nature portfolio

Peer Review File

Defective cell-autonomous signalling and antigenic polyreactivity of B-cell receptors from chronic lymphocytic leukaemia stereotyped subset 1

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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 provides a thorough analysis of the structure of three common subset 1 BCR Fabs in chronic lymphocytic leukemia (CLL). Through the preparation of monoclonal antibodies and the assessment of autoantigen recognition, the study demonstrates that activation signals in these tumor B cells are neither autonomous nor involve self-association, distinguishing them from other subsets (e.g., 2, 4, and 169). Additionally, the absence of Ca^{2+} influx activity in B cell lines expressing subset 1 BCRs suggests that alternative signaling pathways may contribute to BCR-mediated progression in CLL.
Overall, the manuscript is well-written, with rigorous and well-executed experimental approaches. However, several unresolved issues remain that may limit its overall impact.
1. The authors generated recombinant monoclonal antibodies from CLL patients but did not specify how these malignant clones were identified, nor did they provide details regarding the B cell/BCR type from which these malignant clones originated.
2. Although the authors expressed and purified BCR Fabs and achieved crystallization, they did not present data from protein purity assessments during the purification process, limiting our ability to assess the level of protein purity achieved.
3. The subset 1 CLL BCRs demonstrated recognition of multiple autoantigens with restricted light chain usage, which is characteristic of $CD5^+$ B cells. Given that B1 cells typically display low affinity for antigen binding—a factor that can significantly impact signal transduction—this aspect warrants further analysis and discussion in the context of the results.
4. The IGKV1-39/1D-39 light chain used in subset 1 CLL is among the most frequently utilized genes in human kappa chains. The authors attribute this specific usage to optimal interactions with the heavy chain; however, this claim lacks direct supportive data within the results.

Reviewer #2

(Remarks to the Author)
n the Ms of Cocomazzi/Latrou/Minici et al. the U-CLL stereotyped BCR subset 1 is thoroughly investigated on the points of (i) their structure, (ii) antigen binding properties, (iii) self BCR-BCR interaction and (iv) their capacity to induce autonomous BCR signaling, as measured by calcium flux. This Ms is important and provides further insight in the heterogeneity of CLL as a whole group and in the unique structural BCR characteristics of the various stereotyped BCR subsets. Although it is an interesting manuscript, I do have comments, mainly related to the messiness/inconsistencies of the figures and flawed discussion.

1. In Figure 1A, I suppose that the over-all Fab structure is presented, this however does not become clear from the legend. Please emphasize in the legend that the presented structure is based on representation of the three Fabs. For the light chain and heavy chain the colors pink and light blue are used, for H-CDR1, H-CDR2 and H-CDR3 yellow, green and marine are used. The K-CDR1, K-CDR2 and K-CDR3 are not indicated by color. The authors must use colors that are more clearly distinguishable from each other and also show the CDRs of the kappa. In Figure 1B please indicate above the boxed sequences H-CDR1, H-CDR2 and H-CDR3. The three amino acid sequences (or if they are all the same one) of the IGKV1-

39 should also be shown.

2. In Figure 3, the antigen binding of recombinant IgG of subset 1 CLL is shown. This is a very messy figure. The order of the IgG antibodies is different between Figure 3A, 3B left and right. The antibodies should be presented in the same order in all graphs of Figure 3A and 3B to enable the reader to properly judge and compare the binding strength of a particular IgG in the different experiments. For example, the P14197 IgG demonstrated the strongest in all three assays. This also holds for Figure S2; to be able to compare the binding strengths of a specific CLL IgG Ab it is necessary to display the exact reactivities of the various CLL IgGs of the patients. A Figure S2C should be added in which the data is presented in the same manner as in Figure 3B, enabling comparison of the individual binding strengths of the 14 CLL Abs. Now, in Figure S2, Ag binding of the CLL IgGs is shown ordered to the germline IGHV that is expressed by the subset 1 BCRs. This shows that the IgG encoded by the three IGHV gene segments bind similarly polyreactive to the various antigens, although there is a trend of IGHV5 being less polyreactive. This could also be mentioned in the text.

3. In the main text, the recombinant Abs are abbreviated as (rmAbs). This should be renamed in recombinant IgG in order to make clear that IgG is prepared from the subset 1 IgM BCR. I don't know if IgG always fully displays identical antigen binding activity as compared to IgM. Maybe the authors can comment on this.

4. In the legend of Figure 3C it should be mentioned which two CLL IgG Abs were used for the immunohistochemistry. This could also be added in Figure 3C itself.

5. In Figure 4 the protein gene names on the right site of the graph are not legible. This should be corrected. As expected the subset 1 antibodies show a comparable binding profile. Peculiar is the subset 201 P775 IgG, which shows strong binding at the bottom of the graph. Is this real or an artefact ? Maybe this IgG is not good and it would better be deleted as it serves no purpose.

6. Is it not more logical to move the heading “Structural basis for the restricted light chain pairing in subset 1 BCRs” (Page 9, line 195) before the heading “Subset 1 BCR are polyreactive and display intra-subset diversity” (Page 8, line 159).

7. It is informative to also show the size exclusion chromatography of the three Fabs P3129, P10015 and P1173 in a new Figure 6A. Now only P3128 is shown in Figure S4A. Figure 6B would then be the sedimentation velocity centrifugation experiment. Maybe in the supplementary Figure S4 the experiment of subset 2 and subset 4 Fabs can be shown from the Nature Communications Ms of 2017, to illustrate the clear difference of the subset 1 Fabs.

8. In Figure 7A, P3129 and P10015, of which the structure was determined are shown. However, the P1173, of which the structure was also determined, is not shown. Why not? Was there a problem with the TKO transfection? According to the Duhren-von Minden Nature Ms of 2012 a polyreactive IgM (BCR62) also is able to produce Ag-independent Ca flux. Unfortunately, the most polyreactive IgG P14197 is not shown in the TKO system, while this BCR may induce higher levels of Ca than the other subset 1 BCRs.

9. In the discussion at Page 14 Line 329-331, two previous studies on subset 1 antibody reactivities are referred (25 Lanemo, 26 Bergh). However, also the studies of Catera/Chiorazzi, Mol Med.(2008), Hatzi/Chiorazzi, Clin.Imm.(2016), Chu/Chiorazzi, Blood(2010) and Gounari/Ghia, Blood(2015), in which subset 1 reactivities were also studied should be mentioned. In the study of Catera/Chiorazzi (2008), also reactivity with MDA-BSA was shown as a target for subset 1 Abs, however MDA-BSA was not recognized in the Gounari/Ghia(2015) study. These older studies should also be referred and be discussed.

10. The authors claim that BCR-BCR interaction may not represent a ubiquitous mechanism for BCR-mediated signaling in all CLL. This is now is certain as in the Ms it is shown that subset 1 BCRs do not induce calcium influx. This should be changed in ‘BCR-BCR interaction is a mechanism for BCR signaling in subsets of CLL’. It is clear that subset 2, 4 and 169 show BCR-BCR interaction, but I presume also non-BCR-subset VL3-21 R110 CLLs, which is a large subset representing 8 – 10 % of all CLLs, show BCR-BCR interaction. In the Duren-von Minden Nature 2012 Ms 17 CLL were tested including one subset 2, one subset 7E2 and 7C4 and now also a subset 99 CLL (UM-CLL4). The authors should check, which subsets were analyzed in this Ms and add this information in the discussion. Altogether, still a large group of CLL undergo BCR signaling due to homotypic BCR-BCR interaction. Subset 1 IgGs show polyreactive binding to various antigens as shown in the ELISAs (Figure 3) and antigen array (Figure 4). In the Duren-von Minden Nature 2012 Ms, also a polyreactive non-CLL BCR (BCR62) shows Ca flux in the TKO, indicating that strong polyreactive BCRs also possess BCR-BCR interaction (maybe not by the VH-CDR3 VRQ-FR2 interaction). Strong polyreactive CLLs are subset 8, 9 (9A, 9B and 9C) and 28 (Catera/Chiorazzi, Mol Med. 2008), subset 7 (7C2 and 7A3) (Hatzi/Chiorazzi, Clin.Imm. 2016) and (again) subset 8 (Gounari/Ghia, Blood 2015). There are also CLL subsets, which are do not show polyreactivity, such as the IGHV mutated subset 111 (Figure 4), the mutated subset 13 (Kostareli/Stamatopoulos, Leukemia 2012) with rheumatoid factor BCR specificity and other CLL subsets with rheumatoid factor BCR specificity (Hoogeboom/Noesel, Leukemia 2013) and a subset with fungi specificity (Hoogeboom/Noesel, Journal Experimental Med. 2013).
It should be discussed and referenced that (the combined data suggest) we have (i) CLLs with BCR-BCR interaction or likely also strongly polyreactive BCR (such as subset 8), which show autonomous Ca flux, (ii) subsets of moderatly polyreactive CLLs (like subset 1), which do not show autonomous Ca flux but do experience chronic BCR stimulation by the microenvironment due to their polyreactivity and (iii) subsets of CLLs, which have a very specific antigen that they recognize and very likely also do not show autonomous Ca flux, such as subset 111, the rheumatoid factor and the fungi-specific CLL subsets.

Minor comments

1. In the Results section, it is stated that of 11 subset 1 CLL Fab fragments were produced. Yet of three Fabs the crystal structures were made and analyzed. Figure 3 displays 14 recombinantly produced IgGs of subset 1. A table should be provided depicting all 14 subset 1 cases with their IGHV / IGKV rearrangements and their stereotyped VH-CDR3 amino acid sequences. In this table, the three CLLs of which crystal structures were analyzed should be indicated as well as the 11 CLLs of which Fab and the 14 CLLs of which IgG was produced.

2. The order of headings in the results section should at least largely correspond with the order in the method section. As an example the results section starts with the expression of Fab fragments (page 6, line 108), whereas in the methods the production of Fab fragments is at the 7th heading (page 20, line 467).

3. In the text on page 10 line 219, the authors mention their previous work without a reference, the reference should be provided.

4. In the Discussion (Page 15, Line 362), UM-CLL4 is mentioned to expresses IGHV1-2. It actually expresses IGHV1-3 as shown in Figure S7 and in the original Duhren-von-Minden Nature 2012 Ms. This must be corrected.

5. A reference should be provided for the statement that MDA-reactive Abs show a high degree of cross-reactivity.

Reviewer #3

(Remarks to the Author)
The study by Cocomazzi, Iatrou, Minici et al. focuses on the structural and functional characteristics of subset 1 Chronic Lymphocytic Leukemia (CLL) BCRs (5% of cases with unmutated IGHV genes (U-CLL)), revealing that despite their structural conservation, there are significant differences in amino acid sequence that affect antigenic specificity. Using X-ray diffraction, the team examined crystallized IgM BCR Fab fragments from CLL patients, noting similar structural frameworks but with variations in elbow angles and antigen-binding sites, reflecting diverse antigenic reactivities within the subset. These structural variations are paralleled by unique biochemical features at the combining sites of the BCRs, such as differences in electrostatic potential and hydrophobicity, which correlate with their distinct ligand binding profiles demonstrated in biochemical assays. The study further explores the intramolecular interactions and structural basis for the selective pairing of heavy and light chains, pointing out the role of specific amino acids in these interactions. Additionally, unlike other CLL subsets, subset 1 BCRs do not form stable homotypic complexes or activate cell-autonomous signaling (in a B cell model line), suggesting alternative mechanisms of activation and interaction with antigens.

Point 1. The difference between subset 1 and the rest (as described in the paper) is the lack of homotypic BCR-BCR interaction and propensity for ongoing BCR pathway activation. Btk-inhibitors are not only relevant for non-subset 1, but also for subset 1 patients with poor prognosis. It is not improbable that CLL B cells have secretory pathway antigens that deliver a BCR-BTK signal. The stainings of Fig 3c and particularly c and d should be shown with high magnification to discrimate the intracellular structures that stain in some examples. Is this vesicular/membrane stain or cytoskeletal?
Point 2. Please note which Figure 3b) rec mAbs are shown on sections in the 3C. How is the Elisa/cytostain match of dsDNA (Elisa) with nuclear stain, and actin (with cytoskeletal)?
Point 3. Figure 4, the columns are poorly described and the antigen list is not easy to read. It would have been interesting to see positive control SLE sera or mAbs to relate the affinities to what is reported. The authors should show SPR or similar to see the particular curves (on/off rates) of hits that can be assessed in a more relevant manner. It is curious that Fibrinogen IV ("cryo") turns up on this list, what is this protein (or protein mix). I don't think any coagulation deficits are linked to subset 1, the patient P23728 – anything special clinically. Similarly, what is the b2-microglobulin doing here, this would be a good candidate for vesicular BCR ligand (present in vesicles of all class I+ cells), but would also be linked to immune complexes (with shed b2 microglobulin) in patients. The results are curious, but are perhaps all rather low? We really need SPR here. Are any of these subset 1 the same as shown in 3c?
Point 4. IGHV clan I coupled to IGKV1-39/1D-39 is convincing and suggests a model for this obligate pairing.
Point 5. The analysis of self-dimerization (and lack of this in subset 1) is convincing.
Point 6. The lack of Ca2+ in Figure 7 demonstrates that endogenous ligands are not available in this cell model that provides ongoing signaling. Does not CLL subset 1 also have downregulated surface IgM and evidence of ongoing signalling? Is the culpable ligand (besides BCR that obviously is not involved here) not present in this cell type? Is the Figure S5 surface stain or permeabilized cells (I ask as I wonder if any IgM kappa is retained intracellularly and how this compares to subset 2?
The authors should have transfected normal B cells to assess BCR pathway activation (e.g. phosphoflow of Syk and downstream pathway, or Ca2+ flux) and intracellular retention. Could assess lambda positive cells that have been transfected to demonstrate if surface kappa/lambda is equivalent in lambda+ transfectants vs non-subset 1 transfected cells.
Point 7. The results on Supplementary Figure S7 must be moved from the Discussion to the Results
Point 8. As Nat Comm is for a more general reader, the impact of the work should be better presented in the Discussion. The discussion should also provide a better understanding of the field at large.

Overall comment. This is an impressive BCR structure analysis of subset 1 CLL, the autoantigen part is weaker and the results seem to conflict with BCR pathway drug successes in this patient subset. It should be clarified if in subset 1 CLL patient CLL cells have ongoing signaling and this should be presented/discussed. It should be discussed if other antigens other than BCR-BCR can cause similar exogenous antigen-independent signalling. One candidate (according to current results) is b2 microglobulin/class I.

Reviewer #4

(Remarks to the Author)
The authors examine several B cell receptors (BCRs) associated with chronic lymphocytic leukemia (CLL) subset 1 to better understand their signaling mechanism. Unlike what was noted previously for subset 2 and 4 BCRs no conserved interactions are observed between the BCR Fab fragments either in crystalline form or in solution, suggesting that cell independent signaling may not be the mechanism used by subset 1. Three crystal structures are reported for subset 1 Fabs. Validation reports indicate that the crystal structures are well determined and results from the extensive structural analyses are presented in a very clear manner.

I naively would have expected that CLL BCR's utilize rarely used germline genes, but the IgKV1-39 germline gene used in subset 1 is very commonly used in all antibodies in general (~ 40% of light chains in at least one study use 1-39). This might be worth mentioning in the section on the restricted light chain pairing.

Specific comments:
In Fig 1 and the Fig 1 legend, I don't see the glycan on the Cmu1 domain.
In Table S1, please include Rmerge and Rmeas and B values for protein/solvent.
In Table S3 please include the Abangle reference.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the revised manuscript, the authors have addressed most of the questions well. However, I still find it unclear how the monoclonal antibodies were determined to originate from disease-associated malignant clones (original Question 1). I would like this issue to be answered directly.

Reviewer #2

(Remarks to the Author)
Reviewer #2 (Remarks to the Author) Reviewer responses the answers of the authors :

In the Ms of Cocomazzi/Latrou/Minici et al. the U-CLL stereotyped BCR subset 1 is thoroughly investigated on the points of (i) their structure, (ii) antigen binding properties, (iii) self BCR-BCR interaction and (iv) their capacity to induce autonomous BCR signaling, as measured by calcium flux. This Ms is important and provides further insight in the heterogeneity of CLL as a whole group and in the unique structural BCR characteristics of the various stereotyped BCR subsets. Although it is an interesting manuscript, I do have comments, mainly related to the messiness/inconsistencies of the figures and flawed discussion.

We thank the reviewer for the comments, and indeed we strived to present the data in a more linear flow, removing inconsistencies and integrating additional data and discussion, as justly pointed out.

1. In Figure 1A, I suppose that the over-all Fab structure is presented, this however does not become clear from the legend. Please emphasize in the legend that the presented structure is based on representation of the three Fabs. For the light chain and heavy chain the colors pink and light blue are used, for H-CDR1, H-CDR2 and H-CDR3 yellow, green and marine are used. The K-CDR1, K-CDR2 and K-CDR3 are not indicated by color. The authors must use colors that are more clearly distinguishable from each other and also show the CDRs of the kappa. In Figure 1B please indicate above the boxed sequences H-CDR1, H-CDR2 and H-CDR3. The three amino acid sequences (or if they are all the same one) of the IGKV1-39 should also be shown.
Authors' Response to Point #1
We apologize for the lack of clarity in Figure 1. We changed the figure legend to provide more information on the representation of an overall Fab structure, coloured all CDRs differently, and included the IGKV1-39/1D-39 kappa light chain sequence.
Reviewer #2
The colors of the figure are much better, however , the boxes indicating CDR1, CDR2 and CDR3 are gone, please restore.

2. In Figure 3, the antigen binding of recombinant IgG of subset 1 CLL is shown. This is a very messy figure. The order of the IgG antibodies is different between Figure 3A, 3B left and right. The antibodies should be presented in the same order in all

graphs of Figure 3A and 3B to enable the reader to properly judge and compare the binding strength of a particular IgG in the different experiments. For example, the P14197 IgG demonstrated the strongest in all three assays. This also holds for Figure S2; to be able to compare the binding strengths of a specific CLL IgG Ab it is necessary to display the exact reactivities of the various CLL IgGs of the patients. A Figure S2C should be added in which the data is presented in the same manner as in Figure 3B, enabling comparison of the individual binding strengths of the 14 CLL Abs. Now, in Figure S2, Ag binding of the CLL IgGs is shown ordered to the germline IGHV that is expressed by the subset 1 BCRs. This shows that the IgG encoded by the three IGHV gene segments bind similarly polyreactive to the various antigens, although there is a trend of IGHV5 being less polyreactive. This could also be mentioned in the text.
Authors' Response to Point #2
We appreciate the reviewer's comments and suggestions. In response, we have revised the indicated panels (now Figure 4) to ensure that all cases are presented in the same order across all panels, facilitating a clearer comparison of binding strengths.
The individual binding strengths of each subset 1 case are now clearly depicted in Figure 4. Regarding Figure S2 (now Figure S3), its primary objective remains to illustrate the similarity in antigenic reactivity among subset 1 cases utilizing different IGHV genes.
Additionally, as suggested, we have incorporated in the revised Result section a statement regarding the observed trend of rmAbs utilizing IGHV5 genes to be less polyreactive (page 10).
Reviewer #2
I am completely satisfied by the answers and changes.

3. In the main text, the recombinant Abs are abbreviated as (rmAbs). This should be renamed in recombinant IgG in order to make clear that IgG is prepared from the subset 1 IgM BCR. I don't know if IgG always fully displays identical antigen binding activity as compared to IgM. Maybe the authors can comment on this.
Authors' Response to Point #3
We appreciate the reviewer's suggestion. As noted in the Methods section, the term "rmAbs" was used to refer the recombinant IgG monoclonal antibodies throughout the text.
Regarding antigen-binding activity, recombinant monoclonal IgM and IgG antibodies derived from the same BCR source retain identical antigen specificity, as their variable regions remain unchanged. While class switching from IgM to IgG can influence properties such as avidity - given that IgM is typically pentameric - the intrinsic antigen-binding characteristics of the Fab regions are preserved.
That notwithstanding, to address the potential impact of the heavy chain isotype on reactivity raised by the reviewer, we also generated subset 1 recombinant monoclonal antibodies of the IgM isotype and assessed their (auto)antigen reactivity using ELISA and flow cytometry. These experiments revealed consistent reactivity profiles between the recombinant IgG and IgM antibodies, confirming that isotype switching in this context did not affect antigen recognition (page 10). Relevant data are presented in Supplementary Figure S4.
Reviewer #2
In the Methods section it is mentioned that the rmIgG Abs were used in 15 ug/ml. Were the rmIgM Abs used in the same concentrations ? In our hands polyreactive recombinant IgM already plateaus at approximately 2 – 3 ug/ml. If rmIgM was used at the same concentration or used at a different concentration please mention in the Methods section. For the rest I am completely satisfied by the answers and changes.

4. In the legend of Figure 3C it should be mentioned which two CLL IgG Abs were used for the immunohistochemistry. This could also be added in Figure 3C itself.
Authors' Response to Point #4
We appreciate the reviewer's suggestion. The specific CLL IgG antibodies used in the immunohistochemistry experiments are now explicitly mentioned in the legend of the figure (now Figure 4C). Additionally, we incorporated this information directly into the figure to further enhance clarity.
Reviewer #2
I am completely satisfied by the answers and changes.

5. In Figure 4 the protein gene names on the right site of the graph are not legible. This should be corrected. As expected the subset 1 antibodies show a comparable binding profile. Peculiar is the subset 201 P775 IgG, which shows strong binding at the bottom of the graph. Is this real or an artefact? Maybe this IgG is not good and it would better be deleted as it serves no purpose.
Authors' Response to Point #5
We appreciate the reviewer's feedback. In response, we reformatted the antigen list in the figure (now Figure 5) to enhance readability, ensuring that all protein gene names are clearly legible.
Regarding the subset 201 P775 case, we carefully re-examined the data and confirmed that the strong binding signal observed at the bottom of the graph is genuine and not an artefact. However, as this antibody is not central to the main conclusions of the study and may cause confusion, we removed it from the analysis to maintain focus and clarity in the presentation.
Reviewer #2
I am completely satisfied by the answers and changes.

6. Is it not more logical to move the heading "Structural basis for the restricted light chain pairing in subset 1 BCRs" (Page 9, line 195) before the heading "Subset 1 BCR are polyreactive and display intra-subset diversity" (Page 8, line 159).
Authors' Response to Point #6
After reviewing the manuscript, we agree with the reviewer that it is indeed be a more logical positioning, and moved the section as suggested.

Reviewer #2
I am completely satisfied by the answers and changes.

7. It is informative to also show the size exclusion chromatography of the three Fabs P3129, P10015 and P1173 in a new Figure 6A. Now only P3128 is shown in Figure S4A. Figure 6B would then be the sedimentation velocity centrifugation experiment. Maybe in the supplementary Figure S4 the experiment of subset 2 and subset 4 Fabs can be shown from the Nature Communications Ms of 2017, to illustrate the clear difference of the subset 1 Fabs.
Authors' Response to Point #7
We added a new Figure S1 containing the size exclusion chromatography profiles of all BCR Fabs crystallized, together with the calibration curve for the Superdex-200 column used. We also added the SV-AUC profile for a subset 4 BCR in Figure S7, where the SV-AUC data are presented, to provide a visual cue for the formation of dimeric species.
Reviewer #2
I am completely satisfied by the answers and changes.

8. In Figure 7A, P3129 and P10015, of which the structure was determined are shown. However, the P1173, of which the structure was also determined, is not shown. Why not? Was there a problem with the TKO transfection? According to the Duhren-von Minden Nature Ms of 2012 a polyreactive IgM (BCR62) also is able to produce Ag-independent Ca flux. Unfortunately, the most polyreactive IgG P14197 is not shown in the TKO system, while this BCR may induce higher levels of Ca than the other subset 1 BCRs.
Authors' Response to Point #8
We thank the reviewer for these important observations. Unfortunately, we haven't tested the samples P1173 and P14197 in TKO cells for BCR expression and signalling. We would like to clarify that at time of BCR expression and Ca2+ influx experiments we selected two cases of each fully annotated, clan I-derived IGHV genotypes, namely IGHV1-2, IGHV1-3, and IGHV5 (IGHV5-10-1 and IGHV5-51), all paired to IGKV1-39/1D-39 origin kappa light chain sequences. Of relevance, the same IGHD and IGHJ genes, namely IGHD6-19*01 and IGHJ4*02, were utilized in all cases. Later, we added two more cases involving the IGHV1-2 and IGHV1-3 gene segments due to their relatively higher abundance in CLL subset 1 group. Notably, the crystal from sample P1173 that utilized the using IGHV5-10-1*03 gene was similar to that from sample P5092 (highlighted in the supplementary spreadsheet), also using the same IGHV-D-J genes. Furthermore, the study of the BCR from sample P5092 showed evidence for expression and membrane exported to the surface, as well as a good reaction to BCR crosslinking upon anti-Igλ/κ treatment, but didn't show any evidence for autonomous BCR signaling (Figure 7 and S9). Similarly, sample P14197, showing the highest level of polyreactivity, was characterized by the expression of the IGHV (namely, IGHV1-2*02) gene, IGHD and IGHJ genes as in samples P10015 and P3870 (highlighted green in the supplementary spreadsheet), also using the same VDJ recombination. Both P10015 and P3870 reacted well to BCR crosslinking, but failed to induce autonomous Ca2+ influx. Notably, the P10015 BCR was also highly polyreactive in both isotype forms both as (namely, IgG and IgM) (Figure 5 and S4).
The autonomous responsiveness of early immature B cell derived polyreactive BCRs (including BCR62) [DOI: 10.1016/j.immuni.2008.10.013; DOI: 10.1126/science.1086907] and their comparison to CLL #1 BCRs is beyond the scope this MS. We refrain from direct interpretation, as we have no first-hand experimental data on P14197 nor on BCR62. Our current results emphasize the absence of autonomous signaling even among the most polyreactive subset 1 rmAbs tested in our system.
Reviewer #2
It remains very intriguing that the levels (or strengths) of polyreactivity may vary with certain molecules may vary with certain molecules. I understand that this is beyond the current scope, and I am satisfied with the explanations provided.

9. In the discussion at Page 14 Line 329-331, two previous studies on subset 1 antibody reactivities are referred (25 Lanemo, 26 Bergh). However, also the studies of Catera/Chiorazzi, Mol Med.(2008), Hatzi/Chiorazzi, Clin.Imm.(2016), Chu/Chiorazzi, Blood(2010) and Gounari/Ghia, Blood(2015), in which subset 1 reactivities were also studied should be mentioned. In the study of Catera/Chiorazzi (2008), also reactivity with MDA-BSA was shown as a target for subset 1 Abs, however MDA-BSA was not recognized in the Gounari/Ghia(2015) study. These older studies should also be referred and be discussed.
Authors' Response to Point #9
The reviewer is right, and we apologize for the omission. We added a section summarizing and referencing the previous work on subset 1 reactivity (pages 16 and 19) and discuss this in the light of our new results.
Reviewer #2
I am completely satisfied by the answers and changes.

10. The authors claim that BCR-BCR interaction may not represent a ubiquitous mechanism for BCR-mediated signaling in all CLL. This is now is certain as in the Ms it is shown that subset 1 BCRs do not induce calcium influx. This should be changed in 'BCR-BCR interaction is a mechanism for BCR signaling in subsets of CLL'. It is clear that subset 2, 4 and 169 show BCR-BCR interaction, but I presume also non-BCR-subset VL3-21 R110 CLLs, which is a large subset representing 8 – 10 % of all CLLs, show BCR-BCR interaction. In the Duren-von Minden Nature 2012 Ms 17 CLL were tested including one subset 2, one subset 7E2 and 7C4 and now also a subset 99 CLL (UM-CLL4). The authors should check, which subsets were analyzed in this Ms and add this information in the discussion. Altogether, still a large group of CLL undergo BCR signaling due to homotypic BCR-BCR interaction. Subset 1 IgGs show polyreactive binding to various antigens as shown in the ELISAs (Figure 3) and antigen array (Figure 4). In the Duren-von Minden Nature 2012 Ms, also a polyreactive non-CLL BCR (BCR62) shows Ca flux in the TKO, indicating that strong polyreactive BCRs also possess BCR-BCR interaction (maybe not by the VH-CDR3 VRQ-FR2 interaction). Strong polyreactive CLLs are subset 8, 9 (9A, 9B and 9C) and 28 (Catera/Chiorazzi, Mol Med. 2008), subset 7 (7C2 and 7A3) (Hatzi/Chiorazzi, Clin.Imm. 2016) and (again) subset 8 (Gounari/Ghia, Blood 2015). There are also CLL subsets, which are do not show polyreactivity, such as the IGHV mutated

subset 111 (Figure 4), the mutated subset 13 (Kostareli/Stamatopoulos, Leukemia 2012) with rheumatoid factor BCR specificity and other CLL subsets with rheumatoid factor BCR specificity (Hoogeboom/Noesel, Leukemia 2013) and a subset with fungi specificity (Hoogeboom/Noesel, Journal Experimental Med. 2013).
It should be discussed and referenced that (the combined data suggest) we have (i) CLLs with BCR-BCR interaction or likely also strongly polyreactive BCR (such as subset 8), which show autonomous Ca flux, (ii) subsets of moderatly polyreactive CLLs (like subset 1), which do not show autonomous Ca flux but do experience chronic BCR stimulation by the microenvironment due to their polyreactivity and (iii) subsets of CLLs, which have a very specific antigen that they recognize and very likely also do not show autonomous Ca flux, such as subset 111, the rheumatoid factor and the fungi-specific CLL subsets.
Authors' Response to Point #10
We sincerely thank the reviewer for this comment, that prompted us to include a paragraph at the end of the Discussion that summarizes the previously published data, and puts our results on subset 1 into perspective for the general readership (page 19).
Reviewer #2
I am completely satisfied by the answers and changes.

Minor comments

1. In the Results section, it is stated that of 11 subset 1 CLL Fab fragments were produced. Yet of three Fabs the crystal structures were made and analyzed. Figure 3 displays 14 recombinantly produced IgGs of subset 1. A table should be provided depicting all 14 subset 1 cases with their IGHV / IGKV rearrangements and their stereotyped VH-CDR3 amino acid sequences. In this table, the three CLLs of which crystal structures were analyzed should be indicated as well as the 11 CLLs of which Fab and the 14 CLLs of which IgG was produced.
Author's response
We prepared the Table as requested, and incorporated it into the supplementary Material

2. The order of headings in the results section should at least largely correspond with the order in the method section. As an example the results section starts with the expression of Fab fragments (page 6, line 108), whereas in the methods the production of Fab fragments is at the 7th heading (page 20, line 467).
Author's response
We changed the order of the paragraphs in the methods section to better reflect the flow of the results in the manuscript.

3. In the text on page 10 line 219, the authors mention their previous work without a reference, the reference should be provided.
Author's response
The appropriate reference was added.

4. In the Discussion (Page 15, Line 362), UM-CLL4 is mentioned to expresses IGHV1-2. It actually expresses IGHV1-3 as shown in Figure S7 and in the original Duhren-von-Minden Nature 2012 Ms. This must be corrected.
Author's response
We apologize for the oversight; we corrected the IGHV gene of the UM-CLL4 accordingly.

5. A reference should be provided for the statement that MDA-reactive Abs show a high degree of cross-reactivity.
Author's response
We added a reference to the work of Grönwall et al showing that IgGs derived from RA patients reacted with BSA, HSA, histones or vimentin when these proteins were modified through reaction with MDA. We also rephrased the relevant part of the text to better convey our message, that protein-MDA-reactive antibodies may recognize only a small modification irrespective of the protein scaffold, and thus could be accommodated in the subset 1 BCR combining site cavity (page 17).

Reviewer #2
I am completely satisfied by the answers and changes of the Minor Comments.

Reviewer #3

(Remarks to the Author)
I thank the authors for a thorough and comprehensive revision of the manuscript and for their experimental effort to identify an CLL-endogenous antigen X that could cause X-BCR signalling in CLL cells (as a parallel to BCR-BCR). Although unsuccessful in that particular regard (e.g. B2-microglobulin-BCR signalling), the current paper as it stands is an important step forwards in the field. My recommendation is that the paper is appropriate for publication in NatComm, with high priority.

Ludvig Munthe

Reviewer #4

(Remarks to the Author)

Thanks to the authors for successfully addressing my questions and concerns.

We thank all reviewers for their time and thorough review of our manuscript. We carefully considered every point raised and performed experiments to further corroborate our conclusions, and edited the text to make it more accessible to non-specialised readers. Are, we provide a point-by-point response, with the reviewers' comments shown in italic, followed by a description of the action taken to address the issues raised. We hope the revised manuscript is appreciated and found suitable for publication.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript provides a thorough analysis of the structure of three common subset 1 BCR Fabs in chronic lymphocytic leukemia (CLL). Through the preparation of monoclonal antibodies and the assessment of autoantigen recognition, the study demonstrates that activation signals in these tumor B cells are neither autonomous nor involve self-association, distinguishing them from other subsets (e.g., 2, 4, and 169). Additionally, the absence of Ca^{2+} influx activity in B cell lines expressing subset 1 BCRs suggests that alternative signaling pathways may contribute to BCR-mediated progression in CLL.
Overall, the manuscript is well-written, with rigorous and well-executed experimental approaches. However, several unresolved issues remain that may limit its overall impact.

We thank the reviewer for assessing in depth our manuscript. We addressed all the issues raised, and we include new data and a revised discussion to improve the impact of the manuscript.

Reviewer Point #1
1. The authors generated recombinant monoclonal antibodies from CLL patients but did not specify how these malignant clones were identified, nor did they provide details regarding the B cell/BCR type from which these malignant clones originated.
Authors' Response to Point #1
We appreciate the reviewer's request for additional clarity. We have now included a detailed description of how the malignant clones were identified, including BcR sequencing, and analysis of the clonotypic IGHV-IGHD-IGHJ and IGKV-IGKJ gene rearrangements. These details have been incorporated into the revised Methods section (pages 22-23) to enhance transparency and reproducibility.

2. Although the authors expressed and purified BCR Fabs and achieved crystallization, they did not present data from protein purity assessments during the purification process, limiting our ability to assess the level of protein purity achieved.

Authors' Response to Point #2

We apologize for this omission. The revised Results and Methods sections further underscore that the purified Fabs elute as a single peak from size exclusion chromatography, and display a unimodal size distribution in the SV-AUC experiments (page 20). Moreover, we added a Coomassie-stained SDS-PAGE, under both reducing and non-reducing conditions, showing near-homogeneity of the final samples, together with the gel-filtration chromatography elution profiles consistent with high sample purity (Figure S1).

3. The subset 1 CLL BCRs demonstrated recognition of multiple autoantigens with restricted light chain usage, which is characteristic of CD5⁺ B cells. Given that B1 cells typically display low affinity for antigen binding—a factor that can significantly impact signal transduction—this aspect warrants further analysis and discussion in the context of the results.

Authors' Response to Point #3

The reviewer zeroed in an extremely important point that was overlooked in our initial submission. We expanded the relevant section of the Discussion, further underscoring the implications of low-affinity antigen recognition with respect to normal B-cell responses and within haematological malignancies, as was also suggested in our previous study (pages 18-19).

4. The IGKV1-39/1D-39 light chain used in subset 1 CLL is among the most frequently utilized genes in human kappa chains. The authors attribute this specific usage to optimal interactions with the heavy chain; however, this claim lacks direct supportive data within the results.

Authors' Response to Point #4

We understand the reviewer's concern on this matter. Our suggestion for the selective IG chain pairing in subset 1 is based on the analysis of the crystal structures that allow the identification of specific residues in the light chain crucial to ensure chemical and steric complementarity. We understand the limitations of this analysis, and now we also present the results of an *in silico* analysis to study the effect of interface amino acid mutations on the chain pairing. Briefly, we calculated the effect of substitutions at the interface residues using the program FoldX. Our results clearly show that mutation of the IGKV1-39/1D-39-specific amino acids to either alanine or other residues found at these positions in other IGKV genes have a destabilizing effect on the VH-VL pairing (pages 8-9). We feel that these findings further sustain the view that the observed gene usage, both in the IGHV and IGKV and the CDRs arising from the V(D)J recombination, strongly correlates with BCR stability. However, we also acknowledge that alternative factors may contribute to the restricted chain usage, and hence we added a statement regarding the limitations of the analysis and discuss an alternative explanation, specifically the role of these specific genes in creating a specific antigen binding site (page 18).

Reviewer #2 (Remarks to the Author):

In the Ms of Cocomazzi/Latrou/Minici et al. the U-CLL stereotyped BCR subset 1 is thoroughly investigated on the points of (i) their structure, (ii) antigen binding properties, (iii) self BCR-BCR interaction and (iv) their capacity to induce autonomous BCR signaling, as measured by calcium flux. This Ms is important and provides further insight in the heterogeneity of CLL as a whole group and in the unique structural BCR characteristics of the various stereotyped BCR subsets. Although it is an interesting manuscript, I do have comments, mainly related to the messiness/inconsistencies of the figures and flawed discussion.

We thank the reviewer for the comments, and indeed we strived to present the data in a more linear flow, removing inconsistencies and integrating additional data and discussion, as justly pointed out.

1. In Figure 1A, I suppose that the over-all Fab structure is presented, this however does not become clear from the legend. Please emphasize in the legend that the presented structure is based on representation of the three Fabs. For the light chain and heavy chain the colors pink and light blue are used, for H-CDR1, H-CDR2 and H-CDR3 yellow, green and marine are used. The K-CDR1, K-CDR2 and K-CDR3 are not indicated by color. The authors must use colors that are more clearly distinguishable from each other and also show the CDRs of the kappa. In Figure 1B please indicate above the boxed sequences H-CDR1, H-CDR2 and H-CDR3. The three amino acid sequences (or if they are all the same one) of the IGKV1-39 should also be shown.

Authors' Response to Point #1

We apologize for the lack of clarity in Figure 1. We changed the figure legend to provide more information on the representation of an overall Fab structure, coloured all CDRs differently, and included the IGKV1-39/1D-39 kappa light chain sequence.

2. In Figure 3, the antigen binding of recombinant IgG of subset 1 CLL is shown. This is a very messy figure. The order of the IgG antibodies is different between Figure 3A, 3B left and right. The antibodies should be presented in the same order in all graphs of Figure 3A and 3B to enable the reader to properly judge and compare the binding strength of a particular IgG in the different experiments. For example, the P14197 IgG demonstrated the strongest in all three assays. This also holds for Figure S2; to be able to compare the binding strengths of a specific CLL IgG Ab it is necessary to display the exact reactivities of the various CLL IgGs of the patients. A Figure S2C should be added in which the data is presented in the same manner as in Figure 3B, enabling comparison of the individual binding strengths of the 14 CLL Abs. Now, in Figure S2, Ag binding of the CLL IgGs is shown ordered to the germline IGHV that is expressed by the subset 1 BCRs. This shows that the IgG encoded by the three IGHV gene segments bind similarly polyreactive to the various antigens, although there is a trend of IGHV5 being less polyreactive. This could also be mentioned in the text.

Authors' Response to Point #2

We appreciate the reviewer's comments and suggestions. In response, we have revised the indicated panels (now Figure 4) to ensure that all cases are presented in the same order across all panels, facilitating a clearer comparison of binding strengths.

The individual binding strengths of each subset 1 case are now clearly depicted in Figure 4. Regarding Figure S2 (now Figure S3), its primary objective remains to illustrate the similarity in antigenic reactivity among subset 1 cases utilizing different IGHV genes.

Additionally, as suggested, we have incorporated in the revised Result section a statement regarding the observed trend of rmAbs utilizing IGHV5 genes to be less polyreactive (page 10).

3. In the main text, the recombinant Abs are abbreviated as (rmAbs). This should be renamed in recombinant IgG in order to make clear that IgG is prepared from the subset 1 IgM BCR. I don't know if IgG always fully displays identical antigen binding activity as compared to IgM. Maybe the authors can comment on this.

Authors' Response to Point #3

We appreciate the reviewer's suggestion. As noted in the Methods section, the term "rmAbs" was used to refer the recombinant IgG monoclonal antibodies throughout the text.

Regarding antigen-binding activity, recombinant monoclonal IgM and IgG antibodies derived from the same BCR source retain identical antigen specificity, as their variable regions remain unchanged. While class switching from IgM to IgG can influence properties such as avidity - given that IgM is typically pentameric - the intrinsic antigen-binding characteristics of the Fab regions are preserved.

That notwithstanding, to address the potential impact of the heavy chain isotype on reactivity raised by the reviewer, we also generated subset 1 recombinant monoclonal antibodies of the IgM isotype and assessed their (auto)antigen reactivity using ELISA and flow cytometry. These experiments revealed consistent reactivity profiles between the recombinant IgG and IgM antibodies, confirming that isotype switching in this context did not affect antigen recognition (page 10). Relevant data are presented in Supplementary Figure S4.

4. In the legend of Figure 3C it should be mentioned which two CLL IgG Abs were used for the immunohistochemistry. This could also be added in Figure 3C itself.

Authors' Response to Point #4

We appreciate the reviewer's suggestion. The specific CLL IgG antibodies used in the immunohistochemistry experiments are now explicitly mentioned in the legend of the figure (now Figure 4C). Additionally, we incorporated this information directly into the figure to further enhance clarity.

5. In Figure 4 the protein gene names on the right site of the graph are not legible. This should be corrected. As expected the subset 1 antibodies show a comparable binding profile. Peculiar is the subset 201 P775 IgG, which shows strong binding at the bottom of the graph. Is this real or an artefact? Maybe this IgG is not good and it would better be deleted as it serves no purpose.

Authors' Response to Point #5

We appreciate the reviewer's feedback. In response, we reformatted the antigen list in the figure (now Figure 5) to enhance readability, ensuring that all protein gene names are clearly legible.

Regarding the subset 201 P775 case, we carefully re-examined the data and confirmed that the strong binding signal observed at the bottom of the graph is genuine and not an artefact. However, as this antibody is not central to the main conclusions of the study and may cause confusion, we removed it from the analysis to maintain focus and clarity in the presentation.

6. Is it not more logical to move the heading "Structural basis for the restricted light chain pairing in subset 1 BCRs" (Page 9, line 195) before the heading "Subset 1 BCR are polyreactive and display intra-subset diversity" (Page 8, line 159).

Authors' Response to Point #6

After reviewing the manuscript, we agree with the reviewer that it is indeed be a more logical positioning, and moved the section as suggested.

7. It is informative to also show the size exclusion chromatography of the three Fabs P3129, P10015 and P1173 in a new Figure 6A. Now only P3128 is shown in Figure S4A. Figure 6B would then be the sedimentation velocity centrifugation experiment. Maybe in the supplementary Figure S4 the experiment of subset 2 and subset 4 Fabs can be shown from the Nature Communications Ms of 2017, to illustrate the clear difference of the subset 1 Fabs.

Authors' Response to Point #7

We added a new Figure S1 containing the size exclusion chromatography profiles of all BCR Fabs crystallized, together with the calibration curve for the Superdex-200 column used. We also added the SV-AUC profile for a subset 4 BCR in Figure S7, where the SV-AUC data are presented, to provide a visual cue for the formation of dimeric species.

8. In Figure 7A, P3129 and P10015, of which the structure was determined are shown. However, the P1173, of which the structure was also determined, is not shown. Why not? Was there a problem with the TKO transfection? According to the Duhren-von Minden Nature Ms of 2012 a polyreactive IgM (BCR62) also is able to produce Ag-independent Ca flux. Unfortunately, the most polyreactive IgG P14197 is not shown in the TKO system, while this BCR may induce higher levels of Ca than the other subset 1 BCRs.

Authors' Response to Point #8

We thank the reviewer for these important observations. Unfortunately, we haven't tested the samples P1173 and P14197 in TKO cells for BCR expression and signalling. We would like to clarify that at time of BCR expression and Ca^{2+} influx experiments we selected two cases of each fully annotated, clan I-derived IGHV genotypes, namely IGHV1-2, IGHV1-3, and IGHV5 (IGHV5-10-1 and IGHV5-51), all paired to IGKV1-39/1D-39 origin kappa light chain sequences. Of relevance, the same IGHD and IGHJ genes, namely IGHD6-19*01 and IGHJ4*02, were utilized in all cases. Later, we added two more cases involving the IGHV1-2 and IGHV1-3 gene segments due to their relatively higher abundance in CLL subset 1 group. Notably, the crystal from sample P1173 that utilized the using IGHV5-10-1*03 gene was similar to that from sample P5092 (highlighted in the supplementary spreadsheet), also using the same IGHV-D-J genes. Furthermore, the study of the BCR from sample P5092 showed evidence for expression and membrane exported to the surface, as well as a good reaction to BCR crosslinking upon anti-Igλ/κ treatment, but didn't show any evidence for autonomous BCR signaling (Figure 7 and S9).

Similarly, sample P14197, showing the highest level of polyreactivity, was characterized by the expression of the IGHV (namely, IGHV1-2*02) gene, IGHD and IGHJ genes as in samples P10015 and P3870 (highlighted green in the supplementary spreadsheet), also using the same VDJ recombination. Both P10015 and P3870 reacted well to BCR crosslinking, but failed to induce autonomous Ca^{2+} influx. Notably, the P10015 BCR was also highly polyreactive in both isotype forms both as (namely, IgG and IgM) (Figure 5 and S4).

The autonomous responsiveness of early immature B cell derived polyreactive BCRs (including BCR62) [DOI: 10.1016/j.immuni.2008.10.013; DOI: 10.1126/science.1086907] and their comparison to CLL #1 BCRs is beyond the scope this MS. We refrain from direct interpretation, as we have no first-hand experimental data on P14197 nor on BCR62. Our current results emphasize the absence of autonomous signaling even among the most polyreactive subset 1 rmAbs tested in our system.

9. In the discussion at Page 14 Line 329-331, two previous studies on subset 1 antibody reactivities are referred (25 Lanemo, 26 Bergh). However, also the studies of Catera/Chiorazzi, Mol Med.(2008), Hatzi/Chiorazzi, Clin.Imm.(2016), Chu/Chiorazzi, Blood(2010) and Gounari/Ghia, Blood(2015), in which subset 1 reactivities were also studied should be mentioned. In the study of Catera/Chiorazzi (2008), also reactivity with MDA-BSA was shown as a target for subset 1 Abs, however MDA-BSA was not recognized in the Gounari/Ghia(2015) study. These older studies should also be referred and be discussed.

Authors' Response to Point #8

The reviewer is right, and we apologize for the omission. We added a section summarizing and referencing the previous work on subset 1 reactivity (pages 16 and 19) and discuss this in the light of our new results.

10. The authors claim that BCR-BCR interaction may not represent a ubiquitous mechanism for BCR-mediated signaling in all CLL. This is now is certain as in the Ms it is shown that subset 1 BCRs do not induce calcium influx. This should be changed in 'BCR-BCR interaction is a mechanism for BCR signaling in subsets of CLL'. It is clear that subset 2, 4 and 169 show BCR-BCR interaction, but I presume also non-BCR-subset VL3-21 R110 CLLs, which is a large subset representing 8 – 10 % of all CLLs, show BCR-BCR interaction. In the Duren-von Minden Nature 2012 Ms 17 CLL were tested including one subset 2, one subset 7E2 and 7C4 and now also a subset 99 CLL (UM-CLL4). The authors should check, which subsets were analyzed in this Ms and add this information in the discussion. Altogether, still a large group of CLL undergo BCR signaling due to homotypic BCR-BCR interaction. Subset 1 IgGs show polyreactive binding to various antigens as shown in the ELISAs (Figure 3) and antigen array (Figure 4). In the Duren-von Minden Nature 2012 Ms, also a polyreactive non-CLL BCR (BCR62) shows Ca flux in the TKO, indicating that strong polyreactive BCRs also possess BCR-BCR interaction (maybe not by the VH-CDR3 VRQ-FR2 interaction). Strong polyreactive CLLs are subset 8, 9 (9A, 9B and 9C) and 28 (Catera/Chiorazzi, Mol Med. 2008), subset 7 (7C2 and 7A3) (Hatzi/Chiorazzi, Clin.Imm. 2016) and (again) subset 8 (Gounari/Ghia, Blood 2015). There are also CLL subsets, which are do not show polyreactivity, such as the IGHV mutated subset 111 (Figure 4), the mutated subset 13 (Kostareli/Stamatopoulos, Leukemia 2012) with rheumatoid factor BCR specificity and other CLL subsets with rheumatoid factor BCR specificity (Hoogeboom/Noesel, Leukemia 2013) and a subset with fungi specificity (Hoogeboom/Noesel, Journal Experimental Med. 2013).

It should be discussed and referenced that (the combined data suggest) we have (i) CLLs with BCR-BCR interaction or likely also strongly polyreactive BCR (such as subset 8), which show autonomous Ca flux, (ii) subsets of moderatly polyreactive CLLs (like subset 1), which do not show autonomous Ca flux but do experience chronic BCR stimulation by the microenvironment due to their polyreactivity and (iii) subsets of CLLs, which have a very specific antigen that they recognize and very likely also do not show autonomous Ca flux, such as subset 111, the rheumatoid factor and the fungi-specific CLL subsets.

Authors' Response to Point #10

We sincerely thank the reviewer for this comment, that prompted us to include a paragraph at the end of the Discussion that summarizes the previously published data, and puts our results on subset 1 into perspective for the general readership (page 19).

Minor comments

1. In the Results section, it is stated that of 11 subset 1 CLL Fab fragments were produced. Yet of three Fabs the crystal structures were made and analyzed. Figure 3 displays 14 recombinantly produced IgGs of subset 1. A table should be provided depicting all 14 subset 1 cases with their IGHV / IGKV rearrangements and their

stereotyped VH-CDR3 amino acid sequences. In this table, the three CLLs of which crystal structures were analyzed should be indicated as well as the 11 CLLs of which Fab and the 14 CLLs of which IgG was produced.

Author's response

We prepared the Table as requested, and incorporated it into the supplementary Material

2. The order of headings in the results section should at least largely correspond with the order in the method section. As an example the results section starts with the expression of Fab fragments (page 6, line 108), whereas in the methods the production of Fab fragments is at the 7th heading (page 20, line 467).

Author's response

We changed the order of the paragraphs in the methods section to better reflect the flow of the results in the manuscript.

3. In the text on page 10 line 219, the authors mention their previous work without a reference, the reference should be provided.

Author's response

The appropriate reference was added.

4. In the Discussion (Page 15, Line 362), UM-CLL4 is mentioned to expresses IGHV1-2. It actually expresses IGHV1-3 as shown in Figure S7 and in the original Duhren-von-Minden Nature 2012 Ms. This must be corrected.

Author's response

We apologize for the oversight; we corrected the IGHV gene of the UM-CLL4 accordingly.

5. A reference should be provided for the statement that MDA-reactive Abs show a high degree of cross-reactivity.

Author's response

We added a reference to the work of *Grönwall et al* showing that IgGs derived from RA patients reacted with BSA, HSA, histones or vimentin when these proteins were modified through reaction with MDA. We also rephrased the relevant part of the text to better convey our message, that protein-MDA-reactive antibodies may recognize only a small modification irrespective of the protein scaffold, and thus could be accommodated in the subset 1 BCR combining site cavity (page 17).

Reviewer #3 (Remarks to the Author):

The study by Cocomazzi, Iatrou, Minici et al. focuses on the structural and functional characteristics of subset 1 Chronic Lymphocytic Leukemia (CLL) BCRs (5% of cases with unmutated IGHV genes (U-CLL)), revealing that despite their structural conservation, there are significant differences in amino acid sequence that affect antigenic specificity. Using X-ray diffraction, the team examined crystallized IgM BCR Fab fragments from CLL patients, noting similar structural frameworks but with variations in elbow angles and antigen-binding sites, reflecting diverse antigenic reactivities within the subset. These structural variations are paralleled by unique biochemical features at the combining sites of the BCRs, such as differences in electrostatic potential and hydrophobicity, which correlate with their distinct ligand binding profiles demonstrated in biochemical assays. The study further explores the intramolecular interactions and structural basis for the selective pairing of heavy and light chains, pointing out the role of specific amino acids in these interactions. Additionally, unlike other CLL subsets, subset 1 BCRs do not form stable homotypic complexes or activate cell-autonomous signaling (in a B cell model line), suggesting alternative mechanisms of activation and interaction with antigens.

P*oint 1. The difference between subset 1 and the rest (as described in the paper) is the lack of homotypic BCR-BCR interaction and propensity for ongoing BCR pathway activation. Btk-inhibitors are not only relevant for non-subset 1, but also for subset 1 patients with poor prognosis. It is not improbable that CLL B cells have secretory pathway antigens that deliver a BCR-BTK signal. The stainings of Fig 3c and particularly c and d should be shown with high magnification to discrimate the intracellular structures that stain in some examples. Is this vesicular/membrane stain or cytoskeletal?*

Authors' Response to Point #1

The point we wish to convey is that BCR signalling is also important in subset 1: indeed, Ibrutinib shows clinical efficacy in the respective patients. Hence, our finding that subset 1 BCRs do not signal autonomously implies that other antigens, perhaps from the microenvironment, are relevant for CLL cell activation and thus disease progression. In this regard, we also discuss possible antigens based on the combining site structure. That notwithstanding, we agree with the reviewer that the original text could have caused confusion, and we rephrased it to improve clarity (page 16).

In addition, we now provide a higher magnification of the photos of the immunohistochemistry studies as requested. The limited resolution of the technique does not allow for detailed subcellular localization, but the staining appears to be localized at the membrane, thus indicative of extracellular or membrane-associated antigens being recognized.

Point 2. Please note which Figure 3b) rec mAbs are shown on sections in the 3C. How is the Elisa/cytostain match of dsDNA (Elisa) with nuclear stain, and actin (with cytoskeletal)?

Authors' Response to Point #2

We have now specified the CLL subset 1 case depicted in the legend of Figure 4 (numbering has changed due to the changed order of the sections in the manuscript).

Regarding reactivity against autoantigens, this was assessed using an in-house indirect ELISA. Human G-actin was prepared as described following the method described previously by *Dighiero G, et al.* (*The Journal of Immunology*. 1985), and dsDNA was obtained from Sigma-Aldrich (Munich, Germany).

Point 3. Figure 4, the columns are poorly described and the antigen list is not easy to read. It would have been interesting to see positive control SLE sera or mAbs to relate the affinities to what is reported. The authors should show SPR or similar to see the particular curves (on/off rates) of hits that can be assessed in a more relevant manner. It is curious that Fibrinogen IV ("cryo") turns up on this list, what is this protein (or protein mix). I don't think any coagulation deficits are linked to subset 1, the patient P23728 – anything special clinically. Similarly, what is the b2-microglobulin doing here, this would be a good candidate for vesicular BCR ligand (present in vesicles of all class I+ cells), but would also be linked to immune complexes (with shed b2 microglobulin) in patients. The results are curious, but are perhaps all rather low? We really need SPR here. Are any of these subset 1 the same as shown in 3c?

Authors' Response to Point #3

We thank the reviewer for raising this point. First, we wish to underline the fact that the antigen reactivity experiments were performed to ascertain whether subset 1 BCRs showed a distinct pattern of reactivity compared to other CLL subsets. To convey this point in a more effective way, we edited the relevant paragraph to clarify the purpose of the experiments described. Nevertheless, we took to heart this reviewer's comment and performed several experiments to further characterize the nature of the observed reactivity of subset 1 BCR IgGs in the antigen microarrays. To this end, we initiated a collaboration with our colleague Dr. Luca Broggini at the University of Milan to perform biolayer interferometry (BLI) experiments to measure the affinity and kinetic parameters of the IgG-putative antigen interactions. First, we immobilized on the biosensor surface two purified subset 1 IgGs (P14197 and P3870, both also present in the microarray experiment in Figure 5) and used human recombinant beta 2-microglobulin as a ligand at concentrations up to 42 µM. No binding was detected in these experiments. We then repeated the BLI measurements using bovine fibrinogen as an antigen (as found in the microarray), but these experiments were hampered by a strong non-specific interaction of this ligand with the biosensor. Thus, we resorted to mass photometry to demonstrate, at least qualitatively, the binding of fibrinogen to subset 1 BCRs, but no signs of association were detected.

We reasoned that the reactivity observed in the microarray could be due to the presence of both folded and denatured proteins. Thus, we performed a western blot analysis loading fibrinogen on the SDS-PAGE using subset 1 BCR IgGs as primary antibody. Both subset 1 IgGs showed strong reactivity with fibrinogen comparable to the positive control (anti-fibrinogen antibody), while a non-subset 1 BCR did not yield a measurable signal.

Thus, our experiments positively confirmed the reactivity seen in the microarrays, however also demonstrate that subset 1 BCRs bound only to the denatured antigens. We feel that it would be unreasonable to further speculate on the biological relevance of the findings (that may stretch to wondering whether denatured self-antigens may be able to activate the neoplastic cells), and limit the interpretation of the data towards the specific (and weak, as noted by the reviewer) reactivity pattern of subset 1 rmAbs compared to other CLL-derived receptors. We added a thorough description of the methods used, presented the results on page 11 and included the relevant images as Supplementary Figure S5.

Point 4. IGHV clan I coupled to IGKV1-39/1D-39 is convincing and suggests a model for this obligate pairing.

Authors' Response to Point #4

We thank the reviewer for appreciating our modelling effort, which is now further corroborated with an in silico analysis of the energies involved in the intermolecular interaction between the heavy and light chains (page 9).

Point 5. The analysis of self-dimerization (and lack of this in subset 1) is convincing.

Authors' Response to Point #5

We thank the reviewer for this comment.

Point 6. The lack of Ca2+ in Figure 7 demonstrates that endogenous ligands are not available in this cell model that provides ongoing signaling. Does not CLL subset 1 also have downregulated surface IgM and evidence of ongoing signalling? Is the culpable ligand (besides BCR that obviously is not involved here) not present in this cell type? Is the Figure S5 surface stain or permeabilized cells (I ask as I wonder if any IgM kappa is retained intracellularly and how this compares to subset 2?

The authors should have transfected normal B cells to assess BCR pathway activation (e.g. phosphoflow of Syk and downstream pathway, or Ca2+ flux) and intracellular retention. Could assess lambda positive cells that have been transfected to demonstrate if surface kappa/lambda is equivalent in lambda+ transfectants vs non-subset 1 transfected cells.

Authors' Response to Point #6

We thank the reviewer for these insightful suggestions. Regarding former Figure S5 (now S8), we confirm that the analysis represents surface staining only (non-permeabilsed conditions), performed to selectively

evaluate BCR surface expression across the different transfectants. To closely look at the BCR expression level, we analysed the mean fluorescence intensity (MFI) of μ heavy chain (IgM) and λ/κ light chains across multiple subset 1 BCRs, comparing them to healthy donor (HD)-derived IgM.κ and subset 2-derived IgM.λ BCRs (Figure S8 panels B and C). As the reviewer suggested, all subset 1 constructs exhibit intermediate surface IgM expression, lying between the higher levels observed for HD BCRs and the lower levels of subset 2, respectively. We agree that exclusive surface staining data may partly limit insight into potential intracellular retention of IgM. Moreover, it would be speculative at this stage to attribute the observed intermediate expression to either lack of culpable ligands or ongoing signalling in the TKO cells. We fully agree with the reviewer that the source of chronic BCR engagement in subset 1 CLL may reside in exogenous microenvironmental ligands not recapitulated in the TKO model.

We also attempted expression of subset 1 BCRs in transformed human B-cell lines such as CL-01 (Novus Biologicals; now discontinued), which express endogenous BCR isotypes. However, these efforts were unsuccessful in our hand. In parallel, deletion of endogenous BCRs substantially impaired cell viability and transduction efficiency, further limiting our ability to use normal B cells for functional expression studies. Therefore, we refrained from expressing the subset 1 BCRs in normal cells.

We acknowledge the reviewer's suggestion to further assess intracellular retention and pathway activation by performing phosphoflow or Ca^{2+} flux assays in a more physiological B-cell background. However, these experiments go beyond the scope of the current study, which was focused on structure-function relationships using a standardized and comparable model. Nevertheless, we now explicitly discuss this limitation in the revised Discussion (page 16), highlighting the need for future studies to address how subset 1 BCRs may signal in a native B-cell environment, and whether antigen availability or intracellular trafficking differences could influence their signalling behaviour.

Point 7. The results on Supplementary Figure S7 must be moved from the Discussion to the Results

Authors' Response to Point #7

We moved the part of the manuscript concerning the comparative modelling results between subset 1 and subset 99 BCRs to the Results section (page 14).

Point 8. As Nat Comm is for a more general reader, the impact of the work should be better presented in the Discussion. The discussion should also provide a better understanding of the field at large.

Authors' Response to Point #8

We agree with the reviewer that our message should be appreciated by the general audience. Therefore, we edited the Discussion to better position the work presented in the manuscript in the context of BCR signalling in CLL. We also heavily edited the final paragraph (page 19) to summarize the advancement in the field brought forth by the study, and to provide a clearer perspective on the overall relevance.

Overall comment. This is an impressive BCR structure analysis of subset 1 CLL, the autoantigen part is weaker and the results seem to conflict with BCR pathway drug successes in this patient subset. It should be clarified if in subset 1 CLL patient CLL cells have ongoing signaling and this should be presented/discussed. It should be discussed if other antigens other than BCR-BCR can cause similar exogenous antigen-independent signalling. One candidate (according to current results) is b2 microglobulin/class I.

We thank the reviewer for the generous comment, and we feel that the responses to all the points and issues raised resulted in a stronger and accessible manuscript.

Reviewer #4 (Remarks to the Author):

The authors examine several B cell receptors (BCRs) associated with chronic lymphocytic leukemia (CLL) subset 1 to better understand their signaling mechanism. Unlike what was noted previously for subset 2 and 4 BCRs no conserved interactions are observed between the BCR Fab fragments either in crystalline form or in solution, suggesting that cell independent signaling may not be the mechanism used by subset 1. Three crystal structures are reported for subset 1 Fabs. Validation reports indicate that the crystal structures are well determined and results from the extensive structural analyses are presented in a very clear manner.

Author's response

We thank the reviewer for appreciating our work and presentation of the results. The changes and additions we introduced in the manuscript provide some further insights in the structure-function relationship in this group of BCRs from CLL cases .

I naively would have expected that CLL BCR's utilize rarely used germline genes, but the IgKV1-39 germline gene used in subset 1 is very commonly used in all antibodies in general (~ 40% of light chains in at least one study use 1-39). This might be worth mentioning in the section on the restricted light chain pairing.

Authors' Response to Point #1

We thank the reviewer for appreciating our experimental effort. The point raised is well taken, and additional information was added on non-stochastic IG chain pairing in CLL (pages 8-9).

Specific comments:

In Fig 1 and the Fig 1 legend, I don't see the glycan on the Cmu1 domain.

Author's response

The reviewer is right, the sticks were not displayed in the original figure. This has been corrected, and the NAG(FUC)-NAG trisaccharide observed in the electron density map for the P10015 BCR Fab (shown in the figure) is now included in the representation.

In Table S1, please include Rmerge and Rmeas and B values for protein/solvent.

Author's response

The values for Rmerge and Rmeas, and the average B values for protein and solvent atoms were added to Table S1.

In Table S3 please include the Abangle reference.

Author's response

The reference has been added, we apologise for the omitting this in the first version of the manuscript.

Point by point response to reviewers' comments

We thank all reviewers for their enthusiastic comments on our improved manuscript, and we are happy they all feel it is suitable for publication with *Nature Communications*. All reviewers declared their satisfaction with the changes and additions to the manuscript.
Here we provide a response to the remaining comments.

Reviewer 1.

Reviewer's comment. *In the revised manuscript, the authors have addressed most of the questions well. However, I still find it unclear how the monoclonal antibodies were determined to originate from disease-associated malignant clones (original Question 1). I would like this issue to be answered directly.*

Authors' response.

As requested, we added more details about the selection of the subset 1 cases. Specifically, we outlined how patient-derived CLL cells were the source of the mRNA for the amplification of the BCR sequences, and how the gene sequences were used to assign the clones to subset 1 (pages 22-23).

Reviewer 2.

Reviewer's comment, point 1. *[Regarding Figure 1] The colors of the figure are much better, however, the boxes indicating CDR1, CDR2 and CDR3 are gone, please restore.*

Authors' response.

We apologize for the error, the boxes indicating the CDR loops in Figure 1 are now restored

Reviewer's comment, point 3. *In the Methods section it is mentioned that the rmIgG Abs were used in 15 ug/ml. Were the rmIgM Abs used in the same concentrations? In our hands polyreactive recombinant IgM already plateaus at approximately 2 – 3 ug/ml. If rmIgM was used at the same concentration or used at a different concentration please mention in the Methods section. For the rest, I am completely satisfied by the answers and changes.*

Authors' response.

As requested by reviewer 2, point 3, we specified that the concentration of both IgG and IgM antibodies were at 15 µg/mL (page 24).

Reviewer 3.

No further issues were raised by the Reviewer, whom we thank for the very nice comments.

Reviewer 4.

No further issues were raised by the Reviewer, whom we thank for the very nice comments.