nature portfolio

Peer Review File

SUBSTANTIAL ADAPTATION IN HUMAN IMMUNE CELLS RESIDING IN TISSUES AT THE FRONTLINE OF INFECTIONS

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this study, Salvador Martinez & Murga-Moreno et al., have used ABC-MK along with developmental and adult single cell immune cell atlases to analyze adaptation in human immune cells since the divergence from chimpanzees. By examining over 1.2 million cells, they identified specific immune cell types and states with high adaptation rates. The study also investigated the innate immune response to various inflammatory and pathogen exposures using macrophages derived from activated induced pluripotent stem cells. I appreciate the robust approach of the authors in accounting for various potential confounders by matching their gene list with a range of genomic features. However, as written, the paper seems to miss a well-defined conclusion, making it challenging to discern new insights beyond the well-known enrichment of immune-related genes for positive selection signatures.

The authors aim to identify cell types targeted by selection by:

1. Identifying genes expressed differently between specific cell types (marker genes).
2. Checking if these genes show enrichment for selection signatures.

However, I have several concerns regarding these analyses and the interpretation of the results:

1. Variability in Detection Power: The ability to identify significant enrichments likely varies across cell types, depending on the number of differentially expressed (DE) genes. This raises the question of whether the prominence of certain cell types is due to more selection or merely differences in detection power. I realize that the authors used the top 500 DE when possible, but some cell types probably had less than that. Is there are relationship between the number of DE genes uses and the enrichments observed. On a related topic, what was the rational to use p-value < 1x10-4, logFC > 1 to classify DE genes. The authors should instead use an FDR (although I get that the ranks will not change, I find it important to know how many genes included in each group are truly DE). Only genes up-regulated in one cell type vs the others were considered?

2. Significance of Selection Signals: The significance of the selection signals, particularly after adjusting for multiple tests across various cell types, remains unclear. The authors generate an permutation-based p value per cell type, but there is no correction for the number of tests done across all the different cell types and conditions.

3. Contribution of Shared Genes: The extent to which a small subset of shared genes across certain cell types contributes to the observed enrichments is uncertain. I imagine that the list of DE genes are shared across cell types. Are there particular genes that contribute to most of the signal?

4. Relevance of Differentially Expressed Genes: Genes that vary in expression across cell types may not be the most relevant for the function of immune cells. For instance, while T cells express higher levels of CD3 and other markers, their functional relevance becomes apparent only upon TCR engagement and activation of a different gene set.

Reviewer #2 (Remarks to the Author):

*** Summary ***

The manuscript from Salvador-Martinez et al entitled “QUANTIFYING ADAPTIVE EVOLUTION OF THE HUMAN IMMUNE CELL LANDSCAPE“ uses an ABC-based McDonald Kreitman test to analyze adaptive evolution of the immune system in the human lineage. Specifically, it leverages single cell RNA-seq datasets from embryonic and adult immune cells to define cell-type specific genes sets and measures proportion of adaptive non-synonymous substitution across these gene sets. The same approach is then applied to bulk RNA-seq data from iPS-derived macrophages stimulated with a variety of immune stimuli, mimicking either viral or bacterial infections. Finally, the authors test the hypothesis that increase adaptive mutations in specific cell-types could be due to differences in the phylogenetic age of genes between different cell-types, and find that most cell types are characterized by genes that appeared more than 400 million years ago around the time of the emergence of the adaptive immune system.

*** overall appreciation ***

The manuscript is well written and proposes an interesting approach to define and compare the evolutionary importance of specific components of the immune system. The authors provide a number of appealing statements, including the strong adaptation targeting fetal hematopoietic stems cells, high adaptation rate in adult immune cell types localized to barrier tissues (lung, gut, epithelial cells), and high adaptation rate in genes involved in early responses to viral & bacterial pathogens. While I think the analyses performed and the results by themselves are of value and deserve publication, I think that some of the methodological choices and interpretations could be further justified to strengthen the manuscript.

*** Major comments ***

- First, it is not clear form the manuscript if and how multiple testing is accounted for when testing multiple cell types and conditions for excess of adaptations, and what is the percentage of false positives expected among the reported results. I feel that applying a Benjamin-Hochberg correction on the number of cell types / condition-tested would help to establish confidence in the reported results.
- I have concerns that some of the interpretations, in particular related with adaptation in cell-type residing in barriers-tissues, may have more to do with prior expectations, than with data-derived analyses. Indeed, while there is indeed signatures of adaptation in some subset of lung resident memory T-cells & dendritic cells, other cell types that could reasonably be considered as lung-resident such as alveolar macrophages or DC2, do not exhibit such signatures, raising doubts on such an interpretations. I would suggest that the authors try to provide a more systematic view of adaptations in lung resident cell types, by also reporting lung resident cell type that do not exhibit increased adaptations.
- It is unclear how the presented results are influenced by batch/technical variables. In in particular, it is well known that RNA-seq experiments are highly sensitive to batch effects & experimental artifacts, and that subtle change in the experimental design can alter greatly the measured transcriptomes. It would be interesting to see how well the results on adult are replicated when considering for RNAseq profiles from online databases such as DICE (https://dice-database.org/) or data from Monaco et al, 10.1016/j.celrep.2019.01.041).
- On a similar note, it's not clear how powered the proposed approach is for the detection of adaptation, and how cell counts could impact power. This makes it difficult to known

whether differences between adult and embryonic tissues (eg, for Tregs or ILC3) should be interpreted as such, or simply viewed as random fluctuation of results due to low power. I would suggest (i) to show a scatter plot of log Pvalue vs total number of cells in the cell type and (ii) to consider one cell type with high cell counts and significant adaptation results and test how the estimated rate of adaptation α is altered when progressively down sampling this cell type (eg. from 100% to 50, 20, 10, 5, and 1%) prior to the DE analysis. Ideally, I would recommend resampling with replacement to allow some variability in the 100% cases, and perform ~10 resampling for each cell number (assuming that this is computationally tractable).

*** Minor comments ***

- While the authors put a lot of care in adjusting for an important number of technical confounders, it is not clear what the relevance of each confounder is. I would be interested in seeing a supplementary figure where we estimate the impact of each confounder on the estimated adaptation rate. This could be done by comparing adaptation rate between genes within the top/bottom decile of each confounder, while adjusting for all other confounders.
- Finally, it would help the interpretation to provide a supplementary data table with the specific gene sets associated with each cell types in order to allow the identification of specific genes that drive the signal at the cell type level. While my understanding is that the ABC-MK test cannot be directly applied at the gene level, I think providing estimates of dN/dS and pN/pS at gene level together with a few confounding metrics (Background selection, GC content etc…) could still help prioritize genes likely to underlie the cell-type-specific adaptation signals reported in the paper.
- I'm not sure that bootstrapping is the adequate term for the resampling scheme used to assess significance. Bootstrapping usually refers to estimating the properties of an estimand (eg. variance, expectancy, 95% CI), rather than building a null distribution (which is conceptually more akin to permutation testing). I would suggest to refer to the method as "resampling" rather than "bootstrapping".

*** Typos and rephrasing ***

p5 lines 2, 19-20 & 26 + p9 line 34 + p10 line 12 + p11lines 33-34:

Numbers of Supplementary figures and tables are missing

p2, lines 31-32
The sentence "This approach is particularly useful when weakly advantageous alleles segregate along with the evolutionary process known as background selection" is unclear. What does it mean to segregate with an evolutionary process? please rephrase.

P15, lines 35-37
The sentence "For example, adult DCs respond to toll-like receptor stimulation inducing effector T cells, whereas foetal DCs induce Treg cells as a mechanism of tolerance and immune suppression" is unclear. What does it mean to segregate with an evolutionary process? please rephrase.

Fig 3C & supplementary Figure 5
R484 --> R848

*** Additional analyses/suggestions ***
Below are a few additional suggestions, which I think could further strengthen the paper, should the author decide to incorporate them. I am however conscious, that the suggested analyses represent an extension of the present manuscript beyond its initial scope and will thus leave to the authors to decide whether they believe that these analyses are worth pursuing.

- p4 lines 23-24:
"a differential gene expression test for each cell population compared to all remaining cells in the same compartment"
- This suggest selection that affected a whole cell compartment (eg. shared myeloid genes) would not be considered. Have the authors considered doing an analysis where they assess selection directly at the level of cell compartment (contrasting each compartment against all others)?
- While the authors account for overall gene expression in GTEx tissues and expression in

testis, one could argue that some cell-type specific adaptation detected in immune cells, may be driven by adaptation in another unobserved tissue or cell type. As such it could be interesting to compare the signals of adaptation detected in immune cells with those detected in other GTEx tissues. In particular, it could be interesting to assess whether tissues that diverged rapidly at the transcriptomic level such as liver and testis (Brawand et al, Nature, 2011) are associated with higher rate of adaptive non synonymous mutations.
- Finally, it would be interesting to apply the proposed method to assess how adaptation has altered response to cytokines in a more systematic manner. This could be done using the CytoSig database which combines >20,000 transcriptomics profiles to characterize response to cytokines across a wide array of cell types, stimuli and time point (https://doi.org/10.1038/s41592-021-01274-5

Maxime ROTIVAL

Reviewer #3 (Remarks to the Author):
Review of "QUANTIFYING ADAPTIVE EVOLUTION OF THE HUMAN IMMUNE CELL LANDSCAPE" by Salvador-martinez et al.

I found this to be an interesting paper. My background is in more traditional immunology and evolutionary biology so my comments focus on a rather general view of the paper from this viewpoint. It is essential that the paper be reviewed by a couple of systems biologists such as those from the Bustamante or Malik groups who would have expertise on the details and validity of the methods used.

Overall the paper does something interesting. The conventional view is that pathogens and hosts are in an "arms race" and thus one might expect to see increased rates of evolution in "immune-associated genes". For the most part this has been observed (for example see Nielsen et al PMC1088278), but is not always observed as is the case for some genes such as the IFN-g gene see Mary et al PMID 21448974). The advance in the current paper is that it identifies cells types / lineages in which the immune genes that show high rates of evolution are expressed. They do this by combining the information on the genes that show high rates

of adaptation together with developmental and adult immune cell atlases to identify the cell types in which these genes are expressed.

Not surprisingly they find that cell types from both the lymphoid and myeloid compartments to harbour significantly increased adaptation rates, after all the earlier studies found higher rates of adaptation in immune related genes. But more specifically they identified elevated rates of adaptation in:

1. Cells in the adaptive response such as foetal Pre-Pro B cells and adult TRM (resident memory) CD8+ cells show highly elevated rates of adaptation.
2. Cells of the innate immunity responding to early events following infection such as iPSC-derived macrophages also showed elevated rates of adaptation.
3. They suggest that the evolutionary changes involved in 1 and 2 arise from mutations in genes rather than the recruitment of new genes.

All this is relatively new, and very interesting. On this basis I am in favor of publication.

Given limitations in my expertise on the methods used in this paper I would insist that a couple of referees with expertise in the relevant areas also look at the paper.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

MAJOR COMMENTS:

1. Variability in Detection Power:

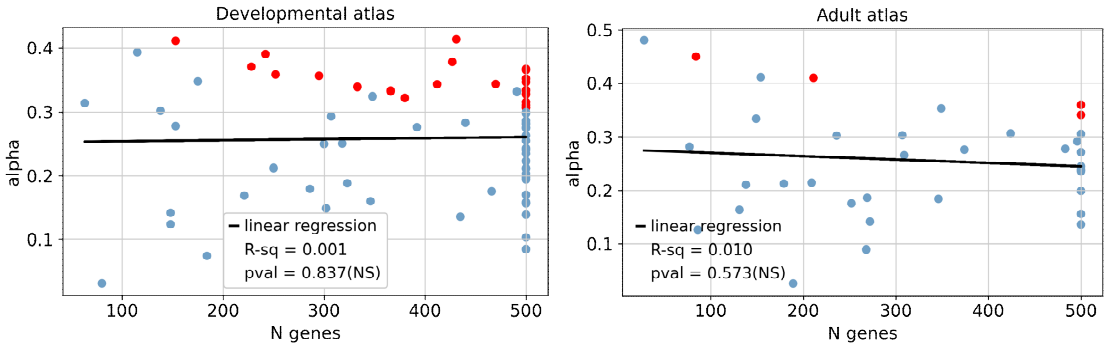
The ability to identify significant enrichments likely varies across cell types, depending on the number of differentially expressed (DE) genes. This raises the question of whether the prominence of certain cell types is due to more selection or merely differences in detection power. I realize that the authors used the top 500 DE when possible, but some cell types probably had less than that. Is there are relationship between the number of DE genes uses and the enrichments observed.

Response: We agree with the reviewer that this is an important point to be addressed. First, we looked into the possible relationship between the number of DE genes and the alpha value using a linear regression. We did not find any significant relationship between both variables. Neither we found a relationship between the number of cells by cell-type and alpha (see comment of reviewer 2). Therefore, we are confident that the selection signatures are not merely due to detection power. In the revised manuscript, we now provide scatter plots with the linear regression summary results as Supplementary Figure 1.

As the reviewer also noted, not all cell types have 500 DE genes, which could lead to a loss of statistical power when performing the MK test. We ran ABC-MK following Murga-Moreno et al. 2024 recommendations (https://academic.oup.com/g3journal/advance-article/doi/10.1093/g3journal/jkae031/7607895). Here, the authors showed that the ABC-MK software could perform robust α estimates, when the dataset accounts for more than 400-500 polymorphic sites depending on the sampling size. Following these results, we excluded datasets with less than 500 polymorphic sites from the analysis and, thus, do not expect a relationship between α estimations and the number of DE genes. Only the activated macrophages' PIC stimuli at an early stage had less polymorphic sites than this threshold. The main text now cites Murga-Moreno et al. 2024 and describes the new threshold used in terms of total number of polymorphic sites (page 4, lines 34-36).

Nonetheless, Figure Supplementary 8 from Murga-Moreno et al. 2024., shows how the confidence interval in α estimation increases when decreasing the total number of genes and polymorphic sites (an expected statistical behaviour with less data), which raises the uncertainty of ABC-MK. Such an effect can be highly increased in human datasets because of the low level of nucleotide diversity. Consequently, the resampling strategy followed to determine α significance could be affected for highly heterogeneous pools of genes in terms of polymorphic data. Therefore, we also estimated an unbiased False Discovery Rate (FDR) for α estimations for each significant cell type. By definition, the FDR fully reflects the variance in results expected under a given sample size, since it provides expectations if the same number of genes as the tested number were randomly sampled. It therefore helps accounting for scenarios where the high confidence intervals on α estimations are expected during the bootstrapping, where the total number of

polymorphic sites is near the established threshold. All the significant cases provided have an FDR less than 0.05, except for the Progenitor CMP cells, which showed an FDR of 0.071. In the next section (2. Significance of Selection Signals), we thoroughly described the FDR to answer the reviewer's concern about multiple testing.



On a related topic, what was the rationale to use p-value < 1x10-4, logFC > 1 to classify DE genes. The authors should instead use an FDR (although I get that the ranks will not change, I find it important to know how many genes included in each group are truly DE). Only genes up-regulated in one cell type vs the others were considered?

Response: We agree with the reviewer on the importance of accounting for multiple hypotheses testing when identifying DE genes. We apologise for the lack of clarity as, although we did perform an FDR correction (Benjamini-Hochberg) when selecting the DE genes, we did not describe it sufficiently in the Main text and Methods. When we mentioned “p-value < 1x10-4” we should have written “Benjamini-Hochberg FDR corrected p-value < 1x10-4”. This FDR correction was performed by the scanpy.tl.rank_genes_groups() function of the *scanpy* package.

Regarding the thresholds for the padj_val and logFC, we used very strict thresholds to guarantee the selection of strongly differentially expressed genes per cell type. Although there is no consensus on what threshold to use for DEG selection scRNA-seq, widely used packages like Seurat (Stuart and Butler et al, 2019) report DEG using pvals_adj<5x10-2 and logFC>0.1. Only genes upregulated in one cell type (with logFC > 1) versus the rest of the cells in the same compartment were considered for the analysis.

We have now specified the use of FDR corrected p-values and logFC selection of upregulated genes in the Methods as follows (changes in bold):

“For differential expression of the different cell types within a compartment, we ran a DE test using the scanpy.tl.rank_genes_groups() function of the scanpy package. **More specifically, we considered genes upregulated in one cell type versus the rest of the cells in the same compartment using the Wilcoxon test and selected the genes with a Benjamini-Hochberg FDR corrected p-value < 1x10-4 and a minimum log2fc value of 1. Finally, we** sorted the DE genes by their log2fold change values. **These thresholds were chosen in an effort to ensure the selection of clearly differentially expressed genes in each cell type. Although there is no consensus on what threshold should be used for DE analysis in**

scRNA-seq, widely used packages like Seurat (Stuart and Butler et al, 2019) report DEG using pvals_adj<5x10-2 and logFC>0.1."

2. Significance of Selection Signals:

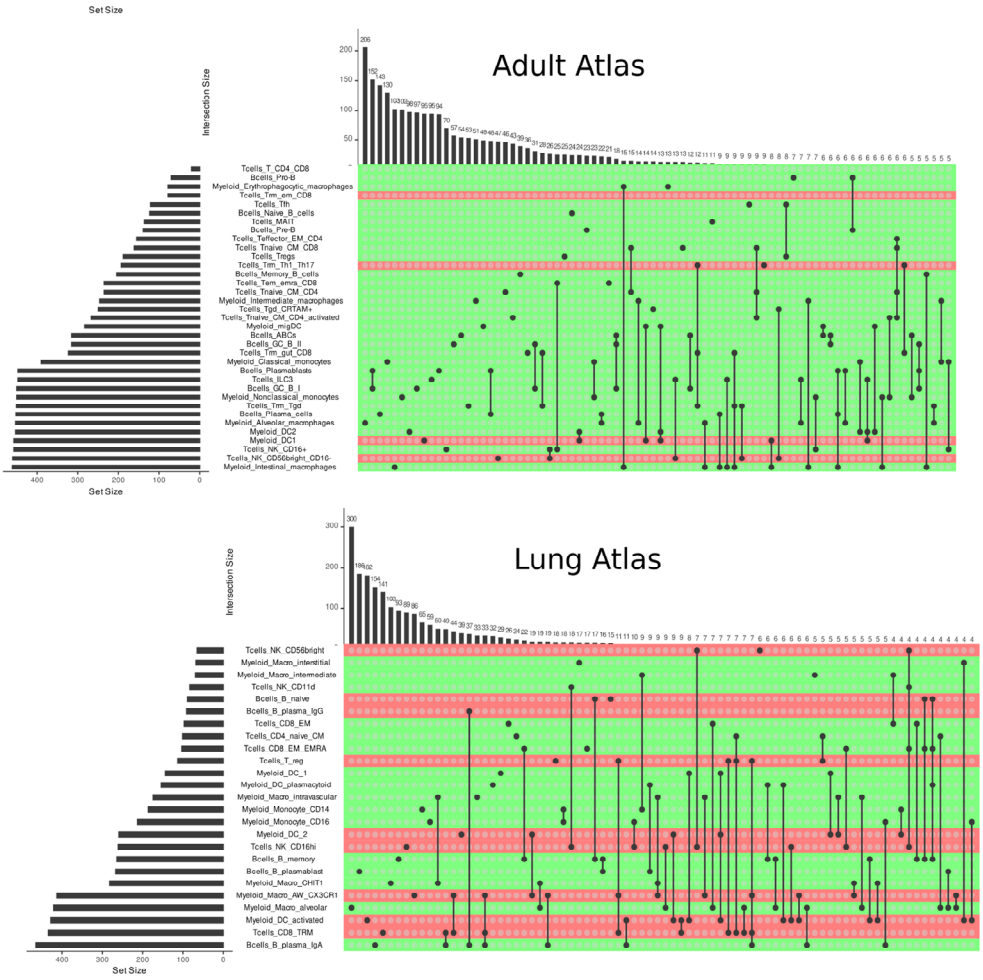
The significance of the selection signals, particularly after adjusting for multiple tests across various cell types, remains unclear. **The authors generate an permutation-based p value per cell type, but there is no correction for the number of tests done across all the different cell types and conditions.**

Response: We thank the reviewer for this very insightful comment. Because the bootstrapping was performed using intrinsic genomic features for each analysed case, the p-values should not be correlated among cell types. Nonetheless, we agree that empirical p-values can be affected and misinterpreted due to the presence of False Positive signals. To test the significance of the increase in adaptation in the immune gene set, we now re-run the bootstrap pipeline to automate an unbiased False Discovery Rate analysis while replicating results. We did not find any difference in the number of significant cell types when re-running the bootstrapping. Hence, we repeatedly performed ABC-MK analyses in gene sets of the same size as the differentially expressed immune genes, but creating 1000 datasets of random genes, made of a random mix of both the differentially expressed gene cases and the control datasets from the bootstrapping. This tested the effect of performing the analysis, but considering immune genes not to be different from random genes and with no impact on immune functions. By measuring the proportion of results for which α was indeed higher than the analysed case, we estimated an unbiased False Discovery Rate by generating a null distribution of estimates of adaptation without impact of the differentially expressed genes. We found that all the significant cases had a FDR less than 0.05 except for the Progenitor CMP cell type, which showed an FDR of 0.071. Adult DC1, developmental MACROPHAGE_LYVE1_HIGH, TYPE_1_INNATE_T, PRE_PRO_B and MAST_CELL, macrophages CIL stimuli at early stage and lung DC_activated and CD8-TRM cell types accounted for FDR values lower or equal to 0.005, being the lowest among the analysed dataset. Such findings indicate that the increase in adaptation rate regarding the base null distribution depends on the action of positive selection. Indeed, unbiased FDRs allow to directly estimate the number of cell types that would be below a given FDR threshold by chance. We tested 162 cell types (including the activated macrophages analyses) and observed eight cell types with their FDR lower than 0.005, which is clearly higher than the expected chance (0.81). Even if the observed number of cell types with a low FDR was similar to the expected number by chance, the enrichment of specific functions, such as early response or tissue-residency, would not be expected. Moreover, following the reviewer's recommendation in section #3 (Contribution of Shared Genes), we tested that there was almost no DEG overlap among cell types for each dataset, which validated and proved the statistical independence of the analyses as well as the FDR approach followed to correct for multiple testing. The main text (pages 6, lines 2-6,and Supplementary Figure 3) now includes results as well as the detailed methodology employed to generate the FDR (page 5, lines 24-32). We also updated figures 2, 3 and 5, supplementary figures 4, 5 and 7 and supplementary tables 2, 3, 5, 6, 7, 9 using the latest bootstrapping results.

3. Contribution of Shared Genes:

The extent to which a small subset of shared genes across certain cell types contributes to the observed enrichments is uncertain. **I imagine that the list of DE genes are shared across cell types. Are there particular genes that contribute to most of the signal?**

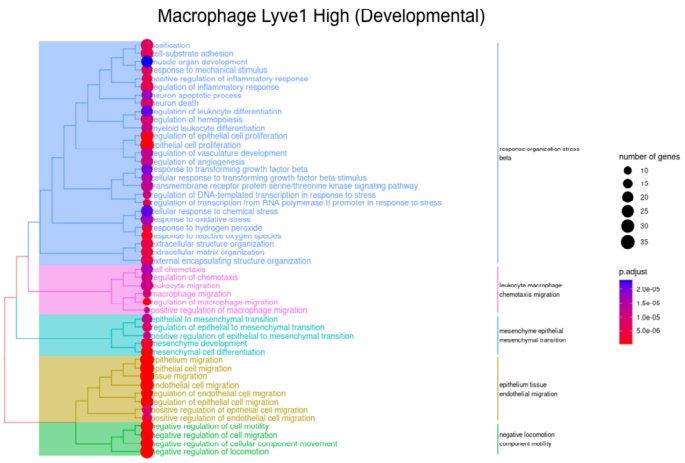
Response. We agree this is an important point to be addressed. As the reviewer rightly points out, the lists of DE genes are not exclusive, therefore leaving the possibility that the cells with significant adaptation share a subset of genes driving the signal. In order to visualise the amount of genes that are shared by different cell types, we generated an upset plot for each cell atlas, shown in the new Supplementary Figure 3. In the plots we can see that although there are many sets of genes shared by different cell types, these are mostly between pairs of lineage-related cell types (for example between B cell subtypes or between Macrophages subtypes). We did not find any considerable set of genes (e.g. greater than 5) shared by cell types with significant adaptation, so we conclude that the signal does not come from a particular set of genes. Additionally, it is essential to mention that because the nucleotide diversity level on the human genome considerably decreases the statistical power to detect adaptation on MK-test analyses at the gene level, it is unlikely that a few genes will lead to significant alpha estimation in a pool of hundreds of genes.

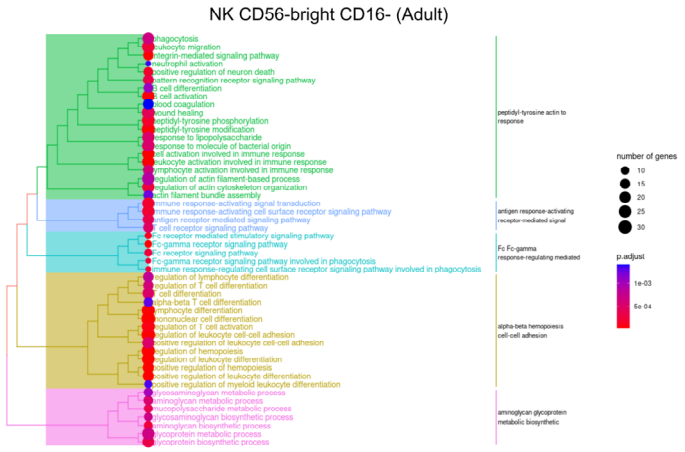


4. Relevance of Differentially Expressed Genes:

Genes that vary in expression across cell types may not be the most relevant for the function of immune cells. For instance, while T cells express higher levels of CD3 and other markers, their functional relevance becomes apparent only upon TCR engagement and activation of a different gene set.

Response. We agree with the reviewer that differentially expressed genes found in a normal physiological state might be relevant both for immune identities and functions, with the former being also under selective evolutionary pressure. However, we expect that many genes expressed under normal physiological conditions will become involved in immune functions after a stimulus/challenge. To test this we ran a GO term analysis using the Biological Process (BP) ontology for each set of differentially expressed genes used for the ABCMK test. For this, we used the package clusterProfiler (version 4.2.2) and the GO.db and org.Hs.eg.db packages for BioConductor version 3.14. Our results show that many cell type-relevant immune functions/processes are enriched in the set of DE genes. As an example, we show in Supplementary Figure 9 summary plots of the enrichment analysis in the DE genes from the cell-types “Macrophage Lyve1 high” and “NK-CD56-bright CD16neg”. In Supplementary Tables 11-13 we provide the results of the GOterm analysis for all the cell-types analysed in this study.





Reviewer #2 (Remarks to the Author):

***** Major comments *****

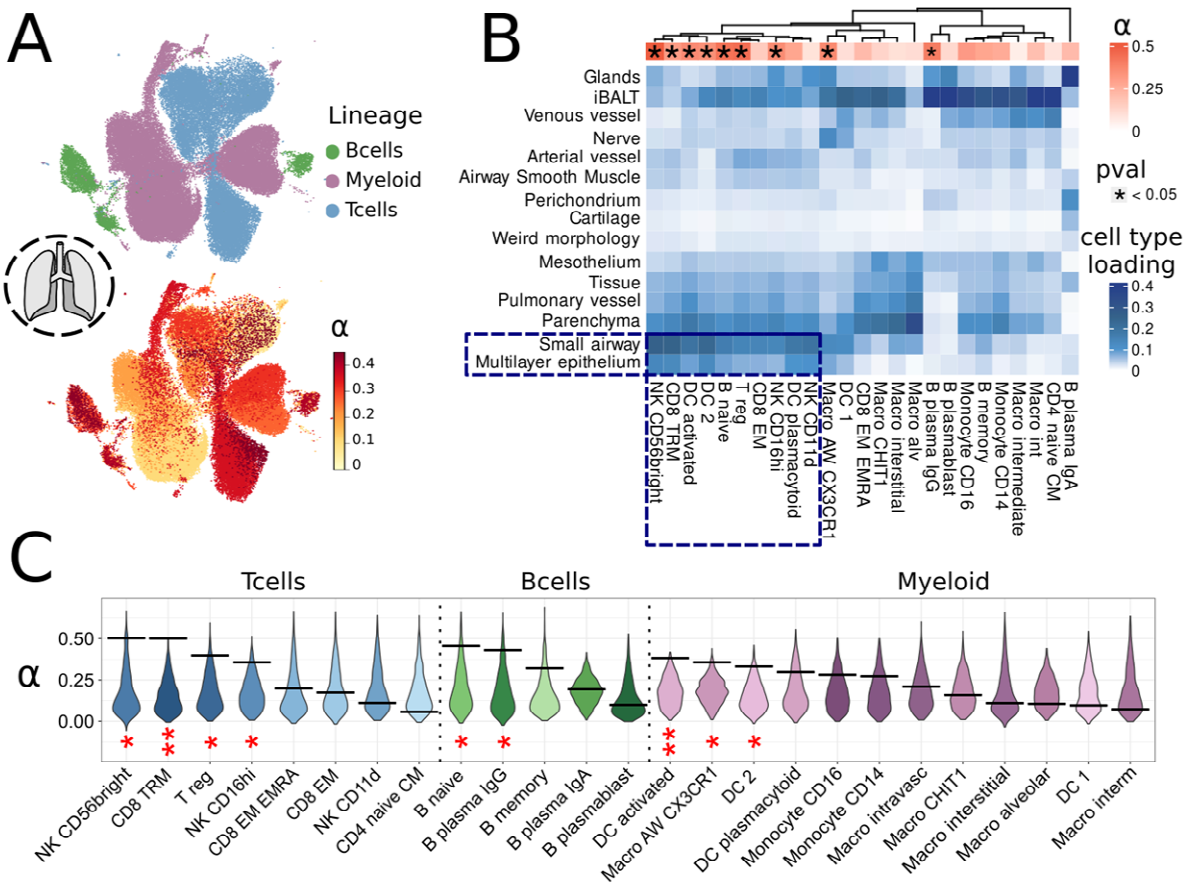
- First, it is not clear from the manuscript if and how multiple testing is accounted for when testing multiple cell types and conditions for excess of adaptations, and what is the percentage of false positives expected among the reported results. **I feel that applying a Benjamin-Hochberg correction on the number of cell types / condition-tested would help to establish confidence in the reported results**.

Response: In the original manuscript, we indeed did not account for multiple testing. Such a decision was based on the resampling technique employed, which used specific and intrinsic genomic features for each cell type and, hence, the resulting p-values should not be correlated. Nonetheless, we agree with the Reviewer that the empirical p-values estimated per cell type can be affected by False Positive signals, misleading the results and interpretation. To test the significance of the increase in adaptation in the immune gene set, we re-run the resampling pipeline to automate an unbiased False Discovery Rate (FDR) analysis, while replicating results. Here, we did not find any difference in the number of significant cell types when re-running the resampling. Hence, we effectively estimated the unbiased FDR by generating a null distribution of estimates of adaptation for each cell type, by measuring the proportion of analysis for which α was indeed higher than the analysed case.

Following the methodology described in Di. et al 2022 (https://www.biorxiv.org/content/10.1101/2022.12.01.518739v1.full), we now also performed ABC-MK analyses on significant cell types, using gene sets of the same size as the differentially expressed immune genes. This involved creating 1000 datasets of random genes that included both the differentially expressed genes and the control datasets obtained from resampling. We found that all the significant cases had an FDR < 0.05 except for the Progenitor CMP cells, which showed an FDR =0.071. Of note, adult DC1, developmental MACROPHAGE_LYVE1_HIGH, TYPE_1_INNATE_T, PRE_PRO_B and MAST_CELL, macrophages CIL stimuli at early stage and lung DC_activated and CD8-TRM cells, all had FDR values < 0.005, being the lowest among the analysed dataset. Such findings indicate that the increase in adaptation rate regarding the base null distribution depends on the action of positive selection. Moreover, as reviewer #1 also noted, the results can also be misinterpreted depending on the contribution of shared genes among cell types. For such a reason, we checked that there was no differentially expressed gene overlap among cell types for each dataset, which proved the statistical independence of the analyses and the FDR approach followed to correct for multiple testing. Figures and text now include the FDR results (pages 5, lines 24-32 and Supplementary Tables 10), and showed the contribution of shared genes among cell types (Supplementary Figure 3). We also updated figures 2, 3 and 5, supplementary figures 4, 5 and 7 and supplementary tables 2, 3, 5, 6, 7, 9 using the latest resampling results.

- I have concerns that some of the interpretations, in particular related with adaptation in cell-type residing in barriers-tissues, may have more to do with prior expectations, than with data-derived analyses. Indeed, while there is indeed signatures of adaptation in some subset of lung resident memory T-cells & dendritic cells, other cell types that could reasonably be considered as lung-resident such as alveolar macrophages or DC2, do not exhibit such signatures, raising doubts on such an interpretations. **I would suggest that the authors try to provide a more systematic view of adaptations in lung resident cell types, by also reporting lung resident cell type that do not exhibit increased adaptations.**

Response: Following the suggestion of the Reviewer, we decided to expand our analysis to include data from the Human Lung Cell Atlas (https://doi.org/10.1038/s41588-022-01243-4). This new dataset served to validate our findings from the Adult Tissue Atlas and to extend the analysis to additional lung-resident cell types and even spatial analysis. In contrast to the Adult Immune Cell Atlas, where the cells derive from different organs/tissues, the Lung Cell Atlas provides an in-depth transcriptomic profile of healthy human lungs, therefore allowing us to provide a systematic view of adaptation on lung-resident cells. These results are now shown in the new Figure 4 and confirm that CD8 TRM and NK CD56 bright cells (named “Trm em CD8” and “NK CD56 bright CD16-” in the adult) have indeed high adaptation in both independent datasets. Significant adaptation was also found in lung-resident cell types in the T cells compartment (Tregs and NK CD 16 high), myeloid compartment (Macrophages CX3CR1+, DC activated, and DC2) and in the B cell compartment (B naive and B plasma IgG). In order to further test our hypothesis that tissue-resident immune cells with high adaptation are preferentially localised in barrier tissues, we integrated spatial mapping of immune cells using Spatial Transcriptomics (ST) of the Lung. These new results represent a "cell type loading", a weighted value that represents the relative abundance of a given cell type in the different tissues areas (eg. parenchyma, epithelia). For all immune cell types, we clustered the mean cell type loadings across all samples (N=11) in order to group cell types with a similar tissue distribution. The new Figure 4B now shows that most of the cell types with significant adaptation cluster together (i.e.similar spatial distribution) and were preferentially localised in “Small airways” and “Multilayer epithelium”. The localisation of immune cell types with high adaptation in these barrier tissue areas further support the hypothesis of high adaptation through barrier tissue immune functions. We have added a new section in the manuscript to describe and discuss the results from the Lung Cell Atlas analysis.

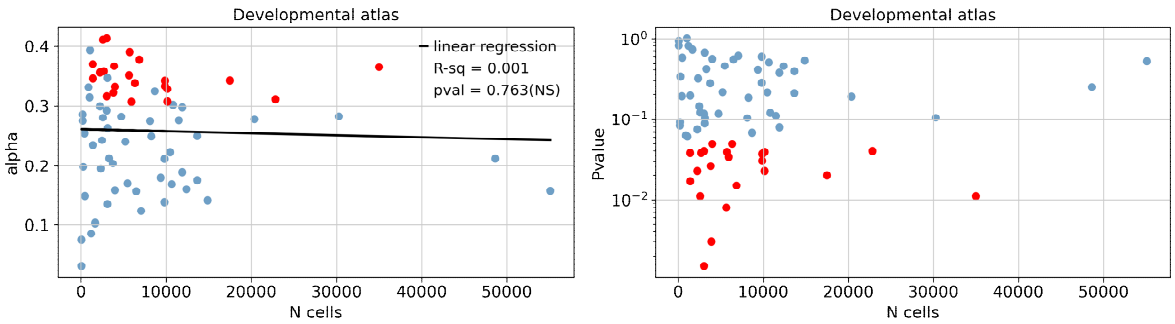


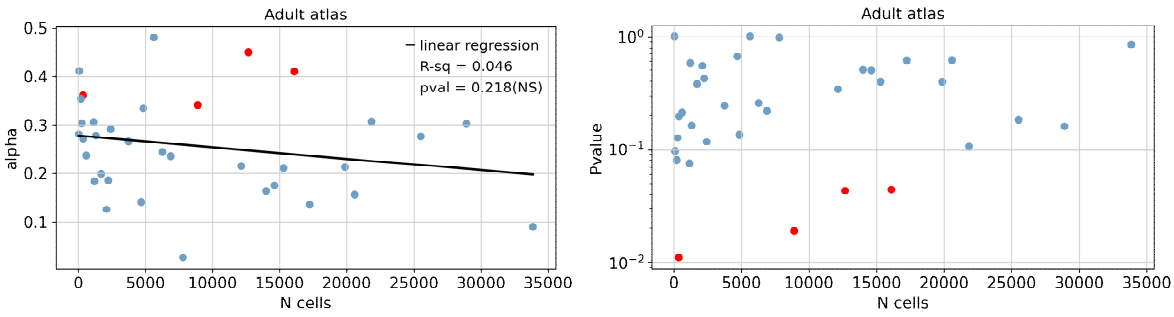
- It is unclear how the presented results are influenced by batch/technical variables. In particular, it is well known that RNA-seq experiments are highly sensitive to batch effects & experimental artifacts, and that subtle change in the experimental design can alter greatly the measured transcriptomes. **It would be interesting to see how well the results on adult are replicated when considering for RNAseq profiles from online databases such as DICE (https://dice-database.org/) or data from Monaco et al, 10.1016/j.celrep.2019.01.041).**

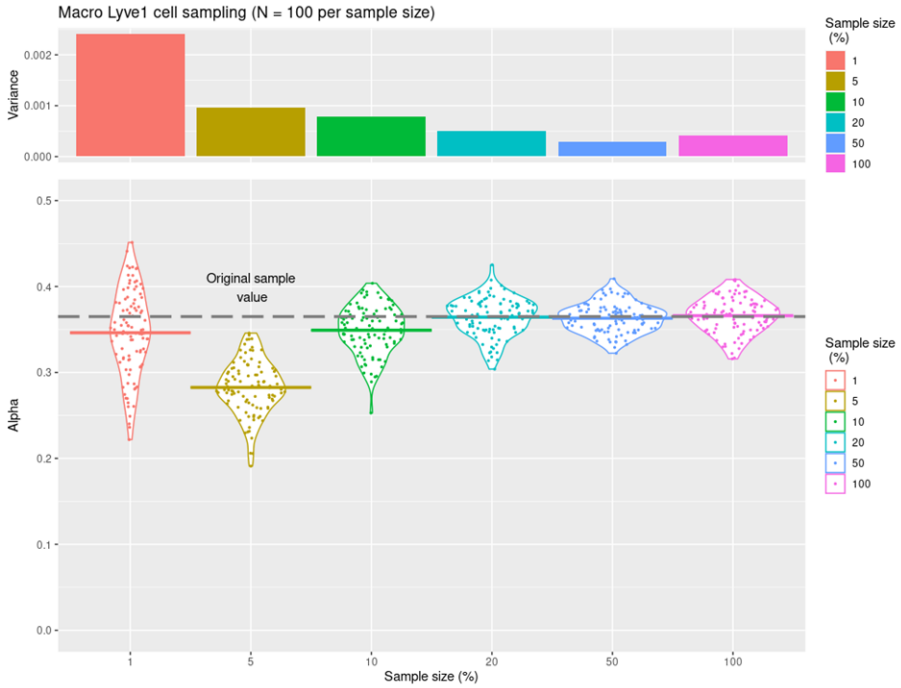
Response: We agree with the Reviewer on the importance of validation with external datasets. However, the suggested datasets are not ideal as they use peripheral blood mononuclear cells (PBMCs) for transcriptional characterisation. Our datasets derived from tissue-resident immune cells from multiple tissues, which is a relevant difference considering the functional specification of immune cells in tissues (https://doi.org/10.1038/d41586-021-01396-y). In the revised manuscript, we now include results from an external dataset of immune cells present in a healthy human organ (see previous comment). We believe this dataset represents an excellent external validation, due to its single-cell resolution and the presence of ample diversity of tissue-resident immune cells, as well as its integration of different technologies (scRNAseq, snRNAseq and spatial transcriptomics). Our results confirm the high adaptation rate and spatial enrichment for CD8 TRM and NK CD56 bright cells and identified additional cell types by leveraging the high cell type resolution after centering on a single organ.

- On a similar note, it's not clear how powered the proposed approach is for the detection of adaptation, and how cell counts could impact power. This makes it difficult to know whether differences between adult and embryonic tissues (eg, for Tregs or ILC3) should be interpreted as such, or simply viewed as random fluctuation of results due to low power. **I would suggest (i) to show a scatter plot of log Pvalue vs total number of cells in the cell type and (ii) to consider one cell type with high cell counts and significant adaptation results and test how the estimated rate of adaptation α is altered when progressively down sampling this cell type (eg. from 100% to 50, 20, 10, 5, and 1%) prior to the DE analysis.** Ideally, I would recommend resampling with replacement to allow some variability in the 100% cases, and perform ~10 resampling for each cell number (assuming that this is computationally tractable).

Response: Following the Reviewer's suggestion, we interrogated the relationship between the number of cells by cell type and *alpha* value using a linear regression. Here, we did not find any significant relationship between both variables. We provide the suggested scatter plot together with the linear regression summary results as new Supplementary Figure 1. Following the second Reviewer's recommendation, we now also performed a resampling with replacement of the cells from the "Macrophage Lyve1 High" cell type with N=100 samples for each sample size (100%, 50%, 20%, 10%, 5% and 1%). For each sample, we ran a DE analysis and estimated *alpha* with the selected DE as before. We selected this cell type as it is the cell type with the highest number of cells (35,001 cells) amongst the cell types with significant adaptation. Our results (provided as new Supplementary Fig X.) show that, compared to the original *alpha* value of 0.365, the mean *alpha* value of the samples remains stable in the 100%, 50% and 20% samples (0.366, 0.363 and 0.364, respectively), showing only a slight decrease in the mean *alpha* in the 10% and 1% samples (0.349 and 0.346 respectively). Only the 5% sample showed a considerable decrease in the mean alpha value (0.283). As expected, we observed that the variance of the sample increases by decreasing the sample size (see new Supplementary Figure 2). In conclusion, we consider that, although there could be fluctuations on the inferred *alpha* value, the *alpha* estimation is robust to cell number variation.







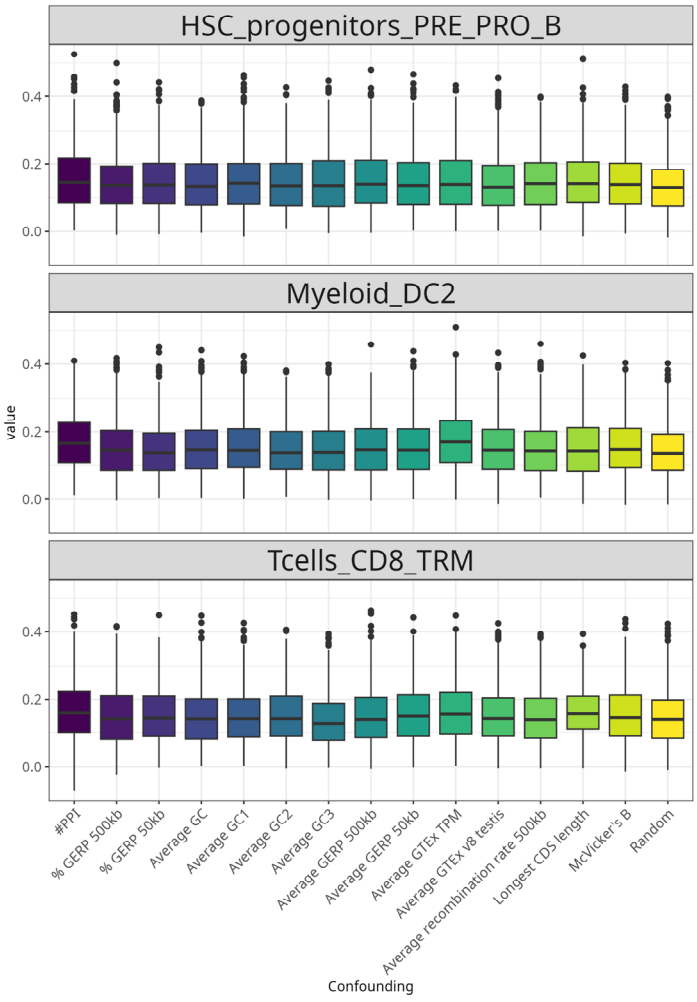
***** Minor comments *****

- While the authors put a lot of care in adjusting for an important number of technical confounders, it is not clear what the relevance of each confounder is. I would be interested in seeing a supplementary figure where we estimate the impact of each confounder on the estimated adaptation rate. **This could be done by comparing adaptation rate between genes within the top/bottom decile of each confounder**, while adjusting for all other confounders.

Response: We agree that discerning the role of each confounder would provide a comprehensive perspective to understand the relationship between natural selection and genomics. There are several studies and MK-test derived methodologies that have such a purpose. However, we have concerns regarding the goals and computational cost of such an analysis. First, the confounders in our study were selected based on several previous studies, comprising the most important features affecting α estimation. In this way, we ensure that significant α results result from the action of natural selection and not a confounder itself. Second, the resampling technique evaluates the confounders' role as much as possible by using the average confounder values for a given set and a tolerance threshold over such

an average. Because each cell type accounts for a different set of genes located randomly in the genome, we do not expect to account for the same average values for all the confounders in all the cell lines regarding the developmental, adult and lung datasets. We checked such a statement for the developmental dataset and found no relationship between cell types and the average confounder values. Hence, not just a confounder is expected to have a significant role in adaptation. However, each case should be affected by a set of confounders, so it is unlikely to provide a list of confounders affecting the adaptation rate measure since a set of different confounders would impact each cell type differently.

Still, we provided resampling estimates for the most significant cell types in developmental atlas, adult and lung datasets for each confounding factor. Similarly to the process already described in the main manuscript (page 25, lines 3-19), we match the gene set with paired controls and set a 5\% matching limit over the gene set average confounding values, but using one confounding at a time instead of the combinations, while restricting each control gene from appearing not more than three times in each control. In addition, to check the effect of each confounding factor, we randomly sampled 500 random genes with replacement from the 13,495 mammal orthologs genes we had information on (page 23, lines 3-16). The plot shows how each confounding affects the mean adaptation independently regarding the random orthologs genes selected.



- Finally, **it would help the interpretation to provide a supplementary data table with the specific gene sets associated with each cell type in order to allow the identification of specific genes that drive the signal at the cell type level.** While my understanding is that the ABC-MK test cannot be directly applied at the gene level, I think providing estimates of dN/dS and pN/pS at gene level together with a few confounding metrics (Background selection, GC content etc…) could still help prioritize genes likely to underlie the cell-type-specific adaptation signals reported in the paper.

Response: As the reviewer noted, ABC-MK is not designed to run at the gene level, so our goal was not to pinpoint individual gene-driving adaptation on the human immune system. We followed results from Murga-Moreno et al. 2024 (https://academic.oup.com/g3journal/advance-article/doi/10.1093/g3journal/jkae031/7607895) when performing ABC-MK test regarding the total number of polymorphic sites. In addition, it is essential to note that it is unlikely to find one or a few genes which lead to the significant α estimation in a pool of 500 genes, especially in the human genome where the level of nucleotide diversity highly decreases the statistical power to detect adaptation on MK-test analyses at the gene level. Nonetheless, we provided a supplementary table including all cell types, the associated top 500 orthologs Ensembl gene id, the confounder values, pN/pS and dN/dS measures in support of other MK-test analyses specialised on gene-by-gene testing. The main text now (page 24, lines 26-32) includes a brief clarification regarding the gene-by-gene testing and cites Murga-Moreno et al 2024 to clarify ABC-MK limitations and we also cite Murga-Moreno et al. 2022 to further interpretation on gene-by-gene analysis. Supplementary Table 14 provides the gene-by-gene information.

- I'm not sure that bootstrapping is the adequate term for the resampling scheme used to assess significance. Bootstrapping usually refers to estimating the properties of an estimand (eg. variance, expectancy, 95% CI), rather than building a null distribution (which is conceptually more akin to permutation testing). I would suggest to **refer to the method as "resampling"** rather than "bootstrapping".

Response: We agree that *resampling* is the proper statistical definition describing the procedure followed in the paper. We change *bootstrapping* by *resampling* accordingly in the main text (Pages 5,25,25,,lines 17,3, 8 respectively and Figures 1, 2, 3 and 5).

*** Typos and rephrasing ***
p5 lines 2, 19-20 & 26 + p9 line 34 + p10 line 12 + p11lines 33-34:
Numbers of Supplementary figures and tables are missing

Response: We apologise for the typing errors. Supplementary Figures and Tables numbers now are properly formatted.

p2, lines 31-32
The sentence "This approach is particularly useful when weakly advantageous alleles

segregate along with the evolutionary process known as background selection" is unclear. What does it mean to segregate with an evolutionary process? please rephrase.

Response: We rephrase the sentence to state clearly that the presence of weakly advantageous alleles, along with the role of BGS, can affect the MK-test, although ABC-MK bypasses it (page 2, lines 34-37 and page 3, lines 1-4):

This approach is particularly useful when the proportion of weakly advantageous alleles segregating in the Site Frequency Spectrum is relatively high and there is the presence of the evolutionary process known as background selection (BGS), like in the case of the human genome \citep{uricchio_exploiting_2019}. BGS decreases neutral genetic diversity when neutral variants are removed together with the deleterious variants they are linked to and can also impact positive selection signatures, especially at high frequencies \citep{uricchio_exploiting_2019}.

P15, lines 35-37
The sentence "For example, adult DCs respond to toll-like receptor stimulation inducing effector T cells, whereas foetal DCs induce Treg cells as a mechanism of tolerance and immune suppression" is unclear. What does it mean to segregate with an evolutionary process? please rephrase.

Response: In order to improve clarity, we have rephrased the mentioned sentence as follows (changes in bold; Page18 , lines 29-3):

For example, **both** adult **and foetal** DCs respond to toll-like receptor stimulation**, but in a different way; in contrast to adult DCs which induce** effector T cells, foetal DCs induce Treg cells **in a proposed** mechanism of tolerance and immune suppression \citep{mcgovern_human_2017}.

Fig 3C & supplementary Figure 5
R484 --> R848

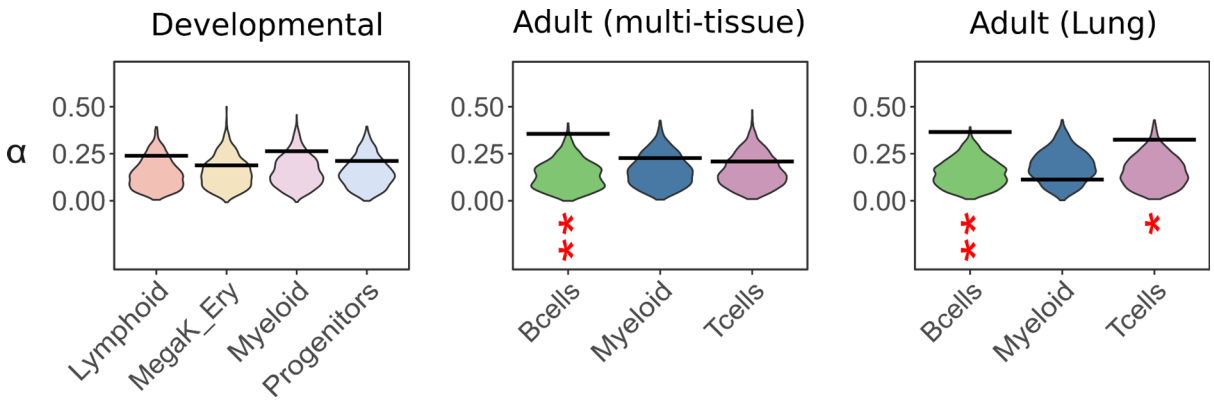
Response: We apologise for the confusion. We changed R484 to R848 in the main text (page 14, line 11), figure 5 and Supplementary Tables 5 and 9.

*** Additional analyses/suggestions ***
Below are a few additional suggestions, which I think could further strengthen the paper, should the author decide to incorporate them. **I am however conscious, that the suggested analyses represent an extension of the present manuscript beyond its initial scope and will thus leave to the authors to decide whether they believe that these analyses are worth pursuing**.

- p4 lines 23-24:
"a differential gene expression test for each cell population compared to all remaining cells in the same compartment"
- This suggest selection that affected a whole cell compartment (eg. shared myeloid genes) would not be considered. Have the authors considered doing an analysis where they assess

selection directly at the level of cell compartment (contrasting each compartment against all others)?

Response: Following the reviewer's suggestion, we inferred alpha at the compartment level for the three different cell atlases. Our results show no significant adaptation at the compartment level in the Developmental atlas. However, we found significant adaptation in the Tcells compartment of the Lung adult dataset and in the Bcells compartment of both the Lung and multi-tissue adult datasets. We include these results as Supplementary Figure 11.



- While the authors account for overall gene expression in GTEx tissues and expression in testis, one could argue that some cell-type specific adaptation detected in immune cells, may be driven by adaptation in another unobserved tissue or cell type. As such it could be interesting to compare the signals of adaptation detected in immune cells with those detected in other GTEx tissues. In particular, it could be interesting to assess whether tissues that diverged rapidly at the transcriptomic level such as liver and testis (Brawand et al, Nature, 2011) are associated with higher rate of adaptive non synonymous mutations.

Response: We agree this is an interesting question, but we believe it falls out of the scope of this paper.

- Finally, it would be interesting to apply the proposed method to assess how adaptation has altered response to cytokines in a more systematic manner. This could be done using the CytoSig database which combines >20,000 transcriptomics profiles to characterize response to cytokines across a wide array of cell types, stimuli and time point (https://doi.org/10.1038/s41592-021-01274-5

Response: We agree this is an interesting question, but we believe it falls out of the scope of this paper.

Maxime ROTIVAL

Reviewer #3 (Remarks to the Author):

Review of “QUANTIFYING ADAPTIVE EVOLUTION OF THE HUMAN IMMUNE CELL LANDSCAPE” by Salvador-martinez et al.

I found this to be an interesting paper. My background is in more traditional immunology and evolutionary biology so my comments focus on a rather general view of the paper from this viewpoint. It is essential that the paper be reviewed by a couple of systems biologists such as those from the Bustamante or Malik groups who would have expertise on the details and validity of the methods used.

Overall the paper does something interesting. The conventional view is that pathogens and hosts are in an “arms race” and thus one might expect to see increased rates of evolution in “immune-associated genes”. For the most part this has been observed (for example see Nielsen et al PMC1088278), but is not always observed as is the case for some genes such as the IFN-g gene see Mary et al PMID 21448974). The advance in the current paper is that it identifies cells types / lineages in which the immune genes that show high rates of evolution are expressed. They do this by combining the information on the genes that show high rates of adaptation together with developmental and adult immune cell atlases to identify the cell types in which these genes are expressed.

Not surprisingly they find that cell types from both the lymphoid and myeloid compartments to harbour significantly increased adaptation rates, after all the earlier studies found higher rates of adaptation in immune related genes. But more specifically they identified elevated rates of adaptation in:

1. Cells in the adaptive response such as foetal Pre-Pro B cells and adult TRM (resident memory) CD8+ cells show highly elevated rates of adaptation.
2. Cells of the innate immunity responding to early events following infection such as iPSC-derived macrophages also showed elevated rates of adaptation.
3. They suggest that the evolutionary changes involved in 1 and 2 arise from mutations in genes rather than the recruitment of new genes.

All this is relatively new, and very interesting. On this basis I am in favor of publication.

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

General appreciation:

The authors have successfully addressed my concerns regarding celltype differences in power to detect adaptation, and claims of preferential adaptation in lung resident cell types. In particular, I would like to commend their work on incorporating spatial data from the lung cell atlas, as I find the the new figure 4b to be extremely convincing and interesting.

I am however still not entirely satisfied with how multiplie testing is handled in the current manuscript (see below).

* Major comment *

Based on their answers, I have the feeling that the authors partly missed the point on the multiple testing problem:

Indeed, in their answers to reviewers, the authors claim that "the resampling technique employed [...] used specific and intrinsic genomic features for each cell type and, hence, the resulting p-values should not be correlated », seemingly implying that this would alleviate the need for multiple testing correction. While it is certainly true that the p-values for the various celltypes are not correlated, this does not preclude multiple testing correction, as the number of tests reaching a given p-value threshold will increase linearly with the number of tests, even when those tests are perfectly independent.

Luckily, the authors state that they have now applied an unbiased FDR. However, I was unable to find any information regarding this unbiased FDR in the method section (nor in the available scripts provided on github, or the Di et al preprint that the authors are referring to). More concerning to me, the authors make several statements which suggest that they are confusing FDR with type I error.

First, the authors say they "estimated the unbiased FDR by generating a null distribution of estimates of adaptation for each cell type, by measuring the proportion of analysis for which

α was indeed higher than the analysed case », from which I understand that the FDR is the proportion of tests under the NULL hypothesis for which alpha is greater than the alpha value of the cell type under consideration. This is the definition of a permutation p-value, and a good measure of type I error. However, it is NOT an FDR, which is defined as the product of (i) the probability to obtain an alpha value greater than the observed alpha under the null (type I error) and (ii) the number of celltypes being considered, divided by (iii) the number of celltype with an alpha value greater than the observed alpha for the current cell type (number of positives).

Second, in their response to reviewer 1 the authors state that "We tested 162 cell types (including the activated macrophages analyses) and observed eight cell types with their FDR lower than 0.005, which is clearly higher than the expected chance (0.81). »
This statement further supports my interpretation that the authors confuse FDR with type I error. Indeed, the probability that any gene reaches an FDR of 0.005 by chance on a null dataset is no more than 0.005. And the expected number of chance findings (i.e. false positives) is obtained by multiplyting the number of tests performed with the type I error, not the FDR. On the upside, if you have 8 cell types out of 162 passing a significance threshold of 0.005 (as estimated by the proposed resampling technique), this corresponds to an FDR of ~ 10%, which is very reasonable, making the results worth reporting in my opinion (especially, given that they seem biologically meaningful and plausible).

If this is only a matter of phrasing, the authors must be more cautious in how they describe the FDR to make sure that their description is accurate. They should also dedicate a proper paragraph to this in the method section (+ provide the code they used).
If, as I suspect, there was a confusion between FDR and type I error, the authors can simply use the Bonferroni Hocheberg procedure on the raw p-values they report; this is straightforward to do in R with p.adjust(* ,'fdr').
The FDR can be computed separately for fetal/adult & lung tissue but should at least be applied across the different cell types in each analysis.

Addressing this point is essential in my opinion in order to make sure that the results are robust and the methods are accurate.

* Minors comment *

• Lines 29-32 p18, the authors have not really addressed my concern. I am however the one to blame here, as my initial comment was truncated by mistake and lacked clarity.

My concern with this sentence was that it’s not clear to me what « inducing" effector T cells or T regs means. Maybe replace with ‘activate’ ?

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

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*** Major comment ***

Based on their answers, I have the feeling that the authors partly missed the point on the multiple testing problem: Indeed, in their answers to reviewers, the authors claim that "the resampling technique employed [...] used specific and intrinsic genomic features for each cell type and, hence, the resulting p-values should not be correlated », seemingly implying that this would alleviate the need for multiple testing correction. While it is certainly true that the p-values for the various cell types are not correlated, this does not preclude multiple testing correction, as the number of tests reaching a given p-value threshold will increase linearly with the number of tests, even when those tests are perfectly independent.

Luckily, the authors state that they have now applied an unbiased FDR. However, I was unable to find any information regarding this unbiased FDR in the method section (nor in the available scripts provided on github, or the Di et al preprint that the authors are referring to). More concerning to me, the authors make several statements which suggest that they are confusing FDR with type I error. First, the authors say they "estimated the unbiased FDR by generating a null distribution of estimates of adaptation for each cell type, by measuring the proportion of analysis for which α was indeed higher than the analysed case », from which I understand that the FDR is the proportion of tests under the NULL hypothesis for which alpha is greater than the alpha value of the cell type under consideration. This is the definition of a permutation p-value, and a good measure of type I error. However, it is NOT an FDR, which is defined as the product of (i) the probability to obtain an alpha value greater than the observed alpha under the null (type I error) and (ii) the number of cell types being considered, divided by (iii) the number of cell type with an alpha value greater than the observed alpha for the current cell type (number of positives).

Response: We thank Reviewer 2 for the constructive comments. We sincerely apologize for not correctly describing the FDR estimation and for not providing the FDR code. The main issue has been a matter of wrong phrasing, as we mistakenly used the term “False Discovery Rate” for a given cell type, when we should have used the term Type I error.

As pointed out by the Reviewer, the simple permutations between test genes and matched control genes for a given cell type provides a measure of the Type I error for that specific type. Such cumulated Type I errors across all cell types provide an estimate of the FDR at a given error threshold. This is what we reported already at the 0.005 Type I error threshold. The language in the manuscript now reflects more precisely, when Type I errors are measured, and where actual FDRs over multiple tests are reported. We apologize again for the unnecessary confusion.

The Reviewer also noted that he was unable to find the related part of the Di et al. 2021 *eLife* paper that described the FDR estimation approach. This was again caused by a lack of clarity on our part. First, in the Di et al. paper, we had more correctly called our estimations of Type I errors estimations of False Positive Rates. Second, in the Di et al. paper the estimation of Type I errors required an elaborated approach to take the clustering of genes in selective sweeps into account. In the present manuscript, the signatures of evolution exploited by our test of selection are independent from each other in neighbouring genes, and we therefore only needed to use simple permutations. Considering the Reviewer’s recommendation, we now added a paragraph in the Methods section that fully explains the simple permutation approach to first obtain an unbiased Type I error for each tested cell type, and then use these estimations to calculate the FDR across the tested cell types (page 26). In addition, we added the following statements to the Results section to clarify the Type I error and the FDR estimation (page 5):

“*However, this process relies on a limited supply of control genes, which can bias the variance of the null controls (Methods). To address this limitation, we also provide an unbiased measure of the type I error, which allowed to estimate the number of cell types below an error threshold by chance. Throughout the manuscript, we report the raw resampling p-values, but also provide a more stringent analysis based on the type 1 error estimation and respective FDR values from multiple testing corrections (Benjamini-Hochberg correction) (Supplementary table 10).*”

Second, in their response to Reviewer 1 the authors state that "We tested 162 cell types (including the activated macrophages analyses) and observed eight cell types with their FDR lower than 0.005, which is clearly higher than the expected chance (0.81). »
This statement further supports my interpretation that the authors confuse FDR with type I error. Indeed, the probability that any gene reaches an FDR of 0.005 by chance on a null dataset is no more than 0.005. And the expected number of chance findings (i.e. false positives) is obtained by multiplying the number of tests performed with the type I error, not the FDR. On the upside, if you have 8 cell types out of 162 passing a significance threshold of 0.005 (as estimated by the proposed resampling technique), this corresponds to an FDR of ~ 10%, which is very reasonable, making the results worth reporting in my opinion (especially, given that they seem biologically meaningful and plausible).
If this is only a matter of phrasing, the authors must be more cautious in how they describe the FDR to make sure that their description is accurate. They should also dedicate a proper paragraph to this in the method section (+ provide the code they used). If, as I suspect, there was a confusion between FDR and type I error, the authors can simply use the Bonferroni Hocheberg procedure on the raw p-values they report; this is straightforward to do in R with p.adjust(* ,'fdr'). The FDR can be computed separately for fetal/adult & lung tissue but should at least be applied across the different cell types in each analysis. Addressing this point is essential in my opinion in order to make sure that the results are robust and the methods are accurate.

Response: Following the suggestion from the Reviewer, we now dedicate a new paragraph in the Method sections (*Resampling and False Discovery rate*) that adequately differentiates between Type I errors and FDR (page 26). In addition, we still cited Enard & Petrov (2020) as the first manuscript where the estimation of Type I errors was conducted, although this was a more complex case of selective sweeps requiring accounting for gene clustering.

As detailed in the revised Methods of the manuscript, we measured Type I errors in each cell type by repeatedly performing ABC-MK analyses in size-matched Differentially Expressed Genes (DEG) sets, but creating 1000 datasets by shuffling DEG and control datasets. Such random sets represent an unbiased null distribution of estimates of adaptation without the impact of the differentially expressed genes. Since the sample size and the genomic features would not differ on the null distribution of randomized genes because of the resampling approach, re-running the entire analysis reproduces the same biases for the randomized genes as the tested cell types. Thus, the strategy effectively estimates an unbiased Type I error with DEG not being different from random genes. Finally, by measuring the proportion of results with *alpha* greater than the analyzed case, we effectively estimate the Type I error,

allowing us to assess the expected number of cell types below a given Type I error threshold by chance.

Moreover, following the Reviewer's recommendations, we performed multiple testing corrections and estimated the FDR using the Benjamini-Hochberg method by applying the R p.adjust(*,'fdr') across the different cell types in each analysed dataset. Such analyses showed that cell types with a Type I error p-value lower than 0.05, have a FDR of around 10 (*mean FDR = 13.2%; range 0.1%-35.2%)*. Here, it is essential to note that the enrichment of specific functions and locations described and discussed in the manuscript and the validation through *in vitro* experiments and independent datasets strongly support highly meaningful results. These limitations are now discussed in the revised manuscript as follows (page 20):

"*A caveat of our analysis is that, although we found several cell types with a high adaptation value (i.e. with raw p-values and type I error lower than 0.05), many of these showed an FDR > 10% (mean FDR = 13.2%; range 0.1%-35.2%) after correcting for multiple testing. Increased FDR values in cell types with elevated rates of adaptation could be a consequence of low statistical power as well as the use of controls that match in multiple covariates. We strongly believe, however, our results to be meaningful as they are supported by biological processes of adaptation to pathogen exposure and were validated through in vitro experiments and in independent datasets (i.e., Lung Cell Atlas).*"

*** Minors comment ***

• Lines 29-32 p18, the authors have not really addressed my concern. I am however the one to blame here, as my initial comment was truncated by mistake and lacked clarity. My concern with this sentence was that it's not clear to me what « inducing" effector T cells or T regs means. Maybe replace with 'activate' ?

Response: The cited reference, McGovern et al. (2017) reports that, similar to adult dendritic cells, fetal dendritic cells migrate to lymph nodes and respond to Toll-like receptor ligation. However, they differ markedly in their response to allogeneic antigens, strongly promoting regulatory T cell induction and inhibiting T cell tumour-necrosis factor-α production through arginase-2 activity. In this context, the term "induction" is used to describe the process by which fetal dendritic cells promote the formation or development of regulatory T cells. While "inducing" and "activating" are related, they are not synonymous. "Inducing" refers to initiating the development or production of regulatory T cells, while "activating" implies stimulating existing cells to become active or responsive.