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Toll-like receptor-mediated IRE1 activation as a therapeutic target for inflammatory arthritis

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 08 January 2013

Thank you for submitting your manuscript to the EMBO Journal. I am sorry for the delay in getting back to you with a decision, but due to the Xmas season it unfortunately took a bit longer to get the paper reviewed. I have now received the comments from three referees that are provided below.

As you can see the referees find the analysis interesting. However, many issues are also raised: important controls are missing, further analysis needs to be done in macrophages to validate findings from cell lines, importantly the contribution of neutrophils to the observed phenotypes is unclear and must be determined. These issues would have to be fully addressed before further consideration here. Should you be able to extend the analysis and address the raised concerns in full then we would consider a revised manuscript. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as

soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

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REFEREE REPORTS:

Referee #1:

In this study, Qiu and colleagues use the transferrable K/BxN serum model to investigate the potential role of IRE1a overactivation in the pathophysiology of rheumatoid arthritis (RA) and report interesting observations suggesting that this arm of the ER stress response may represent a novel therapeutic target for RA. In addition, the study presents data suggesting a new potential role for TRAF6 as key regulator of TLR-mediated IRE1a activation by promoting IRE1a ubiquitination and inhibiting PPA2-mediated dephosphorylation. Specifically, the authors report that cells in the synovial fluid of RA patients exhibit increased mRNA levels of the spliced form of transcription factor XBP1, one of the main targets of IRE1a. Importantly, loss of IRE1a in myeloid cells as well as treatment with an IRE1a inhibitor impaired the development of RA in the transferrable model utilized, effects that correlate with decreased production and secretion of proinflammatory cytokines by macrophages lacking a fully functional form of IRE1a. While this study is interesting from basic and translational perspectives, further experiments could aid in clarifying and/or supporting the conclusions presented. A list of specific major concerns that must be addressed in order to support the conclusions follows:

1. A major flaw of this study was the failure to consider whether IRE1a deficiency or treatment with 4U8C could affect the recruitment/infiltration of different inflammatory cell populations to the ankle joints, a situation that could also explain why decreased joint swelling and lower clinical scores are observed when IRE1a is targeted. Experiments to address/rule out this critical possibility should be carried out. Specifically, total adherent cells obtained from synovial fluid samples were used in Figure 1 to quantify the mRNA levels of XBP1s, XBP1 and IRE1a. Although the authors claim that "a large proportion of adherent cells are macrophages" flow cytometry experiments must be performed in order to determine the real proportion of macrophages in each sample and thus properly interpret the data presented. It could be possible that increased or reduced levels of XBP1s mRNA are just a consequence of having more or less macrophages in each independent case. Conventional XBP1 splicing assays (such as the ones presented in Fig 1B) must be also performed in some of these human samples (preferably on sorted macrophages from synovial fluid) to convincingly demonstrate XBP1s overexpression.

2. Western blot experiments using phos-tag gels should also be performed in order to truly demonstrate IRE1a overactivation only in RA samples. The authors should use a similar time point for phos-tag analysis as they use for XBP-1 splicing by RT-PCR. Is XBP-1 splicing still occurring at 16 hours post-stimulation?

3. The authors must provide additional information on the derivation of the IRE1a flox/flox mouse strain used and how conditional deletion results in a truncated (but still viable) mutant form wherein only the endonuclease domain is compromised.

4. LysM-Cre deletes best in neutrophils, and neutrophils have been demonstrated to play a role in the K/BxN injectable model of RA (Wipke et al, J Immunology 2001). The authors have not ruled out that XBP-1 deletion in neutrophils could be responsible for the observed phenotype. An injection of anti-Gr-1 antibody (RB6-8C5 or 1A8) to deplete Ly6G+ cells would address the contribution of neutrophils to the phenotype.

5. Though the authors demonstrate lack of XBP-1 splicing in bone marrow derived macrophages, deletion efficiency should also be evaluated in vivo by FACS sorting macrophages from the relevant inflamed tissues and/or by sorting peritoneal/splenic macrophages in resting animals

6. The authors need to further explain or speculate on their observation that TRAF6 deletion reduces IRE1 phosphorylation in response to LPS, but not to tunicamycin treatment. This intriguing data raises significant questions about the importance of PP2A to IRE1 activation in this system. Why are TRAF6-/- MEFs, which exhibit constitutive binding of PP2A to IRE1, defective at IRE1 activation selectively in response to TLR stimulation.

7. The authors should repeat the co-IP for TRAF6 and IRE1 in bone marrow macrophages to bolster their data obtained from cell lines.

8. The in vitro data on inflammatory cytokine production in BMDMs (Figure 2A) are striking and deserve more attention in the discussion. The authors mention that the XBP-1 deficient macrophages do not phenocopy the IRE-1 deficient macrophages, but they should elaborate on the differences in magnitude and kinetics.

9. TRAF6 is known to facilitate pro-inflammatory responses. It is therefore difficult to interpret the data in figure 4B. If the authors are using this figure to argue for the importance of IRE1 activation in pro-inflammatory cytokine transcription, they should attempt to rescue the TRAF6-/- phenotype by transfecting or transducing either XBP-1 or IRE1 into the system.

10. The authors should present data or at least speculate on what could be the cue/signal in the RA microenvironment that triggers IRE1a activation and thus XBP1s overexpression. This was not addressed in the paper, and it is critical from the mechanistic perspective. In addition, it would be important to determine if the other arms of the ER stress response (ie. PERK, EIF2a, ATF6 etc) are also overactivated in RA-infiltrating macrophages or if this pathological setting exclusively triggers the IRE1a/XBP1 axis.

11. Figure 4D experiments must be repeated using an irrelevant antibody for IP purposes in order to further confirm the specificity of the pull down assays. Also, since all the interaction experiments were performed via protein overexpression, it would be important to show the endogenous interaction between IRE1a and TRAF6 in LPS-treated RAW or bone marrow-derived macrophages.

12. It would be important to show as control that TRAF6 and PPE2 do not interact with each other in order to further confirm that these proteins directly affect IRE1a activity and stability. In addition, it would be necessary to address whether the expression levels of PP2A are similar in wild type vs. TRAF6-deficient cells.

13. The authors overstate some of their findings as being original, when in fact many of them (i.e. TRAF6 requirement for XBP-1 splicing in response to TLR stimulation) were previously addressed in other work (Martinon et al., Nature Immunology 2010). The relevant passages should be re-worded to reflect this.

- minor concerns that should be addressed:
Please correct typos and grammar errors in the main text.

Referee #2 :

The main finding described here include:
1: that myeloid-specific deletion of the IRE1alpha gene protected mice from inflammatory arthritis,
2. that IRE1-alpha deficiency attenuates TLR induced cytokine production (including inflammasome depedent Interleukin-1 production - not discussed/analysed)
3.that TRAF-6 catalyses IRE1-alpha ubiquitination and blocks PP2A recruitment (the latter beeing known to inhibit IRE1-alpha phosphorylation).
The propensity of ER stress to enhance TLR drivrn innate immune responses has been highlited by recent work of Glimcher's group (see Physiol.Rev,2011,91:1219, or Curr. Op. Immunol, 2011,23:35, not cited) in demonstrating that TLR4 and TLR2 promote the phosphorylation of the ER signalling kinase IRE1 and activation of its downstream target XBP1 (cited: Martinon, et. al.2010). While Glimcher et al based their findings on XBP1 deficient macrophages the authors used here myeloid specifically deleted mice - or cells - for the IRE1 gene. That enabled studies of IRE1 deficiency in

vivo (inflammatory arthitis).
Specific points:
1. The MS appears poor in citation accuracy - and Glimcher's work is cited only once.
2. In reading the cited work of Calfon et al., 2002: and of Shen et. al., 2001 (see pp 9, 4th line)it remains unclear why the authors regard this work as conformation that TLR's activate the IRE1-XBP1-UPR axis. Here, citation of Glimchers work would be more appropiate.
4.The experiments attempting to delineate the molecular basis of TRAF6 mediated IRE1 activation - see pp 10 - would benefit from using a "cell free system". In paricular the proposed competion of TRAF6 with PP2A during IRE1-alpha activation could experimentally be validated
Minor points:
pp10 - 5th line - Fig 2B should read 4C
Conclusion:The hallmark of this work relies on the demonstration that the IRE1-XBP1 pathway is likely to contribute to RA development, a conclusion supported by the effect of the IRE1alpha inhibitor 4U8C.In view of the criticism raised an amended version of the MS appears apropriate.

Referee #3:

In this research article, Qiu and al. demonstrate that IRE1 may be a potential therapeutic target for inflammatory arthritis. Their findings include 1) deletion of IRE1 in mice delays progression of inflammatory arthritis, 2) IRE1 is necessary for pro-inflammatory cytokine production by macrophages, 3) IRE1 activation through TLR/TRAF6 axis in macrophages induce XBP-1 mRNA splicing. In short, the authors reveal a molecular mechanism underlying TLR-induced activation of IRE1 and the potential therapeutic of targeting IRE1 in inflammatory arthritis.

This present study is heavily based on the work published by Martinon and al. in Nature Immunology in 2010. However, rather than investigating the mechanisms behind the activation of IRE1 in a context of innate immunity (like in Martinon et al.), herein the authors evaluate the role of IRE1 in autoimmunity in a context of inflammatory arthritis -this makes it original. Some observations and conclusions remain however confirmatory, not novel per se.

Globally, this is a well-written manuscript containing interesting data. Main concerns regard the rationale behind the focus in vitro studies on one single cell type (macrophages) and the novelty of the research.

Minor comments:

1. Reference formatting required:
-page 3 line 4 .....(Piccinini and Midwood)...year missing
-page 3 line 9 ..van Roon et al. 1997)_ENREF_4.....?
-page 5 line 16....reference missing
-Page 8 line 5.....(Dickout et al.)...year missing

2. In the introduction: "mice with myeloid deletion of IRE1 are resistant to inflammatory arthritis". These mice are not resistant to arthritis rather they demonstrate a delay in the progression of the disease.

3. Original papers re K/BxN serum transfer-induced arthritis (ex. Mathis and Benoist) should be cited.

4. Page 3: "Indeed, Chaperonin 10, a mitochondrial protein that inhibits TLRs, was clinically effective in a small-scale study with RA patients [a]. One reference is in German and cannot be easily assessed, and other references and in fact ´ comments to the editors [a]. Why not citing the original paper in Lancet by Vanags instead?

Major comments:

1. First sentence in the abstract and in the result section: "In rheumatoid arthritis, macrophages are the major source of inflammatory cytokines." This statement seems to form the basis of this focused study on macrophages. No references support this affirmation in the manuscript. What serious study

can support this statement?

2. The authors focus their work on the molecular aspects of IRE1 in macrophages, like in the study by Martinon (where infection was the context and macrophages were highly relevant). Herein, the authors used the K/BxN serum model of arthritis for their study. Although this model is highly relevant to arthritis and well accepted in the field, the role of macrophages in this model remains to precisely establish. In the K/BxN model of arthritis, macrophages can even play anti-inflammatory functions. Rather, neutrophils and mast cells are important players in this model, contribute via TLR4 (Kin 2012), and are most likely cells expressing pathogenic IRE1 . Since IRE1 is deleted in neutrophils and mast cells in IRE1 flox/floxLysM-Cre mice, their respective contribution should be determined.

3. Furthermore, the authors ensured that lymphocytes are intact in IRE1 flox/floxLysM-Cre mice. Lymphocytes are dispensable is the K/BxN model of arthritis. Rather, the authors should make sure the levels of neutrophils and mast cells are intact.

4. Second sentence of the introduction: "The elevated levels of TLR in arthritis... significantly contribute to rheumatoid arthritis." One review is cited, no original papers. What study demonstrates that TLRs indeed contribute to arthritis? What is the background regarding TLRs and the mouse model of arthritis chosen for this study? For instance, TLR2 KO mice develop enhanced arthritis in the K/BxN model; how do the authors reconcile their data with the existing literature on arthritis?

5. Figure 1 A through B:

- Synovial fluids from "acute" and "chronic" rheumatoid arthritis patients were obtained. Where are these data? Were the levels of spliced XBP-1 different in acute compared to chronic patients? Were the chronic patients treated with medications differently?
- According to the authors, "The adherent cells were used to isolate total RNA, as it has been shown that a large portion of adherent cells are macrophages. [a] No citations are given. It is important to know what cells are analyzed in these PCR studies. When OA adherent cells are compared to RA adherent cells, it is likely that different cell types rather than different expression levels are in fact examined. The conclusions that are derived from these experiments are therefore difficult to interpret. MACS purification columns or other methods should be employed to determine the relative expression of mRNA in specific cell types.

6. "IRE1 flox/floxLysM-Cre-, IRE1 flox/+LysM-Cre-, or IRE1 +/+LysM-Cre+, were used as controls [a]. Eight mice are used as controls in figure 1C. What mice are they exactly? Is this a pool of different mice with different genotypes (IRE1 flox/floxLysM-Cre-, IRE1 flox/+LysM-Cre-, or IRE1 +/+LysM-Cre+)? This experimental condition is unclear to the reader.

7. Figure 1 E, F: "Arrows indicate lymphocyte infiltration and cartilage erosion". What arrow indicates what? Different types of arrows should be employed. In addition, the quality of the figure does not permit assessment of the presence of lymphocytes. However, in this model of arthritis, neutrophils (and possibly monocytes/macrophages) are most likely the cells that populate the joint, not the lymphocytes. Why not demonstrating the presence of a cell type relevant to the current study (such as monocytes/macrophages, or LysM+ cells).

7. A better introduction to PP2A in the results section should be made.

1st Revision - authors' response 14 April 2013

Point-to-point Responses to the Reviewers' Comments

We would like to thank all reviewers for their constructive critiques, which led to a significant improvement to our study. We have carefully addressed all critiques in a point-to-point manner as described below as well as in the revised manuscript.

Reviewer #1.

In this study, Qiu and colleagues use the transferrable K/BxN serum model to investigate the potential role of IRE1αoveractivation in the pathophysiology of rheumatoid arthritis (RA) and report interesting observations suggesting that this arm of the ER stress response may represent a novel therapeutic target for RA. In addition, the study presents data suggesting a new potential role for TRAF6 as key regulator of TLR-mediated IRE1α activation by promoting IRE1α ubiquitination and inhibiting PPA2-mediated dephosphorylation. Specifically, the authors report that cells in the synovial fluid of RA patients exhibit increased mRNA levels of the spliced form of transcription factor XBP-1, one of the main targets of IRE1α. Importantly, loss of IRE1α in myeloid cells as well as treatment with an IRE1α inhibitor impaired the development of RA in the transferrable model utilized, effects that correlate with decreased production and secretion of proinflammatory cytokines by macrophages lacking a fully functional form of IRE1α. While this study is interesting from basic and translational perspectives, further experiments could aid in clarifying and/or supporting the conclusions presented. A list of specific major concerns that must be addressed in order to support the conclusions follows:

Comment 1: *A major flaw of this study was the failure to consider whether IRE1α deficiency or treatment with 4U8C could affect the recruitment/infiltration of different inflammatory cell populations to the ankle joints, a situation that could also explain why decreased joint swelling and lower clinical scores are observed when IRE1α is targeted. Experiments to address/rule out this critical possibility should be carried out. Specifically, total adherent cells obtained from synovial fluid samples were used in Figure 1 to quantify the mRNA levels of XBP-1s, XBP-1 and IRE1α. Although the authors claim that "a large proportion of adherent cells are macrophages" flow cytometry experiments must be performed in order to determine the real proportion of macrophages in each sample and thus properly interpret the data presented. It could be possible that increased or reduced levels of XBP-1s mRNA are just a consequence of having more or less macrophages in each independent case. Conventional XBP-1 splicing assays (such as the ones presented in Fig 1B) must be also performed in some of these human samples (preferably on sorted macrophages from synovial fluid) to convincingly demonstrate XBP-1s overexpression.*

Response: We agree with this reviewer that the LysM-Cre mediates gene deletion in all myeloid cells including neutrophils and mast cells, both of which are involved in the inflammatory arthritis development. As pointed out by this reviewer, it has been shown that neutrophils are essential for the inflammatory arthritis development induced by K/BxN serum (Korganow et al, 1999). To determine the effects of IRE1α deletion on neutrophil function and its involvement in inflammatory arthritis, we performed the following new experiments:

First, we analyzed the effect of IRE1α deletion on neutrophil development. As indicated in the new supplemental Fig. 6A & 6B, both the percentages and absolute numbers of Gr1+CD11b- neutrophil populations are indistinguishable between IRE1α conditional knockout ($IRE1\alpha^{f/f}$LysM-Cre+) mice and their wild-type littermate controls. Moreover, IRE1α gene deletion did not alter the percentages of mast cells in mice (supplemental Fig. 6A).

Second, we isolated neutrophils from $IRE1\alpha^{f/f}$LysM-Cre+ mice and their control littermates after K/BxN serum treatment. We analyzed activation of the IRE1α null and control neutrophils in vitro. As shown in

the supplemental Fig. 6C, IRE1α gene deletion indeed impaired TNF-α production by neutrophils. Since TNF-α is one of the critical cytokines that drive inflammatory arthritis development, reduced production of TNF-α by IRE1α-null neutrophils should partially contribute to the protective activities resulted from either LysM-Cre-mediated IRE1α deletion or IRE1α inhibitors in inflammatory arthritis development in our mouse model. This scenario has been described and discussed in detail in the revised manuscript (Pages 9 & 18).

Third, we have used the immunohistochemistry approach to delineate macrophage, neutrophil and mast cell infiltrations in the joint of IRE1α conditional null and control mice after K/BxN serum treatment. As shown in the Figure 1D, a large number of both macrophages and neutrophils were detected in the inflamed joint synovial tissues from the WT control mice, confirming the importance of both macrophages and neutrophils in inflammatory arthritis development. In contrast, only few macrophages and neutrophils were detected in the IRE1α conditional knockout mice, suggesting that both IRE1α null macrophages and IRE1α null neutrophils account for the decreased K/BxN serum-induced arthritis in IRE1α conditional knockout mice. The related data interpretation and discussion was described in the revised manuscript (page 8).

As suggested by this reviewer, it will be important to dissect the contributions of IRE1α-null macrophages and IRE1α-null neutrophils to arthritis suppression phenotype in mice. *Wipke et al (Wipke & Allen, 2001)* reported that anti-Gr-1 Ab-mediated neutrophil depletion fully protected mice from K/BxN serum-induced arthritis, providing an elegant model to study the contributions of neutrophils in the inflammatory arthritis. However, because anti-Gr-1 Ab completely abolished the arthritis development in the treated mice, it would be difficult to evaluate the contributions of IRE1α-null macrophages in mice with neutrophil depletion by anti-Gr-1. We have discussed this in the revised manuscript (page 17).

In addition, we have confirmed the purity of cells isolated from synovial fluids. After a 2-hour culture in 6-well plate, adherent cells were collected and their surface expression levels of CD11b and CD206 (the same condition that we have used for isolating cells for our real-time PCR analysis). As shown in Supplemental Figure 1, over 90% of the isolated cells were conformed to be macrophages.

2. *Western blot experiments using phos-tag gels should also be performed in order to truly demonstrate IRE1α overactivation only in RA samples. The authors should use a similar time point for phos-tag analysis as they use for XBP-1 splicing by RT-PCR. Is XBP-1 splicing still occurring at 16 hours post-stimulation?*

Response: We agree with the reviewer that it would be nice to directly detect IRE1α phosphorylation in macrophages from RA patients. However, the Western blot analysis with phosphor-tag gel has low sensitivity and requires large amounts of cellular lysates for detecting phosphorylated form of IRE1α. However, only limited amounts of macrophages could be obtained from each patient. Based on the cell numbers obtained, we need to pool all macrophages from at least 5 RA and 10 osteoarthritis (OA) patients (as numbers of macrophages from OA patients are usually half less than those from RA patients) for each experiment. Therefore, it is not realistic to analyze phosphorylated IRE1α in macrophages from RA and OA patients using the phosphor-tag gel experimental approach.

As suggested, we confirmed the *XBP-1* mRNA splicing at the time point where we analyze IRE1α phosphorylation (16 hours) (Supplemental Fig 9). The result is consistent with the result indicated by Fig. 3B.

3. *The authors must provide additional information on the derivation of the IRE1α flox/flox mouse strain used and how conditional deletion results in a truncated (but still viable) mutant form wherein only the endonuclease domain is compromised.*

Response: The conditional IRE1α knockout mice were generated by flanking the exon 16-17 of IRE1α gene with two loxP site. Mice with one or two floxed *Ire1α* alleles were viable, fertile, and apparently normal. When mice were breed with LysM-Cre transgenic mice, the Cre recombinase expression in myeloid cells leads to the deletion of IRE1α exon 16-17, resulting in a truncated, nonfunctional IRE1a protein expression as confirmed in Fig. 1B and Supplemental Fig. 2 The exons 16-17 encode the kinase domain of IRE1α (EMBO J. 2011, 30: 1357-1375). The deletion of exons 16-17 leads to a frame-shift translation that produces a truncated, non-functional IRE1αprotein. A detailed explanation of IRE1α floxed mice is now provided in the revised manuscript (Page 6).

4. *LysM-Cre deletes best in neutrophils, and neutrophils have been demonstrated to play a role in the K/BxN injectable model of RA (Wipke et al, J Immunology 2001). The authors have not ruled out that XBP-1 deletion in neutrophils could be responsible for the observed phenotype. An injection of anti-Gr-1 antibody (RB6-8C5 or 1α8) to deplete Ly6G+ cells would address the contribution of neutrophils to the phenotype.*

Response: This issue has been addressed in details in our response to the critique #1.

5. *Though the authors demonstrate lack of XBP-1 splicing in bone marrow derived macrophages, deletion efficiency should also be evaluated in vivo by FACS sorting macrophages from the relevant inflamed tissues and/or by sorting peritoneal/splenic macrophages in resting animals*

Response: New experiments using FACS sorted macrophages have been performed as suggested (Supplemental Fig. 2A). A similar reduction in *XBP-1* mRNA splicing in IRE1α-null macrophages has been observed (Supplemental Fig. 2C & 2D). This result confirmed that IRE1α is required for *XBP-1* mRNA splicing in macrophages upon TLR stimulation.

6. *The authors need to further explain or speculate on their observation that TRAF6 deletion reduces IRE1 phosphorylation in response to LPS, but not to tunicamycin treatment. This intriguing data raises significant questions about the importance of PP2A to IRE1 activation in this system. Why are TRAF6-/- MEFs, which exhibit constitutive binding of PP2A to IRE1, defective at IRE1 activation selectively in response to TLR stimulation.*

Response: Our study indicates that TRAF6 plays an indispensible role in TLR-induced IRE1α activation (Fig. 4). An underlying molecular mechanism by which TRAF6 positively regulates IRE1α activation is through suppressing the recruitment of PP2A to IRE1α (Fig. 6). However, loss of TRAF6 did not affect tunicamycin (TM)-induced IRE1α activation (Fig 5A). We conclude that TM can still activate IRE1α independent of TRAF6. Therefore, it appears that TM and TLR activate IRE1α through different molecular mechanisms.

In addition, IRE1α interaction with PP2A can be detected in TRAF6-/- MEFs, even without TLR stimulation. This is possibly due to, in the absence of TRAF6 suppression, basal physiological stimuli, such as the energy fluctuations (glucose levels) and growth factors in the culture media, can sufficiently

activate IRE1α and PP2a interaction in the absence of TRAF6. A discussion has been incorporated into the revised manuscript (Page 18).

7. *The authors should repeat the co-IP for TRAF6 and IRE1 in bone marrow macrophages to bolster their data obtained from cell lines.*

Response: As suggested, we have repeated the co-IP for the interaction between TRAF6 and IRE1α in mouse bone barrow macrophages using normal mouse IgG as negative controls (Fig. 5B). The result confirmed our conclusion that TRAF6 interacts with IRE1α in macrophages.

8. *The in vitro data on inflammatory cytokine production in BMDMs (Figure 2A) are striking and deserve more attention in the discussion. The authors mention that the XBP-1 deficient macrophages do not phenocopy the IRE-1 deficient macrophages, but they should elaborate on the differences in magnitude and kinetics.*

Response: We have fully addressed this scenario in the revised manuscript**.** We demonstrated that IRE1α deletion impairs the production of a broader spectrum of inflammatory cytokines and leads to more profound inflammatory defects than *XBP-1* deletion in macrophages (Figure 2) (Martinon et al, 2010). Indeed, it has been shown that IRE1α executes its functions through the mechanisms that are independent of XBP-1 in B cell development and in insulin-producing beta cells (Lee et al, 2011; Zhang et al, 2005). While IRE1α is known to be involved in activation of JNK (Urano et al, 2000), a key player that mediates proinflammatory 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, were not affected by IRE1α gene deletion in macrophages upon TLR stimulation (supplemental Fig. 8A). An important finding of this study is that IRE1α interacts with and catalyzes ubiquitin-conjugation onto TRAF6, the adaptor protein of TLR signaling, to render IRE1α activity in regulating pro-inflammatory cytokine production. Apparently, XBP-1 is not the only IRE1α target that is involved in this process. As suggested, we performed new experiments to reconstitute functional XBP-1 into the TRAF6-null cells where IRE1α-mediated pro-inflammatory response is impaired. The results indicated that over-expression of functional/spliced XBP-1 only partially rescue the defect in pro-inflammatory cytokine production and that this incomplete rescue effect is dependent of TLR stimulation (Fig. 4D) (Please also see our response to the critique #9). Therefore, additional XBP-1s-independent IRE1α activity in facilitating TLR-induced cytokine production likely exists. For example, IL-1α secretion requires a cleavage of its precursor through the inflammosome machinery. The possibility that IRE1α regulates inflammosome pathways may exist. A discussion has been now provided in the revised manuscript (Page 17).

9. *TRAF6 is known to facilitate pro-inflammatory responses. It is therefore difficult to interpret the data in figure 4B. If the authors are using this figure to argue for the importance of IRE1 activation in pro-inflammatory cytokine transcription, they should attempt to rescue the TRAF6-/- phenotype by transfecting or transducing either XBP-1 or IRE1 into the system.*

Response: We agree with this reviewer that TRAF6 is known to facilitate production of pro-inflammatory response. Our initial study confirmed the phenotype of *TRAF6* gene deletion in pro-inflammatory cytokines (Fig 4B) but did not automatically lead to the conclusion that the defective production of pro-inflammatory cytokines in the absence of TRAF6 is due to the inactivation of IRE1α.

As pointed out by this reviewer, it is important to evaluate the requirement of IRE1α activation in pro-inflammatory cytokine production in TRAF6-null cells by reconstitution of functional/spliced XBP-1, the target and mediator of IRE1α in driving pro-inflammatory response, in TRAF6 null MEFs . We then ectopically expressed the spliced form of XBP-1 into TRAF6-null MEFs and analyzed the effect of reconstitution of XBP-1 splicing on proinflammatory cytokine production in TRAF6-null MEFs upon LPS stimulation. Surprisingly, only modest, but statistically significant, a partial rescue effect in TNF-α and IL-6 production by XBP-1s reconstitution in TRAF6-null MEFs was observed (Fig. 4D).

For the new XBP-1 reconstitution result, we provide the following interpretations: First, there may exist XBP-1s-independent IRE1α activity in facilitating TLR-induced cytokine production. Since IRE1α is known to be involved in activation of JNK (Urano et al, 2000), a key player that mediates proinflammatory cytokine production through activating transcription factor AP-1 (Brenner et al, 1989), we reasoned that loss of IRE1α activation may impair JNK functions in addition to loss of *XBP-1* mRNA splicing upon TLR stimulation. However, as indicated in the supplemental figure 8A, activation of JNK, as well as other MAPK including p38 and Erk1/2, were not affected by IRE1α deletion (supplemental Fig. 8A). While these findings exclude the possibility that impaired MAPKs, including JNK, p38 and Erk, contribute to the reduced proinflammatory cytokine production by IRE1α-null macrophages, additional unknown IRE1α downstream factor may still exist in macrophages. For example, as pointed out by this reviewer, it is possible that IRE1α regulates IL-1β secretion through inflammosome pathway. Future studies are needed to determine whether IRE1α regulates inflammosome pathways and/or other known targets in macrophages during inflammation.

Second, it is possible that XBP-1s may have synergy with other TRAF6 downstream transcription factors, such as NF-kB, AP-1 and IRFs, in promoting proinflammatory cytokine production. For example, we have observed that while TM induced more than 10 folds or even hundreds of folds more spliced XBP-1 expression levels than TLR stimuli, almost no inflammatory cytokines were produced in the tunicamycin (TM)-stimulated macrophages. One of the possibilities is that, during 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 spliced XBP-1 to elicit their trans-activation effects on pro-inflammatory gene expression. Our laboratory is currently using a ChIP-seq approach to study the genome wide promoter binding profiles of XBP-1s in macrophages during ER stress and TLR stimulation. In addition, we are also determining whether XBP-1s can interact with any family members of transcription factors including NF-kB, AP-1 and IRFs. We are aware that these approaches, which may provide an explanation for the fact that XBP-1 could only partially rescue proinflammatory cytokine productions by TRAF6-null macrophages, are beyond the scope of the current study. A discussion is provided in the revised manuscript (page 16-17).

10. *The authors should present data or at least speculate on what could be the cue/signal in the RA microenvironment that triggers IRE1*α *activation and thus XBP-1s overexpression. This was not addressed in the paper, and it is critical from the mechanistic perspective. In addition, it would be important to determine if the other arms of the ER stress response (ie. PERK, EIF2a, ATF6 etc) are also overactivated in RA-infiltrating macrophages or if this pathological setting exclusively triggers the IRE1*α*/XBP-1 axis.*

Response: Due to the limited macrophage numbers that could be obtained from the synovial fluids of RA and OA patients, we did not analyze the activation of other ER stress arms in the human synovial macrophages. Yoo et al have recently reported that, in consistent to our observation, the elevated phosphorylated-IRE1α and spliced XBP-1 protein levels were detected in the synovial tissues from RA patients than those from OA patients. In addition, the increased CHOP, phospho-eIF2α and ATF6

expression levels were also detected in both the synovial tissues and synovial fluid macrophages (Yoo et al, 2012). Therefore, it is likely that a broad spectrum of ER stress elements is activated in macrophages from RA patients.

11. *Figure 4D experiments must be repeated using an irrelevant antibody for IP purposes in order to further confirm the specificity of the pull down assays. Also, since all the interaction experiments were performed via protein overexpression, it would be important to show the endogenous interaction between IRE1αand TRAF6 in LPS-treated RAW or bone marrow-derived macrophages.*

Response: In our experiments for Co-IP, normal mouse IgG was included as an antibody control. No non-specific pull-down was detected in the interaction experiments via protein over-expression (Fig 5A). As suggested, we performed new co-IP experiments and confirmed the interaction between endogenous IRE1α and TRAF6 in mouse primary macrophages (Fig. 5B).

12. *It would be important to show as control that TRAF6 and PPE2 (we think this should be PP2A) do not interact with each other in order to further confirm that these proteins directly affect IRE1α activity and stability. In addition, it would be necessary to address whether the expression levels of PP2A are similar in wild type vs. TRAF6-deficient cells.*

Response: We have examined the interaction between TRAF6 and PP2A in macrophages. Our result showed that TRAF6 interacts with PP2A in macrophages upon LPS Stimulation (Supplemental Fig. 10A), suggesting that TRAF6 and PP2A may form a complex with IRE1α in macrophages upon TLR stimulation. As indicated in Supplemental Fig. 10B, the protein levels of PP2A in TRAF6-null and wild-type control MEFs are indistinguishable, thus excluding the possibility that the increased PP2A/IRE1α interaction in TRAF6-null cells is due to the altered protein expression levels.

13. *The authors overstate some of their findings as being original, when in fact many of them (i.e. TRAF6 requirement for XBP-1 splicing in response to TLR stimulation) were previously addressed in other work (Martinon et al., Nature Immunology 2010). The relevant passages should be re-worded to reflect this.*

Response: We agree with the reviewer that the study by *Martinon et al* triggered us to analyze TRAF6 in TLR-mediated IRE1α activation as described in the original manuscript as well as the revised version. Our study here has made the discovery that TRAF6 catalyzes IRE1α ubiquitination, by functioning as an E3 ligase of IRE1α, to block the recruitment of protein phosphatase 2A (PP2A), a phosphatase that inhibits IRE1α phosphorylation. This has been clarified in the revised manuscript. The work by Martinon et al. (Nature Immunology 2010) has been cited multiple times in the revised manuscript wherever it is applicable.

-minor concerns that should be addressed:
Please correct typos and grammar errors in the main text.

Response: We have asked professional in scientific English writing to help in English editing. The typos and grammar errors have been corrected.

Referee #2

The main finding described here include:

1. that myeloid-specific deletion of the IRE1α gene protected mice from inflammatory arthritis,
2. that IRE1-alpha deficiency attenuates TLR induced cytokine production (including inflammasome depedent Interleukin-1 production – not discussed/analysed)
3.that TRAF-6 catalyses IRE1-alpha ubiquitination and blocks PP2A recruitment (the latter beeing known to inhibit IRE1-alpha phosphorylation).

The propensity of ER stress to enhance TLR drivrn innate immune responses has been highlighted by recent work of Glimcher's group (see Physiol.Rev,2011,91:1219, or Curr. Op. Immunol, 2011,23:35, not cited) in demonstrating that TLR4 and TLR2 promote the phosphorylation of the ER signalling kinase IRE1 and activation of its downstream target XBP-1 (cited: Martinon, et. al.2010). While Glimcher et al based their findings on XBP-1 deficient macrophages the authors used here myeloid specifically deleted mice - or cells - for the IRE1 gene. That enabled studies of IRE1 deficiency in vivo (inflammatory arthritis).

Response: The publications related to Glimcher's work have been cited in the revised manuscript.

While the only known activator of XBP-1 is IRE1α, accumulated evidences indicate that IRE1α executes its functions in both XBP-1-dependent and independent mechanisms. We demonstrated that IRE1α deletion impairs the production of a broader spectrum of inflammatory cytokines and leads to more profound inflammatory defects than *XBP-1* deletion in macrophages (Figures 2 & 7) (Martinon et al, 2010). Similarly, it has been suggested that that the phenotypes of IRE1α and XBP-1 KO mice are not identical, suggesting the presence of unique functions specific to each gene (Lee et al, 2011). Although both IRE1α and XBP-1 deficiencies ablate the active transcription factor XBP-1s, there are fundamental differences between these two.

First, in addition to XBP-1, IRE1α has been shown to regulate other downstream targets. For example, it has been demonstrated that ER stress activates JNK through IRE1α to induce cell apoptosis (Urano et al, 2000). However, we observed that loss of IRE1α functions did not affect TLR-induced activation of MAPKs including JNK, p38 and Erk1/2, excluding the involvement of MAPKs in IRE1α-mediated proinflammatory cytokine production by macrophages upon TLR stimulation.

Second, IRE1α deficiency does not ablate XBP-1u protein encoded by the unspliced XBP-1 mRNA. Although XBP-1u is unstable and has no transactivation ability (Shen et al, 2001; Yoshida et al, 2001), it might have distinct, yet-to-be explored functions. For example, recent studies have suggested that the unspliced form of XBP-1 protein regulates autophagy (Yanagitani et al, 2011; Zhao et al, 2013).

Last but not the least, XBP-1-deficiency can cause feedback activation of IRE1α (Kaser et al, 2008), which could result in the hyperactivation of IRE1α to enhance the "XBP-1-independent" functions such as insulin mRNA degradation (Han et al, 2009).

A discussion about the possible mechanisms underlying the XBP-1s-independent IRE1a functions in myeloid cells during inflammation is provided in the manuscript (page 17).

Specific points:

1. *The MS appears poor in citation accuracy - and Glimcher's work is cited only once.*

Response: We have carefully checked our citations for accuracy. Glimcher's work (Martinon et al, 2010) has been cited multiple times, wherever it fits, in the revised manuscript.

2. *In reading the cited work of Calfon et al., 2002: and of Shen et. al., 2001 (see pp 9, 4th line)it remains unclear why the authors regard this work as conformation that TLR's activate the IRE1-XBP-1-UPR axis. Here, citation of Glimchers work would be more appropriate.*

Response: The inappropriate citations have been corrected in the revised manuscript. The work from Glimcher's group has been cited.

4. *The experiments attempting to delineate the molecular basis of TRAF6 mediated IRE1 activation -see pp 10 - would benefit from using a "cell free system". In particular the proposed competition of TRAF6 with PP2A during IRE1-alpha activation could experimentally be validated.*

Response: We understand that the *in vitro* ubiquitination assay is helpful to validating the discovery that TRAF6 functions as an E3 ligase of IRE1α. However, since both IRE1α and TRAF6 are big-size proteins, it is technically difficult to obtain purified IRE1α and TRAF6 recombinant proteins for the in vitro assay. Instead, we provide multiple lines of evidences that TRAF6 functions as an E3 ligase of IRE1α. First, TRAF6 interacts with IRE1α in transiently transfected HEK293 cells, in macrophage line RAW264.7 cells and mouse primary macrophages (Fig. 5A-5C). We identified that the linker region of IRE1α and the MATH domain of TRAF6 mediate the interaction between IRE1α and TRAF6 (Fig. 5D & 5E). Second, ectopic expression of TRAF6, but not its E3 ligase catalytic mutant TRAF6/CA, promotes IRE1α ubiquitination (Fig. 6C). Third, topologically, TRAF6 catalyzes IRE1α poly-ubiquitination with a K48-, but not K63-lincage (Fig. 6D &6E). Fourth, TRAF6 promotes IRE1α, but not PP2A, protein degradation (Fig. 6H & 6I). As a consequence, loss of TRAF6 results in the elevated IRE1α interaction with PP2A (Fig. 6A). Collectively, we conclude that TRAF6 inhibits IRE1α interaction with PP2A through catalyzing IRE1α ubiquitination and degradation.

Minor points:
pp10 - 5th line - Fig 2B should read 4C

Response: This has been corrected in the revised manuscript.

Referee #3

In this research article, Qiu and al. demonstrate that IRE1α may be a potential therapeutic target for inflammatory arthritis. Their findings include 1) deletion of IRE1α in mice delays progression of inflammatory arthritis, 2) IRE1α is necessary for pro-inflammatory cytokine production by macrophages, 3) IRE1α activation through TLR/TRAF6 axis in macrophages induce XBP-1 mRNA splicing. In short, the authors reveal a molecular mechanism underlying TLR-induced activation of IRE1α and the potential therapeutic of targeting IRE1α in inflammatory arthritis.

This present study is heavily based on the work published by Martinon and al. in Nature Immunology in 2010. However, rather than investigating the mechanisms behind the activation of IRE1α in a context of innate immunity (like in Martinon et al.), herein the authors evaluate the role of IRE1α in autoimmunity in a context of inflammatory arthritis -this makes it original. Some observations and conclusions remain however confirmatory, not novel *per se*.

Comment1: Globally, this is a well-written manuscript containing interesting data. *Main concerns regard the rationale behind the focus in vitro studies on one single cell type (macrophages) and the novelty of the research.*

Response: The involvement of other myeloid cells, in particular, neutrophils, in our inflammatory RA model, has been addressed in the revised manuscript. Please see the details in our response to the critique 1 from the reviewer #1.

Minor comments:

1. *Reference formatting required:*
-page 3 line 4 .....(Piccinini and Midwood)...year missing
-page 3 line 9 ..van Roon et al. 1997)_ENREF_4.....?
-page 5 line 16....reference missing
-Page 8 line 5.....(Dickout et al.)...year missing

Response: These were all corrected in the revised manuscript.

2. *In the introduction: "mice with myeloid deletion of IRE1α are resistant to inflammatory arthritis". These mice are not resistant to arthritis rather they demonstrate a delay in the progression of the disease.*

Response: Following the comment, we have changed this sentence to "*myeloid deletion of IRE1α gene resulted in a dramatic reduction in the clinical symptoms of inflammatory arthritis in mice".*

3. *Original papers re K/BxN serum transfer-induced arthritis (ex. Mathis and Benoist) should be cited.*

Response: The original paper has been cited in the revised manuscript.

4. *Page 3: "Indeed, Chaperonin 10, a mitochondrial protein that inhibits TLRs, was clinically effective in a small-scale study with RA patients ». One reference is in German and cannot be easily assessed, and other references and in fact « comments to the editors ». Why not citing the original paper in Lancet by Vanags instead?*

Response: As suggested, we have cited the original paper by Vanags in the revised manuscript.

Major comments:

1. *First sentence in the abstract and in the result section: "In rheumatoid arthritis, macrophages are the major source of inflammatory cytokines." This statement seems to form the basis of this focused study on macrophages. No references support this affirmation in the manuscript. What serious study can support this statement?*

Response: To avoid confusion, this sentence has been changed. A rational of this study has also been discussed in the introduction and discussion sections (Pages 2 & 6)

2. *The authors focus their work on the molecular aspects of IRE1α in macrophages, like in the study by Martinon (where infection was the context and macrophages were highly relevant). Herein, the authors used the K/BxN serum model of arthritis for their study. Although this model is highly relevant to arthritis and well accepted in the field, the role of macrophages in this model remains to precisely establish. In the K/BxN model of arthritis, macrophages can even play anti-inflammatory functions. Rather, neutrophils and mast cells are important players in this model, contribute via TLR4 (Kin 2012), and are most likely cells expressing pathogenic IRE1α. Since IRE1α is deleted in neutrophils and mast cells in IRE1αflox/floxLysM-Cre mice, their respective contribution should be determined.*

Response: As response to the critique 1 from the reviewer #1, we have analyzed the activation of neutrophils from IRE1α-null mice. First, IRE1α-deletion has no effect on the neutrophil development as their percentages and absolute numbers were not changed in IRE1α conditional knockout mice (Supplemental Fig. 6A & 6B). Second, loss of IRE1α functions inhibited TNF-α production by neutrophils in the K/BxN serum-treated mice (Supplemental Fig. 6C). Third, a significant reduction in the numbers of infiltrated neutrophils was detected in the joints of IRE1α conditional knockout mice treated with K/BxN sera, compared to that in the control mice (Fig. 1E). These results indicate that the impaired functions of neutrophils contribute to the protective effect of myeloid-specific IRE1α gene deletion in mice. All these scenarios have been fully addressed in the revised manuscript.

3. *Furthermore, the authors ensured that lymphocytes are intact in IRE1αflox/floxLysM-Cre mice. Lymphocytes are dispensable is the K/BxN model of arthritis. Rather, the authors should make sure the levels of neutrophils and mast cells are intact.*

Response: We agree with this reviewer that lymphocytes play a minor or even no role in the disease development in this inflammatory arthritis model. We performed the analysis of lymphocyte in IRE1α conditional knockout and confirmed the specificity of myeloid IRE1α gene deletion (supplemental Fig. 4), and thus excluding the possibility of myeloid IRE1α gene deletion to affect lymphocyte development. We have also analyzed the levels of neutrophils and mast cells in the IRE1α conditional knockout *vs* the control mice. The results confirmed that the levels of neutrophils and mast cells in the IRE1α conditional knockout mice were comparable to those in the control mice (supplemental Figs. 3 & 6)

4. *Second sentence of the introduction: "The elevated levels of TLR in arthritis... significantly contribute to rheumatoid arthritis." One review is cited, no original papers. What study demonstrates that TLRs indeed contribute to arthritis? What is the background regarding TLRs and the mouse model of arthritis chosen for this study? For instance, TLR2 KO mice develop enhanced arthritis in the K/BxN model; how do the authors reconcile their data with the existing literature on arthritis?*

Response: As pointed by this reviewer, it has been recently shown that TLR2 KO mice develop enhanced arthritis in the K/BxN model, and one of the underlying mechanisms is that TLR2 deletion leads to the reduced IL-10 production (Huang et al, 2013). In this study, we show that TLR2 signaling activates IRE1α activation in macrophages to promote inflammatory arthritis in mice. Huang et al utilized a germline TLR2 knockout strain for their study, while we used myeloid-specific IRE1α gene deletion. Although the detailed mechanisms underlying the difference between these two models are not known, the TLR2 knockout model definitely causes a more extensive defect in protective inflammatory signaling originated from plasma membrane (cell surface receptor signaling). Instead, IRE1α mediates an intracellular stress signaling pathway to augment the pro-inflammatory cytokine production by

interacting with TLR signaling under acute inflammatory stress conditions. Our study demonstrated that deletion of IRE1α can alleviate the production of pro-inflammatory cytokines, such as TNF-α, in inflammatory RA model. Therefore, suppression of IRE1α signaling can repress the development of inflammatory RA. Clarification and discussion regarding TLR2 knockout and IRE1α knockout mice in arthritis has been incorporated in the revised manuscript (page 20)

The mice used in our study are at C57/BL6 genetic background. In addition, we have also fixed the related citations.

5. Figure 1 A through B:

- *Synovial fluids from "acute" and "chronic" rheumatoid arthritis patients were obtained. Where are these data? Were the levels of spliced XBP-1 different in acute compared to chronic patients? Were the chronic patients treated with medications differently?*

Response: In the revised manuscript, we provide a supplemental table to summarize the patients' sample information. Our analysis indicated that the levels of spliced XBP-1 mRNA were indistinguishable between acute and chronic RAs (Supplemental Fig. 1C). However, we should clarify that, while chronic RA was diagnosed, synovial fluid can only be obtained during the acute phase. Therefore, the available RA samples can only be collected under acute conditions.

6 *According to the authors, "The adherent cells were used to isolate total RNA, as it has been shown that a large portion of adherent cells are macrophages. » No citations are given. It is important to know what cells are analyzed in these PCR studies. When OA adherent cells are compared to RA adherent cells, it is likely that different cell types rather than different expression levels are in fact examined. The conclusions that are derived from these experiments are therefore difficult to interpret. MACS purification columns or other methods should be employed to determine the relative expression of mRNA in specific cell types.*

Response: In the revised manuscript, we performed new FACS analysis and confirmed that more than 90% of adherent cells are macrophages (Supplemental Fig. 1A & 1B). Therefore, we are confident that the elevated spliced *XBP-1* mRNA levels observed largely reflect IRE1α activation in macrophages. We agree with the reviewer that it will be nice to further purify the cells using MACS purification columns. However, since only limited numbers of cells could be obtained from each human patient, it is technically difficult to do the MACS purification column purification with those human patient samples.

7. *"IRE1αflox/floxLysM-Cre-, IRE1αflox/+LysM-Cre-, or IRE1α+/+LysM-Cre+, were used as controls ». Eight mice are used as controls in figure 1C. What mice are they exactly? Is this a pool of different mice with different genotypes (IRE1αflox/floxLysM-Cre-, IRE1αflox/+LysM-Cre-, or IRE1α+/+LysM-Cre+)? This experimental condition is unclear to the reader.*

Response: In the revised manuscript, we have indicated the specific genetic background of the control mice for each figure. IRE1αflox/floxLysM-Cre-, IRE1αflox/+LysM-Cre-, or IRE1α+/+LysM-Cre+ mice have been used as the control mice for our experiments. The IRE1αflox/floxLysM-Cre- mice have functional IRE1α flox allele but no Cre transgene. This type of control mice can express the functional IRE1α protein at the level comparable to the wild-type mice; the IRE1αflox/+LysM-Cre- mice carry an IRE1α flox allele and a wild-type IRE1α allele but no Cre transgene. These mice also express normal levels of functional IRE1α; the IRE1α+/+LysM-Cre+ mice have wild-type IRE1α allele and a LyM-

CRE transgene. These mice have no difference from the wild-type mice in producing functional IRE1α. We confirmed that there is no difference in the inflammatory macrophage phenotype between IRE1αflox/floxLysM-Cre-, IRE1αflox/+LysM-Cre-, and IRE1α+/+LysM-Cre+ mice. We use these mice as the controls to exclude any potential effect/phenotype caused by IRE1α flox allele and/or LysM-Cre transgene.

8. *Figure 1 E, F: "Arrows indicate lymphocyte infiltration and cartilage erosion". What arrow indicates what? Different types of arrows should be employed. In addition, the quality of the figure does not permit assessment of the presence of lymphocytes. However, in this model of arthritis, neutrophils (and possibly monocytes/macrophages) are most likely the cells that populate the joint, not the lymphocytes. Why not demonstrating the presence of a cell type relevant to the current study (such as monocytes/macrophages, or LysM+ cells).*

Response: The figures have been labeled as suggested. We realized that in the tissue sections it is difficult to define the cells as infiltrated lymphocytes. Accordingly, we changed the description to "the inflamed synovial tissues". A further characterization of infiltrated macrophages, neutrophils, and mast cells has been performed as shown in Fig 1E in the revised manuscript.

9. *A better introduction to PP2A in the results section should be made.*

Response: A discussion about the PP2A has been provided (pages 5 & 13). PP2A is a ubiquitously expressed serine threonine phosphatase that dephosphorylates many key molecule players in cell proliferation, signal transduction and apoptosis (Millward et al, 1999). Recent studies indicate that PP2A interacts with IRE1α through the adaptor protein RACK1 to suppress IRE1α phosphorylation (Qiu et al, 2010) thereby inhibiting glucose-stimulated IRE1α activation and attenuating IRE1α-dependent increases in insulin production.

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2nd Editorial Decision 07 May 2013

Thank you for submitting your revised manuscript to the EMBO Journal. Your paper has now been re-reviewed by referees # 1 and 3.

As you can see below, both referee appreciate the introduced changes. However they also both have some remaining issues that need to be sorted out before acceptance here. Given the referees' comments, I would like to ask you to resolve the comments in a final round of revision.

Thank you for the opportunity to consider your work for publication. I look forward to seeing the final revision.

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REFEREE REPORTS:

Referee #1 :

The authors of this study followed up on the work of Martinon et. al by investigating the role of IRE1alpha in the inflammatory process of rheumatoid arthritis (RA). By using the transferrable K/BxN serum model Qiu and colleagues have investigated the role of IRE1a overactivation in the pathophysiology of RA. The authors show attractive data suggesting that the IRE1a/XBP1 arm of the ER response may represent a novel therapeutic target for RA. Working primarily with macrophages and MEFs, the authors extend previous findings concerning TRAF6 mediated IRE1 activation The study presents a new mechanism by which TRAF6 acts to regulate TLR-mediated IRE1a activation by promoting IRE1a ubiquitination and inhibiting PPA2-mediated dephosphorylation. The authors provide convincing evidence indicating that cells in the synovial fluid of RA patients (predominantly macrophages) exhibit increased mRNA levels of XBP1s, one of the main targets of IRE1a, and that loss of IRE1a in myeloid cells as well as treatment with an IRE1a inhibitor dramatically blocks RA progression in the transferrable model utilized. Though many of our previous comments were satisfactorily addressed in the authors' initial revisions, there are still several outstanding issues that need to be addressed before the study is appropriate for publication..

1. While the effects described correlate with decreased production and secretion of proinflammatory cytokines by macrophages lacking a fully functional form of IRE1a, new experiments included in the revised version (new Figures 1E and 1F) reinforce my initial concern regarding impaired myeloid cell accumulation in the joints of IRE1a KO mice upon transfer of pathogenic serum. The authors now describe that this situation is indeed taking place, but the mechanism behind this interesting phenomenon is not explored. For example, does the IRE1a/XBP1 arm control the expression of chemokine receptors that enable myeloid cell infiltration into the joint? Further experiments are required to rule out/confirm this possibility or others.

2. The relevance of TRAF6 mediated degradation of IRE1alpha via K48 ubiquitination is difficult to interpret given that the authors simultaneously argue that TRAF6 enhances IRE1 activation by interfering with PP2A association with IRE1alpha. If TRAF6 promotes IRE1 degradation through ubiquitination, and LPS stimulation induces binding of TRAF6 with IRE1alpha, how does this ultimately lead to increased activity and XBP-1 activation? Does TLR stimulation decrease the half-life of IRE1? If so, why does it appear as though total IRE1 levels are unchanged (or even increased) after LPS stimulation? These are important questions that the authors can answer with a few simple experiments that would significantly strengthen this part of the paper (measure synthesis and degradation rates for IRE1 with/without LPS treatment).

3. Importantly, the authors still have not addressed why constitutive association of PP2A, a phosphatase previously shown to prevent autophosphorylation and activation of IRE1alpha, under TRAF6-/- conditions does not affect activation of IRE1 upon tunicamycin treatment. Though it is quite possible, and probably likely, that IRE1alpha is activated by different mechanisms when treated with chemical stressors versus LPS, one would still expect that increased basal PP2A association with IRE1 would blunt its activation. This reviewer would like the authors to comment on this finding in the appropriate section of the manuscript.

4. There are several smaller issues as well. There is a conspicuous absence of any mention of IRE1 regulated mRNA decay. As no clear mechanism is provided for why IRE1 enhances inflammation in this model (particularly considering that the phenotype differs from that of the XBP-1 knockout macrophages), the authors should at the very least mention the possibility of other mRNA targets. Moreover, the authors should try to expand on the mechanism by which IRE1 enhances pro-inflammatory cytokine production by overexpressing various mutant IRE1 variants (kinase active/RNAse dead, kinase dead, WT) to determine the relative contributions of the kinase domain and the RNAse domain to their observed phenotype. Finally, the authors observe a decrease in IL-1beta secretion in IRE1alpha knockout macrophages in response to TLR ligands. IRE1alpha has recently been linked to activation of the inflammasome (Lerner et al., Cell Metabolism 2012; Oslowski et al., Cell Metabolism 2012), and these studies should be mentioned and cited accordingly.

5. Finally, the authors ignored my initial question regarding potential cues in the RA microenvironment that may be responsible for promoting IRE1a activation and thus XBP1s upregulation. Again, this reviewer would appreciate further comments on this topic from mechanistic and physiological perspectives.

Referee #3:

Although the authors brought significant improvements to the manuscript, there are still lingering concerns that need attention:
1. The authors claim that the IRE1 deficient mice are "resistant" to arthritis. The mice are not resistant, they rather display a delay in the progression of the disease. While the authors mention that they changed this statement in the revised version, it has not been changed. It has to be corrected to reflect what is observed from the figure/experiment.

2. According to the authors, the adherent cells are mostly macrophages (roughly 90%) (novel suppl. Fig 1). Since PCR can amplify DNA from small "contaminating" cellular populations, it is hard to determine whether the expression of XBP-1 is from macrophages or other minor populations, or both. What is the other 10% cellular population? Does it contain neutrophils, also expressing XBP-1?

2nd Revision - authors' response 02 July 2013

Point-to-point Responses to the Reviewers' Comments

We would like to thank the reviewers for their constructive comments, which undoubtedly further improved the manuscript. We have carefully addressed all the critiques in a point-to-point manner as described below as well as in the revised manuscript.

Reviewer #1:

The authors of this study followed up on the work of Martinon et. al by investigating the role of IRE1alpha in the inflammatory process of rheumatoid arthritis (RA). By using the transferrable K/BxN serum model Qiu and colleagues have investigated the role of IRE1α overactivation in the pathophysiology of RA. The authors show attractive data suggesting that the IRE1α/XBP1 arm of the ER response may represent a novel therapeutic target for RA. Working primarily with macrophages and MEFs, the authors extend previous findings concerning TRAF6 mediated IRE1α activation The study presents a new mechanism by which TRAF6 acts to regulate TLR-mediated IRE1α activation by promoting IRE1α ubiquitination and inhibiting PP2A-mediated dephosphorylation. The authors provide convincing evidence indicating that cells in the synovial fluid of RA patients (predominantly macrophages) exhibit increased mRNA levels of XBP1s, one of the main targets of IRE1α, and that loss of IRE1α in myeloid cells as well as treatment with an IRE1α inhibitor dramatically blocks RA progression in the transferrable model utilized. Though many of our previous comments were satisfactorily addressed in the authors' initial revisions, there are still several outstanding issues that need to be addressed before the study is appropriate for publication.

1. While the effects described correlate with decreased production and secretion of proinflammatory cytokines by macrophages lacking a fully functional form of IRE1α, new experiments included in the revised version (new Figures 1E and 1F) reinforce my initial concern regarding impaired myeloid cell accumulation in the joints of IRE1α KO mice upon transfer of pathogenic serum. The authors now describe that this situation is indeed taking place, but the mechanism behind this interesting phenomenon is not explored. For example, does the IRE1α/XBP1 arm control the expression of chemokine receptors that enable myeloid cell infiltration into the joint? Further experiments are required to rule out/confirm this possibility or others.

Response: We thank the reviewer for this comment. As suggested, we have analyzed the expression levels of chemokine receptors including CXCR2 (Jacobs et al, 2010) and CCR9 (Schmutz et al, 2010), 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 *IRE1α* gene deletion did not affect their expression in either macrophages or neutrophils, thus excluding the possibility that *IRE1α* 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 cell surface Fc receptor CD16 and the complement C5a receptor C5aR in myeloid cells are critical for K/BxN serum-induced arthritis (Ji et al, 2002). Therefore, we compared the expression levels of CD16 and C5aR in IRE1α$^{-/-}$ and control wild-type macrophage, mast cells, and neutrophils. The results show that the expression levels of both CD16 and C5aR are indistinguishable between wild-type and *IRE1α* knockout cells. This is now addressed in the revised manuscript (Page 8-9, Supplemental Figure 3)

2. *The relevance of TRAF6 mediated degradation of IRE1alpha via K48 ubiquitination is difficult to interpret given that the authors simultaneously argue that TRAF6 enhances IRE1α activation by interfering with PP2A association with IRE1alpha. If TRAF6 promotes IRE1α degradation through ubiquitination, and LPS stimulation induces binding of TRAF6 with IRE1alpha, how does this ultimately lead to increased activity and XBP-1 activation? Does TLR stimulation decrease the half-life of IRE1α? If so, why does it appear as though total IRE1α levels are unchanged (or even increased) after LPS stimulation? These are important questions that the authors can answer with a few simple experiments that would significantly strengthen this part of the paper (measure synthesis and degradation rates for IRE1α with/without LPS treatment).*

Response: Ubiquitination-mediated degradation and activation of the same substrate often occurs. For example, the E3 ubiquitin ligase Met30-mediated ubiquitination of the transcription factor VP16 activates VP16 transcriptional activity, but the activation event is followed by VP16 protein destruction (Salghetti et al, 2001). This suggests that ubiquitination plays dual roles in activation and activator destruction. Similarly, we show here that TRAF6-mediated ubiquitination suppresses IRE1α/PP2A interaction, which presumably facilitates both IRE1α activation and IRE1α protein degradation. This is evidenced that TRAF6 is required for both IRE1α activation and degradation (Fig. 4A & Fig. 6H). We speculate that, at the early phase of TLR stimulation, TRAF6-mediated IRE1α ubiquitination blocks the interaction between IRE1α and its inhibitor PP2A, and thus enhances IRE1α activation. This is followed by IRE1α protein degradation mediated through its ubiquitination, in order to terminate the signaling transduction at the late phase of the stimulation. This scenario has been discussed in the revised manuscript (page 20-21).

We agree with the reviewer that TLR stimulation can also promote IRE1α degradation because we have shown that (1) LPS stimulation enhances TRAF6/IRE1α interaction (Fig. 5C) and (2) TRAF6-mediated ubiquitination promotes IRE1 degradation (Fig. 6D). In the revised manuscript, we further analyzed IRE1α protein stability in macrophages with or without LPS stimulation. The result showed that stimulation of macrophages with LPS did not alter the half-life of IRE1α (Supplemental Fig. 14A). As suggested by this reviewer, we examined whether LPS stimulation can increase IRE1α protein expression. As shown in the Supplemental Fig. 14B, stimulation of the cells with LPS for 4 hours did not increase IRE1α protein synthesis as monitored by 35S-M/C incorporation. The unchanged IRE1α protein levels after short-time LPS stimulation may be partially associated with degradation of IRE1α protein triggered by TRAF6. This is consistent with our initial discovery that LPS stimulation promotes IRE1α interaction with TRAF6, which induces IRE1α degradation but does not reduce the total IRE1α protein expression levels (Fig. 5C). However, stimulation of macrophages with LPS for longer time, such as 8-16 hours, increased the total protein levels of IRE1α (supplemental Fig. 14C & D). This result clearly indicates that LPS induces IRE1α protein expression and this induction succeeds TRAF6-mediated degradation during the longer time period of stimulation. Further, we asked whether IRE1α protein synthesis is increased in *TRAF6*-null cells. Pulse-chase analysis showed that both the levels (time 0) and stability (half-life) of the newly synthesized IRE1α protein in *TRAF6*-null cells significantly increased compared to that in the wild-type cells (supplemental Fig. 14E & 14F), thus confirming our initial conclusion that TRAF6 plays a critical role in controlling IRE1α protein stability

Therefore, TLR stimulation, at least by LPS, promotes TRAF6-mediated IRE1α activation and subsequent degradation; on the other hand, it induces IRE1α protein expression. Therefore, LPS stimulation on one hand induces IRE1α protein expression and, on the other hand promotes TRAF6-mediated IRE1α degradation, leading to a relatively stable expression levels of IRE1α during the early stages of stimulation. However, it appears that the induction of IRE1α expression succeeds IRE1α degradation during the later stage of LPS stimulation, because we detected the increased IRE1α protein levels in macrophages after 8 hours of LPS stimulation but not within 4 hours. (page 16-17).

We also noticed that, in the absence of TRAF6, the newly synthesized IRE1α protein was gradually degraded as determined by the pulse-chase experiment (Supplemental Fig. 14E & 14F), which seems contrasting to our original observation that total IRE1α protein levels were stable in *TRAF6*-null cells (Fig. 6H). There are several explanations: first: the newly synthesized proteins may contain misfolded fractions, which can be degraded through the TRAF6-independent manner; second, the cycloheximide treatment, while was used to measure protein stability, may have the off-target effect. Nevertheless, our data clearly indicated that IRE1α protein stability is increased in *TRAF6*-null cells as confirmed by both the pulse chase (newly synthesized) (Supplemental Fig. 14E & 14F) and cycloheximide-treatment (total) experiments (Fig. 6H & 6I).

3. Importantly, the authors still have not addressed why constitutive association of PP2A, a phosphatase previously shown to prevent autophosphorylation and activation of IRE1alpha, under TRAF6-/- conditions does not affect activation of IRE1α upon tunicamycin treatment. Though it is quite possible, and probably likely, that IRE1alpha is activated by different mechanisms when treated with chemical stressors versus LPS, one would still expect that increased basal PP2A association with IRE1α would blunt its activation. This reviewer would like the authors to comment on this finding in the appropriate section of the manuscript.

Response: In the first revision, we discussed that metabolic factors in the culture media, such as glucose, growth factors and amino acids, all of which have been shown to activate IRE1α (references: (Lee et al, 2008; Zhang, 2010; Zhang & Kaufman, 2008), may be sufficient to trigger IRE1α/PP2A interaction in the absence of TRAF6. As shown in the supplemental Fig. 13, only background levels of PP2A/IRE1α 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/IRE1α interaction was detected. However, loss of TRAF6 further enhanced their interaction (supplemental Fig. 13). These results support our initial speculation that existence of the culture media factors is sufficient to trigger PP2A/IRE1α interaction in the absence of TRAF6. Further addition of LPS enhances the interaction in wild-type cells, but the enhancement is minimized in *TRAF6* knockout cells (Fig. 6A & 6B). We described the IRE1α/PP2A interaction in the absence of challenges as "constitutive interaction". These scenarios have been discussed in the revised manuscript.

We concluded that TRAF6-mediated ubiquitination promotes IRE1α activation by suppressing the recruitment of the IRE1α inhibitor PP2A. This conclusion is based on the following findings from our study: 1) TRAF6 promotes IRE1α ubiquitination and degradation (Fig. 6D & 6H); 2) over-expression of TRAF6, but not its E3 ligase-intact mutant, inhibits IRE1α/PP2A interaction; and 3) loss of TRAF6

leads to the elevated IRE1α/PP2A interaction (Fig. 6C). However, we reproducibly detected that LPS stimulation promotes IRE1α interaction both with TRAF6 and with PP2A (Figs. 5B, 5C, 6A & 6B). 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 IRE1α protein in *TRAF6*-null cells significantly increased compared to that in the wild-type cells (supplemental Fig. 14). Nevertheless, the detailed dynamics of IRE1α/PP2A interaction in the presence or absence of TRAF6 under inflammatory stress needs to be further elucidated. The related clarification and discussion have been incorporated in the revised manuscript in page 21.

4. There are several smaller issues as well. There is a conspicuous absence of any mention of IRE1α regulated mRNA decay. As no clear mechanism is provided for why IRE1α enhances inflammation in this model (particularly considering that the phenotype differs from that of the XBP-1 knockout macrophages), the authors should at the very least mention the possibility of other mRNA targets. Moreover, the authors should try to expand on the mechanism by which IRE1α enhances pro-inflammatory cytokine production by overexpressing various mutant IRE1α variants (kinase active/RNAse dead, kinase dead, WT) to determine the relative contributions of the kinase domain and the RNAse domain to their observed phenotype. Finally, the authors observe a decrease in IL-1beta secretion in IRE1alpha knockout macrophages in response to TLR ligands. IRE1alpha has recently been linked to activation of the inflammasome (Lerner et al., Cell Metabolism 2012; Oslowski et al., Cell Metabolism 2012), and these studies should be mentioned and cited accordingly.

Response: To determine whether IRE1α enhances inflammation through unconventional pathways, we first examined whether activation of IRE1α under the inflammatory stimulus of LPS, Pam3, or polyI:C is involved in Regulated Ire1-dependent Decay (RIDD) in macrophages (Han et al, 2009; Hollien et al, 2009; Hollien & Weissman, 2006). We challenged bone marrow-derived macrophages from wild-type and IRE1α conditional knockout mice with LPS, Pam3, or polyI:C (Supplemental Fig.10). Gene expression analysis indicated that the mRNA levels of the major RIDD target genes, including *Blos1, Hgnat, Pmp22, Scara3, Col6* and *PdgfR* were indistinguishable between wild-type control and *IRE1α* knockout macrophages under the inflammatory stimuli. This result suggested that IRE1α is not involved in regulating mRNA levels in macrophages through the RIDD pathway upon LPS stimulation. Note that expression of these RIDD target genes was down-regulated in response to the inflammatory stimuli. However, this regulation is independent of IRE1α. Whether this down-regulation is associated with macrophage inflammation is interesting but beyond the scope of the current study. These data are indicated in the revised manuscript (page 11-12).

As we initially described, our study showed that the *IRE1α*-null mice displayed more severe defect in inflammatory cytokine production compared to the *Xbp-1*-null mice as previously described (Martinon et al, 2010). This implies possible IRE1α activity independent of Xbp-1. As suggested by the reviewer, we analyzed whether reconstitution of RNase-intact IRE1α mutants can partially rescue TLR-induced inflammatory cytokine production in *IRE1α*-null MEFs. As indicated in the Supplemental Fig. 9A, neither the RNase-negative mutant (RM) nor the kinase-negative mutant (KM) showed any significant rescue effect on IL-6 expression in *IRE1α*-null MEFs. As a control, the IL-6 expression in the *IRE1α*-null MEFs is fully rescued by the reconstitution of the wild-type IRE1α. IRE1/RM and IRE1/KM have

been shown to function as dominant-negative factors to suppress IRE1α activities (Lee et al, 2002; Tirasophon et al, 2000). Indeed, IRE1α/RM and IRE1α/KM functioned as dominant-negative forms of IRE1α to suppress IL-6 expression in wild-type MEFs expressing either IRE1α/RM or IRE1α/KM (Supplemental Fig. 9A). However, expression of the spliced form of Xbp-1 was able to partially rescue IL-6 expression in *IRE1α*-null MEFs (Supplemental Fig. 9B). These results suggest that regulation of IL6 expression by IRE1α is partially through spliced Xbp-1s. However, our study also implicated that regulation of inflammatory cytokine production by IRE1α is partially mediated through Xbp1-independent mechanism. The alternative targets of IRE1α in regulating expression of inflammatory cytokines remain to be determined in the future. These new experiments are described in the revised manuscript (page 11).

Additionally, we discovered that IRE1α promotes the production of IL-1β, a cytokine that is regulated by inflammasome. Since the recent studies indicated that IRE1α is involved in inflammasome functions (Oslowski et al, 2012), it is possible that IRE1α regulates IL-1β production partially through the inflammasome pathway. This scenario has been discussed in the revised manuscript. The literatures related to inflammasome have been cited (page 19).

5. Finally, the authors ignored my initial question regarding potential cues in the RA microenvironment that may be responsible for promoting IRE1α activation and thus XBP1s upregulation. Again, this reviewer would appreciate further comments on this topic from mechanistic and physiological perspectives.

Response: While additional efforts are needed to identify the causal factors that directly activates IRE1α/XBP-1 pathway in macrophages and neutrophils in RA patients, we propose that multiple factors in the RA microenvironments are possibly involved in this process: 1) given the facts that TLR signaling activates IRE1α (Fig. 2A) and that endogenous TLR ligands are pathogenic factors of RA, the TRL-signaling, raised under the RA microenvironment, may serve as a major driving force of IRE1α activation in synovial fluid cells of RA patients; 2) in addition to TLR signaling, TNF-α stimulation may directly activate IRE1α-mediated *XBP-1* mRNA splicing in macrophages, as we showed in Supplemental Fig. 8; 3) metabolic and cardiovascular risk factors, including hypertension, obesity, high glucose, and dyslipidemia, some of which like glucose can activate IRE1α, are prevalent in patients with RA (Rostom et al, 2013). These factors, raised under the RA microenvironment, may also be triggers of the IRE1α/XBP1 pathway. This is now discussed in the revised manuscript (page 21-22).

Referee #3 (Remarks to the Author):

Although the authors brought significant improvements to the manuscript, there are still lingering concerns that need attention:

1. The authors claim that the IRE1α deficient mice are "resistant" to arthritis. The mice are not resistant, they rather display a delay in the progression of the disease. While the authors mention that they changed this statement in the revised version, it has not been changed. It has to be corrected to reflect what is observed from the figure/experiment.

Response: As suggested, the word of "resistant" has been removed from the revised manuscript. We agree with the reviewer that mice with *IRE1α* gene deletion are not fully resistant to the arthritis induced by K/BxN sera transfer. Therefore, we described this as "deletion of the *IRE1α* gene attenuated the K/BxN serum-induced inflammatory arthritis".

2. According to the authors, the adherent cells are mostly macrophages (roughly 90%) (novel suppl. Fig 1). Since PCR can amplify DNA from small "contaminating" cellular populations, it is hard to determine whether the expression of XBP-1 is from macrophages or other minor populations, or both. What is the other 10% cellular population? Does it contain neutrophils, also expressing XBP-1?

Response: We showed that an average of 93% of adherent cells in the synovial fluids from RA and OA patients are macrophages (Supplemental Fig. 1). The $CD11b^{+}CD206^{-}$ cells are likely neutrophils (averagely 2-3%). In addition to some dead cells, the $CD11b^{-}CD206^{-}$ cells are possibly fibroblasts and a small number of lymphocytes. We have clarified this issue in our revised manuscript (page 8).

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Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now bee re-reviewed by referee #1 who is happy with the introduced changes. I am therefore pleased to accept the manuscript for publication here.

Also, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this just contact me.

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