EXTENDED REPORT

SNAPIN: an endogenous toll-like receptor ligand in rheumatoid arthritis

Bo Shi, QiQuan Huang, Paul Peter Tak, Margriet J Vervoordeldonk, Chiang-Ching Huang, Andrea Dorfleutner, Christian Stehlik, Richard M Pope

ABSTRACT

Objective The mechanisms contributing to the persistent activation of macrophages in rheumatoid arthritis (RA) are not fully understood. Some studies suggest that endogenous toll-like receptor (TLR) ligands promote the chronic inflammation observed in RA. The objective of this study was to identify endogenous TLR ligands expressed in RA synovial tissue (ST) based on their ability to bind the extracellular domains of TLR2 or TLR4.

Methods A yeast two-hybrid cDNA library was constructed from ST obtained by arthroscopy from patients with RA and screened using the extracellular domains of TLR2 and TLR4 as the bait. Interactions between TLRs and Snapin were demonstrated by reciprocal co-immunoprecipitation. ST was examined by histology, and single- and two-colour immunohistochemistry and quantitative reverse transcriptase PCR. Snapin (SNAP – associated protein) expression in macrophages was examined by Western Blot analysis and confocal microscopy. The ability of Snapin to activate through TLR2 was examined.

Results Employing a yeast two-hybrid system, Snapin was the most frequently identified molecule that interacted with TLR2. These results were confirmed by pull-down of in vitro-expressed Snapin together with TLR2. By immunohistochemistry and quantitative reverse transcriptase PCR, Snapin was highly expressed in RA ST, and it was readily detected in macrophages, where it co-localised in the late endosomes. ST Snapin expression correlated with inflammation and was not disease specific. Finally, Snapin was capable of activating through TLR2.

Conclusion These observations identify Snapin as a novel endogenous TLR2 ligand in RA, and thus support a role for persistent TLR2 signalling in the pathogenesis of RA.

INTRODUCTION

Macrophages are critical to the destruction of cartilage and bone observed in rheumatoid arthritis (RA). RA synovial tissue (ST) macrophages express high levels of tumour necrosis factor α (TNFα), interleukin 1β (IL-1β), granulocyte-macrophage colony stimulating factor, IL-6, IL-8, collagenase (MMP1) and stromelysin (MMP3), all mediators of inflammation and joint destruction. Supporting a central role for macrophages, inhibition of TNFα or IL-1β suppresses synovitis and joint destruction in RA. Further, the number of macrophages in the sublining of RA ST correlates with clinical response. Although the mechanisms contributing to the persistent activation of macrophages are not clear, in addition to immune complexes, other potential mechanisms have been described, including the activation of toll-like receptors (TLRs).

TLRs are pattern-recognition receptors, best characterised for their role in activation of monocytes, macrophages and dendritic cells. TLR2 and TLR4 were both expressed in the RA joint macrophages when ST was examined by immunohistochemistry. We recently documented the increased expression of TLR2 and TLR4 on RA synovial fluid (SF) macrophages and the increased response of these macrophages to microbial TLR2 and TLR4 ligands. In RA, inflammation results in joint damage and the expression and release of a variety of molecules that may be capable of acting as endogenous TLR2 and TLR4 ligands, including heat shock protein (HSP) 60 and 70, extra domain A fibronectin, and High Mobility Group Box chromosomal protein 1 (HMGB1). Recent data suggests a role for the 96-kDa glycoprotein 96 (gp96), an endoplasmic reticulum stress response protein, and the extracellular matrix glycoprotein tenasin C as potential endogenous TLR2 and TLR4 ligands in RA. However, none of these previously identified endogenous TLR ligands were discovered based on their local expression in RA ST and the ability to bind TLR2 or TLR4.

To identify endogenous TLR ligands in RA, we generated a cDNA library constructed with RA ST using the extracellular domains of TLR2 and TLR4. We identified Snapin (SNAP – associated protein), which is important in endoplasmic reticulum stress response and late endosomal transport in neurons, as potential endogenous TLR2 and TLR4 ligands in RA. However, none of these previously identified endogenous TLR ligands were discovered based on their local expression in RA ST and the ability to bind TLR2 or TLR4.

To identify endogenous TLR ligands in RA, we generated and screened a yeast two-hybrid library constructed with RA ST using the extracellular domains of TLR2 and TLR4. We identified Snapin (SNAP – associated protein), which is important in endoplasmic reticulum stress response and late endosomal transport in neurons, as potential endogenous TLR2 and TLR4 ligands in RA. However, none of these previously identified endogenous TLR ligands were discovered based on their local expression in RA ST and the ability to bind TLR2 or TLR4.

MATERIALS AND METHODS

Patients

To synthesise a cDNA library, RA ST from 8 patients with active disease (online supplementary table 1) were obtained at the time of arthroscopy. Patients with RA were diagnosed according to the American College of Rheumatology classification criteria. Additional ST was obtained at the time of joint arthroplasty on patients with RA (n=7),
Figure 1  Snapin co-purifies with TLR2. (A, B) HA-tagged Snapin were incubated with Fc, TLR2-Fc and TLR4-Fc recombinant proteins. Fc and TLR-Fc were purified by immobilised protein-G agarose and Snapin was detected by anti-HA antibody following SDS-PAGE. Both, full-length Snapin (amino acids 1–136) (A) and Snapin (60–136) (B) were used. The lower panels show that equivalent quantities of Fc, TLR2-Fc and TLR4-Fc were loaded and purified by protein-G agarose beads. C. A reciprocal co-isolation of His-tagged Snapin with TLR2 and TLR4 was performed by purifying His-tagged Snapin on TALON magnetic beads. Co-purified Fc and TLR-Fc fusion proteins were detected by anti-Fc antibody (top panel). The bottom panel demonstrates that equivalent amounts of His-tagged Snapin were purified. The results presented are representative of two independent experiments. SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TLR, toll-like receptor.

Yeast two-hybrid assay
Yeast two-hybrid screening was performed using the matchmaker yeast two-hybrid system3 (Clontech, Mountain View, CA, USA). The bait constructs were generated by inserting cDNAs encoding the extracellular domains of human TLR2 (amino acids 18–587), TLR4 (amino acids 25–682), COOH-terminal truncated TLR2 (amino acids 18–453) or TLR4 (amino acids 25 to 419) in frame with the GAL4 DNA binding domain of pGBKT-7, and were transformed into AH109 yeast. For the RA cDNA library, cDNAs were amplified from pooled mRNAs isolated from 8 RA STs, cloned into pGADT7-RecAB, and transformed into Y187 yeast. Mating was performed between AH109 and Y187. Positive yeast colonies were selected twice by X-galactosidase activity and leucine, tryptophan, histidine and adenine auxotrophy. Plasmid DNA was sequenced and TLR-αShi B, Huang Q-Q, Tak PP, et al. Ann Rheum Dis (2012). doi:10.1136/annrheumdis-2011-200899

osteoarthritis (OA, n=8), and from individuals with no apparent arthritis (arthritis-free controls, n=7), at the time of surgery or autopsy, were from Northwestern Memorial Hospital or the National Disease Research Interchange. SFs were obtained from patients with inflammatory arthritis (online supplementary table 2) at the time of therapeutic arthrocentesis.

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Cross-purification assay
TLR2-Fc, TLR4-Fc and the Fc control fusion proteins were generated by cloning of the extracellular domains of human TLR2, TLR4 or the signal peptide of TLR2, as the Fc control, into pcDNA3-His (Invitrogen, Grand Island, NY, USA). All proteins were stably expressed in CHO cells.17 HA-tagged recombinant full-length Snapin (amino acids 1–136) and Snapin (60–136) (amino acids 60–136) were in vitro transcribed and translated (TNT, Promaga, Madison, WI, USA). Co-purification was performed using protein G-bound TLR2-Fc, TLR4-Fc and Fc control incubated with in vitro-translated Snapin and Snapin (60–136). Resulting protein complexes were washed, subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and identified with anti-HA (Cell Signalling, Danver, MA, USA) and anti-human Fc (R&D Systems, Minneapolis, MN, USA) antibodies. Reciprocal experiments employed whole CHO cell lysates containing His-Snapin incubated with CHO supernatants containing Fc, TLR2-Fc or TLR4-Fc. His-tagged Snapin was affinity purified on TALON magnetic beads (Clontech), and co-purified Fc, TLR2-Fc or TLR4-Fc were separated by SDS/PAGE and detected by anti-Fc antibodies.

Immunochemistry, immunoblot analysis, macrophage isolation, immunofluorescence microscopy and detection of Snapin in SF
The details of these procedures are presented in the online supplementary materials.

TLR2 Activation
The human embryonic kidney 293 (HEK-293) cell line stably expressing human TLR2, and a nuclear factor κB (NF-κB) regulated reporter gene (HEK-TLR2)(InvivoGen) was employed to identify TLR2-activating ligands. HEK-TLR2 cells (1×10^5/well) were seeded into 96 well plates and incubated with recombinant Snapin (0–10 μg/ml) (Prospec, Renouot, Israel) for 16 h at 37°C. Where indicated, cells were preincubated with neutralising anti-TLR2 antibody (5–10 μg/ml) for 10 min before adding Snapin, or Snapin was denatured by heat or proteinase K digestion as previously described.17

Activation of macrophages by RA SF
RA SFs were diluted 1:4 then preincubated with monoclonal anti-Snapin antibody or immunoglobulin G (IgG) isotype control (10 μg/ml) for 1 h. The fluids were added to in vitro-differentiated macrophages for 4 h. The cells were isolated, the RNA extracted employing Trizol and quantitative reverse transcriptase PCR (qRT-PCR) was performed to detect TNFα mRNA expression.

Statistical analysis
Experimental data are presented as the means±SE. Comparisons between three or more groups were performed by one-way analysis of variance, followed by the Tukey test. Comparisons between two groups unevenly distributed were performed by the Mann–Whitney U test. The statistical differences between
matched pairs were determined by the paired t test. Pearson’s product-moment correlation was used to define the relationship between variables.

RESULTS

Yeast two-hybrid screen

To identify novel endogenous TLR2 and TLR4 ligands, RNA was isolated from the tissues of eight patients with active RA, transcribed into cDNA and cloned in frame with the GAL4 activation domain. This library was expressed in yeast and mated to yeast expressing the extracellular domains of TLR2 or TLR4 linked to the GAL4 binding domain. With the yeast two-hybrid system, a total of 60 coding sequences representing 11 unique proteins were identified by their interaction with TLR2, while 55 sequences, representing 34 unique proteins interacted with TLR4, including COPS5 and ATP1B3, which were frequently identified. The most frequently identified TLR-interacting molecule was Snapin, a SNARE (soluble-N-ethylmaleimide – sensitive factor accessory protein (SNAP) receptor) complex protein originally described in neurons. Of all coding sequences encoding potential TLR2 interacting proteins, Snapin was identified 33 times, thus representing 55% of all TLR2-binding clones, but it represented only 13% of those interacting with TLR4.

Snapin binds to TLR2

In order to confirm that Snapin specifically interacts with TLR2, either full-length Snapin or Snapin (60–136), lacking the NH₂ terminal region were in vitro expressed and subjected to a binding assay. Recombinant TLR2-Fc, TLR4-Fc and Fc were immobilised on protein-G agarose and incubated with full-length Snapin or Snapin (60–136). Both full-length Snapin and Snapin (60–136) co-purified with TLR2-Fc, but not TLR4-Fc or Fc control.

Figure 2

High expression of Snapin is in RA ST. Snapin expression in RA (F), OA (D) and arthritis-free control (B) STs was examined by immunohistochemistry using mouse monoclonal anti-Snapin antibody, and compared with a mouse IgG isotype control antibody (A, C, E). Scale bar (20 μm) is shown at the bottom right corner, and original magnification is 40× objective lens for all photos. (G) Inflammation score, lining thickness and the number of blood vessels for RA (n=7), OA (n=8) and arthritis-free control (n=7) STs. (H) Percentage of Snapin-positive cells in the lining and sublining of RA, OA and control STs. (I) Snapin expression score was calculated as the product of the intensity of Snapin staining and the percentage of Snapin-positive staining cells. * represents p<0.05, and ** p<0.01 for RA compared with arthritis-free control STs. # represents p<0.05 for RA compared with OA STs. The values in panels G–I represent the mean±1 SEM. IgG, immunoglobulin G; OA, osteoarthritis; RA, rheumatoid arthritis; ST, synovial tissue.
Basic and translational research


In reciprocal experiments, His-tagged Snapin purified using TALON magnetic beads demonstrated interaction with TLR2-Fc, but not TLR4-Fc or the Fc control (figure 1C). Therefore, Snapin specifically binds to TLR2, and this interaction requires the C-terminus (aa 60–136) of Snapin.

Snapin is expressed in RA ST

Studies were performed to determine if Snapin could be detected in RA ST by immunohistochemistry. Snapin was readily detected in RA ST (figure 2E,F), compared with arthritis-free control and OA STs (figure 2A-D). There was no difference in the lining thickness between the groups; however, the inflammation score (p<0.05–0.01) was increased in the RA compared with the control and OA STs, while blood vessels were increased in RA compared with the arthritis-free controls (p<0.05) (figure 2G). The percentage of Snapin-positive cells and the Snapin expression score, reflecting the percent positive and intensity of staining, were both significantly (p<0.05–0.01) increased in the sublining of the RA STs compared with the OA or arthritis-free controls (figure 2H,I). In the lining, the Snapin staining was not significantly different between any of the clinical groups. Although there was no significant correlation when the RA STs were examined alone, when all the tissues were examined together, Snapin in the sublining correlated with blood vessels (r=0.57–0.61, p<0.01) (online supplementary figure 1B). Therefore, Snapin is significantly increased in RA compared with arthritis-free and OA STs, and its expression correlated with inflammation and angiogenesis, and its expression is not disease specific.

Figure 3 Snapin is highly expressed in RA ST macrophages and in vitro-differentiated macrophages. Two-colour immunohistochemistry staining was performed employing anti-Snapin (brown) and anti-CD68 (red) antibodies. Representative figures of double staining for one RA ST are shown in (A), which is representative of 2 individual RA STs. The original magnification used is 20× (left) and 60× (right) objective lens, and the scale bar is shown in the bottom right corner of each panel. The expression of Snapin protein was determined by Western Blot analysis employing 3 sets of (B) normal or (C) RA peripheral monocytes (Mono) and the in vitro-differentiated macrophages (MDs) derived from these monocytes (top panel). β-actin (lower panel) served as the loading control. (D) Co-localisation of Snapin and Rab7, a late endosomal marker in macrophages, was examined by immunofluorescence microscopy. Nuclear staining (Hoechst, blue), Snapin (Green), Rab7 (red) and a merged image are presented from left to right. OA, osteoarthritis; RA, rheumatoid arthritis; ST, synovial tissue.
Snapin is detected in RA SFs
Since TLR2 is expressed on the cell surface, SFs were examined for the presence of extracellular Snapin. By Western Blot, Snapin was detected in the majority of RA SFs examined; however, the level of expression was variable (online supplementary figure 2). Although it was not detected in the four OA or five psoriatic arthritis SFs (data not shown), Snapin was present in the SF of a patient with reactive arthritis (online supplementary figure 2), demonstrating that Snapin in SF is not specific for RA.

Snapin activates TLR2
Since Snapin interacted with TLR2, studies were performed to determine if Snapin is a functional TLR2 agonist. Recombinant Snapin was added to HEK-TLR2 cells in a dose-dependent manner, as indicated. Fold TLR2 activation was calculated by comparison with a non-Snapin-treated control. * represents p<0.05 compared with the no-Snapin control. Snapin was heat-inactivated or pre-treated with proteinase K, before addition to HEK-TLR2 cells. ** represents p<0.01 compared with Snapin alone. HEK-TLR2 cells were pre-incubated with neutralising TLR2 antibodies or IgG control, before Snapin was added, as indicated. * represents p<0.05 compared to the Rat-IgG control. The data in this figure represents the mean±1 SEM of three independent experiments performed in duplicate. HEK, human embryonic kidney; IgG, immunoglobulin G; TLR, toll-like receptor.

Suggesting that TLR2 stimulation was due to Snapin and not a potential microbial contaminant. Further, addition of TLR2 neutralising antibodies, but not control IgG, significantly and dose-dependently suppressed the ability of Snapin to activate HEK-TLR2 cells, demonstrating that the activation was mediated through TLR2.

Experiments were performed to determine if Snapin present in RA SFs was capable of activating macrophages. From another study, four RA SFs were identified based on their ability to activate in vitro-differentiated macrophages determined by the induction of TNFα mRNA. This activation was suppressed by 77±9% (SE) by preincubating the macrophages with neutralising anti-TLR2 antibodies (data not shown). To determine if Snapin might contribute to the observed macrophage activation, the SFs were preincubated with a monoclonal anti-Snapin antibody. The activation of macrophages by three of the four SFs was suppressed by the anti-Snapin antibody a mean of 47±2% (p=0.03) compared with isotype controls (figure 6). When all four fluids were included, the reduction was 44±3% (p=0.09). The SF that did not suppress was from a patient who had recently developed a lymphocytic lymphoma and that fluid only resulted in a 4.3-fold activation of macrophages, which may have limited the ability to observe meaningful change.
**DISCUSSION**

Employing a yeast two-hybrid system, Snapin was the most frequently identified RA ST-expressed protein interacting with TLR2. Snapin was originally described in neurons isolated by its ability to bind to SNAP-25, a member of the SNARE family. The SNARE family consists of at least 38 members that are critical for the transport of vesicles within cells, promoting the docking of lipid bilayers. Snapin-deficient neurons demonstrate defects in synaptic vesicle fusion which impairs the synchronisation of neurotransmitter release. Further, Snapin is critical for neuronal homeostasis through its role in promoting trafficking of late endosomes to lysosomes mediated through interaction with dynein, resulting in reduced lysosomal-degradative capacity. The role of Snapin in macrophages is unclear, although Snapin does co-localise with Rab7 in macrophages, thus indicating its presence within the late endosomes.

The results demonstrate that although Snapin is highly expressed in RA, it is not specific for the disease. Some OA STs also expressed Snapin, and Snapin was detected in the SF of a patient with reactive arthritis. Snapin was readily detected in control and RA in vitro-differentiated and RA ST macrophages and was present in RA ST T cells, consistent with an earlier study identifying Snapin in T cells. Further, Snapin has been detected in a variety of tissues in addition to brain. Even though Snapin expression correlated with inflammation histologically and clinically, incubation of normal macrophages with TNFα or microbial TLR2 or TLR4 ligands failed to further induce the expression of Snapin (not shown). The mechanism by which inflammation induces Snapin expression remains to be determined.

The approach used in this study was novel, based on screening employing the extracellular domains of TLR2 and TLR4 to identify interacting proteins expressed by an RA ST cDNA library. Earlier studies using a candidate molecule approach, identified proteins highly expressed in the RA joints as those that were capable of activating TLR2, and TLR4, including HSP60, HSP70 and HMGB1. However, these studies did not document the ability of the potential endogenous ligands to bind to TLR2, or TLR4, and other investigators were not able to reproduce TLR activation employing very low endotoxin HSP60 and 70. Recombinant serum amyloid A (SAA) was shown to directly interact with and activate through TLR2. While SAA is highly expressed in the circulation of patients with RA, in contrast to recombinant SAA, SAA isolated from the circulation of patients with RA did not activate peripheral blood mononuclear cells. We previously demonstrated that gp96, which is highly expressed in RA ST, was also capable of binding to and activating TLR2. In summary, although a number of potential endogenous TLR2 ligands have been described in RA, not all have been shown to bind and activate TLR2.

In the current study, Snapin was capable of binding TLR2, and recombinant Snapin also specifically promoted TLR2-mediated NF-κB activation. These observations, and the fact that Snapin was identified extracellularly in SF support a role of Snapin as an endogenous TLR2 ligand that is capable of contributing to the pathogenesis of RA. Since it is possible that microbial contamination might contribute to HEK-TLR2 activation, in addition to employing commercially available recombinant Snapin (figure 5), we expressed and purified (His)6-tagged recombinant Snapin, which was isolated and extensively washed employing nickel affinity sepharose. While the level of endotoxin measured in this preparation of Snapin was below that capable of activating HEK-TLR4 cells, it activated HEK-TLR2 cells (not shown). Previous studies demonstrated that heat denaturation and treatment with protease K did not significantly reduce the ability of known microbial TLR2 ligands Staphylococcus aureus lipoteichoic acids and Escherichia coli 0111:B4 peptidoglycans to activate through TLR2. In the current study, heat denaturation and protease K digestion prevented Snapin-mediated TLR2 activation. These observations suggest that the activation of HEK-TLR2 cells was not due to a contaminating microbial ligand.

Employing the yeast two-hybrid screen, we did not detect any of the earlier identified potential TLR2 and TLR4 ligands that have been implicated in RA, including gp96. This may be related to the fact that the molecular weight of the cDNA synthesised from RA ST ranged from 0.8 to 2.3 kb, averaging 1.5 kb. Therefore, the library represented primarily smaller proteins, such as Snapin, or the COOH-terminal regions (including S' untranslated regions) of the larger proteins. It is possible that these regions did not contain the domains responsible for binding TLR2 or TLR4. It is also possible that the previously identified ligands may not interact with TLR2 or TLR4 in the yeast two-hybrid assay, or that they may not have been expressed by our cDNA library.

A number of observations support the role of TLR2 signalling in the pathogenesis of RA. Culture supernatants from RA STs contributed to macrophage activation, although additional TLR ligands and cytokines may also have contributed. In addition, we cannot exclude that Snapin in the SFs may have activated macrophages through a mechanism other than TLR2. Further supporting an important role for TLR2 signalling in RA, a recent study demonstrated that a neutralising monoclonal antibody to TLR2 suppressed the spontaneous release of cytokines from RA ST cultures. Collectively, these observations suggest that endogenous TLR2 ligands are present in inflamed RA ST, and that they are capable of contributing to the perpetuation of chronic inflammation, thus supporting a potential role for neutralising TLR2 signalling as a novel approach to therapy in RA.

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