nature portfolio

Peer Review File

Development of patient-derived lymphomoids with preserved tumor architecture for lymphoma therapy screening

Corresponding Author: Dr Elisa Oricchio

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript submitted by Albert Santamaria and co-authors entitled “Patient-derived lymphomoids preserve the tumor architecture and allow testing response to therapies in lymphoma”, presents an innovative system to culture ex vivo non-Hodgkin lymphoma biopsies for over a week preserving tumor architecture and main cellular subtypes. The authors also show its utility for drug testing and the degree of similarity with clinical responses.
The paper tends to be generalistic, including many types of NHL (all B-NHL but 1 T-cell case), based on biopsy availability. I totally understand the hurdles of this type of study. However, in my view, this holistic approach in terms of NHL subtypes holds limitations in the optimization of culture conditions for each NHL subtype, and in the study of relevant cellular subtypes in each lymphoma. Likewise, although the studies related to clinical response are sound and without doubt they do have value, in a closer look they present important limitations. My main concerns are: 1) the time-of-sampling of the biopsy used and the treatment chosen (most of the time are not aligned), 2) the read-out and method for drug activity evaluation, and 3) the absence of antibody therapy in the panel, when immunotherapy is always combined with another agent in B-NHL.
Thus I believe the system may have utility, but in my view it may need additional refinement to be applied in parallel to clinical trials, which may be the goal, or as a predictive platform for personalized therapy approaches.

Major comments

1.System set up:
a.The authors have used spleen from a FL mouse model to set up the system. These experiments, beautifully performed in terms of technology and results, set the basis for the culture of a heterogenous (by definition) NHL subtypes (including a T – cell lymphoma, where the need of BAFF may be questionable). It would have been interesting instead to perform experiments in non-tumoral LN as a first approach (reactive) or tonsils, based on tissue similarity. In the end one would like to maintain the signaling in the corresponding lymphoma LNs. The tumor B cells in each B-NHL, although it is known their dynamic differentiation state (DOI: 10.1038/s41590-018-0181-4), mainly correspond to specific stages of B cell differentiation, from naïve to Ag-experience B-cells, some are localized in mantle zone, other in the marginal area, germinal center (GC), or post-GC. Hence, although biopsy may function autonomously from a small period, the cytokines that may be suitable for each lymphoma ecosystem may differ. Thus, assuming that BAFF may be optimal for all B-NHL is somewhat simplistic.
b.The election of Vitrogel matrix is not justified or compared with any other material (ie PEG hydrogels have demonstrated its utility in ex vivo NHL culture (DOI:10.1016/j.biomaterials.2015.09.007). Our experience working with this material in B-NHL has been quite disappointing. The authors state the stiffness was adjusted by media dilution, while no stiffness measurements are shown (ie Young modulus or similar). Another question is the degree of cell retention in the biopsy. Some ex vivo systems exhibit cell leakage outside the biopsy during culture. This can be easily tested by flow cytometry of the cell culture supernatants.

2.Drug testing
a.The authors use the changes in proliferating CD20 cells and of total CD20 cells (just in figure 3) as a read-out for drug activity. The use of just one marker to identify tumor cells is always risky. In some B-NHL subtypes, the introduction of an extra marker helps (ie CD5 for MCL o SLL) or at least include the kappa/lambda for light chain restriction. This limitation becomes evident for idelalisib activity in LP8 (figure 3b and 3g) where results do not align when tumor cells are identified by spatial transcriptomics. As it may not be feasible to apply this technique to assess drug profiling routinely, an alternative is to

standardize a well-controlled disaggregation protocol to identify accurately the tumor population by flow cytometry. Moreover, Visium multiplex IF technology allows the analysis of just one section of the lymphomoid, while the whole lymphomoid can be analyzed by total disaggregation followed by flow cytometry analysis. This system may also allow to quantify depletion of tumor cells.
b.Moreover, I do not quite understand why anti-CD20 or anti-CD19 have not been included in figure 4 to really compare the full treatment applied in the clinic. In this case, the read-out should be change in number tumor B cells (not just proliferating ones). Again, in my view flow cytometry may be a better method.
c.In figure 4, 5 out of the 7 lymphomoids from were treated just with part of the treatment received in vivo (apart from LP13 and LP17). Hence, I think it may be too pretentious to state they recapitulate responses in 7/8 of cases, when the full treatment was not applied. Besides the sampling time does not always corresponds to the treatment applied. Changes occur after treatment with R-CHOP, sub clonal populations could be selected and become the predominant population and TME may have also change.

Minor comments

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2.Please revise in page 9 the number of cases for each DLBCL (n= 10, stated 9) and FL (n=3, plus 1 tFL ?, state 2 and 1, respectively).They appear different in supp table 3 and Fig 2a. Also, LP22 is referred as a transformed DLBCL(supp table 3), when it may be a DLBCL arised from a FL, thus transformed FL.

3.Supp Table 3: “biochemistry” may be more accurate that “chemistry” for the heading of LDH and urate values. Also, please correct “par” by “by” in LP18 (CART bridging par ibrutinib).

Below I add my personal opinion on the previous revision.

Major comments reviewer 1
The authors have performed intensive work to satisfy this reviewer, and although the manuscript has improved substantially (I could not see the original, but I understand more cases have been analyzed, spatial transcriptomics workflow was refined, among other improvements) he is still reluctant to appreciate the value of the system. No doubt the duration of response is out of scope for the lymphomoids and needs in vivo testing, and I agree with the authors this ex vivo system may have utility for personalized medicine (comment 1).
I agree with this reviewer that using the spleen from a FL mouse model to set up the system may not be my first approach either (also considering the 3R for animal research), and the reservations as a platform for drug testing.

Major comments reviewer 2
I agree with the reviewer the need of scRNAseq and flow cytometry to assess the similarity with original biopsy. Figure R3C comparing drug efficacy by IF or flow cytometry does not support relying exclusively on imaging.

Version 1:

Reviewer comments:

Reviewer #1

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The authors have addressed all the points raised by the reviewer.
Please find below some feedback to their responses:

Major comments

1.System set up:
a.This reviewer thanks the authors for sharing their approach. My comment was regarding the use of different cocktails and matrixes in human rLN (not from the mice) or tonsils to get closer to human NHL, instead of establishing a protocol with mouse material, and then validate the conditions in lymphoma LN.
b.I do understand, but do not share, the idea of making a simple and operative system, despite the differences among NHL entities. As I said in the previous revision, how is the use of BAFF, mostly a B-factor, justified in the T-cell lymphomoid?
c.This reviewer thanks the authors for sharing their experiences using different matrixes and for the stiffness measurement. However, lymphomoids seem to be pretty soft, as the typical stiffness for a malignant LN ranges between 1500 and 3000 Pa (G´ value).

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a.The reviewer thanks that the authors for the clarification. I understood by the figure that the number of total B cell increased in Fig 3c while tumor decreased in 3g, but it may be not significant.
b.The reviewer thanks the authors for measuring the kappa, Lambda together with CD20. This good correlation indicates that the lymphomoids are enriched in tumor cells.
c.I do understand the reasoning of just focusing in targeted therapies, but considering the lymphomoids preserve quite well the cellular composition and despite the limitation of not being able of accounting for effector recruitment, for future studies I would not discard immunotherapy testing.
d.The reviewer thanks the authors for clarifying this point. I do acknowledge that the samples were adequate. Maybe it is worthy comment in the discussion the authors´ choice of not including immunotherapy in these studies for the reasons discussed in the rebuttal letter.

The reviewer thanks the authors for also addressing the minor points indicated.

Reviewer #4 - Replacement for Reviewer #1 & #2 (Remarks to the Author):

The manuscript submitted by Albert Santamaria and co-authors entitled "Patient-derived lymphomoids preserve the tumor architecture and allow testing response to therapies in lymphoma", presents an innovative system to culture ex vivo non-Hodgkin lymphoma biopsies for over a week preserving tumor architecture and main cellular subtypes.

The authors also show its utility for drug testing and the degree of similarity with clinical responses. The paper tends to be generalistic, including many types of NHL (all B-NHL but 1 T-cell case), based on biopsy availability. I totally understand the hurdles of this type of study. However, in my view, this holistic approach in terms of NHL subtypes holds limitations in the optimization of culture conditions for each NHL subtype, and in the study of relevant cellular subtypes in each lymphoma. Likewise, although the studies related to clinical response are sound and without doubt they do have value, in a closer look they present important limitations.

My main concerns are: 1) the time-of-sampling of the biopsy used and the treatment chosen (most of the time are not aligned), 2) the read-out and method for drug activity evaluation, and 3) the absence of antibody therapy in the panel, when immunotherapy is always combined with another agent in B-NHL.

Thus I believe the system may have utility, but in my view it may need additional refinement to be applied in parallel to clinical trials, which may be the goal, or as a predictive platform for personalized therapy approaches.

We thank the reviewer for the positive assessment of the system we have developed and agree that there are limitations in the proposed model as stated in the manuscript discussion. This study represents an initial proof of principle of the potential utility of lymphomoids and we want to develop this platform side-by-side with clinical trials to further demonstrate its utility. We have included some of the control experiments that this reviewer requested and clarified key results of the study that might have triggered some of the reviewer's major comments. Specifically:

1. ***the time-of sampling*: In Figure 4, we matched the response to therapies obtained *ex vivo* using lymphomoids and in the clinic. In 6 out of the 7 patients, the biopsy was done just before the same treatment started in the clinic and *ex vivo*. In these cases, the time of the treatment *ex vivo* was aligned with the time of the treatment in the clinic. Only one case, patient 18, received immunochemotherapy after the biopsy but rapidly relapsed and was treated with ibrutinib. Ibrutinib was test *ex vivo* on the initial biopsy. The relapse in the case of patient 18 occurred within the first year. Such early relapses have recently been shown to share similar mutation profiles and treatment outcomes as the primary tumor (doi: 10.1200/JCO.23.00570), thus we considered it as a matching sample. We reported all the data for sample collection and clinical treatment in Suppl. Table 3.**

2. ***The read-out method for drug sensitivity evaluation*: We used multicolor fluorescence imaging and spatial transcriptomics and with these two independent methods the read-out matched. In the comment below, the reviewer mentions specifically the case of idelalisib in Figure 3 for LP08, but the results may have been misinterpreted, as the data perfectly match. Moreover, it may not emerge from the figure, but as reported in the methods section and Figure 2a the quantification of B-cell proliferation and total B-cells has been done on multiple different lymphomoids and different sections (different tissue depths). In particular, for LP08 it was done on 3 slides (representing 3 different depths in the tissue), not just one slide (see detailed comment below).**

3. ***The absence of immunotherapies antibodies in the panel*: The reviewer this point as a limitation and we can understand their reasoning. However, together with our clinical collaborators when we designed this study we decided not to include the antibodies**

because the selection of monoclonal antibodies is already standardized in the clinic, while there is no clear path to select small molecule inhibitors. Moreover, we decided to not include rituximab and other therapeutic antibodies because one of the main mechanisms of the actions is through antibody-dependent cellular cytotoxicity and phagocytosis, which typically requires the recruitment of immune cells, but in our system, we can only assess the activity of tissue-resident cells. Based on this discussion we concluded that testing the antibody was not required and it could be a confounding factor. We hope that the reviewer can understand that since these are fresh tissue biopsies treated for a few days, we can not add this treatment to the current cohort, nevertheless, we will take into consideration this comment for the design of future studies.

Major comments

1. System set up:

a.The authors have used a spleen from a FL mouse model to set up the system. These experiments, beautifully performed in terms of technology and results, set the basis for the culture of a heterogenous (by definition) NHL subtypes (including a T – cell lymphoma, where the need of BAFF may be questionable). It would have been interesting instead to perform experiments in non-tumoral LN as a first approach (reactive) or tonsils, based on tissue similarity. In the end one would like to maintain the signaling in the corresponding lymphoma LNs. The tumor B cells in each B-NHL, although it is known their dynamic differentiation state (DOI: 10.1038/s41590-018-0181-4), mainly correspond to specific stages of B cell differentiation, from naïve to Ag-experience B-cells, some are localized in mantle zone, other in the marginal area, germinal center (GC), or post-GC. Hence, although biopsy may function autonomously from a small period, the cytokines that may be suitable for each lymphoma ecosystem may differ. Thus, assuming that BAFF may be optimal for all B-NHL is somewhat simplistic.

Thanks to the reviewer for the positive comment about the initial test that we did using murine models. Regarding the suggestion to use LN (reactive) or tonsils, we did not test this condition in the murine model, but for two lymphoma patients. Indeed, we received two reactive lymph nodes (LP3 and LP10) and multicolor fluorescence imaging shows a similar composition as the original biopsy (Suppl. Figure 4). We have now highlighted these results in the text and included representative pictures in Suppl. Figure 4b-c.

The second comment of the reviewer is about the media composition. We do agree with this reviewer that assuming that BAFF is enough for long-term growth of all lymphoma subtypes is simplistic. However, this system was designed for short-term cultures, and therefore tested multiple conditions (Suppl. Figure 1 and Table S1) and we tried to find the simplest possible conditions that sustain the survival and growth of the tissue explants for a period of a few days/weeks. For long-term experiments, we do agree that probably additional factors should be considered. Further, testing different growth conditions for each patient could limit the applicability of the system in a clinical setting. Thus, we tried to balance the benefits and limitations of the system.

b.The election of Vitrogel matrix is not justified or compared with any other material (ie PEG hydrogels have demonstrated its utility in ex vivo NHL culture (DOI:10.1016/j.biomaterials.2015.09.007). Our experience working with this material in B-NHL has been quite disappointing. The authors state the stiffness was adjusted by media dilution, while no stiffness measurements are shown (ie Young modulus or similar). Another question is the degree of cell retention in the biopsy. Some ex vivo systems exhibit cell leakage outside the biopsy during culture. This can be easily tested by flow cytometry of the cell culture supernatants.

In the initial setting for the culture, inspired by the protocol reported in Neal et al. (2018), we used collagen, but the gel was melting rapidly and we could not maintain the integrity of the tissue.

Then we used Vitrogel and after a few tests briefly described in Supplementary Figure 1a, we were satisfied with the cell viability and tissue composition, thus we did not further test other matrices. We considered the difference in stiffness based on results reported in a manuscript published by the company producing vitrogel (DOI:10.24966/CTB-9107/100030).

Following the reviewer's comment, we have now measured the elastic modulus at different dilution factors and report that the values range between 68 and 790 G'Pa. We included these results in Suppl figure 1.

Regarding the reviewer's questions about the leakage outside of the biopsy during the culture. We did not observe such phenomena in the live culture or when we fixed the cells and analyzed paraffin-embedded tissues. This can be explained because the lymphomoids are cultured using an insert with a mesh of 0.4 μm pore size (Millipore #PICM01250), which is smaller than a mammalian cell diameter which is between 8-10 μm. Also, we do fix and paraffin-embed the gel entirely, without dissolving it, so should any cells be migrating, we would see it in our sections, which is not the case.

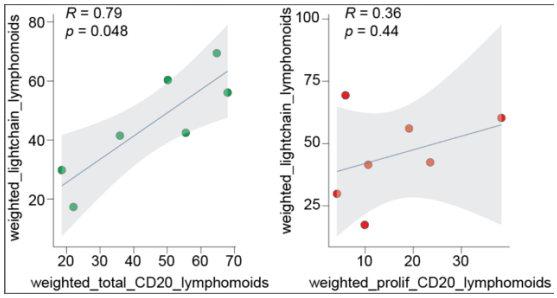
2.Drug testing

a.The authors use the changes in proliferating CD20 cells and of total CD20 cells (just in figure 3) as a read-out for drug activity. The use of just one marker to identify tumor cells is always risky. In some B-NHL subtypes, the introduction of an extra marker helps (ie CD5 for MCL o SLL) or at least include the kappa/lambda for light chain restriction. This limitation becomes evident for idelalisib activity in LP8 (figure 3b and 3g) where results do not align when tumor cells are identified by spatial transcriptomics. As it may not be feasible to apply this technique to assess drug profiling routinely, an alternative is to standardize a well-controlled disaggregation protocol to identify accurately the tumor population by flow cytometry. Moreover, Visium multiplex IF technology allows the analysis of just one section of the lymphomoid, while the whole lymphomoid can be analyzed by total disaggregation followed by flow cytometry analysis. This system may also allow to quantify depletion of tumor cells.

We respectfully disagree with the reviewer's comment that the tumor cells identified by spatial transcriptomic in LP8 do not align with the total number and proliferative B-cells identified by multicolor immunofluorescence. We observe the same results i.e., no changes in total B-cells, no significant reduction in proliferating B-cells using multicolor fluorescent imaging (Figure 3b and c), and no effect of idelalisib in spatial transcriptomic (Figure 3f-g). In addition, the quantification of total and proliferating B-cells reported by multicolor immunofluorescence is based on 3 sections for each condition, not just one section.

We agreed with the reviewer that it is better to consider multiple markers to assess response to therapies. Indeed, we already selected dual markers: CD20+ and proliferation (Ki67+ cells) and we observed a good correlation with clinical response (Figure 4).

To include an additional marker used in the clinic, we followed the reviewer's advice and stained for the kappa/lambda light chain restriction the samples in which we have clinical matching data (Figure 4, n=7). As expected, kappa/lambda staining correlates with total CD20 (r= 0.79 and p=0.048), however, the correlation with proliferating B cells decreases (r= 0.36 and p=0.44) (Rebuttal Figure 1). In particular, using CD20+ Ki67+ cells the effect observed in lymphomoids matched with the clinical data in 8 out of 9 samples, using only CD20+ or kappa/lambda staining this was reduced to 6/9 cases, suggesting that proliferation of B cells is a key parameter to determine the efficacy of the drug. We did not include the kappa/lambda staining data in the manuscript as they seem to be redundant with CD20+ data, but we will follow the reviewer's advice if they think otherwise.



b.Moreover, I do not quite understand why anti-CD20 or anti-CD19 have not been included in Figure 4 to really compare the full treatment applied in the clinic. In this case, the read-out should be a change in the number tumor B cells (not just proliferating ones). Again, in my view flow cytometry may be a better method.

We have already addressed this comment in the general remarks. As mentioned above, when we designed the study we considered the possibility of including rituximab, but since treatment with this antibody is well characterized, we decided to focus on the effect of small molecule inhibitors. Moreover, our system is more suitable to test the effect of therapies on tissue-resident cells, while immunotherapies trigger the recruitment of other immune cells, but we don't model systemic cell trafficking. We hope that the reviewer can understand that since these are fresh tissue biopsies treated for a few days, we can not add this treatment to the current samples cohort and we are not able to perform flow cytometry on these samples. Nevertheless, we will take into consideration this comment for the design of future studies.

c.In figure 4, 5 out of the 7 lymphomoids from were treated just with part of the treatment received in vivo (apart from LP13 and LP17). Hence, I think it may be too pretentious to state they recapitulate responses in 7/8 of cases, when the full treatment was not applied. Besides the sampling time does not always corresponds to the treatment applied. Changes occur after treatment with R-CHOP, sub clonal populations could be selected and become the predominant population and TME may have also change.

We respectfully disagree with the reviewer's comment, because most samples reported in Figure 4 (6/7) at the time of the biopsy received a similar treatment in the clinic and on the lymphomoids. There is no clonal selection, and these were either relapsed cases where a second biopsy was necessary or primary cases that refused chemotherapy treatment. We matched the results a few months apart since the results on lymphomoids were obtained after a few weeks and the clinical response was assessed after a few months of treatment. In supplementary figure 3, we included information about treatment in the clinic and the time of the biopsy.

Minor comments

1.The author refer as "small molecules" all the drugs used. They would be better defined as targeted therapies (BTK, EZH2, HDAC inhibitors) or immunomodulatory agents (lenalidomide). In addition, idelalisib dose of 10μM is quite beyond the range of PI3Kδ inhibition specificity (max 0,5-1μM) (doi.org/10.1517/21678707.2014.978858) and achieved in vivo.

We edited the text accordingly to the reviewer's comment.

About the dose of idelalisib, we agree with the reviewer that we used it at a high concentration, but in most of the cases, we observed no effect on the tumor cells even at this high concentration. Moreover, idelalisib was used at higher concentrations (40uM) both alone or in combinatorial treatments in DLBCL organoid cultures showing only limited cytotoxic effects (Shah et al., 2023).

2.Please revise in page 9 the number of cases for each DLBCL (n= 10, stated 9) and FL (n=3, plus 1 tFL ?, state 2 and 1, respectively).They appear different in supp table 3 and Fig 2a. Also, LP22 is referred as a transformed DLBCL(supp table 3), when it may be a DLBCL arised from a FL, thus transformed FL.

We have revised it and corrected it.

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I agree with the reviewer the need of scRNAseq and flow cytometry to assess the similarity with original biopsy. Figure R3C comparing drug efficacy by IF or flow cytometry does not support relying exclusively on imaging.

Reviewer #1 (Remarks to the Author):

The authors have addressed all the points raised by the reviewer.
Please find below some feedback to their responses:

We thank this reviewer for the constructive feedback and comments.

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a.This reviewer thanks the authors for sharing their approach. My comment was regarding the use of different cocktails and matrixes in human rLN (not from the mice) or tonsils to get closer to human NHL, instead of establishing a protocol with mouse material, and then validate the conditions in lymphoma LN.

b.I do understand, but do not share, the idea of making a simple and operative system, despite the differences among NHL entities. As I said in the previous revision, how is the use of BAFF, mostly a B-factor, justified in the T-cell lymphomoid?
In this particular case, it was justfied by the presence of a proliferating clonal B-cell population in the IALT associated with EBV, but we agree that in regular T-cell lymphomas the BAFF concentration in the media could be reduced or even removed, while other factors such as interleukins may be added.

c.This reviewer thanks the authors for sharing their experiences using different matrixes and for the stiffness measurement. However, lymphomoids seem to be pretty soft, as the typical stiffness for a malignant LN ranges between 1500 and 3000 Pa (G´ value).

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a.The reviewer thanks that the authors for the clarification. I understood by the figure that the number of total B cell increased in Fig 3c while tumor decreased in 3g, but it may be not significant.

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d.The reviewer thanks the authors for clarifying this point. I do acknowledge that the samples were adequate. Maybe it is worthy comment in the discussion the authors´ choice of not including immunotherapy in these studies for the reasons discussed in the rebuttal letter.

We have included this information in the discussion. "In addition, it could be interesting to include combination therapies with monoclonal antibodies, although their efficacy and selection criteria are well established in the clinic. Some patients in our study did indeed receive monoclonal antibodies such as rituximab together with the targeted therapies. Nevertheless, our system is currently designed to test the effect of therapies on tissue-resident cells, rather than immunotherapies, which often trigger the recruitment of other immune cells. For this reason, we did not add combinatorial treatments in our assays."

The reviewer thanks the authors for also addressing the minor points indicated.