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The advent of anti-TNF biologicals has been a seminal advance in the treatment of rheumatoid arthritis (RA) and has confirmed the important role of TNF in disease pathogenesis. However, it is unknown what sustains the chronic production of TNF. In this study, we have investigated the anti-inflammatory properties of mianserin, a serotonin receptor antagonist. We discovered mianserin was able to inhibit the endosomal TLRs 3, 7, 8, and 9 in primary human cells and inhibited the spontaneous release of TNF and IL-6 from RA synovial membrane cultures. This suggested a role for these TLRs in production of TNF and IL-6 from RA synovial cultures. Only stimulation of TLR 3 or 8 induced TNF from these cultures, indicating that TLR7 and TLR9 were of less consequence in this model. The key observation that indicated the importance of TLR8 was the inhibition of spontaneous TNF production by imiquimod, which we discovered to be an inhibitor of TLR8. Together, these data suggest that TLR8 may play a role in driving TNF production in RA. Because this receptor can be inhibited by small m.w. molecules, it may prove to be an important therapeutic target.


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3 Abbreviations used in this paper: RA, rheumatoid arthritis; RASF, RA synovial fibroblast; Mal, MyD88 adaptor-like; 5-HT, serotonin; polyIC, polyinosinic-polycytidylic acid.

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Rheumatoid arthritis (RA),3 a chronic autoimmune inflammatory disease affecting 1% of the population, is characterized by a destructive inflammation of the joints, leading to progressive disability and reduced life expectancy. The synovial membrane is infiltrated by activated immune cells, predominantly macrophages and T cells, resulting in the chronic production of proinflammatory cytokines and matrix metalloproteinases. In turn, these factors lead to inflammation and cartilage and bone degradation (1). The treatment of RA has been revolutionized by the development of biological therapies specifically targeting immune mediators. These include IL-1, IL-6R, B cells (anti-CD20), and activated T cells (CTLA4-Ig). Clinically, the most effective therapies are those that target TNF: infliximab; etanercept; and adalimumab (2).

Despite our understanding of the central role played by TNF in RA, it is still unclear what stimuli are involved in driving its chronic production in disease. Potential candidates for this role include ligands of the TLR family. TLRs form part of a network of receptors that detect pathogen-associated molecular patterns and alert the host to the presence of infection. The family of 10 human TLRs identified to date can be classified into 2 distinct groups based on cellular distribution and ligand repertoire. Cell surface expressed TLRs 1, 2, 4, 5, and 6 recognize ligands of bacterial and fungal origin, whereas TLRs 3, 7, 8, and 9 are expressed predominantly in the endosomal compartment and detect mainly viral products (3). The hallmark of TLR activation is the induction of a strong inflammatory response that is characterized by TNF production among many other mediators. In addition to microbial products, TLRs engage a number of endogenous molecules that can be produced during tissue damage (4) and are likely to be found at the sites of chronic inflammation. Candidate endogenous TLR ligands such as heat shock proteins, hyaluronan, and high-mobility group box protein-1 have all been detected in inflamed joints (5), and the expression of TLRs 2, 3, 4, and 7 have been reported in human RA tissue (6–8). Moreover, TLR3 ligands (generated from freeze-thawing synovial fluid cells to induce necrosis) have been shown to activate cultured RA synovial fibroblasts (RASF) (9). The concept of endogenous ligand-driven activation of TLR signaling has raised interest in these receptors as potential candidates in the induction and/or maintenance of chronic inflammatory conditions such as RA (10).

Our recent studies on TLRs in the human RA tissue model have shown that spontaneous production of cytokines from human RA synovial cell cultures is partly dependent on MyD88 and MyD88 adaptor-like (Mal) protein, intracellular adaptor proteins used by TLRs to engage the intracellular signaling network (11). However, there is still no clear evidence on which TLRs are actually contributing to the TNF production in human RA.

We became interested in reports that some serotonin (5-HT) receptor antagonists can inhibit LPS (TLR4 ligand)-induced TNF production from human monocytes (12) and other studies showing anti-inflammatory properties in animal models of inflammation. One particular example is mianserin, a tetracyclic 5-HT2A/C receptor antagonist which has been shown to decrease PMA-induced edema in the mouse (13) and B1 kinin receptor-induced paw edema formation in rodents (14).
In this study, we observed in primary human cells that mianserin did not inhibit TLR4 activation but was an inhibitor of the endosomal TLR 3, 7, 8, and 9-mediated cytokine production. Moreover, addition of mianserin significantly reduced the spontaneous TNF and IL-6 production from human rheumatoid synovial membrane cultures. Further studies suggested that the suppressive effect on TNF from the human RA membrane cultures might be mediated at least in part through the inhibition of TLR8.

**Materials and Methods**

**Reagents**

Cell culture reagents used were: penicillin-streptomycin, RPMI 1640, and DMEM obtained from Cambrex; indomethacin and cycloheximide from Sigma-Aldrich; and FBS from PAA. The TLR ligands used were chloroform-extracted *Escherichia coli*, LPS, resiquimod (R-848), loxoribine, ss-RNA (RNA40/LyOvec), CpG (oligodeoxynucleotide 2006), and imiquimod from Invivogen. Flagellin (purified) and Pam3Cys-Ser(Lys)4,3-CHCl (Pam3) were from Alexis. Meryerin hydrochloride was purchased from Sequoia Research Products. Chloroquine diphosphate salt was purchased from Sigma-Aldrich. The Abs used for FACS were FITC-conjugated anti-TLR3, anti-TLR8, and anti-TLR9 from Imgenex and an IgG1-FITC control from BD Pharmingen. The 25-D1.16 Ab used to detect SIINFEKL expression was a gift from Professor Ping Wang and Salah Mansour (Institute of Cell and Molecular Science, Queen Mary School of Medicine, University of London, London, U.K.). The Abs used for Western blotting were anti-tubulin Ab from Sigma-Aldrich; Abs recognizing phosphorylated forms of JNK (p46/p54), p38, and ERK (p42/44) were from Cell Signaling Technology. Human CD19 microbeads were purchased from MACS Miltenyi Biotech. All reagents were tested for LPS using the LAL test from Cambrex (15) and were found to have no detectable levels of LPS.

**Cell culture**

RA synovial membrane cells were isolated from patients undergoing joint replacement surgery as previously described (16, 17). Immediately after isolation, cells were used for mRNA analysis, stained by FACS, or cultured at 1 × 10⁶ cells/well in 96-well tissue culture plates (Falcon) in RPMI 1640 containing 5% (v/v) FBS and 100 U/ml penicillin-streptomycin. All patients gave written informed consent, and the study was approved by the local ethics committee. Primary human RASFs and PBMCs were isolated from Sequoia Research Products. Chloroquine diphosphate salt was purchased from Sigma-Aldrich. The Ab used for FACS were FITC-conjugated anti-TLR3, anti-TLR8, and anti-TLR9 from Imgenex and an IgG1-FITC control from BD Pharmingen. The 25-D1.16 Ab used to detect SIINFEKL expression was a gift from Professor Ping Wang and Salah Mansour.

**RT-PCR**

RNA was isolated using a Qiagen RNA Blood isolation kit (Qiagen) and then treated with TURBO DNase (Ambion) according to the manufacturer’s instructions. Total RNA was reverse transcribed with SuperScript II RNase H⁻ reverse transcriptase (Life Technologies) and oligo(dT)₃₄-µlmidlate primer. For human TLR3 amplification, the primers 5′-GCAAACA CAAAGCTCTTGAGATCT-3′ and 5′-TTGAAAGCTTTGGCGAAG CCA-3′ were used with an annealing temperature of 62°C. For human TLR7 amplification, the primers 5′-TCTACCTGGCGCCAACTGT-3′ and 5′-GCGACATGTGAAAGATCTTT-3′ were used with an annealing temperature of 60°C. For human TLR8 amplification, the primers 5′- CGCGACTTGGAGTTCTAGATC-3′ and 5′-AATGCTTCTTGGAT TGTGCT-3′ were used with an annealing temperature of 60°C. For human TLR9 amplification, the primers 5′-GTCCAGCCTTGGAGTCA-3′ and 5′-GGCAATGCTTGGAAATGTTG-3′ were used with an annealing temperature of 55°C. Amplification was performed in a Dyad PCR machine (MJ Instruments). Subsequent PCR amplification consisted of 35 cycles.

**Quantitative RT-PCR**

Steady-state levels of different endogenous mRNA transcripts were quantified by one-step real-time quantitative RT-PCR, after which 5 × 10⁸ macrophages were pretreated with mianserin or imiquimod for 30 min and then stimulated with 10 ng/ml LPS or 1 μg/ml R-848 for 2 h. Total RNA was then extracted using the RNeasy Blood (Qiagen) kits according to the manufacturer’s protocols. Real-time quantitative RT-PCR was performed on a StepOnePlus instrument (Applied Biosystems) using the TaqMan RNA-to-Ct 1-Step Kit (Applied Biosystems) and Assay-On-Demand pre-mixed TaqMan probe master mixes (Applied Biosystems). Each RNA sample was run in triplicate, and relative gene expression was calculated using the ΔCt/ΔCt method (21), with GAPDH as the comparator.

**Western blotting**

Human M-CSF macrophages were precultured with mianserin for 30 min before stimulation with TLR ligands used at previously determined optimum concentrations. Cell extracts were prepared in 100 μl of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 7.5) containing 10 mM EDTA, 10 mM EGTA, 1 mM Na₃VO₄, 5 mM NaF, and a protease inhibitor mixture (Sigma-Aldrich). Extracts were separated on 10% SDS-PAGE gels, and proteins were transferred to polyvinylidene difluoride membrane. Membranes were blocked in 2% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST) and sequentially probed with Abs recognizing phosphorylated forms of JNK (p46/p54), p38, and ERK (p42/44). Blots were stripped of Ab between analysis using Re-blot (Chemicon) and blocked again in 2% BSA-TBST.

**ELISA**

Sandwich ELISAs were used to measure TNF and IL-6 (BD Pharmingen). Optical density was read on a spectrophotometric ELISA plate reader (Lab-systems Multiscan Biochromic) and analyzed using Ascent software V2.6 (Thermo Labsystems). Cell viability was not significantly affected over this time period when examined by the MTT assay from Sigma-Aldrich (22).

**Luciferase assay**

Macrophages and RASFs were infected with a recombinant adenovirus containing an NF-κB luciferase reporter gene (kindly provided by Dr. B. Davidson, University of Iowa, Ames, IA) at a multiplicity of infection of 50. After 24 h, 2 × 10⁵ cells were stimulated with LPS for 6 h with filtered RA supernatants. The cells were washed once in PBS and lysed at 100 μl of CAT lysis buffer (0.65% (w/v) of Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0), and 150 mM NaCl). Fifty microliters of cell lysate were mixed with 120 microliters of luciferase assay buffer (25 mM Tris-phosphate (pH 7.8), 8 mM MgCl₂, 1 mM EDTA, 1% (v/v) Triton X-100, 1% (v/v) glycerol, 0.5 mM DTT, 0.5 mM ATP) in the well of a luminometer cuvet strip. Luciferase activity was measured with a Luminometer (Thermo Labsystems) by adding 30 μl of luciferin (Bright-Glo luciferase assay system; Promega) per assay point.

**Flow cytometry**

Cells were washed, then blocked with 10% human serum in PBS containing 0.01% azide for 30 min at 4°C, or for intracellular staining cells were fixed in 2% paraformaldehyde and permeabilized with 0.1% saponin (Sigma-Aldrich) before blocking. Cells were then incubated with FITC-conjugated anti-TLR3, anti-TLR8, anti-TLR9, or isotype controls for 1 h at 4°C and then washed before analysis on a BD Biosciences LSR flow cytometer.

**Statistical methods**

Mean, SD, SEM, and statistical significance were calculated using GraphPad version 3 (GraphPad Software). For statistical analysis, a one-tailed t test of paired data was used with a 95% confidence interval or a Wilcoxon matched-pairs two-tailed signed ranks test. SEM was used for pooled experimental data, whereas SD was used in graphs showing representative experiments. ***, p < 0.001; ***, p < 0.01; and *, p < 0.05.

**Results**

Mianserin inhibits TLR 3, 7, 8, 9, and 9-induced cytokine production in primary human cells

We tested the ability of mianserin to inhibit TLR function in primary human cells. Mianserin had no effect on TLR1/2, 4, or 5 activity (Fig. 1A) but was found to selectively inhibit R-848 (TLR7/8; Ref. 23) induced TNF production from human macrophages (Fig. 1A) in a dose-dependent manner (Fig. 1B). Further investigation in macrophages revealed that mianserin significantly inhibited the activation of NF-κB by R-848 but not in cells stimulated with LPS (Fig. 1C). Mianserin also inhibited the expression
of the TNF message induced by R-848 but not LPS (Fig. 1D). Activation of TLR3 (by polyinosinic-polycytidylic acid (poly(IC)) does not induce TNF production by human macrophages but does induce a robust IL-6 response from RASFs (24). Mianserin was able to inhibit TLR3-induced IL-6 production in RASFs (Fig. 1E). Primary human macrophages and RASFs do not respond to TLR7 or TLR9 ligands, but B cells do produce IL-6 in response to imiquimod and CpG DNA (25). Mianserin significantly inhibited TLR7- and 9-induced production of IL-6 from B cells (Fig. 1F). Cell viability was measured after all experiments by MTT assay, and no toxicity was observed (data not shown).

**Mianserin inhibits early receptor-associated signaling events in human macrophages and is a competitive inhibitor of R-848**

We decided to investigate the effect of mianserin on early signaling events from TLR8 using R-848 as a control. These TLRs were chosen because they are the most potent inducers of TNF in macrophages. We examined activation of the MAPK families because these are one of the earliest intracellular signaling events induced by TLR activation and they are required for activation of TNF (26). Fig. 2A shows that maximal phosphorylation of p38 MAPK is observed 15 min post-LPS stimulation. This occurs slightly later at 30 min post-R-848 activation, as might be expected given the intracellular localization of the TLR8. Mianserin dose dependently inhibited the R-848 but not the LPS-induced phosphorylation of p38, p54 JNK, and p42/44 Erk (Fig. 2B). These data are consistent with what would be expected for an inhibitor working at the ligand-binding site or a closely associated event. To investigate the mechanism further, increasing concentrations of R-848 were added to macrophages in combination with a fixed dose of mianserin. The results revealed that the inhibitory effect of mianserin decreased with increasing concentrations of R-848 (Fig. 2C).

**Inhibitory effects of mianserin suggest a role for TLRs in TNF and IL-6 production from RA synovial membrane cultures**

Given the inhibitory effect of mianserin on TLRs 3, 7, 8, and 9, we were interested in what effect this drug would have on TNF production in the RA synovial cultures. These cultures are mixed populations of cells that spontaneously release cytokines (11) without the need for exogenous stimulation and are considered an accepted model of human disease. This model was used for the initial studies that identified the importance of TNF in RA (16).

Our previous study (11) suggested a role for TLR signaling in driving TNF and IL-6 production in RA synovial membrane cultures. In light of the data in Fig. 1, and because we were unable to find evidence for a contribution from TLR2 or TLR4, we investigated mianserin in this model. Mianserin was found to dose dependently inhibit spontaneous production of TNF and IL-6 from RA synovial membrane cultures without any effect on cell viability...
Chloroquine and its derivatives are used to treat chronic inflammatory conditions such as RA, although the mechanism of action is unclear (27). They inhibit endosomal acidification, a prerequisite for activation of TLRs 3, 7, 8, and 9 (28–31). Because mianserin inhibits endosomal acidification, it was unclear which TLR(s) were being activated. Because mianserin is able to inhibit spontaneous cytokine release from RA cultures, we were interested in whether inhibition of the endosomal TLRs by chloroquine or mianserin would also inhibit these cytokines in RA synovial membrane cultures.

**Inhibitory effect of chloroquine supports a role for TLRs in TNF and IL-6 production from RA synovial membrane cultures.**

Chloroquine and its derivatives are used to treat chronic inflammatory conditions such as RA, although the mechanism of action is unclear (27). They inhibit endosomal acidification, a prerequisite for activation of TLRs 3, 7, 8, and 9 (28–31). Because mianserin inhibits TLR3, 7, 8, and 9, which are localized to the endosome, and was able to inhibit TNF and IL-6 from RA cultures, we were interested in whether inhibition of the endosomal TLRs by chloroquine would also inhibit these cytokines in RA synovial membrane cultures. At 10 and 100 μM chloroquine, TNF production was inhibited by 51.4 ± 9.6% ($p = 0.0065$) and 82.3 ± 3.25% ($p < 0.0001$), respectively, and IL-6 by 46.1 ± 25.6% ($p = 0.0074$) and 89.8 ± 4.8% ($p = 0.0065$; Fig. 3C). These data are consistent with a role for these TLRs in TNF production from RA synovial membrane cultures.

**Mianserin inhibits activation of NF-κB stimulated by conditioned media from rheumatoid synovial cell cultures.**

We have previously shown that conditioned medium from RA synovial cell cultures contains a ligand(s) that activates NF-κB in human macrophages in a MyD88- and Mal-dependent manner (11), but it was unclear which TLR(s) were being activated. Because mianserin is able to inhibit spontaneous cytokine release from RA culture supernatants, we tested whether it could inhibit NF-κB activation induced by supernatants from RA cultures. Supernatants from rheumatoid cell cultures were collected from cultures after 24 h 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 and RASFs expressing a consensus sequence NF-κB reporter gene. NF-κB activity was measured as a readout as the supernatants used to stimulate the macrophages contained cytokines. The supernatants induced activation of NF-κB that was inhibited by mianserin by 74.7 ± 23.5% ($p = 0.1138$) in macrophages and by 66.7 ± 9.7% ($p = 0.0411$) in RASFs.

**Rheumatoid synovial membranes display a differential response to ligands for TLRs 3, 7, 8, and 9.**

Given the above data, we wanted to confirm the expression and function of TLRs 3, 7, 8, and 9 in RA synovium. Using RT-PCR (Fig. 4A) and FACS (Fig. 4B) when suitable Abs were available, RA synovial cells were examined for the expression of TLRs 3, 7, 8, and 9. All were expressed, and TLRs 3, 8, and 9 were predominantly intracellular in agreement with previous studies in other cell types. We have previously shown the expression of TLR2 and 4 in this tissue (11). The synovial membranes were also tested for the ability to respond to exogenously added TLR ligands. As shown in Fig. 5, R-848 (TLR7/8) produced the largest increase in...
TNF over the spontaneous levels seen in these membranes by 7-fold. Poly(IC) (TLR3) was less effective, significantly increasing the expression of TNF by 3-fold. CpG (TLR9) had no significant effect on TNF. However, unexpectedly imiquimod (TLR7; Ref. 32) significantly inhibited TNF production by 47.3 ± 27% ($p = 0.0202$; Fig. 5), but unlike mianserin it did not inhibit IL-6 production (data not shown).

**Imiquimod inhibits TLR8 activity on human macrophages**

The surprising observation that the TLR7 agonist imiquimod inhibited TNF in RA synovial membrane cultures warranted further investigation. Imiquimod is structurally very similar to R-848, having the same core structure but with a slight modification to the side chains (Fig. 6A). We therefore considered the possibility that imiquimod could act as an antagonist of TLR8 activity. Imiquimod inhibited TNF production induced by R-848 (Fig. 6B), an effect that was dose dependent (Fig. 6C) and statistically significant over multiple donors. In contrast, the drug had no effect on TNF production induced from macrophages by LPS (TLR4), Pam3 (TLR1/2), or flagellin (TLR5; Fig. 6B). Further investigation revealed that imiquimod inhibited R-848 but not LPS-induced expression of the TNF message in macrophages (Fig. 6D). TLR3 induction of IL-6 from RASF was also not inhibited by imiquimod (Fig. 6E).

**The TLR7 ligand loxoribine does not inhibit TLR8 production from RA synovial membrane cultures**

Activation of TLR7 by imiquimod could account for the inhibition of TLR8 signaling. To investigate this possibility, we performed similar experiments in human macrophages and RA synovial membrane cultures using a different TLR7 ligand loxoribine which has less structural similarity to R-848 (Fig. 7A). Loxoribine did not inhibit TNF production from human macrophages induced by R-848 or LPS (Fig. 7B) and had no effect on the spontaneous release of TNF from the RA membrane cultures (Fig. 7C). Alternatively, we also sought to determine whether both imiquimod and mianserin could inhibit an activator of TLR7/8 other than R-848. We used ssRNA and found that both mianserin and imiquimod were able to inhibit TNF production in human macrophages (Fig. 7D).
Mianserin inhibits SIINFEKL presentation on the surface of EG7 cells

Mice expressing a mutation in the endosomal protein UNC93b1 have been demonstrated to have a defect in activation of TLRs 3, 7, and 9 and in the presentation of OVA SIINFEKL peptide on the cell surface of dendritic cells (33). Because mianserin is able to inhibit activation of the same TLRs, UNC93b1 is an attractive candidate target for mianserin. Because it was not possible to investigate a direct interaction between mianserin and UNC93b1, instead we examined whether mianserin would also affect SIINFEKL peptide presentation in murine EL4 cells transfected with chicken OVA cDNA (EG.7 cells). SIINFEKL presentation was measured by FACS. Cycloheximide was used as a control to prevent expression of OVA in EG.7 cells. Mianserin dose dependently inhibited cell surface expression of SIINFEKL peptide, reaching statistical significance at 30 μg/ml mianserin (Fig. 8).

Discussion

TLRs have been suggested to potentially contribute to the pathogenesis of many autoimmune diseases and have thus become prime candidates as targets for new therapeutics (34). In this study, we set out to investigate the anti-inflammatory effect of mianserin and have identified this drug as an inhibitor of the endosomal TLRs 3, 7, 8, and 9 in primary human cells. Mianserin also inhibited the spontaneous release of TNF and IL-6 from RA synovial membrane cultures. Furthermore, these results were supported by the actions of chloroquine, an inhibitor of the endosomal TLRs, which also inhibited TNF and IL-6 in RA cultures and by imiquimod (shown here to be a TLR8 inhibitor), which also significantly inhibited TNF production in the RA synovial membrane cultures, thus suggesting a potential role for endosomal TLRs and in particular TLR8 in RA.

There are several observations from in vivo inflammatory models that mianserin exhibits anti-inflammatory activity (13, 14); however, the mechanism is not known. Our data indicate that the drug blocks the function of the intracellularly located TLR3, TLR7, TLR8, and TLR9 but not the cell surface-located TLRs 1/2, 4, and 5. A potential mechanism by which mianserin may have inhibited the TLRs was possibly through its known effect on 5-HT receptors, but this seems unlikely, given that ~1 nM mianserin (35) is effective on 5-HT receptors whereas between 1 and 10 μM is required to inhibit TLRs and TNF production from RA cultures. Mianserin has also been reported to increase cGMP levels, an event that can be inhibitory to TNF production; however, elevated cGMP inhibits LPS-induced TNF (36), which was not observed in our studies. If this were the mechanism, mianserin should have inhibited TNF from all TLRs which was not observed.

Changes in receptor expression did not account for the inhibition of TLR signaling by mianserin. Protein expression of TLRs 3 and 8 were unaffected by pretreatment (30 min) of macrophages with mianserin (data not shown). The more likely mechanism by which mianserin is operating is through an off-target effect of the drug. Mianserin blocked early signaling pathways and was able to compete with R-848, suggesting that its inhibitory action was close to the receptor level. The mechanism could be through a direct interaction with TLR8, but because it also inhibited TLR3, 7, and 9 it seems more likely that mianserin interacts with a shared accessory receptor or protein that is required for these receptors to signal. A relevant example comes from a murine study in which they generated mice expressing a mutant version of the UNC93b1 protein. Activation of TLRs 3, 7, and 9 was found to be defective (33), and this was later shown to be due to those TLRs no longer being trafficked from the endoplasmic reticulum to the endosomes (37). Tabeta et al. (33) also demonstrated a defect in the presentation of OVA SIINFEKL peptide on the cell surface of dendritic cells carrying the mutation. We examined whether mianserin would also have a similar effect on Ag presentation. Using EG.7 cells, we observed that mianserin dose dependently inhibited cell surface presentation of SIINFEKL peptide. These data support the idea that UNC93b1 may perhaps be a target for mianserin.

Given the growing awareness of the potential for TLRs to promote inflammation in autoimmunity (10, 34) and our previous studies from human RA synovial cultures that indicated a role for TLR signaling adaptors, the ability of mianserin to inhibit TLRs 3, 7, 8, and 9 made it a prime candidate to investigate in these cultures. Examination of the endosomal TLRs in RA synovial membrane cultures revealed that activation of TLR3 and 8 but not TLRs 7 or 9 increased production of TNF. The lack of TNF after TLR7 and 9 stimulation was not surprising in that responsiveness in human systems is mainly confined to plasmacytoid dendritic cells for the production of IFN (38), whereas the majority of TNF production in the rheumatoid synovium is considered to be produced by the monocytes/macrophages (39). All of these TLRs were expressed with TLR3 and 8 being predominantly intracellular, consistent with the belief that these receptors are mainly endosomal. Preliminary data indicated that TLR3 and 8 were expressed on CD68 "TNF" cells from the RA synovial membranes (data not shown). Addition of chloroquine to these cultures significantly decreased the spontaneous release of TNF and IL-6, supporting the hypothesis that chloroquine treatment is beneficial in RA due to inhibition of endosomal TLRs (40). Mianserin also significantly inhibited TNF and IL-6 from these cultures supporting a role for these TLRs in RA.

In a previous study, we demonstrated that conditioned medium collected from RA cultures contained a potential TLR ligand(s) (11). Mianserin inhibited activation of NF-κB in macrophages stimulated with conditioned medium from RA cultures, suggesting there may be a role for the endosomal TLRs, but probably not TLR3, since poly(IC) activation of TLR3 does not activate NF-κB in human macrophages (24). Activation of NF-κB in RASFs by conditioned supernatants was also inhibited by mianserin. TLRs 7, 8, and 9 are not expressed in RASFs (41), thus suggesting the possibility of a TLR3 ligand in the medium. This hypothesis is supported by the observations of Brentano et al. (9), who demonstrated how apoptotic synovial fluid cells release an endogenous ligand that can stimulate RASFs via TLR3.

Further data indicating a role for TLRs 3 and/or 8 came from a short oligodeoxynucleotide. A recent study has shown that phosphothioate modification of oligodeoxynucleotides transforms them...
to become inhibitors of TLRs 7 and 9 and that this inhibitory action is sequence independent instead requiring the modification of the sugar backbone (42). We identified a phosphothioate oligodeoxynucleotide that inhibited TLRs 3 and 8 in primary human cells and was also able to significantly inhibit spontaneous TNF production from the RA synovial membrane cultures (data not shown). In agreement with the publication by Hass et al., this effect appeared to be sequence independent making it hard to control for experimentally.

The key observation that indicated TLR8 as being important in driving TNF in RA synovial cultures came from the TLR7 agonist imiquimod (32). We discovered that imiquimod could inhibit the activation of TLR8, conceivably due to the similarities in the core structure of R-848 and imiquimod. The ability of imiquimod and mianserin to inhibit TLR8 in macrophages was not restricted to R-848 stimulation; similar results were observed with a more physiologically relevant TLR7/8 ligand ssRNA. The ability of imiquimod to inhibit TLR8-induced TNF did not appear to be mediated through activation of TLR7, given that another TLR7 agonist loxoribine was unable to do this. When added to RA synovial membrane cultures, imiquimod inhibited the spontaneous release of TNF by half whereas loxoribine had no effect. Interestingly, unlike mianserin, imiquimod did not inhibit IL-6 production in RA cultures, which may reflect that IL-6 is being produced by one of the other TLRs inhibited by mianserin, possibly TLR3. Activation of TLR3 on synovial fibroblasts leads to high levels of IL-6 production (24).

In the human RA synovial membrane culture system we have previously demonstrated that overexpression of dominant negative versions of MyD88 and Mal suppressed the expression of TNF and IL-6. Although MyD88 is used by all TLRs (except TLR3), Mal is only used by TLR2 and 4 (11). However, we found that neutralizing Abs to TLR2 and 4 failed to inhibit cytokine production in human RA synovial membrane cultures (43). Taken together, the data with mianserin, chloroquine, and imiquimod suggest that TLR8 may be an important contributor to TNF production in human disease. A role for TLR8 is consistent with our previous study with dominant negative MyD88, because this construct also blocks TLR8 function in human macrophages (43). TLR7 and TLR9 do not appear to be involved in this RA model because they are unable to stimulate TNF production.

The other target of mianserin, TLR3, does induce TNF production in RA synovial membrane cultures, and previously we have observed that stimulation of TLR3 can induce low levels of TNF production from RASFs, a major constituent of these cultures (24). A role for TLR3 could explain the unaccounted effect of the Mal dominant negative, because unlike the other TLRs, TLR3 does not use MyD88 but TLR3 signaling in RASF can be blocked by the Mal dominant negative (manuscript in preparation). However, a role for Mal still leaves the possibility of a contribution from TLR2 or 4. Investigation of the precise contribution of TLRs 2, 3, and 4 to the stimulation of TNF and IL-6 production will require more specific reagents. We have been unable to achieve small interfering RNA-targeted knockdown of genes in mixed populations of primary cells as found in the RA synovial membrane cultures.

In summary, these data suggest that TLR8 may be an important contributor to TNF production in RA with a potential contribution from TLR3, although they are certainly not the only factors. Unlike systemic cytokine blockade, inhibiting TLR8 should not lead to an impairment of signaling by the other TLRs or the total inhibition of cytokine function by other signaling mechanisms. Because TLR8 is amenable to inhibition with small m.w. molecules, this receptor may provide a new target for the treatment of RA, bringing potential benefits in safety, in addition to the lower cost and ease of administration provided by oral drugs.

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Disclosures

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