THE EMBO JOURNAL

Manuscript EMBO-2015-93027

Nanoscale organization and dynamics of the siglec CD22 cooperate with the cytoskeleton in restraining BCR signalling

Francesca Gasparrini, Christoph Feest, Andreas Bruckbauer, Pieta Mattila, Jennifer Muller, Lars Nitschke, Dennis Bray, Facundo D Batista

Corresponding author: Facundo D. Batista, The Crick Institute

Review timeline:

Submission date:	08 September 2015
Editorial Decision:	12 October 2015
Revision received:	04 November 2015
Accepted:	09 November 2015

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 October 2015

Thank you for transferring your manuscript with referee reports to The EMBO Journal. I have involved two experts who assessed the technical aspects and the conceptual advance for The EMBO Journal. Their comments are provided below.

As you can see from these comments, the referees find the analysis well done and interesting. Referee #2 raises a few issues that shouldn't involve too much extra work to sort out. I would therefore like to invite you to submit a revised version.

I have also looked at the referee reports from the previous submission and I find that referee #2 and 3 raise very constructive comments that I would like you to respond to as well to in a revised version. We can discuss the specifics more if that is helpful.

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, please visit our website: http://emboj.embopress.org/about#Transparent_Process

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

REFEREE REPORTS

Referee #1:

This is an interesting study looking at the nanoscale organization and dynamics of CD22- a protein with a 2,6-linked sialic acid specific extracellular domain and a cytoplasmic domain that can recruit the phosphatase SHP1 when phosphorylated. The paper uses a combination of SIM, STORM and single particle tracking along with in silico simulation to develop a non-obvious model for how CD22 mediates its negative regulation in B cell activation. The model is convincing. A minor issue is that they need to better explain the complementary aspects of SIM and STORM, which will require discussing more the vulnerabilities of STORM, which the authors may otherwise prefer to avoid. Also the authors should take a quick look at the activity of the Src family kinases. They mechanism they propose in which CD45 and CD22 interact suggests a mechanism to fine tune Src family kinases. They look at Sfk superficially in Figure 1, but they should look at both the inhibitory and activation loop with the major perturbations as they may be altering this. Or call attention to prior data on this if they feel that this is already addressed in the extenstive literature on this system from Cyster and Goodnow, etc.

Referee #2:

This paper addresses a very important question of how the inhibitory receptor CD22 controls B cell receptor function. The quantitation of receptor numbers and the mechanistic understanding the paper provided I feel is huge. The discussions formulated new ideas and the conclusions appear to be based on rigorous and challenging technology. My difficulty comes from the major weakness that I do not understand the technology sufficiently to be be able to fully evaluate the data. However, if experts are happy with the technology and feel the interpretation is correct then I think the biological value of the work is very high. It offers important new insights about how antigen receptors signal.

1st Revision - authors' response 04 November 2015

Point by point reply

Referee #1 (R1):

R1 *stated: This is an interesting study looking at the nanoscale organization and dynamics of CD22- a protein with a 2,6-linked sialic acid specific extracellular domain and a cytoplasmic domain that can recruit the phosphatase SHP1 when phosphorylated. The paper uses a combination of SIM, STORM and single particle tracking along with in silico simulation to develop a non-obvious model for how CD22 mediates its negative regulation in B cell activation. The model is convincing.*

We thank R1 for the positive comments on our manuscript and for their helpful suggestions.

Specific comments:
1. R1 *stated: A minor issue is that they need to better explain the complementary aspects of SIM and STORM, which will require discussing more the vulnerabilities of STORM, which the authors may otherwise prefer to avoid.*

As R1 stated, in our study we use two complementary superresolution microscopy methods, SIM and dSTORM, which allows us to support our conclusions independently. We welcome R1's suggestion and have now included a paragraph in the discussion to better explain the advantages and limitations of each of these novel technologies (page 32, paragraph 8).

2. ***R1*** *stated: Also the authors should take a quick look at the activity of the Src family kinases. They mechanism they propose in which CD45 and CD22 interact suggests a mechanism to fine-tune Src family kinases. They look at Sfk superficially in Figure 1, but they should look at both the inhibitory and activation loop with the major perturbations as they may be altering this. Or call attention to*

prior data on this if they feel that this is already addressed in the extenstive literature on this system from Cyster and Goodnow, etc.

We thank R1 for this suggestion. We have now performed an additional analysis of SFK activity to address his/her advice. The new data are now presented in Figure 1B (Results, page 7). We have also included the suggested references.

Referee #2 (R2):

R2 *stated: This paper addresses a very important question of how the inhibitory receptor CD22 controls B cell receptor function. The quantitation of receptor numbers and the mechanistic understanding the paper provided I feel is huge. The discussions formulated new ideas and the conclusions appear to be based on rigorous and challenging technology. My difficulty comes from the major weakness that I do not understand the technology sufficiently to be able to fully evaluate the data. However, if experts are happy with the technology and feel the interpretation is correct then I think the biological value of the work is very high. It offers important new insights about how antigen receptors signal.*

We thank R2 for the positive comments on our manuscript and helpful suggestions. As requested by R1 above, we have now discussed the advantages and limitations of the novel technologies utilized in our work in more detail (page 32, paragraph 8).

Point by point reply to referee comments from previous submission

Referee #2 (R2):

Specific comments:
1. ***R2*** *stated: The description of CD22 as a 'negative coreceptor' or 'this inhibitory receptor' is over simplistic and should be changed. Two studies cited by the authors (Poe et al., 2000, Otipoby et al., 2001) and others have shown that CD22 recruits both 'positive' and 'negative' signaling elements. A more 4 description of CD22 and its functions would be helpful for and not mislead the reader.*
We thank R2 for pointing this out and we provide now a complete description of CD22 (Introduction, page 4).

2. ***R2*** *stated: Fig. 1: Parts of this figure are confusing. Fig. 1A needs to indicate what is being measured quantitatively on the ordinate. Fig. 1H and 1I show that the increased phosphorylation of Akt and CD19 in CD22-/- cells is still present in the Cd22 Y5/6F mutant B cells. The authors need to discuss this, as it suggests that another site on CD22 distinct from the PTP-1-binding sites regulates Akt and CD19 phosphorylation levels (e.g., Y828). One might predict that a tyrosine phosphatase inhibitor such as bis(maltolato)oxovanadium might have the same effect as LatA. And LatA clearly is not mimicking BCR ligation as implied in Fig. 1B. For instance, in CD22-/- B cells treatment with LatA induces big increases in pAkt and pErk compared to WT B cells (Fig. 1I); yet several studies have reported relatively little differences e.g., in pErk after BCR ligation of WT vs. CD22-/- cells. Therefore, the authors really cannot conclude that 'the molecular events of BCR signalling are similar regardless of whether the activation is triggered by BCR crosslinking or by cytoskeleton disruption.'*

We thank R2 for his/her advice. We have now modified Fig. 1A accordingly. Importantly, we have also extended our study including new data that are completely aligned with our previous conclusions. In brief, we have obtained and performed new experiments with mouse B cells expressing a form of CD22 lacking all 3 tyrosines (CD22-Y2,5,6F) in the intracellular region. These new data is now presented in Figure 1J, K and L; and the results described on page 9. As mentioned

above, this new set of data is in complete agreement with what we had observed with CD22-/- B cells making our conclusion more compelling.

We also agree with R2 that the observations using the CD22-Y5,6F may eventually be of great interest. However, at this point we also believe that they are preliminary, and likely to distract the reader from the main conclusions of our paper. Consequently, we have moved the CD22-Y5,6F data to Figure EV1 (Results, page 9).

*3. **R2** stated: Fig. 2: Are any of the differences in Fig. 2D statistically significant or are they all not significant?*

We thank R2 for pointing this out and we have now added the significance values to Figure 2D.

*4. **R2** stated: Fig. 3: Are there any other significant differences in Fig. 3B like IgM vs. IgD. There is relatively little discussion about the differences between these 4 receptors.*
We have now added the significance values to Figure 3B as requested.

*5. **R2** stated: Fig. 4 is fine as far as it goes in predicting something that could be tested.*

We agree with R2 with this statement. This is indeed what we did in Figure 7 with the CD22-R130E mice.

*6. **R2** stated: Fig. 5 and videos: Terrific in many ways, but per my comments above, the authors should make clear that the LatA treatment is the same as what happens with BCR ligation, e.g., which rapidly increases CD22 cell surface expression. Are the studies of Sherbina et al. (J Immunol. 1996. 157:4390-8) pertinent to the findings and overall approach?*

We thank R2 for this suggestion. We analyzed the surface expression of CD22 upon BCR ligation and LatA treatment in primary murine B cells as in Sherbina et al. and presented the new data in Figure 5L (Results, page 17). Contrary to their study, we did not observe any substantial change after BCR or LatA stimulation.

*7. **R2** stated: Fig. 6. Since CD22 can bind to itself and other ligands in cis, these interactions naturally would be predicted to increase in the absence of a very highly expressed ligand CD45. Why wouldn't they given what we know about CD22? The alterations observed may simply be due to differential expression of ligands for CD22 and not reflect anything terribly surprising. CD45 is one of the most highly expressed molecules on leukocytes and it is known that CD22 interacts with CD45, so the results shown, while nice are predictable and don't tell us that much about what regulates CD22 interactions with CD45 in cis vs. other ligands in cis in normal CD45+ B cells.*

We thank R2 for this comment. However, it is important to point out that no direct evidence so far has been provided regarding CD22 nanoscale *cis*-organization or, more importantly, when CD45 is absent. Indeed, this is one of the main novelties of our manuscript. Similarly, this is the first time that the organization of CD22 on the surface of B cells has been studied by superresolution microscopy. All previous interpretation about CD22 *cis*-association, while important and relevant, has been based on indirect observations.

The data in Fig 7 using the R130E mutant are great.
We thank R2 for their statement.

Reviewer #3:

There are several points that should be addressed by the authors.

Specific comments:
1) The author's conclusions and model are based on the assumption that the nanoclusters themselves are mobile in the plasma membrane. However, multiple examples in the literature have shown that clusters often are stable in their positions while their content can exchange rapidly with their surroundings or neighboring clusters. This should be included in alternative interpretations of

the data and be addressed in the discussion. Ideally, the two possibilities are investigated through an experimental approach, such as
i. Comparison of CD22 nanoclusters in live B cells at different time points using STIM or STORM/PALM imaging. If the clusters move at the speed suggested, two images separated by a several seconds should show significantly different patterns of CD22 cluster. An example for stable clusters has been shown by the Gauss lab for LAT in T cells (Nat Immunology 2011).
ii. Single particle tracking STORM/PALM of sufficient CD22 molecules over time should allow reconstructing the nanoclusters if they do not move or alternatively yield a homogeneous distribution of tracks. An example has been shown for EGFR by the Lippincott-Schwartz lab (J Am Chem Soc 2010).
iii. Overlay tracks with TIRF image of CD22 (only if the clusters are visible by diffraction limited TIRF microscopy). An example was shown for Lat in T cells by the Val lab (Cell 2005)
These are technically and biologically challenging experiments, however, they would add to and support the author's conclusions. At least, the possibility of mobile molecules that form stable nanoclusters through location probabilities or transient interaction (e.g. CD22 multimerization) should be discussed. This would include the possibility that the activity of CD22/SHP-1 is based on the fraction of single and 'free' molecules (fast diffusing) and their movement in relation to IgM, IgD and CD19.

We thank R3 for their comments. As suggested, we have now addressed the point raised by R3 in the Discussion (page 28, paragraph 3).

2) The Latrunculin A (LatA) treatment in the STORM experiments was 4 minutes and showed no effect on nanoclusters distribution. In comparison the single particle tracking was performed with 10 minutes pre-treatment of LatA (2.5 fold different in incubation time). Is the depolymerization of actin comparable between these conditions? LatA does not completely depolymerize actin fibers at this time scale. Therefore it should be mentioned/discussed that the mobility of CD22 molecules could depend on different actin structures than the numbers and sizes of CD22 clusters (e.g. diffusion-barrier vs domain anchoring). The authors show that CD22 diffusion is lower in actin rich areas. Do CD22 clusters co-localize with actin? If so, is clustering the cause for reduced mobility? Another possibility is that the slower CD22 tracks overlap with IgM, IgD or CD19 nanoclusters and reduced mobility is due to the CD22/SHP-1 interaction with these molecules.

We have now included actin staining in the present study showing that the degree of depolymerization is indeed comparable between the different experimental settings (Figure 5K; Results, page 17).

3) It should be stated that in the author's model CD22/SHP-1 interactions with their substrates would only take place at the contact sites between CD22 clusters and BCR areas/clusters.

We thank R3 for this comment. Even if highly plausible and quite predictable, we do not, however, show direct evidence for SHP-1 interaction with its substrates in the current study.

4) In the CD45-/- mice the reduced molecule diffusion might be due to reduced movement of larger cluster or to lower "escape-rates" of single molecules. The later might be explained by lower border length to cluster area ratio.
We thank R3 for this comment. We have addressed this point in the Discussion (page 28, paragraph 3).

