FAT10 localizes in dendritic cell aggresome-like induced structures and contributes to their disassembly

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AUTHORS: Richard Schregle, Stefanie Mueller, Daniel Legler, Wolfgang Krueger, and Marcus Groettrup
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Identifying FAT10 as a potential regulator of DALIS.

Comments for the author

Schregle et al. reported the presence of FAT10, a ubiquitin-like protein, at the DALIS- a p62-enriched foci induced by cytokine (TNF/INF-gamma) and LPS in dendritic cells. The authors presented evidence that unconjugated FAT10 is present at the foci, and FAT10 potentially positively affects the disassembly of DALIS.
Overall, the evidence that FAT10 is present at the DALIS in human MoDC is relatively solid, although it could be strengthened by performing the analysis in FAT10 knockdown MoDC cells. The studies of FAT10 in mouse BMDC, however, is less than ideal, as a transfected version of FAT10 is used (due to the lack of a suitable antibody). In this study, the identification of a mutant FAT10 that cannot reach DALIS would be helpful.
The evidence for a role of FAT10 in DALIS disassembly is less than convincing. This deficiency is, in part due to the slow kinetics of disassembly of DALIS and lack of a secondary biochemical marker to complement the image-based DALIS analysis. The loss of p62 or insoluble ubiquitinated protein aggregates, assessed by immunoblotting, could be considered for this purpose.
Lastly, even if FAT10 is indeed a positive regulator in DALIS disassembly, the study provides little mechanistic insight into how FAT10 might regulate this process and how this putative FAT10 activity is functionally relevant, for example, in MHC processing.
Overall, this study is very preliminary and would require much more mechanistic or functional analysis to support its publication.

Other specific comments:
1. Fig S4A. The data on a weak induction of p62 mRNA in BMDC in response to cytokine requires additional controls. In BMDM, a previous study has demonstrated a robust induction of p62 in response to LPS (>10 fold). In this study, p62 was induced by ~2-4 fold. It is important to rule out PCR conditions as the cause for low p62 mRNA induction. Immunoblotting of p62 would be helpful. If the low induction of p62 reflects the low efficiency of DALIS formation in BMDC, as it was the case in MoDC, it needs to be clearly stated in the text.

Reviewer 2

Advance summary and potential significance to field

This study determines the localization of FAT10, a ubiquitin-like modifier protein induced as part of DC maturation and implicated in antigen processing and presentation, to dendritic cell aggresome-like induced structures (DALIS). The localization of FAT10 to DALIS seems independent of substrate conjugation. DALIS are formed independent of FAT10 and HDAC6, which is an adaptor of aggresomal pathway and identified as a binding partner of FAT10. However, FAT10-deficient BMDC look to have increased numbers DALIS upon cycloheximide treatment which inhibits protein translation, arguing a regulatory role for FAT10 in the clearance of DALIS and thereby suggesting a role for FAT10 in antigen processing in maturing DC.

Comments for the author

The inclusion of primary human cells and primary bone marrow-derived dendritic cells (BMDC) isolated from wild-type and knock-out animals make the study sound and powerful. Multiple approaches and comprehensive analyses of DALIS formation including proper controls and additional supporting data make the study rigorous and robust. Images are high quality and data presentation is in general impressive.
Despite, there are some serious conceptual and experimental gaps that requires a further rigorous investigation. Once they are addressed, the study could be accepted for publication.

Conceptual:

While aggresomes are formed at perinuclear area by the accumulation of undegraded protein aggregates which appear to be ubiquitinylated and trafficked to MTOC along microtubule tracks via HDAC6, which is an aggresomal adaptor and FAT10 binding partner. DALIS are defined as antigen storage sites which are formed within the cytosol of maturing dendritic cells triggered by cytokine (TNF/IFN) or LPS stimuli. Although DALIS and aggresomes are implied as different structures, they look intimately related based on the following previous data from the same group. p62 bodies are formed in HeLa cells upon TNF/IFN stimulation (Aichem 2012).
Are p62 bodies homologous to DALIS structures? On the other hand, aggresomes are formed in HEK-293T cells upon TNF/IFN treatment (Kalveram, 2008)? In both cases, endogenous FAT10 has been localized to both p62 bodies and aggresomes, respectively. The confusion and discrepancy between DALIS and p62 bodies and aggresomes need further clarification and elaboration. Therefore, the following question need to be thoroughly addressed.

- Do BMDC form aggresomes upon proteasomal impairment?

a kinetic experiment with immature and mature BMDC (isolated from wild type and HDAC6) treatment with MG132 at 0, 6, 12, 24 hours without and with cytokine mixture, TNF/IFN should clarify this point?

Experimental:

Figure 5 –the re-expression of Fat10 is expected to recover the impaired disassembly of DALIS in FAT10 knockout BMDC upon cycloheximide treatment. Therefore, to clarify and validate the role of FAT10 in DALIS disassembly, the authors are highly recommended to re-express FAT10-wt, conjugation-defective FAT10 and N- and C- terminal deletion mutants to obtain a clear mechanistic role about FAT10-mediated DALIS disassembly.

Figure 5 - Given the role of FAT10 in the disassembly of DALIS, how are the sizes of DALIS altered in wild type and FAT10 knockout BMDC upon cycloheximide treatment?

Figure 2 – It is highly recommended that endogenous Fat10 needs to be stained with the antibodies in BMDC as in MoDC. Although the group does not have it at the moment, there are a number of various commercially available Fat10 antibodies and the author is encouraged to try to find the optimal one that stains upregulated Fat10 (induced by cytokines) at DALIS vs. basal endogenous Fat10.

Figure1, 2, 3, 4, 5 - How many cells (i.e., MoDC, BMDC) were counted in the experiments? What is the percentage of the counted cells that possess DALIS? A bar graph would help for presentation.
The author needs to count at least 100 cells per experiment to make a firm conclusion. The number of cells counted should be reported in figure legends.

Figure 3 and 4 - Does each circle and filled dot at 0, 8, 24, 48 hours represent only one cell? If so, the author needs to count at least 100 cells per time point to make a stronger conclusion.

Figure 2 – it'd be more insightful if the expression of FLAG-mFAT10 protein detected by immunoblotting using a FLAG antibody without and with MG-132 (4 hours)

Figure 3 – Although not involved in DALIS formation, the role of HDAC6 on DALIS should further be investigated by inhibiting protein neosynthesis, autophagy and proteasome as performed for FAT10 in figure 5.

First revision

Author response to reviewers' comments

Point-to-Point reply to reviewer comments of the manuscript by Schregle et al., entitled 'FAT10 localizes in dendritic cell aggresome-like induced structures and contributes to their disassembly' (JOCES/2019/240085)

Reviewer 1 Advance Summary and Potential Significance to Field: Identifying FAT10 as a potential regulator of DALIS.

Reviewer 1 Comments for the Author:

Schregle et al. reported the presence of FAT10, a ubiquitin-like protein, at the DALIS- a p62-enriched foci induced by cytokine (TNF/INF-gamma) and LPS in dendritic cells. The authors presented evidence that unconjugated FAT10 is present at the foci, and FAT10 potentially positively affects the disassembly of DALIS.

Overall, the evidence that FAT10 is present at the DALIS in human MoDC is relatively solid, although it could be strengthened by performing the analysis in FAT10 knockdown MoDC cells.

Reply: We agree that performing a knockdown in human MoDC would be useful to investigate the effect of FAT10 on DALIS size and number and thereby complement the experiments with mouse BMDC from wild-type and FAT10-deficient mice (Fig. 4). In order to achieve this aim we have established a protocol to knockdown human FAT10 in MoDC using siRNAs. However, while we succeeded to reduce the FAT10 mRNA expression by 70% in MoDC as compared to control siRNAs from two independent blood donors, we found no significant effect of FAT10 protein expression in DALIS during confocal microscopy for both of them. FAT10 still co-localized in DALIS with ubiquitin as we have already shown in Fig. 1. We are showing the data for these two donors at the end of this point-to-point reply with a detailed protocol in the legend for our referee (Ptp Figure 2). In MoDC from four further donors the reduction in FAT10 mRNA after knock down was even less. Hence, we think that a significant down-regulation of FAT10 protein in DALIS by knockdown in MoDC is technically not feasible and also not essential to confirm our data obtained in BMDC.

The studies of FAT10 in mouse BMDC, however, is less than ideal, as a transfected version of FAT10 is used (due to the lack of a suitable antibody).

Reply: We are aware that it is not ideal to use an overexpressed tagged version of mouse FAT10, however, we have extensively tested all commercially available antibodies for mouse FAT10. While most of them could visualize overexpressed mouse FAT10 none of them was able to detect endogenous mouse FAT10 in western blots or confocal microscopy not even in those cell types expressing the highest levels of mFAT10 like mature DC and mature thymic medullary epithelial cells. For the past 10 years we have exhaustively tried to generate polyclonal and monoclonal antibodies in rabbits or FAT10$^{-/-}$ mice using recombinant protein or peptides from mouse FAT10 as vaccines. While several of our FAT10 mAb readily detect overexpressed mFAT10, none of them detected endogenous mFAT10 in western blots or confocal microscopy. According to our titrations and quantifications of mFAT10 mRNA expression, the sensitivity of our mAbs is insufficient to visualize endogenous FAT10 e.g. in TNF/IFN-¤ treated cells. Therefore, we had no alternative than to establish the lentiviral overexpression of FLAG- tagged mFAT10 in dendritic cells. We point out more clearly the lack of antibodies that detect endogenous mouse FAT10 on page 7 of the revised manuscript (all text changes have been marked in red in the resubmitted manuscript)..

In this study, the identification of a mutant FAT10 that cannot reach DALIS would be helpful.

Reply: Recently, we have succeeded after eight years of work to determine the high resolution structure of human FAT10 and found that the two ubiquitin-like domains of FAT10 have an entirely different surface charge distribution than ubiquitin; not even the hydrophobic patch of ubiquitin around Ile44 is conserved in FAT10 (Aichem et al. (2018) Nat. Commun. 9:3321). We had tried by mutagenesis of residues of human FAT10 that are highly conserved in mammals whether we could identify surface residues required for binding to known FAT10-interaction domains in NUB1L,

RPN10, and HDAC6 but failed to do so. To make it worse, receptor proteins that are involved in DALIS formation are not known. Perhaps a random mutagenesis approach could be undertaken using microscopy as read out, but the (very poor) solubility of FAT10 is strongly affected by amino acid replacements. Hence, we think that this suggestion, although scientifically attractive, is not feasible and certainly out of the scope of this study.

The evidence for a role of FAT10 in DALIS disassembly is less than convincing. This deficiency is, in part, due to the slow kinetics of disassembly of DALIS and lack of a secondary biochemical marker to complement the image-based DALIS analysis. The loss of p62 or insoluble ubiquitinated protein aggregates, assessed by immunoblotting, could be considered for this purpose.

Reply: As we mentioned in our results section, we tried to use Western blotting as a biochemical means to complement our results obtained by microscopy but neither two different published methods (Lelouard et al. (2002) Nature 417: 177-182; Rahnefeld et al., (2011) Eur. J. Immunol. 41:2774-2781) nor protocols from our group gave consistent and reproducible results, therefore we could not include it.

Lastly, even if FAT10 is indeed a positive regulator in DALIS disassembly, the study provides little mechanistic insight into how FAT10 might regulate this process and how this putative FAT10 activity is functionally relevant, for example, in MHC processing.

Reply: In Fig.5 A we show that after protein synthesis inhibition there is increased storage of 'retirees' (i.e. readily folded, functional proteins that are targeted for degradation) in DALIS. This is not evident in WT cells, suggesting that FAT10 leads to preferential degradation of retirees that are targeted to DALIS and that FAT10 prevents this accumulation in WT cells as compared to FAT10-deficient cells. FAT10 is a very well documented and potent targeting signal for proteasomal degradation. Bulk FAT10 conjugates are degraded by the proteasome with a half-life of one hour (Aichem et al. (2014) FEBS J. 281:1848- 1859). Although FAT10 directly binds to p62 and co-localizes with p62 and LC3B in cells (Spinnenhirn et al. (2014) J. Cell Sci. 127:4883-4893; Aichem et al. (2012) J. Cell Sci. 125:4576-4585) FAT10 and FAT10-fusion proteins (e.g. with mCherry-EGFP-FAT10 tandem tags) surprisingly do not end up in acidifying autophagosomes (Aichem et al. 2012; Supplementary Figures S2 and S3) in contrast to mChery-EGFP-ubiquitin fusion protein. Moreover, the degradation of FAT10 and FAT10 conjugates is not affected by Bafilomycin A or chloroquine. Hence, disassembly of DALIS by FAT10 very likely is due to targeting conjugated proteins for proteasomal degradation. This notion is also supported by two previous publications showing that the fusion of FAT10 to long-lived viral proteins accelerates their degradation by the proteasome and enhances the presentation of peptide antigens derived from them on MHC class I (Schliehe et al. (2012) J. Virol. 86:9782-9793; Ebstein et al. (2012) Cell. Mol. Life Sci. 69:3443-2454) and by the finding that overexpression of FAT10 affects the pool of peptides eluted from MHC class I (Bürger et al. (2015) J. Immunol. 195:4106-4116). In the discussion of the revised manuscript on page 16 we have included the arguments compiled above.

Overall, this study is very preliminary and would require much more mechanistic or functional analysis to support its publication.

Other specific comments:

1. Fig S4A. The data on a weak induction of p62 mRNA in BMDC in response to cytokine requires additional controls. In BMDM, a previous study has demonstrated a robust induction of p62 in response to LPS (>10 fold). In this study, p62 was induced by ~2-4 fold. It is important to rule out PCR conditions as the cause for low p62 mRNA induction. Immunoblotting of p62 would be helpful. If the low induction of p62 reflects the low efficiency of DALIS formation in BMDC, as it was the case in MoDC, it needs to be clearly stated in the text.

Reply: We repeated the above-mentioned experiments and looked at the RNA and protein level of p62 during the LPS- and cytokine-induced maturation of BMDC. As compared to our previous results, we see increased expression levels of p62 mRNA after cytokine and LPS stimulation by qPCR. As suggested by our referee, we have also performed western blots confirming the induction of p62 during LPS or cytokine-triggered maturation of BMDC. These new data are now shown in Figure S4A

and S4B, respectively. Nonetheless, we again do not observe a more than 10fold increase of p62 expression at the RNA level as stated by reviewer 1, which might be due to the different cell type used. It should be mentioned that these new experiments were performed by a technician using a different qPCR machine in a different lab. Since we still observe similar expression levels, adverse PCR conditions seem very unlikely as a cause of the discrepancy. At the protein level we found a stimulus-dependent increase of p62 which reflects the kinetic of DALIS formation and disassembly which we observed in our microscopy-based analysis. This increase of p62 indicates a decreasing level of autophagy allowing for the efficient DALIS formation in our BMDC preparations in line with previous studies where a decrease of autophagy was found to be necessary for efficient DALIS formation (Wenger et al., (2012) Autophagy 8: 350-363; Terawaki et al., (2015) J. Cell Biol. 210:1133-1152). This suggests that the autophagolysosomal system is the major system for DALIS degradation which is in line with the increase of the number of DALIS upon inhibition of autophagy we have observed in Fig 5B.

Reviewer 2 Advance Summary and Potential Significance to Field:

This study determines the localization of FAT10, a ubiquitin-like modifier protein induced as part of DC maturation and implicated in antigen processing and presentation, to dendritic cell aggresome-like induced structures (DALIS). The localization of FAT10 to DALIS seems independent of substrate conjugation. DALIS are formed independent of FAT10 and HDAC6, which is an adaptor of aggresomal pathway and identified as a binding partner of FAT10. However, FAT10-deficient BMDC look to have increased numbers DALIS upon cycloheximide treatment which inhibits protein translation, arguing a regulatory role for FAT10 in the clearance of DALIS and thereby suggesting a role for FAT10 in antigen processing in maturing DC.

Reviewer 2 Comments for the Author:

The inclusion of primary human cells and primary bone marrow-derived dendritic cells (BMDC) isolated from wild-type and knock-out animals make the study sound and powerful. Multiple approaches and comprehensive analyses of DALIS formation including proper controls and additional supporting data make the study rigorous and robust. Images are high quality and data presentation is in general impressive. Despite, there are some serious conceptual and experimental gaps that requires a further rigorous investigation. Once they are addressed, the study could be accepted for publication.

Conceptual:

While aggresomes are formed at perinuclear area by the accumulation of undegraded protein aggregates which appear to be ubiquitinylated and trafficked to MTOC along microtubule tracks via HDAC6, which is an aggresomal adaptor and FAT10 binding partner. DALIS are defined as antigen storage sites which are formed within the cytosol of maturing dendritic cells triggered by cytokine (TNF/IFN) or LPS stimuli. Although DALIS and aggresomes are implied as different structures, they look intimately related based on the following previous data from the same group. p62 bodies are formed in HeLa cells upon TNF/IFN stimulation (Aichem, 2012). Are p62 bodies homologous to DALIS structures? On the other hand, aggresomes are formed in HEK-293T cells upon TNF/IFN treatment (Kalveram, 2008)? In both cases, endogenous FAT10 has been localized to both p62 bodies and aggresomes, respectively. The confusion and discrepancy between DALIS and p62 bodies and aggresomes need further clarification and elaboration. Therefore, the following question need to be thoroughly addressed.

Reply: One major difference between aggresomes on the one hand and DALIS or ALIS on the other hand is that aggresomes, which are larger aggregates of ubiquitylated or FAT10ylated proteins close to the MTOC outside the nuclear membrane, have to our knowledge only been shown when cells are treated with proteasome inhibitors (Johnston (1998) J. Cell Biol. 143: 1883-1898). Hence, the physiological relevance of aggresomes is not so clear. Aggresome formation is dependent on intact microtubules and HDAC6 whereas the formation of ALIS or DALIS is not (as we show in Fig. 3). The treatment of cells with TNF/IFN-¤ alone does not lead to the formation of aggresomes but requires in addition the inhibition of proteasomes for 4-6 hours (Kalveram et al.(2008) J. Cell Sci. 121:4079-4088). DALIS have been described to transiently form during the maturation of dendritic cells

*(Lelouard et al. (2002) Nature 417:177-182) whereas the closely related ALIS (Szeto et al. (2006) Autophagy 2:189- 199), which have the same properties as 'p62 bodies' (*Bjørkøy *et al. (2005) J. Cell Biol. 171:603-614; Pankiv et al. (2007) J. Biol. Chem. 282:24131-24145), form or accumulate in many cell types after application of stress e.g. upon inhibition of autophagy or induction of protein misfolding by arsenate or heat. Both are smaller aggregates of ubiquitylated or FAT10ylated proteins in the cytoplasm that contain p62. We have better clarified the interrelation between DALIS, ALIS and aggresomes in the introduction on page 4 of our revised manuscript (all text changes have been marked red in the resubmitted manuscript).*

•Do BMDC form aggresomes upon proteasomal impairment? A kinetic experiment with immature and mature BMDC (isolated from wild type and HDAC6) treatment with MG132 at 0, 6, 12, 24 hours without and with cytokine mixture, TNF/IFN should clarify this point?

Reply: As suggested by reviewer 2, we prepared BMDC and tested if aggresomes can form in immature and cytokine-matured BMDC. We only looked at 24 hours after maturation and instead of using HDAC6- deficient BMDC, we treated a sample with impaired proteasomal degradation additionally with nocodazole, which prevents the formation of aggresomes. We included our results in the new Figures 3C and 3D of the revised manuscript. We observed that immature and mature BMDC are able to form aggresomes after six hours of proteasome inhibition, but this does not substantially prevent formation of DALIS. We also observed that aggresomes and DALIS can form in the same cell. Immature and mature cells with inhibited proteasomes that were treated additionally with nocodazole showed only very few aggresomes but DALIS, further supporting the notion that DALIS formation is mechanistically not connected to aggresomes formation.

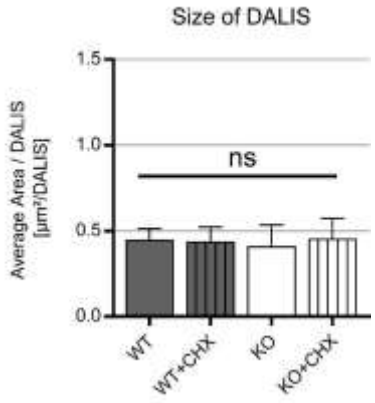
Experimental:

Figure 5 – The re-expression of Fat10 is expected to recover the impaired disassembly of DALIS in FAT10 knockout BMDC upon cycloheximide treatment. Therefore, to clarify and validate the role of FAT10 in DALIS disassembly, the authors are highly recommended to re-express FAT10-wt, conjugation-defective FAT10 and N- and C- terminal deletion mutants to obtain a clear mechanistic role about FAT10-mediated DALIS disassembly.

Reply: We lentivirally re-introduced mFAT10 into FAT10-deficient BMDC and enumerated the number of ubiquitin-positive DALIS in 24 h-matured BMDC treated or not with cycloheximide for two hours (see new panel D in Fig. 5). We observed that the number of DALIS in FAT10-deficient BMDC overexpressing mFAT10 was slightly reduced. Treatment with cycloheximide of these samples prevented that the number of DALIS increased as observed in not transduced FAT10-deficient BMDC (Fig. 5A+D). This strongly suggests that FAT10 counteracts the increased flux of retirees into DALIS most likely by targeting these proteins for degradation by the proteasome.

Figure 5 – Given the role of FAT10 in the disassembly of DALIS, how are the sizes of DALIS altered in wild type and FAT10 knockout BMDC upon cycloheximide treatment?

Reply: Since we have not seen any difference in the size of DALIS in FAT10-proficient and -deficient BMDC in untreated samples (Fig. 4E), we did not analyse the size of DALIS in the experiments with cycloheximide-treated BMDC before. However, we now have analysed the size of DALIS in the experiments newly performed for this revision and found no difference in the size of DALIS between wild type and FAT10-deficient BMDC (see Ptp Figure 1, below). We mention this data in the results section on page 11.



Ptp Figure 1. No change in the size of DALIS of untreated or cycloheximide-treated BMDC from FAT10-deficient or wild type mice. On day 10 of culture BMDC were matured with TNF/IFN-¤ for 24h. Two hours prior to fixation, BMDC were treated with cycloheximide (CHX) where indicated, followed by imaging and analysis as described in Materials and Methods of the revised manuscript. The average area of DALIS was quantitatively determined and is provided as mean +/- SD. The size of DALIS was measured automatically by the feature 'analyse particles' of the ImageJ FIJI software. For this, an intensity threshold was applied onto the image, which was set as high as possible to avoid noise but without changing the signal intensity of DALIS, and the size cut-off was set at 0.15 µm², only above which spots were considered to be DALIS.

Figure 2 – It is highly recommended that endogenous Fat10 needs to be stained with the antibodies in BMDC as in MoDC. Although the group does not have it at the moment, there are a number of various commercially available Fat10 antibodies and the author is encouraged to try to find the optimal one that stains upregulated Fat10 (induced by cytokines) at DALIS vs. basal endogenous Fat10.

Reply: We have extensively tested all reportedly mouse FAT10-specific antibodies commercially available at present. While most of them could visualize overexpressed mouse FAT10 none of them were able to detect endogenous mouse FAT10 in western blots or confocal microscopy, not even in those cell types of mice that express the highest levels of mFAT10 like mature DC and mature thymic medullary epithelial cells. For the past 10 years we have exhaustively tried to generate polyclonal and monoclonal antibodies in rabbits or FAT10$^{-/-}$ mice using recombinant protein or peptides from mouse FAT10 for vaccine preparation. While several of our unpublished FAT10-specific mAb readily detect overexpressed mFAT10, none of them detected endogenous mFAT10 in western blots or confocal microscopy. According to our titrations and quantifications of mFAT10 mRNA expression, the sensitivity of our mAbs is insufficient to visualize endogenous FAT10 e.g. in TNF/IFN-¤ treated cells. Therefore, we had no alternative than to establish the lentiviral overexpression of FLAG-tagged mFAT10 in dendritic cells. We point out more clearly the lack of antibodies that detect endogenous mouse FAT10 on page 7 of the revised manuscript

Figure1, 2, 3, 4, 5 – How many cells (i.e., MoDC, BMDC) were counted in the experiments? What is the percentage of the counted cells that possess DALIS? A bar graph would help for presentation. The author needs to count at least 100 cells per experiment to make a firm conclusion. The number of cells counted should be reported in figure legends.

Reply: We are sorry that it was not clear enough how many cells were counted per experiment. We changed our figure legends and included the minimum number of cells counted per experiment since the number of cells counted ranged from 50 to over 200 depending on the experiment and how the cells survived the different treatments. However, the number of cells showing DALIS for MoDCs are 5-10 fold lower than in BMDC due to the high level of autophagy in these cells that prevents the efficient formation of DALIS as has been previously described in other studies (Wenger et al., (2012) Autophagy 8: 350- 363; Terawaki et al., (2015) J. Cell Biol. 210:1133-1152). Yet, we analysed the

number of cells that showed DALIS in our experiments newly performed for this revision where we looked at the formation of aggresomes and included a bar graph in the new panel C of Fig. 4 showing how many immature and 24h-matured BMDC showed DALIS.

Figure 3 and 4 – Does each circle and filled dot at 0, 8, 24, 48 hours represent only one cell? If so, the author needs to count at least 100 cells per time point to make a stronger conclusion.

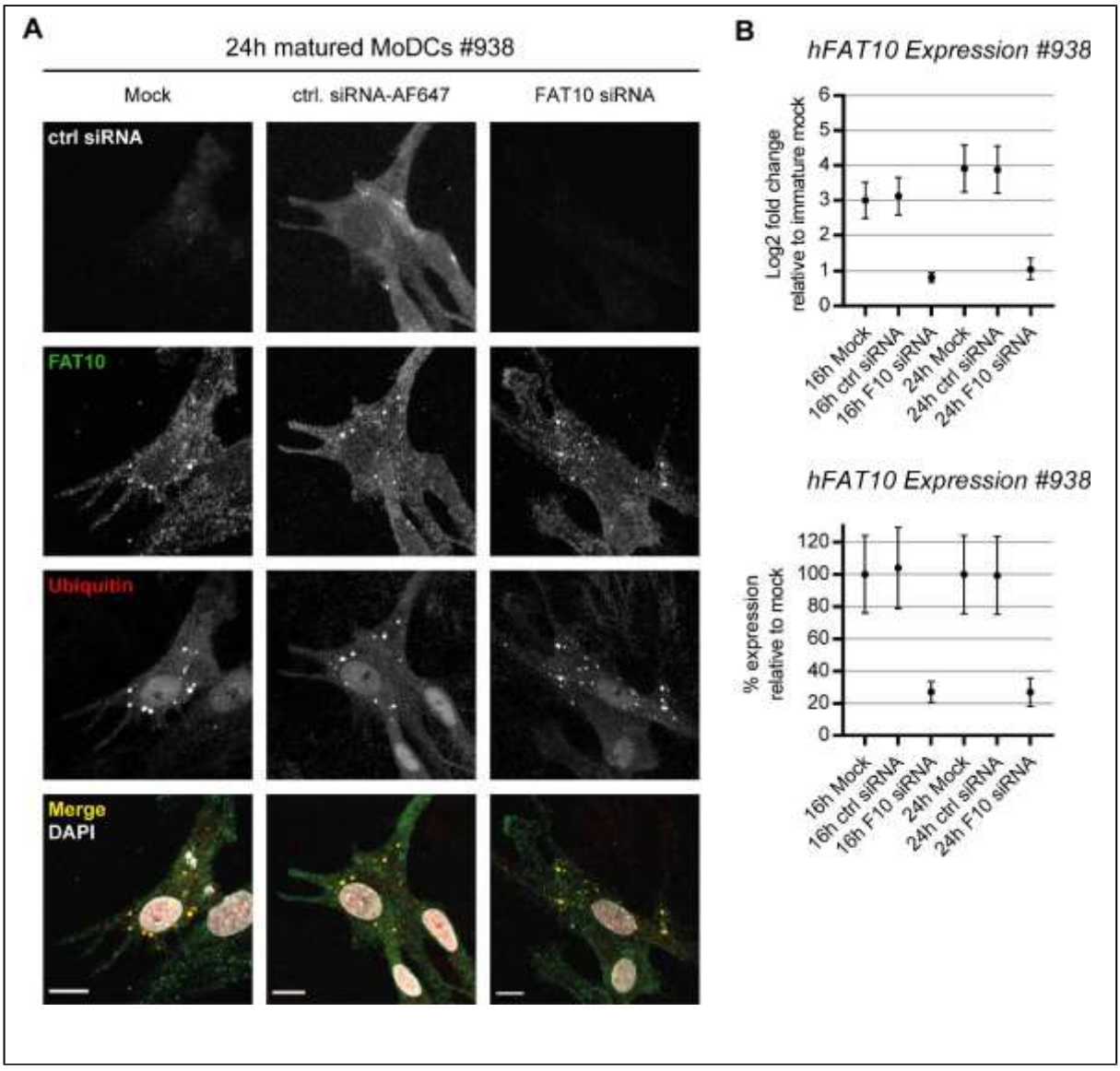
Reply: As mentioned above, we included the number of cells counted in each figure legend. The circles and filled dots in figure 3 and 4 represent the mean number of DALIS per cell in each single experiment performed and do not represent single cells; so one dot stands for data from one of the performed experiments for each of which at least 50-100 cells have been counted.

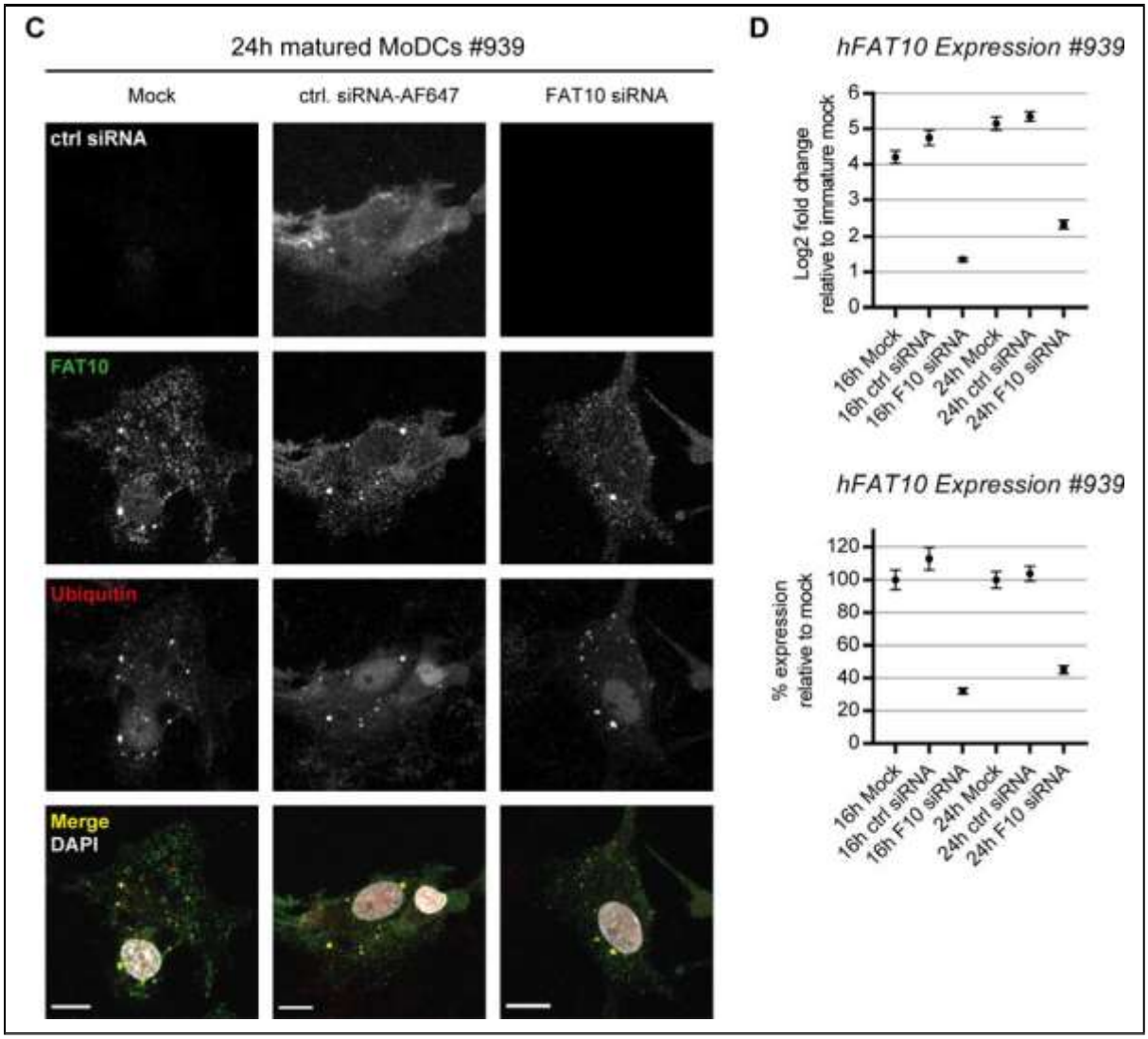
Figure 2 – It'd be more insightful if the expression of FLAG-mFAT10 protein detected by immunoblotting using a FLAG antibody without and with MG-132 (4 hours)

Reply: In order to obtain semi-quantitative data from western blots on the expression of FLAG-mFAT10 in DALIS and not the entire cells, it would be necessary to biochemically isolate DALIS. We have tried this extensively before submitting our study but unfortunately neither two different published methods (Lelouard et al. (2002) Nature 417: 177-182; Rahnefeld et al., (2011) Eur. J. Immunol. 41:2774-2781) nor protocols from our group gave consistent and reproducible results; therefore we could not include the data in our manuscript.

Figure 3 – Although not involved in DALIS formation, the role of HDAC6 on DALIS should further be investigated by inhibiting protein neosynthesis, autophagy and proteasome as performed for FAT10 in figure 5.

Reply: We consider it quite unlikely that these experiments would provide evidence for an HDAC6 dependency of DALIS formation as we already saw no influence of HDAC6 deficiency on undisturbed DALIS formation. Even if we would find a role of HDAC6 in DALIS formation when inhibiting protein neosynthesis, autophagy or the proteasome (which leads to HDAC6 dependent aggresome formation and might confound the results) the physiological relevance of such data would remain uncertain.





Ptp Figure 2. Knockdown of FAT10 using siRNA in human monocyte-derived dendritic cells (MoDCs). We isolated $CD14^+$ monocytes from peripheral blood from two donors and let them differentiate into MoDCs in presence 50 ng/mL human GM-CSF and 50 ng/mL human IL-4. On day 5 of culture, we harvested the cells and adjusted the cell number to 1.5 10^6 cells/mL in complete AIM-V medium. The cells were kept in the cell culture incubator until siRNA transfection. We adapted a transfection protocol from Troegeler, et al. *(2014) Immunol. Cell Biol. 92(8):699-708*. We used a combination of four different siRNAs (Microsynth, Balgach, Switzerland) for the knockdown of human FAT10 and the AllStars Negative Control siRNA labelled with AlexaFluor-647 (Qiagen, Hilden, Germany) for a control knockdown. Each siRNA was used at a final concentration of 400 nM. The different siRNA transfection mixtures were prepared in the sides of the wells of a tilted 6-well plate. First, 30 µL of the HiPerFect transfection reagent was mixed with 470 µL OptiMEM medium. Then, 15 µL of each siRNA was added to the transfection reagent mixture into the respective wells, gently swirled to mix, and incubated for 15-20 min at RT in the tilted 6-well plate. Finally, we added 1.5x10^6 MoDCs to each well (= 1 mL of the MoDC suspension) on top of the siRNA transfection mix and again swirled the 6-well plate to mix. After four hours of incubation at 37°C, the transfection was stopped by adding 2 mL of complete AIM-V medium supplemented with 50 ng/mL human GM-CSF and 50 ng/mL human IL-4. The cells were incubated for 24 h and used as immature MoDCs. For imaging, a glass plate, which was coated with 0.01% (100 µg/mL) poly-L-lysine solution for 30 min at RT, was placed into the wells prior to siRNA transfection. On day 6 of culture, transfected MoDCs were matured for 16 h and 24 h, or not (0h, immature), using a cytokine cocktail consisting of 50 ng/mL GM-CSF, 50 ng/mL IL-4, 20 ng/mL IL-6, 10 ng/mL IL-1β, 400 U/mL TNF, and 200 U/mL IFN-γ. At indicated time points, the cells were washed with PBS. The glass plates were transferred

into 12-well plates, the attached cells were fixed with 4% PFA, and processed for microscopy as described in material and methods of the revised manuscript. The remaining cells were directly lysed inside the wells and lysates analysed by qPCR as described in material and methods of the manuscript. (A, C) Representative images of transfected MoDCs of two donors (#938, #939) after 24 h of maturation showing DALIS that stained positive for FAT10 and ubiquitin regardless of siRNA transfection. The control siRNA was already fluorescently-labelled, and DAPI was used to stain cell nuclei; the scale bar is 10 µm. We excluded the images of the control siRNA shown in the upper panel from the merged images shown in the bottom panel to avoid confusion. (B, D) Expression levels of human *FAT10* mRNA were determined by qRT- PCR in siRNA-transfected MoDCs. Expression levels were normalised to *UBC* and depicted as log2-fold change relative to immature MoDCs (upper graphs). Additionally, we normalised the expression levels to the mock- transfected sample at the respective time point (16 h or 24 h) to show the percentage change of the knockdown at 16 h or 24 h (lower graphs in B and D). Results of two replicates for each donor are shown and depicted as mean ± SEM.

Second decision letter

MS ID#: JOCES/2019/240085

MS TITLE: FAT10 localizes in dendritic cell aggresome-like induced structures and contributes to their disassembly

AUTHORS: Richard Schregle, Stefanie Mueller, Daniel Legler, Jeremie Rossy, Wolfgang Krueger, and Marcus Groettrup
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 2

Advance summary and potential significance to field

This study proposes a potential regulatory role of FAT10 in the clearance of DALIS:

The quality of the study is improved as the following items were rigorously addressed by the authors:

- the conceptual difference between DALIS and aggresomes
- Fat10-mediated rescue of the impaired disassembly of DALIS in FAT10 knockout BMDC upon cycloheximide treatment
- The size alteration of DALIS in WT vs FAT10 KO BMDC upon cycloheximide treatment
- Clarification of cell numbers in the bar graphs

Comments for the author

Despite the technical obstacles (i.e., lack of antibodies that detect endogenous mouse FAT10 and non-reproducible DALIS isolation data) faced during the revision of this study, the authors put great effort and rigorously investigated all the requested points. It is understandable not to include the studies with HDAC6 KO BMDC given the scope of the study. I think this study is ready for publication.