Identification of serine residues in the connexin43 carboxyl tail important for BCR-mediated spreading of B-lymphocytes

Farnaz Pournia, May Dang-Lawson, Kate Choi, Victor Mo, Paul D. Lampe and Linda Matsuuchi

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MS TITLE: Identification of serine residues in the connexin43 carboxyl tail important for BCR-mediated cell spreading of B-lymphocytes

AUTHORS: Linda Matsuuchi, Farnaz Pournia, May Dang-Lawson, Kate Choi, Victor Mo, and Paul D. Lampe
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Pournia and colleagues performed an in-depth analysis of the serine residues at the cytoplasmic tail (CT) of connexin43 (Cx43) in relation with BCR-triggered B cell spreading and signaling. They made a CD8a/Cx43 chimera and a collection of Cx43 mutants, expressed them in B cell lines and compared their ability to promote cell spreading with the wild type Cx43-expressing cell line after BCR stimulation. The experimental approach used is straightforward for this type of study, and gives them robust results for interpretation. The manuscript is well-written, with detailed methodology, and well-defined results in figures and supplementary data that support the main conclusions of the study. The authors showed that the Cx43 CT is enough to regulate B cell spreading, and in particular S282 and S279 have a main role on that; S255 and S262 appear to be dispensable. Take my attention the fact that the mutation of the four serine residues has no effect on cell spreading, in cell lines or primary B cells, neither in the phosphor-Tyr pattern/intensity downstream the BCR. It looks like ones counteract the functions of the others; there is not a predominant dominant-negative effect for S282 or S279. Future studies in relation with the binding partners might shed light on that observation. The reported relevance of the gap junction protein Cx43 in other cell types, not only in B cells, strengths and extends the importance of the manuscript message for the cell biology community.

Comments for the author

Minor points raised.
- Related with figure S2A, the band of CD8a:Cx43 CT seems to shift upon OKT8+anti-IgG stimulation at 15 min, while authors said that there is no shift (lines 144-146). This might be corrected/commented in the text.

- Line 189, it is (Fig S3B) instead of (Fig 3)

- In figure S4B, the cell line expressing WT Cx43 (blue line) showed lower IgM levels at the surface, while in other supplementary figures (S6B, S5B, …) does not. I guess they are different transduced cells, made for each set of experiments??...in relation with this, it would be informative to mention the time after transduction at which B cells are sorted and used in Methods (page 18, line 531).

- Lines 226 and 311, correct “P-Try”

- Comment on the unexpected results of the 4S-mutant might enriched the discussion.

- Lines 698 and 722, repetition of the sentence “Data shown is representative of three independent experiments”.

Reviewer 2

Advance summary and potential significance to field

This study evaluates the role of the C-terminal (CT) of the Connexin 43 (Cx43) protein during early activation of B cells. The authors found that specific serine residues (279 and 282) are required for B cell spreading and signaling, which offers new targets to manipulate B cell activation.
The experiments are well performed and the rationale is correct. Overall, I think the article will be of interesting for the readership of J Cell Sci, the immunology community and the gap junctionÂ’s community. I would recommend publication after addressing some studies on the mechanism, which would make the paper more compelling and strength the interpretations made by the authors.

Comments for the author

Major comments

-A relevant question that emerges from the article's data is whether the different Cx43 mutants are recruited or not towards the synaptic plane upon BCR activation, using the cell spreading assay. Do the Cx43 CT or serine mutants co-localize with actin? It will be very relevant to test this point, because it is conceivable that mutations in the domain might impair the direct interaction between these proteins.
In T cells, actin foci acts as signaling hub (PMID: 25758716). Are there Cx43-actin foci in B cells? If yes, do these foci depend on the phosphorylation of the Cx43 CT? Answering these questions about the mechanism would be a significant advance in the understanding on the mechanism and will be of broader interest for both the immunology community and the gap junction community.
In the same line with the previous paragraph, the authors discuss about possible linkers between Cx43 CT and actin. HS-1 is expressed in B cells and upon BCR engagement is quickly recruited to the immune synapse concomitantly promoting Arp2/3-dependent actin nucleation (PMID: 15166239; PMID: 26987298). Do HS-1 co-localize with Cx43 CT and/or mutants? The demonstration of this interaction will be a considerable advance in the understanding of the role of Cx43 CT during B cell response.

-Figure 1: Quantification of the kinetic for the results shown in panels B and C should be added, something similar to what is shown in Figure S3F.
For the sake of clarity, please include the name of the corresponding cell line used on top of each panel. In addition, the authors should indicate in the figure (for example with an arrow) which band corresponds to each modification.

Figure 2: The authors might add the data not shown about characterization of CD8a:CT-EGFP expression and function in WEHI 231 cells, in order to support their model.
The graph showed in the panel F should be larger (same width if the authors want) in order to better appreciate the differences.

Figure S2, in line 144 the authors state that the shift in MW occurred only after BCR stimulation, but upon treatment with a-CD8 + a-IgG there is a shift after 15 min post-stimulation. While I agree with the authors that the response is different, it would be necessary to add a graph showing the quantification and to rephrase the statement saying that the response was significantly lower than the one observed with anti-BCR stimulation.

Minor comments

Please address the following points.

-The statistical comparisons are well appreciated by this reviewer, but sometimes the graphs look quite busy for the reader. Please try to find an option for statistics representation in the following Figures 4D, 6C, 7C.
-Figure S1: delete "Lorem ipsum"
-Line 108: find an option for "was seen", maybe "found" or "observed"?
-Figure S2: panel "c" is written in lowercase and the legends of the graph are too small. Same for Figure S3, Figure 3.

First revision

Author response to reviewers' comments

Point by Point Response
All the changes in the text were added in red.
The previous text that are going to be removed are highlighted in red while crossed out.

• General:
Title has to be under 120 characters and our previous title counted 124 characters. This was addressed by removing the word “cell” in the title. The current count is 119 characters.

Reviewer 1:

1. In figure S2A, the western blot image has been replaced with another replicate of the experiment to address the band shift noted at 15 min for CD8a: Cx43 CT upon OKT8 + anti-IG stimulation while the text it says there is no shift (line 148-151).
Previous blot was chosen since it was the best replicate that shows the band shift of the CD8a: Cx43 CT (66 kDa) and endogenous Cx43 (43 kDa) upon anti-BCR stimulation on the same blot. The protein isolation method used was optimized for isolating the chimeric protein and the endogenous protein was isolated at a lower level. Therefore, during the revisions we added two panels with shorter and longer exposure times to be able to show the band shifts of both the chimeric (shorter exposure time) and the endogenous Cx43 protein (longer exposure time). Fig S2 A and B respectively.

Name of corresponding cell line along with the shorter vs. longer exposure time were added on top of the panels A and B in FigS2

Actin blots were also added.

Additionally, quantification for the blot (previously S2B and now S2C) showing specific residue phosphorylation of Cx43 CT in the chimeric protein after BCR stimulation was added as Fig S2D.

Respective changes were made in the text and figure legend.

In the text
Line 133 “cellular localization and” was removed.
Line 134 “data not shown” was removed and Fig “S2A,B” was added.
Line 135 “similarly” was removed.
Line 149 “(Fig S2A,B)” was added
Line 152 B was removed and “C” was added due to changes in Fig 2
Line 153 C was removed and “D” was added due to changes in Fig 1
Line 171 C was removed and “E” was added due to changes in Fig S2
Line 172 D was removed and “F” was added due to changes in Fig S2

In the Figure S2 Legend

Lines 18-23 “B) Western blot showing more visual upward shift in MW of the endogenous Cx43 in response to BCR stimulation by anti-mIgM antibodies in WEHI231 cells transfected with the chimeric protein CD8α:Cx43 CT-EGFP. The MW shift only occurred when cells were stimulated with anti-BCR antibodies. Left side of the blot, stimulated with anti-IgM only; Right side of the blot, stimulated with anti-CD8α and anti-IgG.” was added.
Line 23 B was removed and “C” was added
Lines 28-31 C was removed and “D) Sum of band densities presented in C were quantified using ImageJ and plotted using GraphPad Prism 7. The anti-CD8+IgG stimulated parts of the three blots were pooled together and represented in one line.” was added.
Line 31 E) was added
Line 35 D) was removed and “F)” was added.

In the new Fig S2

Panel B was added
Previous Panel B is now C
A new panel C, quantification for panel B was assed
Previous panel C is now E
Previous panel D is now panel F

2. Previously line189 and current line 193-Figure S3B, the S was missing, which was added in the text.

3. Fig S4 B, previous FACS data was replaced with another replicate showing higher level of IgM for WT Cx43 expressing cells. The J558μm3 mouse plasmacytoma B cell line were stably transduced with 4 chains of BCR (membrane IgM (μ, λ), Ig-α and Ig-β) received from Dr. Louis Justement (University of Alabama, Birmingham, AL, USA) and were used in our previous experiments. (Machtaler et al. 2011; Falk et al., 2014). Sometimes we noticed a drop in the surface level of BCR in the cell culture.

In our lab B cell lines were transduced with retrovirus containing WT Cx43, various mutated Cx43-EGFPs, CD8: Cx43CT-EGFP, CD8: EGFP cDNA, or the AP2 plasmid, using the retroviral packing cell line as addressed in the Materials and Methods section. Therefore, stable transduced cell lines were made.

Reviewer also asked about mentioning the time after transduction that cells were sorted for and used in the Materials and Methods section. Transduced cells were sorted one week after transduction, selecting for EGFP and IgM positive ones. The sorted cells were expanded by cell culture and freeze downs were made. The stable cell lines freeze downs were thawed and cultured one week prior to experiments. This was added in the Materials and Methods section as noted below
Line 589 “one week after transduction” was added.
Line 590-591“Freeze downs of the sorted cells were made. The stable cell line freeze downs were thawed and cultured one week prior to experiments.” Was added.

4. In the previous lines 226 and 311, and in the current lines 244 and 351, P-Try was corrected to P-Tyr.

5. Commented on unexpected results of 4S-mutant in the discussion section. Lines 412-418.

6. In the previous lines 699 and 722, the repetition of the sentence “Data shown is representative of three independent experiments” was crossed out.

Reviewer 2:

Major Comments:

1.
a) The reviewer asked “whether different Cx43 mutants are recruited or not towards the synaptic plane upon BCR activation, using the cell spreading assay.”
These experiments are done on glass coverslips and the contact site is technically not a ‘’synaptic plane’’, but the contact site. However it was important to answer this question in the context of our assay.
The cell spreading assay was performed as described before, using the J558μm3 B cell line expressing the controls (Cx43-EGFP or EGFP) or one of S255A, S262A, S279A, S282A, 4S-population A and B Cx43-EGFP. To visualize localization of Cx43 and requirement towards the synaptic plane, optical slices were collected using confocal microscopy and 3D images were reconstructed. Beside the expression of Cx43 and the mutants throughout the cells, we noticed a small aggregate of WT Cx43 and the mutations that did not affect B cell spreading (S255A and S262A as well 4S-both population A and B), at the contact site of the cells and the stimulating coverslips. This small aggregate of Cx43 at the contact site was not observed for the cells expressing Cx43 mutants impeding B cell spreading (S279A and S282A).

These results are presented as Fig 4SC (for individual S mutants) and Fig 5SC for quadruple S mutation. Description in the text are located at lines 228-231 and 261-,265 respectively.
Figure legend was added to the supplementary figure legends lines 58-66 and 86-94 respectively.

Imaging details were added in the materials and methods section, lines 662-667.

b) The reviewer asked “Do Cx43 CT and mutants colocalize with actin?”
The colocalization can be inferred from figures presented in Figs 2E, 3C, 4B, 5B,6B and 7B. In these figures we see a yellow color emerging form green (EGFP fused to WT, mutant and the CT of Cx43), and red (actin) at the cell-coverslip contact site i.e spreading area.

In order to better visualize colocalization of Cx43 mutants and actin at the contact site, spreading assay was repeated and lower concentration of Rhodamine Phalloidin was used for actin staining (1/400 dilution compare to previously 1/200 dilution). We anticipated that a lower concentration of actin staining will better visualize the actin structures such as actin foci at the cell-coverslip contact site. Cells were fixed after 15 min and the contact site was imaged. Colocalization of WT and mutants Cx43 with actin was seen at the cell-coverslip contact site similar to those previously shown in Figs 4B, 5B,6B and 7B. Detecting a difference in colocalization of WT Cx43 vs. different mutants with actin requires more advanced techniques such as super-resolution microscopy which is beyond the scope of this paper.

These results are presented as Fig 4SD (for individual S mutants) and Fig 5SD for quadruple S mutation. Description in the text are located at lines 233-239 and 265-269 respectively.

Figure legend was added to the supplementary figure legends lines 66- 73 and 94-100 respectively.

Imaging details were added in the materials and methods section, lines 669-677.

c) The reviewer asked “Are there Cx43-actin foci in B cells? If yes do these foci depend on the phosphorylation of Cx43 CT?
We did not see actin foci at the contact site of J5588μm3 B cell lines with the stimulatory coverslips. This holds true for earlier experiments where actin was stained with higher concentration of Rhodamine Phalloidin (1/200 dilution) and the new experiments with lower concentration of Rhodamine Phalloidin (1/400 dilution) to address the reviewers’ comments. This might be due to the nature of experimental set up. The reports that visualize actin foci at the contact site or synaptic plane used lipid bilayer or Antigen Presenting cells for their experiments. Perhaps the dynamics of membrane spreading on another membrane vs. solid glass surface will result in different actin structure at the contact site/ synapse. Since our experiments are entirely on glass coverslips, we did not see large actin-foci as observed when cells are examined on lipid bilayers or on antigen presenting cells.

Due to adding a new panel previous Fig S4 panels has been changed as described below.
Previous panel C is now panel E.
Previous panel D is now panel F.
Previous panel E is now panel G.
Previous panel F is now panel H.
Previous panel G is now panel I.

Due to adding a new panel previous Fig S5
Previous panel C is now panel E.
Previous panel D is now panel F.
New panel G was added (see part d below)

d) The reviewer asked “Do HS1colocalize with Cx43 CT and/or mutants?”
To address this point we performed cell spreading assays and stained the cells with anti-HS1 antibody. Images from 15 min time point spreading, where maximum spreading is reached, were collected. HS1 was localized at the cell-coverslip contact site and an enhanced localization around the cell periphery/rim was detected. Additionally colocalization of Cx43 and HS1 was observed around the cell rim, which was more enhanced for cells expressing WT, S255A, S262A and 4S Cx43-EGFP compare to S279A and S282A Cx43-EGFP expressing cells. Detecting a detailed difference in colocalization of WT Cx43 vs. different mutants with HS1 requires more advanced techniques such as super-resolution microscopy which is beyond the scope of this paper Data is shown in Fig S5G and explained in the text, lines 276-288 and 290-292.

Figure legend was added to the supplementary figure legends lines 106-113

A Note about this observation was added in the discussion section, lines 451-454.
Imaging details and antibody information were added in the materials and methods section, lines 680-689 and 516-520 respectively.

2. Figure 1:
a) Quantification for panel B and C were added.
Quantification of panel B is the new panel C.

Previous panel C is now panel D.
Quantification for panel D (previously panel C) is added as panel E.

Additionally, quantification for the blot (previously S2B and now S2C) showing specific residue phosphorylation of Cx43 CT in the chimeric protein after BCR stimulation was added as Fig S2D.

Respective changes were made in the text and figure legend.

In the text
Line 119 ",C" was added beside Fig 1B.
and line 120, "Fig 1D,E" replaced previous Fig number.

In Figure Legends
For Fig 1 lines 723-725,"C) Sum of band densities presented in B were quantified using ImageJ and plotted using GraphPad Prism 7" and lines 730-731 "E) Sum of band densities presented in D were quantified using ImageJ and plotted using GraphPad Prism 7 were added.

b) Name of each corresponding cell line was added on top of the panels Fig1 and 1S
c) bands were identified with arrows and square brackets in Fig 1B and 1D.

3. Figure 2:
a) "Data not shown" was actually presented in Fig S2A. Therefore in the text line 134 "data not shown" was removed and Fig "S2A,B" was added representing the band shift of the CD8a: Cx43 CT (66 kDa) and endogenous Cx43 (43 kDa) upon anti-BCR stimulation in WEHI 231 cells. Actin blots were also added.
b) Panel F, the graph was enlarged
c) In figure S2A, the western blot image has been replaced with another replicate of the experiment to address the band shift noted at 15 min for CD8a: Cx43 CT upon OKT8 + anti-IG stimulation while the text it says there is no shift (line 148-151).
Previous blot was chosen since it was the best replicate that shows the band shift of the CD8a: Cx43 CT (66 kDa) and endogenous Cx43 (43 kDa) upon anti-BCR stimulation on the same blot. The protein isolation method used was optimized for isolating the chimeric protein and the endogenous protein was isolated at a lower level. Therefore, during the revisions we added two panels with shorter and longer exposure times to be able to show the band shifts of both the chimeric (shorter exposure time) and the endogenous Cx43 protein (longer exposure time). Fig S2 A and B respectively.

Name of corresponding cell line along with the shorter vs. longer exposure time were added on top of the panels A and B in FigS2

Additionally, quantification for the blot (previously S2B and now S2C) showing specific residue phosphorylation of Cx43 CT in the chimeric protein after BCR stimulation was added as Fig S2D.

Respective changes were made in the text and figure legend.

In the text
Line 133 "cellular localization and" was removed.
Line 134 "data not shown" was removed and Fig "S2A,B" was added.
Line 135 "similarly" was removed.
Line 134 "(Fig S2A,B)" was added
Line 152 B was removed and "C" was added due to changes in Fig 2
Line 153 C was removed and "D" was added due to changes in Fig 1
Line 171 C was removed and "E" was added due to changes in Fig S2

Lin2 172 D was removed and “F” was added due to changes in Fig S2

In the Figure S2 Legend
lines 18-23 “B) Western blot showing more visual upward shift in MW of the endogenous Cx43 in response to BCR stimulation by anti-mIgM antibodies in WEHI231 cells transfected with the chimeric protein CD8α:Cx43 CT-EGFP. The MW shift only occurred when cells were stimulated with anti-BCR antibodies. Left side of the blot, stimulated with anti-IgM only; Right side of the blot, stimulated with anti-CD8α and anti-IgG.” Was added.
Line 23 B was removed and “C” was added
Line 28 C was removed and “D) Sum of band densities presented in C were quantified using ImageJ and plotted using GraphPad Prism 7. The anti-CD8+IgG stimulated parts of the three blots were pooled together and represented in one line.” Was added.
Line 31 E) was added
Line 35 D) was removed and “F)” was added.

In the new Fig S2

Panel B was added
Previous Panel B is now C
A new panel C, quantification for panel B was assed
Previous panel C is now E
Previous panel D is now panel F

Minor Comments:

1. Reviewer suggested to simplify the stat representation for Fig 4D, 6C and 7C. We did not make any changes here as we’d like to be able to demonstrate the spreading response for the cells expressing Cx43 CT mutatants over a time course as well as a comparison to the controls.

2. Figure S1 “Lorem ipsum” was deleted

3. In previously line 108 and current line 116 “was seen” was “was found”

4. Figure S2 pane “c” (lowercase) was changed to “C” (uppercase). The graph legends in previously C and new E were enlarged and also in Fig S3,

Other Changes

1. In Material and Methods section lines 607-608 “(for data presented in Fig S3-S7)” was added.
2. In Material and Methods section lines 614-617 “Blots presented in Fig 1 were developed and then scanned using a scanner. Quantification was done using ImageJ software (NIH, Bethesda, MD, USA) and plotted with Prism 7 GraphPad software” was added.

3. Supplementary Fig S3 in the figure legend, D was typo and replaced with F

4. In acknowledgement section trainee award to “V.M” was added.

Second decision letter

MS ID#: JOCES/2019/237925

MS TITLE: Identification of serine residues in the connexin43 carboxyl tail important for BCR-mediated spreading of B-lymphocytes

AUTHORS: Linda Matsuuchi, Farnaz Pournia, May Dang-Lawson, Kate Choi, Victor Mo, and Paul D. Lampe

ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. Please address all the minor points raised by referee 2 in your final revised manuscript.

Reviewer 1

Advance summary and potential significance to field

The revised version of the manuscript was improved with new data and discussion. I consider that the revised manuscript is appropriate and now ready for publication in JCS.

Comments for the author

The authors addressed all my previous comments.

Reviewer 2

Advance summary and potential significance to field

This reviewer appreciates that the authors have addressed and answered every single comment I have done, that's why I think the article could be accepted in its current state.
Congratulations for your work.

Comments for the author

Minor comments:

Figure 1B. Please align the "48" on the left and the brackets on the right.
Figure 1C. There is a small dot to be deleted on top of the graph.
Figure 1D. Please also align the MW in the different panels. The same should be applied in the other figures.

Figure 2E. Please align the "E" with the top legends of each panel, namely Cx43-EGFP, CD8:CT-EGFP, etc.

Figure 3A. Please move the "aa 258" from the arrow.
Figure 3C. Please align "C" with the legends on top of the panels.

Figure 4A & B. Please align "A" and "B" with the legends on the corresponding panels.

Figure 5. Please align "A" with "B", "C" with "D" and "E" with "F". The same should be applied to other figures when possible.