Toll-like receptor-mediated IRE1α activation as a therapeutic target for inflammatory arthritis

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In rheumatoid arthritis (RA), macrophage is one of the major sources of inflammatory mediators. Macrophages produce inflammatory cytokines through toll-like receptor (TLR)-mediated signalling during RA. Herein, we studied macrophages from the synovial fluid of RA patients and observed a significant increase in activation of inositol-requiring enzyme 1α (IRE1α), a primary unfolded protein response (UPR) transducer. Myeloid-specific deletion of the IRE1α gene protected mice from inflammatory arthritis, and treatment with the IRE1α-specific inhibitor 4UGC attenuated joint inflammation in mice. IRE1α was required for optimal production of pro-inflammatory cytokines as evidenced by impaired TLR-induced cytokine production in IRE1α-null macrophages and neutrophils. Further analyses demonstrated that tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) plays a key role in TLR-mediated IRE1α activation by catalysing IRE1α ubiquitination and blocking the recruitment of protein phosphatase 2A (PP2A), a phosphatase that inhibits IRE1α phosphorylation. In summary, we discovered a novel regulatory axis through TRAF6-mediated IRE1α ubiquitination in regulating TLR-induced IRE1α activation in pro-inflammatory cytokine production, and demonstrated that IRE1α is a potential therapeutic target for inflammatory arthritis.

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Introduction

Rheumatoid arthritis (RA) is a chronic debilitating disease characterized by leukocyte infiltration, hyper-proliferation of synovial cells, and bone destruction. The elevated levels of toll-like receptors (TLRs) and their endogenous ligands significantly contribute to the initiation and progression of RA. TLRs were found to be highly expressed in macrophages and synovial fibroblasts from patients with RA (Seibl et al, 2003; Proost et al, 2004; Varoga et al, 2006; Ospelt et al, 2008; Yavuz et al, 2008; Kawai and Akira, 2011). Endogenous TLR ligands, such as fibrinogen, heat shock protein 22 (HSP22) (Roelofs et al, 2006), HSP60 (Ohashi et al, 2000), HSP70 (Asea et al, 2002), HSP96 (Huang et al, 2006), HSP60 (Ohashi et al, 2002), HSP96 (Huang et al, 2009) and the high mobility group box chromosomal protein 1 (HMG-1) (Pisetsky et al, 2008; van Beijnum et al, 2008; Wahamaa et al, 2011), are induced or released in the synovial tissue of patients with RA. Upon recognizing specific ligands, TLR signalling transduction is initiated by individual TLRs that recruit adaptor proteins, namely, myeloid differentiation primary response gene 88 (MyD88) or TIR (Toll/IL-1 receptor) domain-containing adaptor inducing interferon (IFN)-β (TRIF). MyD88 is recruited by most of the TLRs to transmit signals culminating in NF-κB and mitogen-activated protein kinase (MAPK) activation and the induction of inflammatory cytokines. Additionally, TLR3 and TLR4 utilize TRIF to activate an alternative pathway that leads to the activation of NF-κB and IRF3 as well as the induction of type I IFN and inflammatory cytokines, such as TNF-α, IL-1β, and IL-6. TLR4 is the only TLR that activates two distinct signalling pathways: the MyD88-dependent and TRIF-dependent pathways (Cao et al, 2008; Gilliet et al, 2008; Egan et al, 2009). Targeting of TLRs to treat RA has shown promise (O’Neill, 2003). Indeed, Chaperonin 10, a mitochondrial protein that inhibits TLRs through blocking their binding to DAMPs, was clinically effective in a small-scale study with RA patients (Vanags et al, 2006).

The endoplasmic reticulum (ER) is an intracellular organelle responsible for protein folding and assembly, lipid and sterol biosynthesis, and calcium storage. Pathophysiological states that increase the demand for protein folding, or stimul...
that disrupt protein folding, create an imbalance between the protein-folding load and capacity of the ER, causing the accumulation of unfolded or misfolded proteins in the ER lumen, a condition referred to as ‘ER stress’ (Kaser et al., 2011). To cope with ER stress, the ER has evolved a group of signal transduction pathways, that is, the unfolded protein response (UPR) pathways, mediated by three major ER transmembrane protein factors, including PKR-like ER kinase (PREEK), inositol-requiring enzyme 1α (IRE1α), and activating transcription factor 6 (ATF6) (Zhang and Kaufman, 2006; Lee and Glimcher, 2009; Hotamisligil, 2010). IRE1α is the most conserved UPR transducer that is known to function as a protein kinase and endoribonuclease (RNase). During ER stress, IRE1α executes an unconventional splicing of the mRNA encoding X-box binding protein 1 (XBP-1) by removing a 26-base intron (Shen et al., 2001; Calfon et al., 2002). Spliced XBP-1 mRNA encodes a potent transcription factor that activates the expression of several genes involved in protein folding, secretion, degradation, as well as inflammation and metabolism (Sha et al., 2011). During ER stress, the cytosolic domain of activated IRE1α recruits tumour necrosis factor receptor-associated factor 2 (TRAF2), which leads to the activation of e-JUN N-terminal kinase (UNK) through apoptosis signal-regulating kinase 1 (ASK1) (Urano et al., 2000).

Although recognition of unfolded proteins is generally regarded as the primary activation model for the IRE1α-mediated UPR, recent studies suggested that IRE1α signalling might not rely solely on ER-luminal sensing of unfolded proteins. In fact, TLR4 and TLR2 can specifically activate IRE1α and its downstream target XBP-1 to augment pro-inflammatory cytokine production in macrophages in the absence of an ER-stress response (Martinon et al., 2010; Hetz et al., 2011; Martinon and Glimcher, 2011). Moreover, high levels of fructose can activate the IRE1α-XBP-1 UPR branch to promote hepatic de novo lipogenesis without inducing classic ER stress-response pathways (Lee et al., 2008). These findings suggest that an unconventional, physiological UPR that is mediated through IRE1α might regulate pathophysiologic processes in specialized cells or organs.

Notably, a recent study showed a link between TLR signalling and activation of XBP-1, the UPR transducer IRE1α, in facilitating the production of pro-inflammatory cytokines by macrophages after acute bacterial infection (Martinon et al., 2010). XBP-1 deficiency resulted in a greater bacterial burden in mice infected with the TLR2-activating human intracellular pathogen Francisella tularensis. However, it is not known whether IRE1α is required for TLR-initiated macrophage inflammation, and if so, how TLR signalling activates IRE1α-mediated UPR. To study those processes, we generated a mouse strain with a genetic deletion of IRE1α in myeloid cells and demonstrated that TLR-mediated IRE1α activation is required for optimal pro-inflammatory cytokine production by macrophages during inflammation and progression of inflammatory RA. Importantly, mice with myeloid IRE1α deletion have reduced inflammatory arthritis. An IRE1α-specific inhibitor suppressed TLR-induced pro-inflammatory cytokine production and protected mice from inflammatory arthritis. Furthermore, our findings provide evidence that TLR signalling promotes IRE1α interaction with TRAF6, a key adaptor protein of TLR2/4 signalling pathways, and that TRAF6 catalyses IRE1α ubiquitination. Interestingly, TRAF6-mediated ubiquitination inhibits the recruitment of protein phosphatase 2A (PP2A), a ubiquitously expressed serine/threonine phosphatase that dephosphorylates many key molecule players in cell proliferation, signal transduction, and apoptosis (Millward et al., 1999). In this study, we revealed a molecular mechanism by which IRE1α is involved in TLR signalling-mediated pro-inflammatory cytokine production in macrophages and identified IRE1α as a therapeutic target for the treatment of inflammatory arthritis.

**Results**

*IRE1α is an important element in the development of inflammatory arthritis*

To investigate the involvement of the UPR signalling pathway in human with RA, we obtained synovial fluids from patients clinically diagnosed with either acute or chronic RA. The synovial fluids from patients with osteoarthritis (OA) were used as controls. The synovial fluids were cultured for 2 h to select adherent cells. Adherent cells were gently dislodged and analysed by flow cytometry for expression of macrophage markers, including CD11b and the macrophage mannose receptor CD206 (Chang et al., 2004). An average of >93% of adherent cells were CD11b and CD206 double positive cells, indicating that the majority of these adherent cells from both RA and OA synovial fluids are human macrophages (Supplementary Figure S1A and B). We next isolated total RNA for quantitative real-time RT–PCR analysis. The level of mRNA encoding the UPR transactivator, spliced XBP-1, in cells from the synovial fluid of RA patients was significantly higher than those from control patients with OA (Figure 1A, top panel). We found that the average XBP-1 mRNA levels were comparable between patients with acute versus chronic RA (Supplementary Figure S1C). As the UPR transducer IRE1α is the sole enzyme that catalyses XBP-1 mRNA splicing (Zhang et al., 2011), the increased production of spliced XBP-1 mRNA reflects the elevated IRE1α activation in macrophages from RA patients. Moreover, levels of total XBP-1 and IRE1α mRNA in synovial fluid cells were comparable between RA and OA patients (Figure 1A, middle and bottom panels), suggesting that the increased production of spliced XBP-1 mRNA was due to enhanced activation, but not expression levels, of IRE1α in the synovial fluid cells from human RA patients. Therefore, the increased IRE1α activation in the synovial fluid cells from human RA patients suggests that IRE1α-mediated signalling might be involved in RA development.

To investigate the roles of IRE1α in arthritis, we generated conditional IRE1α knockout mice by breeding IRE1α<sup>flw/flw</sup> mice with lysozyme M-Cre (LysM-Cre) transgenic mice (IRE1α<sup>flw/flw/LysM-Cre</sup> mice), in which the IRE1α gene was specifically deleted in myeloid cells, including macrophages, monocytes, and neutrophils (Clausen et al., 1999). Western blot analysis confirmed that IRE1α activity in macrophages was efficiently disrupted in the IRE1α conditional knockout mice; the full-length IRE1α protein was not detected in bone marrow-derived macrophages from IRE1α<sup>flw/flw/LysM-Cre</sup> mice (Figure 1B). Because the exons 16–17 of the IRE1α gene are flanked by two loxP sites, the Cre-mediated deletion resulted in a truncated, non-functional IRE1α protein production (Zhang et al., 2011). Moreover, disruption of IRE1α...
activity was evidenced by the defect in splicing XBP-1 mRNA in macrophages derived from IRE1α^{flox/flox}LysM-Cre mice after tunicamycin (TM) treatment (Figure 1B). The non-functional IRE1α mutant protein was detected in CD11b^{+}F4/80^{+} macrophages, but neither in CD3^{+} T cells and B220^{+} B cells, from the conditional knockout mice, nor in the cells from the wild-type mice (Supplementary Figure S2A), thus confirming the specificity of the IRE1α gene deletion in the myeloid lineage. Similarly, IRE1α mutant protein was detected in peritoneal macrophages from the conditional knockout mice (Supplementary Figure S2B). As a consequence, >95% reduction in the TM-induced XBP-1 levels was detected in the peritoneal macrophages from IRE1α conditional knockout mice (Supplementary Figure S2C and D). We then used the IRE1α conditional knockout and control mice to establish a K/BxN serum transfer-induced arthritis model as previously reported (Scatizzi et al., 2010; Mavers et al., 2012). In this study, mice with the following genotypes, IRE1α^{flox/flox}LysM-Cre^{−}, IRE1α^{flox/+}LysM-Cre^{−}, or IRE1α^{+/+}LysM-Cre^{+}, were used as controls because both the expression level and the activity of IRE1α in the macrophages from these mice were indistinguishable from those of wild-type mice. The development of arthritis in those mice, with increased swelling of the ankle joints, was observed in control mice from day 2 after the K/BxN serum transfer. The inflammation reached its peak around day 7 and slightly reversed by day 10 as previously reported (Korganow et al., 1999). In contrast, myeloid-specific deletion of the IRE1α gene in mice delayed arthritis onset and significantly reduced the swelling in the ankle joints (Figure 1C). The clinical RA scores in IRE1α conditional knockout mice were significantly lower than those of the control mice (Figure 1D). Mice were euthanized at day 15 and their joint tissues were characterized by haematoxylin and eosin (H&E) staining (Figure 1E). In the control mice, we observed large amounts of leukocyte infiltration and bone destruction (indicated by arrows) in the ankle
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The CD11b number of lymphocytes. It is possible that IRE1 detected in the joints of both inflammatory arthritis. In contrast, only few mast cells were neutrophil accumulation and infiltration in the joint during myeloid organs of macrophages, mast cells, and neutrophils in conditional IRE1 To gain insight into how inflammatory arthritis is suppressed in production by macrophages and neutrophils knock-out cells (Supplementary Figure S3).

C5aR are indistinguishable between wild-type and results show that the expression levels of both CD16 and expression either on the surface of macrophages or on neutrophils, excluding the possibility that IRE1 gene deletion inhibits myeloid cell trafficking during anti-GPI induced inflammation. In addition, it has been shown that the cell surface expression levels of the Fc receptor CD16 and the complement C5a receptor (C5aR) on myeloid cells are critical for K/BxN serum-induced arthritis (Ji et al., 2002). Therefore, we compared their expression levels between IRE1/−/− and control wild-type macrophage and neutrophils, and the results show that the expression levels of both CD16 and C5aR are indistinguishable between wild-type and IRE1 knockout cells (Supplementary Figure S3).

**IRE1α promotes TLR-induced inflammatory cytokine production by macrophages and neutrophils**

To gain insight into how inflammatory arthritis is suppressed in conditional IRE1α-null mice, we first analysed the percentages of macrophages, mast cells, and neutrophils in IRE1α flox/flox LysM-Cre+ mice. IRE1α is not required for development of myeloid cells, as the percentages of CD11b+/CD206− cells, which are likely neutrophils. In addition to some dead cells, the CD11b−CD206+ cells are possibly fibroblasts and a small number of lymphocytes. It is possible that IRE1α activation in these minor populations may contribute to human RA.

We then analysed the expression levels of chemokine receptors including CXCR2 (Jacobs et al, 2010) and CCR9 (Schmutz et al, 2010) (Supplementary Figure S3), which have been shown to be involved in the myeloid cell trafficking into the inflamed joints in mice after anti-GPI sera administration. Our data indicate that IRE1 gene deletion did not affect their expression either on the surface of macrophages or on neutrophils, excluding the possibility that IRE1 gene deletion inhibits myeloid cell trafficking during anti-GPI induced inflammation. In addition, it has been shown that the cell surface expression levels of the Fc receptor CD16 and the complement C5a receptor (C5aR) on myeloid cells are critical for K/BxN serum-induced arthritis (Ji et al., 2002). Therefore, we compared their expression levels between IRE1−/− and control wild-type macrophage and neutrophils, and the results show that the expression levels of both CD16 and C5aR are indistinguishable between wild-type and IRE1 knockout cells (Supplementary Figure S3).

IRE1α deletion did not alter the percentages of NK1.1+ NK cells, NK1.1+CD3+ NK T cells, B220+ B cells, and CD11c+ dendritic cells in the spleen, and the B-cell subsets in bone marrow from mutant mice are also normal (Supplementary Figure S5E–G).

When bone marrow-derived macrophages from conditional IRE1α knock-out mice were stimulated with TLR agonists, including lipopolysaccharide (LPS, an agonist of TLR4), Pam3CysSK4 (Pam3, an agonist of TLR2/1), and polyinosinic-polycytidylic acid (poly(I:C), an agonist of TLR3), the production of inflammatory cytokines, including IL-1β, IL-6, TNF-α RANTES, and ISG15, was significantly reduced, compared with control macrophages, as determined by quantitative real-time RT–PCR (Figure 2A) and ELISA (Figure 2B–D). These results indicate that IRE1α is required for TLR-mediated pro-inflammatory cytokine production in macrophages. The suppression of inflammatory cytokine production by IRE1α deficiency in macrophages was not caused by increased cell death, as the percentages of Annexin V-positive cells in IRE1α-null and control macrophages were comparable after stimulation with different TLR agonists (Supplementary Figure S6A). Moreover, the expression of TLR2, TLR3, and TLR4 was not affected by IRE1α deficiency (Supplementary Figure S6B), thus excluding the possibility that impaired cytokine production was caused by reduced TLR expression in IRE1α-null macrophages. Similarly, while the percentages and total numbers of neutrophils in the peritoneal cavity of wild-type and IRE1α−/− mice are comparable (Supplementary Figures S7A and B), a significant reduction in TNF-α by IRE1α-null neutrophils was detected (Supplementary Figure S7C). Therefore, our study demonstrated that IRE1α is required for pro-inflammatory cytokine production by both macrophages and neutrophils after acute inflammatory challenges. The reduced production of pro-inflammatory cytokines from the IRE1α-null macrophages and neutrophils might account for the resistance to inflammatory arthritis of the conditional IRE1α-null mice.

As TLR signalling induces XBP-1 mRNA splicing for full-scale pro-inflammatory cytokine production (Martinon et al, 2010), we compared the levels of TLR-induced XBP-1 mRNA splicing between wild-type and IRE1α-null macrophages. As expected, loss of IRE1α significantly impaired TLR-mediated XBP-1 splicing, as determined by semi-quantitative RT–PCR and quantitative real-time RT–PCR (Figure 3A and B). As a positive control, TM induced a significant amount of spliced XBP-1 in wild-type macrophages, whereas only a background level of spliced XBP-1 mRNA was detectable in IRE1α-null macrophages. Note that the background level of spliced XBP-1 mRNA was likely due to contamination by other cells in bone marrow-derived macrophages, as only 93–95% of cells were CD11b+ F4/80+ (Supplementary Figure S4B). Additionally, we demonstrated that LPS stimulation promoted IRE1α phosphorylation in macrophages (Figure 3C). Taken together, these results indicate that TLR signalling induces XBP-1 mRNA splicing through IRE1α activation.

To further confirm TLR-mediated IRE1α activation, we isolated macrophages from ER stress-activated indicator (ERAI) transgenic mice (Iwawaki et al, 2004; Mao et al, 2004), and measured XBP-1 spliced product upon TLR stimuli. The ERAI transgenic mice carry an engineered XBP-1 gene fused with the venus gene, a variant of green fluorescent protein (GFP) (Iwawaki et al, 2004; Mao et al,
Upon induction of the UPR, XBP-1 is transcribed and then spliced and result in the production of an XBP-1–venus fusion protein that can be detected by flow cytometry. After stimulation with TLR agonist, the green fluorescence in the ERAI macrophages increased with the stimulation time (Supplementary Figure S8A and B), confirming our conclusion that TLR stimuli promote XBP-1 mRNA splicing. In addition, TNF-α stimulation also induced ERAI signals in macrophages, suggesting that TNF-α can also activates IRE1. Indeed, the phosphorylation of IRE1α was confirmed in macrophages stimulated with TNF-α (Supplementary Figure S8C). Therefore, our studies show that both TLR and TNF-α can activate IRE1α-XBP-1 pathways, suggesting that the elevated IRE1α activation in RA macrophages is possibly induced by multiple inflammatory factors.

On the basis of the profiles of pro-inflammatory cytokines (Figure 2A), it appears that IRE1 deletion led to more profound impairment in the production of inflammatory cytokines by macrophages, compared with XBP-1 deletion (Martinon et al, 2010). This suggests that additional IRE1α targets, rather than XBP-1, may be involved in TLR-induced inflammatory cytokine productions. Indeed, reconstitution of XBP-1s expression in IRE1-null mouse embryonic fibroblasts (MEFs) partially rescued IL-6 expression (Supplementary Figure S9), confirming that additional factors exist in IRE1-mediated cytokine production. Recent studies suggest that the regulated Ire1-dependent decay (RIDD) of mRNA is involved in regulating ER stress responses and other physiological functions (Hollien and Weissman, 2006; Han et al, 2009; Hollien et al, 2009). However, the mRNA levels of Hgna, Pmp22, Scara3, Col6, and PdgfR, the well-defined RIDD target genes, are indistinguishable between wild-type and IRE1α-null macrophages in the presence or absence of the inflammatory stimuli (Supplementary Figure S10). In addition, it has been reported that IRE1α activates the JNK to mediate ER stress-induced cell death (Urano et al, 2000). As JNK regulates activation of the transcription factor AP-1 in cytokine productions, the robust reduction in the pro-inflammatory cytokine productions in the absence of IRE1 may be due to impaired JNK activation, in addition to lack of functional XBP-1. However, both the phosphorylated and total JNK protein levels, as well as the activation of the MAPKs p38 and Erk1/2, were comparable between wild-type and IRE1α-null macrophages (Supplementary Figure S11A). Moreover, activation of the other branches of ER stress pathways, including PERK/eIF2α and ATF6 pathways, in macrophages was not affected by IRE1α deficiency (Supplementary Figure S11B).
**TLR6 is required for TLR-mediated IRE1α activation**

Knockdown of intracellular adaptor proteins of the TLR pathway, including MyD88, TRIF, and TIRAP, has been shown to partially eliminate TLR-induced IRE1α activation (Martinson et al., 2010). In fact, we found that TRAF6 is essential for TLR-mediated IRE1α activation, as IRE1α phosphorylation and XBP-1 mRNA splicing were attenuated in TRAF6-null MEFs after LPS stimulation. Importantly, TLR-independent TM-induced IRE1α activation was not affected by the loss of TRAF6 functions (Figure 4A–C). This result suggests that TLR and ER stress activate IRE1α through different mechanisms. Consistent with previous observations (Lomaga et al., 1999), the loss of TRAF6 remarkably inhibited TLR-induced production of inflammatory cytokines, including IL-1β, IL-6, TNF-α, and IFN-β (Figure 4B). Interestingly, compared with the wild-type control MEFs, the TRAF6−/− MEFs expressed significantly reduced levels of the splice XBP-1 mRNA in response to 16-h LPS stimulation (Supplementary Figure S12), indicating the requirement of TRAF6 in LPS-triggered IRE1α activation. Notably, TRAF6 deficiency did not affect IRE1α mRNA expression between control and TRAF6−/− MEFs (Figure 4B). Together, our findings suggest that a crosstalk between TRAF6 and IRE1α is involved in TLR-induced IRE1α activation that regulates pro-inflammatory cytokine production.

To test whether the XBP-1 can rescue the defect in inflammatory cytokine production due to impaired IRE1 activation in TRAF6-null cells, we next expressed a spliced form of XBP-1 in TRAF6−/− MEFs. Ectopic expression of spliced XBP-1 only partially rescued TNF-α and IL-6 production by TRAF6−/− MEFs, thus confirming that additional factors are required for IRE1α-mediated inflammatory cytokine productions. Importantly, the partial rescue effect of the functional XBP-1 is dependent on TLR stimulation, as expression of spliced XBP-1 failed to promote inflammatory cytokine production in the absence of LPS treatment, even in the wild-type MEFs (Figure 4D). This result indicates that XBP-1 requires other inflammatory factors, which are presumably driven by TLR stimuli, to promote gene transcription.

**TRAF6 interacts with IRE1α in macrophages**

To further delineate the molecular mechanism by which TRAF6 regulates TLR-mediated IRE1α activation, we tested the interaction between IRE1α and TRAF6 in the presence or absence of inflammatory stimuli. Immunoprecipitation (IP)–western blot analysis with the HEK293 cells transiently expressing exogenous IRE1 and TRAF6 showed that IRE1 protein can interact with TRAF6 (Figure 5A). Further, we demonstrated the interaction between endogenous TRAF6 and IRE1α in primary mouse bone marrow-derived macrophages also by IP (Figure 5B). Importantly, LPS stimulation significantly enhanced TRAF6–IRE1α interaction in macrophages (Figure 5B). A dynamic analysis in the mouse monocyte/macrophage cell line RAW264.7 further confirmed that LPS stimulation significantly enhanced the interaction between IRE1α and TRAF6 (Figure 5C), indicating that the interaction between IRE1α and TRAF6 in macrophages is regulated by TLR signalling. To gain insights into the mechanism underlying IRE1 and TRAF6 interaction, we generated truncated mutations for both TRAF6 and IRE1α proteins to map their interaction domains. Co-IP–western blot analysis revealed that the C-terminal meprin-associated TRAF
after infection, cells were stimulated with or without LPS for an additional 6 h. The levels of standard deviation (s.d.). Source data for this figure is available on the online supplementary information page.

TRAF6 is required for TLR-induced IRE1α activation. (A) TRAF6+/+ and TRAF6−/− MEFs were stimulated with LPS (100 ng/ml) or tunicamycin (TM, 5 g/ml) for 16 h. IRE1α phosphorylation was determined by pho-tag gel (top panel). The parallel-prepared samples were subjected to SDS–PAGE and western blot analysis for IRE1α (second panel), TRAF6 (third panel), and β-actin (bottom panel). (B) Total RNA from LPS-treated MEFs was purified, and the levels of XBP-1s, IL-1β, IL-6, and IFN-β were analysed by quantitative real-time RT–PCR. Student’s t-test was used for the statistical analysis. *P<0.05; **P<0.01; ***P<0.005; error bars represent standard deviation (s.d.). Source data for this figure is available on the online supplementary information page.

Next, we determined how TRAF6 regulates IRE1α activation in which PP2A is involved. A recent report showed that, in pancreatic β cells, the PP2A interacts with the IRE1α linker region through its adaptor protein RACK1 to suppress IRE1α phosphorylation, the key prerequisite for IRE1α RNAse activity (Qiu et al, 2010). Because we found that the IRE1α linker region mediates its interaction with TRAF6 (Figure 5E), we speculated that TRAF6 might activate IRE1α by inhibiting the recruitment of PP2A. Indeed, we detected a significant increase in IRE1α–PP2A interaction in TRAF6-null MEFs (Figure 6A and B). In contrast to LPS-induced IRE1α–PP2A interaction in wild-type MEFs, constitutive interaction between IRE1α and PP2A was observed in TRAF6-null MEFs. It is possible that metabolic signals, such as glucose, growth factors, and amino acids, all of which exist in the culture media and have been shown to activate IRE1 (Lee et al, 2008; Zhang and Kaufman, 2008; Zhang, 2010), may trigger basal IRE1α/PP2A interaction and that TRAF6 inhibits IRE1α–PP2A interaction, and that TRAF6 inhibits IRE1α/PP2A interaction in the absence of TRAF6. Indeed, only background levels of PP2A/IRE1 interaction could be detected in both wild-type and TRAF6-null MEFs under glucose and serum starvation. In cells cultured with normal culture media, PP2A/IRE1 interaction was detected, and loss of TRAF6 further enhanced their interaction (Supplementary Figure S13). These results indicate that TRAF6 suppresses the recruitment of PP2A to IRE1 upon extracellular stimuli, such as LPS and the metabolic factors. Supporting this conclusion, over-expression of TRAF6 inhibited IRE1α interaction with PP2A in a dose-dependent manner (Figure 6C). However, the presence of the E3 ligase catalyticinactive C70A mutant of TRAF6 (TRAF6/CA) failed to suppress the interaction between IRE1α and PP2A, even though the C70A mutation did not affect the interaction between TRAF6 and IRE1 (Figure 6F). These results indicate that the E3 ligase ubiquitin activity of TRAF6 is required for its suppressive effect on the IRE1α–PP2A interaction, and that TRAF6 inhibits IRE1α–PP2A interaction not through its competition for their binding regions in IRE1α with PP2A. These three proteins possibly form a complex because the interaction of TRAF6 with PP2A was also detected (Figure 6G). Furthermore, the expression
levels of PP2A in TRAF6−/− MEFs were comparable to those in the control wild-type MEFs, thus excluding the possibility that increased IRE1α–PP2A interaction is due to the altered PP2A expression levels in TRAF6-null cells (Supplementary Figure S15).

E3 ubiquitin ligases are known to promote ubiquitination of their binding proteins. We speculated that TRAF6 might inhibit the IRE1α–PP2A interaction by catalysing IRE1α ubiquitination. Indeed, transient expression of TRAF6, but not the E3 ligase catalytic activity-negative mutant TRAF6/C70A, enhanced IRE1α ubiquitination, indicating that TRAF6 is an E3 ubiquitin ligase of IRE1α (Figure 6D). This finding implies that TRAF6 inhibits IRE1α–PP2A interaction by specifically catalysing IRE1α ubiquitination. TRAF6 often catalyses K63-linked polyubiquitin conjugation onto its substrates (Deng et al, 2000; Wang et al, 2001; Yang et al, 2009). To characterize TRAF6-mediated ubiquitination of IRE1α, we co-expressed IRE1α with a mutant ubiquitin isoform that carries a single lysine residue at position 63 (K63O) or 48 (K48O) in HEK293 cells (Figure 6E). K63O and K48O ubiquitin mutants carry a single lysine residue, residues 48 and 63, respectively, which allows us to determine the topology of polyubiquitin chains. When Ub/K48O mutant is expressed, IRE1α ubiquitination was detected in a similar level to that in cells expressing wild-type ubiquitin. In contrast, when the Ub/K63O mutant was co-transfected, only a low level of IRE1α ubiquitination was detected. Therefore, the poly-ubiquitin chain conjugation onto IRE1α protein requires the lysine residue 48 (K48) but not the K63, indicating that TRAF6 catalyses IRE1α K48-linked, but not K63-linked polyubiquitination. The weak K63-linkage ubiquitination might have been catalysed by other endogenous E3 ubiquitin ligases, because TRAF6 co-expression did not affect K63-linked IRE1α ubiquitination.

K48-linked polyubiquitination usually mediates protein degradation, whereas K63-linked polyubiquitination regu-
 TLK-mediated IRE1α activation as a therapeutic target

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TRAF6 catalyses IRE1α ubiquitination to suppress IRE1α interaction with PP2A. (A) TRAF6−/− and TRAF6+/− MEFs were treated with 500 ng/ml LPS for 30 min. The interaction between IRE1α and PP2A was determined by co-immunoprecipitation using an anti-IRE1α antibody and western blotting using an anti-PP2A antibody (top panel). The expression levels of IRE1α (middle panel) and TRAF6 (bottom panel) were determined by western blot analysis. (B) The intensities of IRE1α bands that interacted with PP2A were quantified. The relative band intensities are shown. (C) HEK293 cells were transfected with IRE1α (1 g), Myc-PP2A (1 g), and different amounts of Flag-TRAF6 (0, 0.1, and 0.5 g) or its CA mutant (1 g) plasmids. The interaction between IRE1α and PP2A was determined by co-immunoprecipitation and western blot analysis as described in (A). (D) IRE1α, TRAF6, and HA-Ubiquitin expression plasmids were co-transfected into HEK293 cells as indicated. IRE1α ubiquitination was determined by immunoprecipitation using the anti-IRE1α antibody and western blotting using the anti-HA antibody (top panel). The expression level of IRE1α and TRAF6 in whole cell lysates was confirmed (middle and bottom panels). (E) Expression plasmids of IRE1α, TRAF6, and each HA-ubiquitin were co-transfected into HEK293 cells. IRE1α ubiquitination was examined as described in (D). (F) The interactions between IRE1α and TRAF6 or its C70A mutant were determined by co-immunoprecipitation and western blot analysis. (G) Flag-tagged TRAF6 and Myc-tagged PP2A plasmids were co-transfected into HEK293 cells. TRAF6 protein in the lysates of transfected cells was immunoprecipitated with anti-Flag antibody or normal mouse IgG as a control; the bound PP2A was determined by western blotting with anti-Myc Abs (top panel). The expression levels of PP2A and TRAF6 in whole cell lysates were confirmed by western blotting with anti-Myc antibody (middle panel) and anti-Flag antibody (bottom panel), respectively. (H) TRAF6−/− and TRAF6+/− MEFs were treated with 100 μg/ml cycloheximide (CHX) for the indicated time intervals. The protein levels of IRE1α (top panel) and β-actin (bottom panel) in the lysates of treated cells were examined by western blot analysis. (I) The band densities of western blot analysis were quantified, and the relative levels were calculated. Error bars represent data from three independent experiments (mean ± s.d.). **p<0.01, ***p<0.005. Source data for this figure is available on the online supplementary information page.

lates the functions of target proteins. As TRAF6 enhances K48-linked polyubiquitination of IRE1α, we tested whether TRAF6-mediated ubiquitination enhances IRE1α protein degradation. As shown in Figure 6H and I, the half-life of IRE1α in wild-type MEFs is about 1.8 h, but it was significantly prolonged by TRAF6 deficiency. Therefore, TRAF6 promotes IRE1α degradation by catalysing K48-linked polyubiquitination. Since TLR stimulation enhances IRE1α/ TRAF6 interaction, we asked whether LPS stimulation promotes IRE1α degradation. However, neither the half-life nor the levels of newly synthesized IRE1α protein were altered in macrophages upon LPS stimulation for up to 4 h (Supplementary Figure S14A and B). This is likely because TRAF6-mediated degradation compromises the new synthesis. Cultivation of macrophages with LPS for a longer period, such as 8–16 h, increased the expression levels of IRE1α, clearly indicating that LPS induces IRE1 protein expression and this induction succeeds TRAF6-mediated degradation during later time points (Supplementary Figure S14C and D). Supporting this speculation, both the newly synthesized IRE1 protein levels and their stability (half-life) were significantly increased in TRAF6-null cells, compared to that in the wild-type cells (Supplementary Figure S14E and F). Therefore, our studies reveal a previously uncharacterized molecular pathway responsible for TLR-induced IRE1α activation: TLR signalling promotes TRAF6 interaction with and ubiquitinates IRE1α, which blocks the recruitment of IRE1α PP2A and subsequently benefits the activation of IRE1 through auto-phosphorylation. Additionally, the interaction between TRAF6 and IRE1 also contributes to the regulation of IRE1α protein stability under inflammatory stress conditions. The detailed molecular mechanism and dynamics for the fine-tune regulation of IRE1 activation or stability by TRAF6 is an intriguing question to be further elucidated in the future.
IRE1α-specific inhibitor protects mice from inflammatory arthritis

Because elevated IRE1α activation in synovial fluid cells is associated with RA in humans and because deletion of myeloid-specific IRE1α attenuates anti-GP1-induced inflammatory arthritis in mice, IRE1α might be a potential therapeutic target for treating inflammatory arthritis. Recently, 8-formyl-7-hydroxy-4-methylcoumarin (4U8C), which specifically binds to the lysine residue in the ribonuclease catalytic pocket and blocks IRE1α RNase activity, was identified as a novel IRE1α inhibitor (Cross et al., 2012). We tested the ability of 4U8C to suppress TLR-induced IRE1α activation and cytokine production in macrophages. 4U8C significantly inhibited LPS-induced splicing of XBP-1 mRNA with a 50% inhibition efficacy (IE50) of about 150 nM in macrophages from control mice (Figure 7A). Notably, 4U8C treatment significantly inhibited LPS-induced production of IL-6 (Figure 7B) and TNF-α by macrophages (Figure 7C). In IRE1α-null macrophages, 4U8C treatment did not further reduce pro-inflammatory cytokine genes, suggesting that the effect of 4U8C on TLR-induced pro-inflammatory cytokine production is through suppression of IRE1α activity.

To test the therapeutic potential of 4U8C in a mouse model of inflammatory arthritis, we administered 4U8C at a dose of 10 mg per kg body weight per day via intraperitoneal injection. This dose exerted a potent suppressive effect on anti-GP1-induced joint inflammation in control mice. Although myeloid-specific deletion of the IRE1α gene resulted in significantly reduced disease severity in mice after the anti-GP1 serum transfer, treatment of IRE1α conditional knockout mice with 4U8C did not further inhibit joint inflammation after anti-GP1 serum transfer (Figure 7D), suggesting that the suppressive effect of 4U8C on inflammatory arthritis relies on its suppression of IRE1α. Moreover, the levels of inflammatory cytokines in the sera of mice receiving anti-GP1 serum were inhibited by 4U8C treatment (Figure 7E). The activation of IRE1α in the joints of 4U8C-treated mice was inhibited, as measured by the levels of spliced XBP-1 mRNA (Figure 7F). A significant reduction in expression of TNF-α, IL-1β, and IL-6 mRNAs in the joint tissues from 4U8C-treated mice was also detected (Figure 7F). These results indicate that the IRE1α inhibitor 4U8C has promising therapeutic potential for treating inflammatory RA.

Discussion

Our studies demonstrated previously unknown molecular mechanisms underlying TLR-induced activation of IRE1α and identified IRE1α as a potential therapeutic target for inflammatory arthritis. These conclusions are supported by

Figure 7 IRE1α inhibitor suppresses inflammatory cytokine production and protects mice from inflammatory arthritis. (A) Bone marrow-derived macrophages were stimulated with 200 ng/ml LPS in the presence of the IRE1α inhibitor 4U8C (concentrations 0–1000 nM) for 24 h. Total RNA from the stimulated cells was isolated, and the levels of spliced XBP-1 mRNA were analysed by quantitative real-time RT–PCR. (B, C) The levels of IL-6 (B) and TNF-α (C) in the supernatants of stimulated cells in (A) were determined by ELISA. Error bars represent data from three independent experiments (mean ± s.d.). (D) Inflammatory arthritis was induced in IRE1α−/− and control (CTL) mice by intraperitoneal injection of 200 μl K/BxN serum at day 0. Mice were treated daily with 4U8C at 10 mg/kg/day from day 1. The diameters of their ankle joints were measured daily. Error bars represent data from five mice per group (mean ± s.d.). (E) Sera from mice in (D) were collected at day 15. The levels of IL-6 (top panel) and TNF-α (bottom panel) were examined by ELISA. (F) The mice in (D) were euthanized, their ankle joints were collected and homogenized, and total RNA was isolated. The levels of XBP-1s, IL-1β, IL-6, and TNF-α mRNAs were determined by quantitative real-time RT–PCR. Student’s t-test was used for the statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.005. NS, non-significant; error bars represent the standard deviation (s.d.).

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the following discoveries: first, IRE1α is required for optimal production of inflammatory cytokines by macrophages after TLR stimulation; second, elevated IRE1α activation is associated with RA in humans, and myeloid-specific IRE1α deletion largely protects mice from inflammatory arthritis; third, TRAF6 is essential for TLR-mediated IRE1α activation as it catalyses IRE1α ubiquitination and blocks the recruitment of PP2A; and fourth, treatment with the IRE1α-specific inhibitor 4U8C reduces the symptoms of experimental inflammation in mice.

It is well documented that TLR signalling promotes cytokine production by activating downstream transcription factors, including NF-kB and IFN regulatory factors (IRFs), during innate immune responses to pathogens (Mills, 2011). Here, we discovered that IRE1α activation mediates an additional pathway that is required for TLR-induced cytokine production in macrophages. Although a recent study showed that XBP-1, the target of the IRE1α-mediated UPR pathway, is essential for maximizing TLR-mediated innate immune responses (Martinson et al., 2010), our study showed that IRE1α deficiency did not result in a phenotype identical to that of XBP-1 deletion in macrophages. IRE1α deletion impaired the production of a broader spectrum of inflammatory cytokines, and led to more profound inflammatory defects than XBP-1 deletion (Martinson et al., 2010). Indeed, it has been shown that IRE1α executes its functions through mechanisms that are independent of XBP-1 in B-cell development and in insulin-producing β cells (Zhang et al., 2005; Lee et al., 2011). While IRE1α is known to be involved in activation of JNK (Urano et al., 2000), a key player that mediates pro-inflammatory cytokine production through activating transcription factor AP-1 (Brenner et al., 1989), the activation of JNK, as well as other MAPKs including p38 and Erk1/2, was not affected by the IRE1α gene deletion. Therefore, additional XBP-1-independent IRE1α activity in facilitating TLR-induced cytokine production likely exists. A recent study indicates that IRE1 is involved in inflammasome functions (Osłowski et al., 2012). As IRE1 deficiency impairs the production of IL-1β, a cytokine that is regulated by inflammasome, it is possible that IRE1 regulates IL-1β production partially through the inflammasome pathway, an interesting question to be elucidated in the future. In addition to macrophages, neutrophils play critical roles in inflammatory arthritis in both human patients and experimental animal models. In fact, we observed that IRE1α gene deletion dramatically inhibited production of TNF-α by neutrophil and infiltration of neutrophil into the inflamed joints. Therefore, reduced activation of both macrophages and neutrophils contributes to the suppression of K/BxN serum-induced arthritis by myeloid IRE1α gene deletion in mice.

Our findings reveal that TRAF6 activates IRE1α through a linker region between the ER transmembrane domain and the kinase domain of IRE1α. It was recently reported that the PP2A, as well as its adaptor protein RACK1, interacts with IRE1α through this linker region to suppress IRE1α autophosphorylation, a prerequisite for IRE1α activation in pancreatic β cells (Qiu et al., 2010). An increase in PP2A–IRE1α interaction was observed in TRAF6-null cells, which suggests that TRAF6 may activate IRE1α by suppressing the recruitment of the IRE1α inhibitor PP2A. The interaction between IRE1α and PP2A can be detected in TRAF6−/− MEFs, even without TLR stimulation. This is possibly due to basal physiological stimuli, such as glucose, which has been shown to activate IRE1α (Qiu et al., 2010), in the culture media may sufficiently activate IRE1α and PP2A interaction in the absence of TRAF6. Moreover, the TRAF6-mediated suppression of PP2A–IRE1α interaction likely occurs through a K48-linked ubiquitination, as the ubiquitin ligase-active mutant, TRAF6/C70A, failed to suppress PP2A recruitment to IRE1α without affecting IRE1α–TRAF6 interaction. Additionally, TRAF6 appears to promote IRE1α protein degradation, because loss of TRAF6 functions resulted in a significant increase in IRE1α protein stability. Protein ubiquitination has been shown to play dual roles, in both activation and activator destruction (Salghetti et al., 2001). TRAF6-mediated polyubiquitination appears to positively regulate IRE1α activation by suppressing PP2A recruitment and IRE1α protein degradation.

Our study suggests that TRAF6 is also involved in IRE1α protein degradation, because loss of TRAF6 functions resulted in a significant increase in IRE1α protein stability. Recent studies indicated that ubiquitination-mediated degradation and activation of the same substrate occurs. For example, the E3 ubiquitin ligase Met30-mediated ubiquitination of the transcription activation domain of VP16 transcription factor activates VP16 followed by VP16 protein destruction, suggesting that ubiquitination serves as a dual signal for activation and activator destruction (Salghetti et al., 2001). Similarly, we show here that TRAF6-mediated ubiquitination not only suppresses IRE1α/PP2A interaction to enhance IRE1 activation but also catalyses IRE1 protein degradation. Likely, TRAF6 ubiquitinates IRE1 to block the interaction with IRE1 inhibitor PP2A for IRE1 activation at the early phase of TLR stimulation. This is followed by IRE1 protein destruction at the late phase of stimulation to terminate the signalling transduction. TRAF6-mediated polyubiquitination appears to positively regulate IRE1α activation by suppressing PP2A recruitment and IRE1α protein degradation. In addition, we reproducibly detected that LPS stimulation promotes IRE1 interaction both with TRAF6 and with PP2A, which presumably promotes IRE1α ubiquitination and degradation, while the levels of IRE1α protein were not reduced. One explanation is that LPS facilitates IRE1α degradation but also induces its protein expression. Indeed, both the levels (time 0) and stability (half-life) of the newly synthesized IRE1 protein in TRAF6-null cells significantly increased compared to that in the wild-type cells. Nevertheless, the detailed dynamics of IRE1/PP2A interaction in the presence or absence of TRAF6 under inflammatory stress needs to be further elucidated.

It is important to note that reconstitution of the functional/spliced XBP-1 only partially rescued cytokine production by TRAF6-null MEFs upon LPS stimulation. Interestingly, expression of spliced XBP-1 in wild-type or TRAF6-null cells failed to stimulate cytokine production in the absence of LPS stimulation. Clearly, XBP-1s protein alone is not sufficient to promote production of the inflammatory cytokines. One speculation is that XBP-1s may need a crosstalk with other TRAF6 downstream transcription factors, such as NF-kB, AP-1, and IRFs, in driving expression of the pro-inflammatory cytokine genes. It is possible that, under ER stress, XBP-1s binds to the promoter of ER stress responsive genes; in contrast, upon TLR stimuli, the downstream transcription factors, such as NF-kB, AP-1, and IRFs, are activated and interact with the spliced XBP-1 to elicit their trans-activation effects on pro-inflammatory
gene expression. Further studies are needed to test these scenarios.

Recent studies suggest that ER stress is a driving force for, or a consequence of, inflammatory disease (Turner et al, 2005; Colbert et al, 2009; Hara et al, 2009; Nunddall et al, 2010; Yoo et al, 2012). However, a direct connection between IRE1α activation and inflammatory RA had not been established. In light of the facts that TLR signalling activates IRE1α and endogenous TLR ligands are considered to be pathogenic factors of RA, it is not surprising that IRE1α activation is elevated in synovial fluid cells from RA patients. In addition to TLR signalling, we show that TNF-α stimulation can directly activate IRE1α-mediated XBP-1 mRNA splicing in macrophages. In addition, metabolic and cardiovascular risk factors, including hypertension, obesity, high glucose, and dyslipidaemia, some of which like glucose can activate IRE1, are prevalent in patients with RA (Rostom et al, 2013). These factors, raised under the RA microenvironment, may also be triggers of the IRE1/XBP-1 pathway and contribute to RA development. This is supported by our finding that myeloid-specific deletion of IRE1α largely protected mice from inflammatory arthritis triggered by anti-GPI serum from K/BxN mice. The involvement of IRE1α in RA development was further confirmed by the effect of the IRE1α inhibitor 4U8C: it suppressed TLR-induced macrophage inflammation and attenuated the clinical symptoms of inflammatory arthritis in mice. However, it has been recently shown that TLR2 knockout mice develop enhanced arthritis upon the challenge of K/BxN serum (Huang et al, 2013). One of the mechanisms underlying the RA model of TLR2 knockout mice is that TLR2 deletion leads to the reduced IL-10 production. We show here that TLR2 signalling activates IRE1α activation in macrophages and subsequently promotes inflammatory arthritis in mice.

The IRE1α inhibitor 4U8C is a synthetic coumarin derivative that attains its selectivity by forming an unusually stable Schiff base with lysine 907 in the IRE1α endonuclease domain. This inhibitor blocks substrate access to the active site of IRE1 and selectively inactivates both XBP-1 splicing and IRE1α-mediated mRNA degradation (Cross et al, 2012). The coumarin compounds have been widely studied for their potent anti-inflammatory activities in both murine and human inflammation (Yesilada et al, 2001; Pochet et al, 2004; Kontogiorgis et al, 2006; Pan et al, 2010; Shin et al, 2010). Several clinical trials are currently investigating coumarin compounds for treatments of inflammatory diseases, in particular RA. Previous studies showed that coumarin compounds could target several signalling pathways, including NF-kB, MAPK, and protein kinase C in vivo (Shin et al, 2008; Li et al, 2011; Yao et al, 2011).

Our studies suggest that 4U8C suppresses TLR-induced pro-inflammatory cytokine production through IRE1α, as it had no further inhibitory effect on the production of inflammatory cytokines in IRE1α-null macrophages. Further studies are needed to identify additional in vivo targets of 4U8C to gain a better understanding of the molecular mechanisms of 4U8C in anti-inflammation therapy.

Materials and methods

Cells, reagents, and mouse strains are described in the Supplementary Data.

Bone marrow-derived macrophage culture
Bone marrow cells were collected from the femurs and tibias of control and IRE1αfox/foxLy5-M-cre+ mice and cultivated in RPMI medium supplemented with 10% (vol/vol) FCS, M-CSF (5 ng/ml), penicillin (100 U/ml), and streptomycin (100 U/ml). After 1 day of culture, non-adherent precursors were plated for 6 days in 6-well plates at a density of 1 × 10⁶ cells per well in RPMI medium supplemented with 10% FCS, 30 ng/ml M-CSF (eBioscience), and antibiotics. On day 6 or 7, the cells were stimulated with TLR agonist or tunicamycin (Invitrogen), and gene transcription and cytokine production were analysed.

Phos-tag gel analysis of IRE1α phosphorylation
Preparation of the phosphor-tag gel was performed according to the manufacturer’s instructions (Phos-tag acrylamide AAL-107, Wako Pure Chemical Industries). The detailed protocol was followed as previously described (Yang et al, 2010; Qi et al, 2011). In brief, 15–30 g of whole cell lysates were boiled for 5 min prior to loading onto a 25-μM phos-tag 5% SDS-PAGE gel at 100 V for 3 h. The proteins were then transferred onto a PVDF membrane at 100 V for 90 min. Both phosphorylated and non-phosphorylated IRE1 were detected with anti-IRE1 antibody (14C10; Cell Signaling).

Anti-GPI-induced arthritis in mice
Experimental arthritis was induced in control or IRE1 conditional knockout (C57BL/6 genetic background) mice, using the K/BxN serum-transfer model (Scatizzi et al, 2010; Mavers et al, 2012). Mice (8–10 weeks old) were injected intraperitoneally with anti-GPI serum (200 μl/mouse). The diameter of each rear ankle joint was measured daily and the increases were determined based on the size measured the day before serum transfer. Clinical scores were determined as follows: grade 0 = no swelling; grade 1 = mild but definite redness and swelling of an ankle, wrist, or digits; grade 2 = moderate redness and swelling of ankle and wrist; grade 3 = severe redness and swelling of entire paw, including digits; and grade 4 = maximally inflamed limb with involvement of multiple joints. Mice were euthanized 15 days after serum transfer, and their ankle joints were harvested for histological analysis. Joints were fixed in 4% paraformaldehyde for 24 h and decalcified. Tissue sections were characterized by H&E staining, and the inflammation of joint sections was scored as: 0 = no inflammation; 1 = modest inflammation; 2 = severe inflammation, and 3 = severe inflammation with bone or cartilage destruction.

The collection of all human synovial fluid samples used in this study has been approved by the Institutional Review Board of the Harbin Medical University and the Third Military Medical University. For a full description of Materials and methods used in this work, see Supplementary Data.

Supplementary data
Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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Conflict of interest
The authors declare that they have no conflict of interest.
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