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Peer Review File

Agnostic B cell selection approach identifies antibodies against *K. pneumoniae* that synergistically drive complement activation

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this study, van der Lans et. al have devised a new method to screen for antibodies that activate the complement cascade in an unbiased fashion by using whole-cell bacteria (Klebsiella pneumoniae). B cells able to bind whole bacteria were agnostically selected for after binding Klebsiella. Their B cell receptors were then sequenced (not a small task) and ultimately monoclonal antibodies were produced. The majority of these bound either O-antigen or capsule indicating that B cell receptors may not be able to access antigens beneath the surfaces on whole bacterial cells. Additionally, while antibodies against both O-antigen and capsule polysaccharide could both bind quite strongly, only those against O-antigen were able to activate the complement cascade, using their example strain. This manuscript was well-written and the use of Klebsiella as a target is important considering the global significance of this increasingly resistant and virulent pathogen. Overall this method is a powerful tool for finding highly avid antibodies in an unbiased way, although it may be a bit labor intensive upfront. Further, this reviewer has some specific questions about the human samples being used that, if further clarified, could strengthen this work:

-Why did the authors choose to perform this analysis on healthy human serum, rather than choosing serum from patients that have survived a Klebsiella infection. One would expect these survivors to have larger memory B cell populations that were capable of binding (and potentially killing) Klebsiella. By using healthy human sera, aren't the authors selecting for antibodies that may not be as specific or functional? If healthy humans had a large array of pre-existing functional antibodies that bind Klebsiella already circulating, then we would rarely see Klebsiella infections. The authors should justify why they chose healthy subjects and how their results may have differed had they chosen Klebsiella-exposed individuals.

-Despite the detailed description of bacterial dual-labeling with fluorophores (lines 180-195), it remains unclear why double labeling was required. Was this method initially performed with single-labeled bacteria but did not demonstrate good specificity for B cells capable of binding? Why was "no enrichment observed for the single-positive populations" of B cells? Shouldn't a B cell bound to a Klebsiella with even a single fluorophore suggest it may have relevant binding ability? Further, how does unlabeled bacteria "compete" with double-positive B cells? Wouldn't this just decrease the yield of truly relevant B cells capable of binding Klebsiella?

-For the complement activation assays, the authors incubated bacteria, normal human serum (as a source of complement), with their specific antibody in question. However, wouldn't the normal human serum also potentially contain antibodies targeting Klebsiella? The authors discuss the creation of antibody-depleted NHS in the methods but fail to mention it in the results. Using bacteria as a method to deplete antibodies seems like it also may affect the complement components. Why not deplete antibodies another way (using anti-human IgG Abs)?

-The authors include interesting studies looking at mutations in the Fc region that make the antibodies more likely to hexamerize. Interestingly the K-targeted monoclonal antibodies were less likely to hexamerize at baseline unless combined together. Do the authors think this would be the case in a patient that survived Klebsiella infection? Is this representative of the specific monoclonals studied here, or do the authors think this is a more universal phenomenon?

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Reviewer #2 (Remarks to the Author):

The manuscript by van der Lans et al. describes a method to identify and characterise in depth new antibodies directed against specific bacterial strains. The technology seems novel, versatile, elegant and efficient. Using this technology, the authors discover antibodies against different antigens on K. pneumoniae, and characterise their potential to drive complement activation and phagocytosis/killing by human neutrophils. The results and methodology are presented in a logical and accessible manner, and the conclusions drawn are fully supported by the data. As a non-subject expert, I cannot comment on other state-of-the-art studies in this field and the potential impact of this study. Instead my comments mainly focus on methodological aspects and some unexplained observations.
Major points:
1) The rationale behind only the double-positive B cells being enriched in memory B cells (Fig. 1b) is unclear. Theoretically, the two bacterial populations only differ in their staining (actually only in the fluorophore), not in any other aspect which might impact on their binding by B cells. Since the staining efficiency and fluorophore brightness and detector sensitivity might very well differ between the two stained strains, the detection efficiency and the relative brightness of the two populations might be different in absolute numbers but their behaviour should be qualitatively comparable. It appears that the double positive B cells are those who are stained brightest in each of the two channels. If the labeling of bacteria does not affect their binding by B cells, a similar enrichment in Memory B-cells should also be observed when gating for either KpnO2-Cy5 or KpnO2-Atto488 cells with very high fluorescence intensity (=many bacteria bound), e.g. by only analysing I(KpnO2-Cy5)>10,000 or I(KpnO2-Atto488) > 5,000. Is this the case? Otherwise, could the authors explain in more detail what distinguishes these double-positive B-cells from those only binding one stained strain?
2) To understand the technology better, it would be helpful to know how many bacteria are typically bound by one B-cell. This could be estimated based on the fluorescence intensity of single bacteria in the flow cytometer, or visualised by an imaging flow cytometer or, alternatively, by normal fluorescence microscopy of pre-sorted B-cells.
3) To exclude that the staining of the bacteria influenced the binding by antibodies (and thus the selection process), the data with 5x and 25x excess of unstained bacteria (Fig. S1d) could be reanalysed in terms of the gMFI of the stained populations (Q1 and Q3). If unstained bacteria have a similar binding affinity for the B cells as the stained bacteria, the gMFI should roughly reduce by a factor of 5x and 25x, respectively. If this is not the case, the authors should investigate and discuss the potential interference of the staining procedure on the selection process.
4) Figure 5d showed a 100-fold lower survival of KpnO1 at 0.01 µg/mL UKpn72 compared to 10x and 100x higher antibody concentrations (0.1 and 1 µg/mL). This is surprising given that the same antibody at the same concentration hardly induced phagocytosis by neutrophils (Fig. 5b); it did however robustly induce C3b deposition at this concentration (Fig. 4c). Since the authors excluded a neutrophil-independent killing mechanism (Fig. S5c), they should discuss in more detail both, the proposed mechanism of action, and the peculiar dose-response behaviour. Was this behaviour also observed with other O1 antibodies? Would this have implications for possible applications in terms of a therapeutic window?
5) Fig. 6e also shows enhanced binding of fluorescently labeled UKpn3 when competing against 1-10 µg/mL of unlabeled UKpn3. The same effect was seen for UKpn1 (Fig. S6d). What is the explanation for this finding? Shouldn’t the same antibody compete for the same epitope and thus

lead to a decrease in binding (as observed in Figure S6c for UKpn2)?
6) The authors stress that (line 460ff) „Indeed, most antibodies identified in this study could recognize the O1-polysaccharide exclusively in the context of whole bacterial cells and not in its isolated form.", which refers to Fig. 3h. Fig 3g shows that exactly those antibodies which also recognised the antigen in isolated form were those that also recognised other strains. Is there a scientific explanation for this coincidence?
7) The synergism of anti-capsule antibodies is highly interesting, but the underlying mechanism remains mostly elusive, which calls for further investigations. Are capsule antigens more mobile on the bacterial surface than O2-antigen and O1-antigens? What is known about the relative surface concentrations or clustering of capsule proteins vs O2 and O1 glycans? Both, mobility and local clustering, could potentially influence hexamer formation (besides spacing/steric hindrance, as discussed by the authors). Any experiment and/or simulations that could provide more mechanistic insights into the observed synergism would be of high value.
Minor points:
8) Figure 1a: scale bars missing
9) Figure S1c, legend: what does „MOI of 0.5 per fluorophore" mean? Is this referring to Multiplicity of Infection? Or related to the detection sensitivity (0.5 AU per fluorophore)? Please clarify and introduce abbreviation upon first usage.
10) Figure S1 Legend: the last sentence of the legend is duplicate/unneccessary: „(b) Fluorescent intensity (gMFI) and number of events normalized to mode are depicted on the X and Y-axis, respectively."
11) Inconsistency between text and Figure 6a. Line 347f states that „mixing two antibodies recognizing the O2 antigen (UKpn2 and UKpn6) did not affect their capacity to drive C3b deposition (Fig. 6a)." However, Fig. 6 a shows UKpn1+UKpn2. Probably there is a typo in the Figure?
12) Methods: Line 549f: incomplete sentence/information: „Lysis buffer consisted of."
13) Grammar: Line 117f: Clinically, the O1, O2 and O3 O-types are the most relevant as they [verb missing] over 80% of the clinical isolates.
14) Grammar: Line 287: missing „Upon specific deletion [of] wbbY,"
15) Grammar: Line 289: duplicate „suggesting suggests" should be „suggesting" only

Reviewer #3 (Remarks to the Author):

The article "Agnostic B cell selection approach identifies antibodies against K. pneumoniae that synergistically drive complement activation" by van der Lans and Bardoel et al aims to describe antibody characteristics of human blood IgG memory B cells to the surface antigens of Klebsiella pneumoniae. Using an elegant dual staining approach, the authors isolate blood IgG memory B cells from healthy individuals bearing antibodies to K. pneumoniae prevalent O serotypes O1 and O2. Next, they generated monoclonal antibodies from those isolated B cells and showed that a small percentage binds to K. pneumoniae surface glycans. In total they identified 29 unique monoclonal antibodies. Next, they suggest that antibody binding intensity does not always correlate with the capability for C3b deposition, especially capsule, in contrast to O antigen antibodies, are incapable to activate complement. The authors showed that hexamer formation-enhancing Fc mutations allowed capsule antibodies to activate complement and that antibody-dependent bacterial killing did depend on the presence of neutrophils. Last, they show a synergistic binding and complement activation effect of two capsule antibodies.

Novel, notable and interesting things about this paper:

1) Using azide-KDO paired with click-chemistry to isolate memory B cells with specific BCRs is an elegant approach and likely suitable to isolate antibodies to other KDO-expressing bacteria
2) Data suggest that only glycan antigens are accessible on Kp surface
3) The authors show a synergistic binding and C3b deposition effect of two monoclonal antibodies targeting the capsule of Klebsiella pneumoniae

Possible concerns:

1) Fig 2a-d: It seems that O2 O antigen-reactive antibody binding levels do correlate with C3b deposition and only capsule-binders show no correlation. Please confirm. Fig. 2b could be plotted at higher antibody concentration to account for the differences between intermediate and negative binders.
2) The authors should proof that the O1 and O2 O antigen reactive antibodies directly target purified LPS of the respective O serotype (similar to Figure 3h).
3) Fig. 6e is not very intuitive. Why does Ukpn3 bind slightly better if more unlabelled UKpn3 is added? The MFI should drop when more of the same unlabelled antibody is added.
4) Fig S6b: Please confirm that UKpn1 and 3 do not deposit C3b on K00059 and add an isotype control.
5) The authors should show if the synergistic binding effect of UKpn1 and 3 can be observed on isolated capsule glycan or it depends on the intact bacterium.
6) Fig 6g: Can Spa-B bind to UKpn1 and UKpn3 while they bind the surface of KpnO2?
7) The mechanism of UKpn1 and 3 binding synergy is not resolved: Can the Fab fragments of UKpn1 and 3 alone induce the synergistic binding effect?
8) Ultimately, an important question remains: Do UKpn1 and 3 together are protective in an infection model?

Minor comments:

9) Axis labels/ units are frequently missing
10) The authors should change the wording for "active" and "inactive" antibodies. At the end these antibodies are not inactive.
11) Fig. S6b statistics missing
12) Line 340: Uptake by neutrophils was not addressed
13) Line 311: Please change wild-type to unmutated

Point-to-point reply (reference NCOMMS-24-14275)

Reviewer #1:

In this study, van der Lans et. al have devised a new method to screen for antibodies that activate the complement cascade in an unbiased fashion by using whole-cell bacteria (Klebsiella pneumoniae). B cells able to bind whole bacteria were agnostically selected for after binding Klebsiella. Their B cell receptors were then sequenced (not a small task) and ultimately monoclonal antibodies were produced. The majority of these bound either O-antigen or capsule indicating that B cell receptors may not be able to access antigens beneath the surfaces on whole bacterial cells. Additionally, while antibodies against both O-antigen and capsule polysaccharide could both bind quite strongly, only those against O-antigen were able to activate the complement cascade, using their example strain. This manuscript was well-written and the use of Klebsiella as a target is important considering the global significance of this increasingly resistant and virulent pathogen. Overall this method is a powerful tool for finding highly avid antibodies in an unbiased way, although it may be a bit labor intensive upfront. Further, this reviewer has some specific questions about the human samples being used that, if further clarified, could strengthen this work:

POINT 1: Why did the authors choose to perform this analysis on healthy human serum, rather than choosing serum from patients that have survived a Klebsiella infection. One would expect these survivors to have larger memory B cell populations that were capable of binding (and potentially killing) Klebsiella. By using healthy human sera, aren't the authors selecting for antibodies that may not be as specific or functional? If healthy humans had a large array of pre-existing functional antibodies that bind Klebsiella already circulating, then we would rarely see Klebsiella infections. The authors should justify why they chose healthy subjects and how their results may have differed had they chosen Klebsiella-exposed individuals.

ANSWER 1: We focused on healthy donors since it was previously described that healthy individuals are exposed to Klebsiella and are an effective source for identifying memory B cells against purified antigens of Klebsiella. The referee is right that the levels of specific memory B cells in circulation are probably lower in healthy individuals. However, it is much easier to obtain large volumes of blood (and thus more B cells) from healthy donors than from patients. Especially, when using buffy coats derived from 500 ml healthy donor blood. In the revised manuscript, we now clarify our rationale for using healthy donors in (results section page 5, line 181-182; discussion page 13, line 537-540).

We agree that employing a similar strategy on B cells from patients is an interesting follow-up study. Our study demonstrates that healthy donors have functional antibodies, but for patients this is not yet known. One could even argue that healthy individuals could potentially have better antibodies than patients, since they do not get disease. Indeed, there is literature suggesting that infected patients make non-functional antibodies that inhibit complement activation on Gram-negative bacteria. In the discussion, we now elaborate on how the results could have been different when choosing patients (page 13, line 542-546).

POINT 2: Despite the detailed description of bacterial dual-labeling with fluorophores (lines 180-195), it remains unclear why double labeling was required. Was this method initially performed with single-labeled bacteria but did not demonstrate good specificity for B cells capable of binding? Why was "no enrichment observed for the single-positive populations" of B cells? Shouldn't a B cell bound to a Klebsiella with even a single fluorophore suggest it may have relevant binding ability? Further, how does unlabeled bacteria "compete" with double-positive

B cells? Wouldn't this just decrease the yield of truly relevant B cells capable of binding Klebsiella?

ANSWER 2: We used the dual-labeling technique to address the problem of false positive events in rare event sorting. We based this idea based on previous papers in which dual labeling of B cells with purified antigens was presented as a relatively simple method to enhance the signal to noise ratio and thus reduce the number of false positive events (PMID: 28111638). Although we think it would be possible to sort B cells based on fluorescence intensity with a single fluorophore, with one color it could be more difficult to eliminate false-positive events. The rationale for the dual labeling method is now clarified (Results, page 5, line183-184).

In addition, we now performed fluorescence microscopy on the single and dual stained B cells to visualize our selection process (also see answer reviewer 2, point 2). While double-positive B cells indeed have multiple bacteria attached, 95% of single positive B cells have no bacteria bound indicating this signal is not specific (new Fig 1c, S1f & Results, page 5).
When adding unlabeled bacteria there is competition between fluorescently labeled and unlabeled bacteria for binding to B cells (when the cells are saturated). Therefore, we observe a decrease of the double positive B cells when adding unlabeled bacteria in excess. In Fig S1e, we now also included gMFI of Fig S1d to show that when adding an excess of bacteria the specific signal (dual labeled B cells) disappears.

POINT 3: For the complement activation assays, the authors incubated bacteria, normal human serum (as a source of complement), with their specific antibody in question. However, wouldn't the normal human serum also potentially contain antibodies targeting Klebsiella? The authors discuss the creation of antibody-depleted NHS in the methods but fail to mention it in the results. Using bacteria as a method to deplete antibodies seems like it also may affect the complement components. Why not deplete antibodies another way (using anti-human IgG Abs)?

ANSWER 3: Normal human serum (NHS) can indeed contain antibodies against Klebsiella. To exclusively measure the effect of our monoclonal antibodies, we therefore often used serum that is depleted from pre-existing antibodies. This was mainly the case for assays with the *Kpn*O1 strain, since antibody levels against this strain are higher than for *Kpn*O2. For the *Kpn*O2 strain, preexisting antibody levels are low and there is no difference between using NHS or pre-absorbed serum. Therefore, some experiments with *Kpn*O2 are performed with NHS. In the revised manuscript we have now stated more clearly when we used NHS versus depleted serum (Results and legends sections).
Also, we have now clarified the use of our depletion method (Results, page 6, line 223-225). We prefer depleting only bacteria-specific antibodies, since others have shown that total depletion of human IgG negatively affects complement activation (even after adding back polyclonal IgG and IgM (doi: 10.3389/fimmu.2018.02770). We previously showed that the depletion of bacterial antibodies alone specifically depletes strain-specific antibodies while keeping complement activity intact (doi: 10.1038/s41598-023-39613-5).

POINT 4: The authors include interesting studies looking at mutations in the Fc region that make the antibodies more likely to hexamerize. Interestingly the K-targeted monoclonal antibodies were less likely to hexamerize at baseline unless combined together. Do the authors think this would be the case in a patient that survived Klebsiella infection? Is this representative of the specific monoclonals studied here, or do the authors think this is a more universal phenomenon?

ANSWER 4: The fact that the two synergistically acting antibodies (UKpn1 and 3) were isolated from the same healthy donor indeed suggests that cooperation between K-targeting antibodies could also occur in serum of an infected individual. Although future research is needed to determine whether other antibodies can do the same, our study indicates that collaboration between anti-capsular antibodies could be an important, but until now overlooked, mechanism by which antibodies protect against infections. We now elaborate on these points in the discussion section (page 12, line 506-515).

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ANSWER 5: All points above are corrected in the revised manuscript.

POINT 6: Line 203: Upon introduction of the clinical strains, the authors should give further description. What site of infection were they isolated from? Are they classical or hypervirulent strains? Etc.

ANSWER 6: *Kpn*O2 and *Kpn*O1 are classical strains. Their sequence type and isolation site are now included in the manuscript (Results, page 5, line 204; and table S1).

POINT 7: Line 250: the authors demonstrate that capsule-targeting antibodies were all ineffective at activating complement. This contradicts much of the vaccine literature in which capsule-targeted antibodies are highly capable of killing most strains by SBA. Please discuss.

ANSWER 7: We believe that our findings do not contradict previous vaccine literature. Our monoclonal anti-KL110 antibodies were not able activate the complement system. However, as a polyclonal (or rather biclonal) mix, they did efficiently induce complement deposition and opsonophagocytosis. Since a capsule vaccine will induce a polyclonal response, an important aspect of the protective properties of the described capsule vaccines might be due to combined effects antibodies in the polyclonal response. We have now revised the discussion section to better reflect on previous vaccination studies (page 12, line 509-515).

POINT 8: Fig 5: why only neutrophils and no macrophages? (Figure 5)

ANSWER 8: Although macrophages could also be used, we here focused on neutrophils since they are very effective in phagocytosis and killing of bacteria and therefore a relevant cell type to test the effector functions of the here identified antibodies.

Reviewer #2:

The manuscript by van der Lans et al. describes a method to identify and characterise in depth new antibodies directed against specific bacterial strains. The technology seems novel, versatile, elegant and efficient. Using this technology, the authors discover antibodies against different antigens on K. pneumoniae, and characterise their potential to drive complement activation and phagocytosis/killing by human neutrophils. The results and methodology are presented in a logical and accessible manner, and the conclusions drawn are fully supported by the data. As a non-subject expert, I cannot comment on other state-of-the-art studies in this field and the potential impact of this study. Instead my comments mainly focus on methodological aspects and some unexplained observations.

Major points:

POINT 1: The rationale behind only the double-positive B cells being enriched in memory B cells (Fig. 1b) is unclear. Theoretically, the two bacterial populations only differ in their staining (actually only in the fluorophore), not in any other aspect which might impact on their binding by B cells. Since the staining efficiency and fluorophore brightness and detector sensitivity might very well differ between the two stained strains, the detection efficiency and the relative brightness of the two populations might be different in absolute numbers but their behaviour should be qualitatively comparable. It appears that the double positive B cells are those who are stained brightest in each of the two channels. If the labeling of bacteria does not affect their binding by B cells, a similar enrichment in Memory B-cells should also be observed when gating for either KpnO2-Cy5 or KpnO2-Atto488 cells with very high fluorescence intensity (=many bacteria bound), e.g. by only analysing I(KpnO2-Cy5)>10,000 or I(KpnO2-Atto488) > 5,000. Is this the case? Otherwise, could the authors explain in more detail what distinguishes these double-positive B-cells from those only binding one stained strain?

ANSWER 1:

We used the dual-labeling technique to address the problem of false positive events in rare event sorting. We based this idea based on previous papers in which dual labeling of B cells with purified antigens was presented as a relatively simple method to enhance the signal to noise ratio and thus reduce the number of false positive events (PMID: 28111638).

As suggested, we reanalyzed the geoMFI of the double stained bacteria for each fluorophore separately and observed that the levels were higher compared to the single stained bacteria (SFig 1f, right panel). In general, the dual stained B cells bind on average more bacteria (as discussed at point 2 below, new Fig S1f) resulting in higher fluorescence in Cy5 and Atto488 channel (as observed in Fig 1b, S1e). As suggested by the referee, this shows that it would be possible to sort B cells based on fluorescence intensity with a single fluorophore and obtain an enrichment in memory B cells. However, with two colors it is easier to eliminate false-positive events.

As explained in answer 2 below, new microscopy experiments demonstrated that double-positive B cells indeed have multiple bacteria attached. In contrast, single positive B cells have no bacteria bound indicating that this signal is not specific (new Fig 1c, S1f),

POINT 2: To understand the technology better, it would be helpful to know how many bacteria are typically bound by one B-cell. This could be estimated based on the fluorescence intensity of single bacteria in the flow cytometer, or visualised by an imaging flow cytometer or, alternatively, by normal fluorescence microscopy of pre-sorted B-cells.

ANSWER 2: As suggested, we sorted single and double positive B cells for *Kpn*O2-Atto488 and *Kpn*O2-Cy5 into poly-Lysine coated cell imaging chambers. Imaging revealed that most B cells that were sorted as double positive have multiple bacteria in the two different colors attached. For the B cells that stained single positive for *Kpn*O2-Cy5 only some bacteria that were not attached to B cells were observed. We now included an image and quantification of single and double stained sorted cells as Fig 1c and Sfig1f and updated the results section.
Based on the flow cytometry data, we determined that the gMFI of single versus double stained bacteria was 2-3-fold higher (new Fig S1e and f – right panel) indicating that on average there are 5 times more bacteria associated with a B cell. With microscopy we observed that 47% of the double-positive-sorted cells have bacteria bound, indicating that on average around ~10 bacteria bind to a bacterium specific B cell. We included this information as new figure 1c and S1f and described the experiment in the results section (page 5).

POINT 3: To exclude that the staining of the bacteria influenced the binding by antibodies (and thus the selection process), the data with 5x and 25x excess of unstained bacteria (Fig. S1d) could be reanalysed in terms of the gMFI of the stained populations (Q1 and Q3). If unstained bacteria have a similar binding affinity for the B cells as the stained bacteria, the gMFI should roughly reduce by a factor of 5x and 25x, respectively. If this is not the case, the authors should investigate and discuss the potential interference of the staining procedure on the selection process.

ANSWER 3: We reanalyzed the gMFI as suggested. The gMFI of cells in Q1 and Q3 were unaffected by addition of unlabeled bacteria as shown in the new Fig S1e. This indicates that this signal is not specific, which is confirmed with the new microcopy data (new Fig 1c, S1f) of single positive B cells that shows that 95% (S1f, right panel) of the cells have no bacteria bound. In contrast, in the double-positive population many B cells contain multiple bacteria, which explains why adding an excess of unlabeled bacteria results in competition and decrease of gMFI (Fig S1d and S1e). The decrease in fluorescent signal is 2.5-fold when adding 5x or 25x excess of unstained bacteria (Fig S1d). Due to the background signal, the specific signal is already lost at 5x excess of unlabeled bacteria.

POINT 4: Figure 5d showed a 100-fold lower survival of KpnO1 at 0.01 µg/mL UKpn72 compared to 10x and 100x higher antibody concentrations (0.1 and 1 µg/mL). This is surprising given that the same antibody at the same concentration hardly induced phagocytosis by neutrophils (Fig. 5b); it did however robustly induce C3b deposition at this concentration (Fig. 4c). Since the authors excluded a neutrophil-independent killing mechanism (Fig. S5c), they should discuss in more detail both, the proposed mechanism of action, and the peculiar dose-response behaviour. Was this behaviour also observed with other O1 antibodies? Would this have implications for possible applications in terms of a therapeutic window?

ANSWER 4: In figure S5c we show that there is no major effect of UKpn72 on neutrophil-independent killing, however for UKpn72 there is a slight effect at 0.01 µg/ml.
At this concentration, a potential collaboration between MAC and neutrophils (10.1371/journal.ppat.1009227) could lead to better killing. We now better explain this in the results section (page 8, line 345-346). We have also observed this behavior for other O1 antibodies (such as the previously described KPB202 antibody (Patent No.: US 11,117,956 B2)), but we don't know if this behavior is relevant in vivo,

POINT 5: Fig. 6e also shows enhanced binding of fluorescently labeled UKpn3 when competing against 1-10 µg/mL of unlabeled UKpn3. The same effect was seen for UKpn1 (Fig. S6d). What is

the explanation for this finding? Shouldn't the same antibody compete for the same epitope and thus lead to a decrease in binding (as observed in Figure S6c for UKpn2)?

ANSWER 5: We suspect there is no saturation at this point since the capsule is a very abundant antigen on the bacterial surface and therefore there is no competition. In our paper, we focused on the increase in fluorescence when combining UKpn3-A647 with unlabeled UKpn1 since this effect is much stronger than for UKpn3-A647 with unlabeled UKpn3. For the slight increase with unlabeled UKpn3, we have no explanation at this moment.

POINT 6: The authors stress that (line 460ff) „Indeed, most antibodies identified in this study could recognize the O1-polysaccharide exclusively in the context of whole bacterial cells and not in its isolated form.", which refers to Fig. 3h. Fig 3g shows that exactly those antibodies which also recognised the antigen in isolated form were those that also recognised other strains. Is there a scientific explanation for this coincidence?

ANSWER 6: We think this suggests that the antibodies that do not recognize their antigen in bacterial cell lysates recognize a configuration of the O1-antigen that is unique to the *Kpn*O1 strain. Apparently, the epitope(s) that are recognized by these antibodies are affected by making bacterial lysates or recognition requires repetition of the epitope that is not available by western blot. That could be a reason to the impaired binding to other O1 strains that we observed. We now clarified this in the results section (page 8, line 304-305).

POINT 7: The synergism of anti-capsule antibodies is highly interesting, but the underlying mechanism remains mostly elusive, which calls for further investigations. Are capsule antigens more mobile on the bacterial surface than O2-antigen and O1-antigens? What is known about the relative surface concentrations or clustering of capsule proteins vs O2 and O1 glycans? Both, mobility and local clustering, could potentially influence hexamer formation (besides spacing/steric hindrance, as discussed by the authors). Any experiment and/or simulations that could provide more mechanistic insights into the observed synergism would be of high value.

ANSWER 7: We have performed several new experiments to obtain more insights into the mechanism of synergism. Since it was suggested that synergistic binding would depend on Fc-mediated IgG hexamerization, we studied this question specifically. We generated both divalent $F(ab')_2$ and monovalent Fab fragments from UKpn1 and UKpn3. To our surprise, we observed that synergistic binding between the two antibodies was independent of the IgG-Fc tail. Both $F(ab')_2$ and Fab fragments of UKpn1 could strengthen the binding of full-length UKpn3 (and vice versa). Additionally, $F(ab')_2$ fragments of UKpn1 also enhanced complement activation by full-length UKpn3 (and vice versa). Altogether, this new data set demonstrates that synergy between UKpn1 and UKpn3 occurs via a novel mechanism that is different from previously reported IgG hexamerization. In the revised manuscript, we included these 8 extra figures (Fig. 7b-d and SFig. 7c-f) and we made textual revisions of both the Results (page 9&10, line 389-408) and Discussion (page 12, line 489-505) sections to reflect on these new data.
In addition, we have extensively revised our discussion section to better reflect on the existing knowledge concerning physical properties of both capsule and O antigen (page 11, line 462-473).

Minor points

POINT 8: Figure 1a: scale bars missing

ANSWER 8: Scale bars were added to Fig 1a.

POINT 9: Figure S1c, legend: what does „MOI of 0.5 per fluorophore“ mean? Is this referring to Multiplicity of Infection? Or related to the detection sensitivity (0.5 AU per fluorophore)? Please clarify and introduce abbreviation upon first usage.
ANSWER 9: It refers to multiplicity of infection –per B cell 0.5 bacterium labeled with Cy5 and 0.5 bacterium labeled with ATTO488. Text was adjusted for clarification.

POINT 10: Figure S1 Legend: the last sentence of the legend is duplicate/unneccessary: „(b) Fluorescent intensity (gMFI) and number of events normalized to mode are depicted on the X and Y-axis, respectively.“
ANSWER 10: Changed as suggested.

POINT 11: Inconsistency between text and Figure 6a. Line 347f states that „mixing two antibodies recognizing the O2 antigen (UKpn2 and UKpn6) did not affect their capacity to drive C3b deposition (Fig. 6a).“ However, Fig. 6 a shows UKpn1+UKpn2. Probably there is a typo in the Figure?
ANSWER 11: The wrong figure was shown as Fig 6a, this should be as mentioned in the text UKpn2 and UKpn6. We now corrected this in the revised manuscript.

POINT 12: Methods: Line 549f: incomplete sentence/information: „Lysis buffer consisted of.“
ANSWER 12: Corrected in the revised manuscript.

POINT 13: Grammar: Line 117f: Clinically, the O1, O2 and O3 O-types are the most relevant as they [verb missing] over 80% of the clinical isolates.
ANSWER 13: Corrected in the revised manuscript.

POINT 14: Grammar: Line 287: missing „Upon specific deletion [of] wbbY,“
ANSWER 14: Corrected in the revised manuscript.

POINT 15: Grammar: Line 289: duplicate „suggesting suggests“ should be „suggesting“ only
ANSWER 15: Corrected in the revised manuscript.

Reviewer #3

The article "Agnostic B cell selection approach identifies antibodies against K. pneumoniae that synergistically drive complement activation" by van der Lans and Bardoel et al aims to describe antibody characteristics of human blood IgG memory B cells to the surface antigens of Klebsiella pneumoniae. Using an elegant dual staining approach, the authors isolate blood IgG memory B cells from healthy individuals bearing antibodies to K. pneumoniae prevalent O serotypes O1 and O2. Next, they generated monoclonal antibodies from those isolated B cells and showed that a small percentage binds to K. pneumoniae surface glycans. In total they identified 29 unique monoclonal antibodies. Next, they suggest that antibody binding intensity does not always correlate with the capability for C3b deposition, especially capsule, in contrast to O antigen antibodies, are incapable to activate complement. The authors showed that hexamer formation-enhancing Fc mutations allowed capsule antibodies to activate complement and that antibody-dependent bacterial killing did depend on the presence of neutrophils. Last, they show a synergistic binding and complement activation effect of two capsule antibodies.

Novel, notable and interesting things about this paper:

1) Using azide-KDO paired with click-chemistry to isolate memory B cells with specific BCRs is an elegant approach and likely suitable to isolate antibodies to other KDO-expressing bacteria
2) Data suggest that only glycan antigens are accessible on Kp surface
3) The authors show a synergistic binding and C3b deposition effect of two monoclonal antibodies targeting the capsule of Klebsiella pneumoniae

Possible concerns:

POINT 1: Fig 2a-d: It seems that O2 O antigen-reactive antibody binding levels do correlate with C3b deposition and only capsule-binders show no correlation. Please confirm. Fig. 2b could be plotted at higher antibody concentration to account for the differences between intermediate and negative binders.

ANSWER 1: Indeed, there is a correlation between antibody binding and C3b deposition for the mAbs targeting the O2 antigen. Since we did not identify the targets of the antibodies at this stage in the manuscript, we did not specify the different groups yet.
We plotted fig 2b at the same concentration as the fig 2d to make a direct comparison.

POINT 2: The authors should proof that the O1 and O2 O antigen reactive antibodies directly target purified LPS of the respective O serotype (similar to Figure 3h).

ANSWER 2: We performed a western blot (as in Fig 3h for *Kpn*O1) for some of the antibodies directed against *Kpn*O2 on bacterial lysate of *Kpn*O2. This shows that UKpn2, 6, 7 and 8 recognize the O-antigen as illustrated by the smear at around 50 kD indicative for the LPS O-antigen. Antibodies UKpn3 stains at a higher molecular weight, which probably indicate recognition of capsular polysaccharide. We now included the western blot as Fig S3d and included it to the text of the results section (Results, page, 7 line 258-261).

POINT 3: Fig. 6e is not very intuitive. Why does Ukpn3 bind slightly better if more unlabelled UKpn3 is added? The MFI should drop when more of the same unlabelled antibody is added.

ANSWER 3: We suspect there is no saturation at this point since the capsule is a very abundant antigen on the bacterial surface and therefore there is no competition. The slight increase we cannot explain at this moment.

POINT 4: Fig S6b: Please confirm that UKpn1 and 3 do not deposit C3b on K00059 and add an isotype control.

ANSWER 4: We repeated the experiment on 209S and SF0025 (K00059) with an isotype control (aDNP). For K00059 some C3b deposition by UKpn1 and UKpn3 only was observed at 3%, while absent at 1% NHS. We added the updated graphs to the revised manuscript (Fig S6b).

POINT 5: The authors should show if the synergistic binding effect of UKpn1 and 3 can be observed on isolated capsule glycan or it depends on the intact bacterium.

ANSWER 5: UKpn1 and 3 are directed against capsule type KL110. Since isolated glycans are not available for this capsule type, we focused on using whole bacteria.

POINT 6: Fig 6g: Can Spa-B bind to UKpn1 and UKpn3 while they bind the surface of KpnO2?

ANSWER 6: We have performed additional experiments to verify that Spa-B indeed binds to the combination of UKpn1 and UKpn3. We now included these new data as Figure S6c and describe the findings the text of the results section (page 9, line 371-372).

POINT 7: The mechanism of UKpn1 and 3 binding synergy is not resolved: Can the Fab fragments of UKpn1 and 3 alone induce the synergistic binding effect?

ANSWER 7: We have now generated F(ab')2 and Fab fragments of UKpn1 and UKpn3 to study this question. Interestingly, the experiments reveal that the synergistic binding between UKpn1 and UKpn3 is also observed with F(ab')2 and Fab fragments. This shows that synergy between UKpn1 and UKpn3 is independent of the Fc tail. We added these new figures to the revised manuscript (Fig. 7b-d and Fig. S7c-f) and we made textual revisions of both the Results (page 9&10, line 389-408) and Discussion (page 12, line 490-505) sections to reflect on these new data

POINT 8: Ultimately, an important question remains: Do UKpn1 and 3 together are protective in an infection model?

ANSWER 8: Although we agree this would be an interesting study, we think it is outside the scope of the current manuscript.

POINT 9: Minor comments:
Axis labels/ units are frequently missing

ANSWER 9: We tried to clarify this as much as possible in the revised manuscript.

POINT 9: The authors should change the wording for "active" and "inactive" antibodies. At the end these antibodies are not inactive.

ANSWER 10: We changed this or adjusted to complement-inactive.

POINT 11: Fig. S6b statistics missing

ANSWER 11: Statistics was added to Fig S6b

POINT 12: Line 340: Uptake by neutrophils was not addressed

ANSWER 12: In the results section we now clarified that we measured phagocytosis of bacteria by neutrophils

POINT 13: Line 311: Please change wild-type to unmutated

ANSWER 13: We changed this in the revised manuscript.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have done a commendable job addressing my previous concerns. I think the revised manuscript is significantly improved.

Reviewer #2 (Remarks to the Author):

Lans et al have submitted a revised version of their manuscript which includes several control experiments, new experiments exploring the mechanism of synergistic action, as well as edits to text and figures. The authors‘ detailed rebuttal in my opinion addresses the points raised by me and other reviewers in a comprehensive and throrough manner. The revision has clarified the rationale for using a dual-staining approach and substantially strengthened the main conclusions of the paper. While open questions remain about the mechanism of synergy as well as its effectiveness in an infection model, I deem these to be beyond the scope of this manuscript. I thus recommend this manuscript for publication without further request for changes and congratulate the authors on their excellent study.

Reviewer #3 (Remarks to the Author):

I do not have any further comments. Congratulations for the work.

Point-to-point reply

Reviewer #2 raises one point: *While open questions remain about the mechanism of synergy as well as its effectiveness in an infection model, I deem these to be beyond the scope of this manuscript. I thus recommend this manuscript for publication without further request for changes and congratulate the authors on their excellent study.*

Answer: In the discussion section of our revised paper, we now include the limitations mentioned by the reviewer:
page 12, line 498: "*Although open questions about the exact mechanism behind synergistic binding remain,..*"
page 12, lines 511-512: "*Since our study is limited to in vitro experiments, it remains to be assessed whether the combination of two anti-capsule antibodies is effective in an in vivo infection model.*"