nature portfolio

Peer Review File

Personalized neoantigen vaccines as early intervention in untreated patients with lymphoplasmacytic lymphoma: a non-randomized phase 1 trial

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Comments on NCOMMS-23-42495-T
Szymura, SJ, et al « First-in-human clinical trial of personalized neoantigen vaccines as early intervention in untreated patients with lymphoplasmacytic lymphoma »

This manuscript reports the results of a phase I clinical trial (NCT01209871) of a cancer vaccine combined with ancillary studies, primarily scRNAseq, of the molecular/cellular response in these patients. The study enrolled 9 patients with incurable low-grade B cell lymphomas and the personalized DNA vaccine encodes the clonal idiotype (ie antibody variable region) of the malignant cells. The clinical trial reached its primary endpoint (no dose limiting toxicities). One patient achieved a minor response (ongoing), four patients have ongoing stable disease, and four patients progressed. The scRNAseq ancillary studies included pre-vaccination bone marrow samples from all patients and post-vaccination bone marrow samples from all but one patient. The manuscript systematically dissects the main cell types included in this tissue, ie B cells and plasma cells (including the malignant cells), T cells, and myeloid populations, as well as inter-cell signaling pathways inferred from ligand:receptor expression patterns. The primary conclusion is that the main mechanism of resistance is intratumoral heterogeneity – malignant clones include both a "mature B cell" and a "plasma cell" compartment. The plasma cell compartment has primary resistance due to low HLA expression and doesn't change abundance under therapy. The mature B cell compartment is partially reduced in numbers by the vaccine but also changes gene expression patterns to generate secondary resistance.
The clinical trial is relatively straightforward and its results are clearly described. Since the primary endpoint was achieved, presumably this will progress to phase II. The ancillary study is based on a highly informative dataset for such a human clinical study. However, the robustness of the conclusions are difficult to evaluate because many analyses are presented in an unsystematic fashion, there are several references to "data not shown", and some results are over-emphasized when the magnitude of the effect is moderate/minimal but the p value is highly significant (a common problem in genomics studies). Perhaps, with substantial effort these analyses could be re-performed or re-presented in a more clear and compelling manner but it is not clear currently whether the data actually provides insight into the mechanisms of sensitivity/resistance.

Main comments
1. The authors repeatedly highlight, including in the abstract, "significantly reduced numbers of clonal tumor mature B cells." While the p value for cluster 1 in fig 1D is significant, the actual changes are clearly inconsistent across patients with 2/6 showing an increase. Furthermore, clusters 0 and 2 are also "mature B cells" and include high proportions of malignant cells and don't show any significant change in proportions.
2. Relatedly, the text highlights on line 122 that reduction in the tumor clonotype frequency was observed in "all except for three evaluable patients." Since the original cohort was 9 patients and post-treatment sample is not available for one, this means the pattern was observed in 5/8 patients, which is not strikingly consistent.
3. The claim for a "dichotomous response" where mature B cells both drop in levels and also change gene expression while plasma cells are relatively inert, would be more clearly supported if figure 1F (or supplementary material) included volcano plots for all B cell/plasma cell clusters, especially 5 and 10 which include high proportions of malignant cells.
4. Line 154 says there was a trend towards down regulation of HLA class II genes post vaccine in clusters cluster 2 malignant cells, but this is not at all apparent from Fig 1H and some, such as HLA-DPA1 and HLA-DQB1, appear to increase. A different visualization, other than violin plots, might resolve this ambiguity.
5. Lines 171, 195, and 273 all make important and apparently easily demonstrable claims but each say "data not shown" which should not be allowed.
6. Fig 2D,E on TCR expansion changes upon treatment, are very important, but the visualizations are unclear. In 1D, patients 003 and 004 have strong horizontal and vertical patterns – are these from clones with 0 cells (as opposed to very few cells) before or after treatment ? In 1E, the colored swaths linking pre vs post are very unclear. A given color should only be included when there are cells from the corresponding clone both pre and post, but this does not seem to be the case. For example, in patient 003 nearly all colors are present in the middle, but only two or three clones appear to be identified in the pre-vaccine sample. This directly affects the statement on line 188 of the text.
7. The choice of time points and cytokines included in Fig 2I is very unclear/unsystematic. Either a

consistent set of time points and cytokines should be included, or all data should be provided in the supplemental for comparison. Similarly, it would be highly beneficial to perform the same assays on the pre-vaccine samples as a negative control for an endogenous response (not vaccine induced) to the idiotype antigen, which the Nef control does not evaluate.

Minor comments
1. Scale legends in Fig 1F need labels. Are these z-scaled pathway activity scores ?
2. The clustering numbering in Fig 2A is confusing, why are there no clusters 8 or 11 ?
3. Line 178 says an adjusted log p-value of 1.3 was used for significance cutoff. This does not seem to be a standard cutoff (or maybe not a standard logarithm base), can more details/explanation be provided ?
4. The result on line 198/fig2G that more T cells are in G2/M post-vaccine is interesting, but challenging to reconcile with the fact that the T cell "cycling" cluster (cluster 13 in Fig2A) does not change in abundance. How do the authors interpret this discrepancy ?
5. Given the high variation in expression counts (common in scRNAseq) in fig 2H, the interpretation on line 200, that the most abundant TCR clonotypes express markers of activation, differentiation, or proliferation needs a control group of cells not from these clonotypes, where the expression of these genes will presumably be closer. Relatedly, CD39 is often cited as a marker of tumor-specific T cells (eg PMID: 29769722), it could be interesting to add to the figure panel.
6. On line 244, "overall information flow" is not entirely self-explanatory. Although the citation is included, a brief explanation of the calculation/measurement would be helpful.

Reviewer #2 (Remarks to the Author):

This manuscript describes a pilot clinical trial using a novel DNA vaccine platform for patients with LPL. The clinical trial is of an interesting design, and the results are novel. However, there remains several issues that needs to be addressed for this to be published.

In many cases, the reduction in the proportion of cells harboring the dominant B cell idiotype (targeted by the vaccine) was accompanied by a concomitant reduction of other B cell idiotypes (e.g. LPL-101, LPL-102) (Figure 1E). In some cases, there was a reduction in these B cell idiotypes even as the proportion of the vaccine-targeted idiotype increases (e.g. LPL-003, LPL-009). This raises questions about the potential specificity of the vaccine response. The authors should compare the magnitude of T cell response to the vaccine idiotype against other B cell idiotypes before and after vaccination.

Vaccine response was seen predominantly in the mature B cell populations, but not in the lymphoplasmacytic population. The authors attributed this difference to a downregulation of HLA class II genes in the lymphoplasmacytic cells. This should be confirmed at a protein level. Are there other differences in antigen processing and presentation across the different B cell populations? Given the apparent importance of HLA class II expression as a determinant of vaccine response, the authors should confirm whether the idiotype-derived neoantigen targeted by the vaccine is presented on HLA class II. Moreover, the authors should demonstrate whether the immune response seen represents that of a CD4 T cell response.

How does vaccine therapy lead to a suppression of proliferation signaling in lymphoma cells (Figure 1F)? The authors should explain the underlying mechanisms.

In Figure 2I, it is unclear why different cytokines are being measured for different patients. The timing of these measurements also appear to be discrepant across patients, making comparisons rather difficult. Moreover, it is unclear whether CD8 or CD4 T cell responses are being assessed in each case.

In Figure 2E, the authors demonstrate an expansion of specific T cell clonotypes after vaccine therapy. The authors should demonstrate whether these T cell clonotypes are specific for the B cell idiotype targeted by the vaccine. Alternatively, do some of these expanded T cell clonotypes harbor specificity for non-vaccine neoantigens, consistent with epitope spreading?

How does vaccine therapy lead to an obliteration of the signaling pathways and interactions suggested by the authors in Figure 3 (e.g. RESISTIN and APRIL), and the myeloid transcriptional changes in Figure 4D? The authors should as a minimum speculate on the potential underlying

mechanisms.

Reviewer #3 (Remarks to the Author):

This is a phase I study using 3+3 design for two dose levels. It has 3 patients treated at dose level 1 and 6 treated at dose level 2. No DLT. Some minor comments:
1. Table 2: recommended to summarize AEs and severe AEs by dose and overall, respectively.
2. Need to report immune response rate, which is in the protocol, such as 1/9=11% has a response.
3. It's not clear which wilcoxon method was used for the plot. For paired data, like pre and post, it should be Wilcoxon signed rank; for independent data, it should be Wilcoxon rank sum. Not sure where t test was used. Please specify. Some boxplot didn't see large difference but p-value is very significant, like Figure 3S A. Figure 3. A. Please double check.

Reviewer #4 (Remarks to the Author):

The manuscript describes the immunologic and clinical response of 9 patients with lymphoplasmacytic lymphoma of a novel idiotype vaccine involving single chain variable fragment as the antigenic target and CCL20 chemokine to target the vaccine to antigen presenting cells. The study demonstrated that vaccination was associated with relative depletion of the malignant B cell population with preservation of the plasmacytoid tumor cells consistent with resistance in one of the tumor-derived clonal populations. There is associated activation of the T cell response with evidence of clonotypic expansion of idiotype specific T cells. There is a relative depletion of monocytes potentially involved with the immunosuppressive milieu. The manuscript is well written and the experimental results elegantly describe the nature of the immune response. However, the manuscript has several limitations that should be addressed to increase the significance of the findings.
1) The clinical relevance of the findings is not clear. Several of the patients have not shown evidence of progression but with a very small study set it is difficult to assess the clinical impact. It would be helpful to assess whether there is any difference in the immunologic response in those patients with evidence of progression as compared to those with stable disease.
2) The unique aspects of the vaccine design are not explored. Does the immune response and nature of impact on monocyte and accessory populations relate to the antigen or CCL20 expression
3) The manuscript describes the targeting of the malignant B cell population presumably by the T cell clonotypic expansion but then also describes changes in B cell proliferative pathways specific for the B cell and not plasmacytoid population. What is the presumed mechanism for this change-does this relate to a mode of T cell killing, how does the nature of the T cell compartment relate to this.
4) The authors suggest that resistance of the plasmacytoid population may relate to downregulation of class II which is an important finding-it is likely that this not sole mechanism of resistance. What is the explanation for target expressing plasmacytoid cells to not be susceptible to antigen specific T cells.
5) The manuscript suggests that a vaccine capable of appropriate T cell expansion is not necessarily clinically effective-does this relate to T cell phenotype and impact of microenvironment-association of phenotype with clinical course would be of great significance but would potentially require a larger cohort and comparative analysis.

We appreciate the reviewers' comments and suggestions and have performed additional wet lab experiments, as well as extensive reanalysis of the scRNA seq data, as guided by their critiques. We are hopeful that the new data generated satisfactorily addresses their concerns. Please find below our responses on a point-by-point basis, with reviewer comments verbatim in italics, followed by our responses:

Reviewer #1 Main Comments:

1. The authors repeatedly highlight, including in the abstract, "significantly reduced numbers of clonal tumor mature B cells." While the p value for cluster 1 in fig 1D is significant, the actual changes are clearly inconsistent across patients with 2/6 showing an increase. Furthermore, clusters 0 and 2 are also "mature B cells" and include high proportions of malignant cells and don't show any significant change in proportions.

We focused the analysis on cluster 1, because among the three mature B-cell clusters, cluster 1 contains the vast majority of clonal tumor cells, with clusters 0 and 2 containing substantially smaller numbers of clonal tumor (see Fig. 1C, right panel). That said, we have re-analyzed and presented the data as dot plots which make clearer the reduction in cell numbers in post-vaccine samples by individual cluster (Fig. 1D). Moreover, re-analysis shows that statistical significance is retained showing reduced post- vs. pre-vaccine samples when grouping clusters 0 and 1 ($p=0.039$) with a trend towards significance retained when grouping all three mature B-cell clusters (Fig. S2B). These statements have been added to the Results section as clarification (lines 122-125) and to the Fig. S2B legend.

Furthermore, cluster 2 exhibits some differences from clusters 0 and 1 with respect to differential gene expression patterns (Fig. S2C) and may therefore represent a distinct cell subpopulation which shares some characteristics of plasma cell-like cells, as cluster 2 B cells also exhibited downregulation of HLA class II genes (Fig. 1G post-vaccine) (lines 125-129).

2. *Relatedly, the text highlights on line 122 that reduction in the tumor clonotype frequency was observed in "all except for three evaluable patients." Since the original cohort was 9 patients and post-treatment sample is not available for one, this means the pattern was observed in 5/8 patients, which is not strikingly consistent.*

The Wilcoxon matched-pairs signed rank test analysis showed reduction in tumor cells post-vaccine in six out of the eight patients, a substantial majority; and one of these patients later progressed clinically (Fig. S2D). The relevant text has been corrected (lines 131-134).

3. The claim for a "dichotomous response" where mature B cells both drop in levels and also change gene expression while plasma cells are relatively inert, would be more clearly supported if figure 1F (or supplementary material) included volcano plots for all B cell/plasma cell clusters, especially 5 and 10 which include high proportions of malignant cells.

We thank the reviewer for the opportunity to show the data supporting the relative inertness of clonal plasma cell subpopulations and have added the volcano plots for clusters 5 and 10 to revised Fig. 1F.

4. *Line 154 says there was a trend towards down regulation of HLA class II genes post vaccine in cluster 2 malignant cells, but this is not at all apparent from Fig 1H and some, such as HLA-DPA1 and HLA-DQB1, appear to increase. A different visualization, other than violin plots, might resolve this ambiguity.*

We thank the reviewer and the opportunity to more clearly visualize the data. Accordingly, we have deleted the violin plots from original panel H (Fig. 1), and replaced them with dot plots added to Fig. 1G. These now clearly show downregulation of HLA class II genes within cluster 2 cells post-vaccine (line 171-173).

5. *Lines 171, 195, and 273 all make important and apparently easily demonstrable claims but each say "data not shown" which should not be allowed.*

We welcome the opportunity to show all of this data which have been added as Suppl. Fig. S1D and S3C. (lines 196, 224, 232 and 304 in the revised manuscript).

6. *Fig 2D,E on TCR expansion changes upon treatment, are very important, but the visualizations are unclear. In 1D, patients 003 and 004 have strong horizontal and vertical patterns – are these from clones with 0 cells (as opposed to very few cells) before or after treatment ? In 1E, the colored swaths linking pre vs post are very unclear. A given color should only be included when there are cells from the corresponding clone both pre and post, but this does not seem to be the case. For example, in patient 003 nearly all colors are present in the middle, but only two or three clones appear to be identified in the pre-vaccine sample. This directly affects the statement on line 188 of the text.*

We have revised Fig. 2D to include thresholds of detection indicated by lines along both x- and y-axes. Thus, the reviewer is correct that for patients 003 and 004 individual TCR clones which fall below the thresholds indicate clones with 0 cells. Similarly, we have followed the reviewer's suggestion for revising Fig. 2E, and so newly emerging clones (i.e. not present in pre-vaccine samples) are indicated by asterisks for each patient. The reviewer's assessment is thus correct, that expansion of both pre-existing and newly emerging TCR clones were observed. Accordingly, we have modified the conclusion on line 217 to include the additional possibility that newly emerging clones could represent epitope spreading post-vaccination, as suggested by Reviewer #2. Recently, the phenomenon of epitope spreading was conclusively verified in human melanoma patients who had received neoantigen vaccine therapy (ref. 46).

7. *The choice of time points and cytokines included in Fig 2I is very unclear/unsystematic. Either a consistent set of time points and cytokines should be included, or all data should be provided in the supplemental for comparison. Similarly, it would be highly beneficial to perform the same assays on the pre-vaccine samples as a negative control for an endogenous response (not vaccine induced) to the idiotype antigen, which the Nef control does not evaluate.*

We have repeated the functional experiments to analyze tumor idiotype-specific T-cell responses and now show the results for all 30 cytokines detected at a single time point as volcano plots for each patient in revised Fig. 2I. The cytokine profiles of 7 of the 8 patients analyzed showed a clear preponderance of cytokines secreted in response to idiotype, compared with non-transfected antigen presenting cells alone

(negative control). Although each patient exhibited a unique profile of cytokines achieving statistical significance, several of the cytokines were shared across patients, such as GM-CSF, MIPb, IFNg, IL-13, and IL-15. Statements describing these new results are added to the Results section (line 247-253).

In multiple prior clinical trials of vaccines against this target (idiotype), we never observed evidence for pre-existing idiotype-specific immune responses that were detectable above the threshold for ELISPOT or other cytokine-readout assays on pre-vaccine samples (ref. 11-12). Accordingly, we did not feel it necessary to perform the same assays on pre-vaccine samples here (we prefer to use these valuable samples for ongoing discovery research into biology of LPL).

Minor comments:

1. *Scale legends in Fig 1F need labels. Are these z-scaled pathway activity scores?*

   Scale legends have been added to the figure. Yes, indeed they are z-scaled pathway activity scores obtained based on pathway analysis using IPA software with differentially expressed genes as input.

2. *The clustering numbering in Fig 2A is confusing, why are there no clusters 8 or 11 ?*

   This inconsistency has been corrected in the figure.

3. *Line 178 says an adjusted log p-value of 1.3 was used for significance cutoff. This does not seem to be a standard cutoff (or maybe not a standard logarithm base), can more details/explanation be provided ?*

   This was corrected to indicate log10 of p value of -1.3 which represents $p<0.05$ which is an accepted significance cutoff.

4. *The result on line 198/fig2G that more T cells are in G2/M post-vaccine is interesting, but challenging to reconcile with the fact that the T cell "cycling" cluster (cluster 13 in Fig2A) does not change in abundance. How do the authors interpret this discrepancy?*

   Upon re-analysis, because of ambiguity, we have removed this panel from Fig. 2G

5. *Given the high variation in expression counts (common in scRNAseq) in fig 2H, the interpretation on line 200, that the most abundant TCR clonotypes express markers of activation, differentiation, or proliferation needs a control group of cells not from these clonotypes, where the expression of these genes will presumably be closer. Relatedly, CD39 is often cited as a marker of tumor-specific T cells (eg PMID: 29769722), it could be interesting to add to the figure panel.*

   We followed the reviewer's suggestion and added a control group comprised of all T cells, excluding the top 20 TCR clonotypes, and included the data as Fig. S3D. A clause to this effect has

been added (line 226-229). In this control group, there were either no significant differences between the vast majority of pre- and post-vaccine markers (CD4, CD8A, CXCR4, REL, FKBP1A, DUSP2, and TIGIT) or differences in the opposite direction, compared with the top 20 TCR clonotypes (CD27 and LAG3). We also tested expression of CD39 but observed no changes pre vs. post in the top 20 clonotype population.

6. *On line 244, "overall information flow" is not entirely self-explanatory. Although the citation is included, a brief explanation of the calculation/measurement would be helpful.*

   The overall information flow for a given signaling pathway is defined as the sum of the communication probability among all ligand-receptor pairs of cell groups in the inferred cell-cell communication network. This explanation has been added to the Materials and Methods section. (lines 609-611)

Reviewer #2:

1. *In many cases, the reduction in the proportion of cells harboring the dominant B cell idiotype (targeted by the vaccine) was accompanied by a concomitant reduction of other B cell idiotypes (e.g. LPL-101, LPL-102) (Figure 1E). In some cases, there was a reduction in these B cell idiotypes even as the proportion of the vaccine-targeted idiotype increases (e.g. LPL-003, LPL-009). This raises questions about the potential specificity of the vaccine response.*

   We thank the reviewer for pointing out these discrepancies, which prompted us to reanalyze and re-present the data in Fig. 1E. Upon reanalysis, we discovered that some of the unique BCR clonotypes originally assigned to normal B cells were in fact clonally related to the vaccine-targeted tumor idiotype (i.e. shared CDR3 sequences). Thus, no concomitant reduction of normal B-cell idiotypes was observed post-vaccine, and the data do not raise concerns about non-specific vaccine responses.

   LPL-002 was an exception, as there was a second dominant tumor idiotype clone (indicated in orange), identified by shared Ig VH family gene usage (Fig. S2E) which also decreased in frequency post-vaccine. A statement describing this unusual phenomenon has been added to the Results section. (lines 134-137).

   Finally, the reviewer is correct in pointing out that vaccine-targeted idiotype increases were observed in patients LPL-003 and -009. Of note, one of these patients experienced subsequent clinical progression. A statement clarifying this association has been added to the Results section. (lines 131-134).

2. *Vaccine response was seen predominantly in the mature B cell populations, but not in the lymphoplasmacytic population. The authors attributed this difference to a downregulation of HLA*

class II genes in the lymphoplasmacytic cells. This should be confirmed at a protein level. Are there other differences in antigen processing and presentation across the different B cell populations? Given the apparent importance of HLA class II expression as a determinant of vaccine response, the authors should confirm whether the idiotype-derived neoantigen targeted by the vaccine is presented on HLA class II. Moreover, the authors should demonstrate whether the immune response seen represents that of a CD4 T cell response.

We have performed immunohistochemistry experiments on bone marrow slides and show representative images from all patients in revised Fig 1H. In all patients the results confirm the lack of HLA class II in CD138+ plasma cell-like LPL cells at the protein level, while CD79a+ mature B-cell subpopulations uniformly expressed HLA class II. (lines 176-181)

In addition, we studied two well established human WM cell lines, which have previously been characterized to harbor both mature B-cell and plasma cell-like subpopulations. Flow cytometry analysis of both cell lines clearly showed HLA DR expression by mature B-cell, but not plasma cell-like subpopulations. These data have been added to Figure 1, panel H. (lines 176-178)

We also reanalyzed the data to determine expression levels of multiple genes involved in antigen processing and presentation and found no differences except for CD74, which expression was reduced among the plasma cell-like clusters (clusters 5 and 10). Data for CD74 have been added to the heat maps in Fig. 1G and a statement describing these additional results has been added to the Results section.(lines 173-175)

A recent published report established that B-cell tumor idiotypes are predominantly presented on HLA class II, rather than class I (9). (line 165). Finally, the T-cell responses observed consisted of CD4+ (as well as CD8+) responses (Fig. 2H). (also lines 251-255)

3. *How does vaccine therapy lead to a suppression of proliferation signaling in lymphoma cells (Figure 1F)? The authors should explain the underlying mechanisms.*

Experiments to determine the precise mechanisms of immune-mediated suppression of proliferation of tumor cells are outside of the scope of our study. However, one recently recognized mechanism of tumor cell death, ferroptosis, is known to be directly mediated by activated CD8+ T cells (Fig. 1F, heat map). The observation that most of the top 20 T-cell clonotypes identified after vaccination belong to the effector CD8 T-cell cluster is consistent with this possible mechanism. In addition to direct antitumor effects, immune-mediated tumor suppression may occur indirectly; for example, cytokines secreted by T cells can permanently arrest tumor cell proliferation and induce cell cycle arrest (78). To this point, the most dramatic post-vaccine changes we observed in clusters 0, 1 and 2 were reduced expression of molecules involved in protein synthesis (Fig. 1F volcano plots). These possible explanations have been added to the Discussion section (lines 360-368).

4. *In Figure 2I, it is unclear why different cytokines are being measured for different patients. The timing of these measurements also appear to be discrepant across patients, making comparisons rather difficult. Moreover, it is unclear whether CD8 or CD4 T cell responses are being assessed in each case.*

   We have repeated the functional experiments to analyze tumor idiotype-specific T-cell responses and now depict the results for all 30 cytokines detected at a single time point as volcano plots (revised Fig. 2I). Although we did not pre-fractionate T cells into CD8 and CD4 subpopulations, the cytokines detected indicate a mixture of CD8 and CD4 responses. For example, TNFa, Rantes, IP10, MIP1a and b, and MIG are secreted mainly by CD8+ T cells, while IL-2, HGF and IL-13 are secreted mainly by CD4+ T cells, and GM-CSF, IFNg, IL-8, and IL-6 can be produced by both (54-65) A statement has been added to this effect in the Results section. (lines 247-255)

5. *In Figure 2E, the authors demonstrate an expansion of specific T cell clonotypes after vaccine therapy. The authors should demonstrate whether these T cell clonotypes are specific for the B cell idiotype targeted by the vaccine. Alternatively, do some of these expanded T cell clonotypes harbor specificity for non-vaccine neoantigens, consistent with epitope spreading?*

   Taken together with the functional idiotype-specific T-cell responses shown in Fig. 2I, the expansion of top 20 TCR clonotypes post-vaccine in the majority of patients suggests that these expanded clonotypes are against vaccine-targeted idiotypes.

   However, the reviewer raises the additional possibility of epitope spreading which prompted us to re-analyze the data, revealing expansion of both pre-existing and newly emerging clones (i.e. not present in pre-vaccine samples). Such newly emerging clones are more clearly indicated by asterisks in revised Fig. 2E and could represent expanded TCR clonotypes against non-vaccine neoantigens. We have modified the conclusion on lines 217-220 to speculate that newly emerging clones could represent epitope spreading and cite a recent study in melanoma patients treated with neoantigen vaccines which definitively established this phenomenon in humans (ref. 46).

6. *How does vaccine therapy lead to an obliteration of the signaling pathways and interactions suggested by the authors in Figure 3 (e.g. RESISTIN and APRIL), and the myeloid transcriptional changes in Figure 4D? The authors should as a minimum speculate on the potential underlying mechanisms.*

   IL-6 is produced by both stromal and tumor cells in the LPL microenvironment. Our data suggest a possible mechanism of immune-mediated reduction of autocrine IL-6 production by LPL tumor cells (Fig. 3E; and ref 84), which in turn downregulates production of APRIL by myeloid cells (ref. 85). This potential mechanism has been added to the Discussion (lines 408-410).

Reviewer #3:

1. Table 2: recommended to summarize AEs and severe AEs by dose and overall, respectively.

We have re-organized Table 2 by splitting the AEs by dose and grade, which clarifies and provides additional data.

2. Need to report immune response rate, which is in the protocol, such as 1/9=11% has a response.

From the data in revised Fig. 2I, the proportion of patients with profiles showing a preponderance of significantly increased cytokine secretion after idiotype stimulation and thereby, positive immune response, is 7/8 = 87.5%. A statement to this effect has been added to the Results section (lines 249-251).

3. It's not clear which wilcoxon method was used for the plot. For paired data, like pre and post, it should be Wilcoxon signed rank; for independent data, it should be Wilcoxon rank sum. Not sure where t test was used. Please specify. Some boxplot didn't see large difference but p-value is very significant, like Figure 3S A. Figure 3. A. Please double check.

We would like to thank the reviewer for this comment. As explained above, we repeated the experiments in Fig. 2I and represent the entire data set as volcano plots. As suggested by the reviewer, for Fig. 3A and previous Fig. S3A (now Fig. S4A), we used Wilcoxon matched-pairs signed rank test. This test is now clearly indicated in the figure legends.

Reviewer #4:

1. *The clinical relevance of the findings is not clear. Several of the patients have not shown evidence of progression but with a very small study set it is difficult to assess the clinical impact. It would be helpful to assess whether there is any difference in the immunologic response in those patients with evidence of progression as compared to those with stable disease.*

We agree with the reviewer that the sample size is too small to draw definitive correlations and all but one patient (7 out of 8 patients) demonstrated positive immune responses (Fig. 2I). However, the single patient (LPL-007) who did not demonstrate immune response did progress.

2. *The unique aspects of the vaccine design are not explored. Does the immune response and nature of impact on monocyte and accessory populations relate to the antigen or CCL20 expression*

The mechanistic aspects of vaccine design were previously elucidated in preclinical models where the appropriate negative controls (i.e. target antigen or CCL20 alone) could be included. Specifically, in these prior studies constructs encoding chemokine alone were not sufficient to elicit tumor rejection non-specifically. We refer the reviewer to multiple previous publications

which included those mechanistic experiments (refs. 16, 82), and a statement to this effect has been added to the Discussion, lines 386-389.

3. *The manuscript describes the targeting of the malignant B cell population presumably by the T cell clonotypic expansion but then also describes changes in B cell proliferative pathways specific for the B cell and not plasmacytoid population. What is the presumed mechanism for this change-does this relate to a mode of T cell killing, how does the nature of the T cell compartment relate to this.*

One recently recognized mechanism of tumor cell death, ferroptosis, is known to be directly mediated by activated CD8+ T cells (Fig. 1F, heat map). The observation that most of the top 20 T-cell clonotypes identified after vaccination belong to the effector CD8 T-cell cluster is consistent with this possible mechanism. In addition to direct antitumor effects, immune-mediated tumor suppression may occur indirectly; for example, cytokines secreted by T cells can permanently arrest tumor cell proliferation and induce cell cycle arrest (ref. 78). To this point, the most dramatic post-vaccine changes we observed in clusters 0, 1 and 2 were reduced expression of molecules involved in protein synthesis (Fig. 1F volcano plots). These possible explanations have been added to the Discussion section (lines 360-368).

4. *The authors suggest that resistance of the plasmacytoid population may relate to downregulation of class II which is an important finding-it is likely that this not sole mechanism of resistance. What is the explanation for target expressing plasmacytoid cells to not be susceptible to antigen specific T cells.*

In new experiments we have verified the downregulation of HLA class II at the protein level by plasmacytoid subpopulations, providing further support for this mechanism of resistance (Fig. 1H and Fig. S2G). In addition, our CellChat analysis inferred upregulation of the insulin-like growth factor (IGF) signaling axis post-vaccine was by plasma cell, but not mature B cell LPL subpopulations, and scRNAseq data confirmed increased expression of IGF-1 among clonal tumor cells post-vaccine in both plasma cell-like clusters (clusters 5 and 10) but not in any B-cell clusters (0, 1, and 2), suggesting this as an additional mechanism of resistance. These two potential mechanisms of resistance have been summarized in a single paragraph in the Discussion. (lines 368 and following)

5. *The manuscript suggests that a vaccine capable of appropriate T cell expansion is not necessarily clinically effective-does this relate to T cell phenotype and impact of microenvironment-association of phenotype with clinical course would be of great significance but would potentially require a larger cohort and comparative analysis.*

Our results suggest that a vaccine capable of T-cell expansion is not clinically effective because one tumor subpopulation (plasma cell-like) is resistant and generates hypotheses of how to overcome potential mechanisms of resistance (Discussion, last paragraph). We agree that larger studies with the combination therapies suggested are warranted.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have done an excellent job of replying to my initial comments and the manuscript is much improved. The experiments are well done, the data is high quality, and the analyses follow a clear logical progression. I still have a major conceptual confusion with their model and results however. Specifically, the T cell response they see stimulated post-vaccination appears to be primarily a CD8 response. I say this because the top20 expanded TCR clones post vaccine are highly enriched for CD8 compared to CD4 (figure 2H) and the T cell subpopulation showing the strongest activation post vaccine is the effector population (figure 2C) which is CD8+CD4- (supplementary figure S3B). Yet, as the authors remark on line 165-166, "idiotype peptides are predominantly presented on HLA class II, rather than class I" (Khodadoust, MS, et al 2017 Nature). This same reference also identified an endogenous specific response to these tumors by CD4+ T cells. Finally, the authors of the current manuscript invoke the lack of HLA class II on the plasma cell subpopulation of malignant cells at baseline and the downregulation of HLA class II on the mature B cell subpopulation of malignant cells upon treatment (figure 1G) as major mechanisms of resistance. So, assuming CD8+ T cells recognize peptides presented by HLA class I and not HLA class II, I don't understand what these CD8+ T cells are recognizing.

Major comments:
1. Should add to text that normal plasma cells are known to not express MHC-II so this is likely not an aberrant tumor associated phenotype of the LCL-plasma cell subpopulation but rather normal plasma cell biology.
2. Line 211 – 213 says "… expansion of existing clonotypes in all patients except for … LPL-005 and -009 (Fig 2D)." Visually, I don't see how these two patients stand out strongly from the others, and technically all of them have points below the diagonal (ie expansion post vaccine), so was there a specific quantitative calculation that was done to say these two patients are distinct ?
3. Line 249 – 251 says "The cytokine profiles of 7 of the 8 patients … showed a clear preponderance of cytokines secreted in response to idiotype compared with negative control." Similar to my previous comment, I do not see one sample that is clearly distinct from all the others. In particular, LPL-001 and -007 both show only very weak induction. So which 1 of the 8 patients do the authors consider distinct ? Is this based on a quantitative measurement ?

Minor comments
1. In all the UMAPs colored by cluster (eg figures 1C, 2A, 4A), the labels for the cluster numbers are very hard to read and should be made clearer (bigger, bolder, perhaps offset).

Reviewer #2 (Remarks to the Author):

The authors adequately addressed the criticisms raised and substantially improved the manuscript. We have no further comments.

Reviewer #3 (Remarks to the Author):

The authors addressed my comments thoroughly. No further comments.

Reviewer #4 (Remarks to the Author):

The manuscript provides a detailed and thoughtful assessment of immunologic response to LPL following vaccination. The clinical relevance of the study is significantly limited based on small study number and there is a lack of correlation between immunologic findings and clinical outcomes to understand the relative importance of the immune findings. The mechanisms of resistance based on subpopulation of plasma cells and potential for role for HLA deletion are of note although the intersection between these resistance mechanisms and vaccine design are not clear. The authors have responded to the questions raised in the first review.

Reviewer #1 (Remarks to the Author):

…the T cell response they see stimulated post-vaccination appears to be primarily a CD8 response. I say this because the top20 expanded TCR clones post vaccine are highly enriched for CD8 compared to CD4 (figure 2H) and the T cell subpopulation showing the strongest activation post vaccine is the effector population (figure 2C) which is CD8+CD4- (supplementary figure S3B). Yet, as the authors remark on line 165-166, "idiotype peptides are predominantly presented on HLA class II, rather than class I" (Khodadoust, MS, et al 2017 Nature). Finally, the authors of the current manuscript invoke the lack of HLA class II on the plasma cell subpopulation of malignant cells at baseline and the downregulation of HLA class II on the mature B cell subpopulation of malignant cells upon treatment (figure 1G) as major mechanisms of resistance. So, assuming CD8+ T cells recognize peptides presented by HLA class I and not HLA class II, I don't understand what these CD8+ T cells are recognizing.

Response: We apologize for the misunderstanding and welcome the opportunity to clarify. While the top 20 clonotypes consist primarily of effector CD8 T cells, they also contain a smaller but potentially relevant effector CD4 T cell population (updated Fig. 2G, right panel, cluster 3). Moreover, the expression levels of the CD4 marker significantly increased post vaccination (Fig 2H), suggesting that vaccine therapy activated CD4 T cells. Finally, the cytokine secretion results following idiotype stimulation are consistent with cytokine profiles exclusive to CD4 (IL-2, IL-13) or either CD4 or CD8 T cells (GM-CSF, IFNγ, and IL-8) (Fig. 2I and lines 254-256 in the manuscript). Together, these observations suggest that the CD4 T cell population were of particular importance in the response to lymphoma idiotype observed.

In addition, several of the top 20 TCR clones post-vaccination were new clonotypes (not detectable in pre-vaccine samples, Fig. 2E), and reviewer #2 previously pointed out that these new clonotypes could represent epitope spreading; i.e., CD8 T cells in the effector population recognizing novel neoantigens.

We have added a new paragraph connecting these points to the Discussion section (line 397-405).

Major comments:

1. Should add to text that normal plasma cells are known to not express MHC-II so this is likely not an aberrant tumor associated phenotype of the LCL-plasma cell subpopulation but rather normal plasma cell biology.

Response: We have clarified this point in the manuscript (line 372).

2. Line 211 – 213 says "… expansion of existing clonotypes in all patients except for … LPL-005 and -009 (Fig 2D)." Visually, I don't see how these two patients stand out strongly from the others, and technically all of them have points below the diagonal (ie expansion post vaccine), so was there a specific quantitative calculation that was done to say these two patients are distinct?

Response: We have utilized the coefficient of determination (R^2) of Pearson correlation to guide interpretation of our results; specifically, applying a cutoff value of >0.5-0.6 as used by others previously (e.g ref 102). Thus, as pointed out by the reviewer, while technically all patients have points below the diagonal (i.e. expansion post vaccine), the coefficients of determination for LPL-005 (R^2=0.61) and LPL-009 (R^2=0.66) are above this threshold, indicating that clonotype frequencies pre- and post-vaccine are positively correlated and therefore did not change following vaccination. For clarity the R^2 values have been added to each panel in Fig. 2D and this explanation has been added to the figure legend and Methods (lines 563-565).

3. Line 249 – 251 says "The cytokine profiles of 7 of the 8 patients … showed a clear preponderance of cytokines secreted in response to idiotype compared with negative control." Similar to my previous comment, I do not see one sample that is clearly distinct from all the others. In particular, LPL-001 and -007 both show only very weak induction. So which 1 of the 8 patients do the authors consider distinct? Is this based on a quantitative measurement ?

Response: The cytokine profiles of all patients, except patient LPL-007, showed a clear preponderance of cytokines secreted in response to idiotype compared with negative control, indicated by red colored dots on the right side of the volcano plots. Individual chi-square analyses supported this conclusion, and the p-values generated from this test are now added to each panel in Figure 2I. While the p-value for LPL-001 was of borderline significance (p=0.07), we felt the trend showing weak induction of cytokines was sufficient to classify this patient as an immune responder. We also added a statement clarifying that LPL-007 was classified as a non-immune responder (line 251-252).

Minor comments
1. In all the UMAPs colored by cluster (eg figures 1C, 2A, 4A), the labels for the cluster numbers are very hard to read and should be made clearer (bigger, bolder, perhaps offset).

Response: This has been done.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have done an excellent job responding to my earlier comments. I have no more issues to raise.