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Peer Review File

Ectopic NMDAR expression in cancer unmasks germline-encoded autoimmunity

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Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
Here, Kleeman et al. study the links between anti-cancer immunity and autoimmunity. Their study spans scales and diverse methodologies to connect how gene dysregulation in tumors leads to aberrant protein expression, in this case of NMDA receptors in the periphery, and triggers both a helpful anti-tumor immune response, as well as a pathogenic CNS anti-NMDA receptor immune response. The team first surveyed NMDA receptor subunit gene expression in human breast cancers, building on previous findings to detail which subunits are (inappropriately) expressed in which contexts. They next used a mouse system to show a causative relationship between NMDA receptor expression in a tumor and B cell recruitment follow by maturation and somatic hypermutation to generate high affinity anti-NMDA receptor antibodies. Germline sequences were inferred from sequence analyses, which allowed interrogation of the basis of affinity gains. Binding assays (SPR) were used to validate and quantify NMDA receptor interactions. The team then obtained cryo-EM structures of the GluN1-N2B receptor with germline vs. mature antibodies to map epitopes and assess the effect of antibody binding on conformational state of the receptor. These structures defined epitopes, revealing spreading as a result of maturing. Electrophysiology experiments complemented the cryo-EM to understand how binding of a given antibody either antagonizes or potentiates NMDA receptor channel activity (or has no measured effect). Reasoning that a PAM antibody would be most likely to enhance seizures and energy expenditure, hallmarks of NMDA receptor autoimmune encephalitis, they delivered one of these (SK3D) to the CNS through a pump and found that indeed the experimental ANRE antibody lowered the seizure threshold and increased EE compared to a non-NMDA receptor antibody. Together we (2 co-reviewers) found the study to be well written, deeply informative, and very exciting. The scope of the work is huge and the aspects we are experts in (LGIC structure and physiology) appear rigorously performed. The cryo-EM methods and data processing figures and notes are particularly thorough. The immunology aspects are clear (even to us), and seem to constitute major breakthroughs, in part in highlighting the need for patient diagnostic assays representing physiologically assembled NMDA receptor heteromers. We have suggestions/questions to further strengthen the study, none of which would require new experiments.

1. It would be interesting to compare epitopes from the experimental mouse ANRE antibodies from this study and those of the human antibodies in the recent NSMB papers from this group and another, PMIDs 39227719 and 39227720, assuming they are conserved between mouse and hu. (Include in Supplemental Figure S10).
2. Please temper or remove the assertion in line 296 that human anti-GluN2 antibodies have not been found “presumably because the clinical diagnostic test only captures GluN1 reactivity.” In the structural study from the Zhu group, the monoclonal antibodies isolated were derived from a GluN1/GluN2 heteromeric FACS assay that labeled antibody-secreting cells from ANRE patients. They did not isolate any GluN2-binding antibodies. While this is a small dataset to compare with, the fact that the current study is performed in mice does not give sufficient confidence to determine that what the authors see in the antibody response from mice correlates perfectly to humans. The clinical field has at least explored the role of GluN2 in human ANRE. For example, in PMID 22875940, which is cited in the review the authors cite, the authors examined the contributions of GluN2 subunits to a human patient antibody and found it did not affect patient polyclonal antibody staining of cells expressing one or both GluN subunits. To this point, the second study they cite (reference 8) is based on a rat model of ANRE-associated tumors, not human. Please also note PMID 34560071 for contributions of GluN2 vs GluN1 to detection of patient autoantibodies. Most importantly, we suggest these assertions need to be better referenced or removed.
3. Main Fig. 4, and a more general comment about all main figures. These were clearly formatted for a different journal,

which is fine, but it makes it a challenge to guess in some cases what would ‘fit’ into more condensed Nature-specific formatting requirements. Main Fig. 4 has very little labeling on cryo-EM map overviews, but a lot of detail when looking at side chains. We suggest hiding any amino acid side chains that are not directly interacting with the antibodies, or not directly referred to in the text. The maps themselves are not very informative except to illustrate generally the sites of interaction, but they consume precious real estate. There is font that is too large and font that is too small (amino acid labels e.g. in panel c). Labeling subunits in at least a couple of instances, rather than providing only a color legend, would help the colorblind folks. Where dashed lines are drawn, is the resolution of the map in that area (density for those side chains) really good enough to make a high confidence inference about an electrostatic interaction?
4. Nothing is mentioned about ion channel conformations- but, the NMDA receptor’s job is to conduct ions. Are the EM maps good enough to define whether the pores are open or closed? Is there anything about the TMD conformation that would shed further light on antibody mechanisms?
5. Fig. 5, panel a cryoEM subpanel seems to suggest that the non-active to pre-active transition results in shortening of the alpha4-prime to alpha5 distance in the ATD. This is the opposite of what is shown in panel b and summarized in panel c, where the PAM antibodies cause this distance to get longer- or, I am confused. Please clarify.
6. We are not NMDA receptor aficionados, but find the terms non-active and open to be confusing or imprecise. What about resting -> preactive -> activated -> desensitized? A given LGIC can have many non-active states (including resting, multiple desensitized, and any number of substates where the pore is not open). If your terminology is preferred by NMDA receptor people, okay, but it will be a little unclear to the broader audience you are targeting. See lines 312, 319, 325, 366 and more, as examples.
7. Line 339, did SPR lend any insights into glutamate effects on IgG or Fab binding?
8. Final section of the main text, before discussion: SK3D administration did not itself directly cause seizures, in the absence of PTZ?
9. In Figures S15 and S16, the mesh density is not very helpful or clear; please replace with transparent surface or make the mesh smaller grained so readers can more quickly observe quality of the maps in these regions. Please include contour levels in the figure legend.

Very minor:
1. Lines 105 and 109, are those p-values really p=1.13e-170 and p<1e-300?
2. Line 146, missing a space before 100.
3. Line 1029, volume units.
4. Material availability statement: cryo-EM maps and models? Have they been deposited?

Referee #2

(Remarks to the Author)
A. Summary of key results
The authors show that rare cells in breast cancer patients express the NMDAR receptor. To investigate the antibody immune response against this protein, they created a breast cancer cell line that expresses the receptor. Upon triggering receptor expression, the tumors shrink, and antibodies are detected in the mice's serum. The authors then cloned several antibodies from these mice and demonstrated that they bind to NMDAR, with binding dependent on somatic hypermutation. Using structural and functional assays, they further elucidated the mechanism of action of these antibodies.

B. Originality and significance
This is an interesting study that explores an interesting phenomenon. However, it seems that more work and data are needed to strengthen the evidence supporting the authors' claims. Specifically, I'm not entirely convinced that their model is distinct from other types of antigens expressed on tumor cells such as viral antigens or model antigens used to elicit an immune response. In other words, injecting mice with tumors that express a "foreign" antigen would typically induce an immune response. What differentiates this from injecting tumor cells expressing a self-antigen found in the brain? Is there a breakdown of tolerance, or is tolerance maintained, or there is no tolerance at all as might be suggested by the thymic cell analysis? If the NMDAR model has a unique feature that distinguishes it from a foreign antigen on tumor cells, it could enhance the novelty of the paper. However, this doesn't appear to be the case since the authors claim that there is no tolerance to NMDAR.

C. Data & methodology
More monoclonal antibody tests are required. See below for more details.
Some key experiments require negative and positive controls.
Some key experiments lack quantification.

D. Appropriate use of statistics and treatment of uncertainties
OK

E. Conclusions: robustness, validity, reliability
The authors should generate more monoclonal antibodies from multiple mice to make the findings more robust.

F. Suggested improvements: experiments, data for possible revision

Major concerns:

The authors do not provide direct evidence for the presence of anti-NMDAR antibodies in breast cancer patients. In the

introduction, they cite only one reference to support the idea of anti-NMDAR antibodies in breast cancer patients, but this appears to be an abstract rather than a fully published study (unless something is off with the references). Is there more robust evidence available regarding the presence of these antibodies in breast cancer patients? How frequent is it?
If not, the authors should investigate whether these antibodies are present in breast cancer patients, and, if so, determine how frequently they occur. It would be particularly valuable to compare anti-NMDAR antibodies in patients whose tumors express the receptor versus those whose tumors do not. Establishing a clear link between the antibodies and the presence of tumors in patients is crucial and essential for the clinical relevance and physiological significance of the study.

Along the same lines, in Figure 2h, the authors need to prove that the tumor rejection is indeed antibody-mediated. For this they need to ablate B cells and to do serum transfers.

The authors demonstrate the presence of GluN1/2 in cancer cells using scRNA-seq, but they do not provide information on the frequency of cells expressing these subunits. This data should be shown on the UMAP plot, similar to what is presented in Figure 1e. The protein staining presented is unconvincing, and, if anything, it suggests a lack of expression of the subunits in cancer cells. If only 1.5% of the cells express the receptor, this may simply be background noise rather than genuine protein staining. A positive control, such as brain tissue, along with a negative control using non-expressing tissue, would significantly strengthen the findings.

The statement, "The definitive detection but low frequency of NMDAR-expressing cancer cells may be a consequence of immunological elimination," is highly speculative and unsupported by evidence. It raises several questions, such as why the detected cells are not eliminated if this is the case. This is why an additional validation method is needed, such as further analysis of single-cell expression with the existing data the authors have already obtained. A human breast cancer cell line would also be useful to more definitively assess NMDAR expression in this context.

In Figure 3e, the authors present data on only four antibodies. To strengthen their claim, they should analyze a larger number of immunoglobulins. It would be more compelling to show multiple binding antibodies from different mice. In Figures S4b and S4c, the authors indicate that this phenomenon is rare within the tumor itself and suggest that the primary immune response generating the tumor-binding antibodies likely originates from other sites, such as the lymph nodes and spleen. Given this, the amount of data presented is insufficient to support the claim that this is a major phenomenon. In other words, the authors need to show more binding antibodies 10-20 from different mice. Perhaps they should focus on draining lymph nodes for this task, as there are very few B cells in the tumor.

In Figure 3e, controls are missing. How does the binding to BSA look like? Additionally, are the antibodies specific to the subunits of NMDAR? The authors should test binding to other targets and include assays for polyreactive antigens, such as dsDNA, to assess the specificity of the antibodies.

Minor comments
The statement, "To investigate this, we re-analyzed data from a recent atlas of human mTECs, confirming that GRIN1 and GRIN2B were not expressed in the thymus," is unclear. Are the authors implying that there is no central tolerance to NMDAR? Given the established role of the thymus in self-antigen tolerance, this claim raises questions. Further clarification is needed.

“These autoreactive B cells would undergo class switching and affinity maturation within tertiary lymphoid structures35, generating high-affinity IgG autoantibodies that are sufficient to cause neurological disease36”. This a very misleading sentence, and it sounds like TLS in cancer makes antibodies that cause neurological disease. Reference 35 is a review, and I am not sure that antibody-affinity maturation was proven to take place in TLS. In fact, in humans, the target antigens are not known, making it difficult to claim that antibody affinity maturation occurs. Reference 36 is about autoimmune disease.

“The intratumoral recruitment of pre-existing B cells with unmutated NMDAR-binding receptors, leading to NMDAR-directed affinity maturation, indicates that the adaptive immune system is not inherently tolerant to specific NMDAR subunits.” This raises the question: how is an immune response against NMDAR prevented in healthy individuals? It is difficult to believe that no tolerance mechanisms exist to prevent NMDAR-driven autoimmunity.

The study focuses on B cells, yet the data presented indicate that there are very few B cells in the tumor. Could the author use FACS to quantify the number of activated B cells present in the tumor and tumor-draining lymph nodes?

In Figure 2e, can the authors say the frequency of cells expressing protein?

In Figure 2g, can the author show the frequency of immune cells when the NMDAR is not expressed in the tumor? Quantitative data is needed here.

Can the author provide a negative control for NMDAR binding in Figure S3a? For example, how does the staining of unrelated tissues, such as the spleen of mice without tumors, appear? Additionally, how many of the cloned antibodies were specific to NMDAR?

“Using this approach, we idenafied four clonotypes with putative evidence of NMDAR binding (Fig. 3c)”. Which evidence?

“Altogether, these findings demonstrate that our intratumoral B cell isolation workflow allowed us to reconstruct expanded

phylogenies of NMDAR-reactive B cells, originating from germline-configurtion antibodies that bind to NMDAR." I would not emphasize the method here; the authors recovered very few expanded clones. Most of the sequences were not useful.

G. References:
More references about anti-NMDAR antibodies in breast cancer patients are needed.

H. Clarity:
The text is clear to the reader.

Referee #3

(Remarks to the Author)
This is a very interesting paper which uses an impressive array of cutting-edge techniques from immunology, structural biology and electrophysiology, to elucidate autoimmunity to tumor-derived NMDA receptors (NMDARs). The study confirms that a subset of triple negative breast cancer cells express NMDARs, previously implicated in driving progression of cancer. A mouse model of triple-negative breast cancer with inducible NMDAR expression is then developed, and this is used to prove that the adaptive immune system generates autoantibodies against ectopically-expressed NMDARs, which can either potentiate or inhibit NMDAR current, in agreement with structural conformational changes produced by binding of the antibodies to the receptor. Lower-affinity anti-NMDAR antibodies are shown to be germline-encoded, while much higher-affinity antibodies are produced by affinity-maturation. A potentiating antibody is shown to phenocopy symptoms of paraneoplastic anti-NMDAR encephalitis (ANRE), including epileptic seizures consistent with NMDARs being potentiated rather than blocked in ANRE. Finally, it is speculated that the immune system may have germline-encoded anti-NMDAR antibodies in order to detect ectopic expression – in a tradeoff between the need for immune surveillance of NMDAR-expressing tumors and potential autoimmune disease.
The paper is well-written and illustrated, and uses appropriate advanced techniques with expertise. It should be of wide interest to cancer biologists, immunologists and scientists interested in ion channel physiology and structure. I do have a couple of points, though, which I feel would strengthen the paper, and which ought to be readily achievable:
1) It would be nice to see more extensive investigation of the ANRE symptoms, specifically, is this dependent on the potentiation rather than block of NMDARs? Could the experiments of Fig 5i-j (indirect calorimetry and seizure scoring) be performed also with a blocking antibody like SK5G? Does this produce a different outcome – if not, why not?
2) For cancer biology, the particularly intriguing aspect of the paper is the potential antitumour effect of antibodies. This would support the speculation that there is immune surveillance for ectopic NMDARs to suppress tumours. Tumour regression with autoimmunity is cited in the Introduction. However, this is only very slightly touched upon in Fig 2i, where it is suggested that there might be a causal relation between tumour volume reducing following the peak of antibody titre. For example, could growth/invasiveness of cancer cells in 2D or 3D (tumoroid) culture be assayed in the presence or absence of these antibodies, perhaps complemented by imaging with immunostaining for NMDARs to give some insight into what if anything is happening at the cellular level, under the action of the antibodies? Given previous literature on NMDARs in cancer, it might be expected that the blocking antibodies might inhibit, while potentiating antibodies drive cancer progression, i.e. opposite to what their suggested effects on ANRE are.

A more minor point:
It is quite striking that the action of potentiating antibodies (SK3D, Fig 5d and SK5A, Fig S17b) on NMDAR current is reversible, while the action of a blocking antibody (SK5G, Fig 5e) is irreversible. Yet SK5G and SK3D have Kd's of the same order of magnitude. Can the authors suggest what lies behind this difference?

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
We (two co-reviewers) are generally satisfied and impressed by the amount of quality of the additional work the authors performed. This study is a huge accomplishment that connects structural biology, in vitro and in vivo autoimmune antibodies to cancer. The connection to human breast cancer patients is highly meaningful. The bar is now much higher for studies that come after. We have only minor comments and feel this study is an excellent fit for the journal. Having said that, the figures will still need a great deal of refinement to adhere to this journal's format.

Minor comments:
-Lines 114-117 could be condensed, and the details moved to methods, to enhance readability.

-If possible, lined boxes around the panels in 4c and 4f could help readers not familiar with structures to separate the panels and not confuse them as one structure.

-There is an error for the reference in line 357.

-The summary conformational movements in Figure 5c is particularly clear.

-In Figure S21, the density for the a4 helix in panels b and f is not robust. This information is interpreted in order to measure the distance between these two domains for an approximation of conformational change. Perhaps temper the word measured, and rather put estimated? Something to indicate the distance measurements are rough? Especially because in line 438, there are assertions about antibodies triggering a “more inhibited state.” Some ambiguity is present in the resolution of these helices; thus, some error will be present in the measurements, which qualifies the interpretations.

-Our sense is that the paragraph beginning on line 633, regarding the “interoceptive function” of anti-NMDAR antibodies, is very speculative and could be removed. There is no need to provide an “explanation for the evolutionary selection of germline-encoded receptors that specifically target the NMDAR ATD.”

Referee #2

(Remarks to the Author)
I appreciate the author's effort in addressing my concerns. Some issues were more or less solved, but some remain unclear to me.

I do not understand Figure 6b. Why do they show a column based on high and low binders? This is a misleading way to present the data. The right analysis would be a healthy and patient comparison. Using one subject as a control is problematic.

In Figure 6D, a control is missing. Can they show that sera-derived antibodies do not bind cells that do not express the receptor? I cant see it in the figure.

It is still not clear what percentage of patients express the receptor. Figure 6e is very difficult to understand. It says that 75% of the "antibody high" express the receptor; however, it represents only eight patients. It seems like only 10% of all the patients express the receptor. It should be stated clearly.

I do not understand the author's answer regarding B cell or antibody-mediated tumor rejection in Figure 2. They had to ablate B cells, or remove serum from a mouse with a tumor and transfer it to another tumor-bearing mouse, to prove the antibody-mediated tumor rejection mechanism they suggested.

In response to the comment of very few analyzed antibodies - “We performed addi`onal experiments using two pooled mouse cohorts and successfully iden`fied two more NMDAR-binding an`bodies from TDLNs”. It is still not clear how many antibodies were screened and what percentage of NMDR-specific binders they detected.

“Regarding our ELISA approach, since BSA is included in both the blocking buffer and the sample and secondary an`body dilu`on buffers, it is not possible to directly measure binding to BSA, as any conceptual immobilized BSA binding would be blocked by soluble BSA.” This is incorrect and even exacerbates the problem. Which antigen is bound by the antibody, the BSA or the NDMR? Typically, a well coated with only BSA solves this problem.

Checking polyreactivity would help; however, I couldn't find the results of this experiment (described below) - were positive controls included?
“To inves`gate polyspecific binding of our an`body panel, we adopted the established protocol from the Nussenzweig Lab to measure binding to three common autoan`gens by ELISA: dsDNA, insulin and LPS. While we could detect moderate binding (OD 0.3-0.5) across all three an`gens for a posi`ve control polyspecific an`body (G11), we did not detect any signal indica`ng binding for mGO53, SK3D-Matured, SK3D-Germline, SK5A-Germline, SK5A-Matured, SK5B-Germline, SK5B-Matured, SK5G-Germline and SK5G-Matured (all OD<0.1)”.

The correlation in Figure 6f with 4 dots is a poor analysis.

Referee #3

(Remarks to the Author)
The authors have carried out a substantial number of further experiments, effectively addressing the comments of both myself and the other referees. The impact of the paper is now much enhanced by the demonstration of anti-tumor effects of the potentiating antibodies in vitro and in vivo, as well as the demonstration of a correlation between tumor NMDAR expression and antibody titre in human TNBC patients. These improvements in content are described and illustrated very well.

I have no further points arising from the revision - I believe that it is a very clearly written, scientifically solid and interesting paper.

Version 3:

Reviewer comments:

Referee #2

(Remarks to the Author)
Most of the issues I raised were addressed. The precise measurement of how many patients are NDMR-positive is missing. I leave it to the editor to decide if it is essential or not.

We thank the reviewers and editors for their thoughtful and constructive evaluation of our manuscript, and for the opportunity to submit a fully revised version. In response to the comments received, we have undertaken substantial new experiments, expanded datasets, and clarified key mechanistic and translational claims. As a result, the manuscript is now significantly strengthened, with enhanced evidence for human relevance, antibody-mediated tumor control, and structural specificity.

In particular, we prioritized the two key areas highlighted as essential during editorial and reviewer discussions:

1. **Establishing human relevance.** We assembled two new cohorts of 48 and 5 patients with TNBC and quantified anti-NMDAR antibody titers in plasma as well as tumor expression of NMDAR. We find that 8/53 (15%) of patients harbor detectable anti-NMDAR antibody titers, with responses correlating to tumor-intrinsic NMDAR expression and favorable clinical outcomes. These results directly validate the translational scope of our findings.
2. **Demonstrating antibody-mediated tumor control *in vivo*.** We now show that administration of a monoclonal functionally validated anti-NMDAR antibody (SK3D) into B cell–deficient mice is sufficient to induce tumor regression. These data establish causality and support a model in which antibody binding initiates direct cytotoxic effects and broader immune activation.

Additional major improvements include:

- Expanded antibody panel drawn from multiple mice and tissues (tumor-draining lymph nodes), with added controls and specificity assays.
- Comparative epitope mapping across mouse and human antibodies (now Figure S17), including a new human germline and affinity-matured antibody pair, demonstrating structural convergence on the GluN1-GluN2B ATD.
- Characterized phenotypic consequences of intracerebroventricular infusion of NMDAR current-inhibiting SK5G antibody.
- Clarified conformational analysis of antibody-bound NMDAR states, updated structural figures, and revised terminology per reviewer feedback.
- Refined interpretation of tolerance mechanisms and antibody specificity, supported by thymic expression data and polyspecificity controls.

Below, we provide a detailed point-by-point response. Please note that line numbers refer to the manuscript version containing tracked changes.

Referee #1 (Remarks to the Author):

Here, Kleeman et al. study the links between anti-cancer immunity and autoimmunity. Their study spans scales and diverse methodologies to connect how gene dysregulation in tumors leads to aberrant protein expression, in this case of NMDA receptors in the periphery, and triggers both a helpful anti-tumor immune response, as well as a pathogenic CNS anti-NMDA receptor immune response. The team first surveyed NMDA receptor subunit gene expression in human breast cancers, building on previous findings to detail which subunits are (inappropriately) expressed in which contexts. They next used a mouse system to show a causative relationship between NMDA receptor expression in a tumor and B cell recruitment follow by maturation and somatic hypermutation to generate high affinity anti-NMDA receptor antibodies. Germline sequences were inferred from sequence analyses, which allowed

interrogation of the basis of affinity gains. Binding assays (SPR) were used to validate and quantify NMDA receptor interactions. The team then obtained cryo-EM structures of the GluN1-N2B receptor with germline vs. mature antibodies to map epitopes and assess the effect of antibody binding on conformational state of the receptor. These structures defined epitopes, revealing spreading as a result of maturing. Electrophysiology experiments complemented the cryo-EM to understand how binding of a given antibody either antagonizes or potentiates NMDA receptor channel activity (or has no measured effect). Reasoning that a PAM antibody would be most likely to enhance seizures and energy expenditure, hallmarks of NMDA receptor autoimmune encephalitis, they delivered one of these (SK3D) to the CNS through a pump and found that indeed the experimental ANRE antibody lowered the seizure threshold and increased EE compared to a non-NMDA receptor antibody. Together we (2 co-reviewers) found the study to be well written, deeply informative, and very exciting. The scope of the work is huge and the aspects we are experts in (LGIC structure and physiology) appear rigorously performed. The cryo-EM methods and data processing figures and notes are particularly thorough. The immunology aspects are clear (even to us), and seem to constitute major breakthroughs, in part in highlighting the need for patient diagnostic assays representing physiologically assembled NMDA receptor heteromers. We have suggestions/questions to further strengthen the study, none of which would require new experiments.

1. It would be interesting to compare epitopes from the experimental mouse ANRE antibodies from this study and those of the human antibodies in the recent NSMB papers from this group and another, PMIDs 39227719 and 39227720, assuming they are conserved between mouse and hu. (Include in Supplemental Figure S10).

We agree. We have updated Fig. S17 to show the comparative analysis of epitope diversity and convergence, which is discussed in more detail in lines 358-370. We show that the region around GluN1 Lys51 is highly susceptible to anti-NMDAR autoimmune responses in both humans and mice.

2. Please temper or remove the assertion in line 296 that human anti-GluN2 antibodies have not been found “presumably because the clinical diagnostic test only captures GluN1 reactivity.” In the structural study from the Zhu group, the monoclonal antibodies isolated were derived from a GluN1/GluN2 heteromeric FACS assay that labeled antibody-secreting cells from ANRE patients. They did not isolate any GluN2-binding antibodies. While this is a small dataset to compare with, the fact that the current study is performed in mice does not give sufficient confidence to determine that what the authors see in the antibody response from mice correlates perfectly to humans. The clinical field has at least explored the role of GluN2 in human ANRE. For example, in PMID 22875940, which is cited in the review the authors cite, the authors examined the contributions of GluN2 subunits to a human patient antibody and found it did not affect patient polyclonal antibody staining of cells expressing one or both GluN subunits. To this point, the second study they cite (reference 8) is based on a rat model of ANRE-associated tumors, not human. Please also note PMID 34560071 for contributions of GluN2 vs GluN1 to detection of patient autoantibodies. Most importantly, we suggest these assertions need to be better referenced or removed.

We appreciate your question and have removed this claim. Zhu et al. indeed used the intact GluN1-2A NMDAR proteins for B cell isolation. They isolated and published two antibodies that recognize GluN1 ATD.

As the reviewer points out, the sample size here (two antibodies) is insufficient to conclude that anti-GluN2 antibodies do not exist in humans. Furthermore, our analysis of thymic expression patterns shows a strong expression pattern of GluN2A, indicating that anti-GluN2A antibodies do not escape central tolerance. In contrast, the thymus does not appear to express GluN2B, indicating that this subunit is more susceptible to autoimmunity, which is consistent with our results.

3. Main Fig. 4, and a more general comment about all main figures. These were clearly formatted for a different journal, which is fine, but it makes it a challenge to guess in some cases what would 'fit' into more condensed Nature-specific formatting requirements. Main Fig. 4 has very little labeling on cryo-EM map overviews, but a lot of detail when looking at side chains. We suggest hiding any amino acid side chains that are not directly interacting with the antibodies, or not directly referred to in the text. The maps themselves are not very informative except to illustrate generally the sites of interaction, but they consume precious real estate. There is font that is too large and font that is too small (amino acid labels e.g. in panel c). Labeling subunits in at least a couple of instances, rather than providing only a color legend, would help the colorblind folks. Where dashed lines are drawn, is the resolution of the map in that area (density for those side chains) really good enough to make a high confidence inference about an electrostatic interaction?

Thank you for this comment. We have improved the clarity of the figure panels, especially the zoom-in panels for the NMDAR-antibody interaction interfaces in Fig. 4. Specifically, we have carefully re-reviewed all models and maps to ensure that we are only displaying the most relevant interacting side chains. We have further ensured that the side chains and putative electrostatic interactions indicated are matched with clearly resolved densities in the respective maps. Font sizes have been updated to improve readability, and labels have been added for NMDAR sub-units in panels b and e to improve accessibility for colorblind readers.

4. Nothing is mentioned about ion channel conformations, but the NMDA receptor's job is to conduct ions. Are the EM maps good enough to define whether the pores are open or closed? Is there anything about the TMD conformation that would shed further light on antibody mechanisms?

In all structures, the M3 transmembrane helices are sufficiently resolved to determine whether the channels are open or closed. In all cases, the channel gates are closed in comparison to previously reported open channel structures (such as PMID 39085540). We show a representative example in the revised Fig. S20g and have modified the text to state this explicitly in lines 453-458.

5. Fig. 5, panel a cryoEM subpanel seems to suggest that the non-active to pre-active transition results in shortening of the alpha4-prime to alpha5 distance in the ATD. This is the opposite of what is shown in panel b and summarized in panel c, where the PAM antibodies cause this distance to get longer- or, I am confused. Please clarify.

Fig. 5b compares non-active conformation (gray, 9arf) and pre-active2 (colored magenta/deep teal). The alpha4'-alpha5 distances are 12 Å (pre-active) vs. 19.2 Å (non-active) for the ATD dimer #1 (ATD1) and 17.4 Å (pre-active) vs. 19.2 Å (non-active) for ATD2. We apologize that this was not sufficiently clearly; therefore, we revised the figure panel to clarify this point. We also revised Fig. 5b to convey this point more clearly by including dashed lines to indicate the location of each measurement.

6. We are not NMDA receptor aficionados, but find the terms non-active and open to be confusing or imprecise. What about resting -> preactive -> activated -> desensitized? A given LGIC can have many non-active states (including resting, multiple desensitized, and any number of substates where the pore is not open). If your terminology is preferred by NMDA receptor people, okay, but it will be a little unclear to the broader audience you are targeting. See lines 312, 319, 325, 366 and more, as examples.

We thank the reviewer for bringing this to our attention. Indeed, many in the iGluR/NMDAR field regard the 'non-active' state as the desensitized state. While the cryo-EM condition (glycine and glutamate at millimolar concentrations) would stabilize glutamate-mediated desensitization, we, as well as the rest of the field, do not have the direct evidence to state that the conformation represents desensitization, not an agonist-bound closed channel. In the revised manuscript, we have significantly modified the text in lines 387-401 to clarify what the non-active state represents.

7. Line 339, did SPR lend any insights into glutamate effects on IgG or Fab binding?

We did not perform SPR experiments on this. As suggested by the cryo-EM structures that showed antibody binding sites within the allosteric domain, ATD, glutamate that binds to the LBD, does not seem to alter the antibody binding. Furthermore, as discussed in lines 432-446, we performed data collection for SK5A-Matured in complex with NMDAR protein in the gly/apo state (unchanged Fig. 20a-c), and all receptor particles were bound to IgG. That said, there may be correlative effects between the antibody-mediated functional alteration and glutamate concentrations. This would require substantially more extensive electrophysiological analysis, which we would like to pursue in the future.

8. Final section of the main text, before discussion: SK3D administration did not itself directly cause seizures, in the absence of PTZ?

In one case, we observed a spontaneous seizure in an SK3D-treated mouse. However, in general, we did not observe spontaneous seizures in the absence of PTZ.

9. In Figures S15 and S16, the mesh density is not very helpful or clear; please replace with transparent surface or make the mesh smaller grained so readers can more quickly observe quality of the maps in these regions. Please include contour levels in the figure legend.

Thanks for pointing this out. We modified the mesh presentation to a transparent surface as suggested in Fig. S15-16 (now Fig. S20-21) and have included contour levels in the figure legend.

Very minor:
1. Lines 105 and 109, are those p-values really p=1.13e-170 and p<1e-300? Yes, but this is on the basis of treating each bin as an independent statistical event. This is an established approach in the field to compare expression between single-cell RNA sequencing clusters (Seurat 'FindMarkers' function). We recognize that these p-values have limited value and that the type 1 error rate will be increased by this approach. We would be happy to remove the p-values from the manuscript, if this was preferred by the reviewers or editorial team.
2. Line 146, missing a space before 100. Corrected, thank you.
3. Line 1029, volume units. Corrected, thank you.
4. Material availability statement: cryo-EM maps and models? Have they been deposited? All maps (EMDB) and models (PDB) will be deposited prior to manuscript acceptance. We have started the submission process.

Referee #2 (Remarks to the Author):

A. Summary of key results
The authors show that rare cells in breast cancer patients express the NMDAR receptor. To investigate the antibody immune response against this protein, they created a breast cancer cell line that expresses the receptor. Upon triggering receptor expression, the tumors shrink, and antibodies are detected in the mice's serum. The authors then cloned several antibodies from these mice and demonstrated that they bind to NMDAR, with binding dependent on somatic hypermutation. Using structural and functional assays, they further elucidated the mechanism of action of these antibodies.

B. Originality and significance
This is an interesting study that explores an interesting phenomenon. However, it seems that more work and data are needed to strengthen the evidence supporting the authors' claims. Specifically, I'm not entirely convinced that their model is distinct from other types of antigens expressed on tumor cells such as viral antigens or model antigens used to elicit an immune response. In other words, injecting mice with tumors that express a "foreign" antigen would typically induce an immune response. What differentiates this from injecting tumor cells expressing a self-antigen found in the brain? Is there a breakdown of tolerance, or is tolerance maintained, or there is no tolerance at all as might be suggested by the thymic cell analysis? If the NMDAR model has a unique feature that distinguishes it from a foreign antigen on tumor cells, it could enhance the novelty of the paper. However, this doesn't appear to be the case since the authors claim that there is no tolerance to NMDAR.

We thank the reviewer for raising these thoughtful points. To clarify, our study does not model immune responses to non-self antigens, such as viral or engineered proteins (e.g., GFP), nor to modified self-antigens, such as cancer-associated neoantigens. Rather, we are focused on a distinct class of antigens—proteins with highly tissue-restricted expression under normal physiological conditions, specifically those confined to the central nervous system (CNS), an immunologically privileged site.

NMDARs fall into this category for several reasons: (1) their expression is normally restricted to the CNS; (2) they are aberrantly expressed in various cancers, including breast cancer, where they have been implicated in promoting progression and metastasis; and (3) they are the target of anti-NMDAR encephalitis, a well-characterized autoimmune syndrome that often arises in the context of NMDAR-expressing tumors. Furthermore, among NMDAR subunits, we observe heterogeneous thymic expression: GluN2A and GluN2D are detectably expressed in medullary thymic epithelial cells (mTECs), while GluN1 and GluN2B are not (Fig. S1f). The difference in expression between GluN2A and GluN2B is potentially explained by the known developmental switch from GluN2B and GluN2BA during cellular differentiation and organ development (PMID 17785184). This heterogeneity provides a unique opportunity to investigate immune responses to subunits that may escape central tolerance.

Based on these observations, we engineered a tumor model that expresses GluN1-GluN2B heterotetramers, allowing us to probe the immunogenicity of CNS-restricted, developmentally regulated antigens in the tumor context.

Finally, the study reveals broader immunological insights that extend beyond NMDARs per se, including:

- The presence of germline-encoded antibodies capable of recognizing complex neuronal receptors;
- Structural convergence on immunodominant epitopes, suggesting intrinsic biases in antibody recognition.

While these principles may generalize to other multi-subunit neuronal receptors, we have not yet explored this possibility.

C. Data & methodology
More monoclonal antibody tests are required. See below for more details.
Some key experiments require negative and positive controls.
Some key experiments lack quantification.

We thank the reviewer for the constructive suggestions. Modifications to the manuscript have been completed accordingly for all points as discussed further below.

D. Appropriate use of statistics and treatment of uncertainties
OK

E. Conclusions: robustness, validity, reliability
The authors should generate more monoclonal antibodies from multiple mice to make the findings more robust.

We agree with the reviewer. We include more antibodies from mice (Fig. S11, lines 277-287) and also a new germline-encoded and matured anti-NMDAR antibody pair from a patient with encephalitis (Fig. S16, lines 344-356).

F. Suggested improvements: experiments, data for possible revision

Major concerns:

The authors do not provide direct evidence for the presence of anti-NMDAR antibodies in breast cancer patients. In the introduction, they cite only one reference to support the idea of anti-NMDAR antibodies in breast cancer patients, but this appears to be an abstract rather than a fully published study (unless something is off with the references). Is there more robust evidence available regarding the presence of these antibodies in breast cancer patients? How frequent is it?
If not, the authors should investigate whether these antibodies are present in breast cancer patients, and, if so, determine how frequently they occur. It would be particularly valuable to compare anti-NMDAR antibodies in patients whose tumors express the receptor versus those whose tumors do not. Establishing a clear link between the antibodies and the presence of tumors in patients is crucial and essential for the clinical relevance and physiological significance of the study.

We thank the reviewer for highlighting the importance of establishing the translational relevance of our findings. In response, we made this a central focus of our revision, generating multiple lines of data presented in the new Fig. 6 and lines 577-595. We analyzed samples from two independent patient cohorts (n=48 and n=5, respectively), totaling 53 individuals with triple-negative breast cancer. In these cohorts, we detected anti-NMDAR antibodies in a subset of 8 patients (15%). Titers correlated with tumor-intrinsic NMDAR expression, assessed for those patients, where tumor tissue was available, by single-cell RNA sequencing, flow cytometry, and immunofluorescence. We also observed a trend toward improved clinical outcomes in antibody-positive patients.

To further strengthen the clinical link, we characterized a patient-derived affinity-matured anti-NMDAR antibody and its germline-reverted counterpart (Fig. S16, lines 344-356). Both exhibited specific binding to the receptor, supporting the notion that germline-encoded anti-NMDAR antibodies are relevant in the human context and can arise in the setting of breast cancer–associated expression.

Along the same lines, in Figure 2h, the authors need to prove that the tumor rejection is indeed antibody-mediated. For this they need to ablate B cells and to do serum transfers.

This question was raised by multiple reviewers, and we have prioritized this question as part of our revision experiments by performing *in vitro* and *in vivo* experiments. As discussed in the response to reviewer 3, we now include data to show the direct effect of activating and inhibiting NMDAR antibodies raised from our murine model on cell survival *in vitro* (Fig. S23, lines 482-502). We show that activating antibodies substantially increase the rate of cell death, consistent with excitotoxicity, while inhibiting antibodies protect against cell death. Secondly, we leverage a B-cell-deficient mouse line to investigate the effect of the NMDAR current-potentiating SK3D antibody on tumor growth *in vivo*, showing that it significantly reduces tumor growth and is associated with substantial immunological activation. These findings suggest that anti-NMDAR antibodies may mediate tumor control through both direct excitotoxicity and the

promotion of an immune activated tumor microenvironment, a dual mechanism that may explain the durable responses observed.

The authors demonstrate the presence of GluN1/2 in cancer cells using scRNA-seq, but they do not provide information on the frequency of cells expressing these subunits. This data should be shown on the UMAP plot, similar to what is presented in Figure 1e. The protein staining presented is unconvincing, and, if anything, it suggests a lack of expression of the subunits in cancer cells. If only 1.5% of the cells express the receptor, this may simply be background noise rather than genuine protein staining. A positive control, such as brain tissue, along with a negative control using non-expressing tissue, would significantly strengthen the findings.

We appreciate the reviewer's comment and the opportunity to clarify this point. In the original manuscript (Fig. 1d), we presented a dot plot summarizing both the expression level and the percentage of cells expressing each NMDAR subunit across defined cell populations. To address the concern about signal specificity, we have now included both positive (brain tissue) and negative controls in our immunofluorescence experiments, confirming that the signal shown in Fig. 1f reflects specific detection of NMDAR.

In addition, and in response to broader questions regarding translational relevance, we have expanded our analysis using an independent panel of tumor sections. These new data, shown in Fig. 6 and discussed in lines 577-595, include single-cell RNA sequencing, immunofluorescence, and flow cytometry, and further support the presence of NMDAR expression in a subset of tumor cells.

The statement, "The definitive detection but low frequency of NMDAR-expressing cancer cells may be a consequence of immunological elimination," is highly speculative and unsupported by evidence. It raises several questions, such as why the detected cells are not eliminated if this is the case. This is why an additional validation method is needed, such as further analysis of single-cell expression with the existing data the authors have already obtained. A human breast cancer cell line would also be useful to more definitively assess NMDAR expression in this context.

We thank the reviewer for raising these points. We have now incorporated significant additional data regarding validation of NMDAR expression in TNBC tumor sections (as discussed above, see Fig. 6). Nevertheless, we have opted to remove the comment relating to immunological elimination from the manuscript as per the reviewer's suggestion.

In Figure 3e, the authors present data on only four antibodies. To strengthen their claim, they should analyze a larger number of immunoglobulins. It would be more compelling to show multiple binding antibodies from different mice. In Figures S4b and S4c, the authors indicate that this phenomenon is rare within the tumor itself and suggest that the primary immune response generating the tumor-binding antibodies likely originates from other sites, such as the lymph nodes and spleen. Given this, the amount of data presented is insufficient to support the claim that this is a major phenomenon. In other words,

the authors need to show more binding antibodies 10-20 from different mice. Perhaps they should focus on draining lymph nodes for this task, as there are very few B cells in the tumor.

We thank the reviewer for encouraging us to expand our analysis of NMDAR-binding antibodies. In the original submission, we validated specific NMDAR binding for 11 independently derived antibodies, sampled from each of the four B cell phylogenies shown in Fig. 3e. In response to the reviewer's suggestion, we further explored tumor-draining lymph nodes (TDLNs) as a potential site of B cell priming (Fig. S11, lines 277-287). We performed additional experiments using two pooled mouse cohorts and successfully identified two more NMDAR-binding antibodies from TDLNs.

Although the B cell population in TDLNs was skewed toward unmutated, non-class-switched naïve B cells, we view these cells as potentially important precursors that may seed intratumoral affinity-matured clones. The additional TDLN-derived antibodies—both naïve and unmutated—further support our central conclusion that germline-encoded antibodies can recognize NMDARs.

Complementing the murine data, we also identified and characterized a matched pair of affinity-matured and germline-reverted anti-NMDAR antibodies from a patient with confirmed anti-NMDAR encephalitis (Fig. S16, lines 344-356). These were validated structurally using high-resolution cryo-EM.

Together, we feel that these efforts give more certainty to the generalizability of our claims, and we hope that the reviewer agrees.

In Figure 3e, controls are missing. How does the binding to BSA look like? Additionally, are the antibodies specific to the subunits of NMDAR? The authors should test binding to other targets and include assays for polyreactive antigens, such as dsDNA, to assess the specificity of the antibodies.

Regarding our ELISA approach, since BSA is included in both the blocking buffer and the sample and secondary antibody dilution buffers, it is not possible to directly measure binding to BSA, as any conceptual immobilized BSA binding would be blocked by soluble BSA. We believe the SPR traces and cryo-EM structures strongly argue for specific NMDAR binding for all antibodies studied (Fig. 3f, Fig. S10, Fig. 4). We had included in Figure 3e a dashed line to signify the level of signal we observe with the non-binding control mGO53 antibody. When we perform ELISA experiments in the absence of immobilized NMDAR but in the presence of our panel of tumor derived IgGs, the signal is at background levels (OD <0.1). To investigate polyspecific binding of our antibody panel, we adopted the established protocol from the Nussenzweig Lab to measure binding to three common autoantigens by ELISA: dsDNA, insulin and LPS. While we could detect moderate binding (OD 0.3-0.5) across all three antigens for a positive control polyspecific antibody (G11), we did not detect any signal indicating binding for mGO53, SK3D-Matured, SK3D-Germline, SK5A-Germline, SK5A-Matured, SK5B-Germline, SK5B-Matured, SK5G-Germline and SK5G-Matured (all OD<0.1).

Minor comments

The statement, "To investigate this, we re-analyzed data from a recent atlas of human mTECs, confirming that GRIN1 and GRIN2B were not expressed in the thymus," is unclear. Are the authors implying that

there is no central tolerance to NMDAR? Given the established role of the thymus in self-antigen tolerance, this claim raises questions. Further clarification is needed.

As discussed in more detail above, we believe that tolerance to NMDARs varies across the different sub-units that can make up the final expressed heterotetramers. The specific pattern of NMDAR sub-units in the thymus is presented in Fig. S1f.

"These autoreactive B cells would undergo class switching and affinity maturation within tertiary lymphoid structures35, generating high-affinity IgG autoantibodies that are sufficient to cause neurological disease36". This a very misleading sentence, and it sounds like TLS in cancer makes antibodies that cause neurological disease. Reference 35 is a review, and I am not sure that antibody-affinity maturation was proven to take place in TLS. In fact, in humans, the target antigens are not known, making it difficult to claim that antibody affinity maturation occurs. Reference 36 is about autoimmune disease.

We appreciate the reviewer's careful reading and agree that our original wording was too definitive. Our intent was to articulate a hypothesis, rather than claim proven causality. We have therefore modified the introduction (lines 82-85) to highlight the speculative nature of this model and updated the citations to provide more primary evidence for this model. In particular, PMIDs 29406578 and 35680425 specifically characterized tertiary lymphoid structure formation in ovarian teratomas resected from patients with confirmed anti-NMDAR encephalitis. They demonstrate that the TLS-derived B cells secrete GluN1-specific IgG, which is diagnostic for the clinical encephalitis syndrome. The previous reference 36 (now 40) refers to clinical evidence that anti-NMDAR antibody in the CSF (cf. plasma) correlates with disease severity, confirming the importance of blood-brain barrier disruption. We feel that the major accomplishment of our manuscript is to directly address this knowledge gap by providing specific mechanistic data that reconstruct this causal sequence.

"The intratumoral recruitment of pre-existing B cells with unmutated NMDAR-binding receptors, leading to NMDAR-directed affinity maturation, indicates that the adaptive immune system is not inherently tolerant to specific NMDAR subunits." This raises the question: how is an immune response against NMDAR prevented in healthy individuals? It is difficult to believe that no tolerance mechanisms exist to prevent NMDAR-driven autoimmunity.

We agree that this is a critical question in the field. Under normal physiological conditions, NMDAR expression is largely confined to the central nervous system and is not expected to occur at appreciable levels in peripheral tissues. As a result, in healthy individuals, the adaptive immune system is unlikely to encounter NMDAR antigens, and central tolerance mechanisms may not be fully engaged. In our view, malignant transformation and ectopic expression of NMDARs represent a necessary step for unmasking this immune response.

As discussed above, thymic expression data suggest that tolerance is likely established for some NMDAR subunits (e.g., GluN2A), but not all. Notably, GluN2B and GluN1, which comprise the heteromer used in

our model, are not detectably expressed in the thymus, supporting the idea that these subunits may escape central tolerance.

However, peripheral anti-NMDAR antibodies alone are not sufficient to cause encephalitis. In our analysis of TNBC patient plasma, approximately 15% of individuals had detectable anti-NMDAR titers despite no clinical evidence of encephalitis (Fig. 6b). This aligns with clinical observations that serum titers are sensitive but not specific for anti-NMDAR encephalitis (ANRE), and are not sufficient for diagnosis per established guidelines.

We therefore propose a two-step model: (1) tumor-associated NMDAR expression elicits a peripheral antibody response, and (2) clinical encephalitis emerges only if there is concurrent disruption of the blood-brain barrier, allowing pathogenic antibodies to access the CNS. While our current work focuses on step one, we discuss this framework to clarify how tolerance escape may lead to antibody production without necessarily resulting in disease.

The study focuses on B cells, yet the data presented indicate that there are very few B cells in the tumor. Could the author use FACS to quantify the number of activated B cells present in the tumor and tumor-draining lymph nodes?

We agree with the reviewer that it is important to characterize the B cell populations within tumor-draining lymph nodes (TDLNs). We have performed a paired analysis between tumor and matched TDLN, which is shown in the updated Fig. S11 and discussed in the manuscript in lines 213-217 and 277-287.

In Figure 2e, can the authors say the frequency of cells expressing protein?

We have annotated the figure (now Fig. S3d) to display the proportion of cells expressing the mRNA construct.

In Figure 2g, can the author show the frequency of immune cells when the NMDAR is not expressed in the tumor? Quantitative data is needed here.

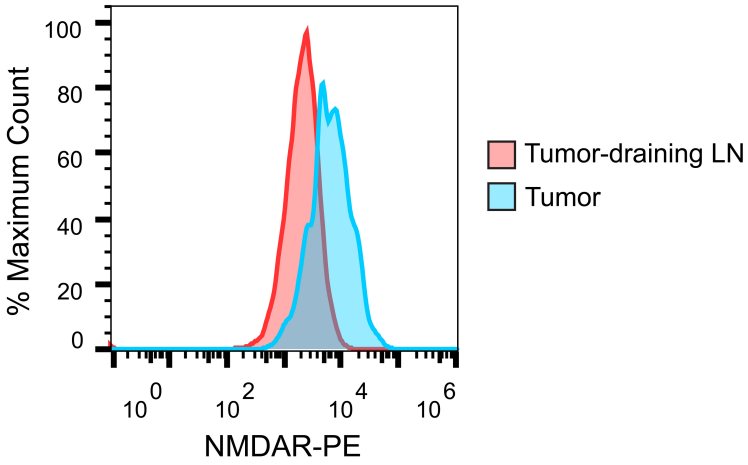
We thank the reviewer for suggesting this highly relevant experiment. We have modified Figure 2 to include a new experiment (Fig. 2h-i) comparing immune activation in tumors with and without doxycycline-induced NMDAR expression, which is discussed in lines 189-209. Through multiple orthogonal analyses, we show that NMDAR expression is highly immunogenic and modifies the spatial architecture of tumor immune infiltrates.

Can the author provide a negative control for NMDAR binding in Figure S3a? For example, how does the staining of unrelated tissues, such as the spleen of mice without tumors, appear? Additionally, how many of the cloned antibodies were specific to NMDAR?

Thank you for raising this important question. The flow cytometry data for the PE-labeled NMDAR channel is designed to reflect NMDAR capture by surface B cell receptors on B cells. In concordance with the

recommendation of 10X Genomics, we include PE-labeled biotinylated mouse albumin in the sequencing reaction to identify highly polyspecific B-cell receptors. NMDAR and mouse albumin are tagged with distinct oligonucleotide sequences, and we use the ratio of NMDAR-oligo counts to albumin-oligo counts to prioritize B cell clones with likely NMDAR binding, as opposed to polyspecific binders.

To investigate whether this channel is likely to be broadly specific to NMDAR, we compared the NMDAR-PE signal in the CD45+ CD3- CD19+ cell population between predominantly naïve B cells in the tumor-draining lymph node and the predominantly antigen-experienced B cells isolated from paired tumor tissue. As shown below, the tumor-derived B cell population is right-shifted, suggestive of increased NMDAR binding in the antigen-experienced tumor-derived subset.



We have discussed the question of cloned antibody specificity to NMDAR in more detail below. Briefly, 4/4 of the cloned tumor-derived antibodies (1 representative matured sequence selected from each clonotype) were specific to NMDAR.

“Using this approach, we identified four clonotypes with putative evidence of NMDAR binding (Fig. 3c)”. Which evidence?

We apologize if this was unclear previously. These clones were prioritized based on a high absolute number of NMDAR-oligo counts (>50) as well as a high ratio of NMDAR-oligo to albumin-oligo counts, altogether suggestive of specific NMDAR binding. The NMDAR-oligo counts are indicated in Fig. 3c and Fig. S7c. We have modified the Methods to include new paragraphs (lines 1404-1431) that specifically detail our approach to prioritizing and validating B cell clones with likely NMDAR reactivity.

“Altogether, these findings demonstrate that our intratumoral B cell isolation workflow allowed us to reconstruct expanded phylogenies of NMDAR-reactive B cells, originating from germline-configuration antibodies that bind to NMDAR.” I would not emphasize the method here; the authors recovered very few expanded clones. Most of the sequences were not useful.

Thank you for this clarification. We have modified the manuscript to reflect the relatively low abundance of NMDAR-reactive B cells (line 289) and have updated the Methods section as discussed above (lines

1373-1400). We would like to clarify the rationale for our approach. Our B cell isolation workflow was designed to have two distinct phases. The flow cytometry sorting phase was specifically designed to have high sensitivity despite low specificity to capture extended phylogenies of B cells, including low-affinity unmutated precursors, irrespective of B cell differentiation status. The computational analysis of the single-cell sequencing was then designed to optimize specificity by determining for each B cell: differentiation status, antibody isotype, and relative capture of NMDAR-oligo in comparison to albumin-oligo (computed as an antigen specificity score). With these multiple lines of information, we were able to precisely prioritize B cells and consequently antibody sequences with a high pre-test probability of binding to NMDAR. As a result of this strategy, when we recombinantly expressed a representative for one mature sequence for each of our 4 clonotypes, 4/4 antibodies demonstrated specific binding by ELISA. The proportion of cloned sequences that were specific to NMDAR was lower in the context of TDLN-derived B cells, which we expected, as in light of the very high abundance of naïve, non-switched B cells, the pre-test probability of NMDAR-binding B cells would necessarily be lower.

G. References:
More references about anti-NMDAR antibodies in breast cancer patients are needed.

We have performed additional experiments to investigate the prevalence of anti-NMDAR antibodies in breast cancer patients as described above and have added one additional reference regarding the diagnosis of anti-NMDAR encephalitis in patients with breast cancer (PMID 23290630).

H. Clarity:
The text is clear to the reader.

Referee #3 (Remarks to the Author):

This is a very interesting paper which uses an impressive array of cutting-edge techniques from immunology, structural biology and electrophysiology, to elucidate autoimmunity to tumor-derived NMDA receptors (NMDARs). The study confirms that a subset of triple negative breast cancer cells express NMDARs, previously implicated in driving progression of cancer. A mouse model of triple-negative breast cancer with inducible NMDAR expression is then developed, and this is used to prove that the adaptive immune system generates autoantibodies against ectopically-expressed NMDARs, which can either potentiate or inhibit NMDAR current, in agreement with structural conformational changes produced by binding of the antibodies to the receptor. Lower-affinity anti-NMDAR antibodies are shown to be germline-encoded, while much higher-affinity antibodies are produced by affinity-maturation. A potentiating antibody is shown to phenocopy symptoms of paraneoplastic anti-NMDAR encephalitis (ANRE), including epileptic seizures consistent with NMDARs being potentiated rather than blocked in ANRE. Finally, it is speculated that the immune system may have germline-encoded anti-NMDAR antibodies in order to detect ectopic expression – in a tradeoff between the need for immune surveillance of NMDAR-expressing tumors and potential autoimmune disease.

The paper is well-written and illustrated, and uses appropriate advanced techniques with expertise. It should be of wide interest to cancer biologists, immunologists and scientists interested in ion channel physiology and structure. I do have a couple of points, though, which I feel would strengthen the paper, and which ought to be readily achievable:

1) It would be nice to see more extensive investigation of the ANRE symptoms, specifically, is this dependent on the potentiation rather than block of NMDARs? Could the experiments of Fig 5i-j (indirect calorimetry and seizure scoring) be performed also with a blocking antibody like SK5G? Does this produce a different outcome – if not, why not?

We agree that this is an important question. We have performed the full suite of experiments to investigate this, directly comparing the effect of mGO53 (non-binding control), SK3D (NMDAR current potentiating) and SK5G (NMDAR current inhibiting), shown as new Figure S25 and discussed in lines 546-553 and 565-567. As we expected, we did not observe any evidence of PTZ-inducible seizure activity in SK5G-treated mice. However, we did observe significantly increased locomotor activity in SK5G-treated mice, a finding that we and others have observed with pharmacological NMDARs inhibitors, such as ketamine and MK-801.

2) For cancer biology, the particularly intriguing aspect of the paper is the potential antitumour effect of antibodies. This would support the speculation that there is immune surveillance for ectopic NMDARs to suppress tumours. Tumour regression with autoimmunity is cited in the Introduction. However, this is only very slightly touched upon in Fig 2i, where it is suggested that there might be a causal relation between tumour volume reducing following the peak of antibody titre. For example, could growth/invasiveness of cancer cells in 2D or 3D (tumoroid) culture be assayed in the presence or absence of these antibodies, perhaps complemented by imaging with immunostaining for NMDARs to give some insight into what if anything is happening at the cellular level, under the action of the antibodies? Given previous literature on NMDARs in cancer, it might be expected that the blocking antibodies might inhibit, while potentiating antibodies drive cancer progression, i.e. opposite to what their suggested effects on ANRE are.

We have taken a multi-step approach to answer this highly relevant question. As discussed in the response to reviewer 2, we now include data to show the direct effect of activating and inhibiting NMDAR antibodies raised from our murine model on cell survival *in vitro* (Fig. S23, lines 482-502). We show that activating antibodies substantially increase the rate of cell death, consistent with excitotoxicity, while inhibiting antibodies protect against cell death. Secondly, we leverage a B-cell deficient mouse line to investigate the effect of the NMDAR current-potentiating SK3D antibody on tumor growth *in vivo*, showing that it significantly reduces tumor growth and is associated with substantial immunological activation.

We recognize that there is evidence in the literature that NMDAR channel blockers such as MK-801 and memantine can reduce tumor growth and invasion *in vitro* and *in vivo*. For example, in PMID 23540692, which is cited in the manuscript, the authors demonstrate a tumor-inhibiting effect in the Rip1-Tag2 model

of pancreatic neuroendocrine cancer but no effect in the MMTV-PyMT mouse model of breast cancer. We have modified the manuscript to reference this point (lines 482-483).

A more minor point:
It is quite striking that the action of potentiating antibodies (SK3D, Fig 5d and SK5A, Fig S17b) on NMDAR current is reversible, while the action of a blocking antibody (SK5G, Fig 5e) is irreversible. Yet SK5G and SK3D have Kd's of the same order of magnitude. Can the authors suggest what lies behind this difference?

Thank you for pointing this out. We do not yet have a clear explanation for this observation. Several factors may be involved. Functionally, SK5G is inhibitory by stabilizing the desensitized state (we called it non-active). Depending on how deeply desensitized the receptor is, its recovery time could be prolonged. We suspect that SK5G induces a deep desensitization state. This would not happen with SK3D, which has a potentiating effect, since active conformation is short-lived compared to the desensitized state. Another possibility is that SK5G contributes to receptor internalization, despite the brief application time.

We thank the reviewers and editors for their thoughtful and constructive evaluation of our manuscript, and for the opportunity to submit a fully revised version.

Referee #1 (Remarks to the Author):

We (two co-reviewers) are generally satisfied and impressed by the amount of quality of the additional work the authors performed. This study is a huge accomplishment that connects structural biology, in vitro and in vivo autoimmune antibodies to cancer. The connection to human breast cancer patients is highly meaningful. The bar is now much higher for studies that come after. We have only minor comments and feel this study is an excellent fit for the journal. Having said that, the figures will still need a great deal of refinement to adhere to this journal's format.

Thank you for your kind comments about our manuscript. We are very grateful for your significant contributions in helping us improve the work. We agree that figures will need to be modified to optimally reflect the Nature formatting specifications, and we will make extensive efforts to achieve this if the manuscript is accepted.

Minor comments:
-Lines 114-117 could be condensed, and the details moved to methods, to enhance readability. Thank you for this suggestion. We have updated the manuscript and methods accordingly.

-If possible, lined boxes around the panels in 4c and 4f could help readers not familiar with structures to separate the panels and not confuse them as one structure. Thank you, have updated the figures accordingly.

-There is an error for the reference in line 357. Thank you, corrected.

-The summary conformational movements in Figure 5c is particularly clear. We are not certain what the reviewer means here and would be keen to seek clarification.

-In Figure S21, the density for the a4 helix in panels b and f is not robust. This information is interpreted in order to measure the distance between these two domains for an approximation of conformational change. Perhaps temper the word measured, and rather put estimated? Something to indicate the distance measurements are rough? Especially because in line 438, there are assertions about antibodies triggering a "more inhibited state." Some ambiguity is present in the resolution of these helices; thus, some error will be present in the measurements, which qualifies the interpretations.

We agree that it would be informative to explain the potential for some uncertainty in the helix distances for lower-resolution volumes. We have added a sentence in the manuscript and updated the legend for Fig. S21 to clarify this point. We have also removed the statement about a "more inhibited state" for SK5G-Matured.

-Our sense is that the paragraph beginning on line 633, regarding the “interoceptive function” of anti-NMDAR antibodies, is very speculative and could be removed. There is no need to provide an “explanation for the evolutionary selection of germline-encoded receptors that specifically target the NMDAR ATD.” We are grateful for this suggestion and have removed this paragraph from the manuscript.

Referee #2 (Remarks to the Author):

I appreciate the author's effort in addressing my concerns. Some issues were more or less solved, but some remain unclear to me.

We appreciate the thoughtful feedback of reviewer 2 to our initial submission and our revised manuscript. Their review identified some areas where we had to add clarity to writing and data presentation and some additional control experiments.

I do not understand Figure 6b. Why do they show a column based on high and low binders? This is a misleading way to present the data. The right analysis would be a healthy and patient comparison. Using one subject as a control is problematic.

We thank the reviewer for this helpful suggestion. We agree that showing the full distribution of antibody titers in the healthy control cohort alongside the TNBC cohort might offer a clearer frame of reference than presenting only the high/low patient stratification. As described in our previous submission, we assembled a cohort of healthy control female plasma samples (n=23) and used these to estimate the 95% confidence interval for anti-NMDAR antibody titers (manuscript lines 563-564, Methods lines 1060-61). The upper limit of this interval (92 ng/ml) was indicated by the dashed line shown in the previous version of Fig. 6b and described in the corresponding figure legend. We had previously designed Fig. 6b in this way with a focus on the transition to Fig. 6c being particularly clear.

In the updated version of Fig. 6b, we now plot the antibody titers for each healthy control sample alongside those from TNBC patients, with the dashed line retained to indicate the upper limit of normal. This presentation more explicitly states that the threshold for “Ab-High” was derived from a healthy cohort rather than a single control subject. The “upper limit of normal” was calculated from the healthy control cohort such that 95% of control values lie below it.

In Figure 6D, a control is missing. Can they show that sera-derived antibodies do not bind cells that do not express the receptor? I cant see it in the figure.

We thank the reviewer for raising this important point. In Fig. 6d, we show the results of immunofluorescence experiments performed with our extensively validated monoclonal antibodies targeting defined epitopes within the GluN1-ATD (‘003-102’) and GluN2B-ATD (‘IgG2’), respectively. We did not stain tumor sections with matched patient serum, as we felt that this approach would not allow us to specifically assess NMDAR binding. Sera from TNBC patients are expected to contain polyclonal antibodies against a broad range of tumor-expressed antigens (see PMIDs 92359, 4108410, 12920480,

18311903), making it challenging to attribute any observed binding to NMDAR-specific reactivity. Furthermore, at present, we have not isolated any monoclonal antibody sequences from TNBC patients. We have revised the Fig. 6d legend and relevant text to clarify that the staining shown is from validated monoclonal antibodies.

Regarding specificity, we include several lines of evidence. Firstly, these antibodies were validated in Fig. S2, where we demonstrate specific binding in NMDAR-expressing tissues (brain) but not control tissues (kidney). Secondly, we have previously solved high-resolution cryo-EM structures to define the precise epitopes for antibody 003-102 (PMID 39227719) and IgG2 (PMID 35177668). Thirdly, we demonstrate that NMDAR staining co-localizes with panCK-positive epithelial cancer cells and is only detected in Antibody-High tumors in our pilot cohort (Fig. 6d).

It is still not clear what percentage of patients express the receptor. Figure 6e is very difficult to understand. It says that 75% of the "antibody high" express the receptor; however, it represents only eight patients. It seems like only 10% of all the patients express the receptor. It should be stated clearly.

We thank the reviewer for this thoughtful comment and appreciate the opportunity to clarify the intent of Figure 6e. In this panel, we present quantitative digital pathology analysis from three representative TNBC patient samples (B1, B2, B3) for which sufficient fresh-frozen OCT-embedded tissue was available. For each sample, the bars indicate the proportion of panCK-positive epithelial cells that are positive for GluN1 only, GluN2B only, or both GluN1 and GluN2B. Thus, the percentages shown in Fig. 6e refer to cells within an individual tumor rather than to the proportion of TNBC patients expressing NMDARs. We have clarified the figure legend to make this distinction clearer.

We agree that determining the prevalence of NMDAR expression across the entire TNBC cohort is an important clinical question. However, a key technical limitation is that reliable immunofluorescent detection of NMDAR protein with our validated antibodies requires fresh-frozen tissue. Standard clinical workflows store surgical specimens as formalin-fixed paraffin-embedded (FFPE) blocks, but the formaldehyde fixation process induces extensive protein crosslinking and epitope masking. Given these constraints, the present study was not designed or powered to make a definitive prevalence estimate. Instead, our aim in Figure 6 is to provide proof-of-principle evidence that (i) NMDAR expression can be robust in a subset of TNBC tumors, and (ii) within this subset, expression is enriched in patients with elevated circulating anti-NMDAR antibodies. In this revision, we have added a statement regarding this limitation of our study, to further reflect this point raised by the reviewer.

Considering the multiple requests for clarification regarding figure 6, we have also provided specific counts for the analyzed samples at the beginning of the figure legend.

I do not understand the author's answer regarding B cell or antibody-mediated tumor rejection in Figure 2. They had to ablate B cells, or remove serum from a mouse with a tumor and transfer it to another tumor-bearing mouse, to prove the antibody-mediated tumor rejection mechanism they suggested.

We appreciate the reviewer's interest in mechanistic clarity, but we respectfully note that we do not claim an exclusively 'antibody-mediated tumor rejection mechanism' in any version of our manuscript. Rather, our data demonstrate that anti-NMDAR antibodies contribute to tumor control within a broader immune response that includes T cells, macrophages, and tertiary lymphoid structure formation (Fig. 2h-i, Fig. S4, Fig. S23f).

Given this multifactorial immune context, we appreciate the reviewer's suggestion regarding B cell depletion and serum transfer experiments. We initially attempted pharmacological B cell depletion using established protocols (anti-CD20 antibody), but encountered technical challenges: while circulating B cells were successfully depleted (by flow cytometry), tumor-infiltrating B cells, including plasma cells, persisted (by IF and RNA sequencing), and anti-NMDAR antibody titers remained detectable at levels sufficient for biological activity. These phenomena have been observed previously: B-1 B cells and plasma cells are well-documented to be particularly resistant to depletion, see PMIDs 18097037, 15778404, 35798357, 23566754. Given that such incomplete depletion would confound interpretation, we opted for an alternative approach and are happy to share our pilot data in full if helpful.

To directly address the reviewer's mechanistic concern while avoiding these potential confounders, we instead used B-cell–deficient (IghJ-/-) mice bearing NMDAR-expressing tumors and administered a single, well-characterized monoclonal antibody (SK3D) with defined NMDAR-binding specificity and Fc isotype. This approach ensured that the only antibody present was anti-NMDAR, with titers matched to physiologic peak levels observed in wild-type mice (Fig. S23c). Furthermore, we connected these *in vivo* effects (Fig. S23d) to *in vitro* data showing SK3D-induced excitotoxicity (Fig. S23a-b), thereby providing a mechanistically specific connection between NMDAR antibody binding and tumor control. In summary, we believe this approach offers several advantages over serum transfer:

1. **Specificity**: Sera from immunized mice contain polyclonal antibodies against multiple tumor antigens, making it impossible to attribute effects specifically to anti-NMDAR antibodies versus other tumor-reactive antibodies.

2. **Reproducibility**: Serum composition would also vary between donors and over time, and antibody titers after passive transfer may not replicate physiological peak levels.

3. **Mechanistic clarity**: By using validated NMDAR-potentiating (SK3D) versus control (mGO53) antibodies in B cell-deficient mice, we can directly test whether an NMDAR-specific antibody drives tumor regression. Pooled serum would likely contain a mixture of NMDAR current-inhibiting and current-potentiating antibodies, complicating interpretation.

4. **Translational relevance**: Our approach better models therapeutic antibody treatment, which would use defined agents rather than polyclonal serum.

In response to the comment of very few analyzed antibodies - "We performed additional experiments using two pooled mouse cohorts and successfully identified two more NMDAR-binding antibodies from TDLNs". It is still not clear how many antibodies were screened and what percentage of NMDR-specific binders they detected.

We thank the reviewer for the opportunity to clarify the number of antibodies screened and the proportion that bound NMDAR. We apologize for not making this clearer in the previous rebuttal.

For tumor-derived B cells:

We selected clonotypes (phylogenies) that met all of the following criteria:

1. ≥50 NMDAR-oligo counts in the single-cell BCR dataset.
2. Evidence of intra-clonotype class switching to IgG.
3. Presence of productive paired single heavy and single light chain sequences.

Based on these criteria, we identified four expanded clonotypes. From these clonotypes, we selected a total of 11 representative antibody sequences for recombinant IgG expression. **All 11/11 (100%)** demonstrated specific binding to NMDAR by ELISA (Fig. 3c/e, Fig. S7b–c).

For tumor-draining lymph node (TDLN)-derived B cells:

We selected antibody clones that met all of the following criteria (there were no expanded clones meeting these criteria):

1. High NMDAR-oligo capture (antigen specificity score >99%). Antibody specificity score is calculated by 10X cellranger and quantifies the probability of NMDAR binding versus non-specific albumin binding.
2. Paired productive single heavy and single light chain sequences.

From two pooled mouse cohorts, this yielded 31 candidate sequences, which we expressed as recombinant IgG. **Two of 31 (7%)** showed specific NMDAR binding by ELISA (Fig. S11e).

The lower yield from TDLNs is consistent with the predominance of naïve, IgM-expressing, unexpanded B cells in this compartment (Fig. S5b, Fig. S11a–d) and the expectation that only a small fraction of these would have NMDAR specificity. In contrast, intratumoral expanded clonotypes presumably reflect antigen-driven selection, explaining the high fraction of binders detected.

“Regarding our ELISA approach, since BSA is included in both the blocking buffer and the sample and secondary antibody dilution buffers, it is not possible to directly measure binding to BSA, as any conceptual immobilized BSA binding would be blocked by soluble BSA.” This is incorrect and even exacerbates the problem. Which antigen is bound by the antibody, the BSA or the NDMR? Typically, a well coated with only BSA solves this problem.
Checking polyreactivity would help; however, I couldn't find the results of this experiment (described below) - were positive controls included?
“To investigate polyspecific binding of our antibody panel, we adopted the established protocol from the Nussenzweig Lab to measure binding to three common autoantigens by ELISA: dsDNA, insulin, and LPS. While we could detect moderate binding (OD 0.3-0.5) across all three antigens for a positive control polyspecific antibody (G11), we did not detect any signal indicating binding for mGO53, SK3D-Matured,

SK3D-Germline, SK5A-Germline, SK5A-Matured, SK5B-Germline, SK5B-Matured, SK5G-Germline, and SK5G-Matured (all OD<0.1).

We thank the reviewer for this important point about BSA controls. We apologize that our previous explanation was inadequate and are grateful for your suggestions regarding direct testing for BSA cross-reactivity. To address this concern, we performed additional ELISA experiments using BSA-only coated wells with our complete panel of tumor-derived anti-NMDAR antibodies. All antibodies showed background-level binding to BSA-only wells (OD <0.1), comparable to buffer-only controls. We have updated the manuscript and methods to incorporate these results. The data are shown in the updated Table S3.

Regarding the polyreactivity screening results discussed in our previous response, we confirm that these experiments included the G11 positive control. G11 has been established in the literature as a positive control antibody for polyspecific binding, see PMIDs 19398190, 21646518, 27730299. G11 showed expected dose-dependent binding (peak OD 0.3-0.5) to dsDNA, insulin, and LPS, while all our NMDAR antibodies showed background levels (OD <0.1) for these antigens. We have now included these data as Fig. S7d and Table S3, and have updated the manuscript and methods accordingly.

We hope that these BSA-specific control and polyreactivity results, combined with our SPR binding kinetics and cryo-EM structural data, provide strong support for specific NMDAR recognition rather than non-specific or cross-reactive binding.

The correlation in Figure 6f with 4 dots is a poor analysis. We appreciate this suggestion and have modified this figure to remove the correlation analysis and instead focus solely on the primary data. The text has also been edited so that no suggestion of correlation is made.

Referee #3 (Remarks to the Author):

The authors have carried out a substantial number of further experiments, effectively addressing the comments of both myself and the other referees. The impact of the paper is now much enhanced by the demonstration of anti-tumor effects of the potentiating antibodies in vitro and in vivo, as well as the demonstration of a correlation between tumor NMDAR expression and antibody titre in human TNBC patients. These improvements in content are described and illustrated very well.

I have no further points arising from the revision - I believe that it is a very clearly written, scientifically solid and interesting paper.

Thank you for your kind comments about our manuscript. We are very grateful for your significant contributions in helping us improve the work.