Molecular requirements of the B-cell antigen receptor for sensing monovalent antigens

Christoph Volkmann, Naema Brings, Martin Becker, Elias Hobeika, Jianying Yang, Michael Reth

Corresponding author: Jianying Yang & Michael Reth, BIOSS Centre for Biological Signalling Studies, University of Freiburg; Max Planck Institute of Immunobiology and Epigenetics

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Thanks for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see below, the referees find the analysis interesting. However, they also find that further revisions are needed in order to strengthen the findings. The concerns raised are clearly indicated below and I suspect that you should be able to address them in a good way. Let me know if we need to discuss anything further. You can use the link to upload the revised version. Please note that it is EMBO Journal policy to allow a single major round of revision only and it is therefore important to resolve the raised issues at this stage.

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

Referee #1:

In this manuscript, Volkmann, Brings and colleagues used a combination of calcium flux and proximity ligation assays (PLA) to assess the response of IgM and IgD BCRs to monovalent antigens. Understanding the early steps in BCR activation for different BCR isotypes remains limited and is of importance to the field of B cell biology. This paper reports several interesting findings in light of the controversial nature of the subject (monovalent activation of the BCR and the DAM hypothesis). However, I feel that the manuscript requires stronger experimental support and controls to better justify its conclusions. Several panels of the manuscript describe experiments that are almost exact repeats of experiments reported in a study from a different group (Minguet et al. 2010) but the findings the authors arrive at in the present ms. are rather different, leading to opposite conclusions. These discrepancies are not discussed in a meaningful manner anywhere in the manuscript and add confusion in a field already controversial. If there are sound reasons to discount the validity of these earlier experiments, then the reader deserves to know why. Many of the suggestions made below probably concern experimental data already in hand.

In fig 1, the authors used calcium flux and proximity ligation assays (PLA) to assess whether monovalent antigens can activate the BCR and whether the nanoscale organization of the BCR is altered. Panels a-c describe experiments that replicate those of Minguet 2010 (fig 2b, 4b-c). The mouse model is the same (B1-8 k-deficient) as well as the peptide antigen (KSKGESAG with NIP on the second K and the first K harbors a fluorescent dye) and the same technique is used (Calcium flux). The Minguet paper claims that B cells were unresponsive to monovalent antigens, as judged from a combination of calcium flux, p-Tyr, P-ERK, activation marker upregulation (CD86, CD69 and MHCII) (fig 2 and 3). In the present study, the opposite result was obtained as reported for calcium flux data. It would be important to include other techniques to report on BCR activation, as well as include a control peptide not modified with NIP. Moreover, the calcium flux response shown in fig 1b for Ac146 Fab is not particularly convincing and is at variance with published data (Minguet et al., 2010, fig 4b). Indeed, the authors do not show the purity of their Fab preparations. Could the delayed response shown in fig 1b due to whole antibody/F(ab)2/aggregated Fab contamination? Another important control to include would be to stimulate the cells in serum-free medium. Serum-containing medium could induce complexation of peptides in solution and thus yield a multivalent ligand.

My second concern is the use of PLA to assess BCR nano-organization. PLA uses fixed samples and presumably evaluates only a small number of receptors per cell, as inferred from the number of dots. Moreover, some of these dots overlap. It thus becomes difficult to assess the number of dots/cell. It was unclear whether the authors used a projection of a Z stack or only a section of cells, which – depending on the focal plane analyzed – could influence the result significantly; not all images seem to have been collected in comparable focal planes). An additional technique such as super-resolution microscopy might be helpful to support the DAM hypothesis in this particular system. In addition, it would be useful to use PLA at different time points to get a better understanding of the dynamics of BCR behavior.

An interesting aspect not mentioned by the authors is the drastic increase of IgD:IgD dots/cell in the cells stimulated with Ac38Fab. Why do IgD BCRs become better targets for PLA but do not show any response as measured by calcium flux? It would be interesting to restimulate B1-8 cells with antigen or cross-linking agents to assess whether these cells become unresponsive after stimulation with Ac38Fab, as was shown for OVA 8-mer peptide in the OB1 model of Dougan et al. (Avalos et al. 2014). Unresponsiveness of B1-8 cells and increased clustering after Ac38Fab stimulation would support the DAM hypothesis. A final aspect that would be interesting to explore is whether the affinity of the BCR for its cognate monovalent antigen could affect its response using B1-8 high and B1-8 low B cells. The affinity of hapten-specific BCRs is often in the lower range of what is seen for protein antigens. Perhaps the authors should comment on the relationship, if any, between affinity and BCR clustering.

In fig 2, the authors repeat the experiment shown in fig 1 in Lyn-deficient primary B cells. It was shown (Mukherjee 2013) that treatment of HEL B cells with SFK inhibitors repressed their stimulation by monovalent antigens, while polyvalent antigen activation was unaffected. Stimulation...
of B1-8 Lyn-deficient primary B cells with a polyvalent antigen shows moderate activation, while monovalent stimulation is completely abrogated as revealed by calcium flux. Again, it would be useful to supplement such experiments with other techniques to support the interpretation of the data. Furthermore, it would be important to assess the level of BCR (IgM and IgD) expression on these cells. The increase in IgD proximity found in fig 1e is not seen in Lyn-deficient cells. How is this explained?

In fig 3 and 4, IgM and IgD BCRs are compared for their capacity to respond to monovalent antigens using the 3046 cell line. In the cell lines that express IgM (with and without SLP65), monovalent antigen does not induce convincing calcium mobilization, although calcium flux is claimed by the authors (3046SM). Furthermore, neither of the anti-idiotypic Fabs induced calcium flux in the IgM-positive cell line, while PLA shows a strong decrease for both monovalent antigen and Ac146Fab. Therefore do not agree with the author's interpretation of the data. If this cell line does not reproduce the results found in primary cells, is it an appropriate model? Can the calcium mobilization seen in fig 1a be reproduced in a 3046 derivative expressing both IgM and IgD? In fig 4, no calcium flux is found in 3046D or 3046SD cells stimulated with monovalent antigen, while polyvalent antigen induces calcium mobilization as expected. There are differences in number of dots/cell in the two IgD cell lines. If the two cell lines express a comparable number of BCRs, does this mean that the presence of SLP65 changes the nano-organization of the BCRs on cells at rest? 3046SD presents on average only 5 dots per cell, a small number when up to ~200,000 BCRs may be found on the surface of B cells. What can the authors say about a possible bias in sampling available BCRs?

In fig 5 the authors assess whether pretreatment of B cell with monovalent antigen enhances the response to subsequent stimulation with cross-linking agents. A useful control would be to pretreat the cells with Ac38Fab which does not alter BCR proximity relationships. What is the impact of Ac38Fab on enhancement of the response to cross-linking agents?

In fig 6, the authors stimulate B cells (B1-8, 3046M, 3046SM, 3046D, 3046SD) with CD19 in addition to monovalent antigens. They show an enhanced response in all cell types (although to different extents) when combined with anti-CD19. In the last set of panels, the experiment is repeated using anti-CD20 antibodies in stead of anti-CD19, with no enhancement of the response of B cells. It would be important to compare the levels of CD19 and CD20 on 3046 cells and know more about the properties (affinities; agonistic vs antagonistic) of the antibodies used. In the present paper, 3046 cell line is described as a pre-B cell line. However, in a previous paper (Yang 2010) this cell line was described as a pre-B cell line. This discrepancy should be resolved. Minor point: As far as the discussion is concerned, OB1 B cells stimulated with 17-mer OVA peptides induced aggregation of the BCRs as revealed by STORM imaging (Avalos, 2014). The authors might want to address these findings.

Referee #2:

This paper investigates the effects of various BCR ligands on BCR cell surface distribution and calcium signaling. The authors particularly focus on the effects of ligand valency (monovalent vs multivalent) and binding site (antigen binding site or constant regions). The authors show that low valency binding induces loss of signal in their PLA assays of IgM and IgD BCR clustering and, in the case of IgM, also produces low levels of intracellular calcium. The calcium signal can be increased by further aggregation of the BCR by multivalent ligands or by aggregation of CD19. The authors conclude that the data is in conflict with a crosslinking model of BCR triggering, but can be explained by the authors' model in which antigen binding dissociates preexisting BCR clusters as a physiologically important step in BCR activation.

The paper is interesting in that it nicely illustrates that we still do not understand how the BCR is triggered by physiologic antigen binding and how the understanding is complicated by differences in ligand valency, the isotype of the BCR and the function of intracellular signaling components. Clearly, something interesting is going on that does not fit with older models of BCR triggering. Thus, the paper has potentially important implications, namely that monovalent antigen binding can be an independent trigger that can be functionally separated from BCR aggregation. This provides further support for the emerging picture that the BCR is not a structurally inert receptor, but undergoes antigen-induced changes that eventually result in reorganization and signaling.
However, the presented data do not provide any new insight into what the inactive and active forms of the BCR are or what exactly are the different antigens inducing upon binding to the IgM and IgD BCRs. BCR triggering with monovalent antigens has been observed before, although it is still controversial whether in these cases the signaling is induced in the complete absence of clustering of the receptors (this needs to be formally addressed here as well, see specific comments). Similarly, differences in signaling induced by reagents binding to different parts of the BCR have been demonstrated. The involvement of Lyn in amplification of BCR phosphorylation after engagement by low valency antigens is in line with previous observations by others and the biochemical mechanism for this has already been proposed. The finding that monovalent antigens open BCR oligomers is interesting, but the opening does not seem to be universally involved in triggering signaling, because the opening itself depends on signaling molecules such as Lyn and Syk, and, under certain situations, the opening does not lead to signaling. Altogether, there are too many unsolved discrepancies in the presented manuscript that suggest that a better model and more data are needed. For example, the authors’ data and model imply that in the inactive oligomeric state of the BCR, the antigen binding site is occluded, thus binding of the monovalent antigen to these dynamic oligomers separates them into monomers. However, the same can be assumed of the constant regions that are presumably involved in isotype specific oligomerization. It would be thus expected that some Fabs to constant region also open the oligomers, which would need to investigated. If the constant regions' engagement by Fab do not dissociate the BCR clusters, it would mean that the constant regions are far apart, which seem to be in conflict with the interpretation of the PLA experiments. Another prediction is that in cells that do not open the BCR clusters because they lack Lyn or Syk, the antigen binding site will not be fully accessible. This does not seem to be the case, but could be investigated by precise binding experiments. When nonstimulatory Fab fragments bound to the BCR are further crosslinked, the authors suggest that they pull apart BCR oligomers, explaining the resulting signaling. Why would this dissociate the oligomers rather then bring two of them together, since, as the authors assume, this also brings several protein islands together. The fact that crosslinking of CD19 increases BCR signaling hints that some reorganization of the cell surface is involved, but the positive effect of CD19 crosslinking on BCR signaling is also already known. How exactly does crosslinking of CD19 enhance signaling in the context of monovalent antigens and the dissociation model?

The authors’ interpretations are sometimes confused by self-conflicting statements such as "We found a strict correlation between the calcium response of the exposed B cells and BCR opening. However, the opening of the BCR oligomers did not always induce a calcium response". Elsewhere, reflections of previous work are inaccurate: the statement "an increased proximity or cross-linking of BCR monomers on activated B cells as predicted by the cross-linking model has never directly been observed " conflicts with published studies: there is increased proximity after multivalent antigen binding in superresolution experiments (Batista and Ploegh labs) and in FRET (Pierce lab).

Specific comments:
The authors use several different antigens to show that it is the binding to the CDR loops of the BCR that is important, not the chemistry of the antigen. However, formal evidence that the peptide and DNA antigens and the Fabs are really monovalent and do not contain aggregated fractions needs to be presented. Low level aggregation is a very common problem, especially in full buffers containing serum.

As presented, the PLA experiments do not distinguish between changes in oligomerization, conformation, localization, or just surface density of binding. Positive controls that detect all the anti-BCR antibodies with the same technique is required. Also a positive control that PLA indeed can detect changes in oligomerization after crosslinking of monomeric proteins is needed.

The choice of the proB cell line is puzzling. These cells seem to express different signaling effectors than mature B cells, as there is SLP65 independent calcium flux. The response to monovalent antigen seems barely detectable. In addition it would be essential to determine if this cell line expresses surrogate light chains as these can pair with the B1-8 heavy chains and alter the behavior of the BCR on the cell surface in a way that is very different from mature B cells.

In the IgD expressing cells, there is much less PLA signal than in any other experiment despite similar surface expression. Is there some constitutive opening? It would also be interesting to understand the relationship of the IgD BCR opening and proximal signaling. Is the BCR phosphorylated after monovalent engagement? Is the phosphorylation still required for opening?
Summary:
This paper focuses on understanding functional and conformational changes of IgM and IgD isotype BCRs in response to monomeric and multimeric antigens as well as anti-idiotype Fab that bind at or near the antigen-recognition domain.

They find that mono- and multi-meric NIP can each induce calcium flux in B1-8-lambda primary murine B cells in vitro. Interestingly, a Fab that can compete away nip-binding also can induce calcium flux, while another anti-idiotype Fab that could not (and presumably bound to a different epitope on the BCR) was not stimulatory.

They go on to show via PLA (proximity ligation assay) that 'opening up' of preformed BCR clusters (read out by loss of PLA signal) by these distinct reagents correlated with calcium flux.

They follow-up reports previously showing SFK-requirement for calcium signaling in response to monomeric antigens, and identify not only Lyn-dependence for fab fragment and monomeric antigen-induced calcium, but also convincing and surprising correlation with PLA result, implying a role for Lyn in opening BCR clusters in response to antigen.

They go on to study antigen specific IgM or IgD-expressing antigen-specific reconstituted pre-B cells. As recently reported by the Jumaa lab (Ubelhart 2015 NI), they show that IgD BCRs do not flux calcium in response to monomeric antigen in reconstituted pre-B cells, while IgM BCRs can trigger a very very slight calcium increase to monomer (that is MUCH weaker than multimer). Interestingly, loss of PLA in this system is profound and discordant with calcium signaling (i.e., not sufficient for signaling).

This result led authors to see whether pretreatment with monomer would "open" BCR clusters and augment calcium response to anti-HC/LC crosslinking, which it did. (however, mechanism for this observation may be related to loss of PLA, but is undertermined)

They then show that anti-CD19 treatment further enhances signaling by monomeric antigen by both IgM and IgD BCRs, albeit more dramatically by IgM.

General critique:
This paper addresses a fundamental topic : mechanism for BCR activation. This was a very well written manuscript, with clear and appropriate background. The data was clear and interesting although I think there are some additions (discussed below) that would be helpful.

That said, a key result using reconstituted spl-deficient B cells is that loss of PLA can be seen in the absence of calcium signaling, disaggregation of pre-clustered BCRs is NOT SUFFICIENT for BCR signal transduction. More time in the discussion should be given to this point; such disaggregation may indeed be necessary (although purely correlative data is presented here) as authors have been proposing for a long time, but it is certainly not sufficient.

Nevertheless, with changes suggested below, this paper is very appropriate for publication in Embo J and will be of great interest to the BCR signaling field.

Specific points:
(1) Would be helpful to include specificity of Fabs for PLA in materials and methods (region of IgM and IgD that they are directed against.
(2) While PLA data from fig 1 shows statistical significance, the enormous scatter of the primary data (for instance outlier values shown by bars) is concerning - although perhaps this represents population distribution of responding and non-responding cells which is seen for all in vitro signaling assays?
(3) It should also be noted that the amplitude of the PLA results do not correlate with the amplitude of the calcium assays. Further, the outlier status of Ac38 fab is seen with IgD PLA but not with IgM PLA. Further, np15 vs. NP1 PLA results don't appear very different from one another in either the images or the quantification for either IgM or IgD. Would be helpful if this nuance was addressed.
(4) Recent work also studied SFK-dependence of NP-specific BCR induced calcium signals to antigens of different valency, and should be cited/discussed together with other references

(5) It would be helpful to have lyn-/- b18 calcium fluxes directly compared to lyn+/+ b18 calcium fluxes. The manuscript refers the reader to compare figures 1 and 2 - but are these run at the same time? If they are, then they can be overlayed. If not, these should ideally be done in parallel. It is interesting in that one might have suspected that other SFKs could provide some redundancy (since non-redundant functions of Lyn have been mostly linked to ITIM-dependent pathways). Use of PP2 in these assays could be revealing.

(6) In Fig 3E the very very subtle change in calcium baseline in the slp-reconstituted cells stimulated with monovalent antigens doesn't look convincingly like a real calcium flux, or like a clear difference from the slp-deficient cells. For this reason the absence of calcium in the IgD-only reconstituted B cells in Fig 4 in response to monomer is less 'specific'. Much rests on the argument that M and D differ in their response to monomer in this system, but the difference is between a flat line and an almost flat line Fig 3E and Fig 4E. Of note, the difference between D-only and M-only cells in response to monomer is at least more visible in Fig 6b, suggesting data in 3e/4e could be improved or at least quantified to make the conclusions justified/convincing. Also including IgD and IgM results in same panel from same experiment would be important to make difference clear.

(7) Interestingly, the remarkably flat calcium with monomeric stimulation of even IgM BCRs (3E) in this system is apparently distinct from Jumaa 2015 NI results which show fairly preserved monovalent-induced calcium by IgM BCRs. Would be important to address and discuss this discordance. It is good to publish distinct results to stimulate further discussion in the field about this model of IgM and IgD responsivenss to monomeric and multimeric antigens. Is this difference due to the density of BCR expression on the surface and/or amount of stimulus provided (titration down?)? Is it due to affinity of antigen (nip v np?). By contrast, the loss of PLA in this system is very clear and convincing, but quite discordant from calcium signaling (see general point above).

Suggestions for improvement:
(a) It would be ideal to use anti-IgM and anti-IgD fabs (or anti-lambda fab which is shown in supplement) to show that these are not only non-stimulatory for calcium (as previously shown) but per model, do not give loss of PLA signal. Otherwise conclusions about mechanism for fab-mediated signaling requiring loss of PLA signal is based on 1 fab only. Would also want titration to high dose of anti-lambda fab for these assays.

(b) In settings where two different reagents are compared (e.g. IgM and IgD, or Lyn+/+ or Lyn-/-) would be ideal to show them side by side (if only in supplement).

(c) Given lyn-/- result, would be good to repeat the calcium and PLA assays using b18 cells and PP2 to clarify whether the role of lyn is kinase-dependent or not in PLA result.

(d) It would be nice to show a BCR-induced signal other than calcium (e.g. pErk) in the conditions used to see if phenomenology across lyn+/+, lyn-/-, and reconstituted cells is similar across signaling pathways downstream or not.

(e) More discussion about discordant (quantitatively) calcium results with reconstituted slp65-deficient B cells from Jumaa study would be productive. What accounts for this difference - nip vs. np, bcr expression (although seems robust in supplemental fig), conc of antigen?

(f) A more balanced and critical discussion about profound discordance between PLA assay and signaling in pre-B cells in discussion would be appropriate.

re: title - consider modifying this as the title includes a direct reference to oligomeric BCR which is really part of a model.
Referee #1:

In this manuscript, Volkmann, Brings and colleagues used a combination of calcium flux and proximity ligation assays (PLA) to assess the response of IgM and IgD BCRs to monovalent antigens. Understanding the early steps in BCR activation for different BCR isotypes remains limited and is of importance to the field of B cell biology. This paper reports several interesting findings in light of the controversial nature of the subject (monovalent activation of the BCR and the DAM hypothesis). However, I feel that the manuscript requires stronger experimental support and controls to better justify its conclusions. Several panels of the manuscript describe experiments that are almost exact repeats of experiments reported in a study from a different group (Minguet et al. 2010) but the findings the authors arrive at in the present ms. are rather different, leading to opposite conclusions. These discrepancies are not discussed in a meaningful manner anywhere in the manuscript and add confusion in a field already controversial. If there are sound reasons to discount the validity of these earlier experiments, then the reader deserves to know why. Many of the suggestions made below probably concern experimental data already in hand.

Indeed the different results of Minguet et al. 2010 using the same mouse system as in our study are apparently confusing. However, there is a simple explanation for this discrepancy. Minguet et al used B220/CD45 staining to gate for the B cells (Minguet et al, 2010) whereas we negatively selected for splenic B cells by removing CD43+ cells with MACS. A major finding of our study is that the BCR dissociation by monovalent antigens requires the presence and activity of the Src-family kinase Lyn and it is well known that the activity of Lyn is regulated by the B220/CD45 phosphatase (Shrivastava et al, 2004). Thus by using B220/CD45 staining, Minguet et al inhibited Lyn activation and did not detect the effect of monovalent antigen. We now repeated our experiment in the absence or presence of anti-B220 antibody and show that in the latter case the calcium response of NIP-specific B cell exposed to monovalent antigens is indeed strongly inhibited (New Fig. EV1D). We think that this resolves the confusion and the controversy.

In fig 1, the authors used calcium flux and proximity ligation assays (PLA) to assess whether monovalent antigens can activate the BCR and whether the nanoscale organization of the BCR is altered. Panels a-c describe experiments that replicate those of Minguet 2010 (fig 2b, 4b-c). The mouse model is the same (B1-8 k-deficient) as well as the peptide antigen (KSKGESAG with NIP on the second K and the first K harbors a fluorescent dye) and the same technique is used (Calcium flux). The Minguet paper claims that B cells were unresponsive to monovalent antigens, as judged from a combination of calcium flux, p-Tyr, P-ERK, activation marker upregulation (CD86, CD69 and MHCII) (fig 2 and 3). In the present study, the opposite result was obtained as reported for calcium flux data. It would be important to include other techniques to report on BCR activation, as well as include a control peptide not modified with NIP.

We resolved this discrepancy as explained above. We also think that our Fab-PLA assay (not available to Minguet et al in their 2010 paper) monitoring directly the (closed-open) conformation of the BCR in nanometer distances is more appropriate for the study of the immediate events in BCR activation than monitoring downstream signaling. Concerning the question of a specificity control with and without NIP, we have shown in Fig. S1D (New Fig. EV1E) a comparison of NIP-coupled and -uncoupled DNA and show that the monovalent antigen-induced BCR signaling is NIP dependent. In addition, we now also show a comparison of calcium response from 1NIP-pep treated 3046SM cells and a non-transduced internal control (New Fig. EV5A, 5B). This clearly demonstrates that the monovalent NIP antigen induced BCR signaling is depending on the expression of a NIP specific BCR.

Moreover, the calcium flux response shown in fig 1b for Ac146 Fab is not particularly convincing and is at variance with published data (Minguet et al., 2010, fig 4b). Indeed, the authors do not show the purity of their Fab preparations. Could the delayed response shown in fig 1b be due to whole antibody/F(ab)2/aggregated Fab contamination?

We now show a non-reducing SDS-PAGE to demonstrate the high purity of the used Fab fragments (New Appendix Fig. S1). Importantly, the Ac146Fab preparation is as clean as the Ac38Fab preparation, but only the Ac146Fab induces a calcium response. Thus it cannot be an aggregation
nor a contamination with undigested antibody (please note that the Ac38 antibody is activating the BCR even better than Ac146 antibody) but rather it is the different binding specificities of these anti-idiotypic antibodies that are responsible for the induced calcium response as further discussed in our manuscript. For a more detailed discussion of the aggregation problem see our response to the similar comments of Referee #2.

Another important control to include would be to stimulate the cells in serum-free medium. Serum-containing medium could induce complication of peptides in solution and thus yield a multivalent ligand.

We have now stimulated B1-8 splenic B cells with 1NIP peptide in FCS-free medium, and measured the calcium flux. Comparing with cells in medium with 1% FCS (our normal calcium assay condition), the B cells in FCS-free medium display a lower baseline of calcium signaling however, they clearly flux calcium upon exposure to 1NIP-pep (New Fig. EV1C).

My second concern is the use of PLA to assess BCR nano-organization. PLA uses fixed samples and presumably evaluates only a small number of receptors per cell, as inferred from the number of dots. Moreover, some of these dots overlap. It thus becomes difficult to assess the number of dots/cell. It was unclear whether the authors used a projection of a Z stack or only a section of cells, which depending on the focal plane analyzed- could influence the result significantly; not all images seem to have been collected in comparable focal planes).

This is a general criticism of the PLA method that is mostly put forward by scientists not familiar with the technique. For the proper quantification of PLA one dilutes the reagents so as to get only about 2-20 dots per cell to allow their quantification by the blobfinder program. It is true that some of the dots overlap and not all cells were collected in the same focal planes as this would produce noise in the PLA signaling counting. However, we are not comparing just one cell or several cells, rather, we count PLA signals in hundreds of cells from several slides for each sample with the same microscopic settings. The differences between samples are verified by statistic analysis. Please also notice that in our Fab-PLA protocol we are using non-permeabilized fixed B cells and thus are specifically monitoring only the BCR conformation on the B cell surface and not inside the cell. For further controls of our technique please see Fig. 1H and 1I of (Kläsener et al, 2014).

An additional technique such as super-resolution microscopy might be helpful to support the DAM hypothesis in this particular system.

We have used super-resolution resolution microscopy techniques before to show that the IgM-BCR and IgD-BCR are localized in different protein islands on the B cell surface but this involved sizes between 30-200 nm (Maity et al, 2015). The difference between a 10 nm closed and a 20 nm opened BCR, however, cannot be reliably monitored by any available super-resolution microscopy technique. Here we can only use our Fab-PLA technique and we previously demonstrated (please see Fig. 2 of (Kläsener et al, 2014)) that this technique clearly distinguishes between an IgM pentamer (positive Fab-PLA signal) and loosely spaced IgM monomers (no Fab-PLA signal).

In addition, it would be useful to use PLA at different time points to get a better understanding of the dynamics of BCR behavior.

PLA at different time points after B cell activation were published before (see Fig. 5 and Fig.5S1 of (Kläsener et al, 2014)). As requested we have now studied the Fab-PLA signal at different time points after monovalent antigen stimulation. We found the loss or a reduced Fab-PLA signal for at least 5 min. See Figure_A_for Referee_#1 as we do not think this is not novel enough to add to the current manuscript.

An interesting aspect not mentioned by the authors is the drastic increase of IgD:IgD dots/cell in the cells stimulated with Ac38Fab. Why do IgD BCRs become better targets for PLA but do not show any response as measured by calcium flux? It would be interesting to restimulate B1-8 cells with antigen or cross-linking agents to assess whether these cells become unresponsive after stimulation with Ac38Fab, as was shown for OVA 8-mer peptide in the OB1 model of Dougan et al (Avalos et al. 2014). Unresponsiveness of B1-8 cells and increased clustering after Ac38Fab stimulation would support the DAM hypothesis.
As suggested we have tested the prestimulation of B1-8 cells with Ac38 Fab, but we do not generate an unresponsiveness of the B cells by this treatment (Data not shown). We think that an increased oligomerization of BCR would not be able to inhibit its signaling here since the Ac38Fab binding could not fix the BCR oligomers. We previously have shown, however, that a fixed oligomerized BCR has lower responsiveness (Yang & Reth, 2010). We also want to point out that we found the increased IgD:IgD PLA signal only on splenic B cells but not on Ac38 Fab-treated 3046SD or 3046D cells with the latter being a more homogenous B cell population.

A final aspect that would be interesting to explore is whether the affinity of the BCR for its cognate monovalent antigen could affect its response using B1-8 high and B1-8 low B cells. The affinity of hapten-specific BCRs is often in the lower range of what is seen for protein antigens. Perhaps the authors should comment on the relationship, if any, between affinity and BCR clustering.

As suggested we cloned the B1-8 high-affinity (W33L) and low-affinity (A24G/S31T/H35Q/R98T) mutants and expressed them on the surface of 3046S cells as IgM-BCR. We indeed found that the affinity affects the calcium response of BCR to multivalent as well as monovalent antigen with high-affinity BCR being more effective. However, since the expression levels of the BCRs with different affinities are not identical, we did not include these data in our manuscript.

In fig 2, the authors repeat the experiment shown in fig 1 in Lyn-deficient primary B cells. It was shown (Mukherjee 2013) that treatment of HEL B cells with SFK inhibitors repressed their stimulation by monovalent antigens, while polyvalent antigen activation was unaffected. Stimulation of B1-8 Lyn-deficient primary B cells with a polyvalent antigen shows moderate activation, while monovalent stimulation is completely abrogated as revealed by calcium flux. Again, it would be useful to supplement such experiments with other techniques to support the interpretation of the data.

We are pleased that our study using B1-8 Lyn-deficient primary B cells are in line with the study of Mukherjee et al. using SFK inhibitors (Mukherjee et al., 2013). So our conclusion that the SFK Lyn is required for the sensing of monovalent antigens is now supported by at least two studies using different techniques. The paper of Mukherjee et al., however, did not study directly BCR cross-linking or BCR opening. We previously have demonstrated the formation of BCR oligomers on the surface of resting B cells with several techniques (BN-PAGE and BiFC). However, to monitor the opening of BCR at 10-20 nanometer distances we currently can only employ the Fab-PLA method. We thus do not know what other techniques we could use to further support the interpretation of our data.

Furthermore, it would be important to assess the level of BCR (IgM and IgD) expression on these cells.

We now conducted a FACScan analysis that shows only subtle difference in the IgM+IgD+ mature B cell pool of B1-8 and Lyn-deficient B1-8 mice (New Appendix Fig. S4).

The increase in IgD proximity found in fig 1e is not seen in Lyn-deficient cells. How is this explained?

As mentioned above we do not see the increase in IgD proximity in the studied 3046 B cells. So this seems to be a phenomenon of mature splenic B cells. On these B cells the IgD-BCR is localized in a class-specific protein island in close proximity to CD19 that also interacts with SFK such as Lyn. In another unpublished study we have found that the IgD-BCR can influence the conformation of CD19 and it might also be true that CD19 can influence the organization of IgD but this requires further experiments.

In fig 3 and 4, IgM and IgD BCRs are compared for their capacity to respond to monovalent antigens using the 3046 cell line. In the cell lines that express IgM (with and without SLP65), monovalent antigen does not induce convincing calcium mobilization, although calcium flux is claimed by the authors (3046SM). Furthermore, neither of the anti-idiotypic Fabs induced calcium flux in the IgM-positive cell line, while PLA shows a strong decrease for both monovalent antigen
and Ac146fab. I therefore do not agree with the author's interpretation of the data. If this cell line does not reproduce the results found in primary cells, is it an appropriate model? Can the calcium mobilization seen in fig 1a be reproduced in a 3046 derivative expressing both IgM and IgD?

We now provide data comparing the calcium signal of BCR<sup>+</sup> and BCR<sup>-</sup> (as internal control) 3046SM B cells exposed to monovalent antigen or anti-idiotypic Fab to validate our claims and also show that the anti-idiotypic AC146Fab induces a calcium flux in the IgM-positive cell line 3046SM (New Fig. EV5 B and C). Furthermore we want to point out that in the presence of anti-CD19 antibodies (that hardly gives a response alone) the monovalent antigen induces a calcium mobilization in 3046SM B cells (Fig. 6).

As we now discuss in more detail in our manuscript and mention in our answer to Referee #3, there is no one-to-one correlation between BCR opening and calcium signaling due to the extensive amplification of the calcium signal and its special requirements. We think that this is an important conclusion from our study that may initiate more research in this matter.

In fig 4, no calcium flux is found in 3046D or 3046SD cells stimulated with monovalent antigen, while polyvalent antigen induces calcium mobilization as expected. There are differences in number of dots/cell in the two IgD cell lines. If the two cell lines express a comparable number of BCRs, does this mean that the presence of SLp65 changes the nano-organization of the BCRs on cells at rest? 3046SD presents on average only 5 dots per cell, a small number when up to ~200,000 BCRs may be found on the surface of B cells. What can the authors say about a possible bias in sampling available BCRs?

We noticed that for the original Fig. 4, we showed data derived from two different batches of anti-IgD Fab used in the Fab-PLA analysis of 3046SD and 3046D B cells. We now show the data from the Fab-PLA experiments using the same batch of Fab, and we get similar number of dots/cell for 3046SD and 3046D (See now modified Fig. 4A, 4C).

In fig 5 the authors assess whether pretreatment of B cell with monovalent antigen enhances the response to subsequent stimulation with cross-linking agents. A useful control would be to pretreat the cells with Ac38Fab which does not alter BCR proximity relationships. What is the impact of Ac38Fab on enhancement of the response to cross-linking agents?

We have now performed this experiment and found that the Ac38Fab treatment did not enhance the response to further anti-Ig cross-linking (see New Appendix Fig. S8).

In fig 6, the authors stimulate B cells (B1-8, 3046M, 3046SM, 3046D, 3046SD) with CD19 in addition to monovalent antigens. They show an enhanced response in all cell types (although to different extents) when combined with anti-CD19. In the last set of panels, the experiment is repeated using anti-CD20 antibodies in stead of anti-CD19, with no enhancement of the response of B cells. It would be important to compare the levels of CD19 and CD20 on 3046 cells and know more about the properties (affinities; agonistic vs antagonistic) of the antibodies used.

We have tested the surface expression of CD19 and CD20 on 3046 cells by surface staining. Their expression was not different between IgD-BCR or IgM BCR producing B cells (See new Appendix Fig. S9). The clone number and source of the antibody are mentioned in our Material and Methods section. The anti-CD19 antibody is known to be agonistic and the anti-CD20 antibody property is unknown.

In the present paper, 3046 cell line is described as a pro-B cell line. However, in a previous paper (Yang 2010) this cell line was described as a pre-B cell line. This discrepancy should be resolved.

The difference between a pro-B and pre-B cell is the HC expression. The original line was lacking Iga and SLP-65 and was clearly a pro-B cell and we are now referring to this in our manuscript.

Minor point: As far as the discussion is concerned, OB1 B cells stimulated with 17-mer OVA peptides induced aggregation of the BCRs as revealed by STORM imaging (Avalos, 2014). The authors might want to address these findings.

In the Avalos paper, the STORM images were acquired 15 min after the treatment of a 17-mer OVA peptide in the presence of 100 mM dynasore. These are quite different conditions to our experiment:
1 min stimulation, without additives. A similar STORM experiment reported by Mattila et al (Mattila et al., 2013), Fig. 6A and B) does not detect a significant difference of BCR clustering between resting and activated MD4 B cells. Furthermore, although Avalos et al. showed that antigen induces BCR clusters, the increase of cluster size (from 22 to 40) is similar or even a bit more than the increase of the number of localization per cluster (from 290 to 400) (Avalos et al., 2014), Fig.1H). Thus at nanoscale distances the BCR in these clusters seems to have a reduced rather than an increased proximity. To our knowledge no super-resolution microscopy technique can reliably distinguish between a closed or more open receptor clusters at 10-20 nm distances. As we mention in our manuscript the loss of the Fab-PLA signal is not excluding the formation of BCR cluster with a more distance spacing of the BCR.

Referee #2:

This paper investigates the effects of various BCR ligands on BCR cell surface distribution and calcium signaling. The authors particularly focus on the effects of ligand valency (monovalent vs multivalent) and binding site (antigen binding site or constant regions). The authors show that low valency binding induces loss of signal in their PLA assays of IgM and IgD BCR clustering and, in the case of IgM, also produces low levels of intracellular calcium. The calcium signal can be increased by further aggregation of the BCR by multivalent ligands or by aggregation of CD19. The authors conclude that the data is in conflict with a crosslinking model of BCR triggering, but can be explained by the authors' model in which antigen binding dissociates preexisting BCR clusters as a physiologically important step in BCR activation.

The paper is interesting in that it nicely illustrates that we still do not understand how the BCR is triggered by physiologic antigen binding and how the understanding is complicated by differences in ligand valency, the isotype of the BCR and the function of intracellular signaling components. Clearly, something interesting is going on that does not fit with older models of BCR triggering. Thus, the paper has potentially important implications, namely that monovalent antigen binding can be an independent trigger that can be functionally separated from BCR aggregation. This provides further support for the emerging picture that the BCR is not a structurally inert receptor, but undergoes antigen-induced changes that eventually result in reorganization and signaling. However, the presented data do not provide any new insight into what the inactive and active forms of the BCR are or what exactly are the different antigens inducing upon binding to the IgM and IgD BCRs. BCR triggering with monovalent antigens has been observed before, although it is still controversial whether in these cases the signaling is induced in the complete absence of clustering of the receptors (this needs to be formally addressed here as well, see specific comments). Similarly, differences in signaling induced by reagents binding to different parts of the BCR have been demonstrated. The involvement of Lyn in amplification of BCR phosphorylation after engagement by low valency antigens is in line with previous observations by others and the biochemical mechanism for this has already been proposed.

We agree with referee #2 that on antigen-exposed B cell “something interesting is going on that does not fit with older models of BCR triggering”.

We do not agree with referee #2 that “the biochemical mechanism for this (our Lyn data) has already been proposed”. Previous studies only used SFK inhibitors whereas we are using antigen (NIP)-specific B cell from Lyn KO mice and study for the first time the nanoscale conformation of the BCR on these cells before and after exposure to monovalent antigens.

The finding that monovalent antigens open BCR oligomers is interesting, but the opening does not seem to be universally involved in triggering signaling, because the opening itself depends on signaling molecules such as Lyn and Syk, and, under certain situations, the opening does not lead to signaling. Altogether, there are too many unsolved discrepancies in the presented manuscript that suggest that a better model and more data are needed. For example, the authors' data and model imply that in the inactive oligomeric state of the BCR, the antigen binding site is occluded, thus binding of the monovalent antigen to these dynamic oligomers separates them into monomers. However, the same can be assumed of the constant regions that are presumably involved in isotype specific oligomerization. It would be thus expected that some Fabs to constant region also open the oligomers, which would need to investigated. If the constant regions' engagement by Fab
do not dissociate the BCR clusters, it would mean that the constant regions are far apart, which seems to be in conflict with the interpretation of the PLA experiments.

We do not understand these arguments. The biological role of the BCR is to sense antigen and it thus seems logical to us this involves the variable parts of the BCR carrying the antigen-binding site and not the constant regions of the BCR that are evolutionary selected to optimally perform the sensing process. Furthermore the hinge regions of the BCR have been implicated in the antigen sensing and signaling process (Ubelhart et al., 2015). If the Fab arms connected by the hinge region become more flexible and change their position upon antigen binding this can induce the conformational change and BCR opening we observe in our Fab-PLA study. We have now tested the reactivity of an anti-lambda constant part Fab fragment and found that it cannot open the BCR (New Appendix Fig. S3).

Another prediction is that in cells that do not open the BCR clusters because they lack Lyn or Syk, the antigen binding site will not be fully accessible. This does not seem to be the case, but could be investigated by precise binding experiments. When nonstimulatory Fab fragments bound to the BCR are further crosslinked, the authors suggest that they pull apart BCR oligomers, explaining the resulting signaling. Why would this dissociate the oligomers rather then bring two of them together, since, as the authors assume, this also brings several protein islands together.

These arguments ignore the critical nanometer distances involved in the BCR activation process and in our Fab-PLA assay. Yes, the BCR can form microcluster upon cross-linking with antigens or anti-BCR antibodies. But that is happening in a scale of 100 nm to 1000 nm. However, in our Fab-PLA studies, we showed that the nanometer spacing of the BCR monomers are different between the resting and antibody cross-linked BCR. We also have clearly shown that these 10-20 nanometer alterations are only detected by Fab-PLA but not by standard PLA (Kläsener et al., 2014). Similarly, some super resolution studies detect BCR cluster upon antigen exposure but these studies cannot determine the critical alterations and the different spacing of the BCR at 10-20 nanometer distances (see also below).

The fact that crosslinking of CD19 increases BCR signaling hints that some reorganization of the cell surface is involved, but the upon positive effect of CD19 crosslinking on BCR signaling is also already known. How exactly does crosslinking of CD19 enhance signaling in the context of monovalent antigens and the dissociation model?

There clearly remains more to learn about the role of CD19 in this process but this is not the focus of this manuscript.

The authors' interpretations are sometimes confused by self-conflicting statements such as "We found a strict correlation between the calcium response of the exposed B cells and BCR opening. However, the opening of the BCR oligomers did not always induce a calcium response".

We apologize for the confusion but our findings are clear. Whenever we monitor a calcium response upon BCR activation we also detect BCR dissociation. However, the opening of the BCR oligomers did not always induce a calcium response. Why is this a self-conflicting statement?

Elsewhere, reflections of previous work are inaccurate: the statement "an increased proximity or cross-linking of BCR monomers on activated B cells as predicted by the cross-linking model has never directly been observed " conflicts with published studies: there is increased proximity after multivalent antigen binding in superresolution experiments (Batista and Ploegh labs) and in FRET (Pierce lab).

We respectfully disagree. There is no direct conflict between our data and published studies. Mattila et al did not detect an increased proximity between resting and activated MD4 B cells in their superresolution experiments ((Mattila et al., 2013), Fig. 6A, 6B). The results of Avalos et al show BCR clustering but no increase in BCR proximity as discussed in our response to referee #1 (see (Avalos et al., 2014), Fig.1H). Most of the FRET data in (Tolar et al., 2005) actually also detect a reduced proximity upon activation (BCR dissociation rather than aggregation). If one ignores the first time point (that is always low and always only one data point!) then one clearly sees a reduction of the FRET signal over time. Interestingly this reduction of the FRET signal is prevented in the...
The presence of the SFK inhibitor PP2 and we have observed the same BCR behavior in our Fab-PLA study (see Figure_for_Referee_#2). We thus do not see a conflict between our data and these publications but rather a conflict in their interpretation. However we agree it would be nice to see more convincing FRET data of the BCR activation process using Fluorescence-lifetime imaging microscopy (FLIM) measurements rather than the intensity based measurements of Tolar et al that can be strongly influenced not only by the proximity but also by the concentration of the fluorescent proteins.

We have now deleted the cited sentence from our manuscript.

Specific comments:

The authors use several different antigens to show that it is the binding to the CDR loops of the BCR that is important, not the chemistry of the antigen. However, formal evidence that the peptide and DNA antigens and the Fab are really monovalent and do not contain aggregated fractions needs to be presented. Low level aggregation is a very common problem, especially in full buffers containing serum.

The fact that many monovalent antigens can indeed activate BCR signaling was a major problem for the cross-linking hypothesis from the beginning. To solve this conflict (a monovalent antigen per se cannot cross-link a receptor) the proponents of this hypothesis always put forward the argument that in case BCR signaling is due to the aggregation of monovalent antigen, a possibility that is difficult to exclude by biochemical experiments. In our study, however, we finally obtained the tools to clarify this issue. First we show that Lyn-deficient B cell can be activated by polyvalent but not by monovalent antigens. Thus any aggregated monovalent antigen or anti-BCR reagent should also activate these Lyn-deficient B cells. This is clearly not the case for our 1NIP-pep or Ac146Fab reagents. Second, we found in our study a striking difference in the activity of the two anti-idiotypic Fab reagents Ac146Fab and Ac38Fab with only the former one able to activate the BCR. Biochemically the two monovalent reagents look identical (see New Appendix Fig. S1). Thus we think it is the different epitope specificity (as discussed in our manuscript) rather than aggregation that explains the activity of Ac146Fab. Finally, as suggested by Referee #1 and #2 we have now performed the calcium measurement with FCS free medium, and we could observe the same calcium response to monovalent antigen (New Fig. EV1C).

As presented, the PLA experiments do not distinguish between changes in oligomerization, conformation, localization, or just surface density of binding. Positive controls that detect all the anti-BCR antibodies with the same technique is required. Also a positive control that PLA indeed can detect changes in oligomerization after crosslinking of monomeric proteins is needed.

The activation of the BCR is accompanied by changes in oligomerization, conformation and localization and there are no techniques that can distinguish between these intimately connected events. Our Fab-PLA study can only monitor changes in the 10-20 nanometer proximity of the BCR that is associated with the BCR activation process. Positive controls that the Fab-PLA technique in contrast to classical PLA can distinguish an IgM oligomer from an IgM monomer are shown in Fig 2 of (Kläsener et al., 2014).

The choice of the pro-B cell line is puzzling. These cells seem to express different signaling effectors than mature B cells, as there is SLIP65 independent calcium flux. The response to monovalent antigen seems barely detectable.

Similar pro-B cell systems have been successfully used in many BCR signaling studies and are regarded as useful model by the B cell field (Storch et al., 2007; Ubelhart et al., 2015; Maity et al., 2015; Minden et al., 2012; Meixelsperger et al., 2007). Also the response to monovalent antigen stimulation is clearly visible in these B cells when directly compared to the internal control (New Fig.EV5). However, we use this pro-B cell system only to study the specific behavior of the IgM-BCR and IgD-BCR as their coexpression on normal B cells excludes such a separated study. Most other studies reported in our manuscript are done with normal splenic B cells from the Bi-8 mouse.

In addition it would be essential to determine if this cell line expresses surrogate light chains as these can pair with the B1-8 heavy chains and alter the behavior of the BCR on the cell surface in a way that is very different from mature B cells.
The cells express some surrogate light chain. However, we now show that the normal LC strongly inhibits the expression of these chains on the B cell surface (New Appendix Fig. S6). Thus most HC expressed on the surface of these B cells are part of the mature BCR.

In the IgD expressing cells, there is much less PLA signal than in any other experiment despite similar surface expression. Is there some constitutive opening?

Please see our answer to Referee #1 for the same question.

It would also be interesting to understand the relationship of the IgD BCR opening and proximal signaling. Is the BCR phosphorylated after monovalent engagement? Is the phosphorylation still required for opening?

In B1-8 cells, after monovalent engagement, we see minor changes of phosphorylation of Iga (New Fig. EV2). When we inhibit the kinase activity of Lyn, monovalent antigen cannot open both IgD and IgM-BCR (New Fig. EV4), suggesting the phosphorylation maybe needed for opening.

Referee #3:

Summary:
This paper focuses on understanding functional and conformational changes of IgM and IgD isotype BCRs in response to monomeric and multimeric antigens as well as anti-idiotype Fabs that bind at or near the antigen-recognition domain.

They find that mono- and multi-meric NIP can each induce calcium flux in B1-8-lambda primary murine B cells in vitro. Interestingly, a Fab that can compete away nip-binding also can induce calcium flux, while another anti-idiotype Fab that could not (and presumably bound to a different epitope on the BCR) was not stimulatory.

They go on to show via PLA (proximity ligation assay) that 'opening up' of preformed BCR clusters (read out by loss of PLA signal) by these distinct reagents correlated with calcium flux.

They follow-up reports previously showing SFK-requirement for calcium signaling in response to monomeric antigens, and identify not only Lyn-dependence for fab fragment and monomeric antigen-induced calcium, but also convincing and surprising correlation with PLA result, implying a role for Lyn in opening BCR clusters in response to antigen.

They go on to study antigen specific IgM or IgD-expressing antigen-specific reconstituted pre-B cells. As recently reported by the Jumaa lab (Ubelhart 2015 N1), they show that IgD BCRs do not flux calcium in response to monomeric antigen in reconstituted pre-B cells, while IgM BCRs can trigger a very very slight calcium increase to monomer (that is MUCH weaker than multimer). Interestingly, loss of PLA in this system is profound and discordant with calcium signaling (i.e., not sufficient for signaling).

This result led authors to see whether pretreatment with monomer would "open" BCR clusters and augment calcium response to anti-HC/LC crosslinking, which it did. (however, mechanism for this observation may be related to loss of PLA, but is undertermined)

They then show that anti-CD19 treatment further enhances signaling by monomeric antigen by both IgM and IgD BCRs, albeit more dramatically by IgM.

General critique:
This paper addresses a fundamental topic : mechanism for BCR activation. This was a very well written manuscript, with clear and appropriate background. The data was clear and interesting although I think there are some additions (discussed below) that would be helpful.

That said, a key result using reconstituted slp-deficient B cells is that loss of PLA can be seen in the absence of calcium signaling, disaggregation of pre-clustered BCRs is NOT SUFFICIENT for BCR
signal transduction. More time in the discussion should be given to this point; such disaggregation may indeed be necessary (although purely correlative data is presented here) as authors have been proposing for a long time, but it is certainly not sufficient. Nevertheless, with changes suggested below, this paper is very appropriate for publication in Embo J and will be of great interest to the BCR signaling field.

We appreciate the opinion of Referee #3 that our manuscript is appropriate for publication in EMBO J and it will be of great interest to the BCR signaling field.

Specific points:
(1) Would be helpful to include specificity of Fabs for PLA in materials and methods (region of IgM and IgD that they are directed against.

As cited in our materials and methods section, we are using commercially available antibodies for the anti-Ig Fab preparation but unfortunately these companies do not know or provide the exact epitope of the reagents.

(2) While PLA data from fig 1 shows statistical significance, the enormous scatter of the primary data (for instance outlier values shown by bars) is concerning - although perhaps this represents population distribution of responding and non-responding cells which is seen for all in vitro signaling assays?

The referee is right, there is some scattering of the PLA data that is likely to be due to heterogeneity of the B cell population plus the noise from the assay itself. Actually, not all B cells respond equally well to stimuli on a cell-to-cell basis in most published studies. Therefore with our Fab-PLA studies we are testing hundreds of B cells in at least three times repeated experiments to obtain statistically valuable data. Although we see some variation in the amount of the detected dots/cell, the assay as such is highly reproducible in that we always see the reduction of the Fab-PLA signal upon NIP-BSA, 1NIP-pep or Ac146Fab treatment but not with Ac38Fab.

(3) It should also be noted that the amplitude of the PLA results do not correlate with the amplitude of the calcium assays. Further, the outlier status of Ac38 fab is seen with IgD PLA but not with IgM PLA. Further, np15 vs. NP1 PLA results don't appear very different from one another in either the images or the quantification for either IgM or IgD. Would be helpful if this nuance was addressed.

Please note that the quantity of the B cell calcium signal is affected by several feedback and feed forward loops. We recently found that B cells with tiny amounts of Syk display a normal calcium signal although they have strongly reduced tyrosine phosphorylation levels (unpublished). Thus due to the extensive amplification of the calcium signal there is no one-to-one correlation between BCR activation and calcium signaling. Therefore it was so important for us to employ with Fab-PLA a method that can directly monitor alterations of BCR conformational in the way no other existing technique can do so.


We now have mentioned this paper in our discussion.

(5) It would be helpful to have lyn-/- b18 calcium fluxes directly compared to lyn+/+ b18 calcium fluxes. The manuscript refers the reader to compare figures 1 and 2 - but are these run at the same time? If they are, then they can be overlayed. If not, these should ideally be done in parallel. It is interesting in that one might have suspected that other SFKs could provide some redundancy (since non-redundant functions of Lyn have been mostly linked to ITIM-dependent pathways). Use of PP2 in these assays could be revealing.
As suggested we have now studied the calcium response of wt and Lyn-deficient B1-8 cells in parallel (New Fig. EV3). We also treated the wt B1-8 cells with the SFK inhibitor PP2. This treatment also inhibits the BCR opening and calcium signaling (New Fig. EV4).

(6) In Fig 3E the very subtle change in calcium baseline in the slp-reconstituted cells stimulated with monovalent antigens doesn't look convincingly like a real calcium flux, or like a clear difference from the slp-deficient cells. For this reason the absence of calcium in the IgD-only reconstituted B cells in Fig 4 in response to monomer is less 'specific'. Much rests on the argument that M and D differ in their response to monomer in this system, but the difference is between a flat line and an almost flat line Fig 3E and Fig 4E. Of note, the difference between D-only and M-only cells in response to monomer is at least more visible in Fig 6b, suggesting data in 3e/4e could be improved or at least quantified to make the conclusions justified/convincing. Also including IgD and IgM results in same panel from same experiment would be important to make difference clear.

We now provide data comparing the calcium signal of BCR+ and BCR- (as internal control) 3046SM B cells exposed to monovalent antigen or anti-idiotypic Fab to validate our claims (new Fig. EV5B, 5C). Please also note that in the presence of anti-CD19 antibodies (that hardly gives a response alone) the effects of monovalent antigen are no longer “very very subtle change in calcium baseline” but huge (Fig. 6B). In the new Fig.EV5D, 5E, we now include results for 3046SD and 3046SM cells in the same panel from the same experiments to better demonstrate the difference between IgD and IgM.

(7) Interestingly, the remarkably flat calcium with monomeric stimulation of even IgM BCRs (3E) in this system is apparently distinct from Jumaa 2015 NI results which show fairly preserved monovalent-induced calcium by IgM BCRs. Would be important to address and discuss this discordance. It is good to publish distinct results to stimulate further discussion in the field about this model of IgM and IgD responsiveness to monomeric and multimeric antigens. Is this difference due to the density of BCR expression on the surface and/or amount of stimulus provided (titration down?) Is it due to affinity of antigen (nip v np?). By contrast, the loss of PLA in this system is very clear and convincing, but quite discordant from calcium signaling (see general point above).

Please note that the study of Jumaa 2015 NI is using a bigger peptide with more positively charged amino acids (20 aa peptide containing 6 Lys residues) as well as a different pro B cell line and these issues could influence the extent of the calcium response. Nevertheless both studies fully agree in their finding that only IgM but not IgD producing B cell respond to monovalent antigens with a calcium flux. Furthermore we also can get an increased calcium response by using higher concentration of the 1NIP-pep (Fig. Ev5E). Concentration, affinity and the exact composition of the monovalent antigen can clearly modify the extent of the calcium response. We thus agree with the reviewer that by measuring BCR proximal event with Fab-PLA we provide more clear data on BCR activation. That there is a discordance between BCR opening and calcium signaling we find actually very exciting as this tells us that there is clearly more to learn about the mechanism connecting BCR opening to calcium channel opening.

Suggestions for improvement:
(a) It would be ideal to use anti-IgM and anti-IgD fabs (or anti-lambda fab which is shown in supplement) to show that these are not only non-stimulatory for calcium (as previously shown) but per model, do not give loss of PLA signal. Otherwise conclusions about mechanism for fab-mediated signaling requiring loss of PLA signal is based on 1 fab only. Would also want titration to high dose of anti-lambda fab for these assays.

We now show that the anti-lambda Fab is not able to open the IgD IgM-BCR and IgD-BCR on B1-8 splenic B cells (New Appendix Fig. S3).

(b) In settings where two different reagents are compared (e.g. IgM and IgD, or Lyn+/+ or Lyn-/-) would be ideal to show them side by side (if only in supplement). For reconstituted cell calcium experiments where monovalent-induced calcium flux is so small (Fig 3 vs. Fig 4), statistical analysis of multiple experiments needs to be presented to make this convincing.
As suggested we have now studied the calcium response of wt and Lyn-deficient B1-8 cells in parallel (New Fig. EV3). Furthermore, we provide data comparing the calcium signal of BCR⁺ and BCR⁻ (as internal control) 3046SM B cells exposed to monovalent antigen or anti-idiotypic Fab to validate our claims. (New Fig. EV5B, 5C).

(c) Given lyn⁻/⁻ result, would be good to repeat the calcium and PLA assays using b18 cells and PP2 to clarify whether the role of lyn is kinase-dependent or not in PLA result.

Using PP2 as a SFK inhibitor we now show that BCR opening by a monovalent antigen and calcium response requires the kinase activity of Lyn (New Fig. EV4)

(d) It would be nice to show a BCR-induced signal other than calcium (e.g. pErk) in the conditions used to see if phenomenology across lyn⁺/⁺, lyn⁻/⁻, and reconstituted cells is similar across signaling pathways downstream or not.

We now show that in comparison to the polyvalent NIP-BSA the monovalent antigens are inducing only a weak AKT, ERK and Iga phosphorylation response in B1-8 cells (New Fig. EV2). We thus did not compare lyn⁺/⁺, lyn⁻/⁻ B cells in these assays.

(e) More discussion about discordant (quantitatively) calcium results with reconstituted slp65-deficient B cells from Jumaa study would be productive. What accounts for this difference - nip vs. np, bcr expression (although seems robust in supplemental fig), conc of antigen?

We have addressed this point above.

(f) A more balanced and critical discussion about profound discordance between PLA assay and signaling in pre-B cells in discussion would be appropriate

We think that there is no discordance between the PLA assay and signaling but rather between BCR opening and the calcium response that we are now discussing in our manuscript in the context of the compartmentalization of the B cell plasma membrane. There is a strong correlation between them: Whenever we measure a calcium response upon BCR activation we also detect BCR opening. However, the opening of the BCR oligomers does not always induce a calcium response. Thus what happens after BCR opening and the communication between the BCR and the calcium channel is clearly a matter of further discussions and experiments.

re: title - consider modifying this as the title includes a direct reference to oligomeric BCR which is really part of a model.

We removed the word oligomer from the title.
**Figure A for Referee #1.** Monovalent anti-IgM binding opens BCR oligomers on splenic B cells.
Proximity between IgM-BCRs and IgD-BCRs on the surface of splenic B cells isolated from B1-8 transgenic mice cells before and after 1 min stimulation with the indicated reagents, assayed by Fab-PLA. (A) Representative microscopy images showing Fab-PLA results measuring the BCR proximity for IgM-BCR (upper) and IgD-BCR (lower) on untreated or treated splenic B1-8 B cells. PLA signals are shown as red dots and the nuclei are visualized by DAPI staining. Scale bar: 5 μm. (B) The Fab-PLA results are quantified by Blobfinder software and presented as box plots. The median values are highlighted as thick lines and the whiskers represent the minimum and maximum value. PLA signals (dots/cells) were counted from at least 100 cells for each sample. Data from treated samples were compared to data from untreated samples, p-values were calculated by Kruskal–Wallis one-way analysis of variance (ANOVA).

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**Figure B for Referee #1.** Monovalent antigen induced BCR signaling is affected by the antigen affinity.
A. FACScan analysis of the expression of low- (A24G/S31T/H35Q/R98T mutant), medium- and high- (W33L mutant) affinity B1-8 IgM-BCR in 3046S cells.
B, C. Calcium response measured by FACScan for 3046S cells expressing the low-, medium- and high-affinity B1-8 IgM BCR upon the stimulation of (B) NIP15-BSA (30 pM) or (C) 1NIP-pep (80 nM).
References:


Maity PC, Blount A, Jumaa H, Ronneberger O, Lillemeier BF & Reth M (2015) B cell antigen receptors of the IgM and IgD classes are clustered in different protein islands that are altered during B cell activation. *Sci Signal* 8:ra93–ra93


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Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the three referees.

The referees appreciate your response, the introduced revisions and support publication here. Referee #2 suggests the inclusion of some additional controls. The PLA control should be feasible. How about the Fab oligomerisation experiment? Take a look at the concerns raised and we can discuss further if needed.

REFEREE REPORTS

Referee #1:

I have looked at the authors' response to my review and find that they have adequately addressed my concerns, several of which were shared by the other reviewers. I think this paper should now be accepted with the modifications included. It is an important contribution to an ongoing debate.

Referee #2:

This is an improved manuscript that rectifies some, but not all the raised issues. First, I strongly suggest that experimental characterization of the valency of the antigens and Fab fragments is documented in the paper with state-of-the-art techniques. I fully agree that the data show that the Fab binding to the idiotypic site gives a different response to the Fab binding outside, and that the NIP-peptide/oligo give a different response to the NIP15-BSA. However, this does not demonstrate that the reagents are monovalent. For example, it is possible that the difference in responses to the Fabs engaging different sites on the BCR occur in conjunction with aggregation. Or that different NIP-antigens induce different size and dynamics of clusters, which could trigger Lyn sufficient and deficient B cells with different efficiency. These are not just theoretical scenarios. There is a precedence for V region-mediated, crosslinking-induced responses, such as the signaling induced by the apparently monomeric cytokinergic IgE on mast cells, which also induces cellular responses that are different from IgE-crosslinking by multivalent antigens (see e.g. Bax HJ et al. Sci Rep. 2015, 5:9538, or Kitaura J et al. Proc Natl Acad Sci USA. 2003, 100:12911-6). Unusual sandwiching of monomeric antigens between antibody binding sites has also been reported (Hattori T, et al. Proc Natl Acad Sci USA. 2016, 113:2092-7) and can lead to dimerization. Therefore, rigorous steps have to be taken to characterize the reagents and their effects. The gel for the Fabs is a step in the right direction, but the state of oligomerization and valency has to be determined in the native form, ideally in the presence of the B1-8 antibody. This is nothing against the validity of the results in this manuscript, just that for the reproducibility of these studies in the future, the properties of the reagents should be documented, whatever the results are. There is a number of techniques that can measure oligomerization of the antigens/Fabs with and without the B1-8, including analytical gel filtration, multi-angle light scattering, or fluorescence studies. For the NIP-reagents, mass spectrometry showing that they contain only one NIP group would also help. Second, controls for the PLA are still missing. All we see is a loss of signal after activation, which may be caused by reduction in average proximity, but also by reduction in the amount of the reagents bound to the cells. This is important to control, because in general, changes in clustering at the nanoscale change the molecular accessibility for the staining reagents. A side by side control that quantifies binding of the reagents independently of their proximity is required to clear this issue. Last, a minor comment is that the gates in the IgM vs IgD plots in the new Fig S4 are not in the same place in the two plots and therefore give the wrong impression that IgM levels are the same between the Lyn +/- and Lyn +/- B cells. It rather appears that the levels of IgM are lower on the Lyn +/- cells, which should be noted.

Referee #3:

Revision of Volkmann et al. for Embo
This revision appropriately responds to reviewer comments. Modifications of the text and references, and new figures with important comparisons and controls are a very nice addition, especially EV3-5. I support publication at this point.

2nd Revision - authors’ response 15 August 2016

Referee #1:

I have looked at the authors’ response to my review and find that they have adequately addressed my concerns, several of which were shared by the other reviewers. I think this paper should now be accepted with the modifications included. It is an important contribution to an ongoing debate.

Referee #3:

Revision of Volkmann et al. for Embo

This revision appropriately responds to reviewer comments. Modifications of the text and references, and new figures with important comparisons and controls are a very nice addition, especially EV3-5. I support publication at this point.

We are pleased to learn that Referee#1 and Referee#3 are now fully satisfied with the modification and the improvement we did to our manuscript according to their suggestions and recommend the publication of our studies.

In particular, referee 1 who raised several important points, which we could address in the revised version of our manuscript, is now clearly stating that our manuscript is an "important contribution to the ongoing debate".

Referee #2:

This is an improved manuscript that rectifies some, but not all the raised issues. First, I strongly suggest that experimental characterization of the valency of the antigens and Fab fragments is documented in the paper with state-of-the-art techniques. I fully agree that the data show that the Fab binding to the idiotype site gives a different response to the Fab binding outside, and that the NIP-peptide/oligo give a different response to the NIP15-BSA. However, this does not demonstrate that the reagents are monovalent. For example, it is possible that the difference in responses to the Fabs engaging different sites on the BCR occur in conjunction with aggregation. Or that different NIP-antigens induce different size and dynamics of clusters, which could trigger Lyn sufficient and deficient B cells with different efficiency. These are not just theoretical scenarios.

There is a precedence for V region-mediated, crosslinking-induced responses, such as the signaling induced by the apparently monomeric cytokinergic IgE on mast cells, which also induces cellular responses that are different from IgE-crosslinking by multivalent antigens (see e.g. Bax HJ et al. Sci Rep. 2015, 5:9538, or Kitaura J et al. Proc Natl Acad Sci USA. 2003, 100:12911-6). Unusual sandwiching of monomeric antigens between antibody binding sites has also been reported (Hattori T, et al. Proc Natl Acad Sci USA. 2016, 113:2092-7) and can lead to dimerization. Therefore, rigorous steps have to be taken to characterize the reagents and their effects. The gel for the Fabs is a step in the right direction, but the state of oligomerization and valency has to be determined in the native form, ideally in the presence of the B1-8 antibody. This is nothing against the validity of the results in this manuscript, just that for the reproducibility of these studies in the future, the properties of the reagents should to be documented, whatever the results are. There is a number of techniques that can measure oligomerization of the antigens/Fabs with and without the B1-8, including analytical gel filtration, multi-angle light scattering, or fluorescence studies. For the NIP-reagents, mass spectrometry showing that they contain only one NIP group would also help.

The possible aggregation of monovalent antigens we have already addressed in our last point to point answer to referee#1. Indeed it is very difficult to exclude a possible antigen aggregation by biochemical experiments. How should one distinguish intra versus inter molecular binding of a small hapten-coupled peptide monomer versus dimer to the 900 kDa pentameric B1-8 IgM
molecule? We are therefore pleased that with the B1-8/Lyn KO B cells we have a genetic tool on hand that can distinguish between polymeric or aggregated antigen and monovalent ones as the later ones are not sensed at all by these B cells and we are puzzled that referee#2 does not find this genetic system more convincing than any of the biochemical assays.

We also want to point out that it requires a lot of hand-waving to suggest that the different reactivity of the two idiotypic Fab fragments that look biochemically identical are due to an aggregation of one of them rather than to the different epitope binding. Referee#2 also mentioned a paper that shows that under certain circumstances a monovalent antigen can be bound by two antibodies molecules (Hattori et al. 2016). This paper reports a quite interesting peptide/antibody structure with a 1:2 stoichiometry. However, the authors of this paper mentioned that such a case is extremely rare and found a single example in 1200 Ag/Ab complex structures in the Protein Data Bank. Anyhow we have carefully examined the known structure of the NP-cap (4-hydroxy-3-nitrophenylacetyl-e-aminocaproic acid)/B1-8 Fab complex (PDB code: 1YUH) and this structure excludes the vague possibility that our monovalent 1NIP-Pep can crosslinking two BCR complexes. We also think that the mentioned papers dealing with the IgE receptor are not relevant for the BCR.

Second, controls for the PLA are still missing. All we see is a loss of signal after activation, which may be caused by reduction in average proximity, but also by reduction in the amount of the reagents bound to the cells. This is important to control, because in general, changes in clustering at the nanoscale change the molecular accessibility for the staining reagents. A side by side control that quantifies binding of the reagents independently of their proximity is required to clear this issue.

Actually we have addressed this concern in the past and showed these controls in our eLife paper (Kläsener et al. eLife 2014;3:e02069. DOI: 10.7554/eLife.02069) by using fluorescent oligos complementary to the Fab-coupled oligos that clearly demonstrate that the Fabs are equally well binding the resting-closed and activated-opened BCR (Figure 2 Figure supplement 1A and B). Anyhow, we have now performed this control experiment again and present the results here as Figure_for_Referee#2

![Figure_for_Referee_#2](https://example.com)

**Figure_for_Referee_#2. Similar binding efficiency of Fab-PLA probes on resting and stimulated B cells.**

A and B FACScan analysis of anti-HC Fab used in PLA assay binding on resting or differently stimulated 3046SM (A) and 3046SD (B) cells. Binding of the anti-HC Fab is detected by PE conjugated mouse anti-rat Ig κ LC (Biolegend, MRK-81). Data are representative of two independent experiments.

Last, a minor comment is that the gates in the IgM vs IgD plots in the new Fig S4 are not in the same place in the two plots and therefore give the wrong impression that IgM levels are the same between the Lyn +/+ and Lyn -/- B cells. It rather appears that the levels of IgM are lower on the Lyn -/- cells, which should be noted.

We gated the cells based on the distribution of the events on the dot plot. We are aware that the gates in the two panels of the Fig. S4 are not in the same place. We did not meant to give the wrong impression. To clarify this issue, we have now added this notion to the figure legend.
Corresponding Author Name: Jianying YANG & Michael Reth

Journal Submitted to: the EMBO Journal

Manuscript Number: REMBO-2016-94177

Reporting Checklist for Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Publishing Preclinical Research issued by the NIH in 2016. Please follow the journal’s author guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n ≤ 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified.
- source data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (e.g. cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range.
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired or unpaired), simple p-tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - use tests one-sided or two-sided;
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P = 0.05 but not P values < 0.05.
  - definition of 'center values' as median or average;
  - definition of error bars as ± s.d. or ± s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. If every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.

2. Describe inclusive/exclusive criteria if sample or animals were included from the analysis. Were the criteria pre-established?

3. Have any steps taken to minimize the effects of subjective bias when allocating animal samples to treatment (e.g. randomization procedure)? If yes, please describe.

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5. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.

6. For animal studies, include a statement about blinding even if no blinding was done.

7. In the case of animal studies, describe any methods used to assess the biological variability of the experimental system.

8. In the case of statistical tests, justify an appropriate test.

9. In the case of the assumptions of the tests (e.g., normal distribution) describe any methods used to assess this.

10. Are there estimates of a variance within each group of data?

11. Is the variance similar between the groups that are being statistically compared?

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.ontobee.org

http://biomodels.net/miriam/

http://biomodels.net/

http://www.ebi.ac.uk/ega


http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumour-marker-prognostic-studies-remark/

http://ClinicalTrials.gov

http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm

http://1degreebio.org

http://www.antibodypedia.com

Page 21, Administrative Region of Fig.7, 7, 9, 16, 54.

Page 21, Administrative Region of Fig.11, 7, 9, 18, 54.

The data did not pass the D’Agostino-Pearson omnibus normality test, box-plots were chosen to present the data and p-values were obtained by Student’s t-test one-way analysis of variance (ANOVA).

Page 23, We use most time ANOVA.

Page 25, We use most time ANOVA.
1. Use the Data Accessibility Document (see link list at top right) if appropriate.

2. For animal models, include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the ARRIVE guidelines and the Department of Health and Human Services Belmont Report.

3. For publication of patient photos, include a statement confirming that consent to publish was obtained.

4. Report any restrictions on the availability (and/or use) of human data or samples.

5. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

6. For phase 1 and II randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under Reporting Guidelines. Please confirm you have submitted this list.

7. If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

10. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal’s data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under ‘Supplemented View’) or in unstructured repositories such as Dryad (see link list at top right) of FigShare (see link list at top right).

11. Access to human clinical and genetic datasets should be provided on a few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public success controlled repositories such as dbGaP (see link list at top right) or EGA (see link list at top right).

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18. Provide accession codes for deposited data. See author guidelines, under Data Deposition.

19. Data deposition in a public repository is mandatory for:
   a. Protein, DNA and RNA sequences
   b. Macromolecular structures
   c. Functional genomics data
   d. Proteomics and molecular interactions
   e. Other data relevant to the study

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21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.

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