nature portfolio

Peer Review File

Defective kinase activity of IKKα leads to combined immunodeficiency and disruption of immune tolerance in humans

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This manuscript has been previously reviewed at another journal. This document only contains reviewer comments, rebuttal and decision letters for versions considered at Nature Communications.

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

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Author comment
The authors have gone to considerable lengths to address the issues arising following the initial review of their manuscript for consideration for publication in Nature Immunology. These additions and clarifications have certainly improved message of the study and lessened some of the more ambiguous data sets. There a still a few outstanding points that I believe need comment by the authors.

1. In vitro culture of naïve B cells and CD4+ T cells to address cell-intrinsic defects. its great the authors did these expts. my concerns are the culture methods and the magnitude of the reported responses. Specifically, it is stated that 150 x 10^3 naive B cells and 250 x 10^3 naïve CD4 T cells were cultured in 96 well plates. This seems a very high density! Beyond this, the levels of Ig secretion induced by CD40L/IL4 also seem very high – this culture does not yield much secretion of switched Ig isotypes especially after only 5 days. It is also well-known that IL-4 can antagonise production of IgA. Similarly, in the absence of polarizing conditions (eg Th1, Th17 etc) naïve CD4 T cells produce very low levels (if any) of cytokines such as IFNg, IL17A, IL-10 in response to anti-CD3/CD28 stimulation. I do not doubt that there are B-cell and CD4+ T cell-intrinsic defects in the IKKa deficient cells, but the readouts of Ig and cytokine secretion in these expts suggest that the isolation of naïve B and naïve CD4+ T cells did not yield a very pure population from the healthy donors and these relatively high levels of secreted IgG/A/E and cytokines reflect the presence of contaminating memory B and CD4+ T cells.

2. The authors state (lines 464) that “serum anti-IgM and anti-IgG antibody levels against IFN-alpha 2a were significantly increased in P1 and P3”. I think the authors mean “levels of autoreactive serum IgM and IgG specific for IFNa2a were significantly increased in P1 and P3 compared to healthy donors”?

3. Along the same lines, can the authors confirm whether these anti-IFNa autoAbs were neutralizing? Also, as the patients were hypogammaglobulinemic, can the authors comment as to when the serum samples were collected in terms of receiving IgRT? Ie could the result be “diluted” by exogenous Ig?

Reviewer #2

(Remarks to the Author)
The concerns of Reviewer #2 have been addressed.

By characterizing the T cells as "potentially" auto-reactive, I think this avoids the stronger conclusion that they are indeed auto-reactive. While oligoclonality would add further evidence, the data presented by the authors is I think sufficient to conclude that there are potentially auto-reactive T cells. This, together with the other data makes a conclusion of autoimmunity quite reasonable.

The concerns regarding selective cytokine expression deficits in T cells have been addressed.

Concerns regarding the FACS plots have been addressed although it would be helpful to add the positive gate in Fig 4E as is present in the other histograms.

The presentation of the transcriptomic data is sufficiently understandable and a reasonable effort has been made at normalization.

One note: The figure legend to Fig 6I-K is mis-labelled for the panels.

Reviewer #3

(Remarks to the Author)
In their revision, the authors have gone at significant length and further improved the quality of the manuscript. In particular, the authors have provided substantial and important new data to further support the claims and findings outlined in the manuscript.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have provided appropriate responses to the issues raised during re-review of the revised manuscript. I believe this paper new represents a strong and solid body of work with conclusions well supported by the presented data. This is worthy of publication in Nat Comms

Point-to-point response for the Reviewer Comments

We thank the esteemed reviewers for their insightful comments and helpful suggestions, which significantly improved our manuscript (NCOMMS-24-24449A). We have carefully considered remaining concerns raised and provide a point-by-point response below.

Reviewer #1:

The authors have gone to considerable lengths to address the issues arising following the initial review of their manuscript for consideration for publication in Nature Immunology. These additions and clarifications have certainly improved message of the study and lessened some of the more ambiguous data sets.

We are delighted to hear that the reviewer appreciates the substantial amount of work we have put into the revision, and that this has resolved most of the concerns previously raised by this reviewer.

There a still a few outstanding points that I believe need comment by the authors.

1. In vitro culture of naïve B cells and CD4+ T cells to address cell-intrinsic defects. its great the authors did these expts. my concerns are the culture methods and the magnitude of the reported responses. Specifically, it is stated that 150 x 10^3 naive B cells and 250 x 10^3 naïve CD4 T cells were cultured in 96 well plates. This seems a very high density!

We agree with the reviewer's comment on the importance of the right cell density for naïve T cell stimulation. Our intention was to maximise the detection of cytokine production in these experiments. Our cell density in culture is 1x10^6/ml (250,000 cells in 250 µl culture medium), which is widely used in the literature for analysis of cytokine production from T cells (such as Sugie et al. PNAS 2004, PMID: 15465914). Independent studies have observed that higher cell densities yield better T cell activation through anti-CD3 and anti-CD28 antibodies. As an example, data from Ma et al. (2010) (PMID: 20625484) shows that a 1x10^6/ml cell density provides the best TCR-based T cell stimulation in culture (6-well plate), consistent with our observations in 96-well plate. In the context of 96-well plates, we identified a range of cell numbers reported, even up to 3x10^5 cells per well (Schultz HS et al., PLoS One, 2017), which is higher than our cell density.

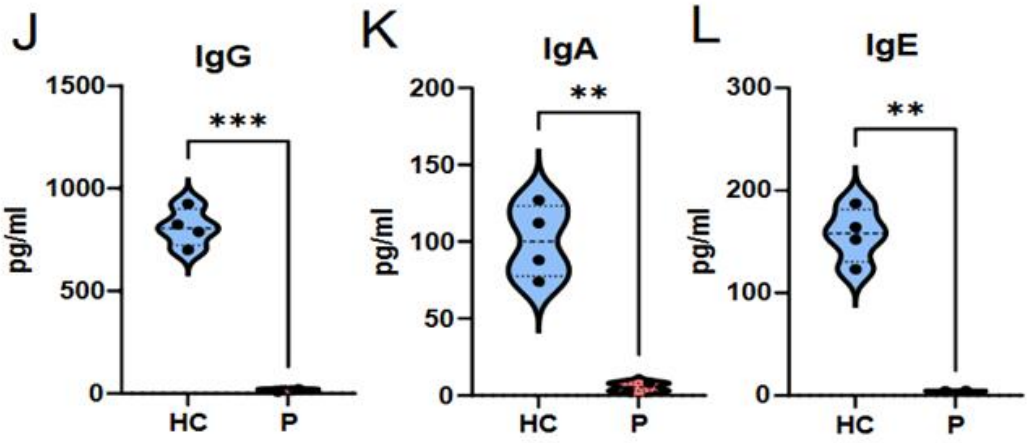
Our naïve B cell culture density is 0.6x10^6/ml (150,000 cells in 250 µl culture medium). While a broad range of cell densities are used in the literature for B cell class switching, some protocols use even much higher concentrations for stimulation. As an example, the protocol by Zaheen et al. 2010 Journal of Visualized Experiments (PMID: 20736917) recommends using 3.2x10^6/ml B cells in culture (96-well plates), and the study by Marasco et al. Eur J Immunol. 2017 (PMID: 27800605) recommends using 2.5x10^6/ml (culture plate not indicated), both of which are much higher than our

culture density. Therefore, we are of the opinion that 0.6x10^6/ml density is within the acceptable range.

Beyond this, the levels of Ig secretion induced by CD40L/IL4 also seem very high – this culture does not yield much secretion of switched Ig isotypes especially after only 5 days. It is also well-known that IL-4 can antagonise production of IgA. Similarly, in the absence of polarizing conditions (eg Th1, Th17 etc) naïve CD4 T cells produce very low levels (if any) of cytokines such as IFNg, IL17A, IL-10 in response to anti-CD3/CD28 stimulation.

The reviewer correctly indicated that IL-4 is not the strongest stimulus for B cell class switching, especially compared to IL-21. We did not have the chance to test CD40L+IL-21, however, in response to the reviewer's concern, we have repeated our experiment with CD40L+IL-4. According to these results, IL-4+CD40L stimulation clearly shows that all IKKα^{G167R} patients have significant cell intrinsic defects in all Ig subtypes. However, the reviewer was indeed correct regarding the IgA response in the culture. Upon using new reagents to prepare standard curves, we observed much lower IgA levels in the culture. Based on our experience, slight changes in standard curves or gating strategies could change the results in the analysis of bead-based assays. We have updated this now in the revised version. We thank the reviewer for their excellent point raised. We also wish to clarify that our naïve B cell culture stimulation was 7 days, not 5. Although correctly indicated in the Figure Legend of the previous version of the manuscript (Line 990), it was mislabelled as 5 days in the Methods (Line 180). We apologize for this oversight, which has now been corrected in the revised version (Line 180). We thank the reviewer for their attention to detail.

Fig-4J-L:



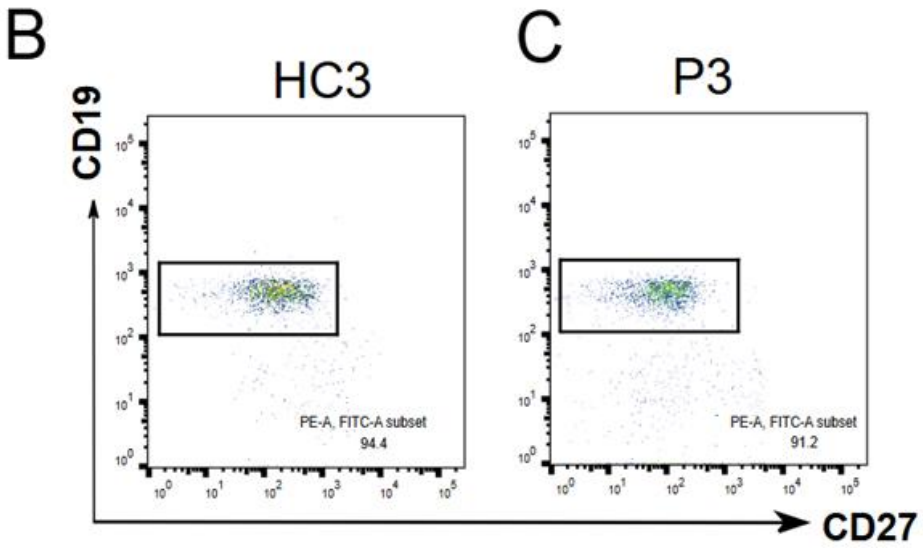
As the reviewer would appreciate, the production of cytokines by naïve T cells upon TCR stimulation in the absence of polarizing conditions is often considered a "baseline" response. As previously reported in the literature (such as Yu et al., JBC 2014, PMID: 25378399), naïve human CD4+ T cells do produce picogram levels of these cytokines, even in the absence of polarizing conditions. Indeed, because they

are at picogram levels from 2.5x10^5 cells in culture, unlike the nanogram levels typically observed in polarizing conditions, this is a low level of induction, consistent with previous findings. However, this response is significantly lower in cells from all three patients compared to healthy paediatric control samples. Furthermore, similar levels of TNFα production clearly indicate that not all responses are non-specifically blunted in the patients' naïve T cells. Overall, we agree that the culture density is high, but this is because we are aiming to look at the baseline response and not to seed, expand, and then analyse polarization as done with the low seeding density cultures.

I do not doubt that there are B-cell and CD4+ T cell-intrinsic defects in the IKKa deficient cells, but the readouts of Ig and cytokine secretion in these expts suggest that the isolation of naïve B and naïve CD4+ T cells did not yield a very pure population from the healthy donors and these relatively high levels of secreted IgG/A/E and cytokines reflect the presence of contaminating memory B and CD4+ T cells.

We agree with the reviewer's comment on the importance of the purity of naïve cells. In the previous version of our manuscript, we provided the purity check of our naïve T cell purification (Fig-S4A) after using BioLegend's MojoSort™ Human CD4 Naïve T Cell Isolation Kit. As shown, CD4+CD45RA+ naïve T cells constitute 94-98% of our culture. Given our culture's high purity of naïve T cells and the negative selection-based purification method, we believe there is minimal risk of culture contamination or unintended antibody activation. Additionally, TNFα production is comparable in the cultures of naïve CD4+ T cells from healthy controls and patients after side-by-side purification, stimulation, and analyses. If memory T cells were present differentially in the cultures of naïve CD4+ T cells from patients and healthy controls, we would expect to see higher levels of TNFα production in healthy controls. Similarly, using BioLegend's MojoSort™ Human Naïve B Cell Isolation Kit, we routinely achieve 92-95% purity from PBMCs. We now provided the purity check of naïve B cells in our revised version (Fig-S2B-C).

Fig-S2B-C:



Nonetheless, we have taken the reviewers comments on board and have amended the text in the results section to state that "While these data show that IKK^{G167R} causes intrinsic differences in naïve CD4+ T cells, which were purified by negative selection, we cannot rule out a contribution from memory T cells to the cytokines detected" (Line 535-538).

Finally, for both B cell immunoglobulin and T cell cytokine production assays, we believe that different commercially available reagents may provide different levels of detection. This is especially true when comparing ELISA vs. bead-based detection assays, which have different sensitivities. However, since our experimental conditions are consistent for cells from both patients and healthy controls, we are confident that our data accurately represent the true biology of the kinase function of IKKα.

2. The authors state (lines 464) that "serum anti-IgM and anti-IgG antibody levels against IFN-alpha 2a were significantly increased in P1 and P3". I think the authors mean "levels of autoreactive serum IgM and IgG specific for IFNa2a were significantly increased in P1 and P3 compared to healthy donors"?

Once again, we thank the reviewer for their attention to detail. The reviewer is right. This is now corrected in the revised version (Line 464-465) as:

"Indeed, as previously reported in many patients with genetic defects in non-canonical NF-κB pathway [54], levels of autoreactive serum IgM and IgG specific for IFNa2a were significantly increased in P1 and P3 compared to healthy donors".

3. Along the same lines, can the authors confirm whether these anti-IFNa autoAbs were neutralizing? Also, as the patients were hypogammaglobulinemic, can the authors comment as to when the serum samples were collected in terms of receiving IgRT? Ie could the result be "diluted" by exogenous Ig?

We agree with the reviewer that further characterization of anti-IFN autoantibodies would be useful. However, unfortunately, we currently lack the resources to deeply study the neutralizing characteristics of these anti-IFNα autoantibodies in patients' serum. However, the landmark study by Le Voyer et al., Nature 2023 clearly demonstrates the neutralizing effects of autoantibodies against IFNα in patients with mutations in the non-canonical NF-κB pathway. We have now indicated in the revised version that we were not able to test this point. However, we anticipate a similar observation in our patients (Line 467-470):

"Although we were not able to test the neutralizing characteristics of these anti-IFNα autoantibodies, we anticipate similar findings to those observed in patients with mutations in other components of the non-canonical NF-κB pathway."

Regarding the collection of serum samples, the reviewer is correct that anti-IFNα autoantibodies might be stronger if not diluted by IVIG treatment. This is now noted in the text (Line 466-467). We thank the reviewer for bringing this to our attention:

“It is also worth noting that anti-IFNα autoantibodies might be stronger if not diluted by IVIG treatment in patients.”

Reviewer #2:

By characterizing the T cells as "potentially" auto-reactive, I think this avoids the stronger conclusion that they are indeed auto-reactive. While oligoclonality would add further evidence, the data presented by the authors is I think sufficient to conclude that there are potentially auto-reactive T cells. This, together with the other data makes a conclusion of autoimmunity quite reasonable. The concerns regarding selective cytokine expression deficits in T cells have been addressed. Concerns regarding the FACS plots have been addressed although it would be helpful to add the positive gate in Fig 4E as is present in the other histograms. The presentation of the transcriptomic data is sufficiently understandable, and a reasonable effort has been made at normalization. One note: The figure legend to Fig 6I-K is mis-labelled for the panels.

We appreciate the reviewer's positive feedback on our manuscript and thank them for their valuable comments and attention to detail. We have addressed their concerns by modifying Figure 4E to include a positive gate and correcting the inadvertent mistake in the Figure 6I-K legends (Lines 1052-1061):

Reviewer #3:

In their revision, the authors have gone at significant length and further improved the quality of the manuscript. In particular, the authors have provided substantial and important new data to further support the claims and findings outlined in the manuscript.

We are grateful to the reviewer for their positive assessment of our manuscript.