichia coli* LPS and $Pam_3Cys\text{-}Ser\text{-}Lys_4$ (Pam3C) were purchased from Alexis (Nottingham, UK), and lipoteichoic acid (LTA) and peptidoglycan (PGN) were from Invivogen (San Diego, CA). The directly conjugated fluorescein isothiocyanate-labeled TLR2 and TLR4 antibodies used for fluorescence-activated cell sorting (FACS) analysis were purchased from Imgenex (San Diego, CA). Anti-CD3-PE and anti-CD68-PE and their isotype controls were purchased from Becton Dickinson (Oxford, UK), and IgG2a-fluorescein isothiocyanate was purchased from Abcam (Cambridge, UK).

Adenoviral Vectors and Their Propagation

Recombinant, replication-deficient adenoviral vectors encoding β-galactosidase (Adβ-gal) or IκBα were kind gifts of Quantum Biotech (Canada) and Dr R. de Martin (University of Vienna, Vienna, Austria). Adenoviruses encoding dominant-negative forms of MyD88 (AdMyD88dn) and Mal/TIRAP (AdMal/TIRAPdn) and the GFP control (AdGFP) were constructed in-house.[12] All viruses used in this study are E1/E3 deleted, belong to the Ad5 serotype, and have been previously used in other studies.[12,17–21] Viruses were propagated in 293 human embryonic kidney cells (American Type Culture Collection, Rockville, MD), purified by ultracentrifugation through two cesium chloride gradients, and viral titers determined by plaque assay as previously described.[22]

Patient Specimens and in Vitro *Culture*

Human macrophages were derived from monocytes after differentiation for 4 days with 100 ng/ml M-CSF as previously described.[23] RA synovial membrane cells were isolated from patients undergoing joint replacement surgery as previously described.[15,16] The study was approved by the Riverside Research ethics committee, and waste tissue (synovium after joint replacement surgery) was obtained only after receiving signed informed consent from the patient and anonymyzing the tissue to protect patient identity. Immediately after isolation, cells were used for mRNA analysis, stained by FACS, or cultured at 1×10^5 cells/well in 96-well tissue culture plates (Falcon, Becton Dickinson) in RPMI 1640 containing 10% (v/v) fetal bovine serum and 100 U/ml penicillin/streptomycin. For exogenous stimulation experiments, RA membrane cell cultures were incubated with 20 μg/ml LTA, 10 μg/ml PGN, 10 ng/ml $Pam_3Cys\text{-}Ser\text{-}Lys_4$, or 10 ng/ml LPS and supernatants collected after 24 hours. For adenoviral gene transfer experiments, cells were incubated with adenoviral vectors at a multiplicity of infection of 100, washed after 2 hours, and cultured in complete medium for 48 hours, at which time supernatants were collected. In all cases, viability of the cells was not significantly affected throughout this time period when examined by the MTT cell viability assay (Sigma, Poole, UK).[24] Supernatants were subsequently examined for the presence of cytokines and MMPs by enzyme-linked immunosorbent assay (ELISA).

Western Blotting

Total RA synovial membrane cells were plated at 2.5×10^6 cells/well in six-well tissue culture plates (Falcon) and were either uninfected or infected with adenovirus. Cytosolic proteins were obtained and subsequently separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% (w/v) polyacrylamide gel and transferred onto polyvinylidene difluoride membrane for Western blotting. Antibodies for MyD88 and IκBα were purchased from QED Bioscience Inc. (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Rabbit polyclonal antibody directed against the N-terminal peptide MASSTSLPAPGSRPK of human Mal/TIRAP was designed in our laboratory.[12]

Analysis of Cytokines by ELISA

Supernatants were analyzed for cytokine levels by ELISA according to the manufacturer's instructions. TNF-α, IL-1β, IL-6, IL-8, and vascular endothelial growth factor (VEGF) ELISAs were purchased from Pharmingen (Becton Dickinson). MMP-1, MMP-2, MMP-3, and MMP-13 ELISAs were purchased from Amersham (Buckinghamshire, UK). Absorbance was read on a spectrophotometric ELISA plate reader (Labsystems Multiscan Biochromic) and analyzed using the Ascent software program (Thermo Labsystems, Altrincham, UK).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA was isolated using a RNA blood isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and then treated with turbo DNase (Ambion, Austin, TX) according to manufacturer's instructions. Total RNA was reverse-transcribed with Superscript II RNase H^- reverse transcriptase and oligo(dT) primer (Life Technologies, Inc., Grand Island, NY). For human TLR2 amplification, the primers 5′-GCCAAAGTCTTGATTGATTGG-3′ and 5′-TTGAAGTTCTCCAGCTCCTG-3′ were used. For human TLR4, the primers 5′-TGGATACGTTTCCTTATAAG-3′ and 5′-GAAATGGAGGCACCCCTTC-3′ were used. Subsequent PCR amplification consisted of 35 cycles with an annealing temperature of 62°C for TLR2 and 58°C for TLR4 and performed in a Dyad PCR machine (MJ Instruments, Waltham, MA).

Luciferase Assay

Macrophages cultured in a 96-well plate were infected with recombinant adenovirus containing a NF-κB luciferase reporter gene (kindly provided by Dr. B. Davidson, University of Iowa, Ames, IA) at a multiplicity of infection of 50:1. The cells were rested at least 4 hours before an additional infection with AdGFP, AdMyD88dn, or AdMaldn at a multiplicity of infection of 100:1. After 24 hours, cells were stimulated for 6 hours with filtered RA supernatants. The cells were washed once in phosphate-buffered saline (PBS) and lysed with 100 μl of CAT lysis buffer [0.65% (v/v) of Nonidet P-40, 10 mmol/L Tris-HCl, pH 8, 0.1 mmol/L ethylenediaminetetraacetic acid, pH 8, and 150 mmol/L NaCl]. Fifty μl of cell lysate were mixed with 120 μl of luciferase assay buffer [25 mmol/L Tris-phosphate, pH 7.8, 8 mmol/L $MgCl_2$, 1 mmol/L ethylenediaminetetraacetic acid, 1% (v/v) Triton X-100, 1% (v/v) glycerol, 1 mmol/L dithiothreitol, and 0.5 mmol/L ATP] in the well of a luminometer cuvette strip. Luciferase activity was measured with a luminometer (Thermo Labsystems) by adding 30 μl of luciferin (Bright-Glo luciferase assay system; Promega, Madison, WI) per assay point.

Flow Cytometry Analysis

Cells were washed, fixed in 2% paraformaldehyde, and then blocked with 10% human serum (PAA, Pasching, Austria) in PBS containing 0.01% azide for 30 minutes at 4°C with or without 0.1% saponin (Sigma, St. Louis, MO) for intracellular or cell surface staining, respectively. Cells were then incubated with α-TLR2, α-TLR4, α-CD3, α-CD68, or isotype control antibodies for 1 hour at 4°C and then washed before analysis on a Becton-Dickinson LSR flow cytometer.

Statistical Methods

Mean, SD, SEM, and statistical tests were calculated using GraphPad version 3 (GraphPad Software Inc., San Diego, CA). For statistical analysis of parametric data, a one-tailed Student's *t*-test for normally distributed data were used. For nonparametric data, a one-tailed Wilcoxon signed rank test was applied.

Results

TLR2 and TLR4 Are Expressed by RA Synovial Membrane Cells

Because most of the endogenous TLR ligands so far described have been for TLR2 and TLR4, the expression of these receptors in RA synovial membrane cells was examined. TLR2 and TLR4 mRNA was detected by RT-PCR in all four RA patients examined (Figure 1A). The presence of TLR2 and TLR4 was also detected by FACS. In addition to the cell surface expression, considerable staining was also found intracellularly, particularly for TLR2 (Figure 1B), in contrast to the belief that these TLRs are mostly cell surface localized. An analysis of the major cell populations showed that macrophages ($CD68^+$) almost universally expressed both TLRs (Figure 1C), whereas a considerable proportion of $CD68^-$ cells (mainly fibroblasts and T cells) did not express TLR2 and/or TLR4 (Figure 1C). Further analysis showed that the TLR2/4-negative population was mostly confined to $CD3^+$ T cells (data not shown).

TLR2 and TLR4 Are Functional on RA Synovial Membrane Cells

The addition of the TLR2 ligands, Pam_3Cys-Ser-Lys_4 (PAM3), PGN, or LTA, and the TLR4 ligand LPS consistently yielded a significant twofold to fourfold increase in the production of TNF-α and IL-8, above that spontaneously produced by these cultures (Figure 2, A and B). For IL-6, the effect of exogenous TLRs was less pronounced, with only PGN (TLR2) and LPS (TLR4) recording a significant increase (Figure 2C).

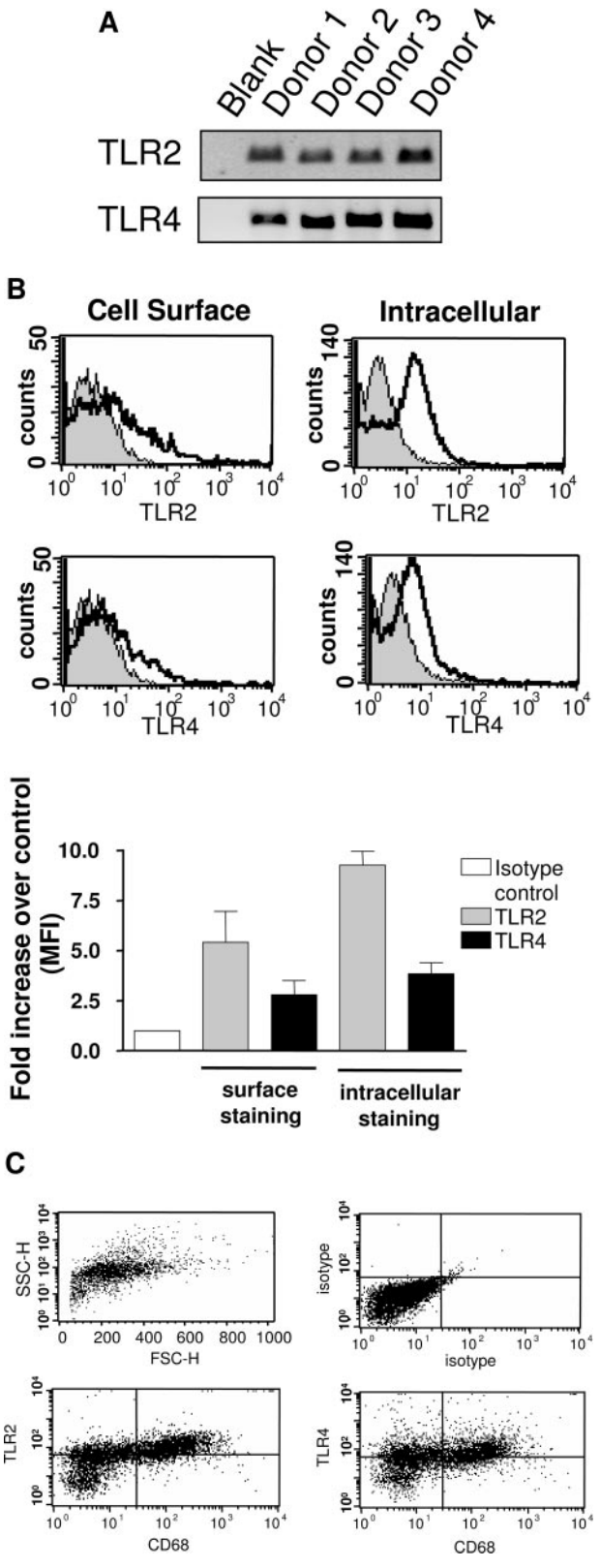


Figure 1. Expression of TLR2 and TLR4 in the RA synovium. **A:** mRNA from synovial tissue was extracted, DNase-treated, and then analyzed by RT-PCR for the presence of TLR2 and TLR4 in four separate donors. **B** and **C:** RA synovial membrane cells from the same donor were examined by FACS for the expression of cell surface or intracellular TLR2 and TLR4. **B:** A representative histogram plot and the fold induction ± SEM of five independent donors is shown. **C:** Permeabilized cells were incubated with isotype control, α-TLR2, or α-TLR4 antibody and co-stained with either α-CD3 or α-CD68.

MyD88 and Mal/TIRAP Are Required for TNF-α, IL-6, IL-8, and VEGF Production in RA Synovial Membrane Cultures

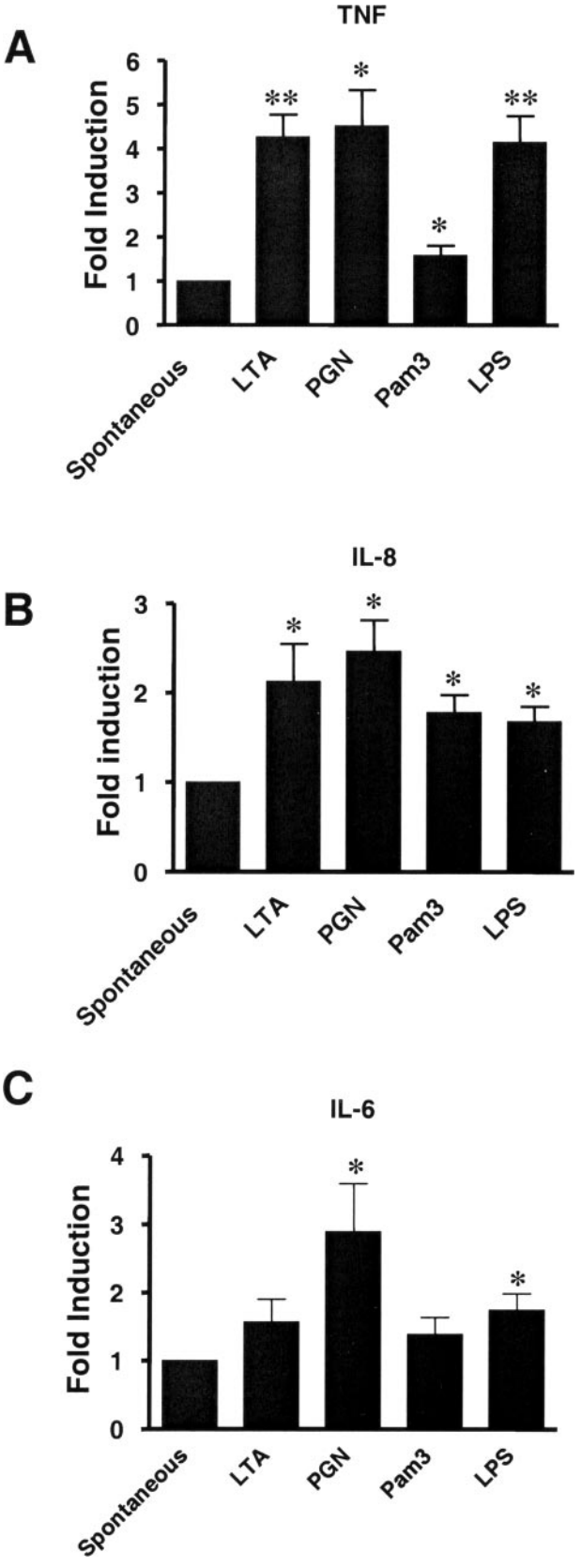
Because the data above showed that functional TLR2 and TLR4 are expressed in RA synovial membranes, the contribution of signaling by these receptors to the endogenous production of inflammatory cytokines and MMPs was examined. This study was performed using adenoviral gene transfer of dominant-negative inhibitory forms of MyD88 and Mal/TIRAP. These constructs have been previously used to examine TLR4 signaling in primary human cells,[12] and this combination of adaptors are used by TLR2 and TLR4.[25,26] An adenoviral construct for IκBα previously used in RA tissue[27] was the positive control because all of the parameters examined have been shown to be NF-κB-dependent.[27,28] A viral construct expressing β-galactosidase was used as the negative control.

Adenoviral gene transfer into RA synovial membrane cultures led to several fold higher levels of expression of MyD88dn and Maldn compared with the endogenous levels (Figure 3A), as previously described for IκBα.[28] The expression of these constructs had a profound effect on the production of most inflammatory cytokines measured. Thus, MyD88dn resulted in a statistically significant but variable decrease in the spontaneous production of TNF-α (28 ± 15% inhibition, $P < 0.05$), IL-6 (73 ± 13% inhibition, $P < 0.05$), IL-8 (67 ± 14% inhibition, $P < 0.01$), and VEGF (44 ± 16% inhibition, $P < 0.01$) but not IL-1β (28 ± 22% inhibition, $P > 0.05$), probably attributable to the wider scatter of IL-1 production (Figure 3, B–F). Expression of Maldn also resulted in a similar inhibition of the spontaneous TNF-α (33 ± 16% inhibition, $P < 0.05$), IL-6 production (70 ± 14% inhibition, $P < 0.05$), IL-8 (48 ± 23% inhibition, $P < 0.01$), and VEGF (40 ± 21% inhibition, $P < 0.05$) but again not IL-1β (20 ± 35% inhibition, $P > 0.05$). IκBα was used as a positive control and resulted in a statistically significant inhibition in TNF-α (48 ± 14% inhibition, $P < 0.05$), IL-1β (47 ± 10% inhibition, $P < 0.05$), IL-6 (94 ± 1% inhibition, $P < 0.05$), IL-8 (72 ± 16% inhibition, $P < 0.05$), and VEGF (59 ± 9% inhibition, $P < 0.01$) (Figure 3, B–F), confirming previous studies.[27,28]

MyD88 and Mal/TIRAP Are Essential in MMP Production in RA Synovial Membrane Cultures

Given the evidence above that MyD88 and Mal were required for part of the inflammatory cytokine production from enzymatically dispersed synovial membrane cultures, the effect of the same constructs was assessed on the spontaneous expression of MMP-1 (collagenase-1), MMP-2 (gelatinase A), MMP-3 (stromelysin-1), and MMP-13 (collagenase-3), four important enzymes considered to be involved in the tissue destruction and remodeling in RA. We found that MyD88dn significantly inhibited MMP-1 (51 ± 13% inhibition, $P < 0.05$), MMP-2 (72 ± 8% inhibition, $P < 0.05$), MMP-3 (54 ± 20% inhibition, $P < 0.01$), and MMP-13 (67 ± 8% inhibition, $P <$

0.01) (Figure 4, A–D). Likewise, Maldn significantly inhibited MMP-1 (48 ± 18% inhibition, $P < 0.05$), MMP-2 (61 ± 14% inhibition, $P < 0.05$), MMP-3 (52 ± 20% inhibition, $P < 0.01$), and MMP-13 (68 ± 10% inhibition, $P < 0.01$) (Figure 4, A–D). The inhibitory effect on MMPs of MyD88dn and Maldn parallels that seen by IκBα overexpression (Figure 4, A–D). The fact that Mal/TIRAP is a specific adaptor for TLR2 and TLR4 signaling and is not involved in IL-1R signaling[29,30] suggests that TLR signaling contributes to MMP expression in the RA synovium.



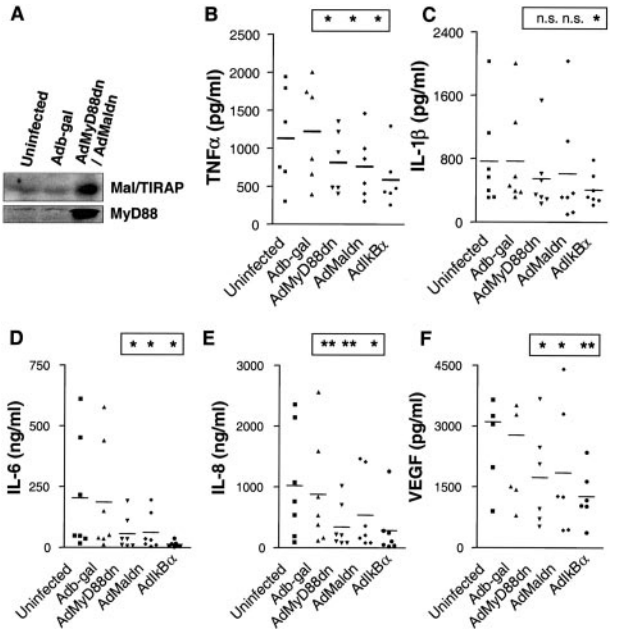


Figure 3. Effect of MyD88dn, Maldn, and IκBα expression in cytokine production in RA synovial membrane cells. RA synovial membrane cells were left uninfected or were infected with Adβ-gal, AdMyD88dn, AdMaldn, or AdIκBα for 2 hours. Cells were subsequently cultured for a further 48 hours. **A:** Cell extracts were obtained and examined for the presence of endogenous or overexpressed MyD88 and Mal/TIRAP by Western blotting. **B–F:** Supernatants were collected and assayed for the presence of TNF-α (**B**), IL-1β (**C**), IL-6 (**D**), IL-8 (**E**), and VEGF (**F**) by ELISA. Mean cytokine production (±SEM) of triplicate cultures from six to eight unrelated patients is shown. For the statistical analysis of these parametric normally distributed data, a one-tailed Student's *t*-test was used to compare uninfected control cells with recombinant adenovirus-infected cells (*$P < 0.05$, **$P < 0.01$).

Conditioned Media from Rheumatoid Synovial Cell Cultures Contains a Ligand(s) that Activates NF-κB in Human Macrophages in a MyD88- and Mal-Dependent Manner

To determine whether a potential TLR ligand(s) was released from the synovial cell cultures, supernatants were collected from cultures after 24 hours and filtered to remove any cell debris. These supernatants were tested for LPS and found to be free from contamination. Supernatants were used to stimulate M-CSF-derived macrophages expressing a consensus sequence NF-κB re-

Figure 2. TLR2 and TLR4 stimulation of RA synovial membrane cultures. RA synovial membrane cells were incubated for 24 hours in the presence of media alone or media containing 20 μg/ml LTA, 10 μg/ml PGN, 10 ng/ml $Pam_3Cys\text{-}Ser\text{-}Lys_4$, or 10 ng/ml LPS. Supernatants were collected after 24 hours and assayed for the presence of TNF-α (**A**), IL-6 (**B**), and IL-8 (**C**) by ELISA. Fold induction of cytokine production ± SEM of triplicate cultures is shown and is representative of five independent experiments from unrelated RA patients.

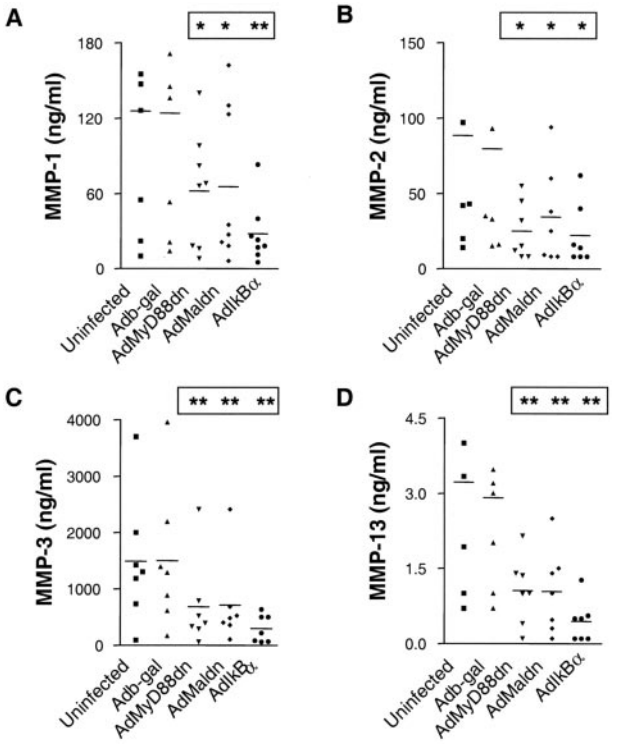


Figure 4. Effect of MyD88dn, Maldn, and IκBα expression in MMP production in RA synovial membrane cells. RA synovial membrane cells were left uninfected or were infected with Adβ-gal, AdMyD88dn, AdMaldn, or AdIκBα for 2 hours. Cells were subsequently cultured for a further 48 hours and supernatants collected and assayed for the presence of MMP-1 (**A**), MMP-2 (**B**), MMP-3 (**C**), and MMP-13 (**D**) by ELISA. Mean cytokine production (±SEM) of triplicate cultures from six to seven unrelated patients is shown. For the statistical analysis of these parametric normally distributed data, a one-tailed Student's *t*-test was used to compare uninfected control cells with recombinant adenovirus-infected cells ($^{*}P < 0.05$, $^{**}P < 0.01$).

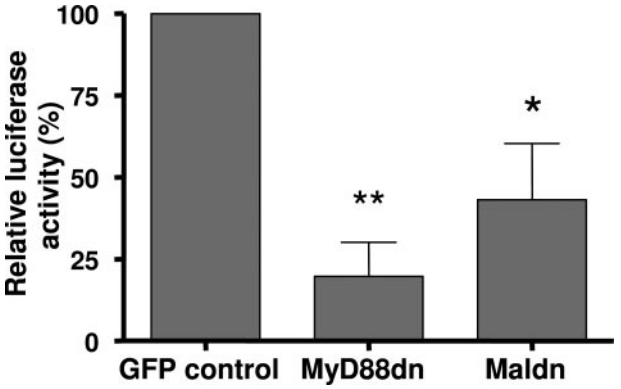


Figure 5. Stimulation of NF-κB activation in macrophages with RA synovial cell culture supernatants. M-CSF-derived human macrophages were infected with an adenovirus containing a NF-κB luciferase reporter gene and AdGFP, MyD88dn, or Maldn. After 24 hours, the cells were stimulated for 6 hours with filtered supernatants harvested from RA synovial membrane cell cultures, after which luciferase activity was measured. Data are shown as relative luciferase activity to the control as the mean (±SEM) ($n = 3$). For the statistical analysis, a one-tailed Student's *t*-test was used to compare ADGFP control cells with ADMyD88dn and AdMaldn cells ($^{*}P < 0.05$, $^{**}P < 0.01$).

porter gene and either a control GFP construct or one containing the MyD88dn or the Maldn. NF-κB activity was measured because the supernatants used to stimulate the macrophages would contain cytokines. The supernatants induced activation of NF-κB that was inhibited by MyD88dn (80 ± 10%, $P < 0.01$) and by Maldn (57 ± 17%, $P < 0.05$), suggesting the presence of a TLR ligand in the conditioned media (Figure 5). Stimulation of macrophages with conditioned media harvested from M-CSF-derived macrophages after 24 hours was unable to stimulate NF-κB activation in unrelated macrophages (data not shown).

Discussion

TNF-α, IL-1, and more recently, IL-6, are recognized as effective targets for the treatment of RA. However, the stimuli that drive the production of these cytokines in the disease, specifically within the synovium are still unclear. This study is the first to provide evidence that TLRs may be involved in regulating inflammatory cytokine production in human RA disease tissue. In addition, it is the first to indicate that the production of MMPs involved in destructive processes could also be dependent on TLR signaling. The fact that MyD88 and Mal/TIRAP appear to have equal roles in driving the production of cytokines and MMPs suggests that TLR2 and/or TLR4 could both contribute, although potential contributions from other TLRs cannot be discounted. In keeping with this concept is the observation that TLR2 and TLR4 are present and functional in RA synovium, and most of the endogenous TLR ligands react with TLR2 and TLR4.

The discovery of the TLR system was a major breakthrough in the understanding of the relationship between infection and the inflammatory response. The subsequent discovery that TLRs have endogenous ligands and thus could potentially detect tissue death or injury has incorporated these receptors into the system capable of detecting stress or danger signals. A question that naturally arises from the existence of endogenous TLR ligands is whether these receptors could be involved in the establishment or maintenance of chronic autoinflammatory diseases. Such a proposition is appealing as many of the endogenous TLR ligands described are likely to be present at sites of tissue injury that occur during chronic destructive inflammatory episodes. One could easily envisage a vicious cycle of inflammatory-induced injury leading to the release of TLR ligands, thus inducing more inflammation and so on. Previous studies have provided some support for this hypothesis because TLR2, -3, -4, and -7 have been detected by immunohistochemistry in RA tissue,[31–33] although the data were not confirmed by FACS analysis and no functional studies were reported. A previous study has indicated that synovial fluid might contain potential TLR4 ligands.[8,9] In addition, a recent study has shown that artificially necrotizing cells from RA synovial fluid causes release of RNA that can stimulate cultured synovial fibroblast, presumably by TLR3.[8] However, neither of these studies directly shows that TLRs are actually driving any part of the inflammatory, destructive, and angiogenic processes in RA. This present study using the well-established human disease model of short-term cultured RA joint synovial membrane cells[15,16] confirmed the presence of TLR2 and TLR4 in RA tissue and

furthermore showed that these receptors are active and capable of up-regulating inflammatory cytokine production. The different levels of cytokines produced by each of the TLR2 ligands most probably reflects their use of different receptor combinations, PAM3 is recognized by a heterodimer of TLR1/2, LTA by TLR2/6, and PGN is recognized by both TLR2 and NOD2. However, it was interesting to note that a significant proportion of receptor expression, especially TLR2, was found to be intracellular. This observation confirmed a recent report in lymphocytes where more TLR2 was detected on the intracellular compartment by FACS staining rather than on the cell surface.[34] Intracellular localization for TLR4 has also been described in pulmonary epithelial cells,[35] further indicating that under certain conditions, TLRs normally found on the cell surface can also be localized intracellularly. It is unclear why this might be the case because these receptors are normally considered to be expressed at the cell surface, unlike TLR3, -7, -8, and -9 that are classically intracellular. One possible explanation could be different locations of the receptor in alternative cell populations or reservoirs of receptors that can shuttle to the surface. TLR2 and TLR4 were detected in both macrophage and nonmacrophage populations. Our previous studies showed TLR2 and TLR4 to be present on the cell surface of human macrophages and demonstrated that cultured synovial fibroblasts respond to both TLR2 and TLR4 ligands.[12]

The inhibitory effect of the dominant-negative versions of MyD88 and Mal/TIRAP on the production of TNF-α, IL-6, IL-8, VEGF, and the MMPs suggests a role for TLR2 and TLR4 in the induction of these cytokines. A role for MyD88 could be expected because the IL-1 receptor family as well as TLRs use this adaptor. However, the role of Mal/TIRAP is so far restricted to TLR2 and TLR4 signaling and the Maldn construct we have used in this study does not inhibit IL-1 signaling.[12] Our data do not preclude that other TLRs and/or the IL-1R contribute to the production of TNF-α and the other cytokines or that other factors that use alternative signaling pathways are also involved. Indeed, the inhibitory effect of blocking Mal and MyD88 signaling were partial and varied considerably depending on the mediator measured, being modest for TNF-α and IL-1 but much more effective for IL-6 and some of the MMPs. It is not surprising that in such a complex inflammatory tissue like the RA synovium multiple factors and pathways are involved in the chronicity of the inflammatory response, all potentially contributing to different extents to driving the inflammatory cycle. In addition, it is difficult to know how much a given effect, for example inhibition of MMP1, is attributable to a direct effect of blocking TLR signaling or a secondary effect of blocking cytokine production.

Interestingly, the RA synovial cell cultures release a ligand(s) that was able to stimulate macrophages in a MyD88- and Mal-dependent manner, presumably by activating TLR2 and/or TLR4 because only these receptors are reported to use Mal as an adaptor protein. The greater inhibition observed with MyD88dn is most probably attributable to combined inhibition of the IL-1 receptor because IL-1 is present in the RA supernatants used to stimulate these cells but could also be through inhibition of other TLR receptors that use MyD88 but not Mal to signal.

An obvious experiment to identify the specific TLR(s) involved would be to use neutralizing antibodies to TLR2 and TLR4 (and others). However, our studies with anti-TLR2 and -TLR4 antibodies so far have proved inconclusive, but the failure of these neutralizing antibodies may not be surprising. We have found that the available antibodies are only effective inhibitors at low concentrations of known TLR ligands. It is unknown what potential endogenous TLR ligands may exist in the RA synovium, and it is not known if these antibodies will be effective in blocking these ligands. On the other hand, it is possible that TLRs other than TLR2 and TLR4 may also contribute to the inflammatory process in the RA synovium. This will not be clear until better means of inhibiting TLR function are available or the nature of the TLR ligand(s) is identified. We checked whether the culture system could have been perturbed by the accidental introduction of LPS during tissue processing but we have not detected any LPS in culture supernatants by using the Limulus amoebocyte assay (data not shown). We also assessed any effect of collagenase on the cells and found that peripheral blood mononuclear cells processed in the same way as the synovial tissue were unaffected by the treatment with respect to TNF production and responsiveness to LPS (data not shown). It seems likely that TLRs may contribute to the vicious cycle of viral infection/tissue damage, up-regulating antigen presentation and promoting autoimmunity as described by Bottazzo and colleagues.[36]

In summary, this study has demonstrated in the human tissue model of RA that there could be a role for TLRs, possibly via TLR2 and/or TLR4, in the up-regulation of the inflammatory response in RA and that the RA synovial membrane cells potentially release a factor(s) that can stimulate TLR signaling. Although this study has not demonstrated directly an unambiguous role for TLRs, the evidence presented provides a new insight into the factors that drive the production of TNF-α and IL-6, both clinically important targets for the treatment of RA, and thus suggest that blocking TLRs or other proximal signaling mechanisms may provide therapeutic targets.

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