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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 TLR2 and TLR4 are functional in these RA synovial membrane cell cultures. 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 HMGB17). 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 lead to a destructive septic arthritis. In addition, activation of 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 Pam3Cys-Ser-Lys4 (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-labeled TLR2 and TLR4 antibodies used for fluorescence-activated cell sorting (FACS) analysis were purchased from Abcam (Cambridge, UK).

Adenoviral Vectors and Their Propagation

Recombinant, replication-deficient adenoviral vectors encoding β-galactosidase (Adβ-gal) or IxBα were kind gifts of Quantum Biotech (Canada) and Dr R. de Martin (University of Vienna, Vienna, Austria). Adenoviruses encod-
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’-GCAAGTTCTGAT-TGATTGG-3’ and 5’-TGAAAGTCTCAGTCCTG-3’ were used. For human TLR4, the primers 5’-TGAGAT-ACGTTCCTTATAG-3’ and 5’-GAATTGGAGGACCCCTTC-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 Trisphosphate, pH 7.8, 8 mmol/L MgCl₂, 1 mmol/L ethylenediaminetetraacetic acid, 1% (v/v) Triton X-100, 1% (v/v) glyceral, 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, Pam₃Cys-Ser-Lys₄ (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).
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, and this combination of adaptors are used by TLR2 and TLR4. An adenoviral construct for IκBα previously used in RA tissue was the positive control because all of the parameters examined have been shown to be NF-κB-dependent. 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. 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 (15% inhibition, P < 0.05), IL-6 (13% inhibition, P < 0.05), IL-8 (14% inhibition, P < 0.01), and VEGF (16% inhibition, P < 0.01) but not IL-1α (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 (16% inhibition, P < 0.05), IL-6 production (14% inhibition, P < 0.05), IL-8 (23% inhibition, P < 0.05), and VEGF (21% inhibition, P < 0.05) but again not IL-1α (35% inhibition, P < 0.05). IκBα was used as a positive control and resulted in a statistically significant inhibition in TNF-α (14% inhibition, P < 0.05), IL-1α (10% inhibition, P < 0.05), IL-6 (1% inhibition, P < 0.05), IL-8 (16% inhibition, P < 0.05), and VEGF (9% inhibition, P < 0.01) (Figure 3, B–F), confirming previous studies.

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% inhibition, P < 0.05), MMP-2 (72% inhibition, P < 0.05), MMP-3 (54% inhibition, P < 0.01), and MMP-13 (67% inhibition, P <
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, \( P < 0.01 \)) (Figure 4, A–D). The inhibitory effect on MMPs of MyD88dn and Maldn parallels that seen by I\( \beta \)B 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 suggests that TLR signaling contributes to MMP expression in the RA synovium.

**Conditioned Media from Rheumatoid Synovial Cell Cultures Contains a Ligand(s) that Activates NF-\( \kappa \)B 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-\( \kappa \)B re-
The fact that MyD88 and Mal/TIRAP appear to 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, 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. In addition, a recent study has shown that artificially necrotizing cells from RA synovial fluid causes release of RNA that can stimulate cultured synovial fibroblasts, presumably by TLR3. 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 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. Intracellular localization for TLR4 has also been described in pulmonary epithelial cells, 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.

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 normally found in the RA synovium and 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 amebocyte 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.

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 signal-mediating mechanisms may provide therapeutic targets.

References


