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TLR9 Sorting by Rab11 Endosome in Enterocyte Maintains Intestinal Epithelial-Microbial Homeostasis

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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.)

Editor: Andrea Leibfried

1st Editorial Decision

6 February 2014

Thank you for submitting your manuscript entitled 'TLR9 Sorting by Rab11 Endosome in Enterocyte Maintains Intestinal Epithelial-Microbial Homeostasis'. I have now received reports from three referees, which are enclosed below.

As you will see, all referees find your study interesting. However, they raise a number of technical concerns that need to be addressed for further proceedings here. Given the comments provided, I would like to invite you to submit a revised version of the manuscript, addressing all concerns of the referees. Please do not hesitate to contact me in case of questions regarding the revision of your manuscript.

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

REFeree COMMENTS

Referee #1:

The manuscript of Yu et al demonstrates that the integrity of the recycling endosome is crucial to maintain the homeostasis of the gut. The authors performed genetic ablation of the small GTPase Rab11a in mouse intraepithelial cells and in *Drosophila* midgut. (Of note, Rab11a expression is essential for the maintenance of the recycling endosome.) In both organisms, this led to high cytokine secretion (IL6), and an inflammatory bowel like disease. According to the authors, the defect in recycling endosomes induces aberrant TLR9 proteolysis and activation, which in turn leads to the inflammatory phenotype observed.

The first half of the paper is very interesting, well performed and clearly written. The data are very clean and convincing. The similarity of the phenotypes induced in mice and *Drosophila* is compelling. The power of the cell type specific knock down in fly is also very well used and elegant. Unfortunately I have major concerns about the part dealing with TLR9 that is surprisingly less convincing and over interpreted. The data presented do not support the conclusions and the title of the paper. Either reformulation of the conclusions or additional experiments are required to strengthen their conclusions.

Full length TLR9 appears absent from the immunoblots as its size is around 140 kD (see papers of the G Barton team for instance). The immunoblots presented exhibit numerous bands but none of the expected size (140 and 80 kD for the cleaved form). The authors should check the specificity of the antibody they used and run samples from wild type animals and other cell types or cell lines known to contain full length and cleaved TLR9. As presented, the identity of the bands revealed by the TLR9 antibody is still uncertain.

With the experiments presented the author cannot conclude as they do that TLR9 missorting in RAB11a deficient animal is responsible for the phenotypes observed. First they should absolutely determine the intracellular localization of TLR9 in wt and Rab11 ko cells.

TLR9 knock down could be performed in RAB11a deficient cells to evaluate the role of this receptor in the activation. Even the nice experiments performed in Fig 6H to I are not conclusive on that matter. Since bacterial DNA and LPS when perfused are able to activate roughly to the same extent the production of IL6 and CXCL1 mRNA expression, the TLR9 specificity of the ligand used should be carefully checked in parallel (using TLR9 KO mice).

Referee #2:

The Rab11 GTPases regulate exocytosis of recycling endosomes and the gene for Rab11A has recently been described as risk locus for Crohns disease (IBD). Here, the authors provide compelling genetic and biochemical evidence that under "steady state" conditions endosomes binding Rab11A protein (usually termed recycling endosomes) sequester TLR9. Inactivation of Rab11A in murine or *Drosophila* IECs caused a IBD-like phenotype associated with activation of NFkB and MAPK - and in the absence of Rab11A - IECs could not tolerate apical TLR9 ligands such as the intestinal microbiota. The data imply that Rab11A deficiency disrupts the IEC tolerance to microbial TLR agonists.

These novel data also raise questions:

1. Recycling of TLR9 to the cell membrane is shown here to feature Caco-BBE cells - yet is not a classical feature of macrophages or Dendritic cells. Do the authors see membrane recycling in Macrophages or DCs? (the issue needs at least to be discussed)
2. Why contain Rab11A "suppressed" Caco-BBE cells 50 fold more IL6 mRNA (Fig 5L) while germ free IEC-organoids contain less compared to controls? - there is a need to discuss whether the more "mature" Caco cells take up apoptotic "DNA" in culture. Inhibitors of endosome acidification and thus TLR9 proteolysis could be informative.
3. Earlier work of Dalpke (JI) implied that the N terminal part of cleaved TLR9 is required for TLR9 signalling. More recent work of the group of Barton (Immunity, 2011) shows that TLR9 transmembrane mutations bypass the requirement for TLR9 proteolysis, and membranes of

granulocytes appear to express "unprocessed" TLR9. Finally, N.Mijaka (Nature Comm.2013) recently reported that the N-terminal part of TLR9 is essential for TLR9 activation. Given this complexity cell-membrane expressed TLR9 may be biologically active - cleaved or uncleaved.

This needs to be discussed since the authors work implies that- as stated in the summary - that "the recycling endosomal compartment maintains IEC tolerance to normal microbiota by preventing aberrant TLR9 proteolysis (and thus biological activation) in late endosomes.

Apart of these critical points the data presented reflect a fine piece of work - congratulation.

Referee #3:

Overall it is a very interesting and data-heavy manuscript that focuses on understanding the role of endosomes, especially Rab11-endosomes in regulating TLR9 subcellular localization and function. Most of Rab11 studies are done using tissue culture cells, thus it is nice to see the study that attempts to test the role of Rab11A in vivo. The confirmation of mice work using fly system is very impressive. However, the manuscript does have several technical issues, especially low quality imaging (see below). If one wants to demonstrate the changes in subcellular TLR9 localization, much better quality and higher magnification images should be used. Colocalization between TLR9 and Rab11 or Rab7 also need to be quantified. Overall, manuscript could also use a lot more quantifications, especially of western blots.

1. BrdU does not really measure proliferation, only cell number in S phase. Increase in BrdU staining can be caused by either increased proliferation or slow-down in cell cycle.
2. Increase in pSTAT3 is not very impressive and need to be quantified as a ratio to total STAT3.
3. Fig. 4D. pIKK westerns are very weak and hard to interpret. Should be replaced.
4. Fig. 5B. If authors want to conclude that TLR9 is present in Rab7-lysosomes/late endosomes, high magnification image (100X) needs to be shown and quantification needs to be provided.
5. Fig. 5E. This experiment is quite puzzling. Since these are IPs from lysates (presumably generated using Triton X-100), do authors want to conclude that TLR9 binds to Rab7? Do TLR9 proteolytically cleaved fragments also bind to Rab7? That seems unlikely. It would seem that TLR9 proteolytic cleavage would occur inside lysosomal lumen. How does it then bind to Rab7? If yes, that would be interesting finding, but much more data is needed to test that.
6. Fig. 5F-G. Images in F are very low quality and difficult to interpret. In G, authors seem to claim that TLR9 binds Rab11a. Does that mean that TLR9 interact with both, Rab7 and Rab11A. If that is the case, the experiment testing other Rab, may be Rab11B or Rab25, that does not bind TLR9 needs to be shown.
7. Fig. 5H-J. Needs to be quantified!

1st Revision - authors' response

05 May 2014

Referee #1:

The manuscript of Yu et al demonstrates that the integrity of the recycling endosome is crucial to maintain the homeostasis of the gut. The authors performed genetic ablation of the small GTPase Rab11a in mouse intraepithelial cells and in *Drosophila* midgut. (Of note, Rab11a expression is essential for the maintenance of the recycling endosome.) In both organisms, this led to high cytokine secretion (IL6), and an

inflammatory bowel like disease. According to the authors, the defect in recycling endosomes induces aberrant TLR9 proteolysis and activation, which in turn leads to the inflammatory phenotype observed.

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Response: We have performed additional experiments – sucrose density fractionation of intestinal vesicular compartments in new Fig. 5D. We showed that in the absence of Rab11a vesicles, the compartmentalization of TLR9 shifted towards endolysosome. In our view, these data strengthened the notion that Rab11a deficiency impacted homeostatic TLR9 distribution. However, we do agree with reviewer 1 that TLR9 may not, and unlikely, be the sole microbial receptor trafficked by Rab11a vesicles in enterocytes. Thus, we modified the title and our discussion/conclusion to suggest that mis-sorting of TLR9 represented one major but may not be the only defect of Rab11a-deficient enterocytes. We hope the reviewer would agree with our revised abstract and discussion.

Full length TLR9 appears absent from the immunoblots as its size is around 140 kD (see papers of the G Barton team for instance). The immunoblots presented exhibit numerous bands but none of the expected size (140 and 80 kD for the cleaved form). The authors should check the specificity of the antibody they used and run samples from wild type animals and other cell types or cell lines known to contain full length and cleaved TLR9. As presented, the identity of the bands revealed by the TLR9 antibody is still uncertain.

Response: We have followed reviewer 1's advice, and performed additional experiments (Fig. S4) to verify the specificity and capacity of 2 different TLR9 antibodies in detecting various forms of TLR9 receptor. The mouse anti-TLR9 antibody (Imgenex, IMG305A, reacts with human, mouse, dog, monkey, and rat TLR9), which was used in our analyses, has been verified in primary macrophages from TLR9-/- mice (Tabeta et al, 2006), in addition to other documentations in Western blot and staining analyses (Lee et al, 2004; Lee et al, 2006; Palladino et al, 2007; Tabeta et al, 2006).

We have further demonstrated in new experiments that the detected receptor fragments were diminished in newly-established stable TLR9-knockdown Caco2 cells (Fig. S4). However, this antibody did not recognize glycosylated full-length TLR9 (predicted to be 130-140 kDa) in human intestine, Ramos, and Caco2 cells (Fig. S4). The highest molecular weight band in our assays was about 95-98 kDa, which were in agreement with the reported TLR9 molecular weight in HEK293 cells overexpressing full-length human TLR9 (containing 1032 amino acid residues) by the same antibody (Chuang & Ulevitch, 2000; Lee et al, 2004). Furthermore, Park et al pointed out that deglycosylated TLR9 was around ~100 kDa, whereas the glycosylated one ran at 140-150 kDa (Park et al, 2008). It appears that TLR9 is one of heavily modified proteins, e.g., glycosylation and proteolysis, which could change its molecular weight in different cell types.

The rabbit anti-TLR9 antibody (Cell Signaling, #5945, reacts with human TLR9) was able to detect glycosylated full-length TLR9 (~140 kDa) in

human Ramos and THP1 cells (2 immune cell types), but failed to detect the same in Caco2 cells (Fig. S4B), suggesting that the glycosylated full length receptor might be unstable in intestinal epithelial cells or different modifications occurred in intestinal epithelial cells. We could not use this antibody in mouse experiments due to its humanspecificity.

With the experiments presented the author cannot conclude as they do that TLR9 missorting in RAB11a deficient animal is responsible for the phenotypes observed. First they should absolutely determine the intracellular localization of TLR9 in wt and Rab11 ko cells.

Response: As stated above, we now performed additional experiments to show TLR9 compartmentalization was impaired in Rab11a deficient intestines. We also revised our discussion/conclusion to suggest potential contribution from other microbial receptors to the phenotype.

TLR9 knock down could be performed in RAB11a deficient cells to evaluate the role of this receptor in the activation. Even the nice experiments performed in Fig 6H to I are not conclusive on that matter. Since bacterial DNA and LPS when perfused are able to activate roughly to the same extent the production of IL6 and CXCL1 mRNA expression, the TLR9 specificity of the ligand used should be carefully checked in parallel (using TLR9 KO mice).

Response: As stated above, we suggested in revised paper that TLR9 might be the major but unlikely the sole microbial receptors trafficked by Rab11a vesicles in enterocytes. Nevertheless, our new data strengthened the point that Rab11a did impact on TLR9 distribution, stability, and activation. Chasing down various TLRs or even downstream adaptors (e.g., Myd88) will be out of this paper's scope. Thus, we have revised our interpretation to leave the exploration open-ended. Furthermore, the TLR9 agonist we used was commercial, ultrapure, and endotoxin-free ssDNA/LyoVec™ from Invivogen. They are highly TLR9 specific ligands as it binds directly and sequence-specifically to single-stranded unmethylated CpG-DNA. These ssDNAs are complexed with the cationic lipid LyoVec™ to allow a better internalization of the immunostimulatory DNA to the acidic compartment where TLR9 is present.

Referee #2:

The Rab11 GTPases regulate exocytosis of recycling endosomes and the gene for Rab11A has recently been described as risk locus for Crohns disease (IBD). Here, the authors provide compelling genetic and biochemical evidence that under "steady state" conditions endosomes binding Rab11A protein (usually termed recycling endosomes) sequester TLR9. Inactivation of Rab11A in murine or drosophila IECs caused a IBD-like phenotype associated with activation of NFkB and MAPK - and in the absence of Rab11A - IECs could not tolerate apical TLR9 ligands such as the intestinal microbiota. The data imply that Rab11A deficiency disrupts the IEC tolerance to microbial TLR agonists.

These novel data also raise questions:

1. Recycling of TLR9 to the cell membrane is shown here to feature Caco-BBE cells - yet is not a classical feature of macrophages or Dendritic cells. Do the authors see membrane recycling in Macrophages or DC's ? (the issue needs at least to be discussed)

Response: Following reviewer's advice, we have discussed and cited literatures showing TLR9 cell surface localization. For instance, Onji et al used a newly developed TLR9 antibody showed surface localization of the receptor in splenic dendritic cells (Onji et al, 2012). Lee et

al showed TLR9 on the surface of human colon epithelial cells using vectorial biotinylation assay and flow cytometry assay (Lee et al, 2006).

2. Why contain Rab11A "suppressed" Caco-BBE cells 50 fold more IL6 mRNA (Fig 5L) while germ free IEC-organoids contain less compared to controls? - there is a need to discuss whether the more "mature" Caco cells take up apoptotic "DNA" in culture.

Response: There are multiple possibilities. First, ATCC stated that Caco2 cells express heat stable enterotoxin (Stx, E. coli), which might potentiate its susceptibility to microbial ligand stimulation. Second, Caco2 cells harbor mutations in beta-catenin and APC, which might genetically predispose them to robust transcriptional activation. In contrast, the mouse organoids contained no genetic alteration other than Rab11a. Third, as suggested by reviewer 2, cultured Rab11a-KD Caco2 cells might be prone to activation by apoptotic self-DNA, as their apical-basolateral polarity was impaired. We have incorporated above discussion into the main or supplementary portions of the paper.

Inhibitors of endosome acidification and thus TLR9 proteolysis could be informative.

Response: We have treated Caco2 cells with a recycling endosome inhibitor – Monensin, and showed that it caused the same TLR9 fragmentation change as the one caused by transient RAB11A knockdown.

3. Earlier work of Dalpke (JI) implied that the N terminal part of cleaved TLR9 is required for TLR9 signalling. More recent work of the group of Barton (Immunity, 2011) shows that TLR9 transmembrane mutations bypass the requirement for TLR9 proteolysis, and membranes of granulocytes appear to express "unprocessed" TLR9. Finally, N. Mijaka (Nature Comm. 2013) recently reported that the N-terminal part of TLR9 is essential for TLR9 activation. Given this complexity cell membrane expressed TLR9 may be biologically active - cleaved or uncleaved. This needs to be discussed since the authors work implies that - as stated in the summary - that "the recycling endosomal compartment maintains IEC tolerance to normal microbiota by preventing aberrant TLR9 proteolysis (and thus biological activation) in late endosomes. Apart of these critical points the data presented reflect a fine piece of work - congratulations.

Response: Thanks for these important points, some of which were missed in our previous version. We have now carefully introduced and discussed these previous works by including substantial amount of texts. Since our current data could not precisely map out the exact processing defects, we made it clear that future studies are necessary to determine the exact molecular impacts of Rab11a deficiency on TLR9 and potentially other microbial receptors. Thanks for generously sharing with us the great ideas.

Referee #3:

Overall it is a very interesting and data-heavy manuscript that focuses on understanding the role of endosomes, especially Rab11-endosomes in regulating TLR9 subcellular localization and function. Most of Rab11 studies are done using tissue culture cells, thus it is nice to see the study that attempts to test the role of Rab11A in vivo. The confirmation of mice work using fly system is very impressive. However, manuscript does have several technical issues, especially low

quality imaging (see below). If one wants to demonstrate the changes in subcellular TLR9 localization, much better quality and higher magnification images should be used. Colocalization between TLR9 and Rab11 or Rab7 also need to be quantified. Overall, manuscript could also use a lot more quantitations, especially of western blots.

1. BrdU does not really measure proliferation, only cell number in S phase. Increase in BrdU staining can be caused by either increased proliferation or slow-down in cell cycle.

Response: We have fixed these statements.

2. Increase in pSTAT3 is not very impressive and need to be quantified as a ratio to total STAT3.

Response: New Western blots have been performed and quantified.

3. Fig. 4D. pIKK westerns are very weak and hard to interpret. Should be replaced.

Response: we would like to have a better blot for pIKK, but we have tried this particular assay more than 3 times with various lysis/blocking/detecting conditions. The blot shown in original version was the most satisfactory one.

4. Fig. 5B. If authors want to conclude that TLR9 is present in Rab7-lysosomes/late endosomes, high magnification image (100X) needs to be shown and quantification needs to be provided.

Response: New images with high magnification have been provided with Pearson correlation analysis (Fig. 5B).

5. Fig. 5E. This experiment is quite puzzling. Since these are IPs from lysates (presumably generated using Triton X-100), do authors want to conclude that TLR9 binds to Rab7? Do TLR9 proteolytically cleaved fragments also bind to Rab7? That seems unlikely. It would seem that TLR9 proteolytic cleavage would occur inside lysosomal lumen. How does it then bind to Rab7? If yes, that would be interesting finding, but much more data is needed to test that.

Response: Sorry for not having provided a clearer interpretation. First, we used 0.1% NP40 in lysis buffer (Supplementary Methods). Second, the homogenization we performed on tissues preserved vesicular integrity. We monitor the lysates via microscope. Therefore, for the co-IP results, we meant to suggest that TLR9 (and processed fragments) was contained by Rab7+ vesicles rather than directly binding to Rab7 small GTPase. We have made this very clear in revised text.

6. Fig. 5F-G. Images in F are very low quality and difficult to interpret. In G, authors seem to claim that TLR9 binds Rab11a. Does that mean that TLR9 interact with both, Rab7 and Rab11A. If that is the case, the experiment testing other Rab, may be Rab11B or Rab25, that does not bind TLR9 needs to be shown.

Response: We agree with Reviewer 3 and removed these low quality IF images. As stated above, we did not mean to suggest that TLR9 binds to Rab7 or Rab11. Instead, TLR9 traffics through these vesicular compartments.

7. Fig. 5H-J. Needs to be quantified!

Response: We fixed these issues.

Chuang TH, Ulevitch RJ (2000) Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. *European cytokin network* 11: 372-378

Lee HK, Dunzendorfer S, Tobias PS (2004) Cytoplasmic domain-mediated dimerizations of toll-like receptor 4 observed by beta-lactamase enzyme fragment complementation. *The Journal of biological chemistry* 279: 10564-10574

Lee J, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, Lee HK, Shen C, Cojocaru G, Shenouda S, Kagnoff M, Eckmann L, Ben-Neriah Y, Raz E (2006) Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. *Nature cell biology* 8: 1327-1336

Onji M, Kanno A, Saitoh S, Fukui R, Motoi Y, Shibata T, Matsumoto F, Lamichhane A, Sato S, Kiyono H, Yamamoto K, Miyake K (2012) An essential role for the N-terminal fragment of Toll-like receptor 9 in DNA sensing. *Nature communications* 4: 1949

Palladino MA, Johnson TA, Gupta R, Chapman JL, Ojha P (2007) Members of the Toll-like receptor family of innate immunity pattern-recognition receptors are abundant in the male rat reproductive tract. *Biology of reproduction* 76: 958-964

Park B, Brinkmann MM, Spooner E, Lee CC, Kim YM, Ploegh HL (2008) Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. *Nature immunology* 9: 1407-1414

Peter ME, Kubarenko AV, Weber AN, Dalpke AH (2009) Identification of an N-terminal recognition site in TLR9 that contributes to CpG-DNA-mediated receptor activation. *Journal of immunology* 182: 7690-7697

Qi J, Buzas K, Fan H, Cohen JI, Wang K, Mont E, Klinman D, Oppenheim JJ, Howard OM (2011) Painful pathways induced by TLR stimulation of dorsal root ganglion neurons. *Journal of immunology* 186: 6417-6426

Tabeta K, Hoebe K, Janssen EM, Du X, Georgel P, Crozat K, Mudd S, Mann N, Sovath S, Goode J, Shamel L, Herskovits AA, Portnoy DA, Cooke M, Tarantino LM, Wiltshire T, Steinberg BE, Grinstein S, Beutler B (2006) The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. *Nature immunology* 7: 156-164

2nd Editorial Decision

20 May 2014

Thank you for your swift reply to my recent correspondence and the draft point-by-point response to the criticisms raised by the referees who have seen the revised version of your manuscript. Based on this, I would like to invite you to submit a revised version of the manuscript, addressing all remaining concerns of the referees as you've indicated. Please be reminded that we generally allow 90 days for revisions, so there is no need to rush through the additional experiments.

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

Referee #1:

The revised version of the paper by Yu et al. has been improved since the first submission. Experiments have been added that complement the study very well and clear up certain points that were critical during the first submission.

Nonetheless, a few points still need to be addressed before accepting the paper:

1. In Fig 5C, the authors interpret the absence/reduction of the cleaved bands of TLR9 as a reduction in proteolytic processing of TLR9. However, one cannot talk about the reduced cleavage if the levels of total TLR9 (full length and cleaved) of the protein are not comparable. The authors must document that the total levels of TLR9 are not modified in the absence of Rab11a by RT-PCR since the antibody used by western blot fails to detect full length TLR9. They might already have this information in their microarray data presented in figure 2.
2. In Fig 5C, the authors need to clearly mention whether the low molecular weight bands revealed by the anti-TLR9 antibody are non-specific. In their test of the antibody on Caco cells with or without TLR9 (fig S4), these low molecular weight bands are not revealed and thus we do not know if these are specific to TLR9.
3. In Fig 5F, it seems that the molecular weight markers have been misplaced, i.e. there are too low. In addition, it would have been nice to analyse on the same blot cells from mice at SPF condition of the same age. This is a minor point but if the authors have the data they should present them.
4. In Fig 6J, the authors observe an increase in LPS responsiveness in the absence of Rab11a in the intestinal perfusion assay *in vivo*. They interpret these results as a lack of immunosuppressive control by apical TLR9 signalling. Although, this might be true, other hypotheses should not be excluded. For instance, TLR4 signalling in itself might be affected in the absence of Rab11a. We know that upon internalisation TLR4 recruits other adaptors and induces a different signalling pathways. Rab11a might be involved in this process of internalisation. Since the effect of Rab11a on TLR4 trafficking and biology has not been documented, this hypothesis cannot be excluded and needs to be added in the discussion.

Overall, the study is of wide interest and has been very elegantly carried out.

Referee #3:

In this revised manuscript authors attempted to address some of my concerns. Unfortunately, many of my original concerns have not really been addressed (see below). Many of these concerns are crucial to the final conclusions of the manuscript, thus should be resolved before it is published. Since overall, I do like the manuscript, authors should be encouraged to make a more serious attempt to address its problems.

1. Addressed.
2. Authors state that they now provide quantifications of pSTAT3 gels. I cannot find them. The only thing I did find (in figure legend) is a statement that pSTAT3 increased three fold. That is not quantification. Where are the actual numbers and statistical analysis with normalization to total levels of STAT3? The data should also be derived from at least three independent experiments.
3. If authors cannot produce better pIKK gels, the conclusions regarding pIKK levels need to be removed from the manuscript. As it is shown now, the gels are not publishable quality.
4. Authors state that they now include high magnification images of TLR9 and Rab7 colocalization. However, the shown images are hardly high-resolution. As the result, it is very difficult to determine whether TLR9 actually colocalizes with lysosomes.
5. I am still quite puzzled with this experiment. Authors state that during this preparation vesicles remain intact. I do not see how is that possible, since prep is done in 0.1% NP40, which is a detergent! If vesicles are not actually intact, the entire interpretation of this experiment is flawed.
6. Addressed.
7. Addressed.

Referee #1

(Report for Author)

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Nonetheless, a few points still need to be addressed before accepting the paper:

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Response: We have included both microarray and real-time RT-PCR data showing no change of TLR9 at mRNA levels. Fig. S4.

2. In Fig 5C, the authors need to clearly mention whether the low molecular weight bands revealed by the anti-TLR9 antibody are non-specific. In their test of the antibody on Caco cells with or without TLR9 (fig S4), these low molecular weight bands are not revealed and thus we do not know if these are specific to TLR9.

Response: We cannot conclude the identities of these low molecular weight bands (below 40 kDa). We have clearly made this statement in Fig.5 legend (page 27, bottom 2 lines). These bands appeared in mouse intestinal tissues (Fig. 5), and one of them (~30 kDa) appeared in human intestinal tissue lysates from an independent commercial source (Fig. S4). These bands were absent from human Ramos cells, suggesting that they represent certain intestinal proteins. We still wanted to show the full-length gel to show their presence.

3. In Fig 5F, it seems that the molecular weight markers have been misplaced, i.e. there are too low. In addition, it would have been nice to analyse on the same blot cells from mice at SPF condition of the same age. This is a minor point but if the authors have the data they should present them.

Response: Thanks for identifying this error. All the molecular weight labels were shifted upwards while we composited the panel; now fixed. The germ free mice used were 4-wk old (please see label at the bottom of original Fig. 5F). SPF mice of same age were shown in original Fig. 5C. We will make this clear that we compared SPF and germ-free mice of same ages.

4. In Fig 6J, the authors observe an increase in LPS responsiveness in the absence of Rab11a in the intestinal perfusion assay in vivo. They interpret these results as a lack of immunosuppressive control by apical TLR9 signalling. Although, this might be true, other hypotheses should not be excluded. For instance, TLR4 signalling in itself might be affected in the absence of Rab11a. We know that upon internalisation TLR4 recruits other adaptors and induces a different signalling pathways. Rab11a might be involved in this process of internalisation. Since the effect of Rab11a on TLR4 trafficking and biology has not been documented, this hypothesis cannot be excluded and needs to be added in the discussion.

Response: Thanks for the helpful suggestion. We have included these discussions addressing alternative possibilities (page 12, from bottom 2 lines).

Overall, the study is of wide interest and has been very elegantly carried out.

Referee #3

In this revised manuscript authors attempted to address some of my concerns. Unfortunately, many of my original concerns have not really been addressed (see below). Many of these concerns are crucial to the final conclusions of the manuscript, thus should be resolved before it is published. Since overall, I do like the manuscript, authors should be encouraged to make a more serious attempt to address its problems.

Response: We would like to apologize for not having elaborated our points clearly in addressing some of the concerns. For point #4, we realized that our newly collected confocal images still did not reach reviewer's standard. We have now performed additional confocal imaging with both Rab7 and Lamp2 antibodies (please see below). We do want to emphasize that in our previous revision, we had seriously taken each of Reviewer 3's comment into consideration.

1. Addressed.

2. Authors state that they now provide quantifications of pSTAT3 gels. I cannot find them. The only thing I did find (in figure legend) is a statement that pSTAT3 increased three fold. That is not quantification. Where are the actual numbers and statistical analysis with normalization to total levels of STAT3? The data should also be derived from at least three independent experiments.

Response: We have now included a graphed quantification in Fig. 2F, with detailed legend (page 26, first 2 lines). The data were quantified from 3 independent animals of each genotype (please see legend).

3. If authors cannot produce better pIKK gels, the conclusions regarding pIKK levels need to be removed from the manuscript. As it is shown now, the gels are not publishable quality.

Response: We then have to remove it. The activation of NFκB pathway in Rab11a-deficient intestines was also supported by increased nuclear p65 levels (Fig. 4D), elevated downstream cytokine levels (Fig. 2), and Bay11-0782 (NFκB inhibitor) rescue experiment (Fig. 4H-I).

4. Authors state that they now include high magnification images of TLR9 and Rab7 colocalization. However, the shown images are hardly high-resolution. As the result, it is very difficult to determine whether TLR9 actually colocalizes with lysosomes.

Response: We recognize that the previously collected images were still not convincing due to the weak Rab7 signal in intestinal tissues. We have now co-stained TLR9 and Lamp2 (a marker of lysosome). We acquired and quantified new confocal images (Fig. 5B). In addition, we performed TLR9 and Rab7 co-staining in control and RAB11A-KD Caco2 cells treated with CpG (Fig. S5B). Of note, these images were acquired by Zeiss LSM 510 at a resolution of 2048x2048. By showing individual green and red channels (Fig. 5B, Fig. S5B), we hope these images provided good examples of large TLR9 aggregations in Lamp2+ or/and Rab7+ compartments in Rab11a-deficient IECs and RAB11A-depleted Caco2 cells.

5. I am still quite puzzled with this experiment. Authors state that during this preparation vesicles remain intact. I do not see how is that possible, since prep is done in 0.1% NP40, which is a detergent! If vesicles are not actually intact, the entire interpretation of this experiment is flawed.

Response: In contrast to strong detergent Triton-X 100, 0.1% NP40 was used to only weakly permeabilize cell membranes for cell lysis. As stated in previous response, we monitor vesicular integrity after tissue/cell lysis using microscopes. We have provided a DIC image of preserved vesicles in Caco2 lysate supernatants following a lysis by 0.1% NP40 buffer (Fig. S7E). In addition, TLR9 contains a transmembrane domain that threads the receptor through vesicular membranes. The receptor ectodomain cleavage will not alter its membrane association (please see diagram depicting TLR9-vesicle association in Fig. 7A). Rab11 and Rab7 small GTPases contain a membrane-insertion domain attaching them to the vesicles (please see diagram depicting Rab-vesicle association in Fig. 7A). The co-IP experiments were used to test whether TLR9 attached to (or trafficked through) Rab11+ and/or Rab7+ vesicles. Even after the vesicles were broke, if the vesicular membrane that holds both TLR9 and Rab7 were present, these membrane-associated proteins (TLR9 and Rab7) would be co-precipitated with the membranes.

- 6. Addressed.
- 7. Addressed.

3rd Editorial Decision

12 June 2014

Please find below again the comments of the two referees who have reported on the revised version of your manuscript. As discussed on the phone, I would like to invite you to address the last remaining concern of referee #3 in a response letter and to amend the manuscript text and DIC image description accordingly.

I am looking forward to receiving the final version of your manuscript!

Referee #1:

The authors have addressed most of my concerns. The paper has been very much improved since the first submission. This nice study is now ready for publication

Referee #3:

The authors addressed all my concerns except #5. Based on provided data, I do not believe that investigators actually immunisolating intact vesicles. For starters, if authors expect that detergent will solubilize plasma membrane, it will also solubilize vesicles (not just brake vesicles open). That means that all membrane embedded proteins will become soluble, making the interpretation of the experiment impossible. The DIC images (in Fig. S7E) makes no sense. The shown round things (I have no idea that they are) cannot be vesicles because they are way to large. Based on the scale bar, some of them are as large as 10 microns. Considering that that is the cell is about 10-20 microns in size, they would not fit more then one or two of those putative "vesicles". Actually, Rab11-containing endosomes should be more like 50-200 nm in size, which would not be visible by DIC.

3rd Revision - authors' response

12 June 2014

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Response: First, we used non-ionic detergent (NP-40) at 0.1%, a concentration well below the point

that all plasma and vesicular membrane proteins will be solubilized. Significant resistance of cellular membranes to various detergents has been documented in literatures (Schuck et al, 2003; Yu et al, 2008). Our intention of using mild detergent at low concentrations was to rupture the cells rather than to largely solubilize membrane proteins. Simons K's group showed that integral membrane proteins, e.g., VIP17, PLAP, caveolin-1, stomatin, etc. resisted 1% Triton extraction (Schuck et al, 2003). Transferrin receptor, a transmembrane protein recycled by Rab11a endosome resisted extraction by 0.5% Lubrol WX (Schuck et al, 2003). In addition, Knepper MA's group showed that detergent-resistant membranes (DRMs) following 3 hr extraction by 1.0% Triton X-100 retained Rab11a in addition to a large array of other membrane proteins in the DRM fractions (Yu et al, 2008). The microscopic analysis of lysate supernatants after extraction was to ensure a preservation of vacuolar/vesicular structures, which, in agreement with previous reports, suggested that the lipid bilayers of the membrane compartments remained intact under our lysis conditions, and that membrane proteins were unlikely to have been completely solubilized.

Second, the DIC image demonstrating a variety of vacuole/vesicle with heterogeneity in size served to make the point that the cellular membrane compartments after lysis remained intact. Therefore, the membrane proteins were unlikely to have been completely solubilized (please see above). We agree with the reviewer that, based on the sizes, some of the large vacuole-like structures may not be Rab11 endosomes; we did not make that statement in previous response. Yet, we do want to emphasize that Caco2 cells frequently contain large intracellular inclusion bodies surrounded by membranes. The supernatants we took and loaded on the slides could have been selectively enriched for those vacuolar compartments. Nevertheless, the micrograph was to display membrane integrity in cell lysates.

We are sincerely thankful for these critical comments by reviewer 3; above discussions have been incorporated into methods and figure legend sections of revised paper.

Schuck S, Honsho M, Ekroos K, Shevchenko A, Simons K (2003) Resistance of cell membranes to different detergents. Proc Natl Acad Sci U S A 100(10): 5795-5800

Yu MJ, Pisitkun T, Wang G, Aranda JF, Gonzales PA, Tchapyjnikov D, Shen RF, Alonso MA, Knepper MA (2008) Large-scale quantitative LC-MS/MS analysis of detergent-resistant membrane proteins from rat renal collecting duct. American journal of physiology Cell physiology 295(3): C661-678

4th Editorial Decision

13 June 2014

I appreciate the introduced changes and I am pleased to accept the manuscript for publication here. Thank you for contributing to the EMBO Journal!