nature portfolio

Peer Review File

COVID-19 progression and convalescence in common variable immunodeficiency patients show dysregulated adaptive immune responses and persistent type-I interferon and inflammasome activation

Corresponding Author: Dr Esteban Ballestar

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 authors used single-cell RNA sequencing to decipher global landscape of immune cell response alterations in CVID patients infected with SARS-CoV2. The authors tracked disease progression and recovery in these patients and compared gene expression in different cell subsets to those in normal controls with severe and mild COVID. Sc RNAseq is a relatively new technology and this is the first time CVID patients were being investigated with this technology.

The authors found sustained type 1 IFN response in all cell types except monocytes. Prolonged inflammatory gene expression was detected in monocytes of the CVID patients after recovery. Great effort in putting forward a well presented manuscript.

I have a few questions for the authors:

1. CVID is a heterogeneous disorder. Can the authors comment on whether the 5 patients are representative CVID patient cohort? The authors highlighted fold changes expression of certain genes in CVID patients in each of the pre-infected, infected and recovery phase. Are there any variation in those gene expression within the 5 patients in each phase?

2. Are all patients and normal controls infected with the same SARS-CoV2 variant? Would the more recent variants, being more infectious but have less severe clinical outcomes, induce different molecular signatures compared to the original SARS-CoV2?

3. Is there a sentence in the manuscript that mentioned all CVID patients were vaccinated with mRNA vaccine along with booster shots, the time lapse between vaccination and infection?

4. The first CVID patient had non-conventional COVID treatment HQC and Ivermectin. Any reason for the treatment?

5. The authors described many upregulated gene expression during the COVID progression from infection to recovery in CVID patients. Are there any genes that were significantly downregulated?

Reviewer #2

(Remarks to the Author)
In this manuscript Rodríguez-Ubreva et al seek to define the molecular basis of the altered immune responses caused by SARS-CoV-2 infection in CVID patients. They generated single-cell datasets of peripheral blood immune cells along viral infection and recovery. We observed that COVID-19 CVID patients show defective canonical NF-κB pathway activation and dysregulated expression of BCR-related genes in naïve B cells, as well as enhanced cytotoxic activity but incomplete cytokine response in NK and T cells. Moreover, monocytes from COVID-19 CVID patients show persistent activation of

several inflammasome-related genes, including the pyrin and NLRC4 inflammasomes. These conclusions are based on comparison of scRNAseq of CVID patients pre – during and post-infection or alterrnatively by comparison with publicly available data on healthy donors.

Although the question is interesting, there are several methodological issues.

First, the choice of the CVID patients included is hard to understand.
CVID is the most common primary immunodeficiency, and the Freiburg Center follows a very large cohort of patients and searches for genetic diagnoses in these. However, only 5 patients are included here, and two of these have absent B cells, which is not typical for CVID. Moreover, it limits the analysis to only three patients for some of the results sections, which severely hampers the validity of the conclusions.
Then, only patients with mild COVID were included. Inclusion of patients with severe COVID would have strengthened the data.
Also, no genetic data are given except for TNFRSF13B c. p.A181E in one. Did no other patient receive genetic testing ? Were the others maybe potentially harboring pathogenic variants in NF-KB1 / NF-KB2 ? Also, as CVID is a black box, it would be important to exclude any other PID by at least targeted NGS PID Panel sequencing, as I am sure these patients have received. Also, there is a paucity of data on the actual pre-infectious state and the infection course. Were the patients vaccinated ? If so, did they receive boosts ? Which vaccines ? What viral strain was involved ? Was this the same strain in all patients and all controls ? Did the authors measure anti-IgN antibodies in the patients ? if the patients were on IVIG / SCIG, it is important to know when these samples were obtained as some recent SCIG and IVIG batches will contain plasma donors' anti-COVID antibodies after natural infection / vaccination. In other words, this may have influenced the responses and even cellular phenotypes. From supplemental table 1 I get the impression that the CVID cohort is older than the controls. Can the authors present median and SD for age ? The authors should if possible also include viremia levels for SARS-CoV2 or maybe (comparable) logs for the nasaopharyngeal PCRs. I am presuming that COVID diagnosis was based on nasopharyngeal PCR, but this is not stated in he methodology. Also, it is unclear why in some patients the sampling during convalescence was very distant from the actual infection. Was this because of logistic problems or were several PCRs tested in between and was this the first negative obatined ? All of this needs to be specified in the methodology. Without stringent criteria, it feels like comparing apples to oranges. The entire set-up leaves this reviewer unconvinced.

Second, from the methodology it is unclear why deconvolution of scRNA-seq data was used.
Was it used in the CVID patients or for the healthy donors from the publicly available data sources? Also, if I understand well from the methodology, the controls were not necessarily involving longitudinal samples in the same individuals ? This is unclear from the methodology section.

One of the first conclusions is that CVID patients have longer type I IFN response upregulation in all cell subsets except monocytes. However, this is surprising, especially since all included patiens had mild COVID. Did the authors measure anti-type I IFN auto-antibodies in the patients presented here ?

Then, the authors investigate the B cell subset (n=3 patients). They found reduced p50 and p65 activity in the CVID patients, but a higher activity of NFKB1A. Therefore it is all the more important to know the NF-KB1/2 mutation status. To fully understand and conclude on the BCR repertoire and IGHV gene usage, we really need to have info on the SCIG/IVIG use and on the vaccination status of both patients and controls.

Interestingly, the NK and T cells of COVID19 CVID patients show impaired IFN-gamma and TNF expression. However NK and T cells do have a higher activation state and express high levels of genes associated with cytotoxic responses, which is maintained during convalescence. When comparing the monocyte subsets, inflammasome complex assembly, pyroptosis and IL-1β production were more enriched in monocytes from COVID-19 CVID patients compared to those from COVID-19 control individuals. More specifically there was a
significant upregulation of the NLRC4 gene and MEFV gene in CVID versus controls. The expression of many inflammasome related genes was maintained during convalescence in CVID versus controls. When investigating TLR7 and TLR8 gene expression, this was upregulated in CVID patients and persisted into convalescence. Finally, anti-inflammatory gene upregulation was impaired in CVID. The signature that emerges is one of increased sensing, increased inflammasome activation and deficient regulation in monocytes.

A few questions arise here.
How specific is this finding for COVID19? Is this comparable to observations during other (viral) infections in CVID? Also, how do the authors explain the increased TLR7 expression to the description of severe COVID19 in decreased TLR7 function? Did the authors go back to PBMCs and actually verify this at the protein level? This is more difficult for inflammasome function but it's feasible for the TLR7 and TLR8 findings. I have the same question for the NK cell and T cell findings: do the DEG signatures indeed result in increased cytotoxicity?

In all, the authors present potentially interesting data. However, in this reviewer's opinion, there are several methodological issues which significantly undermine the significance of the results: my most important concern is the lack of detail on the COVID CVID cohort. Although the scRNA seq comparisons are potentially interesting, validation of the hypotheses generated here by other scientific methods is needed, eg. Formal cytotoxicity testing, TLR7 expression assay etc. Also, the hypothesis of viral persistence in CVID versus healthy donors is premature and needs confirmation.

Reviewer #3

(Remarks to the Author)
The manuscript entitled “COVID-19 progression and convalescence in common variable immunodeficiency patients shows incomplete adaptive responses and persistent inflammasome activation” by Rodrigue-Ubreva et al. is attempting to address potential differences of CVID patients in comparison to non-CVID individuals in coping with COVID-19. While certainly an interesting topic and also approach to learn more about COVID-19, but also about CVID itself, the presented draft of the manuscript is characterized by numerous shortcomings that all would have to be addressed carefully before major statements can be made. One also needs to consider that there is a huge number of datasets in the public that has revealed many important aspects about COVID-19 already and this huge knowledge has not been adequately and sufficiently utilized in this manuscript. Almost three years into the pandemic with a meriad of information and also scRNA-seq datasets, this was a little bit surprising to the reviewer, why this was not leveraged further. In this context, it also might be helpful for certain aspects to team-up with some of the leading centers/groups in COVID-19 scRNA-seq projects.

Following are major and minor concerns in the order as they appear in the text with an emphasis on the result section.

Line 67-70. Sentence seems to miss something, not clear what is meant.

Line 115: Provide a graphical representation of the time axis of sampling for all samples used within the generated datasets. Like this, the reader can directly see for each individual when sampling was performed. This cannot be judged from the current information provided in this manuscript. E.g. the term progression is not specified sufficiently. When exactly after onset of symptoms were the samples taken? Same for convalescence.

Line 117f: Wouldn't the authors have expected more severe courses of the disease? Any explanation, why all CVID patients only had mild disease?

Line 121: The chosen published COVID-19 cohort is one of the worst datasets in the field and should be avoided at all means. This analysis needs to be repeated and validated with other datasets that have much higher qualities. Also, if using other datasets, the authors need to be very careful in not including patients that have been under corticosteroid therapy. It will dramatically impact on the interpretation of the findings.

Line 126: The Ren et al dataset has ~1.46 million cells, but only ~140,00 cells were chosen without describing the criteria for inclusion or exclusion of samples and cells from the dataset. This needs clarification. But even better would be to exclude the Ren dataset, use two of the much more reliable datasets in the field and compare both independently in this study (like an in-project validation cohort). This is important, because when using previously generated datasets without CVID patients, the differences might come only from technical differences. This is not yet excluded entirely when validating by a second previous dataset, but it would already be mitigated to a certain extend. As is, this is not the case. Differences might just come from technical aspects differing between the different cohorts compared here directly.

Line 132: This is one of the more reliable datasets that should be used instead of Ren et al. However, it is important to ensure that when using the COVID-19 data from this study, the patients with corticosteroid therapy need to be excluded. One additional other large COVID-19 dataset should be used as validation (not integration).

Line 133: define "census". There are so many ways of cell labeling. The authors should just name the method they used.

Line 134: In Figure 1b, the total number of cells in the analysis needs to be shown. Also, classical QC parameter of the integrated dataset are missing. Need to be added, see other high-level scRNA-seq data papers in the field.

Line 134 - 141: The listing of the cell type should be avoided in the text, this is legend within Fig 1b. Describe findings, e.g. cell population level and compare to known findings in the field concerning this high-level analysis.

Line 142ff: Why did the authors chose to directly focus on IFN response. Make a serious effort to provide information about the overall changes (major pathways and mechanisms changed) and only then start to zoom in on certain aspects like IFN signaling. Also, what is exactly meant with analyzed? In Fig 1C a Go-Term is presented, was this a kind of enrichment analysis. Further, a FC is shown: which groups were compared? Did you correct for different cell numbers per patient group etc. etc. Please, the authors need to be more precise.

Line 146ff: This is probably the most important finding. From the text, it was not entirely clear to this reviewer, whether the non-CVID groups also have samples before and during COVID-19, or if in these groups the comparison is between COVID-19 patients and a historical control group of samples derived from before the pandemic? If so, can the authors better describe, whether this difference in study design between the two groups exists. And if so, how can the authors exclude that a historical group of healthies might just differ in signal to COVID-19 due to other reasons (e.g. technical reasons, or lower baseline levels of these genes, etc. etc.). As this finding seems to be central, some more information to the study design for this questions and an independent validation of this finding is required.

Line 153: This is very interesting. How would the authors explain, that despite the fact of a complete lack of IgA and almost complete lack of IgG, these patients made it through COVID-19 with mild disease? Does this mean that antibodies after all are not that important? Make an attempt to address this most important aspect of CVID in context of COVID-19. For example, would there be experiments to address this further?

Line 155ff: Apparently in Fig. 2a, the B cells were subsetted, but there is no information about from which dataset this was started, how many cells remained, which samples are now part of this subset of cells, if samples were excluded and if so, which criteria were used. This needs update.

Line 163ff: Again, very interesting, but was this analysis corrected for different cell numbers per group? What is the cellular distribution of the signals, how many cells / subset are positive? Some more information is required.

Line 173f: Isn't this already known, if so please cite respective reference.

Line 177ff: well, this reviewer would argue that the previous findings (ref 33) is just not complete and therefore with the scRNA-seq data, one now can see that the dysregulation of the canonical NF-kB pathways is detectable in both naive and CD21low B cells. Along these lines, the suggestive sentence should be rephrased accordingly. It is not so much an issue about whether this is a consequence of presence or not of expanded CD21low B cells, it is more about the effect is seen in both cellular compartments, which is the power of scRNA-seq.

Line 180ff: The authors chose an approach to zoom into a particular TF directly, probably because it has been described before to be associated with CVID (see reference 36). More informative would have been a modeling approach to define the most important TFs involved in the observed deviations, which requires to apply a pipeline including TF expression analysis, TF differential expression analysis and TF binding prediction analysis to key genes (e.g. those altered in a certain cell subset). Such analysis should be added to achieve several things: first, it classifies the importance of HIF1 in context of other potentially important TFs, second, it makes much better use of the data at hand. Thirdly, it might identify the really important TFs involved in the pathophysiology observed.

Line 184ff: What is the connection of this sentence with the aspect of HIF1 one sentence before. Remains unclear.
Line 187: This is certainly of interest, but the reader cannot judge from the data representation, whether these terms are among the most enriched terms, whether those terms were chosen manually, and if so, how. Needs further improvement of text and/or figure.

Line 190ff: Similar here again: "... of several genes encoding members of ...". The authors are requested to make a better case of how they choose to show the data, how they choose the genes, what are the rules behind the selection? As is, it could be rather arbitrary and this reviewer thinks that it cannot be the intention of the authors to make arbitrary decisions during the analysis process. E.g. which other non-BCR related genes have the same pattern? What is their role, etc. etc.

Line 197ff: Again, this would be another interesting aspect. However, it remains unclear to the reviewer, which cells are compared here. Earlier in the manuscript it is clearly stated that switched B cells and PCs are reduced in CVID. But these would be the interesting once to be compared. If the comparison would be all B cells, then it would not be surprising that COVID-19 non-CVID patients would have higher SARS-CoV-2 associated BCR sequences in their blood. This needs further clarification, what actually was compared here.

Line 201 and throughout the manuscript: The term COVID-19 controls could be misleading. For CVID it is the control, but in fact these are also COVID-19 patients. since there are also controls without COVID-19, maybe the better term would be COVID-19 non-CVID as it is stated in several figure panels. Optimize throughout the manuscript, text, figures, legends.

Line 206: It is indeed fascinating to see that naive B cells in CVID are showing transcriptional deviations. But it would have been also very important to provide at least some evidence how this could be explained mechanistically, or which pathways might be involved, which TFs. Also, how do the authors explain impairment of NF-kB activation, yet highly increased expression of BCR-pathway related molecules. Some more explanatory insights based on some additional experiments here would significantly improve this part of the manuscript.

Line 212ff: similar as before, the subsetting of the T cell and the NK cell compartments need more clarification, following statements made for the B cells above. Supp Figure 2a: It would be much more informative, if the most relevant and cluster-defining genes for the clusters rather than genes related to certain literature-based T cell types would be shown. As is, it is not convincing, because many genes are expressed in many cell types not really classifying the cell types here. This is expected, but it is therefore also not very informative.

Line 214ff, Figure 3b: The reader needs more information about how these terms were retrieved from the analysis. Are these the Top terms, are these chosen manually from a list? Just more precise information. Also, the authors should take effort to compare the NK cell annotation provided here with previous publications on NK cells in COVID-19, e.g. there have been reports entirely focusing on NK cells, e.g. in Immunity, 2021 already. This needs more work and more inclusion of already existing knowledge in the field about NK cells in COVID-19.

Line 219: This is an interesting statement. Probably similar to other extended expression deviations in CVID patients, it would be really of great interest to see, if CVID patients just have a prolonged inflammatory state and if so, is this reflected by any clinical observations and also, if these phenomena are turning to normal at later time points. Since the authors seem to sample these patients regularly, an even later additional time point to address these questions should be possible to be added.

Lines 220ff and Sup Fig 2b: This reviewer would argue slightly different: If looking at the CD56high NK cells in CV: GZMB

and GZMA are also still upregulated in non-CVID patients. Maybe focus only on those genes that are really different, like PRF1 and NKG7. If so, the question also is, why were only these four genes chosen, why not making a more data-driven analysis and report on those genes that are still dysregulated in CVID patients at CV but not at CV in non-CVID patients. Like this, the reader would get a more complete picture about what the differences really are. To some extend the authors do this with the cytotoxic score, but this still leaves out other potential molecular changes, pathways and programs. Concerning the score: while mentioning that the Methods section shows details about the score, the reader should be guided here already, whether the score is based on manually picking genes or a data- and algorithmic-based approach.

Line 224ff: Now the authors introduce some other pathways, but overall, it looks a little bit like cherry picking. A more data driven approach first and then highlighting some specific findings would make this analysis more robust and would guide the reader better about the importance of the reported findings in respect to the overall changes observed by scRNA-seq. One important question concerning the non-CVID patients: What do the authors think is the reason for such a huge upregulation of IFNG genes during convalescence? The question is, are these patients already truly convalescence or do these patients show prolonged signs of disease? Either clinically or molecularly or both. Would be important to report on this in the patient description section of this manuscript. Overall, it goes towards the question: what does this difference really mean? Is it solid and would this be similar in a validation cohort? To really make these statements some additional analyses would be required.

Lines 228ff: The figures are oriented along the genes, but the text is oriented along the different cell types, which really makes it somewhat difficult for the reader to quickly see the message in the figures. Either change the text to describe the figures or change the figures to better follow the flow first NK cells than T cells.

Lines 236 and Figure 3e: Along the same lines as already stated for NK cells and CD8 cells, the increased expression of inflammatory cytokines during CV in non-CVID COVID-19 patients requires additional validation, either by including another cohort of such patients' scRNA-seq data or alternative experimental approaches, but it needs to be excluded that these non-CVID patients chosen for this study are not the exception rather than the rule for convalescence.

Line 245ff and the whole paragraph before concerning cytotoxicity: There has been a recent report in Cell concerning CD16+ cytotoxic T cells with detrimental function in severe COVID-19 patients. What is the relationship of the cytotoxic T cells seen here in CVID patients and these CD16+ T cells previously reported? The authors should make an attempt to bring this together with previous knowledge in the field.
Supp Figure 2c: Could the authors turn the figure by 90° so that the cell names can be easily read? Also the terms used in the figure and the text need to be equal. The reviewer presumes that Tprolif are Tcell cycling in the figure. Is this correct? If so, correct accordingly.

Lines 255ff: Actually, this might come from the subsetting strategy. An alternative to subset NK cells and T cells together is to do this separately. Very often, this leads to a better separation of NK cells and T cells and then allows to clean the subsetted datasets of T cells and NK cells respectively and then, such observations are usually excluded. Either the authors change the last sentence and focus on the overall findings of this paragraph or they test the alternative subsetting strategy, or both.

Line 260ff: reference 10 missing here. As also mentioned for the other cell types, the authors need to bring their data into context of previously described subsetting of the different immune cell types in COVID-19, here the myeloid cell compartment. Otherwise, it will be impossible to make definite conclusions from this new dataset and the comparison with non-CVID COVID-19 patients. All previous comments and suggestions stated earlier for the other figures apply here as well.

Line 262ff: As also stated for the other cell types, the authors need to provide a date-driven full picture of all the major pathways being altered. As presented here, it looks like cherry-picking. Focusing on certain pathways once the whole picture is provided might be ok, but as is, this would not be a meaningful description of the overall findings within the myeloid cell compartment of CVID patients in relationship to COVID-19 disease.

Line 268ff: This is another example of not being precise enough. There is a difference in what is here now called non-classical monocytes during CV. Regulation does not seem to be completely identical.

Lines 270ff: When looking at what is termed here classical monocytes, there is not much difference between non-CVID mild and CVID patients during PG, only during CV, where only CVID patients still show elevated signals. Again, important would be to provide further evidence that these changes are not due to the group of non-CVID patients included in the analysis (goes again towards an additional non-CVID COIVD-19 CV population to be used for comparison). For what is called non-classical monocytes, this is rather similar. The biggest difference is seen in what is called intermediate monocytes. Here, the reviewer asks the authors to explain, how they derived these cell state, because this cell state is known to be rather difficult to pinpoint in scRNA-seq data (goes back to the issue of bringing cell type annotation into the context of prior knowledge within the COVID-19 scRNA-seq blood landscape.

Line 279ff: Everything said to the inflammasome genes above, also applies to the TLR7 genes. A revised analysis will clarify this here as well.

Line 286ff: This is another interesting aspect. It is a little bit surprising to the reviewer that the authors did not make an attempt to experimentally show, if such inhibitors could repress or at least blunt the proposed prolonged inflammatory state in CVID patients during CV. Incubating cells from these patients during CV with inhibitory ligands and reading out transcriptional changes could have addressed this rather easily. It would at least hint towards a potential molecular

mechanism of the phenomenon of prolonged inflammatory immune cell responses (if validated) in these patients and thereby would have increased the novelty and impact of the findings. The authors need to think about such experiments to be added to the manuscript.

Lines 414ff: All patient samples used in this study, including those from previous studies require a full table with clinical metadata, this is missing. For examples, see descriptions of previous large COVID-19 datasets (except for Ren et al, which is also insufficient in this respect).

Line 570: Accession number at EGA as well as accession procedure are missing

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
There is substantial reworking of the manuscript following reviewers comments. FIgure 7 is particularly useful for summarizing their findings in CVID patients during COVID infection and convalescence. CVID patients lacking B cells similarly to agammaglobulinemia patients have milder course of infection has been reported. Overall there are new insights into these patients. One would have thought such a heterogenous group of CVID patients lacking genetic diagnoses would have rendered the scRNAseq results uninterpretable. However the authors managed to present a snapshot of the dynamic gene expression in the adaptive and innate immunity in CVID patients. It is likely there will be more insights to be unearthed from the scRNAseq data.

There is currently no scRNAseq in CVID published in literature so this manuscript is novel for the field of CVID.

Reviewer #2

(Remarks to the Author)
In their revised manuscript, Javier Rodríguez-Ubreva and colleagues have provided additional information on the methodology and the collection of data enhancing our interpretation of the presented data.
At this point still, this reviewer's concern is that conclusions are based on massive data production from a limited number of patients with a heterogeneous condition.

Reviewer #3

(Remarks to the Author)
The manuscript now entitled "COVID-19 progression and convalescence in common variable immunodeficiency shows dysregulated adaptive responses and persistent type-I interferon and inflammasome activation" by Rodriguez-Ubreva at al is a first revision of a recently submitted paper. The authors have revised the large number of necessary and requested major and minor concerns which has significantly improved this manuscript. Overall, the data are now much more clear and due to additional validation analyses, which eliminated some of the findings reported in the first submission, the findings described in this manuscript are now - in general - more valid. The addition of further experiments validating some of the initial findings with other experimental approaches also contributed to more sound results.

There are some minor aspects that should be clarified.

Lines 459-461. In this sentence it is stated that a comparison was performed between non-CVID and CVID patients. In which figure is the comparison shown. This was not clear enough to this reviewer.

Line 581: Severe COVID-19 patients display functional and phenotypic alterations, mild COVID-19 not. Add "severe".

Supplemental Figure 2d, 3e, 4a, 5e, 6e: color code for showing scores: very colorful, maybe a little bit too colorful: therefore, try to use simpler color scheme

The discussion section is rather long now, if necessary, the authors could shorten.

Point-by-point Responses to the Reviewers' comments:

Reviewer #1:

The authors used single-cell RNA sequencing to decipher the global landscape of immune cell response alterations in CVID patients infected with SARS-CoV2. The authors tracked disease progression and recovery in these patients and compared gene expression in different cell subsets to those in normal controls with severe and mild COVID. Sc RNAseq is a relatively new technology and this is the first time CVID patients were being investigated with this technology.

The authors found sustained type 1 IFN response in all cell types except monocytes. Prolonged inflammatory gene expression was detected in monocytes of the CVID patients after recovery. Great effort in putting forward a well presented manuscript.

We would like to thank the reviewer for their positive comments on the quality and value of our study, and for the constructive comments, which we have addressed as detailed below.

I have a few questions for the authors:

1. CVID is a heterogeneous disorder. Can the authors comment on whether the 5 patients are representative CVID patient cohort? The authors highlighted fold changes expression of certain genes in CVID patients in each of the pre-infected, infected and recovery phase. Are there any variation in those gene expression within the 5 patients in each phase?

Heterogeneity is a key aspect when studying CVID and, therefore, we understand the request of the reviewer. All patients included in the study had a longstanding diagnosis of CVID (age range 32-70 years old). All patients except CVID4 belonged to the "complex form of CVID" with signs of immune dysregulation listed in the Suppl. Table 1a. The EUROclass distribution showed mostly low B cells or an expansion of CD21low B cells, as expected in this clinical subgroup of patients, especially in older individuals, since B cell numbers in this subgroup often decrease. Thus, the patients in this study were typical CVID patients. We have added details about how representative these patients are in relation to CVID (see page 36 of the revised marked manuscript) at the beginning of the Methods section of the revised manuscript. In addition, the revised version of the manuscript includes samples from 4 additional complex CVID patients analyzed by spectral flow cytometry, which have increased the representation of CVID heterogeneity. Supplementary Table 1 includes additional clinical and demographic information of the cohort.

Regarding the variation in gene expression among CVID patients, given the individual differences, we have highlighted only the significantly differentially expressed genes between CVID and non-CVID individuals across the stages of COVID-19 (statistical significance obtained by Wilcoxon test). Nevertheless, gene and protein expression, as well as cell frequencies of individual patients, have now been included in the box plots of the revised figures (For instance, see Figure 2h-2j, and Supp. Figure 2e, 2i, 2k and 2l in the case of the B cell compartment).

2. Are all patients and normal controls infected with the same SARS-CoV2 variant? Would the more recent variants, being more infectious but have less severe clinical outcomes, induce different molecular signatures compared to the original SARS-CoV2?

This is a very relevant point. None of the CVID nor non-CVID donors were infected with any of the recent SARS-CoV-2 variants. According to the clinical records, the CVID patients in our cohort were all infected between April 2020 and May 2021, when the wild type strain and the alpha variant of SARS-CoV-2 were predominant in Germany. Regarding the non-CVID donors, they were infected between January 2020 and April 2021, again when the wild type strain and the alpha variant were predominant. We have now added a sentence referring to this aspect in the revised manuscript (page 36 of the revised marked manuscript). In addition, we have now added a column in Supp. Table 1a, indicating the most frequent SARS-CoV-2 variant at the time of infection.

3. Is there a sentence in the manuscript that mentioned all CVID patients were vaccinated with mRNA vaccine along with booster shots, the time lapse between vaccination and infection?

This is another important point. None of the samples analyzed were taken from CVID patients that had received anti-SARS-CoV-2 vaccination at the time of sample collection. As the aims of our study were to investigate the impact of a first time viral encounter on the immune homeostasis of patients with an impaired humoral immunity, the study of non-vaccinated patients is particularly relevant. We have now clarified this point in the text (page 36 of the revised marked manuscript). In this regard, our cohort is unique including COVID-19 CVID patients in a pre-vaccination state, and we believe that our results may be relevant for patient care/treatment, targeting for instance the prolonged type I IFN response observed in these patients, including the immune response of CVID patients to novel infectious agents.

4. The first CVID patient had non-conventional COVID treatment HQC and Ivermectin. Any reason for the treatment?

At the time of infection of the patients included in this study, no known antiviral was available, and various drugs, including HCQ and ivermectin, were administered under compassionate use before their efficacy or lack thereof was demonstrated. This is now mentioned in the text (page 36 of the revised marked manuscript).

5. The authors described many upregulated gene expression during the COVID progression from infection to recovery in CVID patients. Are there any genes that were significantly downregulated?

In addition to upregulated genes, we have also detected numerous downregulated genes associated with CVID patients across the stages of SARS-CoV-2 infection. For instance, both upregulated and downregulated genes are represented in Figure 2e. This pattern is also evident at the protein level, as seen in Figure 3h and Figure 4e. We have now included a table (Supp. Table 3) that incorporates the total number and names of significantly differentially expressed genes (both up or downregulated) in each comparison and cell cluster.

Reviewer #2:

In this manuscript, Rodríguez-Ubreva et al seek to define the molecular basis of the altered immune responses caused by SARS-CoV-2 infection in CVID patients. They generated single-cell datasets of peripheral blood immune cells along viral infection and recovery. We observed that COVID-19 CVID patients show defective canonical NF-κB pathway activation and dysregulated expression of BCR-related genes in naïve B cells, as well as enhanced cytotoxic activity but incomplete cytokine response in NK and T cells. Moreover, monocytes from COVID-19 CVID patients show persistent activation of several inflammasome-related genes, including the

pyrin and NLRC4 inflammasomes. These conclusions are based on comparison of scRNAseq of CVID patients pre – during and post-infection or alternatively by comparison with publicly available data on healthy donors.

Although the question is interesting, there are several methodological issues.

We appreciate the reviewer's positive feedback on the interest and significance of our study. We would like to thank the constructive criticism and detailed suggestions provided, which we have thoroughly considered and addressed as follows:

First, the choice of the CVID patients included is hard to understand.
CVID is the most common primary immunodeficiency, and the Freiburg Center follows a very large cohort of patients and searches for genetic diagnoses in these. However, only 5 patients are included here, and two of these have absent B cells, which is not typical for CVID. Moreover, it limits the analysis to only three patients for some of the results sections, which severely hampers the validity of the conclusions.

In this study, we aimed to investigate the effects of a viral infection on the immune homeostasis in patients with an impaired humoral immunity, and with no preformed immunity against SARS-CoV-2 either by vaccination or the antibody preparation for immunoglobulin replacement therapy (IgRT). Our cohort included the first five patients we had access to at the very beginning of the pandemic in 2020. As there were increasing restrictions, sample collection from CVID patients was more difficult throughout the pandemic as most patients were treated close to their home. The challenge to obtain such samples is also evident by the very limited number of other published studies on this topic, which makes our work highly relevant and unique. Additionally, the investigations of these early patients have allowed us to study the effects of the COVID-19 course on the immune homeostasis without concerns about secondary effects due to vaccination or treatment with antiviral drugs. Nevertheless, in the revised version of the manuscript, we have included additional samples from CVID samples (infected with SARS-CoV-2 during 2021), without adding additional confounding factors from vaccination. In this regard, our cohort allows us to explore unresolved questions in the fields of immunology and virology. Our results are also relevant for patient treatment, targeting for instance the prolonged type I IFN response observed in these patients, including countries where vaccination rates are low, and potentially for future breakthrough infections.

Regarding the reviewer's concern about the representation of B cells in CVID, we respectfully disagree. Most patients with a complex form of CVID undergo a loss of B cells as they age. In fact, the two patients without B cells correspond to the archetypical CVID group with secondary complications. While it is true that the absence of B cells is characteristic of some CVID individuals, to avoid limiting our study of B cells in these patients, we have now incorporated the analysis of four additional COVID-19 CVID patients for a further examination of the B cell compartment (along with other immune cells) using spectral flow cytometry. We truly believe that our results are highly relevant since they have allowed us to have a comprehensive picture of the general dysregulated response of the immune system to a viral challenge in complicated CVID patients who, indeed, many of them may show absence of B cells.

Then, only patients with mild COVID were included. Inclusion of patients with severe COVID would have strengthened the data.
Also, no genetic data are given except for TNFRSF13B c. p.A181E in one. Did no other patient receive genetic testing? Were the others maybe potentially harboring pathogenic variants in NF-KB1 / NF-KB2 ? Also, as CVID is a black box, it would be important to exclude any other PID by at least targeted NGS PID Panel sequencing, as I am sure these patients have received. Also, there is a paucity of data on the actual pre-infectious state and the infection course. Were the patients vaccinated? If so, did they receive boosts ? Which vaccines ? What viral

strain was involved? Was this the same strain in all patients and all controls ? Did the authors measure anti-IgN antibodies in the patients? if the patients were on IVIG / SCIG, it is important to know when these samples were obtained as some recent SCIG and IVIG batches will contain plasma donors' anti-COVID antibodies after natural infection / vaccination. In other words, this may have influenced the responses and even cellular phenotypes. From supplemental table 1 I get the impression that the CVID cohort is older than the controls. Can the authors present median and SD for age ? The authors should if possible also include viremia levels for SARS-CoV2 or maybe (comparable) logs for the nasopharyngeal PCRs. I am presuming that COVID diagnosis was based on nasopharyngeal PCR, but this is not stated in the methodology. Also, it is unclear why in some patients the sampling during convalescence was very distant from the actual infection. Was this because of logistic problems or were several PCRs tested in between and was this the first negative obtained ? All of this needs to be specified in the methodology. Without stringent criteria, it feels like comparing apples to oranges. The entire set-up leaves this reviewer unconvinced.

The criteria for inclusion in this study were:
- well defined CVID patients with typical complications and immunological phenotypes, as defined by the EUROclass classification.
- no severe immunosuppression.
- under regular IgRT.
- at a time of no additional variation caused by the presence of vaccination or spike specific antibodies in the IgG preparations used for IgRT.

It is true that our COVID-19 CVID cohort only includes patients with mild symptoms. In fact, during the course of the pandemic, over 90% of the patients in the Freiburg cohort had a mild course, similar to the CVID patients presented in this study, with only very few patients with a severe course of the disease. This is consistent with the clinical data of other CVID cohorts around the world. An intriguing observation was that most CVID patients infected with SARS-CoV-2 experienced a longer course of the disease rather than a more severe one. Therefore, the main question in our study was not the distinction of the immune response in mild vs severe COVID-19 in CVID patients, but the analysis of the effects of SARS-CoV-2 infection on the immune homeostasis in CVID patients.

Regarding genetic testing, all patients except CVID4 were initially tested by whole exome sequencing and the only mutation identified related to CVID was the TNFRSF13B c. p.A181E in patient CVID2. For the revisions, we have now performed WGS on the DNA of patient CVID4 and no PID-associated mutation has been identified. We have now added this information to Supp. Table 1a.

In addition, we have expanded the clinical data in Supp. Table 1a to include information on treatment and additional secondary complications. Because of the early time of investigation, none of the commercial products contained anti-SARS-CoV-2 antibodies. One difference is, however, that patients CVID3 and CVID5 received plasma therapy before they were evaluated during their convalescent phase as stated in Supp. Table 1a and Supp. Figure 1a.

The samples analyzed were collected from CVID patients who had not been vaccinated against SARS-CoV-2 at the time of sample collection. This clarification has now been added to the text (page 36 of the revised marked manuscript). Consequently, our cohort is unique in including COVID-19 CVID patients in a pre-vaccination state.

According to our records, the CVID patients in our cohort were infected between April 2020 and May 2021, a period during which the wild-type strain and the alpha variant of SARS-CoV-2 were prevalent in Germany.

Similarly, the non-CVID donors were infected between January 2020 and April 2021, also during the predominance of the wild-type strain and the alpha variant. We have now included a sentence addressing this issue in the revised manuscript (page 36 of the revised marked manuscript). Additionally, we have added a column to Supp. Table 1a, indicating the most common SARS-CoV-2 variant at the time of infection. All the viral tests refer to nasopharyngeal swabs. This information has been added to the Methods section (page 36 of the revised marked manuscript).

Anti-IFN antibodies were tested in all CVID patients but none of them were positive. We have now added this information to the manuscript (page 8, line 199-200 of the revised marked manuscript).

The non-CVID samples were selected in order to match in age and sex with the CVID cohort. We have now added a table showing the age mean and SD in each group, together with the t-test p-values, indicating that there are no significant differences in age between groups (Supp. Table 1b-1c).

The time point of testing for convalescent state was influenced by the time of the first negative test as well as logistics. The information of the time point of the first negative test was included in Supp. Table 1a and Supp. Figure 1a.

We hope that providing the additional data clarifies the raised concerns about the validity of the comparison in this study.

Second, from the methodology it is unclear why deconvolution of scRNA-seq data was used. Was it used in the CVID patients or for the healthy donors from the publicly available data sources? Also, if I understand well from the methodology, the controls were not necessarily involving longitudinal samples in the same individuals? This is unclear from the methodology section.

We apologize for the lack of clarity in this regard. We have now indicated in the Methods section that we pooled several samples in the same Chromium 10x reaction to optimize the use of reagents and minimize batch effects (page 37, lines 965-966 of the revised marked manuscript). For that reason, we had to deconvolute the samples in order to properly assign donor identity to each single-cell.

In addition, we have now clarified in the Methods section that only CVID samples involve longitudinal samples, whereas the non-CVID samples correspond with samples at the three stages of the viral infection (before, during and after) but are not paired samples (page 36 of the revised marked manuscript).

One of the first conclusions is that CVID patients have longer type I IFN response upregulation in all cell subsets except monocytes. However, this is surprising, especially since all included patients had mild COVID. Did the authors measure anti-type I IFN auto-antibodies in the patients presented here?

Anti-type I IFN auto-antibodies have been associated with severe but not with mild and prolonged COVID-19 (PMID: 32972996), as observed in our patients. As mentioned earlier, none of the investigated CVID patients had detectable anti-type I IFN auto-antibodies. We have now added this information into the revised manuscript (page 8, line 200-201 of the revised marked manuscript).

Then, the authors investigate the B cell subset (n=3 patients). They found reduced p50 and p65 activity in the CVID patients, but a higher activity of NFKB1A. Therefore, it is all the more important to know the NF-KB1/2 mutation status. To fully understand and conclude on the BCR repertoire and IGHV gene usage, we really need to have info on the SCIG/IVIG use and on the vaccination status of both patients and controls.

As stated above, the WES analysis of the CVID patients included in this study discard the possibility that they carry mutations in *NFKB1* and *NFKB2*. Please see the information in regard to vaccination and IgRT in Supp. Table 1a.

In addition, to increase the power and robustness of our analysis on the B cell compartment, we have now performed spectral cytometry analysis of an independent cohort of COVID-19 CVID and non-CVID individuals in the revised manuscript. These independent cohorts included samples from additional CVID patients (3 samples at baseline, 4 samples at progression, and 4 samples at convalescence), as well as additional non-CVID individuals (4 samples at baseline, 6 samples at progression, and 4 samples at convalescence) (Supp. Table 1a).

All CVID patients analyzed are comparable as they have not been vaccinated and were on regular IgRT. Non-CVID individuals were not vaccinated either. We have clarified that point in the text (page 36-37 of the revised marked manuscript).

Interestingly, the NK and T cells of COVID19 CVID patients show impaired IFN-gamma and TNF expression. However, NK and T cells do have a higher activation state and express high levels of genes associated with cytotoxic responses, which is maintained during convalescence. When comparing the monocyte subsets, inflammasome complex assembly, pyroptosis and IL-1β production were more enriched in monocytes from COVID-19 CVID patients compared to those from COVID-19 control individuals. More specifically there was a significant upregulation of the NLRC4 gene and MEFV gene in CVID versus controls. The expression of many inflammasome related genes was maintained during convalescence in CVID versus controls. When investigating TLR7 and TLR8 gene expression, this was upregulated in CVID patients and persisted into convalescence. Finally, anti-inflammatory gene upregulation was impaired in CVID. The signature that emerges is one of increased sensing, increased inflammasome activation and deficient regulation in monocytes.

A few questions arise here.
How specific is this finding for COVID19? Is this comparable to observations during other (viral) infections in CVID? Also, how do the authors explain the increased TLR7 expression to the description of severe COVID19 in decreased TLR7 function? Did the authors go back to PBMCs and actually verify this at the protein level? This is more difficult for inflammasome function but it's feasible for the TLR7 and TLR8 findings. I have the same question for the NK cell and T cell findings: do the DEG signatures indeed result in increased cytotoxicity?

This is a very interesting point. Previous studies have indicated that SARS-CoV-2 infection triggers transcriptional changes distinct from those seen in other infections caused by influenza or other pathogens [PMID: 32651212, PMID: 34424199]. Consequently, we assume that SARS-CoV-2 results in a specific response also in CVID patients. There is currently no publicly available data on CVID patients infected with other viruses for comparison with our findings. We agree that, in the future, it will be of interest to analyze the effects of other viral infections, such as influenza, on the immune system of CVID patients, but this will be more difficult as the infection is often not as well documented and common as it was for SARS-CoV-2 during the pandemic. Here, we aimed to analyze the *in vivo* effects of a viral infection on the impaired immune system of CVID patients, since it was speculated that viral infections could underlie some of the observed chronic immune deviation in CVID patients.

Following the recommendations of Reviewer #3, we needed to modify our initial conclusions regarding TLR7 and TLR8 changes, as they were not validated following the inclusion of cohort non-CVID 2 and the adjustment for cell counts in each sample. These adjustments are crucial to mitigate potential biases arising from unequal

cell numbers in each sample and to capture biological phenomena rather than technical signals resulting from sample processing across datasets.

We agree with the reviewer that it is crucial to verify certain findings by confirmatory assays of respective protein expression. To answer some of the legitimate questions, we have now performed spectral cytometry analysis to assess the levels of 57 different proteins (revised Supp. Table 7) including, for instance, granzyme B and perforin in the cytotoxic compartments (revised Figure 4d-e, and Figure 5k), as well as several members of the BCR signaling pathway (revised Figure 2i). In addition, we have performed caspase-1 assays in order to estimate the activity of inflammasomes within the monocytic compartment (revised Figure 6h).

In all, the authors present potentially interesting data. However, in this reviewer's opinion, there are several methodological issues which significantly undermine the significance of the results: my most important concern is the lack of detail on the COVID CVID cohort. Although the scRNA seq comparisons are potentially interesting, validation of the hypotheses generated here by other scientific methods is needed, eg. Formal cytotoxicity testing, TLR7 expression assay etc. Also, the hypothesis of viral persistence in CVID versus healthy donors is premature and needs confirmation.

We thank the reviewer for recognizing the interest and relevance of the data and hope that we were able to clarify their questions in regard to the cohort by providing additional data (Supp. Table 1). We thank the reviewer for requesting the validation of some of our findings which allowed us to confirm most of them and be more cautious when we could not. We believe that the inclusion in the revised manuscript of the analysis of 57 molecules at the protein level, including cytotoxic and BCR-related signaling pathway proteins, together with inflammasome activity assay within the monocytic compartment of these patients, have strengthened the robustness and validity of our study.

Remarkably, the slower recovery was an observed clinical feature for most of the COVID-19 CVID patients included in our cohort, and it has been also recently described by others [PMID: 37148422]. Our hypothesis is that the unbalanced immune responses described in our study might be related to such delayed recovery. We agree with the reviewer that this claim needs to be confirmed in future studies with a larger cohort of patients. We have now acknowledged this idea in the discussion section of the manuscript (page 34, lines 908-911 of the revised marked manuscript).

Reviewer #3:

The manuscript entitled "COVID-19 progression and convalescence in common variable immunodeficiency patients shows incomplete adaptive responses and persistent inflammasome activation" by Rodriguez-Ubreva et al. is attempting to address potential differences of CVID patients in comparison to non-CVID individuals in coping with COVID-19. While certainly an interesting topic and also approach to learn more about COVID-19, but also about CVID itself, the presented draft of the manuscript is characterized by numerous shortcomings that all would have to be addressed carefully before major statements can be made. One also needs to consider that there is a huge number of datasets in the public that has revealed many important aspects about COVID-19 already and this huge knowledge has not been adequately and sufficiently utilized in this manuscript. Almost three years into the pandemic with a myriad of information and also scRNA-seq datasets, this was a little bit surprising to the reviewer, why this was not leveraged further. In this context, it also might be helpful for certain aspects to team-up with some of the leading centers/groups in COVID-19 scRNA-seq projects.

Following are major and minor concerns in the order as they appear in the text with an emphasis on the result section.

We would like to express our gratitude to the reviewer for their thorough revision, their comments about the interest of the topic, and for providing constructive and highly detailed comments, which we have addressed in full to prepare a revised version of the manuscript. In particular, we appreciate the suggestions regarding relevant scRNA-seq datasets and studies on COVID-19, which we have carefully considered and incorporated into our work. Below, we outline how we have addressed the concerns raised by the reviewer:

1 Line 67-70. Sentence seems to miss something, not clear what is meant.

We have improved the wording of this sentence and hope the message is clearer now (page 4, lines 80-86 of the revised marked manuscript).

2 Line 115: Provide a graphical representation of the time axis of sampling for all samples used within the generated datasets. Like this, the reader can directly see for each individual when sampling was performed. This cannot be judged from the current information provided in this manuscript. E.g. the term progression is not specified sufficiently. When exactly after onset of symptoms were the samples taken? Same for convalescence.

We agree that the time axis of sampling was not sufficiently clear and therefore we have included a plot depicting the timeline of sample collection for all samples used in the generated dataset (Supp. Figure 1a-b).
In addition, the days after COVID-19 symptoms onset when the samples were taken (for both progression and convalescence) are now indicated in Supp. Table 1a.

3 Line 117f: Wouldn't the authors have expected more severe courses of the disease? Any explanation, why all CVID patients only had mild disease?

Indeed, at the beginning of the COVID-19 pandemic, there was a general concern about a potentially higher severity of COVID-19 in CVID patients in relation to the general population. Fortunately, during the course of the pandemic, we observed that over 90% of the patients in the Freiburg cohort had a mild course, similar to the CVID patients presented in this study, with only very few patients with a severe course of the disease. This has been consistent with the clinical data of other CVID cohorts around the world. Based on our observations, we believe the immune system's response—specifically the expansion and enhanced cytotoxic activity of CD8+ T cells, the skewed Th1 polarization with higher levels of IFN-γ, and the robust type I IFN response—might have

contributed to controlling the viral infection in CVID patients. The lack of a specific antibody response may have led to a prolonged rather than a more severe disease course, similar to patients with agammaglobulinemia. Additionally, the absence of anti-IFN autoantibodies, which are linked to COVID-19 severity, might be a beneficial consequence of this lack of humoral response [PMID: 32972996]. This concept is now explicitly incorporated into the Discussion section of the revised manuscript (pages 33-34, lines 891-903 of the revised marked manuscript).

4 Line 121: The chosen published COVID-19 cohort is one of the worst datasets in the field and should be avoided at all means. This analysis needs to be repeated and validated with other datasets that have much higher qualities. Also, if using other datasets, the authors need to be very careful in not including patients that have been under corticosteroid therapy. It will dramatically impact on the interpretation of the findings.

We apologize for not having been precise enough detailing the references of the publicly available datasets that we used in our analysis of non-CVID individuals. Indeed, we have used single-cell datasets from three different published studies [PMID: 32788748 (Zhang et al, 2020, Nat Immunology), PMID: 32759967 (Yu et al, 2020, Cell Res), PMID: 32377375 (Wen et al, 2020, Cell Discov)], which were used in the manuscript by Ren and colleagues. However, we have not used any of the datasets specifically generated by Ren et al. We have now clarified the original studies from which we obtained the non-CVID datasets used in our study, and we have also included a table indicating the original studies (Supp. Table 1a).

All these studies offer valuable datasets of non-CVID samples before, during, and after SARS-CoV-2 infection and, therefore, serve as important references for our observations in COVID-19 CVID patients. Furthermore, it is worth noting that the samples were collected at the very onset of the pandemic, thereby minimizing additional confounding factors associated with vaccination or the specific SARS-CoV-2 strain. In any case, following the reviewer's recommendation in order to validate our previous observations, we have now incorporated the analysis of additional publicly available datasets (including samples from Stephenson E et al., 2021 Nat Med., Georg P et al., 2022 Cell, Khoo WH et al., 2023 Clin Immunol., and Yao C et al., 2021, Cell Rep), but excluding samples from patients under corticosteroid treatment ('validation non-CVID 2 cohort', Supp. Table 1a). This validation non-CVID 2 cohort, included 14 samples at baseline, 19 samples during progression (11 mild and 8 severe COVID-19), and 13 samples at convalescence (8 mild and 5 severe COVID-19) (Supp. Table 1a).

5 Line 126: The Ren et al dataset has ~1.46 million cells, but only ~140,000 cells were chosen without describing the criteria for inclusion or exclusion of samples and cells from the dataset. This needs clarification. But even better would be to exclude the Ren dataset, use two of the much more reliable datasets in the field and compare both independently in this study (like an in-project validation cohort). This is important, because when using previously generated datasets without CVID patients, the differences might come only from technical differences. This is not yet excluded entirely when validating by a second previous dataset, but it would already be mitigated to a certain extent. As is, this is not the case. Differences might just come from technical aspects differing between the different cohorts compared here directly.

The samples selected from non-CVID donors from the mentioned datasets (PMID: 32788748 (Zhang et al., 2020, Nat Immunology), PMID: 32759967 (Yu et al., 2020, Cell Res), PMID: 32377375 (Wen et al., 2020, Cell Discov) were chosen to match our CVID cohort in relation to age and sex (see Supp. Table 1b-1c). Therefore, while the number of cells is lower than the entire datasets, this approach minimizes potential biases related to age and sex.

Please see the previous point in which we refer to the specific publicly available scRNA-seq datasets used for this study. We have now specified the studies from which we obtained those datasets of non-CVID individuals (Supp. Table 1a).

As mentioned before, we have now used additional publicly available datasets (non-CVID 2 cohort) in order to validate our observations.

6 Line 132: This is one of the more reliable datasets that should be used instead of Ren et al. However, it is important to ensure that when using the COVID-19 data from this study, the patients with corticosteroid therapy need to be excluded. One additional other large COVID-19 dataset should be used as validation (not integration).

We appreciate the suggestion regarding the dataset by Stephenson et al., 2021 Nat Med., PMID: 33879890. Integrating additional publicly available datasets can indeed enhance the validity and robustness of our study. Therefore, we have now generated a validation cohort, termed non-CVID 2, which includes samples from Stephenson E et al., and also from other studies [PMID: 35032429 (Georg P et al., 2022 Cell), PMID: 36539107 (Khoo WH et al., 2023 Clin Immunol.), PMID: 33357411 (Yao C et al., 2021, Cell Rep)]. This validation cohort (non-CVID 2 cohort) includes samples from baseline, progression, and convalescence stages, derived from mild and severe COVID-19 patients not treated with corticosteroids (see Supp. Table 1a).

7 Line 133: define "census". There are so many ways of cell labeling. The authors should just name the method they used.

For clarity, we have now removed the term census and now it is simply mentioned as ‘transcriptomic analysis’ (line 167, page 7, revised marked manuscript). Cells were manually annotated based on known cell markers and marker genes used in other studies in the field. This is specified in page 40 on the revised marked manuscript.

8 Line 134: In Figure 1b, the total number of cells in the analysis needs to be shown. Also, classical QC parameter of the integrated dataset are missing. Need to be added, see other high-level scRNA-seq data papers in the field.

We have now indicated the total number of cells in the analysis in Figure 1b (and Supp. Figure 1h), as well as in the subsequent UMAPs corresponding to the different immune cell compartments. In addition, we have now included QC parameters of the integrated dataset (Supp. Figure 1c-g), similar to several high level scRNA-seq data papers in the field.

9 Line 134 - 141: The listing of the cell type should be avoided in the text, this is legend within Fig 1b. Describe findings, e.g. cell population level and compare to known findings in the field concerning this high-level analysis.

Following the reviewer’s recommendation, we have now removed the list of cell types in the Results section to avoid duplication with the figure legend. In addition, we have now contextualized our findings of cell population levels with previous literature.

10 Line 142ff: Why did the authors choose to directly focus on IFN response. Make a serious effort to provide information about the overall changes (major pathways and mechanisms changed) and only then start to zoom in on certain aspects like IFN signaling. Also, what is exactly meant with ‘analyzed’? In Fig 1C a Go-Term is presented, was this a kind of enrichment analysis. Further, a FC is shown: which groups were compared? Did you correct for different cell numbers per patient group etc. etc. Please, the authors need to be more precise.

Following the reviewer's suggestion, we have now conducted a systematic and unbiased gene ontology analysis, identifying all the significant altered pathways and responses for each of the immune cell compartments analyzed (including B cells, NK cells, CD4+ and CD8+ T cells, and various monocyte subsets). This global analysis is included in the revised Supp. Table 4. Only then, we have zoomed in on specific dysregulated relevant pathways based mainly on a list of immunological features described in the methods section (page 42, revised marked manuscript).

The term 'analyzed' is no longer applicable since we have now removed the initial global type I IFN response analysis.

For the calculation of GO terms scores related to several immunological categories, a list of genes related to those GO categories was obtained from the GSEA molecular Signatures Database. First, in order to normalize the data excluding potential biases in raw counts due to different sequencing depth, we calculated counts per million (CPM). Second, in order to correct for different cell numbers per patient group, we calculated gene expression scores in each single-cell and depicted the mean of these scores by PID status (CVID, non-CVID), stage (baseline, progression or convalescence) and cell population. We have now specified this aspect in the revised manuscript (page 42-43, lines 1118-1121, revised marked manuscript).

11 Line 146ff: This is probably the most important finding. From the text, it was not entirely clear to this reviewer, whether the non-CVID groups also have samples before and during COVID-19, or if in these groups the comparison is between COVID-19 patients and a historical control group of samples derived from before the pandemic? If so, can the authors better describe, whether this difference in study design between the two groups exists. And if so, how can the authors exclude that a historical group of healthies might just differ in signal to COVID-19 due to other reasons (e.g. technical reasons, or lower baseline levels of these genes, etc. etc.). As this finding seems to be central, some more information to the study design for these questions and an independent validation of this finding is required.

Thanks for highlighting the importance of the findings related to the persistence of type I IFN response. In the case of non-CVID individuals, the samples prior to the infection were not paired with those from the progression and convalescence time points. This contrasts with the CVID patients, for which the entire baseline-progression-convalesce sets are paired. To the best of our knowledge, there is not any study using paired samples at baseline and progression in non-CVID individuals, and this is why our CVID cohort is very unique. We have now improved the description of this difference in the study design between the two groups (page 36-37, lines 959-961, revised marked manuscript).

To avoid potential biases due to the different origin of the datasets, we performed an integration and batch correction approach (lines 1044-1054,, page 40, revised marked manuscript). This is a strategy used in several studies where samples from different datasets are integrated to generate a single object [PMID: 34331874; PMID: 38552005, PMID: 36720220, PMID: 34548323]. Furthermore, in our analysis, we considered counts per million (CPM) which correct for potential differences in raw counts due to different sequencing depth.

As suggested by the reviewer, to validate our findings and to exclude technical or cohort-associated bias, we have now included 4 additional and independent scRNA-seq datasets. This validation non-CVID 2 cohort, included 14 samples at baseline, 19 samples during progression (11 mild and 8 severe COVID-19), and 13 samples at convalescence (8 mild and 5 severe COVID-19) (Supp. Table 1a).

Finally, we have also performed a validation at the protein level using spectral cytometry, where both CVID and non-CVID individuals were analyzed (Supp. Table 1a and 1c). These independent cohorts included samples from additional CVID patients (3 samples at baseline, 4 samples at progression, and 4 samples at convalescence), as well as additional non-CVID individuals (4 samples at baseline, 6 samples at progression, and 4 samples at convalescence) (Supp. Table 1a).

12 Line 153: This is very interesting. How would the authors explain, that despite the fact of a complete lack of IgA and almost complete lack of IgG, these patients made it through COVID-19 with mild disease? Does this mean that antibodies after all are not that important? Make an attempt to address this most important aspect of CVID in context of COVID-19. For example, would there be experiments to address this further?

We agree with the reviewer that this is an unexpected outcome. At the beginning of the pandemic, we would not have been able to predict this either. Remarkably, it has been reported that agammaglobulinemia patients also suffer from prolonged but not more severe disease, as in our case [PMID: 37124420, PMID: 34159904, PMID: 32333914]. Nevertheless, the defective humoral response in CVID patients might be relevant not for the severity of COVID-19 but for the increased risks of both initial infection and subsequent reinfection [PMID: 36211430].
As indicated in our response to point 3, we have now discussed why these patients might display only mild symptoms during SARS-CoV-2 infection (pages 33-34, lines 891-903 of the revised marked manuscript).

13 Line 155ff: Apparently in Fig. 2a, the B cells were subsetted, but there is no information about from which dataset this was started, how many cells remained, which samples are now part of this subset of cells, if samples were excluded and if so, which criteria were used. This needs update.

We agree that this was not well described. We have now clarified that we zoomed in on annotated B cells and performed a reclustering of all the B cells contained in the initial object, excluding the two patients lacking B cells for downstream analysis. We have clarified this matter in the revised manuscript (lines 200-204, page 8, revised marked manuscript). In addition, we have now included the total number of cells analyzed in the B cell compartment (revised Figure 2a, and revised Supp. Figure 2b).

14 Line 163ff: Again, very interesting, but was this analysis corrected for different cell numbers per group? What is the cellular distribution of the signals, how many cells / subset are positive? Some more information is required.

As mentioned above, first we considered counts per million (CPM) which correct for potential differences in raw counts due to different sequencing depth. This is clarified in lines 1047-1049, page 40 of the revised manuscript.
Second, we calculated the mean of the gene expression per group by PID (CVID or non-CVID), stage (baseline, progression or convalescence) and cell population. We have now clarified this in the Methods section (see page 41, line 1086-1088 in the revised manuscript). In addition, we have now included the percentages (pct) of cells expressing each gene in the revised Supp. Table 3.

15 Line 173f: Isn't this already known, if so please cite respective reference.

We have indicated the reference of previous studies where a defective NF-kB pathway activation in the B cell compartment of CVID patients has been described [PMID: 27461466].

16 Line 177ff: well, this reviewer would argue that the previous findings (ref 33) is just not complete and therefore with the scRNA-seq data, one now can see that the dysregulation of the canonical NF-kB pathways is detectable in both naive and CD21low B cells. Along these lines, the suggestive sentence should be rephrased accordingly. It is not so much an issue about whether this is a consequence of presence or not of expanded CD21low B cells, it is more about the effect is seen in both cellular compartments, which is the power of scRNA-seq.

We agree with the reviewer and now the reference to the CD21low compartment has been removed in the revised manuscript. In this regard, the new re-analysis of the TF activities indicates that the activation of the canonical NF-kB pathway is impaired in both naïve and US-memory B cells from CVID patients.

17 Line 180ff: The authors chose an approach to zoom into a particular TF directly, probably because it has been described before to be associated with CVID (see reference 36). More informative would have been a modeling approach to define the most important TFs involved in the observed deviations, which requires to apply a pipeline including TF expression analysis, TF differential expression analysis and TF binding prediction analysis to key genes (e.g. those altered in a certain cell subset). Such analysis should be added to achieve several things: first, it classifies the importance of HIF1 in context of other potentially important TFs, second, it makes much better use of the data at hand. Thirdly, it might identify the really important TFs involved in the pathophysiology observed.

In response to the reviewer's suggestion, we have now incorporated the tool decoupleR [PMID: 36699385] to estimate TF activities (see page 11 line 273 in the revised marked manuscript). This tool infers the TF activity, at single-cell level, based on the weighted expression of the target genes of each TF (regulons). In addition, instead of focusing on selected TFs, we have now included the entire list of TFs whose activity is significantly altered and could contribute to the observed differences.
The activity of many TFs is influenced not only by protein or mRNA levels but also by post-translational modifications (such as phosphorylation, acetylation, etc) and subcellular localization (translocation from cytoplasm to nucleus). Therefore, we believe that analyzing TF activities using decoupleR, rather than TF gene expression, can better address the reviewer's concern.

18 Line 184ff: What is the connection of this sentence with the aspect of HIF1 one sentence before. Remains unclear.

We have now removed this sentence since HIF1 findings were not validated with the improved re-analysis of TFs activities.

19 Line 187: This is certainly of interest, but the reader cannot judge from the data representation, whether these terms are among the most enriched terms, whether those terms were chosen manually, and if so, how. Needs further improvement of text and/or figure.

We have now performed a more comprehensive gene ontology (GO) analysis, which is presented in the revised version of the manuscript. First, we have calculated differentially expressed genes (DEGs) between baseline and progression in the CVID cohort or in the non-CVID 1 cohort (revised Supp. Table 3). Then, we selected only those DEGs in the non-CVID 1 cohort that were also validated in the non-CVID 2 cohort. We then clustered them based on their expression profile during SARS-CoV-2 infection, and grouped them in three categories: 'BL equal', comprising genes that exhibit similar expression levels in CVID and non-CVID individuals at BL; 'BL CVID up', for genes that are upregulated in CVID patients compared to non-CVID individuals at BL; 'BL CVID down', including genes that are downregulated in CVID patients compared to non-CVID individuals at

BL. Next, we performed GO category enrichment analysis of the genes in the different clusters of DEGs. We have included a complete list of all the significant GO categories for the reader's perusal (revised Supp. Table 4). Finally, we have focused on specific significant GO categories that matched with a list of terms related to immunological features. We have included the list of terms in the Methods section (see page 42 of the revised marked manuscript).

20 Line 190ff: Similar here again: "... of several genes encoding members of ...". The authors are requested to make a better case of how they choose to show the data, how they choose the genes, what are the rules behind the selection? As is, it could be rather arbitrary and this reviewer thinks that it cannot be the intention of the authors to make arbitrary decisions during the analysis process. E.g. which other non-BCR related genes have the same pattern? What is their role, etc. etc.

We agree with the reviewer that we had not properly exploited the global analysis in a more comprehensive manner in the initially submitted version of the manuscript. Therefore, we have now performed a comprehensive analysis showing additional dysregulated pathways in CVID beyond the BCR. In this regard, we have depicted the global analysis of all differentially expressed genes between CVID and non-CVID during SARS-CoV-2 infection, together with a complete list of significant GO categories that are enriched (revised Supp. Table 4).

21 Line 197ff: Again, this would be another interesting aspect. However, it remains unclear to the reviewer, which cells are compared here. Earlier in the manuscript it is clearly stated that switched B cells and PCs are reduced in CVID. But these would be the interesting once to be compared. If the comparison would be all B cells, then it would not be surprising that COVID-19 non-CVID patients would have higher SARS-CoV-2 associated BCR sequences in their blood. This needs further clarification, what actually was compared here.

We have clarified that this comparison of the BCR repertories is made for naïve and un-switched memory B cells at all stages of SARS-CoV-2 infection (line 283-285, page 11, revised marked manuscript).

We respectfully disagree with the notion that comparing switched memory B cells and plasma cells (PCs) would be the most pertinent approach in the context of CVID. Primarily, these B cell subsets are typically absent in many CVID patients, rendering such comparisons unfeasible. Moreover, given that these populations originate from naïve B cells, examining dysregulated responses in pre-GC B cells can provide clues about alterations that might contribute to the humoral immune response.

22 Line 201 and throughout the manuscript: The term COVID-19 controls could be misleading. For CVID it is the control, but in fact these are also COVID-19 patients. Since there are also controls without COVID-19, maybe the better term would be COVID-19 non-CVID as it is stated in several figure panels. Optimize throughout the manuscript, text, figures, legends.

We agree with the suggestion and we have now replaced the term 'COVID-19 controls' with 'COVID-19 non-CVID' throughout the entire manuscript and figures.

23 Line 206: It is indeed fascinating to see that naive B cells in CVID are showing transcriptional deviations. But it would have been also very important to provide at least some evidence how this could be explained mechanistically, or which pathways might be involved, which TFs. Also, how do the authors explain impairment of NF-kB activation, yet highly increased expression of BCR-pathway related molecules. Some more explanatory insights based on some additional experiments here would significantly improve this part of the manuscript.

Our data reveal that several BCR-related genes are upregulated in the B cell compartment of CVID patients during SARS-CoV-2 infection. Following the reviewer's recommendation for performing a global analysis of the TF activities, our results suggest that this increase in BCR-related genes could be linked to the higher activity of PAX5 [PMID: 22669466] observed in CVID patients during infection (Supp. Figure 2g). However, these BCR-related genes upregulation affects both activator and inhibitor molecules. Indeed, we confirmed through spectral cytometry that CD22, one of these inhibitory molecules, is also upregulated at the protein level. It is important to note that since we did not exclusively examine the fraction of B cells generating SARS-CoV-2 specific responses, our findings reflect overall alterations in B cells during viral infection and recovery, including activation by TLR ligands and cytokines. Therefore, we cannot attribute the impaired activation of the NF-kB pathway exclusively to defective BCR signaling. Based on our observations of the persistent activation of naïve and un-switched memory B cells, we hypothesize that the chronic immune-dysregulated background characteristic of complex CVID patients, may contribute to a higher basal activity of the NF-kB pathway, hindering its proper activation during an immunological challenge such as SARS-CoV-2 infection. We have indicated this idea on page 11, lines 280-282, revised marked manuscript.

24 Line 212ff: similar as before, the subsetting of the T cell and the NK cell compartments need more clarification, following statements made for the B cells above. Supp Figure 2a: It would be much more informative, if the most relevant and cluster-defining genes for the clusters rather than genes related to certain literature-based T cell types would be shown. As is, it is not convincing, because many genes are expressed in many cell types not really classifying the cell types here. This is expected, but it is therefore also not very informative.

As mentioned in point 13 for the B cell compartment, we have now included the total number of cells analyzed in the T cell and NK cell compartments (page 13 and 20, together with new Figure 3a, 5a, and new Supp. Figure 3b and 5c). Moreover, we explain that we zoomed in on annotated T or NK cells, and performed a reclustering of all the cells contained in the initial object. We have now clarified this matter in the manuscript (pages 13 and 20 in the revised manuscript).
Also, as requested, we now show the most cluster-defining genes for the different cell clusters in an unsupervised manner for all the immune compartments.

25 Line 214ff, Figure 3b: The reader needs more information about how these terms were retrieved from the analysis. Are these the Top terms, are these chosen manually from a list? Just more precise information. Also, the authors should take effort to compare the NK cell annotation provided here with previous publications on NK cells in COVID-19, e.g. there have been reports entirely focusing on NK cells, e.g. in Immunity, 2021 already. This needs more work and more inclusion of already existing knowledge in the field about NK cells in COVID-19.

As indicated in point 19, we have now performed a more comprehensive gene ontology (GO) analysis, and we have included a complete list of all the significant GO categories for the readers' perusal (revised Supp. Table 4). Finally, we have focused on specific significant GO categories that matched with a list of terms related to immunological features. We have included the list of terms in the Methods section (see page 42 of the revised marked manuscript).

We appreciate the reviewer's suggestion regarding the incorporation of previous knowledge on NK cell compartment annotation in the context of COVID-19. The study referenced provides insights into a cohort similar to ours concerning the SARS-CoV-2 viral strain, which we found immensely useful. As per the recommendation, we have conducted a reclustering of the NK compartment and annotated six populations of NK cells, mirroring the approach employed in the aforementioned study. Moreover, we have aligned our

annotations with those from the referenced study (Supp. Figure 5b). Additionally, we have now included the analysis of pro-fibrotic features in NK cells (Supp. Figure 5h), which is one of the main findings by Krämer et al. 2021.

26 Line 219: This is an interesting statement. Probably similar to other extended expression deviations in CVID patients, it would be really of great interest to see, if CVID patients just have a prolonged inflammatory state and if so, is this reflected by any clinical observations and also, if these phenomena are turning to normal at later time points. Since the authors seem to sample these patients regularly, an even later additional time point to address these questions should be possible to be added.

In relation to the possibility of including additional samples at later time points, this would have been a very interesting addition. However, the inclusion of such samples may have introduced confounding factors, such as vaccination and Ig replacement treatments with plasma containing anti-SARS-CoV-2 antibodies, which could potentially impact the observed immune responses.

27 Lines 220ff and Sup Fig 2b: This reviewer would argue slightly different: If looking at the CD56high NK cells in CV: GZMB and GZMA are also still upregulated in non-CVID patients. Maybe focus only on those genes that are really different, like PRF1 and NKG7. If so, the question also is, why were only these four genes chosen, why not making a more data-driven analysis and report on those genes that are still dysregulated in CVID patients at CV but not at CV in non-CVID patients. Like this, the reader would get a more complete picture about what the differences really are. To some extent the authors do this with the cytotoxic score, but this still leaves out other potential molecular changes, pathways and programs. Concerning the score: while mentioning that the Methods section shows details about the score, the reader should be guided here already, whether the score is based on manually picking genes or a data- and algorithmic-based approach.

As indicated in point 25, we have now performed a comprehensive gene ontology analysis for NK cells, identifying and showing additional dysregulated pathways and programs in COVID-19 CVID patients beyond cytotoxicity (Figure 5d and Supp. Table 4).
We agree with the reviewer that an unsupervised analysis, without restricting the selection to a limited number of genes, provides a more accurate insight into the process. Therefore, we have now calculated a cytotoxicity score for both CVID patients and non-CVID individuals at different stages of SARS-CoV-2 infection (baseline, progression, and convalescence). This score considers the expression of a comprehensive list of genes included in the GO category *'leukocyte mediated cytotoxic-related genes GO:0001909'*. We have elaborated on this approach in the Methods section (page 42-43, revised marked manuscript), and we have also specified the details of the analysis in the Results section in order to facilitate the reading (page 21, revised marked manuscript).

28 Line 224ff: Now the authors introduce some other pathways, but overall, it looks a little bit like cherry picking. A more data driven approach first and then highlighting some specific findings would make this analysis more robust and would guide the reader better about the importance of the reported findings in respect to the overall changes observed by scRNA-seq. One important question concerning the non-CVID patients: What do the authors think is the reason for such a huge upregulation of IFNG genes during convalescence? The question is, are these patients already truly convalescence or do these patients show prolonged signs of disease? Either clinically or molecularly or both. Would be important to report on this in the patient description section of this manuscript. Overall, it goes towards the question: what does this difference really mean? Is it solid and would this be similar in a validation cohort? To really make these statements some additional analyses would be required.

As mentioned before, we agree with the reviewer that it is very useful to show a more comprehensive picture of the dysregulated responses. Therefore, we have now generated new analyses covering all significantly enriched pathways beyond those previously selected.

According to the publicly available studies used for generating our non-CVID 1 cohort [PMID: 32788748 (Zhang et al, 2020, Nat Immunology), PMID: 32759967 (Yu et al, 2020, Cell Res), PMID: 32377375 (Wen et al, 2020, Cell Discov)], all samples from convalescent COVID-19 non-CVID patients were obtained from recovered individuals, with no mention of prolonged signs of disease by the authors.

Regarding the gene expression of *IFNG*, the inclusion of an additional non-CVID cohort (non-CVID 2 cohort) following the reviewer's recommendation, shows that, although the revised analysis did not validate the upregulation of this gene observed in non-CVID 1 cohort (Supp. Figure 3g), there was a general higher expression of *IFNG* in the CD4+ memory T cell compartment of CVID patients compared with non-CVID individuals from both cohorts.

We sincerely appreciate the suggestion of including an additional validation cohort. This inclusion has allowed us to mitigate, to some extent, certain artifacts in the analysis that might be associated with specific samples rather than the biological process itself.

29 Lines 228ff: The figures are oriented along the genes, but the text is oriented along the different cell types, which really makes it somewhat difficult for the reader to quickly see the message in the figures. Either change the text to describe the figures or change the figures to better follow the flow first NK cells than T cells.

That figure is no longer included in the revised manuscript.

30 Lines 236 and Figure 3e: Along the same lines as already stated for NK cells and CD8 cells, the increased expression of inflammatory cytokines during CV in non-CVID COVID-19 patients requires additional validation, either by including another cohort of such patients' scRNA-seq data or alternative experimental approaches, but it needs to be excluded that these non-CVID patients chosen for this study are not the exception rather than the rule for convalescence.

The inclusion of the validation non-CVID 2 cohort indicates that the behavior of *TNF* gene is not equivalent in both non-CVID cohorts. Therefore, we decided to remove this finding in the revised version of the manuscript.

31 Line 245ff and the whole paragraph before concerning cytotoxicity: There has been a recent report in Cell concerning CD16+ cytotoxic T cells with detrimental function in severe COVID-19 patients. What is the relationship of the cytotoxic T cells seen here in CVID patients and these CD16+ T cells previously reported? The authors should make an attempt to bring this together with previous knowledge in the field.
Supp Figure 2c: Could the authors turn the figure by 90° so that the cell names can be easily read? Also the terms used in the figure and the text need to be equal. The reviewer presumes that Tprolif are Tcell cycling in the figure. Is this correct? If so, correct accordingly.

To enhance the clarity of our analysis for readers, we have chosen to annotate the T cell compartment using conventional labels such as naïve, central memory, effector memory, and TEMRA. However, upon the reviewer's suggestion, we have also re-annotated T cells according to the article by Georg et al., published in Cell in 2022, to provide context for our results in relation to previous findings in the field. This re-annotation has enabled us to identify a population of CD8+ T cells expressing CD16 (FCGR3A+), which appears to be expanded in non-CVID individuals with severe COVID-19 as described by Georg and colleagues (see revised

Supp. Figure 4c). Interestingly, this expansion is not observed in patients with mild COVID-19, regardless of their CVID status (revised Supp. Figure 4c). We have incorporated these findings into the revised manuscript (pages 422-430, pages 16-17, revised marked manuscript).

The aforementioned Supp Figure 2c is not present in the new version of the manuscript. Now we have used the term Proliferating MKI67++ to label this proliferating subset of T cells.

32 Lines 255ff: Actually, this might come from the subsetting strategy. An alternative to subset NK cells and T cells together is to do this separately. Very often, this leads to a better separation of NK cells and T cells and then allows to clean the subsetted datasets of T cells and NK cells respectively and then, such observations are usually excluded. Either the authors change the last sentence and focus on the overall findings of this paragraph or they test the alternative subsetting strategy, or both.

As suggested by the reviewer, we have now performed a separate subsetting of NK and T cells (see Figures 3, 4 and 5, and their corresponding sections in Results).

33 Line 260ff: reference 10 missing here. As also mentioned for the other cell types, the authors need to bring their data into context of previously described subsetting of the different immune cell types in COVID-19, here the myeloid cell compartment. Otherwise, it will be impossible to make definite conclusions from this new dataset and the comparison with non-CVID COVID-19 patients. All previous comments and suggestions stated earlier for the other figures apply here as well.

We have now included the reference. As suggested, we have now used the annotation strategy described by Schulte-Schrepping, J. et al., Cell 2020 (Supp. Figure 6b) in order to bring our data into context with previously described subsettings of monocytes.
We have also followed the previous recommendation made for the other immune compartments to myeloid cells regarding comprehensive analysis of dysregulated pathways and programs, correction of number of cells, and use of a validation cohort of non-CVID individuals.

34 Line 262ff: As also stated for the other cell types, the authors need to provide a date-driven full picture of all the major pathways being altered. As presented here, it looks like cherry-picking. Focusing on certain pathways once the whole picture is provided might be ok, but as is, this would not be a meaningful description of the overall findings within the myeloid cell compartment of CVID patients in relationship to COVID-19 disease.

Following the reviewer's recommendation, we have now generated new analyses covering all significantly enriched pathways beyond the selected molecules (revised Supp. Table 4).

35 Line 268ff: This is another example of not being precise enough. There is a difference in what is here now called non-classical monocytes during CV. Regulation does not seem to be completely identical.

We agree with the reviewer that the previous statement was not sufficiently accurate so we have now clarified the paragraph in order to be more precise in the explanation of the observed results regarding inflammasome-related gene expression in the different monocyte subsets (page 24, lines 624-632, revised marked manuscript).

36 Lines 270ff: When looking at what is termed here classical monocytes, there is not much difference between non-CVID mild and CVID patients during PG, only during CV, where only CVID patients still show elevated signals. Again, important would be to provide further evidence that these changes are not due to the group of non-CVID patients included in the analysis (goes again towards an additional non-CVID COIVD-19 CV

population to be used for comparison). For what is called non-classical monocytes, this is rather similar. The biggest difference is seen in what is called intermediate monocytes. Here, the reviewer asks the authors to explain, how they derived these cell state, because this cell state is known to be rather difficult to pinpoint in scRNA-seq data (goes back to the issue of bringing cell type annotation into the context of prior knowledge within the COVID-19 scRNA-seq blood landscape.

Please refer to the revised analysis of the data, now presented in Figure 6f and Supp. Figure 7a, where CVID and both non-CVID 1 and non-CVID 2 cohorts are shown. The results indicate that the persistent activation of some inflammasome-related genes occurs specifically in the CVID group.

We identified classical, intermediate, and non-classical monocytes based on the expression of *CD14* and *FCGR3A* (CD16) markers, in line with previous scRNAseq studies (PMID: 32377375). Cluster 5, annotated as intermediate monocytes, exhibits expression of both CD14 and FCGR3A genes (see Figure 6b).

37 Line 279ff: Everything said to the inflammasome genes above, also applies to the TLR7 genes. A revised analysis will clarify this here as well.

In the revised version of the manuscript, we decided to remove the *TLR7* and *TLR8* findings as we were unable to validate them, following the suggestions of this reviewer, through the reanalysis involving an additional validation non-CVID 2 cohort.

38 Line 286ff: This is another interesting aspect. It is a little bit surprising to the reviewer that the authors did not make an attempt to experimentally show if such inhibitors could repress or at least blunt the proposed prolonged inflammatory state in CVID patients during CV. Incubating cells from these patients during CV with inhibitory ligands and reading out transcriptional changes could have addressed this rather easily. It would at least hint towards a potential molecular mechanism of the phenomenon of prolonged inflammatory immune cell responses (if validated) in these patients and thereby would have increased the novelty and impact of the findings. The authors need to think about such experiments to be added to the manuscript.

During the manuscript revision, we conducted caspase-1 activity assays as a surrogate measure of inflammasome activation. Evaluation of caspase-1 activity in monocytes revealed an increased inflammasome activity in CVID patients compared to non-CVID individuals during SARS-CoV-2 infection (Figure 6h). Interestingly, the increased inflammasome activity observed in CVID patients during progression did not persist at convalescence, despite the elevated expression of inflammasome genes. This suggests a partial inflammasome priming at that stage of COVID-19. Consequently, as we did not validate the increased inflammasome activity at convalescence in CVID patients, we did not find it necessary to conduct the suggested assay. Additionally, due to the limited cell quantity in each sample, we prioritized the new spectral cytometry analysis along with the inflammasome activity assay.

39 Lines 414ff: All patient samples used in this study, including those from previous studies require a full table with clinical metadata, this is missing. For examples, see descriptions of previous large COVID-19 datasets (except for Ren et al, which is also insufficient in this respect).

We have now updated the clinical metadata table initially provided (revised Supp. Table 1a), which includes age, sex, disease stage, CVID EUROclass classification, levels of anti-SARS-CoV-2 IgM, IgA and IgG, treatments, genetic status, time of sample collection after onset of COVID-19 symptoms, etc.

40 Line 570: Accession number at EGA as well as accession procedure are missing

We have now included the required accession number under the EGA dataset accession EGA50000000354 (page 46, revised marked manuscript).

REVIEWERS' COMMENTS

Reviewer #1:

There is substantial reworking of the manuscript following reviewers comments. FIgure 7 is particularly useful for summarizing their findings in CVID patients during COVID infection and convalescence. CVID patients lacking B cells similarly to agammaglobulinemia patients have milder course of infection has been reported. Overall there are new insights into these patients. One would have thought such a heterogenous group of CVID patients lacking genetic diagnoses would have rendered the scRNAseq results uninterpretable. However the authors managed to present a snapshot of the dynamic gene expression in the adaptive and innate immunity in CVID patients. It is likely there will be more insights to be unearthed from the scRNAseq data.

There is currently no scRNAseq in CVID published in literature so this manuscript is novel for the field of CVID.

We thank the reviewer for their supportive comments and for recognizing both the challenges and potential of scRNAseq in this context, as well as the novel insights our manuscript provides into the field of CVID.

Reviewer #2:

In their revised manuscript, Javier Rodríguez-Ubreva and colleagues have provided additional information on the methodology and the collection of data enhancing our interpretation of the presented data.
At this point still, this reviewer's concern is that conclusions are based on massive data production from a limited number of patients with a heterogeneous condition.

We thank the reviewer for their constructive feedback and for recognizing the improvements in methodology and data presentation. We understand the concern regarding conclusions drawn from a large dataset based on a limited and heterogeneous patient sample.
To address this, we have revised the discussion to explicitly acknowledge these limitations, highlighting how sample size and patient heterogeneity may impact the generalizability of our findings. We also detail the inclusion of additional samples from CVID patients analized by spectral cytometry to ensure the robustness of our observations. We hope this additional transparency adequately addresses the reviewer’s concerns.

Reviewer #3:

The manuscript now entitled "COVID-19 progression and convalescence in common variable immunodeficiency shows dysregulated adaptive responses and persistent type-I interferon and inflammasome activation" by Rodriguez-Ubreva at al is a first revision of a recently submitted paper. The authors have revised the large number of necessary and requested major and minor concerns which has significantly improved this manuscript. Overall, the data are now much more clear and due to additional validation analyses, which eliminated some of the findings reported in the first submission, the findings described in this manuscript are now - in general - more valid. The addition of further experiments validating some of the initial findings with other experimental approaches also contributed to more sound results.

We sincerely appreciate the referee's thoughtful review. The insightful feedback and valuable suggestions provided have been crucial in improving the quality of the manuscript. The revisions and additional validation experiments, guided by these comments, have strengthened our findings, and the authors are grateful for the referee's role in this process.

There are some minor aspects that should be clarified.

Lines 459-461. In this sentence it is stated that a comparison was performed between non-CVID and CVID patients. In which figure is the comparison shown. This was not clear enough to this reviewer.

The comparison mentioned in this sentence is shown in Figure 3g and Supp. Figure 4d, as previously indicated in paragraph 433-439 of the revised marked manuscript.

Line 581: Severe COVID-19 patients display functional and phenotypic alterations, mild COVID-19 not. Add "severe".

We have now added "severe" to the sentence.

Supplemental Figure 2d, 3e, 4a, 5e, 6e: color code for showing scores: very colorful, maybe a little bit too colorful: therefore, try to use simpler color scheme

We have now used a simpler color palette.

The discussion section is rather long now, if necessary, the authors could shorten.

Following the reviewer's suggestion, we have removed some redundant paragraphs in the discussion to shorten that section.