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Beclin1-driven autophagy modulates the inflammatory response of microglia via NLRP3

Judith Houtman, Kiara Freitag, Niclas Gimber, Jan Schmoranzer, Frank L. Heppner, Marina Jendrach

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Transaction Report:

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

1st Editorial Decision 3rd May 2018

Thanks for submitting your manuscript to The EMBO Journal. I am sorry for the slight delay in getting a decision back to you but in this case it took some extra time to get the referee comments back due to busy schedules.

Your study has been reviewed by two good experts and their comments are provided below. As you can see both referees appreciate the analysis and suggests a number of experiments that will strengthen the analysis. The suggested experiments should be doable within a reasonable timeframe. I would like to invite you to submit a revised version.

Referee #2 brings up an important issue concerning the mouse NDP52. It looks like mouse (and also rat) express a truncated form of NDP52 that contains the SKICH domain, but apparently nothing else. I don't know what your antibody recognizes, but this questions the data that mouse NDP52 might act as an autophagy receptor for NLRP3. To be honest I don't think that you have very strong data to support such a claim as it is based upon co-localization studies and there is no loss of function analysis to support this. I also realize that this aspect of the study is not a key part of the paper and that the paper stands on its own without the NDP52 data. However, before deciding to remove the NDP52 part I do think it is important to address if the antibody indeed recognises NDP52 and if NDP52 has a function in your system.

REFEREE REPORTS:

Referee #1:

In this manuscript by Houtman et al. the authors describe the role of autophagy in modulating the activation of the NLRP3 inflammasome in microglia. This manuscript utilizes several different distinct methods to demonstrate the interplay between NLRP3 and autophagy. The paper is novel and exciting. I have several major and minor points that the authors should consider to improve the overall study:

Major comments:

1. The authors utilize bafilomycin to inhibit autophagy to show that there is autophagic flux occurring in these cells and that the Becn1+/- cells are undergoing less autophagy (Fig. 1) and that inhibiting autophagy can lead to increased caspase-1 activation (Fig. EV2). The authors however do not show if inhibition of inflammasome activation can play a role on either the extent of autophagy or the colocalization of NLRP3 and LC3.
2. It is well established that during NLRP3 inflammasome activation that much of the available NLRP3 colocalizes with the mitochondria. Therefore the authors should determine if the LC3/NLRP3, which is colocalized, is also colocalized with mitochondria.
3. These findings are very relevant within the context of neurodegenerative diseases. With that said the authors only describe finding theses structures in isolated microglia in vitro. The authors should consider both if this phenomenon occurs in cells in the brain other than microglia and if this occurs in vivo in the context of a neurodegenerative disease model such as Alzheimer's or MS.

Minor comments:

1. As the use of NLRP3 and caspase 1 antibodies throughout the literature has been questionable the authors need to validate that the NLRP3 and caspase 1 antibody they are using are indeed specific to NLRP3 and caspase 1.
2. The authors need to include molecular weight markers on all of their immunoblots.
3. In figure 1 the number of LC3+ vesicles/cell should also be evaluated.
4. In figure 2 LPS and ATP only controls should be shown.
5. In figure EV1C TNF and IL-6 levels should be shown for cells cultured in both normal media and HBSS similar to what is shown in figure 2C.
6. In figure 3 the authors show that there is more NLRP3 in the Becn1+/- microglia. The authors need to also consider subcellular localization of the NLRP3 protein.
7. In figure 3 the authors make a point to say that there are non-cell associated ASC-specs present following pyroptosis. This statement is a bit controversial and the authors do not prove that the specs are from cells that have undergone pyroptosis rather than the result of cells ejecting the specs.
8. Related to comment 7, the authors should assess whether there is a higher percentage of extracellular specs in the cultures of the Becn1+/- cells than the WT cells. This would support the idea that the Becn1+/- cells are less able to recycle the specs via autophagy.

Referee #2:

Beclin1-driven autophagy modulates the inflammatory response of microglia via NLRP3

Judith Houtman, Kiara Freitag, Niclas Gimber, Jan Schmoranzer, Frank L. Heppner, Marina Jendrach

Summary

This study by Houtman et al., describes the effects of heterozygous deficiency of autophagy protein Beclin1 in inducing NLRP3 inflammasome activation and maturation of IL-1β and IL-18 in microglial cells. In APPPS1 mouse model of tauopathy, the microglia with Beclin 1 deficiency show increased protein levels of IL-1β and IL-18 compared to non-transgenic mice. The Beclin 1 deficient-microglial inflammasomes were functional with increased NLRP3, cleaved Caspase 1, aggregation of NLRP3, especially with LC3 positive vesicles, which were shown by high resolution microscopy. Overall, this is an interesting study aimed towards assessing the role of microglial autophagy on inflammasome activation and IL-1β and IL-18 maturation. However, there are numerous major and minor issues in terms of the novelty of the study, true relevance of microglial-Beclin 1 deficiency on amyloid pathology and Alzheimer's disease, and identity of the proteins. Related to the latter, authors seem to be unaware of the well-known fact that mice have a truncation that inactivates NDP52/CALCOCO2), and yet claim that "the best of our knowledge, we have shown for the first time that CALCOCO2 is involved in selective autophagy of a specific protein" using mouse cells. Other issues are listed below.

Major comments

1. This appears to be a mostly descriptive study without exploration of any specific mechanisms. For example: the authors describe that CALCOCO2 (NDP52) - NLRP3 interaction plays a role in NRLP3 degradation which is impaired due to Beclin-1 deficiency. An siRNA against CALCOCO2 would have been helpful to demonstrate the functional relevance of this interaction. Another example is the observation that heterozygous deficiency of Beclin 1 elevates NLRP3 recruitment and IL-1β maturation. It would be helpful to confirm this by restoring Beclin 1 levels (via overexpression etc.) and demonstrate that restoring Beclin-1 prevents inflammasome assembly and activation of IL-1β via inducing autophagy in microglia (rescue experiment).
2. While the inflammasome/IL-1β activation appears to be significantly higher in APPPS1/Becn+/- mice, however, there is no functional measure on what does this mean to amyloid pathology? This is important because increased phagocytic function of microglia linked to elevated IL-1β has been shown to reduce amyloid burden and Aβ plaque count in several previous studies. Does Beclin-1 deficiency in microglia make microglia more phagocytic (with enhanced IL-1β) against amyloid?
3. Did the authors run HBSS-only condition? Why can't it be serum-free DMEM to achieve serum starvation? Also, why is the LC3 in serum starved (HBSS) in wild type condition not elevated (in fact shows slight reduction without Baf A1)?
4. Fig. EV1D-E: Why are the TNF and IL-16 levels reduced from 4- to 8- months of age? If IL-1β were to induce neuroinflammation via NFkB, one would expect overall elevated levels of TNF and IL-6 as well. Any explanation would help.
5. NLRP3 is a target of autophagy, as already shown by several reports (e.g. PMID: 28786745). It is not clear what is new in Fig. 4 and Fig. EV4 other than showing it is in microglia and using high resolution microscopy.
6. While many of the protein-protein interactions (eg. NLRP3 - CALCOCO2) have been shown by SIM and super-resolution microscopy. It might help to validate that the physical interactions indeed are occurring via IP/Co-IP methods for at least key interactions like NLRP3-CALCOCO2.

Minor comments

1. Fig. 1c: Not sure what is the purpose of the DAPI if they are shown in color.
2. Fig. EV1 and elsewhere: Molecular weight markings are needed to determine full-length vs active IL-1β.
3. Fig. 2D: It is not clear if the pictures shown are with full medium or HBSS (with and without 3-MA). If it is with only HBSS as said in the figure legend, then, the results should be edited accordingly.
4. Fig. EV2: A representative western blot should be shown for panel A. It looks like Beclin 1+/- microglia may show elevated pro-IL1β.
5. Fig. 3A: When comparing multiple groups with different genotypes, it may be better to use one-way ANOVA than a t test.
6. It is not clear about the novelty of the data presented in Fig. EV3B-C. NLRP3-ASC interaction has been very well established.

1st Revision - authors' response | 5th Sep 2018

-Point-by-point-reply to referee's comments:

General statement:
We are very grateful for the time the referees took to thoroughly review our manuscript and glad to learn that the referees overall were very positive with respect to the results of our study. While referee #1 wrote "*This manuscript utilizes several different distinct methods to demonstrate the interplay between NLRP3 and autophagy. The paper is novel and exciting.*", referee #2 stated "*Overall, this is an interesting study aimed towards assessing the role of microglial autophagy on inflammasome activation and IL-1β and IL-18 maturation.*"

Referee #1:

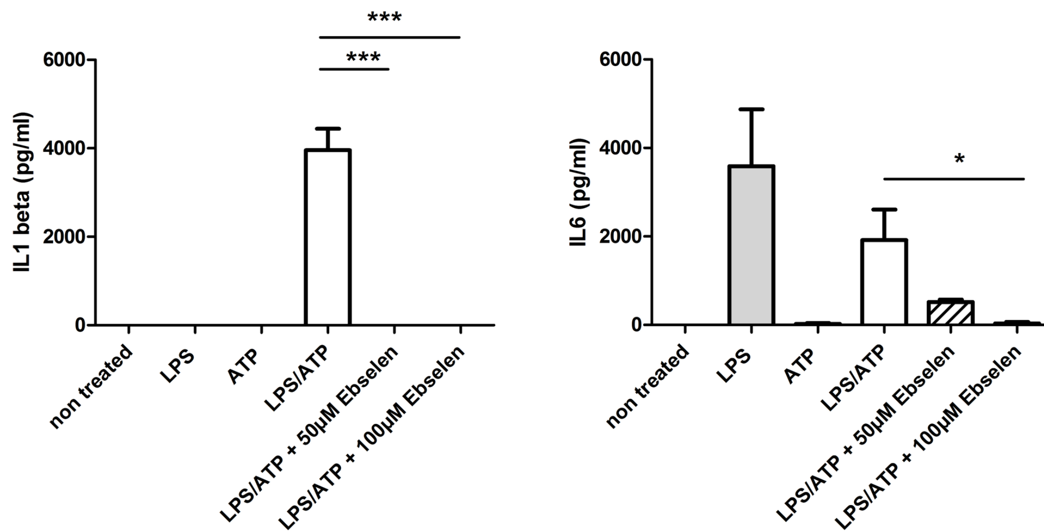
In this manuscript by Houtman et al. the authors describe the role of autophagy in modulating the activation of the NLRP3 inflammasome in microglia. This manuscript utilizes several different distinct methods to demonstrate the interplay between NLRP3 and autophagy. The paper is novel and exciting. I have several major and minor points that the authors should consider to improve the overall study:

We are very grateful for referee's #1 critical and well-balanced evaluation of our data. To address his/her critiques, we performed additional experiments and rephrased the manuscript accordingly (changes in the manuscript are highlighted by blue font color). Overall, we believe that these additional data helped substantially to improve our understanding of the topic in question.

Major comments:

1. The authors utilize bafilomycin to inhibit autophagy to show that there is autophagic flux occurring in these cells and that the Becn1+/- cells are undergoing less autophagy (Fig. 1) and that inhibiting autophagy can lead to increased caspase-1 activation (Fig. EV2). The authors however do not show if inhibition of inflammasome activation can play a role on either the extent of autophagy or the colocalization of NLRP3 and LC3.
Reply: The referee raises a valid point, which we decided to address experimentally. We therefore treated primary wild type microglia with the established anti-inflammatory agent Ebselen (Jabaut, Ather et al., 2013, Tewari, Sharma et al., 2009) along with LPS. After 3 h ATP was added as second stimulus. Subsequently, the release of IL1b and IL6 was measured by ELISA showing a complete absence of these pro-inflammatory cytokines in the supernatant upon adding 100 µM Ebselen (Fig. R1A, see below).

The autophagy status of non-treated, LPS/ATP-treated and LPS/ATP + Ebselen-treated microglia was determined by western blotting, where the amount of LC3-II was normalized to the level of ACTIN expression (Fig. R1B, see below). Interestingly, we were not able to detect an inflammatory response or an effect of its inhibition by Ebselen with respect to the expression of LC3-II. This is in contrast to data published by Francois et al. (François, Terro et al., 2013), who described an induction of autophagy in primary microglia (either cultured as pure microglial culture or in co-culture with neurons) after a 48 h treatment with LPS or IL1beta. We assume that the induction of pro-inflammatory cytokines in our setup failed to modulate LC3-II levels compared to non-treated microglia due to the rather short period of treatment we choose (3 h LPS followed by 45 min ATP) in contrast to the rather long stimulation period employed by Francois et al.. In view of the change in autophagy in neurodegenerative diseases it appears possible that autophagy is altered in the course of a rather long-lasting, chronic inflammatory process rather than in the short-term, acute response to an inflammatory stimulus.



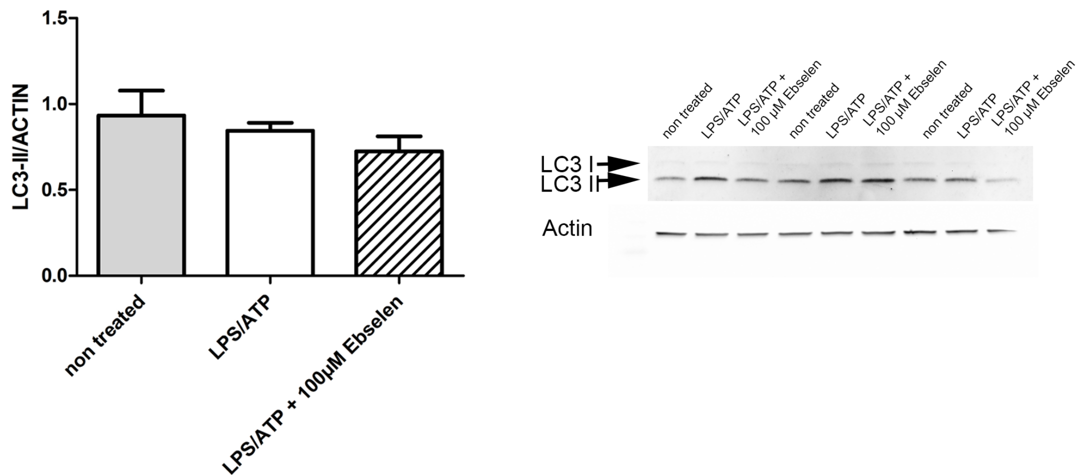


Fig. R1 Microglia from new-born wild type mouse pups were either non-treated, treated with ATP or LPS only or kept for 3 h in LPS with the concentrations of Ebselen as indicated. Afterwards, ATP was added and the amount of IL1beta and IL6 in the supernatant was determined by ELISA (**A**). Addition of 100 µM Ebselen abolished cytokine release; n = 3-6, ANOVA with Tukey's multiple comparison *post hoc* test for LPS/ATP, LPS/ATP + 50 µM Ebselen and LPS/ATP + 100 µM Ebselen *: $p < 0.05$, ***: $p < 0.001$.
B Cell lysates of non-treated, LPS/ATP-treated and LPS/ATP + 100 µM Ebselen treated cells were probed for LC3 and ACTIN protein expression (right). Quantification (left) showed no effect of LPS/ATP or Ebselen on LC3-II expression; n = 3, ANOVA with Tukey's multiple comparison *post hoc* test, ns.

2. It is well established that during NLRP3 inflammasome activation that much of the available NLRP3 colocalizes with the mitochondria. Therefore the authors should determine if the LC3/NLRP3, which is colocalized, is also colocalized with mitochondria.
Reply: To address this valuable point we looked into the localization of mitochondria upon the expression of pro-inflammatory cytokines: wild type microglia were stained with the mitochondrial dye Mitotracker CMX ROS (MTR) and afterwards challenged with LPS/ATP. Endogenous NLPR3 and LC3 were visualized by means of immunocytochemistry. We observed, as described before, that NLRP3 and LC3 indeed colocalize. Furthermore, in agreement with data from Zhou et al., 2011, Subramanian et al., 2013 and Bracey et al., 2014 ((Bracey, Gershkovich et al., 2014, Subramanian, Natarajan et al., 2013, Zhou, Yazdi et al., 2011), NLPR3 colocalizes with MTR-labelled mitochondria. Moreover, NLRP3 aggregates/punctae were often found in very close vicinity to the mitochondria without direct colocalization. However, no colocalization occurred between NLRP3, LC3 and mitochondria indicating that mitochondria are not involved in the degradation of NLRP3 or

that NLRP3 has no substantial impact on mitophagy, respectively. These data are now depicted in the new **Fig. EV3E** of the revised manuscript.

3. These findings are very relevant within the context of neurodegenerative diseases. With that said the authors only describe finding theses structures in isolated microglia in vitro. The authors should consider both if this phenomenon occurs in cells in the brain other than microglia and if this occurs in vivo in the context of a neurodegenerative disease model such as Alzheimer's or MS.

Reply: To address this valid point we assessed the occurrence of NLRP3 aggregates and their degradation by autophagy also in the following settings:

1. As suggested by referee #1 we chose a neurodegenerative disease model mimicking aspects of Alzheimer's disease, namely *APPPS1* mice. *APPPS1* mice at the age of 8 months exhibit strong amyloid beta plaque deposition pathology along with a severe neuroinflammatory reaction (Prokop, Miller et al., 2015, Radde, Bolmont et al., 2006, Wagner, Gilling et al., 2017). Confocal microscopy of microglia isolated from 8 months old *APPPS1* mice revealed NLRP3 aggregates as well as LC3-positve vesicles, while age-matched wild type microglia did not. Furthermore, NLRP3 was found to colocalize with LC3-positve vesicles in these *APPPS1* microglia indicating NLRP3 degradation by autophagy. These data are now depicted in the new **Fig. EV5A** of the revised manuscript.

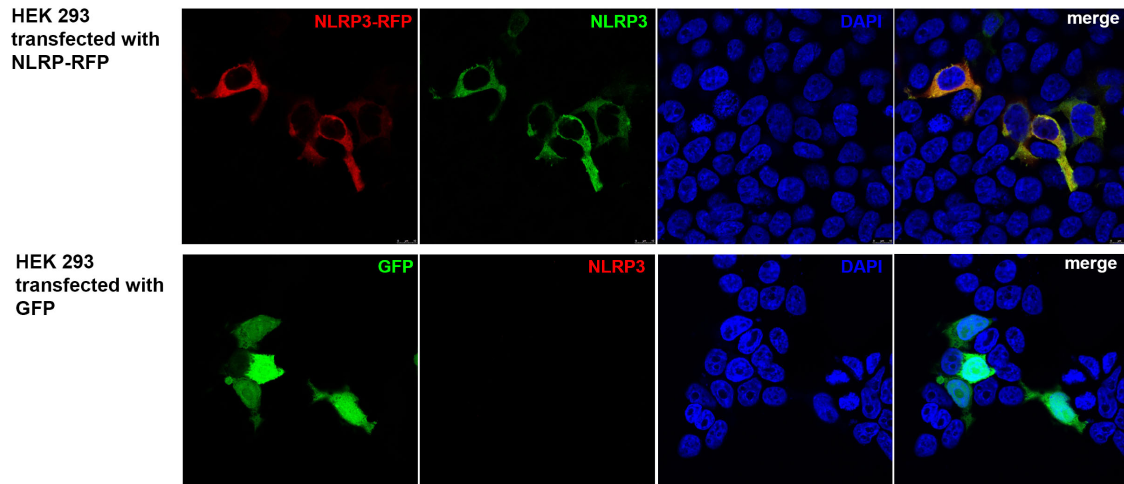
2. To demonstrate the specificity of our findings for microglia, astrocytes as yet another brain cell capable of producing cytokines under certain conditions were isolated from neonatal wild type mice and stimulated with LPS/ATP. In contrast to what was found in microglia, we failed to detect NLRP3 in astrocytes by means of western blotting or by immunocytochemistry. These data are now depicted in the new **Fig. EV5BC** of the revised manuscript.

Minor comments:

1. As the use of NLRP3 and caspase 1 antibodies throughout the literature has been questionable the authors need to validate that the NLRP3 and caspase 1 antibody they are using are indeed specific to NLRP3 and caspase 1.

Reply: To address this valid critique, we confirmed the specificity of the NLRP3 and caspase-1 antibody used in our study as follows:

NLRP3: HEK293 cells, which do no express NLRP3 endogenously, were transfected with the plasmid NLRP3-RFP (kind gift of E. Latz, University of Bonn, Germany) or a GFP-coding plasmid as control. Transfected cells were analyzed by western blotting and immunocytochemistry. Both methods revealed a NLRP3 signal exclusively in cells transfected with NLRP3-RFP (Fig. R2, see below). Moreover, the specific detection of endogenous NLRP3 induced by LPS/ATP treatment (Fig. 3A) further confirmed the specificity of the antibody.



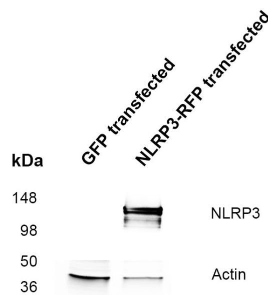


Fig. R2 HEK293 were transfected with plasmids coding for GFP or NLRP3-RFP. Only NLRP3-transfected cells gave rise to a NLRP3 signal upon probing with the NLRP3 antibody (immunocytochemistry (top rows) and western blot (bottom row)).

Caspase1: As for NLRP3, the induction of cleavage of Pro-Caspase1 by LPS/ATP treatment supports the specificity of the antibody. As shown in Fig. R3 (below) only LPS/ATP-stimulated cells contained the cleaved Caspase1 in their lysates, while Pro-Caspase1 levels were not significantly altered.

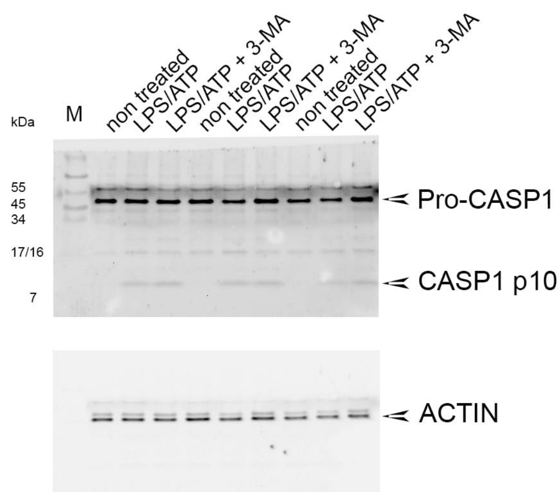


Fig. R3 Wild type microglia were either not treated or treated with LPS/ATP in the presence or absence of 3-Methyladenine (3-MA). Western blotting of cell lysates for CASP1 showed the precursor as well as the cleaved p10 fragment of Caspase1 exclusively in LPS/ATP treated cells.

2. The authors need to include molecular weight markers on all of their immunoblots.

Reply: This was done.

3. In figure 1 the number of LC3+ vesicles/cell should also be evaluated.
Reply: As suggested we also determined the number of LC3 positive vesicles/cell (Fig. R4, see below). Due to a higher variance, most likely due to the various microglia cell morphologies/activation status/phenotypes known to occur *in vitro*, at best a trend towards reduced LC3 positive vesicles/cell in *Becn1*$^{+/-}$ microglia was detectable, which did not reach statistical significance ($p = 0.143$), in contrast to the microglial cell area, which gave rise to a significant difference, as shown in Fig. 1C of the manuscript.

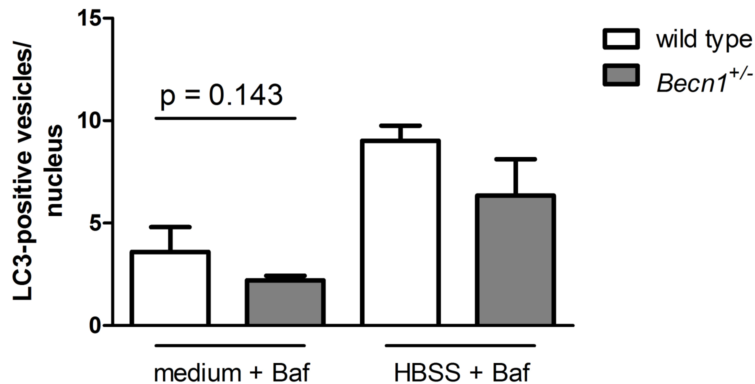


Fig. R4 Microglia from newborn *Becn1*$^{+/-}$ and wild type mouse pups were kept for 2 h either in full medium or HBSS with Bafilomycin A1 (Baf) and stained for endogenous LC3. The number of autophagosomes/nucleus was not significantly altered by means of counting LC3-positive vesicles in microglia from *Becn1*$^{+/-}$ mice; wild type n = 4, *Becn1*$^{+/-}$ n = 4, 3-5 fields per view/n and condition and 73-271 cells/n and condition; two-tailed t-test, ns.

4. In figure 2 LPS and ATP only controls should be shown.
Reply: Microglia were not treated, treated either with LPS or with ATP, or with LPS followed by ATP. As shown in Fig. R1 (see above), treatment either with LPS or with ATP did not result in the release of IL1beta. **Fig. 2A** has been changed accordingly.

5. In figure EV1C TNF and IL-6 levels should be shown for cells cultured in both normal media and HBSS similar to what is shown in figure 2C.
Reply: As suggested by referee#1 we included the quantification of TNFalpa and IL6 expression of HBSS- and 3-MA-treated wild type microglia. While HBSS reduced the levels of both cytokines, 3-MA treatment reduced expression only of TNFalpha. These data are now added to **Fig. EV1C** of the revised manuscript.

6. In figure 3 the authors show that there is more NLRP3 in the Becn1+/- microglia. The authors need to also consider subcellular localization of the NLRP3 protein.
Reply: To address this question we analyzed the distance of NLRP3 aggregates to the nucleus in the SIM images in 3 dimensions. No significant differences of the respective distances between the genotypes became apparent. These data are now included in the new **Fig. EV4B** of the revised manuscript.

7. In figure 3 the authors make a point to say that there are non-cell associated ASC-specs present following pyroptosis. This statement is a bit controversial and the authors do not prove that the specs are from cells that have undergone pyroptosis rather than the result of cells ejecting the specs.
Reply: We agree with referee #1 and changed the sentences accordingly, namely by exchanging “pyroptosis” with “pyroptosis/ejection” in the revised manuscript.

8. Related to comment 7, the authors should assess whether there is a higher percentage of extracellular specs in the cultures of the Becn1+/- cells than the WT cells. This would support the idea that the Becn1+/- cells are less able to recycle the specs via autophagy.
Reply: To address this point, we re-analyzed the ASC stainings of stimulated microglia and discriminated between ASC foci inside vs. outside the cells. Although we found a higher amount of ASC foci in the *Becn1*$^{+/-}$ microglia in general (Fig. 3C), the percentage of the ASC foci located inside vs. outside of microglial cells was

not significantly different in wild type vs. *Becn1*$^{+/-}$ microglia (Fig. R5). While we assume that the referee's statement appears to imply that the presence of a higher amount of extracellular ASC foci in *Becn1*$^{+/-}$ microglia indicates a reduced capacity of *Becn1*$^{+/-}$ microglia to recycle ASC foci, we interpret this finding – also based on the increase in IL1beta and IL18 (Fig 2A and 2B) – somewhat differently, namely that the reduced ability to recycle ASC foci rather leads to a more active inflammasome pathway.

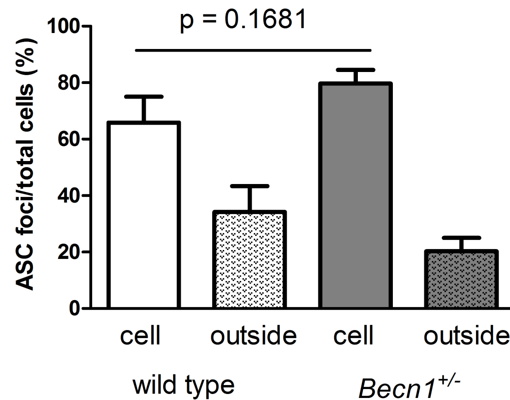


Fig. R5 LPS/ATP treated wild type and *Becn1*$^{+/-}$ microglia were immunolabeled for ASC (see Fig. 3C) and imaged by confocal microscopy to investigate the assembly of inflammasomes. Quantification of ASC foci and separation in inside the cell (inside) and outside the cell (outside) showed no difference between *Becn1*$^{+/-}$ and *wild type* microglia. Percentages were calculated based on the number of foci per genotype; wild type n = 4, *Becn1*$^{+/-}$ LPS/ATP n = 7; t-test ns.

Referee #2:

This study by Houtman et al., describes the effects of heterozygous deficiency of autophagy protein Beclin1 in inducing NLRP3 inflammasome activation and maturation of IL-1β and IL-18 in microglial cells. In APPPS1 mouse model of tauopathy, the microglia with Beclin 1 deficiency show increased protein levels of IL-1β and IL-18 compared to non-transgenic mice. The Beclin 1 deficient-microglial inflammasomes were functional with increased NLRP3, cleaved Caspase 1, aggregation of NLRP3, especially with LC3 positive vesicles, which were shown by high resolution microscopy. Overall, this is an interesting study aimed towards assessing the role of microglial autophagy on inflammasome activation and IL-1β and IL-18 maturation. However, there are numerous major and minor issues in terms of the novelty of the study, true relevance of microglial-Beclin 1 deficiency on amyloid pathology and Alzheimer's disease, and identity of the proteins. Related to the latter, authors seem to be unaware of the well-known fact that mice have a truncation that inactivates NDP52/CALCOCO2), and yet claim that "the best of our knowledge, we have shown for the first time that CALCOCO2 is involved in selective autophagy of a specific protein" using mouse cells.

We thank referee #2 for his/her thorough evaluation of our manuscript. We addressed all comments and suggestions by performing additional experiments and/or by rephrasing the respective parts of the manuscript (changes in the revised version of the manuscript are shown in blue color font).

Major comments:

1. This appears to be a mostly descriptive study without exploration of any specific mechanisms. For example: the authors describe that CALCOCO2 (NDP52) - NLRP3 interaction plays a role in NRLP3 degradation which is impaired due to Beclin-1 deficiency. An siRNA against CALCOCO2 would have been helpful to demonstrate the functional relevance of this interaction. Another example is the observation that heterozygous deficiency of Beclin 1 elevates NLRP3 recruitment and IL-1β maturation. It would be helpful to confirm this by restoring Beclin 1 levels (via overexpression etc.) and demonstrate that restoring Beclin-1 prevents inflammasome assembly and activation of IL-1β via inducing autophagy in microglia (rescue experiment).
Reply: We indeed are aware that murine cells express only a truncated version of NDP52/CALCOCO2, whose function and functionality is a matter of an ongoing scientific debate. However, the murine protein still contains the SKICH domain and has been shown to bind phosphorylated tau (Jo, Gundemir et al., 2014). Using cDNA of murine microglia amplification of the coding sequence of murine CALCOCO2 resulted in a band of around 1000 bp (Fig. R6). In western blots (with an antibody raised against the N-terminal domain of human NDP52/Calcoco2) a band of about 34 kDa was detected (new **Fig. 5E**), which is in agreement with what is depicted for murine NDP52/Calcoco2 in the NCBI gene bank (NM_001271018.1 and NP_001257947.1).

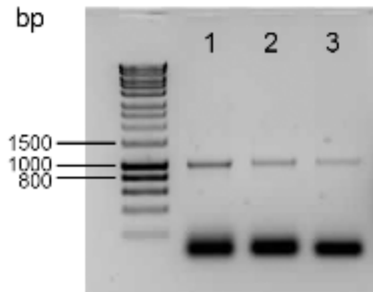


Fig. R6 Three different cDNA libraries obtained from primary mouse microglia were used in a PCR with primers binding to the 5´ and 3´end of the coding sequence of murine CALCOCO2. Amplification resulted in a band of about 1000 bp, correlating with the predicted size of 995 bp for the coding sequence.

Thus, as suggested by referee#2, we used a siRNA approach to reduce CALCOCO2/NDP52 expression. 6 days after transfection CALCOCO2 protein levels were reduced by around 40% compared to cells transfected with scrambled siRNA. When CALCOCO2 siRNA transfected cells were stimulated with LPS/ATP, IL1beta protein release in the cell supernatant was significantly increased when compared to microglia transfected with scrambled siRNA. In contrast, TNFalpha and IL6 protein release of microglia transfected with CALCOCO2 siRNA was unchanged to microglia transfected with scrambled siRNA, indicating that CALCOCO2 downregulation fairly specifically affects the IL1beta signaling pathway. These data speak in favor of a rather defined function of CALCOCO2 in murine microglia. We added these results to the manuscript (new **Fig. 5E,F** and **Fig**. **EV5D,E** of the revised version).

We also tried to overexpress Beclin1, as suggested by this referee, by transducing *Becn1*$^{+/-}$ microglia with the plasmid pBOBI BECN1 (kind gift of T. Wyss-Coray, Stanford University School of Medicine/USA). While we were not successful in establishing a detectable increase in BECN1expression at 3 and 6 days upon transduction as mandatory experimental prerequisite for performing the proposed overexpression/rescue experiment within the given time for revising this manuscript, we believe that the above mentioned pathway-specific modulation of IL1beta upon siRNA-based CALCOCO2 downregulation along with the results from neonatal *Becn1*$^{+/-}$ and adult *APPPS1 Becn1*$^{+/-}$ mice, plus the SIM and super-resolution microscopy data as well as the evidence from neonatal HBSS and 3-MA treated wild type microglia sufficiently confirm the relevance of Beclin1 in the regulation of the IL1beta pathway.

2. While the inflammasome/IL-1β activation appears to be significantly higher in APPPS1/Becn+/- mice, however, there is no functional measure on what does this mean to amyloid pathology? This is important because increased phagocytic function of microglia linked to elevated IL-1β has been shown to reduce amyloid burden and Aβ plaque count in several previous studies. Does Beclin-1 deficiency in microglia make microglia more phagocytic (with enhanced IL-1β) against amyloid?
Reply: To address this point, we quantified the amount of amyloid beta 1-40 and amyloid beta 1-42 in protein lysates of brains from age-matched *APPPS1* and *APPPS1 Becn1*$^{+/-}$ mice at 3, 4 and 8 months by means of electrochemiluminescence (MesoScale) as well as by morphometric analyses of immunostained histological sections from brains of 4 months old mice. Since we were not able to detect a significant difference in the amyloid burden as well as in plaque size and plaque distribution in *APPPS1* vs. *APPPS1 Becn1*$^{+/-}$ mice (now included as new **Figs. 2F** and **EV2A-C** in the revised manuscript), we next compared the phagocytic capacity of microglia in acute brain slices from 4 months old *Becn1*$^{+/-}$ mice to those of microglia in brains slices derived from wild type mice. Again, no significant differences with respect to the amount of phagocytic cells, the overall phagocytic index (new **Fig. EV2D-F** of the revised manuscript), or the number of phagocytosed beads (Fig. R7) were found in microglia derived from *Becn1*$^{+/-}$ mice when compared to wild type microglia.
While this certainly explains the above-mentioned lack of a difference in the amyloid burden in *APPPS1* mice with one vs. two alleles of *Becn1*, it appeared to be counter-intuitive at first sight, also in light of previous data showing that IL1beta release can drive the phagocytic capacity of microglia. However and importantly, Lucin et al. (Lucin, O'Brien et al., 2013) demonstrated that knocking down Beclin1 in the microglial cell line BV2 reduces their phagocytic capacity. Based on this we assume that the Beclin1-mediated decrease in phagocytosis, which should also apply to

Becn1$^{+/-}$ microglia, may be compensated by the IL1beta-driven reverse effect in these cells, i.e. that exclusively in heterozygous *Becn1*$^{+/-}$ microglia the opposing effects modulating phagocytosis outcompete each other. Along this line, one would speculate that *APPPS1* mice with a homozygous deletion of *Becn1* are expected to harbor microglia with reduced phagocytic microglia, supposedly resulting in an increase in amyloid pathology. The respective experimental *in vivo* proof of this hypothesis is, however, difficult to provide given that homozygous Beclin1 knock out mice are lethal (Qu, Yu et al., 2003, Yue, Jin et al., 2003).

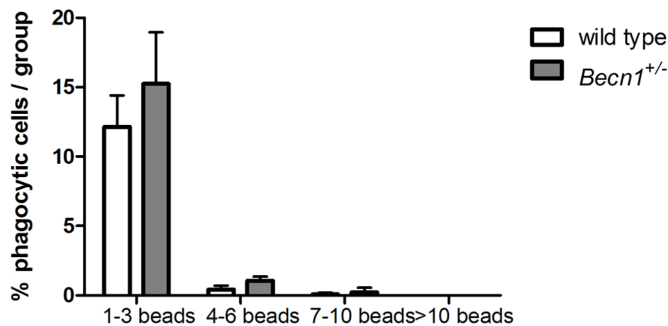


Fig. R7. Phagocytosis assay of microglia in acute brain slices from 4 months old wild type and *Becn1*$^{+/-}$ mice using fluorescent microbeads. Microglia in *Becn1*$^{+/-}$ slices were capable of phagocytosing fluorescent beads alike microglia in wild type slices. Wild type n = 5, *Becn1*$^{+/-}$ n = 4, 3 slices per mouse, 2 fields of view per slice.

3. Did the authors run HBSS-only condition? Why can't it be serum-free DMEM to achieve serum starvation? Also, why is the LC3 in serum starved (HBSS) in wild type condition not elevated (in fact shows slight reduction without Baf A1)?

Reply: HBSS in contrast to DMEM contains no amino acids and is a standard medium to evoke maximal autophagic flux (Shang, Chen et al., 2011). Under condition of HBSS the amount of autophagosome-bound LC3-II is known to be reduced due to the fact that autophagosomes are maturating faster to autolysosomes (maturation occurs by fusion of autophagosomes and lysosomes), where LC3-II gets degraded. Only when formation of autolysosomes is inhibited by Bafilomycin A1 treatment and autophagic flux is thus impaired, differences in autophagosome formation become apparent (KlionskyAbdelmohsen et al., 2016).

4. Fig. EV1D-E: Why are the TNF and IL-6 levels reduced from 4- to 8- months of age? If IL-1β were to induce neuroinflammation via NFkB, one would expect overall elevated levels of TNF and IL-6 as well. Any explanation would help.

Reply: We deliberately performed only statistics **within the age-groups** and not between the age groups as samples were taken, processed and analyzed at different time points. For the same reason we included wild type mice as reference (and nominator) in all age groups. As shown in Figs. 2E and EV1D-E, the cytokine levels of wild type mice varied over time as well. To overcome this methodological problem we set the respective cytokine amount of all wild type mice to 1 and calculated the cytokine values of *Becn1*$^{+/-}$, *APPPS1* and *APPPS1 Becn1*$^{+/-}$ mice accordingly (for a better overview we combined both protein fractions). As shown in Fig. R8, levels of TNFalpha and IL6 do not decline from 4 to 8 months of age.

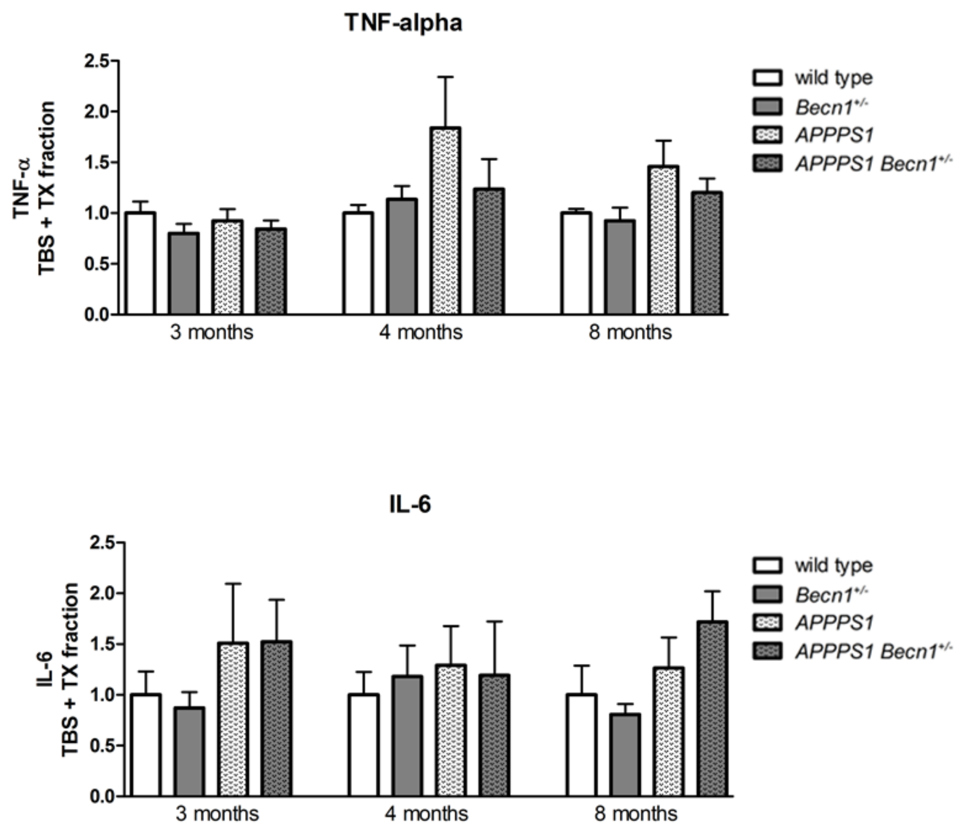


Fig. R8 Proteins were extracted from brains of wild type, *Becn1*$^{+/-}$, *APPPS1* and *APPPS1 Becn1*$^{+/-}$ mice, the respective age is indicated and the cytokines measured by electrochemiluminescence (MesoScale). Cytokine values from TBS and TX fraction were combined and TNFalpha and IL6 values of wild type mice in each age group were set as 1. The values (fold-increase) of *Becn1*$^{+/}$-, *APPPS1* and *APPPS1 Becn1*$^{+/-}$ from the respective age groups were calculated accordingly. No significant changes over time were apparent; wild type: n (per age group) = 2/6/3 *Becn1*$^{+/-}$: n = 2/5/2 *APPPS1*: n = 5/5/6 *APPPS1 Becn1*$^{+/-}$: n = 7/9/6; ANOVA with Dunnett´s *post hoc* test with wild type 3 months old as control group.

5. NLRP3 is a target of autophagy, as already shown by several reports (e.g. PMID: 28786745). It is not clear what is new in Fig. 4 and Fig. EV4 other than showing it is in microglia and using high resolution microscopy.

Reply: Indeed, the impact of autophagy on inflammation, mostly in macrophages and very recently in microglia, lately became a matter of intense interest in the science community. However, a detailed understanding of the mechanism, especially in microglia – which appears to be distinct from other myeloid cells in a variety of physiological as well as pathophysiological settings (Prinz, Tay et al., 2014) - particularly in the setting of neurodegeneration, has not been fully put forward. As stated in the discussion (page 15) "*A fundamental question is the mechanism by which reduced BECN1 levels and autophagy modulate the Il1beta/IL18 production. Proposed mechanisms include degradation of pro-IL1beta (Harris, Hartman et al., 2011), degradation of inflammasomes (Shi, Shenderov et al., 2012) and an indirect activation of the inflammasome via accumulation of dysfunctional mitochondria and oxidative stress due to impaired mitophagy.(Lee, Kim et al., 2016, Lodder, Denaes et al., 2015, Saitoh, Fujita et al., 2008, Ye, Jiang et al., 2017, Zhou et al., 2011)*". In contrast to published data on myeloid subtypes other than microglia, pro-IL1beta levels were not altered in microglia with reduced BECN1 expression (**Fig. EV3A**). On the contrary, Shi et al. (Shi et al., 2012) assessed IL1beta levels in THP-1 cells, a macrophage cell line, which differs significantly from primary microglia, while Ye et al.(Ye et al., 2017) observed an increase in TNFalpha and IL6 upon knockdown of *Becn1* in the microglial cell line BV2 (apart from enhanced IL1beta levels),

which is different from the IL1beta/IL18-specific effect we observed in primary microglia as well as *in vivo*.

Thus, we are able to demonstrate some new findings:

(1) Stimulated microglia from *Becn1*$^{+/-}$ mice released a higher amount of IL1beta and IL18, while TNFalpha and IL6 remained unchanged

(2) IL1beta but not TNFalpha and IL6 was also enhanced in an AD-like mouse model with reduced BECN1 expression (*APPPS1 Becn1*$^{+/-}$ mice)

(3) Crucial components of the IL1beta/IL18 processing pathway were increased (number of inflammasomes/cell, levels of cleaved Caspase1, as well as the amount of NLRP3, the sensor component of the inflammasome)

(4) Super resolution microscopy (SIM) showed the localization of NLRP3 aggregates in LC3-positive vesicles

(5) The autophagic linker protein CALCOCO2 but not p62/SQSTM1 colocalizes with NLRP3 and its downregulation results in increased IL1beta but not TNFalpha or IL6 production.

(6) No influence of mitochondria on NLPR3 degradation in LPS/ATP stimulated microglia

We therefore believe that our data contribute to the expanding and ongoing research on autophagy in neuroinflammation, thus justifying presenting our data to the scientific community.

6. While many of the protein-protein interactions (eg. NLRP3 - CALCOCO2) have been shown by SIM and super-resolution microscopy. It might help to validate that the physical interactions indeed are occurring via IP/Co-IP methods for at least key interactions like NLRP3-CALCOCO2. Reply: Despite the fact that we consider our data, including SIM and super-resolution microscopy, as appropriate to confirm the interaction between NLRP3 and CALCOCO2, we set out to expand our analyses, as proposed by referee #2. We therefore aimed at immunoprecipitation (IP) of overexpressed as well as of endogenous NLRP3. Since there are only a few colocalisation events per cell detectable by means of immunocytochemistry, it was not surprising that IP of endogenously expressed NLRP3 in microglia did not yield a detectable band of CALCOCO2. Our approaches to overexpress NLRP3-RFP (kind gift from E. Latz) and CALCOCO using a CALCOCO-HA plasmid (Jo et al., Nat Comm. 2014; kind gift from G. Johnson) by means of transfection – a prerequisite for performing an IP - within the given time for revising this manuscript – unfortunately were also not successful. However, we believe that our data showing an interaction of NLRP3 and CALCOCO2, namely by means of confocal microscopy and by SIM (Fig. 5 A and B), by the nearest neighbor analysis of SIM data (Fig. 5 C and D), as well as on a functional level by the siRNA-based knock down of CALCOCO2 leading to an increased IL1beta production (new Figs. 5 E and F) are sufficient to support our conclusions even in the absence of a successful IP.

Minor comments:

1. Fig. 1c: Not sure what is the purpose of the DAPI if they are shown in color.
Reply: The DAPI images are supposed to give a point of reference for the LC3 staining. Given that fine structures such as autophagosomes can be detected more easily in monochrome images it seemed reasonable to us to maintain the monochrome setting also for the DAPI stainings.

2. Fig. EV1 and elsewhere: Molecular weight markings are needed to determine full-length vs active IL-1β.
Reply: As proposed by Referee #2 molecular weight markings were added.

3. Fig. 2D: It is not clear if the pictures shown are with full medium or HBSS (with and without 3-

MA). If it is with only HBSS as said in the figure legend, then, the results should be edited accordingly.
Reply: We apologize for this inaccuracy. Images were taken from HBSS and HBSS + 3-MA treated cells. We rephrased the respective parts in the manuscript accordingly.

4. Fig. EV2: A representative western blot should be shown for panel A. It looks like Beclin 1+/- microglia may show elevated pro-IL1β.
Reply: The difference in Pro-IL1beta levels between wild type and *Becn1*$^{+/-}$ microglia was not significant when performing an unpaired t-test (p = 0.1208). However, as this may implicate a trend towards more Pro-IL1beta in *Becn1*$^{+/-}$ microglia, we analyzed 3 additional wild type and 4 additional *Becn1*$^{+/-}$ microglia samples. While the p value was further decreased (0.1088), significance was not reached, indicating that there is no (significant) modulation of Pro-IL1beta by reduction of BECN1. As proposed by Referee #2 a representative blot was added (**Fig. EV3A**).

5. Fig. 3A: When comparing multiple groups with different genotypes, it may be better to use one-way ANOVA than a t test.
Reply: We agree with referee#2 on the importance of utilizing the correct statistical test. Thus, as suggested we analyzed the data in Fig. 3A and 3B using one-way ANOVA followed by Tukey´s *post hoc* test. The legends of Fig. 3A and 3B were changed accordingly.

6. It is not clear about the novelty of the data presented in Fig. EV3B-C. NLRP3-ASC interaction has been very well established.
Reply: Indeed, the interaction of NLRP3 and ASC is well established. However, we consider this co-staining as an important confirmation validating our methodical setup as well as our conclusions, as indicated in the results section (page 9): "*To confirm the specificity of the NLRP3 staining stimulated microglia were co-labeled with ASC (Fig. EV4C,D). As described previously (Walsh, Muruve et al., 2014), we observed a very close association and partial overlap of NLRP3 and ASC signals using confocal and super resolution microscopy*."

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2nd Editorial Decision 19th Nov 2018

Thanks for your patience with the re-review process - one of those unfortunate situations where it just took a bit longer than anticipated to get the full input on the study.

I have now heard back from both referees and as you can see below. Referee #1 is satisfied with the revised version pending some minor text changes. Referee #2 still has issues with the NDP52 data. I have not looked at the NCBI entry, but would appreciate if you could comment on this.

One option is to remove the NDP52 data from the paper and publish the paper without it. I feel a bit reluctant to remove sound data from a manuscript, because I think we should be open to publish well-controlled experiments that do not always fit into how we see things as long as one has a very careful and balanced presentation and discussion of the data. Your input on Ref #2's comment is important.

Can we discuss further what to do - either by phone or email works for me.

REFEREE REPORTS:

Referee #1:

The authors have addressed my concerns and the revised manuscript now provides an important contribution that advances our knowledge of the impact of autophagy in microglial response to Abeta. There are three minor point that should be addressed
1. Page 5, First paragraph: "LC3-II levels of Becn1-/- microglia......lower when the flux was block with Bafilomycin A". THE AUTHORS SHOULD COMMENT THE PANEL IN WHICH CELLS ARE INCUBATED WITH HBBS.
2. Page 5. "resulted in increased presence of IL1b and IL18 in the supernatant of Becn1-/- microglia....COMPARED TO WHAT?
3. Figure 4. There is no quantification. It is unclear if the differences presented are statistically significant or represent just a trend

Referee #2:

My concerns re NDP52 in murine cells remain the same. The authors have now referenced an NCBI entry but that entry shows a hypothetical protein with multiple 4 amino acid repeats and looks nothing like human NDP52.

Additional correspondence from Authors 19th Nov 2018

First of all we are happy that referee #1 thinks that the manuscript "provides an important contribution" and we will address the minor points he raised.

Regarding referee #2`s comment we are a bit surprised, because the provided link to the protein sequence does not show a hypothetical protein and a sequence comparison of this murine NDP52 sequence and the human one using blastp suite showed 55% identity and 68% positives.
We would very much appreciate your thoughts on this matter either by phone or we could send you in the next days an "unofficial" reply to referee #2, whatever is more convenient for you.

Additional correspondence from Editor 20th Nov 2018

Thanks for your input - so the way I see the mouse NDP52 data is the following:

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- Mouse express a truncated form - I think it is fair to say that if this truncated form can functional as an autophagy receptor is not fully clear. You guys know more about this field than I do, so my question for you is can it act as an autophagy receptor with the domains it has?? Please advise

- You do see co-staining of mouse CALCOCO2 and inflammasome

- You do show that the Ab recognizes mouse CALCOCO2

- You do show that partial kd of CALCOCO2 affect IL-1beta expression.

So I think it is fair enough to say that you show that CALCOCO2 affects IL-1beta expression, I think what one might have to be cautious about is if CALCOCO2 indeed functions in this context as an autophagy receptor. What is your take on this?

I am OK with leaving the data in because I find it insightful, but maybe you just have to be careful in stating that it functions as an autophagy receptor. This is something that you can discuss. I would also be interested in your input in my above question if CALCOCO2 with its domain can function as an autophagy receptor? If it can then I would feel more comfortable stating it is an autophagy receptor, if it can't then you should be more cautious

Additional correspondence from Authors 22nd Nov 2018

We completely agree with your summary and interpretation of our data.

Regarding the subject of murine NDP52 we re-analyzed all published data. In short, three different publications showed that the SKICH domain of human NDP52 can bind different cargos which is in accord with our data showing a colocalization of murine NDP52 (containing a SKICH domain) and NLRP3. As downregulation of NDP52 results in reduced IL1beta release, the protein seems to have a function in the IL1beta pathway despite its truncation. Due to the lack of a ubiquitin-binding domain it is unclear if murine NDP52 can act alone as an autophagic receptor/adaptor protein, therefore we decided to avoid the term “autophagic receptor/adaptor protein” when mentioning NDP52, were rather descriptive regarding the functional impact and the implication of our NDP52-related findings and discussed its implications (see detailed answer in the cover/rebuttal letter and the new version of the manuscript, both attached). We hope that this approach will meet your expectations.

2nd Revision - authors' response 28th Nov 2018

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Revision II - Detailed response to the referees
Changes in the text are depicted in violet

Referee #1
The authors have addressed my concerns and the revised manuscript now provides an important contribution that advances our knowledge of the impact of autophagy in microglial response to Abeta.
We thank Referee #1 for his/her time and consideration reviewing our rebuttal letter as well as the revised version of our manuscript and are pleased by his/her appraisal of our work.

There are three minor point that should be addressed
1. Page 5, First paragraph: "LC3-II levels of Becn1-/- microglia......lower when the flux was block with Bafilomycin A". THE AUTHORS SHOULD COMMENT THE PANEL IN WHICH CELLS ARE INCUBATED WITH HBBS.
We regret this inaccuracy and rephrased the text accordingly: “Whilst the HBSS cultured cells showed a trend of decreased autophagic flux in the Bafilomycin A1 treated *Becn1*$^{+/-}$ microglia, LC3-II/MAP1LC3B-II levels of *Becn1*$^{+/-}$ microglia were significantly lower when autophagic flux was blocked with Bafilomycin A1 in cells cultivated with full medium in accordance with the reduced BECN1 content (Fig 1B).”

2. Page 5. "resulted in increased presence of IL1b and IL18 in the supernatant of Becn1-/- microglia....COMPARED TO WHAT?
We’d like to apologize for this inaccuracy. The supernatant of *Becn1*$^{+/-}$ microglia was compared to the supernatant of wild type microglia; this has been added in the revised version of the manuscript.

3. Figure 4. There is no quantification. It is unclear if the differences presented are statistically significant or represent just a trend
We apologize this was not clear. The quantification of Figure 4C is presented in Figure 5D, where we compare the distance to NLRP3 of both LC3 and CALCOCO2, and it shows a significant difference between CALCOCO2 and LC3. A reference to Figure 5D has been added to the revised manuscript.

Referee #2:
We also like to thank Referee #2 for his/her interest in our work and the time he/she spend on reviewing the rebuttal letter and the revised version of our manuscript.

My concerns re NDP52 in murine cells remain the same. The authors have now referenced an NCBI entry but that entry shows a hypothetical protein with multiple 4 amino acid repeats and looks nothing like human NDP52.
We understand the importance of the concern of Referee #2 regarding the existence and the functionality of murine CALCOCO2/NDP52. Therefore, we did an in-depth study of all published data on murine (m)NDP52 and came to the following results and conclusions regarding the existence and functionality of mNDP52:

Existence of murine NDP52

1. NCBI records of the mRNA (NM_001271018.1) and protein (NP_001257947.1) of murine NDP52/Calcoco2 in the NCBI gene bank are validated as demonstrated in the provided links; thus, mNDP52 appears to be a genuine rather than a hypothetical protein.
   https://www.ncbi.nlm.nih.gov/nuccore/NM_001271018.1
   https://www.ncbi.nlm.nih.gov/protein/NP_001257947.1

2. Comparison between the protein sequence of human (isoform1) and murine NDP52 illustrates 128 identical amino acids in the N-terminal region (Fig R1) indicating that the murine NDP52 protein sequence as presented in the link above is related to the human one (see also (Tumbarello, Manna et al, 2015)).

```
Sequence ID: Query_136495  Length: 470  Number of Matches: 3

Range 1: 13 to 251 Graphics                                   ▼ Next Match  ▲ Previous Match
Score           Expect  Method                          Identities     Positives      Gaps
244 bits(623)   8e-82   Compositional matrix adjust.    128/239(54%)   159/239(66%)   34/239(14%)

Query  8    TLLEHGNFSQVLFNNVEKFYAPRGDIMCYYTLTEKFIPRRKDWIGIFK------------  55
             LL+H +FSQV+FN+VEKFY P GD+ C+YT T+ FIPRRKDWIGIF+
Sbjct  13   VLLDHCHFSQVIFNSVEKFYIPGGDVTCHYTFTQHFIPRRKDWIGIFRAFKCFQDKLEQE  72

Query  56   ------------VGWKTTQEYYTFMWAPLPKDQNKDSATQQEIQFKAYYLPKDVERYQFC  103
                        VGWKTT+EYYTFMW  LP D N  SA QQE+QFKAYYLPKD E YQFC
Sbjct  73   LLKWRSQGQKLQVGWKTTREYYTFMWVTLPIDLNNKSAKQQEVQFKAYYLPKDDEYYQFC  132

Query  104  YVDEDGLVRGTSVPFQFCPDPDEDIMVVINKEKVEEMEQLSEELYQQNQELKD-------  156
            YVDEDG+VRG S+PFQF P+ +EDI+VV  + +VEE+EQ ++EL ++NQELKD
Sbjct  133  YVDEDGVVRGASIPFQFRPENEEDILVVTTQGEVEEIEQHNKELCKENQELKDSCISLQK  192

Query  157  KYADLHEQLQRKQVALEATQRVNKTLEHKV-EEKASWEKEKASWEE--EKASWEEEKAS  212
            + +D+  +LQ+KQ  LE  Q +NK LE KV E+K  WE E    +E  +K S E EK
Sbjct  193  QNSDMQAELQKKQEELETLQSINKKLELKVKEQKDYWETELLQLKEQNQKMSSENEKMG  251
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Fig R1 Alignment of the N-terminal 239 amino acids of human (isoform 1; sbjct) and murine NDP52 (query) using blastp suite-2sequences.

3. Expression of murine NDP52 mRNA and protein has been detected in the brains of mice mimicking certain aspect of Alzheimer’s disease (Kim, Lee et al, 2014).

4. Using cDNA of murine microglia, amplification of the coding sequence of murine CALCOCO2 resulted in a band of around 1000 bp (Fig R2). In Western blots (with an antibody raised against the N-terminal domain of human NDP52/Calcoco2) a band of about 34 kDa was detected (**Fig. 5E**), which is in agreement with the published coding sequence.

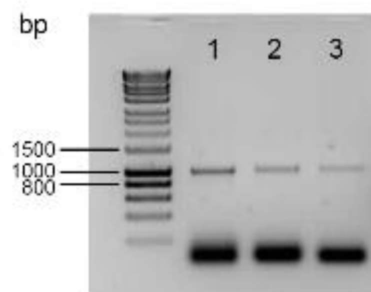

Fig R2 Three different cDNA libraries obtained from primary mouse microglia were used in a PCR with primers binding to the 5´ and 3´end of the coding sequence of murine CALCOCO2. Amplification resulted in a band of about 1000 bp, correlating with the predicted size of 995 bp for the coding sequence.

Functionality of murine NDP52

hNDP52 consists of a C-terminal **LIM**-like domain (zinc fingers), a **Galectin-8** domain, an intermediate **coiled coil** domain, a non-canonical LC3-interacting region motif for LC3C interaction (**CLIR**), and an N-terminal **SKICH** domain.

mNDP52 is truncated, but still contains the intermediate **coiled coil** domain, a non-canonical LC3-interacting region motif for LC3C interaction (**CLIR**), and a N-terminal **SKICH** domain.

1. The non-canonical **LIR motif** selectively binds LC3C, which recruits all ATG8 orthologues to the autophagosome (von Muhlinen, Akutsu et al, 2012).

2. The **SKICH domain** of hNDP52 has been shown to bind:

- phosphorylated tau and amyloid-beta (Jo, Gundemir et al, 2014)
- damaged mitochondria (Furuya, Kakuta et al, 2018)
- TRIF and TRAF6 (Inomata & Into, 2012)

3. In the protein TAX1BP1/CALCOCO3 the **SKICH** domain is thought to be required for autophagy (Yang, Wang et al, 2015).

4. Autophagic degradation of tau and amyloid-beta is suggested to be **ubiquitin-independent** (Jo, Gundemir et al, 2014).

Based on the above-cited literature, we conclude that - despite its truncation resulting in the lack of the ubiquitin-binding zinc finger domains - mNDP52 is not necessarily non- or dysfunctional. We assume that mNDP52 may still play a role in autophagy - most likely in a slightly different way due to its lack of ubiquitin-binding capacity. This "slight difference" of its function could be that mNDP52 might require a second protein or a protein complex to connect the cargo to the phagophore. Future studies will have to unravel the precise molecular and biochemical underpinnings.

However, the domains that are present in mNDP52 have been shown to be relevant in autophagic degradation of different cargo types. We propose that the role of mNDP52 in decreasing IL1beta release – which we can demonstrate - is mediated via autophagy. We consider autophagic degradation of the inflammasome by binding of NLRP3 to the SKICH domain and subsequent binding of LC3-II on the phagophore by the LIR domain present in mNDP52 or in complex with one or more additional proteins.

To be more precise and reflect the valid point of referee #2, we refrained to dub CALCOCO2/NDP52 an autophagic receptor/adaptor in the revised version of the manuscript and extended the discussion on this subject:
"Murine cells express only a truncated version of CALCOCO2 and its functionality is under discussion. However, the murine protein still contains the SKICH and LIR domain and several domain specific knock-out studies in CALCOCO2 showed that the SKICH domain is essential for binding TRIF, TRAF6 (Inomata & Into, 2012), phosphorylated tau, amyloid beta (Jo, Gundemir et al, 2014) and damaged mitochondria (Furuya, Kakuta et al, 2018). The LIR domain specifically binds LC3C on the autophagosomal membrane, which then attracts all other ATG8 orthologues (von Muhlinen, Akutsu et al, 2012). Our data show that CALCOCO2 is located between NLRP3 and LC3 in a vesicle resembling the size of an autophagosome. While it appears that murine CALCOCO2 is able to bind NLRP3 via its SKICH domain, it is not yet entirely solved whether it acts as an autophagic adaptor protein by also binding LC3, or

if additional proteins are involved in the degradation process to compensate for the missing ubiquitin-binding domain. However, the downregulation of CALCOCO2 by siRNA in microglia resulted in an increase of IL1beta expression, while TNFalpha and IL6 were not affected, implying that murine CALCOCO2, despite its truncation, is involved in regulating IL1beta production in murine microglia."

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3rd Editorial Decision 5th Dec 2018

Thanks for submitting the revised version. I have looked at it and all looks good. I am therefore very happy to formally accept the manuscript for publication here.

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Corresponding Author Name: Marina Jendrach
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-99430

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.
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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.
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- 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 χ2 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.

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B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	n = 3/ genotype was the minimal sample size. Due to Beclin1+/- x Beclin1+/- pairings, the amount of Beclin1+/- pups excededed the amount of wild type pups.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Based on experience of amyloid beta and cytokine measurement of APPPS1 mice and their crossings, 6 animals/genotype were analyzed.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All samples with the right genotype were included apart from samples determined as outliers by Grubbs test.
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	Since the animals used in this study were not treated, no randomization was applied. The only difference between the groups was a genetic alteration.
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.	When analyzing immunocytochemistry fields of view were selected based on the DAPI staining, thus the relevant staining was not visible during the selection process.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The investigator analyzing the samples was blinded to the genotypes of the mice.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution was analyzed using the software Graph pad.
Is there an estimate of variation within each group of data?	Variance within each group was analyzed using the software Graph pad.
Is the variance similar between the groups that are being statistically compared?	Variance between groups was analyzed using the software Graph pad. Due to normalization on e.g. wild type microglia (set as 1) variance between groups did differ in some experiments.

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).	BECN1 (Novus, NB500-249 or Cell Signalling, 3495), LC3 (Sigma, L8918), CASP1 and pro-CASP1 (Abcam, ab179515), IL1beta and pro-IL1beta (eBioscience, 88701388), NLRP3 (AdipoGen, AG-20B-0014), p62/SQSTM1 (MBL, PM045), CALCOCO2 (Proteintech, 12229-1-AP) and ACTIN (Sigma, A1978) ICC: LC3 antibody (Sigma, L7543), p62 (Santa Cruz, sc-25575) respectively CALCOCO2 antibody (Proteintech, 12229-1-AP), NLRP3 antibody (AdipoGen, AG-20B-0014), antibody ASC (AdipoGen, AG-25B-0006, 1:500)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A - only primary cells were used

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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.	APPPS1+/- mice were described before (Radde et al., 2006). Male APPPS1+/- mice were crossed to female Bl6J mice and APPPS1+/- mice were compared to littermate wild type controls. Becn1+/- mice have also been described before (Qu et al., 2003) and were a gift from Tony Wyss-Coray (Stanford University School of Medicine/USA). Mice were bred from heterozygous matings. Becn1+/- mice were compared to littermate wild type controls. Animals were housed together and kept in individually ventilated and enriched cages with a 12 h light cycle with food and water ad libitum.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were conducted in accordance with animal welfare acts and were approved by the regional office for health and social service in Berlin (LaGeSo) #0132/09.
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.	Our manuscript complies with the ARRIVE guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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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	N/A
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