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FoxO1 Regulates Tlr4 inflammatory pathway Signalling in Macrophages

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Editorial Decision 11 March 2010

Thank you for submitting your manuscript to the EMBO Journal. I have now had a chance to read your manuscript carefully and to discuss it with the other members of our editorial team. In addition, I have also sought external advice on the study from a good expert in the field and I am sorry to inform you that we find that the present analysis is not well suited for publication in the EMBO Journal and that we therefore have decided not to proceed with its handling and peer review.

Your analysis reports that FoxO1 directly regulates TLR4 expression in macrophages and thereby enhances TLR4 signaling and inflammation. A feedback mechanism is also reported whereby TLR4 signaling inactivates FoxO1 by promoting its nuclear exclusion. We appreciate the potential interest of the findings reported. However, the advisor and we also find that some further in vivo work to support the physiological significance of these findings would be needed to consider publication here. Should you be able to provide such additional data then we would be happy to consider another version.

I am sorry that I cannot be more positive on the current submission, but I hope nevertheless that you might find these comments useful.

Yours sincerely,

Editor
The EMBO Journal

Resubmission 04 May 2010

As we discussed by phone, we have now followed through on your suggestions to add an in vivo component to our studies on the proinflammatory effects of FoxO1, the FoxO1 cistrome, and activation of the TLR4 pathway. To accomplish this, we have focused our experiments on in vivo studies as well as on bone marrow-derived macrophages from FoxO1 haploinsufficient mice (the FoxO1 null mutation is lethal).
For the primary macrophage studies, it's technically not possible to do loss-of-function studies in thioglycollate-elicited peritoneal macrophages from FoxO1+/- mice, since these once-activated primary cells exhibit constitutive AKT activation (which inactivates FoxO1), even after rigorous serum starvation. However, we were able to carry out studies with bone marrow derived macrophages (BMDMs) as an alternative.
We found that LPS-induced mRNA expression of major inflammatory cytokines, including TNFα, IL-6 and IL-1β, were all reduced in the BMDMs from FoxO1+/- mice, and that LPS-induced phosphorylation of JNK and p65 was also attenuated. We believe these studies provide a nice ex vivo demonstration that our more basic in vitro results translate to a reduced inflammatory state when FoxO1 function is physiologically reduced.
In addition, we conducted formal in vivo studies to test the idea that FoxO1 loss-of-function would translate into reduced pro-inflammatory responses and improved glucose homeostasis. Thus, it is know that injection of a non-lethal dose of the TLR4 ligand LPS (1 mg/kg) leads to systemic activation of inflammatory pathways and glucose intolerance in normal mice and we have used this model in the past. When we administered LPS ip to WT and FoxO1+/- mice, we found that the rise in circulating TNFα and IL-6 levels was attenuated in the FoxO1+/- group. This strongly supports the ideas generated from our in vitro and ex vivo experiments indicating that FoxO1 mediates proinflammatory signaling via TLR4 (the receptor for LPS). Taking this one step further, we also performed glucose tolerance studies (GTTs) in WT and FoxO1+/- mice 1 hour after LPS injection. Interestingly, the WT mice exhibit the expected effect of LPS to exaggerate the glycemic response to glucose injection, while, in contrast, LPS did not lead to a hyperglycemic response in the FoxO1+/- mice. These differences in glycemia were somewhat modest, but highly significant, and manifested at the peak glycemic time (15 minutes) of the GTT. In terms of the magnitude, we were quite pleased to see this effect, since, we were only able to study heterozygous mice (complete FoxO1 KO is lethal), and a modest effect is all that could be expected. Taken together, these data demonstrate that endogenous FoxO1 is required for the full LPS TLR4 inflammation insulin resistance response in vivo, and FoxO1 haploinsufficiency attenuates the inflammatory response and insulin resistance induced by LPS.
The manuscript has been revised, and the new results are included in Fig 3 and in new supplemental figure 3. We believe these new results strengthen our overall concepts and have led to a substantially improved paper. We hope that these new data have addressed your important concern about the in vivo significance of FoxO1 regulation of TLR4 signaling.

1st Editorial Decision 08 June 2010

Thank you for submitting your manuscript to the EMBO Journal. This is an invited resubmission of manuscript # 74134. Your manuscript has now been seen by three referees and their comments to the authors are provided below. As you can see, the referees find the analysis interesting and are supportive of publication here pending adequate revisions. The concerns raised are clearly indicated below, but some further insight into the implication of the FoxO1 pro-inflammatory response for obesity and insulin resistance is needed. Should you be able to address the raised concerns then we would consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single major round of revision only and that it is therefore important to address the raised concerns at this stage. 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication.

I look forward to your revision.

Editor
The EMBO Journal

REFEREE REVIEWS

Referee #1 (Remarks to the Author):

It is shown in this study that FoxO1 participates in triggering the immune response. In particular the authors show that FoxO1 overexpression induces the Tlr4 pathway, including phosphorylation of NFkB. These effects were shown to be dependent on the transactivation capability of FoxO1. Using ChIP sequencing analysis it is convincingly shown that FoxO1 is a transcriptional regulator of the expression of several genes implicated in the Tlr4 pathway, including Tlr4 itself. There is no doubt about the originality and novelty of this study. The identification of the Tlr4 pathway as a FoxO1 target is very relevant. The physiological implication of these finding for obesity and diabetes research remains however limited, and required further analysis. In particular this reviewer has the following concerns and suggestions

1. The authors show in Figure 3 that FoxO1+/- mice are protected to LPS-induced glucose intolerance. This is correlated to decreased expression and secretion of IL-6 and TNFa. The correlation is clear, although it does not demonstrate that FoxO1 mediates, in this model, glucose intolerance. Rescue of wt macrophages in FoxO1+/- mice could prove that these effects are mediated by FoxO1 haploinsuficiency.

2. The novelty of this study relies on the implication of FoxO1 in the inflammatory response associated to obesity-induced insulin resistance. The authors only demonstrate, however that FoxO1 mediates an inflammatory response in non-relevant models, such as LPS-induced insulin resistance. Other models would be more relevant to demonstrate the participation of FoxO1 in obesity-induced insulin resistance. Activation of FoxO1 in isolated macrophages from different models of obese-diabetic mice could be indicative of this implication. Could it be feasible to replace FoxO1+/+ with FoxO1+/- macrophages in mice models of obesity-induced insulin resistance? Moreover, are obese FoxO1+/- mice protected from insulin resistance, compared to +/+ mice?

3. Similarly, the presence of FoxO1 in the Tlr4 promoter in adipose tissue-derived macrophages isolated from obese, insulin resistant mice would prove the real implication of the pro-inflammatory effects of FoxO1 in this pathological condition.

4. The authors claim that the major effects of FoxO1 in inflammatory processes are mediated by the transcriptional regulation of Tlr4 expression. They consider, however that the effects on the other genes observed in the ChIp experiments should not be underestimated. The effects of FoxO1 in Tlr4-/- cells or in Tlr4 knock down cells would help to further prove the relative importance of Tlr4 in FoxO1-mediated immune response.

Minor points:

Results in figure 3H-I showing that the pro-inflammatory effects of FoxO1 are not mediated by PPARγ inhibition are out of the scope of the study. This reviewer suggests withdrawing this data.

The discussion section should be more focused, and the authors should avoid describing again the results.

Referee #2 (Remarks to the Author):

The authors show that ectopic expression of constitutively active (CA) FoxO1 in RAW 264.7 cells or wt macrophages enhances LPS driven TLR4 signaling as measured by several "read outs". While FoxO1 haploinsufficient mice - termed here k.o. (why?) display a somehow reduced glucose

tolerance and PPARY mediated transrepression appears not to be operative, global assessment of FoxO1 DNA binding sites via ChIP-sequencing revealed that TLR4 but not TNF-α signaling displayed the most abundant FoxO1signature. Interestingly the "proinflammatory" FoxO1 appears to be counteracted by TLR4 induced AKT activation (which also activates mTOR!).

While ChIP combinded with massively parallel sequencing provided compelling data that FoxO1 transactivates TLR4 gene expression in macrophages, questions remain:
1. Given that Akt-signaling "deactivates" FoxO1 (this manuscript) how does TLR4 Akt activated "proinflammatory" mTOR signaling (Immunity 2008, Nat. Immunol. 2008, Europ. J. Immunol.) influence these signalpathways? (not discussed)
2. Use as TLR4 ligand of commercially obtained FFSs (Fig. 2F) raises the question of its potential LPS content (not addressed, see also February issue of Nat, Immunol. 2010, describing CD36 mediated TLR4-6 heterodimerization being required for recognition of oxidized LDL).
3. Dependent on the individual experiments, either AD-GFP or DBD mutant FoxO1 are used as controls and FoxO1 wt or FoxO1 CA as "active" reagents. The variations preclude a comparison of the different experiments.
4. While, via quantitation, the data of supplemental Fig. 1 (effect of Ad-GFP versus Ad-FoxO1 CA on p65 (P)) appears significant, the data in Fig. 2E and 2F are difficult to be compared with those of Fig. A - D, since the former are based on primary macrophages and the latter on thioglycolate induced and thus activated macrophages.

Referee #3 (Remarks to the Author):

In these studies, Fan et al. report that FoxO1 controls Tlr4 driven inflammatory response in macrophages. Using ChIP-Seq they found that FoxO1 is bound to different locations in the Tlr4 gene. As a consequence of Tlr4 increase, its signaling induces the inflammation response. In this loop, it appears that Tlr4 signaling induces activates that inactivates FoxO1 establishing an attenuation mechanism to the inflammatory response. In general, these are interesting and to a certain extend novel studies, especially the identification of Tlr4 as a new target of FoxO1. These studies also represent further understanding of how insulin signaling can interfere with inflammatory pathways and vice-versa. However, there are several important points that are weakly addressed and can compromise the overall conclusions of this manuscript.

• One of the critical points is to establish in what conditions, especially after LPS, or FFA stimulation FoxO1 plays a role. As it is described it appears that temporally LPS induces rapidly phosphorylation of Akt that will inactivate FoxO1. It would be of interest to put this signaling in a physiological context, for example in insulin resistant states, are these mechanisms altered and it is when FoxO1 might play a role.

• How the authors can reconcile that LPS and inflammatory FFA -known to induce insulin resistance- can induce AKT, which in theory should mimic insulin action.

• Since FoxO1 also induces an attenuation pathway on Akt, the interpretation of some of the results should be taken with caution and should be indicated in the discussion.

• Related to the previous point, in the absence or expression of the CA FoxO1 how Akt changes in response to LPS.

• The inflammatory pathway is though to be one of the causative mechanisms of insulin resistance. A crucial point, to what extent insulin signaling in the macrophage interferes with the LPS, or FFA response. The authors should experimentally address this point.

1st Revision - authors' response 16 September 2010

REVIEWER #1

1. In this comment, the Reviewer is asking for additional evidence that the protection from

LPS-induced glucose intolerance in vivo can be traced to macrophages in the FoxO1+/- mice. This is an important point, and we have several comments plus additional new data to fortify this conclusion.
1. Firstly, since LPS signals through Tlr4, it is evident that the phenotype is based on the Tlr4 signaling pathway, which we find is a key target of FoxO1 and represents an important difference between the wild type and FoxO1+/- mice.
2. The primary cellular targets of LPS are immune cells, in this case macrophages, rather than somatic cell types.
3. As the Reviewer acknowledges, the phenotype is strongly correlated with the reported differences in circulating levels of TNFa and IL-6, and there is already significant published evidence that the predominant source of these cytokines is immune cells rather than some other cell type. We now provide additional evidence for this assertion. Firstly, previous papers have already demonstrated that in adipose tissue, it is the ATM-containing stromal vascular fraction, which elaborates the great majority of these cytokines. In fact, Weisberg et al. (J. Clin. Invest. 112, 1796, 2003) suggested that essentially all the TNF in adipose tissue comes from macrophages. In our own studies (Oh et al. Cell 2010; 142(5): 687-98. Supplemental Figure 7. And Fig 1 of the Referee-only material), we find that 80-90% of adipose tissue TNF , IL6, and IL- is in SVCs with only a minor part coming from adipocytes.
4. We have now performed similar analyses in the liver. In this experiment (new Supplemental Fig. 4) we measured TNF , IL-1B and IL-6 expression in different cellular fractions from HFD liver and find that essentially all of the TNF , IL-1B and IL-6 mRNA is expressed in the Kupffer cell (CD11b+) fraction, with none in hepatocytes and very little in the CD11b- non-hepatocyte cell fraction. In these experiments, we prepared isolated hepatocytes in the conventional manner and then separated Kupffer cells from the non-Kupffer cell containing fraction by purifying cells on CD11b antibody magnetic beads. The results indicate that Kupffer cells are the LPS targets in liver. As an additional point, we have already published data in the context of bone marrow transplantation (BMT) of IKK knockout macrophages that the glucose intolerance induced by LPS is due to the behavior of macrophages and not some other somatic cell type (Arkan MC et al. Nat Med. 2005 Feb;11(2):191-8.).
BMT, as the Reviewer mentions, could also be an approach to this problem. However, we have had a great deal of experience with BMTs, and find that it is extremely difficult to demonstrate in vivo phenotypes with BMTs when comparing WT to the heterozygous state (FoxO1+/- donor cells). This is particularly challenging when one is looking for gain-of-function results. In addition, given the time for bone marrow reconstitution and subsequent HFD feeding, it takes 6 months to perform an experiment, and this doesn't take into account the months required to breed up a colony of animals. Therefore, this would be a very substantial expenditure of resources and time, and most likely would not advance the story. Furthermore, we think the evidence cited about cell types by LPS, the source of inflammatory cytokines, coupled with all of the other data in the paper, provides a reasonable case that the protection from the in vivo LPS-induced inflammatory/glucose intolerant phenotype is macrophage related.

These issues are now further discussed in the page 9, line 9-15 of the text.

2. In this comment, the Reviewer raises an excellent point concerning the LPS-induced insulin resistant model. He/She wonders whether similar findings would be seen in a more chronic model. In fact, we have already published the experiment the Reviewer has requested. Thus, our earlier paper showed that HFD/obese FoxO1+/- mice are protected from insulin resistance compared to WT mice (Kim et al., Diabetes. 2009 Jun;58(6):1275-82.). We have now discussed this point on page 20, line 5 to 6. Another point central to the Reviewer's question relates to our choice of the acute LPS inflammation model. In our paper, we provide evidence that FoxO1 is a key factor promoting Tlr4 mediated inflammation. We also show that chronic inflammatory stimulation leads to FoxO1 phosphorylation, with nuclear exclusion, which provides a negative feedback mechanism to mitigate the proinflammatory effects of FoxO1. We conclude that an important role for FoxO1 is to provide an amplification signal for the acute inflammatory response and that as inflammation becomes more chronic, the negative feedback mechanism comes into play, as a protective mechanism. Obviously, sustaining the effects of an acute inflammatory response would be extremely detrimental to tissue and organismal health and homeostasis. This is why we chose the LPS model, since it gives us the opportunity to assess the role of FoxO1 in the context of acute inflammatory stimulation (LPS) and the corresponding metabolic defects (glucose intolerance). In the more chronic state, much of the FoxO1 becomes phosphorylated and, therefore, excluded from the nucleus, as mentioned above. In this situation, BMTs or other maneuvers would be unlikely to

discriminate subtle differences related to FoxO1 activity. In response to this point, we have now included new experiments (new Supplemental Fig. 18a-b) in which we show that endogenous FoxO1 in ATM of lean mice is predominantly located in the nuclei, where it is active and primes the cells to respond to any new incoming inflammatory stimuli. In HFD/obese mice, the situation is chronic and endogenous FoxO1 is predominantly cytoplasmic in ATMs and, as mentioned above, we view this as part of a feedback mechanism, which serves to avoid inappropriate over-activation of the immune system, perpetuating the acute inflammatory state. Given the importance of a straining acute inflammatory response, there are undoubtedly other feedback mechanisms which exist. In fact, we have recently published (Oh et al. Cell. 2010 Sep 3;142(5):687-98.) that omega 3 fatty acids, working through macrophage GPR120 can serve as an additional negative feedback break on inflammation. Resolvins/protectins and other mechanisms, probably play additional roles.

Interestingly, the nuclear/cytoplasmic disposition of FoxO1 is different in adipocytes compared to macrophages. We have shown previously that compared to lean mice, there is more FoxO1 expressed in the nuclei of obese adipocytes (Fan W et al, J Biol Chem. 2009 May 1;284(18):12188-97.). This is a reflection of impaired insulin action with decreased insulin stimulated FoxO1 phosphorylation in these cells, compared to the cytokine mediated FoxO1 phosphorylation, which occurs in macrophages. Importantly, we also now show that adipocyte Tlr4 mRNA levels are higher in HFD compared to lean mice, fully consistent with the rest of our data demonstrating that FoxO1 induces Tlr4 (new Supplemental Fig. 18c).

We completely agree with the Reviewer's general comment that in addition to the acute LPS challenge model, it would be of interest to study the role of macrophage FoxO1 in other models of insulin resistance. Our approach to this is to generate mice with FoxO1 gain-of-function by employing a macrophage-specific doxycycline- inducible constitutively active FoxO1 transgenic system. In this case, the FoxO1 can be turned on and off by the administration of doxycycline and this can be done after the HFD/obese state is well established. In this project, we are using a constitutively active form of FoxO1, which cannot be phosphorylated (inactivated) and therefore, will retain within the nucleus. However, we are just establishing these founder mice, and it will take 6-12 months before experimental animals are available for study. While these experiments are important, we believe they are outside the scope of the current manuscript and will form the basis of a future effort.

In response to the Reviewer's comment, please see the new data in Supplemental Figure 18, and the changes to the manuscript on pages 18, lines 1 to 7.

3. In this comment, the Reviewer notes that the presence of FoxO1 on the Tlr4 promoter in ATMs from HFD/obese mice would be an important observation. However, as discussed above, in the chronic HFD/obese situation, FoxO1 is largely phosphorylated and, therefore, excluded from the nucleus (please see new Figure 6 and new Supplemental Figures 18) and, therefore, chip assays for FoxO1 on the Tlr4 promoter would not be revealing.

4. In this point the Reviewer makes an excellent suggestion, requesting studies of FoxO1 in the context of Tlr4 knockout cells. We have now performed these requested experiments and the new data are provided in new Figures 5I and 5J, and new Supplemental Figure 11. Thus, peritoneal macrophages were harvested from Tlr4 KO and WT mice. The cells were then transfected with an adenovirus expressing constitutively active (CA, non-phosphorylatable) FoxO1 or controll GFP adenovirus. The cells were then treated for 6 hours with LPS, TNF , TPA, or PBS; and mRNA levels of inflammatory genes were then analyzed by qPCR. The results show that CA-FoxO1 substantially enhanced LPS-induced expression of all of these genes but the CA-FoxO1 was without effect on TNF or TPA-induced inflammatory gene expression. In contrast, in the Tlr4-/- cells, LPS was without effect (as expected) and the CA-FoxO1 had no effect to enhance LPS, TNF , or TPA-induced gene expression. These new data are now provided in new Figs 5I and J and Supplemental Fig. 11 and are discussed on page 15, lines 11 to 17.

MINOR POINTS:

1. These data have now been moved to Supplemental Fig. 5 as requested.
2. We have substantially reworked the Discussion section and hope that it has now been improved.

REVIEWER #2

1. In this point, the Reviewer asks how Tlr4/Akt activated ìproinflammatoryî mTOR signaling

influences our results. In response to the Reviewer's comments, we have carefully reviewed this issue, including the three papers the Reviewer cites. However, our reading is that Weichhart et al (Immunity 2008 Oct 17;29(4):565-77) report that Akt/mTOR signaling is actually anti-inflammatory and not proinflammatory. In specific, they address Tlr4 signaling in monocytes and macrophages and reported that mTOR, which is activated by the Tlr4/PI3K/Akt cascade attenuated Tlr4- induced NF B signaling. They went on to show that inhibition of mTOR by Rapamycin was proinflammatory, leading to increased production of LPS-induced inflammatory cytokines. They also found that activation of mTOR by deletion of TSC2 impaired LPS-induced inflammatory responses. Finally, they found that inhibition of PI3 kinase by Wortmannin enhanced the LPS response. Thus, we do not believe there is any discrepancy between our findings and conclusions and those of Weichhart et al. and, in fact, believe that their data are quite consistent with our idea that Tlr4/PI3K/Akt is a negative feedback circuit dampening chronic activation of Tlr4 signaling. In the Nature Immunology (2008 Oct;9(10):1157-64) paper, Cao et al. addressed the effect of mTOR on Tlr9 activation in plasmacytoid dendritic cells. We have no data concerning FoxO1 or the PI3K/Akt cascade on Tlr9 signaling in this cell type. Also, our ChIP Seq data did not reveal any FoxO1 occupancy on the Tlr9 gene in macrophages. In a paper by Schmitz et al. (Eur J Immunol. 2008 Nov;38(11):2981-92.,) data are reported that various Tlrs, including Tlr4, can mediate activation of mTOR via PI3K/Akt. These authors also show that in BMDMs and PBMCs, Rapamycin mediated inhibition of mTOR enhances LPS-induced inflammatory responses and suppresses anti-inflammatory responses (IL-10), again, showing that mTOR activation is anti-inflammatory and, more importantly for our paper, suppresses Tlr4 signaling. They further showed that both in vitro and in vivo inhibition of mTOR increased the lethality of endotoxin mediated shock and this is correlated with increased levels of IL-1B. Again, these authors make the case that the PI3K/Akt/mTOR cascade is anti-inflammatory, not proinflammatory, in the context of Tlr4 signaling. Based on these papers, we think that the data in our current manuscript is well supported and believe that our results show that inactivation of FoxO1 by Tlr4-mediated PI3K/Akt signaling is the likely mechanism for the anti-inflammatory effects of PI3K/Akt. In response to the Reviewer's comment, we have now revised our paper and included a discussion of this issue with citations to the papers the Reviewer mentions (please see pages 23, lines 5 to 8).

2. In this comment, the Reviewer raises an excellent point concerning potential contamination of BSA preparations with endotoxin. In many previous studies, FFAs are often complexed with BSA, so contamination of BSA with LPS is an issue which has been discussed in recent papers. However, we think this issue is not problematic for our current studies for the following reasons: (1) The FFAs used in this study are in the sodium salt form and, therefore, dissolved in methanol so that there is no complex with BSA. (2) We used BSA for serum starvation of cells, but the BSA we used is endotoxin free (Sigma Catalogue #A9430; less than 1 EU/mg endotoxin) and we have already reported that this BSA preparation does not stimulate inflammatory responses in macrophages (Nguyen MT et al. J Biol Chem. 2007 Nov 30;282(48):35279-92.). In addition, in the current experiments, both the control and FFA treated cells were serum starved in the exact same BSA containing medium. (3) The individual FFAs manifested differential effects on Akt activation in RAW 264.7 macrophages (now seen in Supplemental Fig. 12), indicating that the results are due to intrinsic effects of different FFAs rather than to the BSA, which is constant throughout all experiments.

We agree with the Reviewer that the recent discovery of CD36 as a mediator of Tlr4-6 heterodimerization provides an interesting alternative mechanism by which FFAs can activate Tlr4 signaling. This point has now been addressed and the paper cited (page 21, lines 8 to 12).

3. In this point, the Reviewer comments on the various vectors used as controls. We used Ad-GFP as a general control vector for adenovirus infection. The DBD FoxO1 was employed not so much as a control, but as a comparator in several of our plasmid-based promoter reporter assays (Fig. 1C, 1D, 3H, and 3I; Fig 3H and 3I are now Supplemental Fig. 5a and 5b, respectively) because it lacks transactivational capacity due to the absence of the DNA binding domain. In Figure 1C and 1D, the basal control for these luciferase reporter assays is the pcDNA, the backbone plasmid in which all the FoxO1 variants are cloned. The DBD FoxO1 is included here as an additional comparator/control to directly demonstrate that the transactivational function of FoxO1 is critical for its ability to enhance LPS responsiveness in macrophages. Since DBD FoxO1 does not bind to DNA and is, therefore, unable to transactivate FoxO1 target genes, the lack of function of DBD FoxO1, compared to WT or CA FoxO1 re-enforces our conclusions about FoxO1 transactivation and Tlr4/inflammation.

In Figures 3H and 3I (now Supplementary Fig. 5 in the revised manuscript), the DBD FoxO1 is used to fortify the conclusion that FoxO1-mediated transpression of PPAR is not the mechanism by which FoxO1 enhances Tlr4 signaling. This is important since we have previously reported that DBD FoxO1, although transactivationally incompetent, retains full capacity to bind to and transrepress PPAR . Thus, DBD FoxO1 is as effective as wild type FoxO1 at binding to and transpressing PPAR and given the established literature showing that PPAR attenuates inflammatory responses, it seemed important to determine whether transpression of PPAR in macrophage might play a role in FoxO1-mediated enhanced LPS responsiveness. In this context, we think that DBD FoxO1 provides the ideal control/comparator. Based on our previous work (Fan W et al, J Biol Chem. 2009 May 1;284(18):12188-97.) and the new data in this paper, we conclude that FoxO1 transrepresses the transactivation function of PPAR , but that FoxO1 has no effect to block the transrepression function of PPAR . In response to the Reviewer's question, we have elaborated on this point in the revised manuscript (1, page 7, line 14-18., 2, page 10, line 8-12., 3, page 19, line 22 to page 20, line 1., 4, page 20, line 7-8.)

4. We completely agree with this comment by the Reviewer. Indeed, the kinetics and relative magnitude of LPS responsiveness, and the effects of FoxO1, are not always identical between the three different types of macrophages we have used. Thus, direct quantitative comparison between different macrophage cell types must be viewed with this in mind. Nevertheless, all the qualitative patterns of LPS responsiveness and the effects of FoxO1 are identical across all the macrophage types used in this study (RAW264.7 cells, Thioglycolate-elicited peritoneal primary macrophages, and BMDMs) and all conclusions about the relative interplay between FoxO1, Tlr4, and inflammatory pathway signaling are the same. In specific, throughout the manuscript, in each macrophage cell type, enhancement of Tlr4 signaling by FoxO1 is observed by means of gain- or loss-of-function studies, or both. We believe that using multiple cell types to show the same phenomenon operating in each strengthens our study. In response to the Reviewer's point, we have now modified our manuscript and have further discussed this issue (page 19, lines 16 to 19).

REVIEWER #3

1. This is an excellent point and we completely agree with the Reviewer's comment. Please see our response to Reviewer #1, point #2. As the Reviewer describes, our data show that FoxO1 can induce the Tlr4 gene and is a potentiator of the broader Tlr4 signaling pathway. In this way, nuclear FoxO1 primes the macrophage, augmenting their ability to respond to an acute Tlr4-mediated stimulus with a proinflammatory response. As time goes on, Tlr4 stimulation mediates phosphorylation of Akt, which then phosphorylates and inactivates FoxO1. We believe this provides a negative feedback system to dampen the ongoing inflammatory response so that the chronic inflammatory state is tempered, rather than perpetuating the robust acute inflammatory response, which would be highly detrimental. In the context of chronic insulin resistant states in obesity, it is important to distinguish between the effects of Tlr4/Akt FoxO1 in macrophages versus those in other cell types, such as adipocytes. Clearly, Akt can have many independent effects in insulin target tissues, which are not seen in macrophages, and the same is true for FoxO1. Tlr4 is much more restrictive to macrophages. As stated above, we now provide new data showing that Tlr4 expression is 2-3 fold higher in ATMs compared to adipocytes, and in the liver, Tlr4 is expressed exclusively in Kupffer cells (new Supplemental Figure 4d-e). Please see page 9, line9 to 15.

2. Here the Reviewer raises an interesting issue, it is well accepted that cytokines or Tlr4 signaling can activates Akt in macrophages. This is less studied in adipocyte, and insulin target cells are not the focus of our studies. Clearly, Akt can induce many different biologic effects in insulin target cells and we have concentrated on inflammation in macrophage in this paper. In direct response to the Reviewer's point, differential functions of Akt in different tissues may relate to isoform specificity. We have now included new Supplemental Fig. 15a, which shows that Akt1 is the exclusive isoform in macrophages, whereas Akt2 is reported as the important form in the insulin action cascade in insulin target tissues, particularly adipocytes. These separate roles for Akt1 and 2 were demonstrated by Akt1, 2 or combined Kos, showing the importance of Akt2 in the insulin receptor signaling pathway (J Clin Invest. 2003 July 15; 112(2): 197ñ208.). Please see page 17 lines 4-6 , and new Supplemental Figures 15a on this point.

3. Here, the Reviewer comments on the attenuation, or negative feedback pathway, of Akt on FoxO1 and asks for additional discussion. Please refer to discussion on page 22, line 18-20 for this point.

4. In this point, the Reviewer asks for data on the effects of CA FoxO1 on Akt. In response to this, we have now performed new experiments measuring the effects of CA FoxO1 on Akt in macrophages in different experimental situations. As shown in new Supplemental Fig. 15b, when CA FoxO1 is expressed in RAW264.7 macrophages, we observed enhanced Akt phosphorylation/activation in response to LPS. These data further support the concept of Tlr4-mediated Akt activation and FoxO1 phosphorylation. Please see page 17 lines 6-8, and new Supplemental Figures 15b on this point.

5. The Reviewer raises an important issue in this comment related to the effects of insulin signaling in macrophages and inflammatory responses. We have now performed several new experiments to address this question. We appreciate this important suggestion, which has led to some important new findings highly relevant to the current study. This has allowed us to define the anti-inflammatory effects of insulin in macrophages and to elucidate the key role of FoxO1 phosphorylaiton as an underlying mechanism. In specific, we have studied insulin signaling in RAW264.7 macrophages. As shown in new Supplemental Fig. 16a, insulin stimulates phosphorylation of IR, IRS1 and Akt, and these data indicate the presence of a functional insulin signaling system in these cells. To address how insulin action might affect Tlr4 signaling, cells were pretreated with insulin for 16 hours and then LPS-induced mRNA expression of inflammatory genes was quantitated by qPCR. As shown in new Supplemental Fig. 16b-e, pretreatment with insulin attenuated LPS-induced TNF , IL-6, MCP1 and iNOS expression compared to untreated control cells, demonstrating attenuation of LPS responsiveness by insulin. This shows that insulin, through Akt activation, provides an anti-inflammatory signal. Next we studied whether FoxO1 is involved in this insulin mediated attenuation of LPS responsiveness by employing BMDM primary macrophages from FoxO1+/- mice. In new Supplemental Fig. 17a, we show that LPS-induced IKK and JNK activation are attenuated by insulin pretreatment in wild type cells. As shown, the BMDMs from FoxO1+/- mice exhibit less LPS induction of IKK and JNK compared to wild type cells, but, importantly, insulin pretreatment was less effective at attenuating LPS responsiveness in the FoxO1+/- BMDMs. This suggests a role for FoxO1 in the insulin-mediated anti-inflammatory response. We also measured mRNA levels of LPS-induced inflammatory cytokines to show that LPS-induced TNF , IL-6, MCP1, and iNOS are all inhibited by insulin pretreatment. Furthermore, FoxO1 haploinsufficiency leads to decreased levels of LPS-mediated gene expression but also blunted the insulin effect (Supplemental Fig 17b-e).

From these studies, we conclude that insulin signaling is functional in macrophages, and through a canonical signaling pathway (IR/IRS/PI3K/Akt1), insulin treatment inhibits Tlr4 proinflammatory effects. Finally, we show the novel finding that phosphorylation/nuclear exclusion/deactivation of FoxO1 plays a key mechanistic role in this insulin effect. Please see page 17 line 8 to 23, and new Supplemental Figures 16 and 17 on this point.

Referee-only material

Fig. 1

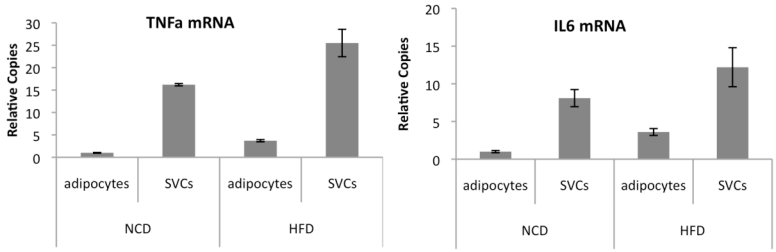

Fig. 1. mRNA expression of TNFα and IL6 in adipocytes and SVCs fractions from adipose tissue of normal chow diet (NCD) or 60% high fat diet (HFD) fed mice. Expression levels in NCD adipocytes are set to 1.

Additional Correspondence 01 October 2010

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by the original three referees and their comments are provided below. As you can see all three referees find that the revised version has addressed their original concerns and they all strongly support publication here. I am therefore very pleased to proceed with the acceptance of the paper for publication in the EMBO Journal. Your will receive the formal acceptance letter shortly.

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The authors have fully addressed my concerns. I therefore suggest to publish this manuscript without any further revision.

Referee #2

This MS provides novel and exciting data on how FoxO1 promotes TLR4 driven inflammation in macrophages either by LPS, the pathogen derived TLR4 ligand LPS, or by endogenous ligands such as saturated fatty acids. In elegant experiments it is delineated how FoxO-1 functions as key determinant able to integrate inflammatory responses in the context of obesity and insulin resistance. The amended version integrates well all the comments of the three referees. Overall, the MS contributes significantly by unravelling the molecular basis of inflammation in obesity and type 2 diabetes. This is an important area in today's biomedical research.

Referee #3

The authors have adequately addressed the main critiques raised in the first revision.

