Syndecans and glycosaminoglycans influence B cell development and activation

Craig McKenzie, Alexandra Dvorscek, Zhoujie Ding, Marcus Robinson, Kristy O'Donnell, Catherine Pitt, Daniel Ferguson, Jesse Mulder, Marco Herold, David Tarlinton, and Isaak Quast

Corresponding author(s): Isaak Quast (isaak.quast@monash.edu) , Isaak Quast (isaak.quast@monash.edu), Craig McKenzie (craig.mckenzie@monash.edu)

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Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
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Referee #1:

McKenzie et al describe "The role of syndecans in B cell development, activation and plasma cell formation".

Plasma cells, by secreting antibodies, are obviously central to humoral immunity. Plasma cells are characterized by high expression of CD138, but the role of this glycosaminoglycan-containing protein besides serving as a marker of plasma cells for immunologists is incompletely understood.

Here, the authors study the expression and function of CD138 (Syndecan-1) and of another syndecan expressed in B cells (syndecan-4) using state of the art techniques in at least 10 B cell stages between pre-pro B cells and plasma cells. It is noteworthy that their study stopped at plasma cell generation and was not designed to study how PCs are maintained in the long-term. The work is divided in 4 main blocks.

In a first part, the detail surface expression of CD138 and Syndecan4 is studied. In a second part, authors monitor, using bone marrow chimera, how stem cells knock-out for Sdc1 (CD138) or Sdc4 compare to WT to reconstitute B cell populations. Results show that neither Sdc1 nor Sdc4 are essential for B cell development. Indeed plasma cells can be produced and are alive in the absence of one or the other. In both ko, B cell development is impaired about 50% usually at the stage where these molecules are most highly expressed. This indicates a positive but non-essential function of Sdc1 and Sdc4 during development.

In a third part, regulation of SDC1 and SDC4 expression was studied in naïve B cells that express SDC4 but not SDC1. Direct stimulation of the BCR permanently decreased SDC4 expression and transiently increased SDC1 expression. Other stimuli tested (IL4, IL21, anti-CD40 [all produced by or mimicking T helper cells]) had no effect alone, but tended to maintain the status quo of SDC1 and 4 expression upon BCR stimulation (and therefore go against the BCR effect on SDC1 and 4, but without preventing proliferation). The exception was IL21 that further promoted transient BCR-mediated CD138 expression. Consistent

results of SDC4 downregulation and SDC1 expression were observed when B cells were stimulated with antigen and T helper cells in vivo.

In the fourth part, authors studied the effect of adding moderate or excess amounts of soluble glycosaminoglycans to naïve B cells {plus minus} cognate antigen. In a "reverse" experiment, they digested glycosaminoglycans on B cells with heparinase. Although effects were present and sometimes spectacular (addition of glycosaminoglycans increased B cell number up to almost 10-fold; addition of glycosaminoglycans reduced activation markers CD69 or Fas or loss of SDC4 induced by BCR stimulation; removal of cell-bound glycosaminoglycans with heparitinase increases CD69 expression post BCR stimulation), I found it difficult to understand how authors conclude that glycosaminoglycans and in particular SDC4 have an inhibitory function on B cells, and to figure out by which mechanism this could be.

In summary, the experiments are very well performed, figures are of high quality, the introduction is to the point and some important conclusions are fully supported by data (SDC4 and CD138 are not essential for B cell development and production of plasma cells). The conclusion that syndecans (at least SCD4) are inhibitory for naïve B cells is probably supported by data, but this could be better explained. Often, conclusions are written in vague (or cautious) terms that do not help the reader understand what is going on (eg title: "The role of syndecans in B cell development, activation and plasma cell formation." OK, but what is this role? Eg abstract: "Collectively, our results reveal SDCs as regulators of the B cell response to antigen, with GAGs modulating B cell activation and survival." The terms "regulator" and "modulating" gives no indication on the direction or amplitude of the modulation. Etc...

Major point 1: The authors should clarify their descriptions of Fig 5 and Fig 6 investigating the role of GAGs in naïve B cell activation. For example, Fig 5A shows that heparin has no effect on the number of naive B cells in the absence of antigen stimulation, but that in the presence of the antigen (HEL), there are about 10-fold more B cells in the presence of heparin than in its absence. It is very difficult for the reviewer to reconcile this result with the final conclusion of the authors that GAGs have an inhibitory role on B cells. One possibility is that under these conditions, the high affinity ligand induces death of the stimulated cells (AICD) which would explain the strongly reduced cell number of the condition "no GAGs" in the presence of antigen compared to the absence of antigen. AICD could be due to upregulation of FAS which is counteracted to some extent by the presence of heparin. As FAS is an activation marker, then heparin would negatively regulate activation, keep cells under the threshold of AICD and prevent cells from dying. If this is the case, it could be explained better, eg in a wrap up sentence. But then it is unclear why in Fig 5C that tests the effect of short-term naïve B cells exposure to heparin there is no difference in B cell number in the "No GAG" condition minus or plus antigen, and that this is also not seen in Fig 5F in No GAG minus or plus antigen. Fig 6A however resembles Fig 5A in that naïve B cell number is drastically reduced upon stimulation with antigen (probably by AICD). This defect is attenuated by heparin, but never to the extent seen in Fig 5A. Experimental variability? Different experimental conditions?

Also, it would be useful for the reader if authors could integrate results of Fig 4 with their conclusion that syndecans have an inhibitory role on B cells. For example, is it conceivable that T cell help would prevent premature differentiation of cycling B cells in antibody secreting cells and plasma cells by among others, preventing CD138 expression and preventing SDC4 down-regulation, with the aim of achieving a better cell amplification before terminal differentiation?

Minor point 2: In the experiment of Figure 4, naïve B cells not only express anti-HEL BCR, but also come from a Rag1-ko background. It would be helpful in all figures using B cells with anti-HEL BCR to mention if they are also Rag1-ko or not.

Minor point 3: In Fig 4A, the CTV profile indicates that CD138 that is not expressed in naïve B cells was induced by HEL even before division, and then fully lost again after 3 divisions. This profile looks exactly like the one of Fig 4B with SDC4, except that here SDC4 was already present and is just lost upon HEL stimulation within 3 divisions. This could be better explained in the text. What justifies this very transient expression is a mystery for me. It cannot be directly linked to a differentiation to PC. Are Fig 4A,B comparable in terms of cells and HEL activation to Fig 5 and 6? If yes and if CD69 and/or FAS staining were included in these experiments (of Fig 4A,B), and if SDC4 indeed prevents activation, one would expect to see CD69 go up as cells divide in the Media only + HEL condition, but not in the anti-CD40 + HEL condition. If this data is available, please show the results (and possibly revise the conclusion of the inhibitory role of SDCs as necessary). If these stainings were not included, I let authors decide whether they would like to do it, but I do not insist that the experiment should be done.

Minor point 4: It is unclear how addition of heparin, or digestion with heparinase would interfere with SDC4 to exert its effect on B cells. A) GAGs of SDC4 inhibit B cell activation directly (e.g. by binding the BCR). Adding heparin allows to inhibit B cells even more (e.g. by binding more BCR). The digestion with heparinase removes GAGs and less partner is inhibited. B) GAGs of SDC4 bind a positively charged partner (APRIL, IL6, IL21?) that is displaced by an excess of heparin and thus stops activating B cells. What happens if naïve B cells are digested with heparinase, then washed, then stimulated with heparin? If heparin can bind and inhibit the same target as SDC4, this should cancel the action of heparitinase (I do not insist to have this experiment done).

Referee #2:

Syndecans can play important roles as modulators of cytokine and growth factor responses and can mediate attachment of cells. They can also act as co-receptors for GPRs. This report aims at addressing the abundance and function of two Syndecans, Sdc1 and 4, in B cells. To this end, bone marrow of Cas9tg mice was infected with lentiviral vectors encoding specific sgRNAs and a BFP reporter. Infected BM along with RAG1-/- BM was transplanted into irradiated WT mice and B cell development and activation were analyzed. While CD138 peaks at pre B cells and in plasma cells, Sdc4 becomes up-regulated in pre and immature B cells and down-regulated upon B cell activation as well as in memory B cells, however, its expression differs among memory B cell subsets.

Functionally, deletion of both CD138 and Sdc4 disfavoured early B cell development. While Sdc4 B cells reached control frequency, the disadvantage of CD138 targeted B cells remained until the immature B cell stage. Immunization of recipient mice with NP-KLH did not reveal any effects of CD138 or Sdc4 deletion on the frequency of deleted germinal center B cells or plasma cells.
As to B cell activation, the SwHEL system was used in combination with IL-4, IL-21 and anti CD40. While IL-21 promoted HEL induced CD138 upregulation, IL-4 and CD40 suppressed it. The reverse was observed for Sdc4.

To address functional effects by putative Sdc ligands such as Polybrene and Heparin, activated B cells were co-cultured with either substance or treated with Heparinase. Heparin acted as B cell mitogen and is discussed as putative TLR4 ligand, hence being not so much relevant for human B cells in that respect. On the other hand, Heparin (HEL stimulation) suppressed AICD and CD69 expression. Heparinase (3xHEL) had no effect on AICD but increased CD69 expression. Sdc4 limited pS6 in resting and anti Ig activated B cells.

In summary, the presented data serve as a good starting point to pinpoint the role of CD138 and Sdc4 in B cells.

1. There are only few functional data regarding CD138 or Sdc4 knock-out, but both appear to affect early B cell development, which is interesting. What is the mechanism behind the disadvantage of CD138 and Sdc4 deficient pre/pro/pre B cells? PS6 needs to be downregulated to ensure proper small pre B cell development and Sdc4 appears to modulate pS6 in activated B cells. Given that the authors outstanding expertise in BM and stem cell cultures, would it not be feasible to set up stem cell / IL-7 cultures with CD138 or Sdc4 targeted stem cells and analyze pre B cell differentiation in vitro, defining proliferation/apoptosis/signaling/small pre B cell differentiation (using CD19/B220/CD2/IgM staining)? Pre BCR ligands should be discussed. It is also possible that in vitro cultrues show no effect as the potential Sdc ligand may only be present in the bone marrow. However, I believe that the role of Sdcs in B cell development should be explored more here.

2. Was the pS6 in activated B cells normalized to total S6? Can pS6 be blocked by Rapamycin? I think the conclusion that Sdc4 is a negative regulator of BCR signaling is a bit premature. Is BCR induced pSyk/PLCg2/Ca2+ affected or are the observed effects downstream of proximal BCR signaling?

3. Conceptually, it would be important to assess the effects of Heparin and Heparinase also on Sdc4KO B cells.

4. What happens if B cells are incubated with both Polybrene and Heparin?

5. Why did the authors use HEL in Figure 6 A and 3 x HEL in Figure 6B? This is a bit misleading and hard to grasp.

Referee #3:

The manuscript of McKenzie and colleagues provides a comprehensive overview on expression of the Glycosaminoglycan (GAG) containing cell surface proteins SDC1 and SDC4 during differentiation and activation of B lymphocytes. Based on this overview, the impact of genetic ablation of SDC1 or SDC4 in mice, and the impact of soluble GAGs on human and murine B cell activation is analysed, with the key claims that SDC4 is a negative modulator of B cell activation and SDC1 (CD138) is not relevant for differentiation of activated B cells into plasma cells. As such, the manuscript adds important information on the regulation of B cell differentiation and activation by GAGs, of interest to the readership of EMBO Reports, in light of the central role of B cells in providing humoral immunity and autoimmunity. However, some issues have to be clarified upfront. The major drawback seems to be that the entire readout of expression is based on cytometric data, and not adjusted to (single cell) transcriptome data, for staging of B cell differentiation. This would clearly put the manuscript on a different level.

The descriptive part of the manuscript, i.e. Figs 1 and 2, and EVFigs 1 and 2, is rather clear, as far as the expression pattern of SDC1 and SDC4 in the general steps of B cell differentiation is concerned. However, when analysing the bimodal expression of SDC1 and 4 in experienced B cells, and using CD62L and CD44 to discriminate between recent GC emigrant MBC (CD62L+CD44-) and established MBC (CD62L+CD44+), the data shown in EVFig 2C show that the mice analysed did not have any CD62L+CD44+ MBC, for whatever reason, maybe the mice were just too young and immunologically inexperienced. However, in Fig. 2A bimodal distributions are shown for CDF62L-CD44- MBC and CD44-CD62L+ MBC, not for CD44+CD62L+, but then in Fig. 2B, frequencies are provided for these MBC. These data are not convincing. The differential expression of SDC1 and 4 on MBC remains enigmatic, the data provided are inconsistent, and mice with significant numbers of advanced MBC

should be analysed.

When it comes to the controversial point, and as the authors claim key statement, of whether SDC1 (CD138) does impact on plasma cell differentiation, as suggested by an earlier publication (McCarron et al., 2017), the authors of the present manuscript claim that their data do show that SDC1 ablation does not impact on the generation of antibody-secreting plasma cells. The difficulty in enumerating plasma cells deficient for SDC1 is that SDC1 is a key cytometric marker for plasma cells. In particular here single cell transcriptome signatures would have been extremely helpful. In their absence, the authors base their classification on surrogate markers like CD19 and size (EVFig. 4) with erratic gate settings which are not defining separate populations (lymphocyte, FSA and CD19 gates). While in conjunction with CD138, these gates may define plasma blasts or even plasma cells, in the absence of CD138, i.e. in the SDC1-deficient B lineage cells, they obviously do not. There is considerable spillover from the adjacent populations and the true numbers and differentiation stages of antibody-secreting B lineage cells remain enigmatic. Even more so, the authors also show the numbers of antigen-binding "plasma cells" in Figs. 3F and I, which at least for Ig-secreting cells means that these cells are not yet fully mature plasma cells, which would not express membrane-bound antibodies, but rather either B cell blasts or plasma blasts, thus weakening the claim that SDC1 does not impact on plasma cell differentiation even further.

The authors then analyse the regulation of SDC expression in activated B cells by the costimulators IL21, IL4 and CD40L. Here they claim that SDC1 is upregulated and SDC4 downregulated in antigen-activated B cells, and IL21 enhances, while IL4 and CD40L inhibit these SDC regulations. What is not clear from the data shown is to what extent IL21, IL4 and antiCD40 impact on the differentiation of activated B cells into plasma cells versus their prolifaration as B cells, i.e. whether their effect is directly on SDC expression or indirectly on B cell activation (Fig. 4).

Finally, in Figs 5 and 6, the authors use heparin (Fig. 5) and heparinase (Fig. 6) to demonstrate that the GAG heparin sulfate can (down)regulate B cell activation. These data are not directly linked to SDC1 or 4, and thus remain a bit preliminary. As are the data on S6 phosphorylation. As a net result, the molecular mechanism of SDC4 attenuation of antigen-mediated activation of B cells remains to be elucidated.

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In the fourth part, authors studied the effect of adding moderate or excess amounts of soluble glycosaminoglycans to naïve B cells {plus minus} cognate antigen. In a "reverse" experiment, they digested glycosaminoglycans on B cells with heparinase. Although effects were present and sometimes spectacular (addition of glycosaminoglycans increased B cell number up to almost 10-fold; addition of glycosaminoglycans reduced activation markers CD69 or Fas or loss of SDC4 induced by BCR stimulation; removal of cell-bound glycosaminoglycans with heparitinase increases CD69 expression post BCR stimulation), I found it difficult to understand how authors conclude that glycosaminoglycans and in particular SDC4 have an inhibitory function on B cells, and to figure out by which mechanism this could be.

We conclude that SDC4 and glycosaminoclycans (GAG) limit B cell activation based on three independent lines of evidence:
1. GAG reduce CD69 upregulation, activation-induced cell death (Fig. 6A) and FAS expression (Fig. 4E) in response to antigen. This indicates a role for GAG in BCR signalling, which is in line with the potent modulation of Syndecan expression by antigen encounter (Figure 3).
2. GAG removal by heparinase resulted in increased activation (CD69 upregulation) in response to low-affinity (HEL3x) antigen stimulation (Fig. 5B)
3. SDC4 deletion increased baseline S6 and Syk phosphorylation and antigen-induced S6 phosphorylation (Fig. 5C and new Fig. EV6H).
While our experiments do not directly link SDC-linked GAG to these effects, an aspect we highlight in the discussion, we think that these data convincingly support the novel conclusion that GAG and SDCs limit BCR signalling.

In summary, the experiments are very well performed, figures are of high quality, the introduction is to the point and some important conclusions are fully supported by data (SDC4 and CD138 are not essential for B cell development and production of plasma cells). The conclusion that syndecans (at least SCD4) are inhibitory for naïve B cells is probably supported by data, but this could be better explained. Often, conclusions are written in vague (or cautious) terms that do not help the reader understand what is going on (eg title: "The role of syndecans in B cell development, activation and plasma cell formation." OK, but what is this role? Eg abstract: "Collectively, our results reveal SDCs as regulators of the B cell response to antigen, with GAGs modulating B cell activation and survival." The terms "regulator" and "modulating" gives no indication on the direction or amplitude of the modulation. Etc...

We thank the reviewer for their positive assessment of our work and the suggested improvements. We have changed the title and re-phrased the abstract and conclusions to better reflect the findings of our study.

Major point 1: The authors should clarify their descriptions of Fig 5 and Fig 6 investigating the role of GAGs in

naïve B cell activation. For example, Fig 5A shows that heparin has no effect on the number of naive B cells in the absence of antigen stimulation, but that in the presence of the antigen (HEL), there are about 10-fold more B cells in the presence of heparin than in its absence. It is very difficult for the reviewer to reconcile this result with the final conclusion of the authors that GAGs have an inhibitory role on B cells. One possibility is that under these conditions, the high affinity ligand induces death of the stimulated cells (AICD) which would explain the strongly reduced cell number of the condition "no GAGs" in the presence of antigen compared to the absence of antigen. AICD could be due to upregulation of FAS which is counteracted to some extent by the presence of heparin. As FAS is an activation marker, then heparin would negatively regulate activation, keep cells under the threshold of AICD and prevent cells from dying. If this is the case, it could be explained better, eg in a wrap up sentence. But then it is unclear why in Fig 5C that tests the effect of short-term naïve B cells exposure to heparin there is no difference in B cell number in the "No GAG" condition minus or plus antigen, and that this is also not seen in Fig 5F in No GAG minus or plus antigen. Fig 6A however resembles Fig 5A in that naïve B cell number is drastically reduced upon stimulation with antigen (probably by AICD). This defect is attenuated by heparin, but never to the extent seen in Fig 5A. Experimental variability? Different experimental conditions?

In the absence of antigen-induced activation, SW_{HEL} B cells die over the course of 3 days and the extent of this death slightly varied between experiments, something we can't explain as yet. However, the key point we make in these experiments is that a dose of heparin equal to that used for therapeutic purposes did not effect the survival/expansion of cells in the absence of antigen but greatly increased B cell numbers in the presence of antigen. Mechanistically, this is due to the reduction in AICD, an instant effect of high-affinity antigen encounter. We discuss AICD later in the manuscript, in conjunction with Figure 5A (formerly 6A). We agree it is important to highlight the experimental variability and have added the following sentence:

"Of note, the survival of SW_{HEL} B cells in the absence of antigen varied between experiments (Fig. 4A, C) but the effect of heparin was highly consistent across experiments."

Also, it would be useful for the reader if authors could integrate results of Fig 4 with their conclusion that syndecans have an inhibitory role on B cells. For example, is it conceivable that T cell help would prevent premature differentiation of cycling B cells in antibody secreting cells and plasma cells by among others, preventing CD138 expression and preventing SDC4 down-regulation, with the aim of achieving a better cell amplification before terminal differentiation?

We have added the following sentence to the discussion:
"T cell help via cytokines or CD40 signaling stimulated or retained SDC4 expression over consecutive cell divisions following naïve B cell activation and it is conceivable that this mechanism facilitates pre-GC B cell expansion."

Minor point 2: In the experiment of Figure 4, naïve B cells not only express anti-HEL BCR, but also come from a Rag1-ko background. It would be helpful in all figures using B cells with anti-HEL BCR to mention if they are also Rag1-ko or not.

We have adjusted the Figure legends accordingly as all SW_{HEL} B cells used in this study were RAG1 deficient.

Minor point 3: In Fig 4A, the CTV profile indicates that CD138 that is not expressed in naïve B cells was induced by HEL even before division, and then fully lost again after 3 divisions. This profile looks exactly like the one of Fig 4B with SDC4, except that here SDC4 was already present and is just lost upon HEL stimulation within 3 divisions. This could be better explained in the text. What justifies this very transient expression is a mystery for me. It cannot be directly linked to a differentiation to PC. Are Fig 4A,B comparable in terms of cells and HEL activation to Fig 5 and 6? If yes and if CD69 and/or FAS staining were included in these experiments (of Fig 4A,B), and if SDC4 indeed prevents activation, one would expect to see CD69 go up as cells divide in the Media only + HEL condition, but not in the anti-CD40 + HEL condition. If this data is available, please show the results (and possibly revise the conclusion of the inhibitory role of SDCs as necessary). If these stainings were not included, I let authors decide whether they would like to do it, but I do not insist that the experiment should be done.

We did not include FAS and CD69 in this experiment and have added 2 sentences to better describe the data:

"As expected, naïve SW_{HEL} B cells lacked CD138 but expressed high levels of SDC4."
…
"PC express high levels of CD138 but the transient CD138 expression induced by antigen stimulation is not associated with the initiation of the PC program, which only starts after > 5 cell divisions (Dvorscek et al, 2022; Hasbold et al, 2004; Scharer et al, 2018)."

Minor point 4: It is unclear how addition of heparin, or digestion with heparinase would interfere with SDC4 to

exert its effect on B cells. A) GAGs of SDC4 inhibit B cell activation directly (e.g. by binding the BCR). Adding heparin allows to inhibit B cells even more (e.g. by binding more BCR). The digestion with heparinase removes GAGs and less partner is inhibited. B) GAGs of SDC4 bind a positively charged partner (APRIL, IL6, IL21?) that is displaced by an excess of heparin and thus stops activating B cells. What happens if naïve B cells are digested with heparinase, then washed, then stimulated with heparin? If heparin can bind and inhibit the same target as SDC4, this should cancel the action of heparitinase (I do not insist to have this experiment done).

We thank the reviewer for these interesting thoughts but consider it very unlikely that soluble heparin only acts on molecules that also interact with SDC4-bound proteoglycans, in particular given the comparatively strong effect of heparin.

Referee #2:

Syndecans can play important roles as modulators of cytokine and growth factor responses and can mediate attachment of cells. They can also act as co-receptors for GPRs. This report aims at addressing the abundance and function of two Syndecans, Sdc1 and 4, in B cells. To this end, bone marrow of Cas9tg mice was infected with lentiviral vectors encoding specific sgRNAs and a BFP reporter. Infected BM along with RAG1-/- BM was transplanted into irradiated WT mice and B cell development and activation were analyzed. While CD138 peaks at pre B cells and in plasma cells, Sdc4 becomes up-regulated in pre and immature B cells and down-regulated upon B cell activation as well as in memory B cells, however, its expression differs among memory B cell subsets.

Functionally, deletion of both CD138 and Sdc4 disfavoured early B cell development. While Sdc4 B cells reached control frequency, the disadvantage of CD138 targeted B cells remained until the immature B cell stage. Immunization of recipient mice with NP-KLH did not reveal any effects of CD138 or Sdc4 deletion on the frequency of deleted germinal center B cells or plasma cells.
As to B cell activation, the SwHEL system was used in combination with IL-4, IL-21 and anti CD40. While IL-21 promoted HEL induced CD138 upregulation, IL-4 and CD40 suppressed it. The reverse was observed for Sdc4.

To address functional effects by putative Sdc ligands such as Polybrene and Heparin, activated B cells were co-cultured with either substance or treated with Heparinase. Heparin acted as B cell mitogen and is discussed as putative TLR4 ligand, hence being not so much relevant for human B cells in that respect. On the other hand, Heparin (HEL stimulation) suppressed AICD and CD69 expression. Heparinase (3xHEL) had no effect on AICD but increased CD69 expression. Sdc4 limited pS6 in resting and anti Ig activated B cells.

In summary, the presented data serve as a good starting point to pinpoint the role of CD138 and Sdc4 in B cells.

1. There are only few functional data regarding CD138 or Sdc4 knock-out, but both appear to affect early B cell development, which is interesting. What is the mechanism behind the disadvantage of CD138 and Sdc4 deficient pre/pro/pre B cells? PS6 needs to be downregulated to ensure proper small pre B cell development and Sdc4 appears to modulate pS6 in activated B cells. Given that the authors outstanding expertise in BM and stem cell cultures, would it not be feasible to set up stem cell / IL-7 cultures with CD138 or Sdc4 targeted stem cells and analyze pre B cell differentiation in vitro, defining proliferation/apoptosis/signaling/small pre B cell differentiation (using CD19/B220/CD2/IgM staining)? Pre BCR ligands should be discussed. It is also possible that in vitro cultrues show no effect as the potential Sdc ligand may only be present in the bone marrow. However, I believe that the role of Sdcs in B cell development should be explored more here.

We thank the reviewer for this excellent suggestion. We have now performed *in vitro* B cell differentiation assays using bone marrow from *Sdc1* or *Sdc4* targeted mice. These cultures solely depend on the presence of IL-7 and resulted in a strong (10-fold) expansion of B cell precursors (new Figure EV4C), which was associated with a loss of pro-B cells and an accumulation of large and small pre-B cells (new Figure EV4D). As shown in new Figures EV4E-F, the effects of *Sdc1* and *Sdc4* deletion closely resembled *in vivo* results, suggesting a role for SDCs in IL-7 signalling. This is also in line with studies by Paul Kincade and colleagues (PMID 9864155) who showed a key role of heparan sulfate proteoglycans in IL-7-dependent B cell development.
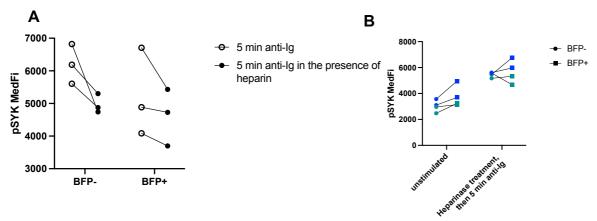
2. Was the pS6 in activated B cells normalized to total S6? Can pS6 be blocked by Rapamycin? I think the conclusion that Sdc4 is a negative regulator of BCR signaling is a bit premature. Is BCR induced pSyk/PLCg2/Ca2+ affected or are the observed effects downstream of proximal BCR signaling?

pS6 was not normalized to total S6. As shown in NEW Figure EV6G, S6 phosphorylation can be blocked by rapamycin treatment. To assess BCR signalling, we sorted BFP+ and BFP- cells from Sdc1 and Sdc4 targeted mice and analyzed Syk phosphorylation by phosphoflow. As shown in NEW Figure EV6F, baseline Syk

phosphorylation was increased in *Sdc4* but not *Sdc1* targeted mice while pSyk following BCR cross-linking was similar. The latter could be due to the high affinity of BCR cross-linking antibodies.

3. Conceptually, it would be important to assess the effects of Heparin and Heparinase also on Sdc4KO B cells.

The effect of heparin was much stronger than the deletion of Sdc4, making it highly likely that heparin treatment will not additionally affect Sdc4ko cells. In keeping with this, the presence of heparin reduced Syk phosphorylation irrespective of SDC4 (Reviewer Figure 1A). We also analysed the effect of heparinase treatment on Syk phosphorylation, with the data showing no difference between non-targeted and Sdc4 targeted cells (Reviewer Figure 1B). A detailed investigation of the effect of heparinase on antigen-mediated activation of SDC4 deficient cells would require the generation of SDC4 deficient SW_HEL mice to stimulate B cells with antigens of various affinity. This is associated with several months of significant mouse breeding efforts (generation of Cas9 transgenic SW_HEL mice) and goes beyond what can be done within the timeframe for manuscript revision. We did not include the results shown in Reviewer Figure 1 in the manuscript as they add little additional information.

*Reviewer Figure 1. (**A**) Syk phosphorylation in the presence or absence of heparin comparing non-targeted (BFP-) to Sdc4 targeted (BFP+) cells. (**B**) Syk phosphorylation in heparinase treated non-targeted (BFP-) or Sdc4 targeted (BFP+) B cells. Colours indicate cells from individual mice, assayed in duplicates.*

4. What happens if B cells are incubated with both Polybrene and Heparin?

We have now performed an experiment in which we incubated B cells with antigen, heparin and polybrene. As shown in new Figure EV6F, polybrene reversed the effect of heparin in a dose-dependent manner.

5. Why did the authors use HEL in Figure 6A and 3 x HEL in Figure 6B? This is a bit misleading and hard to grasp.

HEL has high affinity for the SW_HEL BCR and maximally activates B cells making the use of HEL as an antigen not ideal to study molecules that promote BCR signalling. We therefore used the lower affinity HEL3x antigen to investigate if heparinase treatment promoted antigen-mediated activation.

Referee #3:

The manuscript of McKenzie and colleagues provides a comprehensive overview on expression of the Glycosaminoglycan (GAG) containing cell surface proteins SDC1 and SDC4 during differentiation and activation of B lymphocytes. Based on this overview, the impact of genetic ablation of SDC1 or SDC4 in mice, and the impact of soluble GAGs on human and murine B cell activation is analysed, with the key claims that SDC4 is a negative modulator of B cell activation and SDC1 (CD138) is not relevant for differentiation of activated B cells into plasma cells. As such, the manuscript adds important information on the regulation of B cell differentiation and activation by GAGs, of interest to the readership of EMBO Reports, in light of the central role of B cells in providing humoral immunity and autoimmunity. However, some issues have to be clarified upfront. The major drawback seems to be that the entire readout of expression is based on cytometric data, and not adjusted to (single cell) transcriptome data, for staging of B cell differentiation. This would clearly put the manuscript on a different level.

The descriptive part of the manuscript, i.e. Figs 1 and 2, and EVFigs 1 and 2, is rather clear, as far as the expression pattern of SDC1 and SDC4 in the general steps of B cell differentiation is concerned. However, when

analysing the bimodal expression of SDC1 and 4 in experienced B cells, and using CD62L and CD44 to discriminate between recent GC emigrant MBC (CD62L+CD44-) and established MBC (CD62L+CD44+), the data shown in EVFig 2C show that the mice analysed did not have any CD62L+CD44+ MBC, for whatever reason, maybe the mice were just too young and immunologically inexperienced. However, in Fig. 2A bimodal distributions are shown for CDF62L-CD44- MBC and CD44-CD62L+ MBC, not for CD44+CD62L+, but then in Fig. 2B, frequencies are provided for these MBC. These data are not convincing. The differential expression of SDC1 and 4 on MBC remains enigmatic, the data provided are inconsistent, and mice with significant numbers of advanced MBC should be analysed.

We have now performed experiments to study defined, antigen-specific MBC. As such, we immunized mice with the model antigen NP-KLH and analyzed MBC three weeks thereafter. We identified MBC subsets based on CD62L/CD44 expression and on a more common approach using PD-L2 and CD80. NP-binding MBC were clearly identified and showed an expected subset distribution (new Fig. EV2). All MBC subsets showed low, heterogenous SDC4 expression, with around twice as many PD-L2-CD80- MBC expressing SDC4 compared to all other subsets.

When it comes to the controversial point, and as the authors claim key statement, of whether SDC1 (CD138) does impact on plasma cell differentiation, as suggested by an earlier publication (McCarron et al., 2017), the authors of the present manuscript claim that their data do show that SDC1 ablation does not impact on the generation of antibody-secreting plasma cells. The difficulty in enumerating plasma cells deficient for SDC1 is that SDC1 is a key cytometric marker for plasma cells. In particular here single cell transcriptome signatures would have been extremely helpful. In their absence, the authors base their classification on surrogate markers like CD19 and size (EVFig. 4) with erratic gate settings which are not defining separate populations (lymphocyte, FSA and CD19 gates). While in conjunction with CD138, these gates may define plasma blasts or even plasma cells, in the absence of CD138, i.e. in the SDC1-deficient B lineage cells, they obviously do not. There is considerable spillover from the adjacent populations and the true numbers and differentiation stages of antibody-secreting B lineage cells remain enigmatic. Even more so, the authors also show the numbers of antigen-binding "plasma cells" in Figs. 3F and I, which at least for Ig-secreting cells means that these cells are not yet fully mature plasma cells, which would not express membrane-bound antibodies, but rather either B cell blasts or plasma blasts, thus weakening the claim that SDC1 does not impact on plasma cell differentiation even further.

To confirm that our gating strategy indeed identifies PC, we have applied that approach to prdm1-tdTomato reporter mice. In these mice all PC are identified by tdTomato expression and >90 % of cells identified as PC by our gating strategy expressed tdTomato (new Fig. EV4B). This provides clear evidence that our approach is applicable for PC analysis.

Regarding the analysis of NP-specific PC, we would like to note that most early PC retain some surface BCR expression, and NP-specific PC can be readily detected (please see for example Fig1A from PMID 27924814 or Fig. 2A from PMID 22529295). We agree that the NP-specific PC are unlikely to be mature, long-lived PC given the analysis was done 7 days post immunization and PC maturation occurs gradually over time. To highlight this important point, we have now included the following sentences on page 5 of the revised manuscript:

"Similarly, Sdc1 targeting did not influence the representation of these cells among splenic GC and PC compared to naïve B cells when total (Fig. 2E) or NP-binding GC B cells and PC (surface immunoglobulin expression by early PC allows detection of antigen-specific cells (Blanc et al, 2016; Bortnick et al, 2012)) were compared (Fig. 2F), again arguing against a role of CD138 for B cell activation or PC differentiation. It is important to note that our data do not allow conclusions about the potential role of CD138 in regulating PC lifespan as the total PC pool is constantly replenished and long-lived PC make up only a small fraction of all PC (Robinson et al., 2023; Robinson et al., 2022). However, at the level of analysis conducted here, and aside from a possible drop in small pre-B cells, CD138 had no or only a minor role in B cell development and was dispensable for antigen-induced activation and PC differentiation."

The authors then analyse the regulation of SDC expression in activated B cells by the costimulators IL21, IL4 and CD40L. Here they claim that SDC1 is upregulated and SDC4 downregulated in antigen-activated B cells, and IL21 enhances, while IL4 and CD40L inhibit these SDC regulations. What is not clear from the data shown is to what extent IL21, IL4 and antiCD40 impact on the differentiation of activated B cells into plasma cells versus their proliferation as B cells, i.e. whether their effect is directly on SDC expression or indirectly on B cell activation (Fig. 4).

Plasma cell differentiation has yet to occur at the time point analyzed. We have now added the following sentence to clarify that:

"PC express high levels of CD138 but the transient CD138 expression induced by antigen stimulation is not associated with the initiation of the PC program, which only starts after > 5 cell divisions (Dvorscek et al, 2022; Hasbold et al, 2004; Scharer et al, 2018)."

Finally, in Figs 5 and 6, the authors use heparin (Fig. 5) and heparinase (Fig. 6) to demonstrate that the GAG heparin sulfate can (down)regulate B cell activation. These data are not directly linked to SDC1 or 4, and thus remain a bit preliminary. As are the data on S6 phosphorylation. As a net result, the molecular mechanism of SDC4 attenuation of antigen-mediated activation of B cells remains to be elucidated.

Analyzing the detailed molecular mechanism of how SDC4 regulates B cell activation goes beyond the scope of this study. We do think, however, that our data add valuable information about the role of SDCs in B cell biology and the effects of soluble proteoglycans provide an important advance in our understanding how B cell activation can be regulated.

Dear Dr. Quast,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the report from the three referees that were asked to re-evaluate the study, you will find below. As you will see, the referees now fully support the publication of the study in EMBO reports. Please correct the typo mentioned by referee #2.

Before I can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- Please provide the abstract written in present tense.

- Please reduce the number of keywords to 6 and order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Methods - Data availability section - Acknowledgements (including the funding information) - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

- Please upload individual production quality figure files as .eps, .tif, .jpg (one file per figure), also for the EV figures. Please upload these as separate, individual files upon re-submission. Moreover, please use the nomenclature Figure EVx for these in their legends and check again all the callouts.

- Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file. Presently, grants 'Monash University Research Training Program stipend and Career Advancement Award provided by the Australian and New Zealand Society for Immunology' and 'Monash University Future Leader Fellowship' are missing from the submission system. Please check.

- The Data availability section (DAS) is restricted for information on primary datasets produced in a study (e.g. RNA-seq, ChIP-seq, structural and array data) that are deposited in an public database. If no primary datasets have been deposited, please state this here (e.g. 'No primary datasets have been generated and deposited'). Please remove all further text not related to externally deposited datasets from this section.

- Please check again that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends. Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams. Presently, several diagrams have no or only partial statistics or 'n.s.' is missing (e.g. please check panels 1D, 1F, 2D-I, EV4D-F, EV5C and EV6B-I). Moreover:
- Please define the annotated p values ****/***/**/* as well as provide the exact p-values for the same in the legend of figure 5A, EV4 C as appropriate.
- Please note that the exact p values are not provided in the legends of figures 1F, 2B, C; 3C, D, 4A-F; 5A-C; EV5 B.
- Please note that information related to n is missing in the legends of figures 3C, D; 4F, EV5 C, EV6 B, C, D, E, F, G.
- Please note that the error bars are not defined in the legends of figures 1F, 4B, D; EV5 C, EV6 B, C, D, E, F.

- There seems to be a re-use of panels between Figure EV2A (firtst row, 'All events' and 'Live') and Figure EV5D (firtst row, 'All events' and 'Live'). If this is intended, blease clearlyc explain and indicate this in the respective figure legends.

In addition, I would need from you uploaded separately:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short (!) bullet points highlighting the key findings of your study (two lines each).
- a schematic summary figure as separate file that provides a sketch of the major findings (not a data image) in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Achim Breiling
Editor
EMBO Reports

Referee #1:

I thank authors for their answers and I have no further comments

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Referee #2:

I thank the authors for addressing the comments adequately.

I found one typo: l 144, PLIMP-1.

Referee #3:

The authors have responded perfectly to my previous concerns and modified the manuscript accordingly, added new data, in particular on MBC, to clarify previous inconsistencies. From my point of view, the manuscript should now be accepted.

Dear EMBO Reports team, dear Achim,

Please find below a point-by-point-response to all requests.

- Please provide the abstract written in present tense.
The abstract is now written in present tense.

- Please reduce the number of keywords to 6 and order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Methods - Data availability section -
Acknowledgements (including the funding information) - Disclosure and Competing Interests Statement -
References - Figure legends - Expanded View Figure legends
Keywords were reduced to 5. Section order has been changed accordingly.

- Please upload individual production quality figure files as .eps, .tif, .jpg (one file per figure), also for the EV
figures. Please upload these as separate, individual files upon re-submission. Moreover, please use the
nomenclature Figure EVx for these in their legends and check again all the callouts.
Nomenclature changed accordingly, individual HQ figures uploaded and callouts re-checked.

- Please make sure that all the funding information is also entered into the online submission system and that it is
complete and similar to the one in the acknowledgement section of the manuscript text file. Presently, grants
'Monash University Research Training Program stipend and Career Advancement Award provided by the
Australian and New Zealand Society for Immunology' and 'Monash University Future Leader Fellowship' are
missing from the submission system. Please check.
Checked and added to the online system.

- The Data availability section (DAS) is restricted for information on primary datasets produced in a study (e.g.
RNA-seq, ChIP-seq, structural and array data) that are deposited in an public database. If no primary datasets
have been deposited, please state this here (e.g. 'No primary datasets have been generated and deposited').
Please remove all further text not related to externally deposited datasets from this section.
Changed accordingly.

- Please check again that the number "n" for how many independent experiments were performed, their nature
(biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-
values is indicated in the respective figure legends. Please also check that all the p-values are explained in the
legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please
avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates.
Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2,
please show the data as separate datapoints without error bars and statistics. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis
Nature and amount (n) of replicates clearly stated.

If n<5, please show single datapoints for diagrams. Presently, several diagrams have no or only partial statistics
or 'n.s.' is missing (e.g. please check panels 1D, 1F, 2D-I, EV4D-F, EV5C and EV6B-I). Moreover:
- Please define the annotated p values ****/***/**/* as well as provide the exact p-values for the same in the
legend of figure 5A, EV4 C as appropriate.
We now show all exact p values in the figures, and I have removed all 'stars'. I have added additional statistical
comparisons but for some data, we have chosen not to perform statistical analysis for the following reasons:
   -    1 B and D show Syndecan expression levels of on cell types and I do not think statistical analysis is
        relevant but can of course be provided if that is preferred.
   -    Figure 1F, statistical analysis of relevant comparisons provided.
   -    Figure 2D-I and EV4E, F show the representation of the cell population over consecutive differentiation
        stages and the key point is the trend over time rather than comparison within individual differentiation
        stages.
   -    EV5B,C: The most relevant statistical analysis would be comparing panels B and C which would require
        changing the figure outline. If requested, I could prepare a separate graph showing 'division number 0'
        only data and perform statistical analysis but I do not think this adds information.
   -    EV6B-E: Statistical analysis provided.
   -    EV6F: This is supporting data for Figure 4F and shows that the effect of polybrene is titratable. To
        analyze this statistically, I would have to do a correlation analysis which is difficult given the data is
        multi-dimensional (division number, cell-count and polybrene dose). I think the correlation between
        increased polybrene and reduced cell count is very clear and does not require statistical analysis.
   -    EV6G: There are not enough data points for statistical analysis. This data was provided in response to a
        reviewer request and is simply confirmatory, showing the well-known fact that rapamycin inhibits S6
        phosphorylation.

- Please note that the exact p values are not provided in the legends of figures 1F, 2B, C; 3C, D, 4A-F; 5A-C; EV5 B.
Exact p values are now shown for all statistical analysis (all * removed).
- Please note that information related to n is missing in the legends of figures 3C, D; 4F, EV5 C, EV6 B, C, D, E, F, G.
Information added.
- Please note that the error bars are not defined in the legends of figures 1F, 4B, D; EV5 C, EV6 B, C, D, E, F.
Error bars defined (there are no error bars in 1F)
- There seems to be a re-use of panels between Figure EV2A (firtst row, 'All events' and 'Live') and Figure EV5D (firtst row, 'All events' and 'Live'). If this is intended, blease clearlyc explain and indicate this in the respective figure legends.
Thank you for highlighting this, we have prepared the gating strategy figures on the same day and must have accidentally forgotten to exchange this panel because FSC/SSC look very similar for every sample. We have now provided the correct exemplary image for Figure EV5D.

In addition, I would need from you uploaded separately:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short (!) bullet points highlighting the key findings of your study (two lines each).
- a schematic summary figure as separate file that provides a sketch of the major findings (not a data image) in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.
Provided.

Achim Breiling
Editor
EMBO Reports

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The typo has been corrected.

Referee #3:

The authors have responded perfectly to my previous concerns and modified the manuscript accordingly, added new data, in particular on MBC, to clarify previous inconsistencies. From my point of view, the manuscript should now be accepted.

We thank the reviewers for their valuable contributions to improving our study.

Dr. Isaak Quast
Monash University
Immunology and Pathology
89 Commercial Road
Melbourne, Victoria 3004
Australia

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Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

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Manuscript Number: EMBOR-2024-59818V2

Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
→ ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
→ plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
→ if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
→ Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
→ a specification of the experimental system investigated (eg 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 vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
- are tests one-sided or two-sided?
- are there adjustments for multiple comparisons?
- exact statistical test results, e.g., P values = x but not P values < x;
- definition of 'center values' as median or average;
- definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	not applicable

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgment

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figures
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Materials and Methods
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants**: For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples**: State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III 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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	