NBR1 is a new PB1 signaling adapter in Th2 differentiation and allergic airway inflammation in vivo

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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 25 May 2010

Thank you for submitting your manuscript to the EMBO Journal. Your manuscript has now been seen by three referees and their comments to the authors are provided below. As you can see, the referees find the analysis interesting and insightful, but also find that some further analysis is needed for the manuscript to have the full impact of an EMBO Journal paper. In particular both referees #1 and 2 find that some further data in support of the mechanism whereby NBR1 affects Th2 differentiation and responses is needed for further consideration here. They suggest a number of different ways to address this concern. All three referees also raise some specific experimental concerns that should be addressed. Should you be able to address the raised concerns then we would consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single major revision only and it is therefore important to address the points raised if you wish the manuscript ultimately to be accepted.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Editor
The EMBO Journal
REFEREE REVIEWS

Referee #1 (Remarks to the Author):

In this manuscript entitled "NBR1 is a new PB1 signaling adapter in Th2 differentiation and allergic airway inflammation in vivo", Yang et al. reported that NBR1 is an important adapter for Th2-inducing signaling both in vitro and in vivo by using conditional Nbr1 knockout mice in which the floxed Nbr1 alleles in activated CD4 T cells are deleted by OX40-cre. They further showed that NBR1 deletion affects GATA3 and NFATc1 activation. The results are interesting, however, the mechanism through which NBR1 regulates Th2 responses requires further investigation. In addition, there are several concerns on the experiment designs.

Specific comments:

1. The mechanism of Th2 defect in NBR1 deficient cells is not clear in the current form of the paper. What is the linkage between NBR1 and GATA3? It was shown that the expression and/or translocation of GATA3 were impaired in NBR1 deficient cells. However, Stat6 activation was also diminished. Authors argued that this could result from lower amount of IL-4 made by NBR1 deficient cells. But whether Stat6 activation and GATA3 expression can be rescued by adding IL-4 exogenously was not tested. This is important for distinguishing at least two possibilities: NBR1 deficient cells have a global defect in Th2 differentiation versus NBR1 only affects IL-4 production including initial production by naïve cells. It is interesting that NBR1 regulates NFATc1 activation. However, whether this is the main cause of Th2 defect is not proven. Can an active form of NFAT restore all the defects?

2. The levels of Th2 cytokines were measured by ELISA. Since ELISA results depend on total cell numbers, the degree of cell expansion should be compared between WT and KO cells especially at day 3. Furthermore, assays with intracellular staining of Th2 cytokines should be performed to provide solid evidence at single cell level. These experiments will substantiate the mechanism.

3. Authors mentioned that naïve CD4 T cells were used. However, in the methods, the "naïve" cells were actually isolated by Miltenyi CD4 purification kit, thus they were total CD4 cells, which include CD25+ regulatory T cells and CD44hi memory CD4 T cells. The staining profile of cell surface markers of these cells (such as CD44, CD62L and CD25) from WT and KO mice was not shown anywhere in the paper. Since Tregs and memory cells are included in the assay, their contribution to the finding should be considered. Sorting for real naïve CD4+CD25-CD62LhiCD44lo cells should address the concern.

4. The appropriate reference for OX40cre mice should be J. Immunol. 182:4581-4589 in which the details of mice generation are described. In that report, OX40cre-mediated deletion efficiency is ~70% in CD4+Foxp3-CD44hi cells at steady state. Since OX40 is only expressed on a proportion of activated CD4 T cells especially at early stages of T cell activation (less than 50% at 8 hr after T cell activation), authors should measure the deletion efficiency of NBR1 by OX40cre in their study, particularly at 14 hr after TCR activation as shown in fig.5. OX40cre mice are OX40+/-, therefore, caution should be taken when interpreting the data.

5. Which cytokine is responsible for STAT4 nuclear translocation in 14hr activated cells (Fig. 5c) if IL-12 is not present?

Referee #2 (Remarks to the Author):

The authors have previously characterized the role of several PB1-containing proteins during Th helper cell differentiation. In this manuscript evidence is presented that NBR1, another PB-1 signaling adapter, also regulates Th2 differentiation. Using a conditional KO model in which deletion of NBR1 is coupled to OX40 expression, the data in the paper shows that CD4+ T cells from those produce reduced levels of Th2 cytokines which correlates with a diminished response in a model of OVA-induced allergic airway inflammation. At the molecular level NBR1-deficient cells show defects in Gata3 and NFATc1 activation which correlate with altered activation-induced T cell
polarity. A model that involved interaction/co-recruitment of several PB1 proteins is proposed to integrate the function of this family of protein in T helper differentiation. These results are very novel and significant as they may contribute to gain a better understanding on how these adaptor proteins regulate T cell activation and differentiation.

While the data clearly shows that, as previously reported by the authors for p62, the absence of NBR1 affects Th2 differentiation, the mechanisms that underlies this defect is not clear. How do the general defects in synapse formation/cell polarity shown here correlate with specific signaling defects that affect only Th2 but not Th1 cells? Does NBR-1 regulate the recruitment of specific mediators of TCR or cytokine signaling to the immunological synapse? Or is the altered T helper differentiation a consequence of the NBR-1-mediated regulation of the general strength of the signals activated downstream of the TCR?

Two of the consequences of the lack of NBR1 expression that can explain the defect in Th2 differentiation are the defects in Gata3 and NFATc1 activation. Regarding Gata3, the authors claim that "GATA3 was localized to the nucleus in activated wild-type CD4+ T cells, but that it was nearly totally excluded from the nucleus in activated NBR1-deficient cells". Expression of Gata3, which is up-regulated in Th2 cells in response to TCR and IL4R signals, seems to be normal then. The level of regulation the data shows is exerted on Gata3 nuclear translocation. How is this defect established in NBR1-deficient cells? Nuclear translocation of Gata3 has been shown to be dependent on p38-mediated phosphorylation, are Gata3 phosphorylation status or p38 activity altered in these cells?

The defect in NFATc1 activation seems to also reside on the control of its nuclear translocation, as levels of NFATc1 expression seem to be normally up-regulated in activated NBR1-deficient T cells. Differences in the activation of NFATc2 and NFATc1 in these cells could explain some of the aspects of their phenotype. It would be interesting to show if these cells show differences in the levels of phosphorylation of these two proteins that could explain their distinct behavior in the absence of NBR1 (maybe through activation of specific NFATc1-kinases).

Ox40 has also been shown to be expressed in resting and activated Tregs. Have the authors checked if NBR1-deletion also occurs in Tregs? Is Treg function altered? Can this have any effect on the airway inflammation model?

Referee #3 (Remarks to the Author):

Yang et al have generated conditional knock out mice to delete the PB1 containing protein NBR1 in activated T cells (NBR1fl/fl). For this Cre was expressed in the Tnfrsf4 locus that is almost exclusively expressed in activated CD4+ T cells (NBR1fl/fl CreOX40). T cells from these mice produce diminished Th2 cytokines, whereas production of Th1 cytokines appears normal. Consistent with a defective Th2 response these mice are less sensitive to OVA induced airway inflammation. Further, expression of Th2 transcription factors Gata3, NFATc1 and STAT6 is impaired in activated CD4+ T cells from NBR1fl/fl CreOX40 mice. Mechanistically, recruitment of the PB1 protein p62 and the polarity markers Talin and Scrib into the immunological synapse (IS) is diminished in NBR1 deficient T cells.

The manuscript convincingly shows that NBR1 is required for Th2 responses upon stimulation and in a model of allergic airway inflammation. This in itself is a very interesting finding. How NBR1 is mediating this effect remains rather obscure. The data suggest a mutual recruitment of NBR1 and p62 to the IS and a partial distortion of the IS after deletion of NBR1. Whether these effects are indeed responsible for the reduced activation of Th2 transcription factors and induction of cytokines is not so clear. As NBR1 and p62 are involved in autophagy, it may well be that the effects observed in NBR1 deficient T cells are caused by deregulated autophagy and not a primary signaling defect. This needs to be discussed much more open.

Specific points:

Figure 1 and Tables I-III could be largely moved to the supplement without significant loss of information.
Figure 2: Looking at panel A it seems that all PB1 proteins analyzed are exclusively expressed in activated T cells. Given the different time points of all the experiments, the authors should perform a kinetic analysis of NBR1 induction. Is NBR1 equally expressed in Th1 and Th2 cells? Also IL-2 induction in Th1 cells should be determined in the absence of NBR1 to get a better idea about the effect in Th1 cells. As NBR1 was described as an autophagy receptor, it should at least be determined if CD3/CD28 surface expression is comparable in NBR1fl/fl and NBR1fl/fl CreOX40 Th2 cells.

Figure 5: Induction of Gata3 expression (and not only nuclear translocation) seems to be completely gone in NBR1fl/fl CreOX40 T cells. Blotting of whole cell lysates should be performed. Comparing the extremely strong effects in panel A with the somewhat weaker effects in the quantification (panel B), it is sort of questionable if these cells are really representatives. It would be helpful to show more then one cell. This also holds true for Figures 6 and 7.

Figure 6 and 7: Some effects are very mild and in general the relevance of these recruitment defects for the observed Th2 phenotype is unclear. Is the impaired recruitment Th2 specific? If not, why are Th1 cytokines not affected. Further, interaction of NBR1 and p62 has been shown previously and it seems unlikely that this is dependent on IS recruitment. Especially P/I stimulation in Jurkat T cells bypasses upstream signaling (7C). These results need to be repeated with CD3/CD28 stimulation. The lack of PKCl/I and NBR1 co-IP is a negative result and it needs to be shown that in the same experimental setting (e.g. PKCl IP) p62, but not NBR1 is precipitated. Figure legend 7C is missing.

Discussion:

Page 13: It is stated:’... unlike p62, NBR1’s translocation is independent of PKCl/i.’ This is confusing, because from the figure 6Cit seems clear that p62 translocation is also independent of PKCl/i.

The whole discussion is confusing and biased and needs to be revised. As the authors state themselves’...a common link between cell polarity defects, ..., and T-cell signaling is not readily apparent.’ Never the less they spend about 1.5 pages in discussing links between cell polarity and signaling. Possible effects of autophagy are not mentioned at all.

Minor points:

The language of the text should be revised, because the description is very biased. For instance: Recurring expressions like ‘important’, ‘this is important, because’ or ‘interestingly’ are found throughout the text. In the first page of the discussion the authors state: ‘Cell signaling through PB1-containing adaptors and kinases is attracting great interest because it constitutes a novel paradigm for the control of cell signaling specificity and diversity in several systems, including T cells (Moscat & Diaz-Meco, 2009; Moscat et al, 2006; Moscat et al, 2007; Moscat et al, 2009).’ If PB1 adaptors are attracting great interest, it seems awkward that all cited references are from the authors themselves. I also wonder what this novel paradigm would be.

1st Revision - authors’ response 15 July 2010

Referee #1:

We are happy to see that this reviewer finds our data interesting and appreciate his/her comments that have certainly helped to improve our manuscript.

Specific comments:

1. ‘The mechanism...But whether Stat6 activation and GATA3 expression can be rescued by adding IL-4 exogenously was not tested. This is important for distinguishing at least two possibilities: NBR1 deficient cells have a global defect in Th2 differentiation versus NBR1 only affects IL-4 production including initial production by naive cells.’
Please note that, following this reviewer’s comment we have performed the requested experiment (new Fig. 5A). We show now that the exogenous addition of IL-4 in TCR activated naïve T cells cannot rescue the defect in the activation of Gata3 in KO cells, despite a potent activation of Stat6 nuclear translocation, suggesting that the ability of IL-4 to synergize with TCR signals for the activation of Gata3 is impaired in NBR1-deficient T cells. Please note that previously published data demonstrate that NFATc1 KO T cells were unable to activate Gata3 and differentiate towards Th2 even in the presence of IL-4 (Yoshida et al., Immunity 8, 115, 1998; and Ranger AM et al., Immunity 9, 627, 1998). These results reinforce the notion that NBR1 is a bona fide novel regulator of NFATc1. We believe that our original interpretation that Stat6 activation is defective in NBR1-deficient T cells due to the hypoproduction of IL-4 is correct but the new IL-4 reconstitution experiment shows that NBR1, like NFATc1, is required for Gata3 activation even in the presence of exogenous IL-4. This will be consistent also with our own data (Fig. 1K and L), demonstrating that NBR1 T cells differentiate poorly under Th2-polarizing conditions, which rely heavily on the presence of exogenous IL-4.

“It is interesting that NBR1 regulates NFATc1 activation. However, whether this is the main cause of Th2 defect is not proven. Can an active form of NFAT restore all the defects?”

Following this reviewer suggestion, we have expressed a retroviral GFP-tagged version of an NFATc1 active mutant previously characterized by Anjana Raois laboratory (Monticelli S et al., Eur. J. Immunol. 32, 2971, 2002) into WT and KO T cells. Results of Fig. 5B and C demonstrate that the expression of this mutant rescues the defective activation of Gata3 in the NBR1 KO T cells.

2. “The levels of ... ... the degree of cell expansion should be compared between WT and KO cells especially at day 3.”

Please note that new Fig. S2A demonstrate that NBR1-deficient T cells do not have impaired proliferation, which rules out this as a potential cause of reduced cytokine production in the KO cells.

“Furthermore, assays with intracellular staining of Th2 cytokines should be performed to provide solid evidence at single cell level. These experiments will substantiate the mechanism.”

Following this reviewer suggestion, we have performed the requested experiment. Please see new Fig. 1G-I.

3. “Authors mentioned that naive CD4 T cells were used. However, in the methods, the "naive" cells were actually isolated by Miltenyi CD4 purification kit, thus they were total CD4 cells, which include CD25+ regulatory T cells and CD44hi memory CD4 T cells. The staining profile of cell surface markers of these cells (such as CD44, CD62L and CD25) from WT and KO mice was not shown anywhere in the paper.

Please note that we have corrected the paper to make clear that we are working with total CD4+ T cells. However, also note that the experiments of new Fig. S2B-D show that the loss of NBR1 does not affect the staining profile of cell surface markers, neither under resting nor under activation conditions.

“Since Tregs and memory cells are included in the assay, their contribution to the finding should be considered. Sorting for real naive CD4+CD25-CD62LhiCD44lo cells should address the concern.”

As there are not changes in the populations of these cells (please see new Fig. S3A-C), we believe that sorting is not necessary. Also please note the data in new Fig. S3D demonstrating that the activation of Tregs is not affected by the lack of NBR1.

4. “The appropriate reference for OX40cre mice should be J. Immunol. 182:4581-4589 in which the details of mice generation are described.”

The manuscript has been modified accordingly to include this reference. We apologize for the mistake.
“In that report, OX40cre-mediated deletion efficiency is ~70% in CD4+Foxp3-CD44hi cells at steady state. Since OX40 is only expressed on a proportion of activated CD4 T cells especially at early stages of T cell activation (less than 50% at 8 hr after T cell activation), authors should measure the deletion efficiency of NBR1 by OX40cre in their study, particularly at 14hr after TCR activation as shown in fig.5.”

Please note that Fig. 4D shows efficient deletion of NBR1 in knock-out cells at 14 h after TCR stimulation.

OX40cre mice are OX40+/-, therefore, caution should be taken when interpreting the data.

Please note that in the reference describing the generation of OX40cre mice is stated that “Loss of one copy of Ox40 gene does not detectably impair immune responses or the representation of lymphocyte subpopulations, so heterozygous OX40cre mice were expected to behave immunologically as if they were wild type mice” (Klinger et al., J. Immunol. 182, 4581, 2009). Furthermore, in our previous publication using the same cre line, we showed that control OX40cre mice were completely normal from the point of view of T cell activation and differentiation and no Cre-mediated effects were detected when PKC wt/wtCreOX40 and PKC flo/fl mice were compared (Yang et al., PNAS 106, 1099, 2009).

5. “Which cytokine is responsible for STAT4 nuclear translocation in 14hr activated cells (Fig. 5c) if IL-12 is not present?”

We do not know the answer to this question. Presumably, as the incubation times are rather long, cytokines might be produced capable of activating Stat4. As we see no effect on this parameter in the KO cells and this result might be confounding, we have removed that blot in the revised manuscript (now Fig. 4C).

Referee #2:

We are very happy to see that this reviewer finds that “These results are very novel and significant as they may contribute to gain a better understanding on how these adaptor proteins regulate T cell activation and differentiation.”

This reviewer raises a number of interesting comments and suggestions that we have used to improve our manuscript in this revised version.

“While the data clearly shows that, as previously reported by the authors for p62, the absence of NBR1 affects Th2 differentiation, the mechanisms that underlies this defect is not clear. How do the general defects in synapse formation/cell polarity shown here correlate with specific signaling defects that affect only Th2 but not Th1 cells?”

This is a very interesting question, which we have tried to address in our discussion. The role of cell polarity in T cell activation is not clear despite the elegant work of several investigators. This is even less known in T cell differentiation towards Th1 vs. Th2. For example, the lack of Crtam affects late T cell polarity (like in the case of lack of p62, or PKC δ, or NBR1), but inhibits Th1 differentiation, whereas deficiency in the latter three adapters, selectively affects Th2. We believe that these molecules not only affect cell polarity but also signaling as they are scaffolds of several signal transduction molecules. For example, recent evidence demonstrate that the levels of some of the bona fide “polarity regulators” such as Par-6, Crumbs, or aPKCs, can be manipulated affecting signaling pathways involved in cell growth without detectable effects on polarity parameters. See for example Nolan et al., Cancer Res 68, 8201, 2008; Nicola et al., Curr. Biol. 20, 573, 2010). We think that something similar is happening in the T cell system.

“Does NBR-1 regulate the recruitment of specific mediators of TCR or cytokine signaling to the immunological synapse?”

It is possible, although we have no evidence on defects in the recruitment of specific mediators of
TCR or cytokine signaling.

"Or is the altered T helper differentiation a consequence of the NBR-1-mediated regulation of the general strength of the signals activated downstream of the TCR?"

We do not think it is a matter of “general signaling strength” as a reduced activation response correlates with a strengthen Th2 response not with a reduced one, as we show here. Please see the excellent review of Constant and Bottomly in the Ann.Rev.Immunol. 15, 297, 1997, on this particular topic.

“Two of the consequences of the lack of NBR1 expression that can explain the defect in Th2 differentiation are the defects in Gata3 and NFATc1 activation. Regarding Gata3, the authors claim that "GATA3 was localized to the nucleus in activated wild-type CD4+ T cells, but that it was nearly totally excluded from the nucleus in activated NBR1-deficient cells". Expression of Gata3, which is up-regulated in Th2 cells in response to TCR and IL4R signals, seems to be normal then. The level of regulation the data shows is exerted on Gata3 nuclear translocation. How is this defect established in NBR1-deficient cells?”

We believe there is some confusion in the interpretation of our results on this specific question. We have rewritten the manuscript and provided new data to make this clearer. We do not think that there is a selective role of NBR1 in regulating the nuclear translocation of Gata3. What we show in Fig. 4A is that the loss of NBR1 inhibits Gata3 induction, which is mostly nuclear. In fact, the new WB data shown in new Fig. 4E demonstrate that NBR1 actually controls Gata3 induction. The new results of Fig. 4F further support this notion as NBR1-deficient cells display reduced induction of Gata3 mRNA levels. Therefore, we think that the primary defect is in NFATc1 regulation as the expression of an NFATc1 active mutant rescues the Gata3 defects in the KO cells (please see new Fig. 5B and C).

“Nuclear translocation of Gata3 has been shown to be dependent on p38-mediated phosphorylation, are Gata3 phosphorylation status or p38 activity altered in these cells?”

We have not found any mobility shift in our Gata3 blots that could suggest changes in phosphorylation. Likewise, we did not find any alterations in p38 activity in the KO cells (Fig. 4E). Also, please note that the time point of stimulation is too long for p38 activity as it has been shown that phospho-p38 is only detected up to 6-8 h after TCR stimulation. As NBR1 is induced in later time points, our study is focused on later rather than earlier activation times. In any case, in Jurkat T cells with NBR1 knockdown and 30 min stimulation, we do not see any defect in p38 activity, as measured by its substrate phospho-ATF2 (Fig. 4E).

“The defect in NFATc1 activation seems to also reside on the control of its nuclear translocation, as levels of NFATc1 expression seem to be normally up-regulated in activated NBR1-deficient T cells. Differences in the activation of NFATc2 and NFATc1 in these cells could explain some of the aspects of their phenotype.”

We attempted to determine whether the loss of NBR1 leads to defects in NAFTc2 phosphorylation and/or nuclear translocation. However, it should be noted that the stimulation times used in the experiments reported here are rather long (14-72h) since NBR1 induction is a long-term event, but at those times we do not see a clear stimulation of NFATc2 as we do for NFATc1. Again, in the experiments with the NBR1 knockdown in Jurkat we did not detect any defect on NFATc2 (Fig. S5C).

“It would be interesting to show if these cells show differences in the levels of phosphorylation of these two proteins that could explain their distinct behavior in the absence of NBR1 (maybe through activation of specific NFATc1-kinases).”

This is a very interesting question. However, as there are not clearly defined phosphorylation sites with available antibodies for NFATc1, this is a very difficult matter to address. Also, note that WB detection of NFATc1 gives a smear. However, in contrast to NFATc2 in which phosphorylation/dephosphorilation can be easily followed by immunoblotting of the shifted band, in the case of NFATc1 this is not possible as the multiple bands shown in the WB are due to the
expression of alternative splicing forms that obscures the possible detection of phosphorylation by electrophoretic shift.

“Ox40 has also been shown to be expressed in resting and activated Tregs. Have the authors checked if NBR1-deletion also occurs in Tregs? Is Treg function altered? Can this have any effect on the airway inflammation model?”

Please see our new Fig. S3, in which we show that NBR1 deletion occurs in Tregs (Fig S3B and C), and that the lack of NBR1 has no effect on the proportion of Tregs (Fig. S3A) or in their activation state (Fig. S3D).

Referee #3:

We are happy to see that this reviewer thinks that “The manuscript convincingly shows that NBR1 is required for Th2 responses upon stimulation and in a model of allergic airway inflammation. This in itself is a very interesting finding”.

This reviewer raises a number of interesting questions that we have addressed in our revised manuscript, which we believe strengthen the paper and improve its understanding.

“The data suggest a mutual recruitment of NBR1 and p62 to the IS and a partial distortion of the IS after deletion of NBR1. Whether these effects are indeed responsible for the reduced activation of Th2 transcription factors and induction of cytokines is not so clear.”

We agree that the link between changes in polarity and the control of cell signaling is not clear. Please see our Discussion. Also, as we comment in our response to Reviewer#2: “This is a very interesting question, which we have tried to address in our discussion. The role of cell polarity in T cell activation is not clear despite the elegant work of several investigators. This is even less known in T cell differentiation towards Th1 vs. Th2. For example, the lack of Crtam affects late T cell polarity (like in the case of lack of p62, or PKCδ, or NBR1), but inhibits Th1 differentiation, whereas deficiency in the latter three adapters, selectively affects Th2. We believe that these molecules not only affect cell polarity but also signaling as they are scaffolds of several signal transduction molecules. For example, recent evidence demonstrate that the levels of some of the bona fide “polarity regulators” such as Par-6, Crumbs, or aPKCs, can be manipulated affecting signaling pathways involved in cell growth without detectable effects on polarity parameters. See for example Nolan et al., Cancer Res 68, 8201, 2008; Nicola et al., Curr. Biol. 20, 573, 2010). We think that something similar is happening in the T cell system.”

“As NBR1 and p62 are involved in autophagy, it may well be that the effects observed in NBR1 deficient T cells are caused by deregulated autophagy and not a primary signaling defect. This needs to be discussed much more open.”

This is actually a very provocative possibility. However, we have been unable to see defects in autophagy in the NBR1 KO T cells, at least under the conditions of these experiments (please see new Fig. S6). There are no defects in LC3 punctuate staining, as determined by immunofluorescence (Fig S6A and B), or in LC3 levels (Fig. S6C). It is hypothetically possible that the loss of NBR1 could impair the degradation of some unidentified molecule, which indirectly could impact in NFATc1 activation. However, at this point this is highly speculative. In fact, our results in EFS and macrophages (not reported in this paper) have so far failed to show a role for NBR1 (using KO cells) in the autophagic functions proposed for p62 or p62/NBR1, such as the shuttling of polyubiquitinated aggregates. We rather favour the notion that NBR1, like p62, is an important cell signaling scaffold.

Specific points:

“Figure 1 and Tables I-III could be largely moved to the supplement without significant loss of information.”

Following this reviewer’s suggestion, Fig. 1 and Tables 1 to III are now part of the Supplemental
Figure 2: Looking at panel A it seems that all PB1 proteins analyzed are exclusively expressed in activated T cells. Given the different time points of all the experiments, the authors should perform a kinetic analysis of NBR1 induction. Is NBR1 equally expressed in Th1 and Th2 cells?

Please see new Fig. 1A in which we show a kinetic analysis of NBR1 induction and the other PB1 proteins, p62 and PKC, as requested by this Reviewer. From that, it is clear that NBR1 is induced upon chronic T cell activation. Interestingly, when the expression of NBR1 is investigated under skewing conditions, it is selectively induced in Th2 cells but not in Th1 cells, reinforcing its role in Th2 cells. Please see new Fig. 1J.

Also IL-2 induction in Th1 cells should be determined in the absence of NBR1 to get a better idea about the effect in Th1 cells.

Please see new Fig. 1F showing that the loss of NBR1 have no effect on IL-2 production.

As NBR1 was described as an autophagy receptor, it should at least be determined if CD3/CD28 surface expression is comparable in NBR1fl/fl and NBR1fl/fl CreOX40 Th2 cells.

Please see new Fig. S6D in which the lack of effect of NBR1 deficiency in CD3 or CD28 surface expression is shown.

Figure 5: Induction of Gata3 expression (and not only nuclear translocation) seems to be completely gone in NBR1fl/fl CreOX40 T cells. Blotting of whole cell lysates should be performed.

Please see new Figs. 4E and 4F in which we show Gata3 levels in total extracts, as well as mRNA levels of Gata3. It is clear that the lack of NBR1 impairs Gata3 synthesis. We have modified the manuscript to make this point more clear.

Comparing the extremely strong effects in panel A with the somewhat weaker effects in the quantification (panel B), it is sort of questionable if these cells are really representatives. It would be helpful to show more than one cell. This also holds true for Figures 6 and 7.

Please note that the quantification shown in Fig. 5, 6 and 7 (now new Fig. 4, 6 and 7, are not a reflection of the intensity of the signal in the cells but the percentage of cells that have a phenotype like the one shown in the immunofluorescence panel.

Figure 6 and 7: Some effects are very mild and in general the relevance of these recruitment defects for the observed Th2 phenotype is unclear.

We respectfully are in disagreement with this reviewer. Please note that the defects shown in Fig. 6 and 7 are comparable to those reported for Crtam (Ye et al., Cell 132, 846, 2008). However, we agree with this reviewer that the link between defects in polarity and defects in T cell differentiation are clearly not linear. Please see our Discussion and our response above.

Is the impaired recruitment Th2 specific? If not, why are Th1 cytokines not affected?

Please note that NBR1 is only induced in Th2 cells and not in Th1 cells (new Fig. 1J).

Further, interaction of NBR1 and p62 has been shown previously and it seems unlikely that this is dependent on IS recruitment. Especially P/I stimulation in Jurkat T cells bypasses upstream signaling (7C). These results need to be repeated with CD3/CD28 stimulation.

Please see new Fig. 7E with the requested experiment.

The lack of PKCl/i and NBR1 co-IP is a negative result and it needs to be shown that in the same experimental setting (e.g. PKCl IP) p62, but not NBR1 is precipitated.

Please see new Fig. 7D with the requested control.
“Figure legend 7C is missing.”

We apologize for the mistake. This has now been amended.

Discussion:

“Page 13: It is stated: ‘... unlike p62, NBR1’s translocation is independent of PKCl/i.’ This is confusing, because from the figure 6C it seems clear that p62 translocation is also independent of PKCl/i.’”

This is a mistake (we apologize for that) that has been amended in the revised version of the paper, in which now we state the following: “the translocation of p62 and NBR1 are mutually dependent, but, like p62, NBR1 is translocation is independent of PKCl/i.”

“The whole discussion is confusing and biased and needs to be revised. As the authors state themselves ‘...a common link between cell polarity defects, ..., and T-cell signaling is not readily apparent.’ Never the less they spend about 1.5 pages in discussing links between cell polarity and signaling.”

Following this reviewer’s suggestion, we have shortened the discussion about polarity and signaling in T cells. However, we believe that it is important that this question is discussed in some extent in our paper as the link between polarity proteins and polarity-independent signaling is a question that is becoming to emerge in several recent studies and needs to be addressed here as our data clearly fall into that debate.

“Possible effects of autophagy are not mentioned at all.”

Following this reviewer’s suggestion, we have included some discussion about the purported role of NBR1 in autophagy. Please also note that new data in Fig. S6 demonstrate that the deletion of NBR1 has no effect on autophagy, at least in T cells and under these experimental conditions.

Minor points:

“The language of the text should be revised, because...... are from the authors themselves. I also wonder what this novel paradigm would be.”

We have revised the language of the manuscript and included some more references about PB1-mediated signaling, as requested by this reviewer.

Additional Correspondence 09 August 2010

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original three referees to review this version and I have now received their comments. As you can see below, all three referees appreciate the introduced changes and support publication in the EMBO Journal. I am therefore very pleased to proceed with the acceptance of the paper for publication here. Before doing so there are a few minor comments raised that I would like you to comment on. Referee #3 has a question regarding fig 4D and Referee #2 has one regarding FigS6. Could you take a look at these issues, comment on them and let me know if you need to make any changes to the manuscript that we have on file. If so then you can send me modified files by email, you can send it via the manuscript tracking system. If not, then we will proceed with the acceptance of the manuscript that we have on file.

Editor
REFEREE REVIEWS

Referee #1

Authors have adequately addressed all the concerns that this reviewer had.

Referee #2

This new revised manuscript by Yang et al has addressed most of the concerns raised in the previous round of reviews. New data has been included to determine how NBR1 may control the TCR-NFAT-Gata3-Stat6 signaling axis and regulate T helper cell polarization. Although the mechanisms that underlie the specific control of Th2 differentiation by changes in cell polarity caused by NBR1 are still not clear, I agree with the authors that this may be a complex question to answer at this point. This manuscript presents, nonetheless, a very exciting set of results that characterizes the specific involvement of NBR1 in Th2 differentiation and should help lead to the way to the uncovering of those mechanisms.

Just a couple of minor comments:

- I assume that the authors are detecting the conjugated form of LC3 in their blots. If so, it should be clearly indicated in Fig S6. If the WB is measuring total unconjugated LC3, this would have then little meaning as indicator of autophagy activation. However, the quantification of LC3+ puncta shown in the same figure would still clearly support the authors' point.
- The paper clearly shows now that NBR1 is also deleted in Tregs, and although this does not seem to affect Treg generation or TGFβ production, it would have made a more compelling argument if a suppression assay would have been included to show no defects in Treg function.

Referee #3

The issues that I have raised have been addressed and some additional experiments have considerably strengthened the model that NBR1 acts as a signaling adaptor in Th2 cells. The mechanism how NBR1 in the IS connects to the downstream targets is still not so clear, but I guess future studies will need to clarify this issue.

Fig. 4D: The panel looks like p38 is constitutively phosphorylated. Is this the right blot?

Additional Correspondence 09 August 2010

Thanks very much for your message and the excellent news about our paper. We are happy to see that all the reviewers are now supportive of its publication.

In regard to the minor comments raised by Reviewer#2, her/his interpretation is right, the LC3 band shown in the western blot of Fig. S6D is obviously the conjugated form and the data are consistent with the immunofluorescence results, as s/he clearly points out. So, unless you think otherwise, we do not think that we need to amend the manuscript. In regard to her/his comments on Treg cells, the fact that we do not find defects in the production of TGFβ by NBR1-negative cells, together with all the amount of results we present supporting the role of NBR1 in Th2, makes us to think that a very time-consuming and expensive experiment just to confirm that the loss of NBR1 in Treg has no effect on the phenotype shown here is not justifiable at this point. We respectfully feel that this would unnecessarily delay the publication of our paper.

In regard to the comment of Reviewer#3 on p38, please note that what we show is that at 14 h of
stimulation, p38 is not activated, as well as that the lack of NBR1 has no effect on this parameter. The gel is overexposed precisely to show this lack of effect. It should be noted that p38 activation in T cells is a relatively early event. Therefore, as NBR1 induction is late and its role is in late events, our data are perfectly consistent with p38 not being relevant in NBR1 actions, at least in this system.

We hope that these responses are satisfactory and that our paper is definitively accepted now.