EMBO reports

The fungal ligand chitin directly binds TLR2 and triggers inflammation dependent on oligomer size

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Review timeline:

Submission date:	6 March 2018
Editorial Decision:	26 March 2018
Revision received:	26 June 2018
Editorial Decision:	25 July 2018
Revision received:	9 August 2018
Editorial Decision:	14 August 2018
Revision received:	31 August 2018
Accepted:	10 September 2018

Editor: Achim Breiling/Martina Rembold

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 26 March 2018

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think the manuscript is of interest, but requires major revision to allow publication in EMBO reports. All three referees have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript. As the reports are below, I will not detail them here. I think, however, that in particular the points by referee #1 (purity of samples, synthetic oligomers, effect of TLR2 loss on characteristics and recognition of chitin, also in vivo), and referee #3 (fungal chitin, heterodimers) need to be addressed experimentally. Also criticisms regarding novelty (referee #2 point 1, referee #3 third major concern) need to be addressed.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature.

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Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable. See:
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We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures)

Please also note that we now mandate that all corresponding authors list an ORCID digital identifier that is linked to their EMBO reports account!

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The nature of the chitin receptor has been long debated in the literature, and several candidates have been proposed. In this manuscript, TLR2 is proposed as a novel receptor. The data are extensive and address the problem logically, however a fundamental issue remains....the purity of the sample used.

In the methods, it is stated that the reagents are >95% pure and endotoxin free. Yet this does not exclude the possibility that there is a contaminant in this preparation that underlies the activity they observe. To be completely convincing, they should show similar properties with synthetic oligomers.

In addition, they should show that loss of TLR2 affects some of the characteristics of chitin (such as allergic responses, eosinophil/basophil IL-4/IL-13 accumulation...see Locksley's papers for example). Does chitinase treatment of zymosan lead to loss of activity? Loss of TLR2 only partially accounts for activities described, especially in vivo. This should be made more explicit. How does loss of TLR2 affect chitin particle recognition in vivo? There are strains of Candida with altered chitin cell wall compositions, these would serve as good controls.

I cannot comment on the structural aspects as this is outside my area of expertise.

Referee #2:

This manuscript identifies a minimal chitin chain length able to stimulate immune activation through TLR2. Furthermore, they demonstrate direct chitin binding to TLR2 for the first time. The data is of high quality, but it is also over-interpreted at times, and the novelty and importance of some aspects of the work is oversold. Specific concerns are listed below.

1. Does the presence of large chitin particles in crude extracts as shown in Fig 1A necessarily mean that smaller immunostimulatory fragments are not also present? Thus, is chitin detection by TLR2 really novel? This seems to be already shown in the literature using the crude extracts.
2. TLR2 does not appear to be the only receptor for chitin. Figure 2c indicates that there is not a statistically significant difference in cytokine secretion between WT, TLR2, and MyD88 KO cells. In vivo experiments also indicate that TLR2 is not the sole receptor for chitin, and this does not seem to be adequately acknowledged in the manuscript. I would also note that while TLR2-overexpressing HEK293T cells give the clearest data, these cells likely lack the other receptors involved in chitin detection.
3. Since the data presented in the manuscript indicate that TLR2 is not the sole receptor for chitin (though it is the sole receptor for Pam ligands), the rationale for and description of Figure 3 is misleading. This is presented as though different ligands signaling solely through TLR2 induce different outcomes. However, it should be no surprise that chitin activates different signaling pathways and gene transcription as compared to Pam given that they activate different receptors.
4. A major conclusion listed in the abstract is that short chitin chains inhibit TLR2, yet the text describes these experiments as "preliminary."

Minor points

1. The manuscript needs extensive copy editing for clarity. Many of the sentences are excessively long and difficult to read.
2. Flow cytometry data in Figure 4c is not convincing. It is hard to believe that the barely perceptible shift is a 25% increase when quantified.

Referee #3:

In this work Fuchs et al. report TLR2 as the immune receptor that recognizes chitin. They show that TLR2 is activated by a minimum of 6 subunits of N-acetyl-glucosamine (NAG) to induce a gene expression pattern distinct from other TLR2-ligands. Smaller chitin fragments, with 5 or fewer subunits of NAG antagonizes TLR2 activation.

This is a topic of interest as chitin is an abundant polysaccharide found in the cell wall of fungi, as well as in the house dust mite allergen. Thus, it is important to understand the inflammatory responses regulated by chitin in the context of fungal infections or allergies and asthma.

This is a well written manuscript. Experiments were performed using all the proper controls, for instance, endotoxin contamination of the chitin preparations was evaluated using polymyxin B and TLR4 KO macrophages. TLR2 specificity has been assessed using a wide range of in vitro/in vivo mouse and human methods: TLR2 KO mice, blocking antibodies, siRNA, CRISPR, HEK293T transfected cells and human samples with TLR2 polymorphisms. Signaling molecules downstream of TLR2, such as MyD88 and NF-kB, have also been assessed, and other PRRs were evaluated (TLR4, TLR9, NOD2 and Dectin-1).

Major concerns:

- Most experiments use crab chitin. Does fungal chitin also induce TLR2-mediated responses?
- TLR2 forms heterodimers with TLR1 or TLR6, but TLR1- or TLR6-dependence has not been addressed. This is an important aspect for TLR2 activation and signaling, which the authors should address. They could perhaps use the HEK293T cells transfected with TLR2 and block/delete TLR1 and TLR6 using blocking antibodies, siRNA or CRISPR.
- A recent paper in Molecular Simulation (https://doi.org/10.1080/08927022.2015.1124102) reported a binding model of chitin and TLR2. How does this work relate to the current study?

Minor concerns:

- It should be clearly noted in the text that crab chitin has been used for most of the experiments.
- Fig. 1a. Magnification boxes in the electron micrograph are misleading given that everything else is to scale. I suggest plotting the lines from a point instead of a box.
- Fig. 1j and 1k. How are the chitin oligomers applied to the seedlings and to the leaf pieces? It should be stated in methods and perhaps also in the main text.
- Fig. 2c. Reduced TLR2 responses to Pam2 or Pam3 should be significant. If the TLR2 knockdown is not very efficient I suggest removing this figure from the manuscript.
- Fig. 2c and 2e. Controls should be shown to demonstrate reduction of TLR2 expression or CRISPR-deletion respectively.
- Fig. 2e. Are IL-6 measurements mRNA or protein levels? please explain in the figure legend.
- Fig. 3e. Gene expression similarities with LPS, but not the other TLR2 agonists, are shown. Is the C10-15 expression profile more similar to Pam2, Pam3 or LPS? How many overlapping genes are there between C10-15 and Pam2, Pam3 or Pam2/3?
- Page 9, 4th line starting from the end. Figures references are wrong. It should be Fig. 3f and S3c instead of Fig. 3g and S3b.
- Fig. 4c. TLR2 binding to C. albicans cells measured by flow cytometry is not very convincing. Images in Fig. 4d are more supportive.

1st Revision - authors' response 26 June 2018

Point-by-point reply

Editor's specific comments

Comment #1: As you will see, all referees think the manuscript is of interest, but requires major revision to allow publication in EMBO reports. All three referees have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript. As the reports are below, I will not detail them here. I think, however, that in particular the points by referee #1 (purity of samples, synthetic oligomers, effect of TLR2 loss on characteristics and recognition of chitin, also in vivo), and referee #3 (fungal chitin, heterodimers) need to be addressed experimentally. Also criticisms regarding novelty (referee #2 point 1, referee #3 third major concern) need to be addressed.

Author reply #1: We thank the editor for the opportunity to revise the current manuscript and in this point-by-point reply we address all the raised questions and concerns. Regarding novelty, not only do we provide the first conclusive evidence for TLR2 to be the direct receptor, we also show

that small oligomers of chitin can be activating or antagonistic and thus probably play a vital role in fungal sensing, contrary to all previous assumptions. Previous concepts of size-dependent immune activation of chitin need to be completely re-drawn. We also show for the first time that this size-dependent chitin sensing in mammals resembles its recognition by plants and therefore probably has a broad implication in anti-fungal defences in many organisms. Taken together, we contend that the manuscript provides sufficient novelty to attract attention within the readership of EMBO Reports and beyond.

Please note, due to additional data added, the numbering of certain figures in the revised manuscript has changed (tracked changes) and was generally adapted to EMBO Reports nomenclature (e.g. EV items).

Referee #1:

Comment #2: The nature of the chitin receptor has been long debated in the literature, and several candidates have been proposed. In this manuscript, TLR2 is proposed as a novel receptor. The data are extensive and address the problem logically, however a fundamental issue remains....the purity of the sample used.
Author reply #2: We appreciate the reviewer's favorable assessment as well as the concern about purity. This is understandable especially since in most other papers on chitin purity was not sufficiently addressed or even considered (see Table S1). This also highlights that obtaining sufficient quantities of high purity chitin is indeed not trivial. That is why we went to great lengths to ensure a defined purity (see next comment), which is in fact appreciated by reviewer 3, see comment 15.

Comment #3: In the methods, it is stated that the reagents are >95% pure and endotoxin free. Yet this does not exclude the possibility that there is a contaminant in this preparation that underlies the activity they observe. To be completely convincing, they should show similar properties with synthetic oligomers.

Author reply #3: As shown in Fig. S1a and S1b we can exclude TLR4 agonists in general or LPS in particular as contaminants. Analyses e.g. in HEK293T cells additionally ruled out TLR5, TLR9, NOD2 and Dectin-1 activating contaminants (cf. Fig. 2f and Expanded View figs. 2e, f). The presence of other TLR2 activating contaminants, e.g. lipopeptides, is of course difficult to assess since chitin and lipopeptides involve the same receptor. Based on the high purity of our preparations, >95%, one could – hypothetically - assume that there could be up to 5% of contaminant present, some of which may – hypothetically – be TLR2 agonists. If what we refer to as "C10-15" was not a TLR2 agonist and all the effects observed in our experiments across the multiple experimental systems shown here were attributable to this contaminant, we would expect the following: at equimolar concentrations of Pam2, Pam3 and chitin, C10-15 would consistently induce a lower cellular response as only 5% of the added sample had TLR2 activity, unless this contaminant was considerably (>20x) more potent than known TLR2 agonists (which is unlikely). This was, however, not the case: in fact responses were similar, or for certain readouts (e.g. *IFNB1* mRNA, see Fig. 3g) even higher for C10-15 at equimolar preparation. Although theoretically possible, our data thus provide no evidence for a non-chitin TLR2 activating moiety. The most plausible explanation also corroborated by the direct binding studies and molecular docking is that chitin itself is responsible for the effects.

We appreciate the reviewer's request for the use of synthetic chitin oligomers which naturally had also occurred to us. Unfortunately, there is no commercial source of synthetic chitin oligomers. We made contact to Prof Peter Seeberger, from the Max Planck Institute of Colloids and Interfaces, Department of Biomolecular Systems, a world-leading pioneer in oligosaccharide synthesis. He indicated the theoretical possibility to synthesize 6-mers (which are barely active) but held the synthesis of 12-mers for highly challenging and a separate project in itself. Consequently, he could not supply such materials. As desirable as this reagent would be, its non-availability and the comments of Prof. Seeberger indicate that this theoretical possibility represents a control experiment far outside the scope of this present work. Compared to dozens of chitin papers in which contaminants were never even analyzed or discussed, we consider the efforts we have undertaken to exclude the effects of contaminants as sufficient for obtaining valid and reliable results. The

extensive data and control experiments included in the manuscript as a whole in our opinion will put the reader into a strong position to assess the issue of purity adequately.

Comment #4: In addition, they should show that loss of TLR2 affects some of the characteristics of chitin (such as allergic responses, eosinophil/basophil IL-4/IL-13 accumulation...see Locksley's papers for example).
Author reply #4: We appreciate and have cited the work by Locksley et al and share the reviewer's curiosity in this regard. However, we consider such an analysis to go way beyond the scope of the present work which was aimed at investigating size dependence and receptor usage on a molecular level, not immunological effects in asthma and allergic responses. These are questions that will be interesting to address in future studies. We would like to point out that in the studies by Locksley and colleagues (Van Dyken, Mohapatra et al. 2014; Van Dyken, Liang et al. 2017) , crude chitin beads (from NEB, no information on purity or endotoxin given by the authors or the manufacturer) led to recruitment of eosinophils in the lungs but not neutrophils at day 2 after chitin instillation. In our data, we observe significant recruitment of neutrophils within 12 hour post infiltration but not eosinophils. Results similar to ours were obtained by Da Silva et al with crude chitin, showing recruitment neutrophils but not eosinophils after 6 hours post treatment (Da Silva, Hartl et al. 2008). It will be difficult to compare the effects observed in Locksley's studies with the current study due to different time points and different chitin preparations. Given potential confounders in previous work with crude chitin, we expect performing such experiments with crude chitin (used previously) and chitin oligomers (used here) would yield both overlapping but also discrepant TLR2-dependent effects which would be difficult to pin to the different stimulus composition per se or size-dependent differences. To satisfy a similar potential curiosity readers of our manuscript may have, we have taken the reviewer's suggestion as a prompt to include a short paragraph on this issue in the results section (pages 6 and 7).

Comment #5: Does chitinase treatment of zymosan lead to loss of activity? Loss of TLR2 only partially accounts for activities described, especially in vivo. This should be made more explicit.
Author reply #5: We had already performed treatments of zymosan and chitin with recombinant bacterial exochitinase from *Streptomyces griseus*. We could see generation of mono- and dimers of NAG by mass spectrometry (*cf.* Fig. S3a) and a reduction in cytokine production from primary PMN and HEK cells. However, additional controls showed that the chitinase itself was immunogenic despite sterile handling, probably due to endotoxins or softeners in the preparation, which were impossible to remove. Thus we can positively answer the reviewers question but feel it is not helpful to show the data referred to above. The only partial loss of activity for zymosan in Fig. 2b is expected as chitin only accounts for ~1% of zymosan (Di Carlo and Fiore 1958) and the fungal cell wall in general and several other MAMPs, e.g. glucans, are present and can engage their cognate PRRs. We have included a sentence to clarify this point on page 9.

Comment #6: How does loss of TLR2 affect chitin particle recognition in vivo? There are strains of Candida with altered chitin cell wall compositions, these would serve as good controls.
Author reply #6: The essential role of TLR2 in protection from fungal infection, including systemic candidiasis and invasive pulmonary aspergillosis in mice, has been confirmed by multiple studies, e.g. (Bellocchio, Montagnoli et al. 2004; Villamon, Gozalbo et al. 2004; Balloy, Si-Tahar et al. 2005), some of which were cited in our manuscript. Although no mechanistic rationale was provided, a TLR2 effect on chitin *particle* responsiveness in vivo was also noted by Da Silva et al (Da Silva, Hartl et al. 2008). Using strains with altered cell wall chitin (either by chitin synthase knock-out or modifying cell wall composition by growth in presence of glucosamine or CFW, $CaCl_2$ or echinocandins) in vivo is unfortunately problematic: the fungal cell wall contains multiple other MAMPs (see comment above), is highly dynamic (see e.g. (Hall 2015) for a review) and the relative dependence on particular PRRs can vary in dependence on the external environment. For example, changes in chitin content were shown to modulate the degree of β-glucan exposure in the fungal cell wall i*n vivo* (Marakalala, Vautier et al. 2013), thereby rendering the interpretation of experiments with such strains regarding the dependence of a specific PRR impossible. Moreover, the strongly impaired virulence of strains such as the ΔCHS3 mutant of C. albicans (that displays reduced chitin content) makes it further unsuitable for *in vivo* infection experiments. We therefore consider the proposed experiments not practically possible but have added a short comment to the results to engage with this point for the benefit of the reader (page 11).

Referee #2:

Comment #7: This manuscript identifies a minimal chitin chain length able to stimulate immune activation through TLR2. Furthermore, they demonstrate direct chitin binding to TLR2 for the first time. The data is of high quality, but it is also over-interpreted at times, and the novelty and importance of some aspects of the work is oversold. Specific concerns are listed below.
Author reply #7: We thank the reviewer for the generally positive evaluation and have scrutinized the interpretation of our data again to avoid the impression of over-interpretation/-selling. In response to the reviewer's comment regarding novelty, we also would like to stress reviewer 1's assessment in comment #1 that the question of chitin receptor identity has until now been strongly debated and thus not been definitively answered. We also show that small oligomers of chitin are active and thus probably play a vital role in fungal sensing, contrary to all previous assumptions. We also show for the first time that chitin sensing in mammals resembles its recognition by plants. Taken together, we contend that the manuscript provides sufficient novelty which we have sought to highlight to the reader.

Comment #8: 1. Does the presence of large chitin particles in crude extracts as shown in Fig 1A necessarily mean that smaller immunostimulatory fragments are not also present? Thus, is chitin detection by TLR2 really novel? This seems to be already shown in the literature using the crude extracts.
Author reply #8: The reviewer's question is difficult to assess for previously published papers as the analysis of purity in crude chitin is only possible upon de-polymerization using trifluor acetic acid hydrolysis. This would break down large particles but also smaller oligomers so that, for participles, typically given "purity" values do not give an indication of the relative abundance of large and oligomeric chains. A very comprehensive and technically challenging analysis that has never been conducted but may be informative in the future would have to be applied. Since the purification of the particles used previously involved centrifugation we consider the content of small oligomers to be minor but it cannot be excluded.

In any case, for all the studies using crude particles of unknown purity (see Table S1) it could never be safely concluded that effects originating from the particulate state or contaminants could obscure the receptor dependence deducted. But given the same basic motif it is plausible and expected that both large particles and oligomers share TLR2 dependence.

We appreciate the reviewer's resulting concern with regard to novelty based on a 2008 publication in which TLR2 has been implicated in chitin sensing (Da Silva, Hartl et al. 2008). However, anyone who closely examines this paper will have to acknowledge that the paper deals with the question of receptor identity only in a superficial way, involving only phenomenological studies in knock out animals that have very limited mechanistic and conceptual value. Direct binding was never attempted or observed here or elsewhere.

Therefore it is not surprising that in the field, many leading researchers view the question of receptor dependence as open (Bueter, Specht et al. 2013). This is also evidenced by the still ongoing search for the chitin receptor by leading fungal labs, e.g. by Wagener et al (Aberdeen fungal group) who proposed TLR9 and NOD2 instead to be bone fide receptors (Wagener, Malireddi et al. 2014), again based on non-mechanistic evidence and not binding studies.

Thus the mere study of knockout phenotypes with ill-defined chitin preparations as ligands has so far yielded contradictory results, as evidenced by reviewer 1's comment. Not only does our paper provide unequivocal proof to this controversial question and directly rule out several previously discussed candidates such as Dectin-1, NOD2 and TLR9 using a well-defined chitin ligand; it also provides a coherent concept of chitin sensing that involves oligomers and translational avenues for exploitation of this concept that have never been put forward before. Although at a relatively superficial level, evidence for TLR2 in relation to chitin sensing has been provided by a single study and since neither reviewers 1 and 3 raise strong concerns regarding novelty, we trust the reader will take a similar view and regard our results as highly interesting and novel regarding the chitin sensing and binding immuno-receptor.

Comment #9: 2. TLR2 does not appear to be the only receptor for chitin. Figure 2c indicates that there is not a statistically significant difference in cytokine secretion between WT, TLR2, and MyD88 KO cells. In vivo experiments also indicate that TLR2 is not the sole receptor for chitin, and this does not seem to be adequately acknowledged in the manuscript. I would also note that while TLR2-overexpressing HEK293T cells give the clearest data, these cells likely lack the other receptors involved in chitin detection.
Author reply #9: We agree that there is room for interpretation in our data, especially in the murine system, that other molecules may also contribute to chitin sensing, whether these are TLR2 co-receptors or separate sensing molecules (not NOD2, TLR9 or Dectin-1). Although the possibility for an additional receptor had been included in the original manuscript (page 10 and page 12), following the reviewers' comments, we have expanded these sections slightly. Additionally, some of the experiments requested by reviewer 3 regarding TLR1 and TLR6 were performed (see comment 17 below). Regarding Fig. 2c the trend follows what would be expected (and is specific for TLR2 since the TLR8 ligand and LPS, not shown, were unaffected) but statistical significance is not reached due to the high inter-donor-variability which is well known for working with human primary samples. Human primary cells are, on the other hand, necessary for understanding problems relevant for human health, and more informative than mouse immune cells or human cell lines in this regard. We therefore consider the data nevertheless insightful and significant, albeit not strictly in a statistical sense.

Comment #10: 3. Since the data presented in the manuscript indicate that TLR2 is not the sole receptor for chitin (though it is the sole receptor for Pam ligands), the rationale for and description of Figure 3 is misleading. This is presented as though different ligands signaling solely through TLR2 induce different outcomes. However, it should be no surprise that chitin activates different signaling pathways and gene transcription as compared to Pam given that they activate different receptors.
Author reply #10: Our data conclusively (see reviewer 3's comment #15) and for the first time shows that chitin directly binds TLR2, so that the experiment in our opinion is reasonable in order to elucidate differences. Of course the previously discussed involvement of a co-receptor needs to be taken into account for the interpretation, which we have now expanded (page 10) for the reader's benefit. We now include the possibility more clearly that some of the observed differences may be due to a co-receptor or receptor "X". However, the notion that different ligands may signal through the same receptor and induce different outcomes has been shown before for TLR7/8 (Colak, Leslie et al. 2014) or TLR4 (Pieterse, Rother et al. 2016) and would thus also be conceivable for TLR2.

Comment #11: 4. A major conclusion listed in the abstract is that short chitin chains inhibit TLR2, yet the text describes these experiments as "preliminary."
Author reply #11: Since the experimental results are preliminary we have removed this statement from the abstract.

Comment #12: Minor points 1. The manuscript needs extensive copy editing for clarity. Many of the sentences are excessively long and difficult to read.
Author reply #12: We appreciate the reviewer's feedback and sought to shorten sentences where possible. Since reviewer #3 finds the manuscript well written (comment 15), we hope this will lead to an altogether acceptable presentation.

Comment #13: 2. Flow cytometry data in Figure 4c is not convincing. It is hard to believe that the barely perceptible shift is a 25% increase when quantified.
Author reply #13: Since the scale in Fig. 4c is logarithmic, the ~25% MFI shift observed for TLR2 relative to isotype consistently observed and depicted in Fig. 4c, unfortunately, is not very evident. However, this small shift is consistent with the low fraction of chitin in fungal cell (~3-4% according to (Gow, Latge et al. 2017)) walls and we have now pointed this out in the revised manuscript. As indicated by Referee #3 (see comment #14), microscopy pictures, which were taken from the same preparations, are visibly more convincing, but due to a lower number of analyzed cells, we felt quantification was statistically more reliable by flow cytometry. Nevertheless in our opinion the quantified flow cytometry data are representative since 4 out of 4 independent experiments showed the same result with an acceptable scattering of data points (range of MFI difference relative to IgG1-Fc control is 20.5% to 29.5%). We have sought to visible enhance presentation and highlight the log/linear scale difference in the revised Fig. 4c.

Referee #3:

Comment #15: In this work Fuchs et al. report TLR2 as the immune receptor that recognizes chitin. They show that TLR2 is activated by a minimum of 6 subunits of N-acetyl-glucosamine (NAG) to induce a gene expression pattern distinct from other TLR2-ligands. Smaller chitin fragments, with 5 or fewer subunits of NAG antagonizes TLR2 activation. This is a topic of interest as chitin is an abundant polysaccharide found in the cell wall of fungi, as well as in the house dust mite allergen. Thus, it is important to understand the inflammatory responses regulated by chitin in the context of fungal infections or allergies and asthma.
Author reply #14: We thank reviewer 3 for her/his favorable appreciation of the importance of the current work.

Comment #16: This is a well written manuscript. Experiments were performed using all the proper controls, for instance, endotoxin contamination of the chitin preparations was evaluated using polymyxin B and TLR4 KO macrophages. TLR2 specificity has been assessed using a wide range of in vitro/in vivo mouse and human methods: TLR2 KO mice, blocking antibodies, siRNA, CRISPR, HEK293T transfected cells and human samples with TLR2 polymorphisms. Signaling molecules downstream of TLR2, such as MyD88 and NF-kB, have also been assessed, and other PRRs were evaluated (TLR4, TLR9, NOD2 and Dectin-1).
Author reply #15: We are glad the efforts we have undertaken to rule out contaminations and to employ multiple, complementary approaches have been noted by this reviewer. The reviewer's assessment also relates to comment 2 of reviewer 1 which is concerned with purity.

Comment #17: Most experiments use crab chitin. Does fungal chitin also induce TLR2-mediated responses?
Author reply #16: Indeed the oligomers we used were purified from crab chitin or generated from chitosan oligomers also derived from crab chitin (detailed in Expanded View methods). We are not aware of similar oligomers from a fungal source being commercially available. Given the basic motif GlcNAc motif is the same and that at the level of oligomers the secondary structure of chitin (α, β or γ fibrils) will become less important with decreasing chain length, we think that oligomers purified from fungi would yield identical results. That both particle (immediate sensing in situ) and oligomer (distal sensing of a diffusible ligand) detection by TLR2 could work together in fungal sensing is part of the discussion on page 13.

Comment #18: TLR2 forms heterodimers with TLR1 or TLR6, but TLR1- or TLR6-dependence has not been addressed. This is an important aspect for TLR2 activation and signaling, which the authors should address. They could perhaps use the HEK293T cells transfected with TLR2 and block/delete TLR1 and TLR6 using blocking antibodies, siRNA or CRISPR.
Author reply #17: Following the reviewer's suggestion, we first interrogated this question using blocking Abs in TLR2-HEK-Dual cells (which express both co-receptors). This new data (included as new Fig. 3c) points to a preference for TLR1. This is in good agreement with our mutagenesis data (Fig. EV4) and a study that appeared after our submission to BiorXive and EMBO, in which crude chitin microparticles were shown to precipitate TLR2 and TLR1 (Davis, Cirone et al. 2018), discussed and cited on page 13.

We have also tried to conduct experiments in WT, *TLR2, TLR1* and *TLR6* BMDM (n=4 mice/group). Since the mouse strains were not available locally, bones from differently aged mice for BM isolation had to be shipped from a collaborator abroad for local BMDM generation. We think that this may account for the substantial inter-individual variations observed within each group and leading to the fact that not even for Pam2 or Pam3 differences are statistically significant. We therefore would not like not show this data in the manuscript but append a figure for the Editor and reviewers (FIGURE FOR REFEREES NOT SHOWN). These preliminary data mirror the results of the blocking Abs shown in Fig. 2c and support the general involvement of TLR1, but would need to be repeated under more standardized conditions before inclusion in the manuscript.

Comment #19: A recent paper in Molecular Simulation (https://doi.org/10.1080/08927022.2015.1124102) reported a binding model of chitin and TLR2. How does this work relate to the current study?
Author reply #18: We thank the reviewer for flagging up this study which we find in generally good agreement with our dockings of Chitin DP3. However the mentioned paper is limited to DP3, which is not 'active', and since it is relatively small, may be accommodated more readily in any hydrophobic cavity. Whether the entrance of the TLR2 pocket was large enough to allow access for chitin chains was not assessed in the study. We happened to use the same crystal structure for our study and found that the entrance to the pocket was large enough to allow easy access for the alkyl chains of the lipopeptide ligand but for entrance of the slightly bulkier chitin the side chain conformation of Phe349 ('gate keeper') needed to be flexible. Thus the suggested study is in agreement with our work but only provided a limited explanation how longer chains may get access to and be bound by TLR2. Additionally, the study was purely computational with no experimental evidence supporting the binding model provided. Because of its limitations we had not cited this study but have changed this in the revised version of the manuscript.

Comment #20: Minor concerns: It should be clearly noted in the text that crab chitin has been used for most of the experiments.
Author reply #19: This has been added to the materials and methods section (page 14) and the beginning of results (page 6).

Comment #21: Fig. 1a. Magnification boxes in the electron micrograph are misleading given that everything else is to scale. I suggest plotting the lines from a point instead of a box.
Author reply #20: We thank the reviewer for this helpful comment and have changed Fig. 1a accordingly.

Comment #22: Fig. 1j and 1k. How are the chitin oligomers applied to the seedlings and to the leaf pieces? It should be stated in methods and perhaps also in the main text.
Author reply #21: This information has been added accordingly in the methods section of the main text and in the expanded view methods.

Comment #23: Fig. 2c. Reduced TLR2 responses to Pam2 or Pam3 should be significant. If the TLR2 knockdown is not very efficient I suggest removing this figure from the manuscript.
Author reply #22: As discussed for comment 9, the donor-to-donor variability underlying these results affects both C10-15 and Pam2/Pam3. We have verified the knock-down efficiency in these samples to be significant for *TLR2* and have included this information in Expanded View Fig. S2c. Although knock-down also shows variation, we would suggest keeping the data in the manuscript as this provides an insight into the situation in human primary cells. We would of course be prepared to remove the figure in the final version if the Editor or reviewer wishes.

Comment #24: Fig. 2c and 2e. Controls should be shown to demonstrate reduction of TLR2 expression or CRISPR-deletion respectively.
Author reply #23: For Fig. 2c see comment above and the new Expanded View figure S2c. Re Fig. 2e, these cells were previously generated by Hornung et al and validated based on comprehensive sequencing (Schmid-Burgk, Schmidt et al. 2014). We nevertheless now included data showing the reduced TLR2 expression in these cells by both immunoblot and flow cytometry (new Expanded View figure S2d). Of note, the expression level of TLR2 protein is relatively low even in parental THP-1 cells.

Comment #25: Fig. 2e. Are IL-6 measurements mRNA or protein levels? please explain in the figure legend.
Author reply #24: IL-6 protein (by ELISA) is shown. We apologize for the omission and have added the information to the figure legend.

Comment #26: Fig. 3e. Gene expression similarities with LPS, but not the other TLR2 agonists, are shown. Is the C10-15 expression profile more similar to Pam2, Pam3 or LPS? How many overlapping genes are there between C10-15 and Pam2, Pam3 or Pam2/3?
Author reply #25: For most genes, up- or downregulation has the same direction across treatments, but the log-fold change often significantly differs. More information can be found on pages 10 and

15 of the Extended View Code ("R code supplement"). In an already dense manuscript, we decided to first illustrate this overall trend using PCA (Fig. 3d) then to show chitin-specific deviations from it using Fig. 3E, and additionally to provide a comprehensive "R code supplement" intended e.g. to help other investigators in their potential follow-up studies. To summarize, most genes overlap qualitatively (if up in chitin, then also up in LPS/PAM2/PAM3), but overall C10-15 is more similar to Pam2 and Pam3 than to LPS, as indicated by PCA. Distinct from Pam2/3 is for example the induction of *IFNB1* (*cf.* 3f). We have expanded the results section accordingly to guide the reader more precisely and have included the direct reference to the Expanded View Code supplement which had not been referred to in results. If a Venn diagram of differentially regulated genes is of interest, this could be generated and included as an Expanded View element.

Comment #27: Page 9, 4th line starting from the end. Figures references are wrong. It should be Fig. 3f and S3c instead of Fig. 3g and S3b.
Author reply #26: We apologize for this mistake and thank the reviewer for this helpful feedback. We have corrected the figure references in this section accordingly.

Comment #28: Fig. 4c. TLR2 binding to C. albicans cells measured by flow cytometry is not very convincing. Images in Fig. 4d are more supportive.
Author reply #27: This point was already discussed above, please refer to comment and author reply #29 from reviewer #2.

References cited in point-by-point reply

Balloy, V., M. Si-Tahar, et al. (2005). "Involvement of toll-like receptor 2 in experimental invasive pulmonary aspergillosis." Infect Immun 73(9): 5420-5425.

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Van Dyken, S. J., A. Mohapatra, et al. (2014). "Chitin activates parallel immune modules that direct distinct inflammatory responses via innate lymphoid type 2 and gammadelta T cells." Immunity 40(3): 414-424.

Villamon, E., D. Gozalbo, et al. (2004). "Toll-like receptor-2 is essential in murine defenses against Candida albicans infections." Microbes Infect 6(1): 1-7.

Wagener, J., R. K. Malireddi, et al. (2014). "Fungal chitin dampens inflammation through IL-10 induction mediated by NOD2 and TLR9 activation." PLoS pathogens 10(4): e1004050.

2nd Editorial Decision 25 July 2018

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study (you will find enclosed below). As you will see, the referees now support the publication of your manuscript in EMBO reports, however, referees #1 and #3 have further suggestions to improve the manuscript, and some remaining concerns, we ask you to address in a final revised version of your manuscript, or in a detailed point-by-point response. In particular, we ask you discuss the physical relevance of the findings as indicated by referee #1.

Further, I have the following editorial requests:

- The title is currently rather convoluted. Could you provide a simpler version (with not more than 100 characters including spaces)? How about:
'The fungal ligand chitin directly binds TLR2 and triggers inflammation dependent on oligomer size'

- Please provide the abstract written in present tense.

- We would like to publish the paper as Scientific Report. For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do that for your manuscript. Please make sure that the combined character count for title, abstract, introduction and results & discussion is not more than 30000 (including spaces). For a short report, you could have up to 5 main figures and up to 5 EV figures, thus the present setup would be fine. For more details please refer to our guide to authors:
http://embor.embopress.org/authorguide#manuscriptpreparation

- It seems that the authors Felix Frauhammern, Lloyd Miller and Thorsten Nürnberger are not mentioned in the section 'author contributions'. Please check that all authors are mention in this section. There are two authors DH. Please indicate their specific contributions (who is who). Finally, as there number of authors is rather high, we would ask you to consider the ICNJE authorship recommendations:

http://www.icmje.org/recommendations/browse/roles-and-responsibilities/defining-the-role-of-authors-and-contributors.html

- Please check all the figure/table callouts in the manuscript and adjust these to our nomenclature. There are callouts like 'Figure Sx', which might point to an Appendix Figure, and should therefore be termed 'Appendix Figure Sx' (or it should refer to an EV figure - i.e. Figure EVx), or EVSx. There are also callouts for Expanded View Table S4 and S5 (which should read Appendix Table S4 and S5, I guess - however, it seems there are no Appendix Tables S4 and S5 in the Appendix). See also: http://embor.embopress.org/authorguide#figureformat

See also our guide for figure preparation:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

- There are two EV tables (both called EV2), which are datasets as they cannot be shown in the online version of the manuscript (as table EV1). Please call these files Dataset EV1 and Dataset EV2 and adjust the respective callouts in the manuscript text.

- For the movie files, please remove their legend from the manuscript text. Please provide a legend and a shirt description as a text file, ZIP these together with the movie file, and upload the combined ZIP file as movie.

- Please call the uploaded R code Code EV1, and check that it is called out like this in the manuscript text.

- Could statistical testing be provided for the diagrams in Figs. EV1a and EV2a?

- Please provide the accession numbers for the PRIDE and GEO datasets in the methods section.

- Please format the references according to our journal style. See:
http://embor.embopress.org/authorguide#referencesformat

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of about 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors have not really addressed any if my earlier criticisms. I understand their argument about purity, but still think they should provide some additional evidence. If they add chitinase does their activity disappear? This would be a simple test. They mention contaminants in these enzyme preparations..but there are ways around this eg: coupling to beads etc. Also, does chitinase treated zymosan show reduced staining with FC-TLR2 and calcoflour white? OR do their oligo complete Fc-TLR binding to yeast cell/zymosan etc. We need something more to make this iron-clad!
Their response to my comment (#4), makes me wonder if their Oligo's are representative of chitin particles? I disagree that more study on these is beyond the scope of this manuscript. What they need to show is that these short chains physiologically relevant.
IN terms of comment #6..while I understand their comments about difficulties working with altered Candida strain in vivo, they should be able to show this in vitro such as in fig 4d. In fact this data (and fig4c and d) is not convincing at all....and there is very little overlap between the Fc-TLR2 and the chitin (fig 4d).
I am also concerned by the data shown in the response to referee 3...TLR KO mice are widely available, and so could not be difficult to obtain, and why don't even their controls and C10-15 work in these experiments?

Referee #2:

My previous concerns have been addressed. Of note, the cytokine data from TLR2 KO human monocyte cell lines strongly support the conclusion that TLR2 is an essential chitin receptor in human cells.

Referee #3:

The authors addressed most of my previous comments, although I have some minor concerns. I still have problems with Fig. 2c. It is not clear how many human samples were used. If inter-donor-variability is a concern, perhaps normalizing the IL-6 values from the silenced samples to its own donor NT control would be a better representation. Additionally, I don't find any improvement in Fig. 4c. Flow cytometry is still not convincing. Statistics are missing in the quantification graph.

2nd Revision - authors' response 9 August 2018

Point-by-point reply

Editor

Comment #30: Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study (you will find enclosed below). As you will see, the referees now support the publication of your manuscript in EMBO reports, however, referees #1 and #3 have further suggestions to improve the manuscript, and some remaining concerns, we ask you to address in a final revised version of your manuscript, or in a detailed point-by-point response. In particular, we ask you discuss the physical relevance of the findings as indicated by referee #1.

Author reply #28: We thank the editor for this positive assessment of our manuscript and the opportunity to respond to these comments as follows. Of note, we have added additional experimental data (including some of the suggested control experiments) to Fig. EV1 and EV3.

Referee 1

Comment #31: The authors have not really addressed any if my earlier criticisms. I understand their argument about purity, but still think they should provide some additional evidence. If they add chitinase does their activity disappear? This would be a simple test. They mention contaminants in these enzyme preparations..but there are ways around this eg: coupling to beads etc. Also, does chitinase treated zymosan show reduced staining with FC-TLR2 and calcoflour white? OR do their oligo complete Fc-TLR binding to yeast cell/zymosan etc. We need something more to make this iron-clad!

Author reply #29: Chitinase/specificity. We have performed experiments with chitinase treatment of zymosan as referee 1 suggested. We think the TLR2 activity induced by the chitinase is difficult to circumvent, but the effect is that the chitinase reduces zymosan stimulatory potential significantly down to the level of chitinase only. Given the many additional controls already in the manuscript, we hope that this experiment suggested by referee 1 would answer his/her remaining concern. The new experimental data was added as a new Fig. EV3a.

Comment #32: Their response to my comment #4 makes me wonder if their oligo's are representative of chitin particles? I disagree that more study on these is beyond the scope of this manuscript. What they need to show is that these short chains are physiologically relevant. **Author reply #30:** Physiological relevance of small fragments vs particles. We have conducted experiments showing that large particles induce chitinase expression in human whole blood (new Fig. EV1a) and that murine BALF (new Fig. EV1b) and human macrophage supernatants (new Fig. EV1c) degrade polymeric chitin using polymeric ‘chitin azure’, a well-known indicator of chitinase activity. As a control recombinant *S. griseus* chitinase was used. This new and additional data has been included in the revised version of the manuscript. Generally, it is very difficult to detect such small fragments in a complex mixture (BALF, supernatant, in vivo) as the fragments are poorly soluble and cannot easily be ionized for mass spectrometry, which is the only method suited to identify the fragments in such a complex mixture. We have conducted some preliminary tests together with two professional mass spec facilities here at Tübingen University. But we could not even detect defined C7 fragments spiked in using this setup with culture supernatants. This means

the technical aspect of such an experiment is highly challenging. We are planning to work on this further by finding experts on lipid/sugar MS elsewhere but this is will be a major research effort. That is why we kept the focus of the paper to the fragments. We concede that the physiological relevance of fragments remains to be established further and have therefore also amended this in the discussion of the revised manuscript (page 12).

Comment #33: In terms of comment #6..while I understand their comments about difficulties working with altered Candida strain in vivo, they should be able to show this in vitro such as in fig 4d. In fact this data (and fig4c and d) is not convincing at all....and there is very little overlap between the Fc-TLR2 and the chitin (fig 4d).

Author reply #31: Also in order to address referee 3's comment #8, we have adjusted the presentation of Fig. 4c in which a small mistake was corrected and statistics were added. For Fig. 4d one needs to also bear in mind that TLR2-Fc is much bigger than CFW (https://pubchem.ncbi.nlm.nih.gov/compound/21065046#section=2D-Structure) in terms of accessibility to the chitin layer (buried under a cross-linked mesh of other glucans). Our data also indicate TLR2 binds only free chitin ends (*cf.* Fig. 4e), which will make out only a fraction of the highly cross-linked chitin-containing areas. Additionally, these end regions may be stained less by CFW. Thus co-localization is expected to be only partial at best. We had hoped to have pointed this out in the previous rebuttal letter and in the Results section and have made every effort to clarify this in the revised version of the manuscript, so that the reader can appreciate that the limited amount of co-staining is consistent.

Comment #34: I am also concerned by the data shown in the response to referee 3...TLR KO mice are widely available, and so could not be difficult to obtain, and why don't even their controls and C10-15 work in these experiments?

Author reply #32: TLR1/2/6 KO mice. The mice are indeed available but to get regulatory approval for introduction of the mice into our own local facility and to be able to do experiments on them would take months, at least in Germany, where things are highly regulated. Since the new Fig. 2c is clear and the co-receptor usage is not the main focus of the paper and additional experiments on this point will not fundamentally change the insights of the paper. Although including data on the co-receptors was the suggestion of referee 3, we would be prepared to remove figure 3c and the question of co-receptors again since this is not the main focus. We await the response of the editor on this point but assume that the blocking antibody data is sufficiently clear to leave as is.

Referee 2

Comment #35: My previous concerns have been addressed. Of note, the cytokine data from TLR2 KO human monocyte cell lines strongly support the conclusion that TLR2 is an essential chitin receptor in human cells.
Author reply #33: We thank the reviewer for this positive assessment.

Referee 3:
Comment #36: The authors addressed most of my previous comments, although I have some minor concerns. I still have problems with Fig. 2c. It is not clear how many human samples were used. If inter-donor-variability is a concern, perhaps normalizing the IL-6 values from the silenced samples to its own donor NT control would be a better representation.
Author reply #34: For Fig. 2c the number of donors was given in the figure legend. The suggestion to normalize the values to the respective NT for each donor has indeed reduced the variation somewhat and the use of statistics (one-sample t-test) indicates a significant reduction for C10-15. We have modified the figure accordingly and thank the referee for this helpful suggestion.
Comment #37: Additionally, I don't find any improvement in Fig. 4c. Flow cytometry is still not convincing. Statistics are missing in the quantification graph.
Author reply #35: See comment #4 above re Fig. 4c. The scales were adjusted and stats included.

Comments Editorial Office:
Further, I have the following editorial requests:

Comment #38: - The title is currently rather convoluted. Could you provide a simpler version (with not more than 100 characters including spaces)? How about:

'The fungal ligand chitin directly binds TLR2 and triggers inflammation dependent on oligomer size'

Author reply #36: We thank the reviewer for this suggestion and have adopted the new title.

Comment #39:- Please provide the abstract written in present tense.

Author reply #37: This has been amended.

Comment #40:- We would like to publish the paper as Scientific Report. For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do that for your manuscript. Please make sure that the combined character count for title, abstract, introduction and results & discussion is not more than 30000 (including spaces). For a short report, you could have up to 5 main figures and up to 5 EV figures, thus the present setup would be fine. For more details please refer to our guide to authors:

Author reply #38: We have shortened the relevant sections accordingly.

Comment #41:- - It seems that the authors Felix Frauhammern, Lloyd Miller and Thorsten Nürnberger are not mentioned in the section 'author contributions'. Please check that all authors are mention in this section. There are two authors DH. Please indicate their specific contributions (who is who). Finally, as there number of authors is rather high, we would ask you to consider the ICNJE authorship recommendations:

http://www.icmje.org/recommendations/browse/roles-and-responsibilities/defining-the-role-of-authors-and-contributors.html

Author reply #39: We apologize for these omissions which were now added. After reviewing these guidelines we conclude that all authors significantly contributed to the present work and that their participation as authors is justified.

Comment #42: - Please check all the figure/table callouts in the manuscript and adjust these to our nomenclature. There are callouts like 'Figure Sx', which might point to an Appendix Figure, and should therefore be termed 'Appendix Figure Sx' (or it should refer to an EV figure - i.e. Figure EVx), or EVSx. There are also callouts for Expanded View Table S4 and S5 (which should read Appendix Table S4 and S5, I guess - however, it seems there are no Appendix Tables S4 and S5 in the Appendix). See also: http://embor.embopress.org/authorguide#figureformat

See also our guide for figure preparation:

http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

Author reply #40: This has been corrected.

Comment #43: - There are two EV tables (both called EV2), which are datasets as they cannot be shown in the online version of the manuscript (as table EV1). Please call these files Dataset EV1 and Dataset EV2 and adjust the respective callouts in the manuscript text.

Author reply #41: This has been corrected.

Comment #44: - For the movie files, please remove their legend from the manuscript text. Please provide a legend and a shirt description as a text file, ZIP these together with the movie file, and upload the combined ZIP file as movie.

Author reply #42: This has been corrected.

Comment #45: - Please call the uploaded R code Code EV1, and check that it is called out like this in the manuscript text.

Author reply #43: This has been corrected.

Comment #46: - - Could statistical testing be provided for the diagrams in Figs. EV1a and EV2a?

Author reply #44: For Fig. EV1a this is not possible as only 2 donors were measured, but statistics were added for Fig. EV2a.

Comment #47: - Please provide the accession numbers for the PRIDE and GEO datasets in the methods section.

Author reply #45: These were now included in the methods section.

Comment #48: - Please format the references according to our journal style. See: http://embor.embopress.org/authorguide#referencesformat

Author reply #46: This has been corrected.

In addition I would need from you:

Comment #49: - a short, two-sentence summary of the manuscript

Author reply #47: We suggest the following but are open for editorial suggestions:
The fungal ligand chitin directly binds to the innate immune receptor TLR2 and triggers inflammation dependent on chitin oligomer size. This requires a minimum motif of 6 N-acetyl-glucosamine units for nanomolar binding. Since blocking of the chitin-TLR2 interaction effectively prevents chitin-mediated inflammation *in vitro* and *in vivo*, our study highlights the chitin TLR2 interaction as a potential target for developing novel therapies in chitin-related pathologies and fungal disease.
Comment #50: - two to three bullet points highlighting the key findings of your study

Author reply #48: We suggest the following:

- Oligomeric chains of fungal chitin directly bind to the innate immune receptor TLR2 and trigger inflammation
- Blocking of the chitin-TLR2 interaction prevents chitin-mediated inflammation *in vitro* and *in vivo*
- Size-dependent chitin recognition based on oligomers is found in both plants and humans

3rd Editorial Decision 14 August 2018

Thank you for the submission of your revised manuscript to our editorial offices. After going through your point-by-point response, I consider the remaining points by the referees as adequately addressed.

Before I can proceed with formal acceptance, I have these final editorial requests:

- We do not permit the term 'Data not shown' (presently found on page 9 of the manuscript). All significant data should be displayed in the main figures, the Expanded View information, or in the Appendix. Thus, please show these data, or rephrase the sentence. See also: http://embor.embopress.org/authorguide#unpublisheddata

- It seems that author Lloyd Miller is still missing from the author contributions. Further, Maria A.

Schlöffel seems to be listed as MAS. However, there is also an author MS mentioned. Please adjust this.

- Please upload the final file for Code EV1 with your final submission (this one was missing from the submission V3).

- Please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries (comments), we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

3rd Revision - authors' response 21 August 2018

The authors performed all minor editorial changes.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Alexander Weber, PhD
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-46065-T

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	As the effects observed were novel, sample size calculations based on pre-specified effect sizes were not calculated for in vitro experiments; for animal studies, sample sizes were calculated based on the effects observed for known TLR2 agonsits and also submitted to the local authorities for approval; For certain mouse experiments and all studies involving human subjects, sample size primarily depended on the availability of suitable donors.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample sizes were calculated based on the effects observed for known TLR2 agonsits and also submitted to the local authorities for approval but subject to the availability of mice of suitable genotype and age
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Samples were only exclued from the analysis if appropriate controls significantly deviated compared to published work or experience in the lab (ROUT test was used with a stringency set to 1%)
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Not used for in vitro or animal studies; in studies involving genotype-dependent effects in human donors, corresponding DNA samples were genotyped after measurement and data then stratified into groups to minimize bias.
For animal studies, include a statement about randomization even if no randomization was used.	Randomization was not employed
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	see 3.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not used.
5. For every figure, are statistical tests justified as appropriate?	For each dataset normality was tested using Shapiro-Wilk; subsequently, a parametric or non-parametric test was used as indicated in each figure legend; biological or technical replicates are also given for each figure/dataset
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	see above, Shapiro Wilk test
Is there an estimate of variation within each group of data?	Variation is given in the form of error bars
Is the variance similar between the groups that are being statistically compared?	Yes; appropriate tests were used to determine normal vs non-normal distributions

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Suppliers and catalog numbers are generally given in Appendix; where possible antibodies were validated in the lab using appopriate conditions such as siRNA KD, CRISPR-deletion or murine gene KO; for key reagents, e.g. anti-TLR2 blocking Abs, the appropriate reference was provided.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines were regularly tested for mycoplasma contamination which was always negative; profiling was not done; sources are given in methods

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Information is given in the manuscript; housing and husbandry was performed in accordance with local rules and regulations, as follows: Myd88 and Tlr2 KO mice (both on a C57BL/6 background, a gift from H. Wagner, Ludwigs-Maximilian University, Munich) and C3H/HEJ (Tlr4LpsD, Jackson) and their respective WT counterparts (WT C57BL/6 or C3H/HEN, respectively) were used between 8 and 20 weeks of age in accordance with local institutional guidelines on animal experiments and under specific locally approved protocols for sacrificing and in vivo work. All mouse colonies were maintained in line with local regulatory guidelines and hygiene monitoring. Further details are available upon request.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Is included in the manuscript as follows: "Myd88 and Tlr2 KO mice (both on a C57BL/6 background, a gift from H. Wagner, Ludwigs-Maximilian University, Munich) and C3H/HEJ (Tlr4LpsD, Jackson) and their respective WT counterparts (WT C57BL/6 or C3H/HEN, respectively) were used between 8 and 20 weeks of age in accordance with local institutional guidelines on animal experiments and under specific locally approved protocols for sacrificing and in vivo work. All mouse colonies were maintained in line with local regulatory guidelines and hygiene monitoring."
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	The ARRIVE guidelines were followed in reporting animal experiments, except for parameters such as time of day for treatments, weight, additional details on housing (e.g. bedding material, light-dark cycle). These are available upon request.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	University Hospital Tübingen/Tübingen University Medical Faculty
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Is included in the manuscript as follows: All healthy blood donors included in the analyses of immune cells for this study provided their written informed consent before study inclusion. Approval for use of their biomaterials was obtained by the local ethics committee at the University of Tübingen, in accordance with the principles laid down in the Declaration of Helsinki. Buffy coats were obtained from blood donations of healthy donors and received from the Center for Clinical Transfusion Medicine (ZKT) at the University Hospital Tübingen and whole blood from voluntary healthy donors was obtained at the University of Tübingen, Department of Immunology.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n/a
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n/a
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	n/a
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	n/a

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Microarray data (Gene Expression Omnibus (GEO) repository at www.ncbi.nlm.nih.gov/geo/ with the dataset identifier GSE103094), Proteomics data (ProteomeXchange Consortium via the PRIDE partner repository at www.ebi.ac.uk/pride with the dataset identifier PXD007542), both given in the manuscript
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Data have been deposited, see 18
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	n/a
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Only published bioinformatics tools were used and the appropriate references given; for R processing of microarray data an Expanded View file "Code EV1" was submitted

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	n/a