Grb2 regulates B cell maturation, B cell memory responses and inhibits B cell Ca2+ signalling

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(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 10 December 2010

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by two referees and their comments to the authors are provided below.

As you can see both referees find the analysis interesting and suitable for publication here, pending adequate revisions. Referee #1 raise relative minor concerns with the paper, while referee #2 finds that some further data is needed to support the conclusions drawn. In particular further data to support that Grb2 regulates B cell memory would strengthen the paper. Given this input, I would therefore like to invite you to submit a suitably revised manuscript that addresses the raised concerns. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that it is therefore important to resolve the major concerns at this stage. 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.

Best wishes

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This manuscript makes an important contribution to our understanding of B cell receptor (BCR) signaling. Previous work in cell lines had shown that Grb2 functions as a negative regulator of Ca2+ signaling. In this manuscript the authors have selectively disrupted the Grb2 gene in B cells and made a number of novel findings about the role of Grb2 in BCR signaling as well as B cell development and function. In addition to confirming in primary cells the role of Grb2 in limiting BCR-induced Ca2+ signaling, the authors identify other signaling pathways that Grb2 contributes to. More importantly, they provide the first functional data that Grb2 is important for the development of mature B cells and memory B cell function. Elegant approaches are used including competitive repopulation of Rag KO mice. The data are generally of very high quality and the findings are novel and interesting. Although the underlying mechanisms by which Grb2 controls B cell development and function are not explored in detail, this work will likely form the basis for a number of future studies, therefore making it a valuable contribution to the field. Given the multiple roles of Grb2 in B cells that the authors show here for the first time, and the fact that Grb2 has important functions in many cell types, I feel that this manuscript would be of interest to a general audience.

I have a few comments about experiments in which the effects are small but these do not affect the overall conclusions of the manuscript. Most of my other comments are suggestions for making the figures easier for the reader to interpret.

Main comments
1. Figure 3C, lower panel. The authors should comment on whether this 20% difference in BAFF-R expression between mature and immature wild type B cells is similar to what has been reported and whether there is any data suggesting that the smaller maturation-associated increase in BAFF-R levels in the mutant B cells would result in impaired survival of mature B cells. The changes in expression are all quite small. In this graph, it would also be good to present statistical analysis of BAFF-R levels in mature versus immature mutant B cells. There are higher BAFF-R levels in the mature Grb2/-/- cells than in the immature Grb2/-/- B cells but the question is whether or not this increase is sufficient to provide the required survival signals.

2. Figure 5B. A better quality blot could be shown. The authors should indicate whether blotting was done with a pan-Ras Ab or an Ab that recognizes specific Ras proteins (e.g. K-Ras). Also, blotting with an anti-Ras Ab would be a more appropriate loading control than the actin blot. The Y-axis should have hash marks indicating values, even if they are arbitrary units.

3. Page 10 and Figure 5C. The authors indicate that there was "a slightly impaired tyrosine phosphorylation of CD22" but should comment on whether the difference was statistically significant (the graph suggests that the difference did not reach P<0.05).

4. Page 11. The authors state: "We also observed a strong increase of IgMhi plasma cells in the red pulp of the spleen of B-cell specific Grb2/-/- mice" but it is not clear where this data is shown. If this is in fact depicted in Figure 6C, some arrows pointing to these cells would be useful.

Minor points
1. In Supplemental Figure 1C, don't the CD5+ spleen cells also include B-1a cells? It may not be appropriate to refer to this population as T cells on page 6 of the text.

2. Figure 1C, top panels. "M" should be defined in the figure legend as "mature B cells".

3. Figure 2D. In the 1D histograms, the cell type identification is reversed for R2 and R3. R2 should be the B2 cells and R3 should be the CD5+ B-1 cells.

4. Figure 2. It would help the reader if the labels on the bar graphs of %CD45.1+ cells indicated the cell type rather than the region (or both).
5. Page 8: Where the authors state "We observed no difference in BrdU incorporation between mature B cells (IgMloIgDhi) and T2 B cells (IgMhiIgDhi) from B-cell specific Grb2/-/- mice and control mice" it should read "in BrdU incorporation in mature B cells (IgMloIgDhi) or T2 B cells (IgMhiIgDhi)..." since the comparison is between the same cell populations from the wild type and mutant mice, not between mature B cells and T2 cells.

6. In the legend for Figure 3, the word "experiment" should be spelled out.

7. Page 10. CD22 is not an enzyme, so it is not correct to say "CD22 activation". "CD22 phosphorylation would be more appropriate".

8. In the legend for Figure 5, it should be blotting with "anti-phosphotyrosine" not "anti-tyrosine".

9. Figure 4. The panels could be organized better. The two cell gating FACS plots should be side by side and the Ca2+ traces should be below them. Otherwise it looks like the lower Ca2+ traces are for the mutant cells and the top two Ca2+ traces are for the wild type cells. It would also be good to refer to both pairs of Ca2+ traces in the text and indicate the rationale for choosing these two concentrations of anti-IgM should be discussed. If the intention was to show that there was higher Ca2+ signaling in the mutant cells at both saturating and sub-saturating concentrations of anti-IgM then a lower concentration of anti-IgM (e.g. 1 µg/ml) might be more appropriate for the sub-saturating concentration. The lower anti-IgM concentration used here (7.5 µg/ml) seems to elicit a response that is quite similar to that elicited by the higher concentration.

a) In the figure legend, it should be "time point".

10. Figure 6C. Scale bars would be preferable to indicating the magnification.

11. Figure 7B. P-values should be shown.

12. Page 19. "anti-I-Ab"-should this be "anti-MHC-II Ab"?

Referee #2 (Remarks to the Author):

This study has aimed at clarifying the B cell intrinsic function of Grb2. The approach is quite reasonable, the overall data quality is pretty high, and the Ms is well written. However, I have three major concerns about this Ms.

1) BrdU pulse experiments in Fig. 3 are nice, but more importantly, authors are encouraged to do pulse-chase experiments for determination of turn-over ratio of peripheral B cells, because authors think that the decreased numbers of mature B cells are due to survival defect.

2) Given that both BCR and BAFF-R signaling are required for development or maintenance of marginal zone (MZ) B cells, the normal number of MZ B cells is quite puzzling. At least, authors should intensively discuss the possibility of why the number of MZ B cells is normal.

3) One of the important messages in this Ms is that Grb2 regulates B cell memory. In this regard, analysis of this part is poor. Authors should do more experiments to make their argument clear and strong. In this sense, the interpretation of Fig. 8 is particularly problematic.

First, authors should calculate Ag-specific IgM and IgG B cells (as shown in JI 2010, Weisel et al). And, in the case of Ag-specific IgG B cells, authors should use CD38 to distinguish memory versus GC B cells. The Fig. 8B data are suggestive, but strictly speaking, do mean nothing.

Second, since VLP contains DNA, one of the big differences between NP-KLH and VLP immunization systems is that NP-KLH utilizes T-dependent system only, whereas VLP utilizes probably TL9 or other DNA sensor system, in addition to TD system. Thus, one of the interpretations of Fig. 8C is that IgG memory B cells have same ability in terms of TD secondary responses (in the case of NP-KLH) even in the absence of Grb2, whereas the VLP system requires Grb2. Grb2 could be required for activating innate-dependent activation system or synergy between
innate and acquired systems in memory responses. To test this possibility, authors can do the Rag reconstitution experiments using activated T cells, memory B cells, and soluble Ag for measuring the TD manner. If the similar levels of response in the absence or presence of Grb2 will be obtained, this possibility is reasonable. Then, authors should examine more seriously the effects of Grb2 deletion on innate signaling (proliferation, survival, differentiation). Together, I feel that through the above suggested experiments, they can make a stronger conclusion about memory part.

Minor points;
1. Although the authors showed the activation of BCR-mediated MAPKs, syk and Akt signaling by examining the quantification of band intensities (phospho-specific bands divided by loading controls) as shown in Figure 5, they should examine their protein loading controls using anti-total Erk, JNK, p38, syk and Akt Abs instead of anti-actin Ab.
2. PLC 2 is a key regulator of Ca2+ mobilization in B cells. The authors require data about the activation of BCR-mediated PLC 2 signaling in Figure 5.
3. The authors showed that Grb2-deficient mature B cells have an impaired BAFF-R upregulation as shown in Figure 3C, suggesting that an impaired BAFF response may contribute to a reduced survival rate of Grb2-deficient mature B cells. How about survival and proliferation rates of immature and mature B cells in response to BAFF?
4. Statistical significance is required in Figure 7B (right bar graph).
5. In Material and Methods section, the authors should describe clone names of antibodies used in this manuscript.

We thank referee 1 for the overall very positive evaluation. Below we will answer his comments in detail.

Main comments
1. Figure 3C, lower panel. The authors should comment on whether this 20% difference in BAFF-R expression between mature and immature wild type B cells is similar to what has been reported and whether there is any data suggesting that the smaller maturation-associated increase in BAFF-R levels in the mutant B cells would result in impaired survival of mature B cells. The changes in expression are all quite small. In this graph, it would also be good to present statistical analysis of BAFF-R levels in mature versus immature mutant B cells. There are higher BAFF-R levels in the mature Grb2-/- cells than in the immature Grb2-/- B cells but the question is whether or not this increase is sufficient to provide the required survival signals.

Response: It has been shown by several groups that upregulation of BAFF-receptor between the transitional and mature B cell stage is crucial for survival of the B cells. The upregulation is never large between these two populations, similar to the upregulation which we see in mature wild type, but not mutant B cells (see Meyer-Bahlburg JEM, 205, 155 or Hsu JI 168, 5993). We have in all our graphs only shown statistics for those changes which were statistically significant. The small upregulation of BAFF-R in mature versus immature Grb2-/- B cells is not significant.

2. Figure 5B. A better quality blot could be shown. The authors should indicate whether blotting was done with a pan-Ras Ab or an Ab that recognizes specific Ras proteins (e.g. K-Ras). Also, blotting with an anti-Ras Ab would be a more appropriate loading control than the actin blot. The Y-axis should have hash marks indicating values, even if they are arbitrary units.
Response: We have exchanged the blot by another blot of better quality. As loading control we have added anti-Ras bots. The antibody recognizes all isoforms of Ras. This was now written in the materials and methods section (page 22). All changes in the text are shown in red.

3. Page 10 and Figure 5C. The authors indicate that there was "a slightly impaired tyrosine phosphorylation of CD22" but should comment on whether the difference was statistically significant (the graph suggests that the difference did not reach P<0.05).

Response: the reviewer is right, this difference was not significant. This is now mentioned in the results section (page 10).

4. Page 11. The authors state: "We also observed a strong increase of IgMhi plasma cells in the red pulp of the spleen of B-cell specific Grb2-/- mice" but it is not clear where this data is shown. If this is in fact depicted in Figure 6C, some arrows pointing to these cells would be useful.

Response: Arrows showing IgM+ plasma cells are now added in Fig. 6C

Minor points
1. In Supplemental Figure 1C, don’t the CD5+ spleen cells also include B-1a cells? It may not be appropriate to refer to this population as T cells on page 6 of the text.

Response: The T cells were FASC sorted as CD5hi B220-. This is now indicated in the Suppl fig. 1C. B-1a cells are CD5low B220low and are not included in the sort.

2. Figure 1C, top panels. "M" should be defined in the figure legend as "mature B cells".

Response: now added to the legend

3. Figure 2D. In the 1D histograms, the cell type identification is reversed for R2 and R3. R2 should be the B2 cells and R3 should be the CD5+ B-1 cells.

Response: this was corrected.

4. Figure 2. It would help the reader if the labels on the bar graphs of %CD45.1+ cells indicated the cell type rather than the region (or both).

Response: The name of the cell types were added to the bar diagrams.

5. Page 8: Where the authors state "We observed no difference in BrdU incorporation between mature B cells (IgMloIgDhi) and T2 B cells (IgMhiIgDhi) from B-cell specific Grb2-/- mice and control mice" it should read "in BrdU incorporation in mature B cells (IgMloIgDhi) or T2 B cells (IgMhiIgDhi)..." since the comparison is between the same cell populations from the wild type and mutant mice, not between mature B cells and T2 cells.

Response: this was corrected

6. In the legend for Figure 3, the word "experiment" should be spelled out.

Response: this was corrected

7. Page 10. CD22 is not an enzyme, so it is not correct to say "CD22 activation". "CD22 phosphorylation would be more appropriate".

Response: this was changed
8. In the legend for Figure 5, it should be blotting with "anti-phosphotyrosine" not "anti-tyrosine". Response: this was corrected

9. Figure 4. The panels could be organized better. The two cell gating FACS plots should be side by side and the Ca2+ traces should be below them. Otherwise it looks like the lower Ca2+ traces are for the mutant cells and the top two Ca2+ traces are for the wild type cells. It would also be good to refer to both pairs of Ca2+ traces in the text and indicate the rationale for choosing these two concentrations of anti-IgM should be discussed. If the intention was to show that there was higher Ca2+ signaling in the mutant cells at both saturating and sub-saturating concentrations of anti-IgM then a lower concentration of anti-IgM (e.g. 1&≤00B5;g/ml) might be more appropriate for the sub-saturating concentration. The lower anti-IgM concentration used here (7.5 &≤00B5;g/ml) seems to elicit a response that is quite similar to that elicited by the higher concentration.

a) In the figure legend, it should be "time point".

Response: we agree and changed the order of the panels. We have seen the differences in Ca2+ responses between Grb2-/- B cells and control B cells at several concentrations of anti-IgM. We chose two concentrations and would like to show both of them, because we feel a titration of the stimulating antibody is always more convincing to show than one arbitrary chosen concentration. Both conc. were subsaturating.

10. Figure 6C. Scale bars would be preferable to indicating the magnification.
Response: we have given the magnification (x50) for both Fig.6C and Fig. 7C, because our analysis software does not easily allow scale bar allocation. We think this should be fine.

11. Figure 7B. P-values should be shown.
Response: p-value symbols were added.

12. Page 19. "anti-I-Ab"-should this be "anti-MHC-II Ab"?
Response: yes, the more general term for the antibody is now used.

Response to referee 2

We thank referee 2 for the positive evaluation and for the constructive suggestions for new experiments. Responses to the specific concerns follow below.

1) BrdU pulse experiments in Fig. 3 are nice, but more importantly, authors are encouraged to do pulse-chase experiments for determination of turn-over ratio of peripheral B cells, because authors think that the decreased numbers of mature B cells are due to survival defect.

We agree that a pulse-chase BrdU experiment was important to do. In fact, we had started this experiment already during the first submission phase. We have now finished the experiment and added the results as a new figure (fig.3B) to the manuscript. Interestingly, the result was different to our expectations. We had expected a higher turnover of peripheral B cells in the chase phase, i.e. a quicker decline of BrdU+ B cells in the Grb2/-/- mice. Instead, the mutant B cells show a lower turnover in the chase phase, i.e. more BrdU+ cells remain than in the control mice. We discuss these new results in the results section (page 8) and in the discussion (page 13) as a result of a lower proliferation rate of preB cells or a lower exit of immature B cells from the bone marrow. We conclude that there are two effects contributing to the smaller number of mature B cells in the periphery: lower proliferation of precursor cells (predominantly seen in the BrdU experiment) and a lower survival of peripheral B cells. All changes in the text are shown in red.

2) Given that both BCR and BAFF-R signaling are required for development or maintenance of marginal zone (MZ) B cells, the normal number of MZ B cells is quite puzzling. At least, authors should intensively discuss the possibility of why the number of MZ B cells is normal.
We agree that MZ B cells should also be sensitive to changes in BAFF-R signalling. We discuss possible reasons why this could be not the case now in our discussion (page 19).

3) One of the important messages in this Ms is that Grb2 regulates B cell memory. In this regard, analysis of this part is poor. Authors should do more experiments to make their argument clear. In this sense, the interpretation of Fig. 8 is particularly problematic. First, authors should calculate Ag-specific IgM and IgG B cells (as shown in Ji 2010, Weisel et al). And, in the case of Ag-specific IgG B cells, authors should use CD38 to distinguish memory versus GC B cells. The Fig. 8B data are suggestive, but strictly speaking, do mean nothing. Second, since VLP contains DNA, one of the big differences between NP-KLH and VLP immunization systems is that NP-KLH utilizes T-dependent system only, whereas VLP utilizes probably TL9 or other DNA sensor system, in addition to TD system. Thus, one of the interpretations of Fig. 8C is that IgG memory B cells have same ability in terms of TD secondary responses (in the case of NP-KLH) even in the absence of Grb2, whereas the VLP system requires Grb2.
Grb2 could be required for activating innate-dependent activation system or synergy between innate and acquired systems in memory responses. To test this possibility, authors can do the Rag reconstitution experiments using activated T cells, memory B cells, and soluble Ag for measuring the TD manner. If the similar levels of response in the absence or presence of Grb2 will be obtained, this possibility is reasonable. Then, authors should examine more seriously the effects of Grb2 deletion on innate signaling (proliferation, survival, differentiation).
Together, I feel that through the above suggested experiments, they can make a stronger conclusion about memory part.

We agree that it would strengthen the study to do more adoptive transfer experiments to work out more clearly the mechanism of the B cell memory defect. These could be experiments using antigen-specific sorted memory B cells in the VLP system (as in Weisel et al. 2010) or a similar adoptive transfer experiment for the NP-KLH system with transfer of purified memory B cells and primed T cells. We will do these experiments in the future, but prefer not to do them for this manuscript, because they will take 3-4 months and we know of a competitor group which has also submitted a manuscript in parallel to us. Instead we have done 3 more experiments to strengthen our argument of a Grb2-dependent memory B cell defect in the VLP system.
1) After the transfer of VLP primed memory B cells and VLP challenge in the RAG1-/- recipients we have now also done an ELISSPOT assay to measure IgG1-secreting cells 21 days after the challenge. After transfer of Grb2-/- memory B cells, the IgG1 secreting cells were greatly reduced, compared to the recipients with control memory B cells. This again strengthens the argument of a defective memory B cell response also on a cellular basis. These data have been added as Fig. 8D to the manuscript.
2) The referee was concerned, that the specialty of the VLP antigen may cause the defect in response due to CpG dependent TLR responses. This would imply a defective TLR9 response of the Grb2-/- mice contributing to the defective immunisation response with VLPs. We have measured TLR9 and TLR7 responses of Grb2-/- B cells and they are even increased (new suppl. Fig.5). Thus, contributions of potentially remaining viral DNA containing CpGs cannot explain the defect in VLP antibody responses after the adoptive transfer.
3) Finally, to exclude that Grb2-/- B cells can simply not switch in vitro we have done an in vitro class switch experiment after stimulation of B cells by LPS and IL-4 and found a normal switch to IgG1 (new suppl. Fig.6)
Thus we conclude from these new experiments, that at least after virus-like particle immunisation there is a true memory B cell defect.

Minor points:
1. Although the authors showed the activation of BCRmediated MAPKs, syk and Akt signaling by examining the quantification of band intensities (phospho-specific bands devided by loading controls) as shown in Figure 5, they should examine their protein loading controls using anti-total Erk, JNK, p38, syk and Akt Abs instead of anti-actin Ab.
We also used the suggested specific antibodies as loading controls with generally similar results as for the actin controls. We used the actin stainings for the figures because of a generally better quality of the actin blots. This is widely used in the literature as well.
2. PLC\textgamma{}2 is a key regulator of Ca\textsuperscript{2+} mobilization in B cells. The authors require data about the activation of BCR-mediated PLC\textgamma{}2 signaling in Figure 5. We agree that it would be important to look for PLC\textgamma{}2 activation. We planned to include this already in the first version of the Ms. However, the commercially available phospo-PLC\textgamma{} antibody (Cell Signalling Tyr759-specific) did not detect an increased phosphorylation after anti-kappa or anti-IgM stimulation (in both types of mice), in about 5 experiments. Therefore, we left out these results.

3. The authors showed that Grb2-deficient mature B cells have an impaired BAFF-R upregulation as shown in Figure 3C, suggesting that an impaired BAFF response may contribute to a reduced survival rate of Grb2-deficient mature B cells. How about survival and proliferation rates of immature and mature B cells in response to BAFF? Again, we tried those experiments. For technical reasons they did not work, i.e. we did not see better survival of B cells with added BAFF in vitro at concentrations of BAFF which were used in similar assays by other groups.

4. Statistical significance is required in Figure 7B (right bar graph). This is now added to the graph.

5. In Material and Methods section, the authors should describe clone names of antibodies used in this manuscript. The clone names are now added.

2nd Editorial Decision

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #2 to review the revised version. I have now heard back from the referee and the comments are provided below. As you can see the referee appreciates the introduced changes. The referee also requests a few additional experiments: 1) To estimate the number of IgG type memory B cells after VLP primary immunization (not requiring adoptive transfer experiments). The reason of why I think that it is important is that authors showed the increase of IgG positive B cells (Fig. 8B), suggesting the increased number of class-switched CD38\textsuperscript{+} B cells, like shown in Weisel's paper) after VLP primary immunization and 2) to determine the activation state of PLC\textgamma{}2 by measuring the IP3 content. Regarding point #2, while this is a good suggestion it is also not essential to address this point at this stage. If you want to discuss this issue in the discussion feel free to do so. Regarding point #1, do you have any data on hand to address this point? If so, it would be good to include in. We can discuss it further if this is helpful. Once we get these last issues resolved, we will proceed with the acceptance of the paper for publication here.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

Best regards
Editor
The EMBO Journal

REFEEEREE REPORTS:

Referee #2 (Remarks to the Author):

Although authors have done several suggested experiments, I think that two key experiments are still missing. First, authors should calculate the number of IgG type memory B cells (class-switched gB-specific CD38\textsuperscript{+} B cells, like shown in Weisel's paper) after VLP primary immunization (not requiring adoptive transfer experiments). The reason of why I think that it is important is that authors showed the increase of IgG positive B cells (Fig. 8B), suggesting the increased number of class-switched
memory B cells in the absence of Grb2. But, the Ag-specific Ab titer after secondary challenging was inhibited, raising the question of why (Fig. 8A). Obviously, detection of the number of Ag-specific memory B cells is very informative. Second, activation status of PLCγ2 is also key. Since phospho-PLCγ2 Ab does not work, authors should measure IP3 content after BCR stimulation.

2nd Revision - authors’ response 22 February 2011

Referee #2 (Remarks to the Author):
Although authors have done several suggested experiments, I think that two key experiments are still missing.
First, authors should calculate the number of IgG type memory B cells (classswitched gB-specific CD38+ B cells, like shown in Weisel’s paper) after VLP primary immunization (not requiring adoptive transfer experiments). The reason of why I think that it is important is that authors showed the increase of IgG positive B cells (Fig. 8B), suggesting the increased number of class-switched memory B cells in the absence of Grb2. But, the Ag-specific Ab titer after secondary challenging was inhibited, raising the question of why (Fig. 8A). Obviously, detection of the number of Ag-specific memory B cells is very informative.
Second, activation status of PLCγ2 is also key. Since phospho-PLCγ2 Ab does not work, authors should measure IP3 content after BCR stimulation.

Response
To 1) We agree that it would be important to determine the number of antigen-specific (gB-specific) memory B cells after VLP immunisations, such as done in Weisel et al. We tried to do the IgG/ gB double staining already for our experiment shown in fig.8, but were not able to detect any gB-specific cells. The reason is probably a technical one. As described by Weisel et al. mice need to be immunized several times with gB protein in adjuvants in order to be able to detect enough memory B cells in FACS. So we would have to repeat the immunisation experiment with gB instead of VLP and challenge 2 or 3 times in order to be able to detect the gB specific IgG+ cells. Since this would take at least another 2 months and since we feel that we have worked on the mechanism substantially by adding 3 new experiments addressing the memory B cell defect for the first revision, we would rather not wait for such a long new experiment. We plan, however, to do such an experiment in future work.

To 2) We think that PLCγ2 activation experiments are not key experiments for our study. We have shown increased Ca2+ signalling of Grb2-/- B cells. To work on the mechanism, we have analysed Syk and CD22 phosphorylation, which both would suggest a mechanism of how the higher Ca2+ signalling is regulated. PLCγ2 phosphorylation or IP3 levels would probably be increased, since Ca2+ is increased, but this would not necessary tell the regulatory mechanism by Grb2.