

Revealing population-specific heterogeneity in ontogeny of the broadly-conserved blood transcriptional program during the first week of life

Corresponding Author: Dr Robert Hancock

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)

In this manuscript, the authors explored the details of early transcriptomic responses and distinct population-specific molecular mechanisms that define neonatal immune development during the first week of life.

This work was done in a cohort of 90 infants from The Gambia and New Guinea. The newborn cohort had no vaccine exposures prior to or during the period when blood samples used for the analysis were obtained. 45 neonates enrolled at each site and assigned randomly to 3 groups.

- a. D0, D1
- b. D0, D3
- c. D0, D7

My questions are primarily about the cohort on which the work and results are based. The reader may or may not be aware of the health access in the regions where the neonates were born - that could be added to the methods. Table 1 presents sex, BW and mom's age. The reader would necessarily assume that they are all "similar" newborns outside of these 3 covariates including the assumption that all were born vaginally at term and healthy, fed mother's milk without supportive signs of infection or signs that would support exposure to in utero infection. True? Antibiotics in mothers or newborns? Are there any clinical features in the newborn the authors can provide that might have contributed to the RNAseq differences noted that were not presented?

Blood processing, RNA stabilization, RNAseq, and stat analysis acceptable. Results are clearly presented.

Discussion

Interpretation of results is reasonable. The novelty of the author's work may preclude integration of their findings into the existing literature, but where possible perhaps they can modify paragraphs where they have few references to other works (paragraphs 2, 3, 5, 6 in discussion).

Reviewer #2

(Remarks to the Author)

General assessment

The authors have collected blood from neonates at two distinct locations at birth and at one further timepoint during the first week of life. Then have performed bulk RNAseq on the samples and do a series of statistical analyses with those. Overall, the results are in line with previously described characteristics of neonatal immune development, such as activation of IFN-signaling during the first week (described by the authors previously). Some novelty lies in the relatively little differences between the distinct geographical locations, confirming a previously suggested stable postnatal developmental program. However, the majority of provided information is neither new nor unexpected, e.g. changes in white blood cell composition in the first week of life.

It is surprising that the study lacks any information on the neonates beyond geographical location, birth weight and sex. This

could have added some novelty, i.e. correlation of the RNAseq data to general health data, demographic data, birth characteristics and so on.

Overall, this study contains relatively little and solely descriptive data, is simple in methodology and lacks innovation. The focus on analyzing RNAseq data from neonatal samples lacks the novelty that one would expect from a study published in this journal.

Suggestions

Abstract

1. Consider mentioning that it concerns the immune system either in the title or in the first sentence of the abstract?

Introduction

1. Citations seem to be biased towards their own work, ignoring some other relevant studies on neonatal immune development

Methods

1. The sentence “the Wilcoxon test was used to identify groups with statistically different proportions.” Is unclear. Was it a paired Wilcoxon signed-rank test? And DOL-specific?

2. I think DESeq2 does not recommend filtering results by arbitrary FC thresholds, but rather apply FC-shrinkage and then filter by significance.

Results

1. Fig. 1 should be placed to the supplement.

2. Fig. S2: What is the meaning of different types of macrophages circulating in the blood? Is this a mis-attribution by the algorithm?

3. Fig. S6: It is unclear what the arrows pointing up and down at the same datapoint mean. Also why do the authors speak of IFN γ “dysregulation”, instead of simply upregulation?

4. Fig. 3: Again, why are genes “dysregulated” here? In Fig. 3C maybe further shorten the list of terms, the relevance of some remains unclear.

5. In my opinion figure 4 is uninformative and can be removed

6. The whole section Changes in nutritional status and indicators of disease is very speculative and rather belongs into the discussion. In fact, relating these pathways to vitamin deficiency or viral infections is a very far stretch.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

Neonates are highly susceptible to infections, a leading cause of infant morbidity and mortality. Effective public health interventions, including vaccination strategies, require a clear understanding of neonatal immune ontogeny, which begins in utero and continues to mature postnatally to provide protection against pathogens and maintain immune homeostasis

In this manuscript, the authors explored the details of early responses and distinct population-specific molecular mechanisms that define neonatal immune development during the first week of life from two geographically distinct populations. This work is important because deeper understanding of early period of immune development can expand our understanding of neonatal susceptibility to infectious diseases, as well as the impact of early-life interventions such as immunization. This work lays a crucial foundation for future research endeavors. However, there are several areas that require attention in this article.

Major

1. The authors use bulk mRNA-seq to profile early neonatal immune cell development and claim cell type-specific gene expression. However, many genes are shared across immune cell subsets, so the conclusions cannot be fully supported by bulk data alone. Validation using single-cell transcriptomics or orthogonal methods such as flow cytometry is recommended.

2. It is expected that neonates predominantly exhibit naïve immune cell profiles, making the reported dynamic changes in gene expression at these early stages notable. However, perinatal factors—such as the stress of delivery and sudden exposure to the external environment—could contribute to these observed changes. The authors should discuss the potential impact of these factors on their findings.

3. The study lacks mechanistic insight into the regulation of the observed gene expression changes. The authors could

strengthen their analysis by performing ATAC-seq for epigenomic profiling or, at a minimum, conducting in silico analysis of the promoters of differentially expressed genes. Mapping transcription factor binding profiles on these promoters would provide molecular insight into the regulation of dynamic gene expression changes.

4. Importantly, though this study advanced their previous published observations, this study failed to bring the novelty from the previous work. I believe that addition of distinct layer of omics dataset would have revealed molecular aspect to their conclusions.

Minor

1. The authors should include a section for authors contributions.
2. On page 8, line 267, 'TFH marker FOXP3' should be 'Treg marker FOXP3'.
3. It is recommended that all figures be reviewed for clarity and accuracy in labeling.
4. Use of 'in vitro' or 'in vivo' should be italicized.
5. The authors should clearly specify whether the newborns were delivered via C-section or vaginal delivery, as mode of delivery can influence early immune development.

Reviewer #5

(Remarks to the Author)

Summary

The authors present bulk RNA sequencing of peripheral blood from healthy neonates in two geographically distinct locations (Africa and Australasia) during the first week of life, aiming to delineate and compare transcriptional programs of ontogeny. By applying block randomisation strategies, they successfully reduced sequencing bias and reproduced results from their previous multi-omics study, this time using RNA-seq alone. The analysis identified more differentially expressed genes and transcriptional programs during the first week of life compared with their earlier study. These additional changes are explored in greater detail and are mainly attributed to the maturation of adaptive immunity. Additionally, the authors compare these programmatic changes between the two cohorts.

Significance to the field

This study builds on and refines previous findings on postnatal transcriptional changes in the peripheral blood of healthy newborns across different regions of the world. It provides further evidence that early postnatal changes follow a shared developmental program. Importantly, the authors demonstrate that, with appropriate experimental design, bulk RNA-seq can provide insights comparable to multi-omics approaches. The study also highlights how robust and harmonised workflows facilitate high-quality research in low- and middle-income countries (LMICs). On the other hand, bulk RNA-seq inherently limits the ability to resolve transcriptomic changes at the level of specific cellular subsets.

Conclusions, claims, and data analysis

The presented data support the existence of common postnatal changes in neonates across diverse populations. However, the reviewer questions whether computationally inferred cell subset distributions are sufficient to explain observed differences in differentially expressed genes or pathways (from the same sequencing data). This issue could potentially be addressed by incorporating data from the authors' previously published multimodal analyses, which include flow cytometry.

While preserved postnatal programs are evident, population-specific differences are also suggested. These are described in parts of the manuscript and presented in Supplementary Figure S5, but no systematic analysis of inter-cohort differences is provided, particularly with respect to metadata that could influence transcriptional patterns. Sex, in particular, warrants further analysis, given the unequal distributions between cohorts (44% vs. 67% female). Although sex does not appear to be a major driver in PCA, sex distributions at later time points are not presented.

Because this is an observational study based solely on transcriptomic data, the conclusions remain hypothesis-generating. The "intervention" here is effectively the sampling location. The study impact might be strengthened by first analysing postnatal transcriptomic programs across the full dataset (to provide a more granular analysis than previous studies) and subsequently assessing the systematic effects of location and other metadata. Although the relatively small sample size per group (n = 15 per time point) may limit statistical power, such an approach would provide deeper insights into population-specific trajectories. Finally, the authors could attempt to integrate published datasets from other populations to confirm both shared and unique developmental programs.

Methodologies and documentation

The study applies state-of-the-art, publicly available bioinformatics tools to analyse the bulk RNA-seq data. Methods appear sufficiently documented to enable reproducibility once sequencing data are released.

Overall assessment

This manuscript leverages rare and valuable samples collected through a carefully harmonised strategy to investigate the variation and commonality of neonatal postnatal transcriptional trajectories. However, the current version primarily provides impressions of shared processes rather than a systematic exploration of cohort-specific differences. In the reviewer's opinion, the manuscript would benefit substantially from a deeper and more structured analysis of differences between the groups, including integration of relevant metadata. Given the quality of the data and the thoughtful study design, such an analysis would greatly strengthen the impact of the work.

Specific issues

The authors note dynamic changes in neutrophil frequencies within the first 6–12 hours of life. Since not all samples on day 0 are likely taken at the same postnatal age in hours, could this be incorporated into the analysis?

The previous study (Lee 2019) included $n = 30$ per group (GAM and PNG). The present manuscript ($2 \times n = 45$) should not claim to have more than double the sample size (lines 102–103), especially since line 167 refers to $n = 50$ in the earlier study, which appears inconsistent.

Figure 3C: Consider including enrichment scores in addition to p-values. Clarify the meaning of arrows pointing up and down within the same line.

Figure 4 and lines 229 ff: The described differences between GAM and PNG are not obvious from the figure. The color scheme for nodes is not explained, the cited genes are difficult to locate, and the selection criteria are not described in the methods.

Figure 5B: Why was an absolute log-fold change (LFC) of 1.5 used as a threshold, while log2FC values are plotted? For clarity, a consistent scale should be applied.

Line 267: FOXP3 is a Treg marker, not Tfh.

Line 314: Why are read counts now used to support the stated effect?

Line 317: Could differences in HLA haplotype distributions between cohorts underlie observed differences?

Supplementary Figure 1: Dot plot presents only p-values. Consider including effect sizes or other quantitative measures. In the right panel, the "percent variance" values are all <1 , are those percentages?

Supplementary Figure 2: Are these a subset of the cell types computed by CIBERSORTx? Would it be possible to include all major T, B, and NK subsets? Analysing more abundant populations could clarify the extent to which gene expression differences are driven by cell composition. Was correction for multiple testing applied?

Supplementary figures are cited out of order (e.g., Supp. Fig. 6 before Supp. Fig. 3; Supp. Fig. 5 before Supp. Fig. 1).

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The addition of the flow cytometry data strengthens the manuscript substantially. However, some key information on this method is missing in the method section. It doesn't describe how cells were preserved for flow cytometry, which is important especially given that the panel contains live neutrophil staining, unless a fixable viability dye was used, either way this needs explanation. The full list of antibodies used is required. When this information is provided, I would suggest to make a stronger point out of this interesting dataset: Please consider putting a shortened version of figure S11 to the main manuscript (it is much more informative than current figure 3).

I don't think "pathway rewiring" is the right term for what we see in figure 3. Instead, what it shows is that the DE gene set in GAM vs PNG implicates different interacting modules/pathway engagement. There is no "re" involved here.

In figure 4C-D, are all green nodes upregulated? If yes, please state so in the legend, if not, please add this info somehow to the figure.

Fig. S15 is not mentioned in the result section. In fact this is potentially interesting data. I would encourage the authors to also perform a direct sex-comparison (either merged for all locations or as a gene-wise 2x2 linear model considering sex and location). Even if this reveals only few genes, it is worth exploring and mentioning.

The following papers should be included in the discussion

Ulas et al. 2017, PMID: 28459433

Olin et al. 2018, PMID: 30142345

Ariyakumar et al. 2022, PMID: 35711432

McDavid et al. 2022, PMID: 35310935

Dzanibe et al. 2024, PMID: 38744812

Minor:
line 407 should point to fig. S11

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

Reviewer #5

(Remarks to the Author)

General comments

Structure and flow of the analysis

Although the manuscript has substantially improved following revision, the overall narrative remains somewhat difficult to follow due to the order in which the analyses are presented. The readability might benefit from a restructuring in which the authors first describe the general features of postnatal immune ontogeny, followed by a more focused analysis of population-specific differences. Such an organization could help guide the reader more clearly from global developmental patterns to the study's comparative aspects.

Positioning and impact of the study

While the manuscript presents interesting and carefully generated data, it remains somewhat unclear what specifically elevates the work to the level expected for publishing in this highly ranked journal. This concern may be addressed if the authors further clarify and more explicitly highlight the conceptual advances arising from their findings — in particular, the biological implications and broader relevance of the observed differences between the two studied populations. Strengthening the interpretation and contextualization of these comparative results would help emphasize the manuscript's overall impact.

Minor comments

Overall, the authors have done an excellent job revising the manuscript and addressing the major concerns raised previously. I only have a few remaining minor points:

Line 216:

The reference to the supplementary figure appears inconsistent ("Supplementary Figure SX??"). Please verify whether this should refer to Figure S3.

Line 228:

Please confirm whether the correct reference is Supplementary Figure S4.

Lines 239 onwards (environmental factors):

While I agree that genetic factors are controlled for in this context, I do not fully agree with the statement that variation in nutrition, environment, microbiota composition, and pathogen exposure is eliminated. These factors are unlikely to be fully established or in a steady state at day 0 and cannot be assumed to remain constant over time within an individual. The wording of this conclusion should therefore be moderated or clarified.

FACS cell counts:

Please clarify whether cell numbers are reported as counts per μL of blood. Values below ~ 20 T cells/ μL would indicate severe T-cell lymphopenia (within the range observed in SCID), which would require explanation or contextualization.

Line 518 (PCA interpretation):

Later time points appear to cluster more toward the lower right region of the PCA plot. Given this distribution, the stated conclusion may be somewhat strong and could benefit from more cautious wording.

Figure S9:

Figure S9 is intuitive and convincing. The authors may consider moving these data to the main figures (e.g., replacing or complementing Figure 3), as they substantially support the central message.

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RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors explored the details of early transcriptomic responses and distinct population-specific molecular mechanisms that define neonatal immune development during the first week of life.

This work was done in a cohort of 90 infants from The Gambia and New Guinea. The newborn cohort had no vaccine exposures prior to or during the period when blood samples used for the analysis were obtained. 45 neonates enrolled at each site and assigned randomly to 3 groups.

a. D0, D1

b. D0, D3

c. D0, D7

My questions are primarily about the cohort on which the work and results are based. The reader may or may not be aware of the health access in the regions where the neonates were born - that could be added to the methods.

All mothers and infants received care at single centres in The Gambia (GAM) and Papua New Guinea (PNG) respectively. All mothers and newborns were healthy and did not require special care beyond this standard at the time of enrollment and biosampling. In GAM, newborns were recruited at one secondary level care institution (The Kanifing General Hospital) and one primary health facility (The Banjullinding Health Center). In PNG, newborns were recruited at Goroka Hospital, the only tertiary hospital in the Eastern Highland Province of PNG. A detailed clinical protocol is provided in a separate publication that is now cited with additional detail in the Methods section (Idoko *et al.* 2020).

Table 1 presents sex, BW and mom's age. The reader would necessarily assume that they are all "similar" newborns outside of these 3 covariates including the assumption that all were born vaginally at term and healthy, fed mother's milk without supportive signs of infection or signs that would support exposure to in utero infection. True?

This is correct and we have added a comment about this to the text at line 97 "Mothers and neonates were healthy and no antibiotics or special care beyond local standards was required at time of enrolment or biosampling.". There were no clinical concerns for any participants (mom or baby), and adjusting for potential variables beyond sex, birthweight and maternal age would not allow meaningful analysis given the modest sample size.

Antibiotics in mothers or newborns? Are there any clinical features in the newborn the authors can provide that might have contributed to the RNAseq differences noted that were not presented?

No antibiotics were used in this cohort of newborns or in the birth mothers, as noted at line 97. Clinical features were broadly similar. Furthermore, we minimized any unrecognized clinical, demographic and genetic differences by comparing each infant to themselves, e.g. comparing infants at D1, D3, and D7 to D0 (birth). This is further clarified at lines 193-201, "Use of a paired-design strategy in which each infant was compared to themselves at birth, largely eliminated sources of variation such as differences in genetics, epigenetics, environment, nutrition, microbial load, exposure to pathogens, or other external factors which are likely to have shown some variation over the first week of life in any given infant. Importantly, the use of this paired-design

by indexing each neonate's DOL1/3/7 sample to DOL0, and employing both overall ontogeny modeling (indicated by "All" in **Figure 2C**) and population-specific modeling (indicated by "GAM" and "PNG" in **Figure 2C**) was key to detecting large numbers of both shared and population-specific changes at the gene expression level."

Blood processing, RNA stabilization, RNAseq, and stat analysis acceptable. Results are clearly presented.

Thank you.

Discussion

Interpretation of results is reasonable. The novelty of the author's work may preclude integration of their findings into the existing literature, but where possible perhaps they can modify paragraphs where they have few references to other works (paragraphs 2, 3, 5, 6 in discussion).

We have added additional references to these paragraphs, e.g. refs 49-53.

Reviewer #2 (Remarks to the Author):

General assessment

The authors have collected blood from neonates at two distinct locations at birth and at one further timepoint during the first week of life. Then have performed bulk RNAseq on the samples and do a series of statistical analyses with those.

Overall, the results are in line with previously described characteristics of neonatal immune development, such as activation of IFN-signaling during the first week (described by the authors previously). Some novelty lies in the relatively little differences between the distinct geographical locations, confirming a previously suggested stable postnatal developmental program. However, the majority of provided information is neither new nor unexpected, e.g. changes in white blood cell composition in the first week of life.

Previous studies that have addressed immune ontogeny have analyzed infant groups at time points >1 month, and to our knowledge, the only study that focuses on neonatal time points during the first week of life was our previous pilot study³. The current findings build on the previous pilot study with a significantly larger sample size. The careful design of this study, including block randomization and paired-model for head-to-head comparison of GAM and PNG in the same RNA-Seq assay runs, enabled deeper evaluation of differences between the two different racial groups of infants in addition to finding common core ontogeny trajectories. These topics, to our knowledge, have not been addressed in the past.

We have further improved our Results section to centre the novelty of this study around the population-specific differences that were not revealed previously by any studies. We further refined our subsequent common program analysis using an approach suggested by Reviewer #5 wherein we consider all samples together regardless of location and then compare the results against using a location-specific model. This allowed us to focus in on the novel findings of population specific heterogeneity during the first week of life, and hope that this has become clearer throughout the manuscript, rather than being masked within summarizing core ontogeny changes that have already been described previously in detail. We also altered the bioinformatic methods (including some that we developed, e.g. joint DyNet-based networks of population-specific genes, NetworkAnalyst-based protein:protein interaction networks, and pathlinkR-based pathway networks and heat maps) used to reveal changes consistent with pathway and network

remodelling. We have now provided excellent evidence that favours pathway and network remodelling in Gambian cf. Papua New Guinea infants.

It is surprising that the study lacks any information on the neonates beyond geographical location, birth weight and sex. This could have added some novelty, i.e. correlation of the RNAseq data to general health data, demographic data, birth characteristics and so on.

Although these infants were from two different populations/countries/continents, they received health care according to local standards and mothers or newborns that required care beyond standard delivery medical care were not included in this study (now clearly stated at line 97; "Mothers and neonates were healthy and no antibiotics or special care beyond local standards was required at time of enrolment or biosampling."). Part of the strength of this paper is the geographical diversity represented by infants from two diverse Lower and Middle Income (LMIC) Countries, and the implications of understanding neonatal ontogeny beyond High Income Countries (HIC). Furthermore, if we had not compared each infant (at D1, D3 or D7) to themselves (at D0), we would have been exposed to several major confounders including race, genetics, microbiota, etc., and we confirmed the importance of including this comparison by setting up the analyses using a paired-design (since otherwise the confounders would severely eliminate most differences due to ontogeny). In previous studies, samples were not collected simultaneously, and differences could not be directly evaluated due to batch effects between sequencing runs. We would argue that differences that were not observable in any previous study were revealed here because of the study design and block randomization, and that these differences are likely as important as the strong trajectories observed.

Overall, this study contains relatively little and solely descriptive data, is simple in methodology and lacks innovation. The focus on analyzing RNAseq data from neonatal samples lacks the novelty that one would expect from a study published in this journal.

We appreciate the reviewer's concerns. We now also have included additional data modality (flow cytometry). This not only supports the RNA-Seq findings but expands the insight that can be gleaned. Specifically, the flow cytometry measurements using specific flow cytometry automated gating strategies as previously published (Montante et al. 2024) represent a more accurate method of specific cell-typing. Therefore, we have now replaced data in the manuscript that was derived from CIBERSORTx predictions with direct measurement of cell types.

Importantly, it is our understanding that except for our pilot study, no other study has looked at the developmental trajectory over the first hours/days/week of life. This is the period across all of human life where most deaths happen in any given week of life, yet it is completely unknown what a constitutes 'normal' developmental path (thus we cannot identify the abnormal or at-risk individual). Our dataset helps to define 'normal' and do so not just in a population in e.g. North America but in areas of the world where the most newborn deaths occur (LMIC). Furthermore, by contrasting two populations each from high-mortality populations, but different ones, we can immediately identify the more likely 'universal' normal vs variables that could differ between populations. We believe this is an important strength of this study.

Suggestions

Abstract

1. Consider mentioning that it concerns the immune system either in the title or in the first sentence of the abstract?

Agreed we added this to the first sentence in the abstract.

Introduction

1. Citations seem to be biased towards their own work, ignoring some other relevant studies on neonatal immune development

We are sorry if we gave this impression. If we ignore reviews, only 3 papers from our consortium were cited as these are the only studies we know of that evaluate immune ontogeny at timepoints during the first week of life. We would appreciate it if the reviewer could provide suggestions of any additional studies that we may have missed.

Methods

1. The sentence “the Wilcox test was used to identify groups with statistically different proportions.” Is unclear. Was it a paired Wilcoxon signed-rank test? And DOL-specific?

Thank you for noting this lack of clarity in the methods for comparing CIBERSORTx predictions of cell type proportions across populations (GAM and PNG). The Wilcoxon rank-sum test was used in this case and these tests were run separately for each DOL and were not run paired. However, we have removed CIBERSORTx results and have updated them with the more accurate results from a flow cytometry myeloid cells panel but still using the Wilcoxon test.

2. I think DESeq2 does not recommend filtering results by arbitrary FC thresholds, but rather apply FC-shrinkage and then filter by significance.

We appreciate your feedback on the methodology of identification of differentially-expressed genes. The approach of applying FC-shrinkage is important in order to account for cases where low count genes or genes with high dispersion have inflated and noisy fold-changes however the add a degree of uncertainty since they might reveal differences that are statistically significant but not biologically important. Based on our understanding of DESeq2, the shrinkage methods would not change any p-value calculations of significance, but would rather make fold-change estimations more precise for a modest number of outlier cases. In fact, we have a list of all significantly dysregulated genes but chose to adopt 2-fold and 1.5 fold changes as per most studies. In our study approach, as part of our data pre-processing, we removed high and low count genes prior to finalizing a count matrix that could be used for several different downstream analysis pipelines (such as multi-omic integration). We decided that it was important to use a consistent set of genes in the transcriptomic count matrix in all study analyses. After removing these less-informative/noisy gene counts, and using an additional stringency filter on fold-changes ($\geq \pm 2$ fold), it is our hope that we are focusing our analysis on the most accurate and informative data points. Genes with smaller (possibly noisy) effect sizes may be harder to interpret in the context of bulk RNA-Seq data, and were not considered at all for these reasons. Even so, there was a considerable number of differentially-expressed genes identified that passed these stringent filters and allowed for a detailed dive into higher-level enrichment of pathways and mechanisms.

Results

1. Fig. 1 should be placed to the supplement.

Done.

2. Fig. S2: What is the meaning of different types of macrophages circulating in the blood? Is this a mis-attribution by the algorithm?

It is not a misattribution. CIBERSORT assigns cells by using cell-specific markers. M1 macrophages are so-called classically activated, typically by IFN- γ or lipopolysaccharide (LPS), and produce proinflammatory cytokines, phagocytize microbes, initiate an immune response, and produce nitric oxide (NO) or reactive oxygen intermediates (ROI) to protect against bacteria and viruses. M2 are alternatively activated by IL-4, IL-13, IL-10, immune complexes, and glucocorticoids, and promote angiogenesis, tissue remodeling, parasite defences, and can act as immunoregulators by suppressing the inflammatory response. M0 are uncommitted macrophages. Our data show that M1 macrophages represent a relatively large fraction in our samples, while the other macrophage populations are only a very small proportion of cells. However, we have now replaced CIBERSORTx results with more accurate flow cytometry measurements of cell types in the blood (Supplementary Figure S2, S11, Supplementary Table S9) and have updated the manuscript text accordingly.

3. Fig. S6: It is unclear what the arrows pointing up and down at the same datapoint mean. Also why do the authors speak of IFN γ “dysregulation”, instead of simply upregulation?

We have added to the Figure legend: “Directionality of arrows signifies direction of differential-expression, wherein arrows pointing up signify up-regulation and down signify down-regulation compared to expression at DOL0. Arrows pointing both up and down for any given pathway signify a mixed response with a significant enrichment of sets of genes that were up and down regulated.”

4. Fig. 3: Again, why are genes “dysregulated” here? In Fig. 3C maybe further shorten the list of terms, the relevance of some remains unclear.

Thank you for asking us to clarify this. Dysregulation means up or down regulated and the explanation for the arrows has been added to the legend. We have shortened the list somewhat although this is just a subset of pathways that describe key ontogeny pathways. We have added an expanded legend to Supplementary information that describes in more detail the functional significance of each pathway included.

5. In my opinion figure 4 is uninformative and can be removed

We do not agree with this because this Figure reflects a key point of the manuscript, i.e. pathway rewiring. However, we have moved this Figure to Supplementary Figure S8 and have instead added more specific Networks that directly compare on a single Network the population-specific up and down regulated genes, which turn out to be linked, indicating they are likely alternative ways of achieving the same biological response. The point is that Networks represent biological wiring since protein:protein interactions (including physical, biochemical and regulatory interactions) drive biology and such depictions cannot be represented by linear pathways or simple pathway analysis. This highlights that there are multiple routes connecting the various proteins and population specific patterns are in fact interwoven (new Figure 3). We have added this explanation to the text to provide greater impact and an explanation for the inclusion of network graphs (Lines 221-225 and 234-236). We also now provide data about similarities and differences of hubs (new Table 3) since as explained at lines 234-6 “Networks are characterized by connectivity and key to this are hub proteins that are highly connected to other proteins and are considered as key biological intermediates that receive and transmit signals from interconnected proteins.”

6. The whole section Changes in nutritional status and indicators of disease is very speculative

and rather belongs into the discussion. In fact, relating these pathways to vitamin deficiency or viral infections is a very far stretch.

We agree and have abbreviated and moved this to discussion.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you for your contributions. We truly hope you benefitted from the experience.

Reviewer #4 (Remarks to the Author):

Neonates are highly susceptible to infections, a leading cause of infant morbidity and mortality. Effective public health interventions, including vaccination strategies, require a clear understanding of neonatal immune ontogeny, which begins in utero and continues to mature postnatally to provide protection against pathogens and maintain immune homeostasis

In this manuscript, the authors explored the details of early responses and distinct population-specific molecular mechanisms that define neonatal immune development during the first week of life from two geographically distinct populations. This work is important because deeper understanding of early period of immune development can expand our understanding of neonatal susceptibility to infectious diseases, as well as the impact of early-life interventions such as immunization. This work lays a crucial foundation for future research endeavors. However, there are several areas that require attention in this article.

We thank the reviewer for this strong summary of the importance of our work.

Major

1. The authors use bulk mRNA-seq to profile early neonatal immune cell development and claim cell type-specific gene expression. However, many genes are shared across immune cell subsets, so the conclusions cannot be fully supported by bulk data alone. Validation using single-cell transcriptomics or orthogonal methods such as flow cytometry is recommended.

Unfortunately, single cell transcriptomics is expensive and thus beyond the scope and resources of this study and often sacrifices transcriptomics depth since it detects the most highly-expressed transcripts per cell. Our main goal was to generate a comprehensive overview of immune developmental trajectories rather than resolve cell-type specific transcription, which would be very complex indeed. However, as suggested by the reviewer we have performed flow cytometry and have now added this . This data set provides a more accurate representation of cell types in the blood rather than our previous use of CIBERSORT predictions (Supplementary Figure S2, S11, Supplementary Table S9).

2. It is expected that neonates predominantly exhibit naïve immune cell profiles, making the reported dynamic changes in gene expression at these early stages notable. However, perinatal factors—such as the stress of delivery and sudden exposure to the external environment—could contribute to these observed changes. The authors should discuss the potential impact of these factors on their findings.

We have added the following sentences to the discussion at Lines 449-453: "It is possible that perinatal factors, such as the stress of delivery and sudden exposure to the external environment,

could contribute to these observed changes. However, all babies were delivered vaginally without any major medically-observed complications and any such changes would likely be obscured by our use of aggregate data from multiple infants and by the paired design."

3. The study lacks mechanistic insight into the regulation of the observed gene expression changes. The authors could strengthen their analysis by performing ATAC-seq for epigenomic profiling or, at a minimum, conducting in silico analysis of the promoters of differentially expressed genes. Mapping transcription factor binding profiles on these promoters would provide molecular insight into the regulation of dynamic gene expression changes.

Thank you for this suggestion, however, we cannot perform ATAC-seq because the samples no longer exist for any further experiments. We agree a more in-depth analysis of regulation of these gene expression changes may provide invaluable insights and could be completed in the near future but our prior experience with such methods have revealed that they are only modestly informative revealing dozens to hundreds of transcription factors.

4. Importantly, though this study advanced their previous published observations, this study failed to bring the novelty from the previous work. I believe that addition of distinct layer of omics dataset would have revealed molecular aspect to their conclusions.

We agree that we failed to describe well the novelty of the work with the previous manuscript. Thus, we have considerably reorganized the manuscript to place the population specific differences first since these were enabled by our better experimental design and clearly point to population-specific pathway and network remodelling. Furthermore we have added new layers of computational analysis to provide more intuitive ways of describing this including joint DyNet-based networks of population-specific genes, NetworkAnalyst-based protein:protein interaction networks, and pathlinkR-based pathway networks.

We appreciate your feedback to improve this study in adding distinct layers of omics studies, and now include flow cytometry results, that represent accurate measurements of cell-types that are circulating in the blood. This is based on previously published work (Montante et al. 2024) now applied our ontogeny study cohort. The innate myeloid compartment (primarily mature neutrophils, monocytes and T cells) had the most pronounced changes across the first week and were included in this study analysis (Supplementary Figure S2, S11, Supplementary Table S9).

In addition to this, we have improved our Results section to reveal more of the novel findings, specifically the unique heterogeneity in each population, rather than highlighting the core common ontogeny trajectory, as this was a common concern from reviewers. We hope that the updated analyses address this concern.

Minor

1. The authors should include a section for authors contributions.

This has been included now.

2. On page 8, line 267, 'TFH marker FOXP3' should be 'Treg marker FOXP3'.

Amended as requested.

3. It is recommended that all figures be reviewed for clarity and accuracy in labeling.

Reviewed and found to be accurate as is.

4. Use of 'in vitro' or 'in vivo' should be italicized.

Nature Communications policy is not to italicize these terms.

5. The authors should clearly specify whether the newborns were delivered via C-section or vaginal delivery, as mode of delivery can influence early immune development.

Agreed and sorry we omitted this. All newborns used in this study were delivered vaginally as now specified in the text.

Reviewer #5 (Remarks to the Author):

Summary

The authors present bulk RNA sequencing of peripheral blood from healthy neonates in two geographically distinct locations (Africa and Australasia) during the first week of life, aiming to delineate and compare transcriptional programs of ontogeny. By applying block randomisation strategies, they successfully reduced sequencing bias and reproduced results from their previous multi-omics study, this time using RNA-seq alone. The analysis identified more differentially expressed genes and transcriptional programs during the first week of life compared with their earlier study. These additional changes are explored in greater detail and are mainly attributed to the maturation of adaptive immunity. Additionally, the authors compare these programmatic changes between the two cohorts.

Significance to the field

This study builds on and refines previous findings on postnatal transcriptional changes in the peripheral blood of healthy newborns across different regions of the world. It provides further evidence that early postnatal changes follow a shared developmental program. Importantly, the authors demonstrate that, with appropriate experimental design, bulk RNA-seq can provide insights comparable to multi-omics approaches. The study also highlights how robust and harmonised workflows facilitate high-quality research in low- and middle-income countries (LMICs). On the other hand, bulk RNA-seq inherently limits the ability to resolve transcriptomic changes at the level of specific cellular subsets.

Thanks for this excellent summary and recognition that there is value in bulk RNA-Seq. We also recognize the limitations of lack of cellular specificity for events. This has to be balanced off against the very substantial difference in cost and limited gene expression data one achieves from single cell sequencing.

Conclusions, claims, and data analysis

The presented data support the existence of common postnatal changes in neonates across diverse populations. However, the reviewer questions whether computationally inferred cell subset distributions are sufficient to explain observed differences in differentially expressed genes or pathways (from the same sequencing data). This issue could potentially be addressed by incorporating data from the authors' previously published multimodal analyses, which include flow cytometry.

We agree with your concerns and have decided to include flow cytometry data, specifically using a myeloid panel that has been shown previously to be the most informative (Montante *et. al* 2024). We have evaluated the relationship between the myeloid cell type measurements via flow cytometry and gene expression variance (Supplementary Figure S3) and also directly compared

measurements of cells/ μ l of blood for each cell type for each DOL between the two locations (Supplementary Figure S11, Supplementary Table S9). We believe this has improved our ability to interpret the results of the RNA-Seq analysis in the context of these measurable changes in cell types in the blood.

While preserved postnatal programs are evident, population-specific differences are also suggested. These are described in parts of the manuscript and presented in Supplementary Figure S5, but no systematic analysis of inter-cohort differences is provided, particularly with respect to metadata that could influence transcriptional patterns. Sex, in particular, warrants further analysis, given the unequal distributions between cohorts (44% vs. 67% female). Although sex does not appear to be a major driver in PCA, sex distributions at later time points are not presented.

Certainly, there are many factors that can influence transcriptional patterns between cohorts and we appreciate that sex is a very important consideration. However, given that this study cohort was already split into 15 participants per randomization group, splitting further would greatly reduce the sample size and may bias any interpretation of findings. We have provided additional information about a disaggregated analysis in Supplementary Figure S15, and a breakdown of samples sizes in each group in Table 1. However, we caution against drawing conclusions from these findings since the smallest group included only 4 samples.

Because this is an observational study based solely on transcriptomic data, the conclusions remain hypothesis-generating. The “intervention” here is effectively the sampling location. The study impact might be strengthened by first analysing postnatal transcriptomic programs across the full dataset (to provide a more granular analysis than previous studies) and subsequently assessing the systematic effects of location and other metadata. Although the relatively small sample size per group ($n = 15$ per time point) may limit statistical power, such an approach would provide deeper insights into population-specific trajectories. Finally, the authors could attempt to integrate published datasets from other populations to confirm both shared and unique developmental programs.

Thank you for this thoughtful suggestion in the approach to understanding this topic. We agree with this and ran an analysis of the entire study cohort without considering any effect of location and have used this as a base to describe core ontogeny development), allowing us to tease apart population-specific DE genes from the location-specific modeling. We have included all DE gene lists and pathway enrichment results as supplementary tables (Supplementary Tables S1-2) and weaved results into Figure 2. This suggestion was extremely powerful and allowed us to clearly and confidently extract population-specific changes. Because of this additional comparison, we revised Figures 3 and 4, and several Supplementary Figures to provide more focus on the key findings from this additional analysis step.

Unfortunately, there are no other published datasets that we are aware of from other populations that have performed transcriptomics during similar timepoints within the first week of life, other than our published pilot study. If the reviewers are aware of any such studies, we are willing to confirm observations of shared/unique pathways in immune development.

Methodologies and documentation

The study applies state-of-the-art, publicly available bioinformatics tools to analyse the bulk RNA-seq data. Methods appear sufficiently documented to enable reproducibility once sequencing data

are released.

Thanks for this.

Overall assessment

This manuscript leverages rare and valuable samples collected through a carefully harmonised strategy to investigate the variation and commonality of neonatal postnatal transcriptional trajectories. However, the current version primarily provides impressions of shared processes rather than a systematic exploration of cohort-specific differences. In the reviewer's opinion, the manuscript would benefit substantially from a deeper and more structured analysis of differences between the groups, including integration of relevant metadata. Given the quality of the data and the thoughtful study design, such an analysis would greatly strengthen the impact of the work.

This is an essential part of this study analysis and we appreciate the reviewer bringing a focus on differences between groups. We have attempted to improve the flow of the manuscript to bring more attention to key differences and highlight them better in the discussion, including the differences in IFN gamma signaling, antigen processing and indicators of T cell regulation and activity. We also provide substantial new support for the concept of pathway rewiring using multiple illustrations.

Specific issues

The authors note dynamic changes in neutrophil frequencies within the first 6–12 hours of life. Since not all samples on day 0 are likely taken at the same postnatal age in hours, could this be incorporated into the analysis?

This is a very interesting observation and we have included this in Supplementary Figure S14 wherein the age of the sample (in hours) is depicted on a PCA plot of all DOL0 samples. In addition, there were modest gene expression changes from DOL0 to DOL1 compared to other timepoints, indicating that gene expression changes in the first 24 hours are more modest than at DOL3/7.

The previous study (Lee 2019) included $n = 30$ per group (GAM and PNG). The present manuscript ($2 \times n = 45$) should not claim to have more than double the sample size (lines 102–103), especially since line 167 refers to $n = 50$ in the earlier study, which appears inconsistent.

Yes, there were 30 infants in the previous study which of course suffered from the two datasets from GAM and PNG being prepared and sequenced more than a year apart and so any differences between cohorts could not be identified because they were confounded by the sequencing batch. The number 50 is a typo and has been corrected. This previous study was only 10 participants in each follow-up DOL group. The current study is 15 participants per group for each country which is 1.5-fold increase from the previous study (and this has also been corrected now). However following Reviewer 4's suggestion to examine these samples overall (i.e. the combined data from the two populations, as enabled by the block randomization design) has enabled an analysis with 30 individuals per DOL, which is 3-fold larger than our previous study. We have corrected the inconsistencies in the text to provide further clarity in the manuscript text as to the difference in cohort sizes between this study and the previous pilot study.

Figure 3C: Consider including enrichment scores in addition to p-values. Clarify the meaning of arrows pointing up and down within the same line.

We could include enrichment scores but this would make the Figure impossible to read, instead

we have used statistics to define pathways according to the methods utilized here. We have added to the Figure legend “Directionality of arrows signifies direction of differential-expression, wherein arrows pointing up signify up-regulation and down signify down-regulation compared to expression at DOL0. Arrows pointing both up and down for any given pathway signify a mixed response with a significant enrichment of sets of genes that were up and down regulated.”

Figure 4 and lines 229 ff: The described differences between GAM and PNG are not obvious from the figure. The color scheme for nodes is not explained, the cited genes are difficult to locate, and the selection criteria are not described in the methods.

We have moved this Figure to Supplementary Figure S8 (allowing the reader to enlarge the diagram) and updated the figure using updated DE gene lists using a higher threshold cut-off for fold change, thus reducing the number of nodes in the figures. This is replaced by DyNet-generated Networks of population specific genes which are much easier to see and support the concept of network remodeling much better. We have also explained the colour scheme in the legend. Unfortunately, it is really difficult to control where the different nodes appear within the network since the drawing of networks is stochastic, which is why we switched to DyNet in the current Figure 3. We hope these updated and new figures provides more clarity.

Figure 5B: Why was an absolute log-fold change (LFC) of 1.5 used as a threshold, while log₂FC values are plotted? For clarity, a consistent scale should be applied.

Sorry we failed to explain this was a log₂FC of (log₂1.5) (hence why we simply stated fold-change 1.5 for clarity), and we have now kept it consistent throughout the manuscript. The scale was actually consistent.

Line 267: FOXP3 is a Treg marker, not Tfh.

Agreed and fixed.

Line 314: Why are read counts now used to support the stated effect?

Thank you for pointing out this. We have now revised this section to include supporting data from the flow cytometry panel, though the gating of T cell populations was very limited. We hope that it would be helpful for the reader to observe patterns in changes of expression of key marker genes of T helper cell populations, but agree that this observation should be evaluated further using a more detailed T cell gating strategy. Unfortunately, there are no remaining samples for this cohort to run additional flow cytometry experiments.

Line 317: Could differences in HLA haplotype distributions between cohorts underlie observed differences?

Some changes in HLA were observed but these were population and DOL specific so we think likely not. This section was largely deleted in the amended manuscript.

Supplementary Figure 1: Dot plot presents only p-values. Consider including effect sizes or other quantitative measures. In the right panel, the “percent variance” values are all <1, are those percentages?

The reviewer is correct that it is proportional variance is presented in the percent variance.

Supplementary Figure 2: Are these a subset of the cell types computed by CIBERSORTx? Would it be possible to include all major T, B, and NK subsets? Analysing more abundant populations could clarify the extent to which gene expression differences are driven by cell composition. Was

correction for multiple testing applied?

These data have now been replaced by flow cell data and the gating strategies used enable subsetting of T, B, NK and monocyte cells. However, it must be said that the rather low amounts of any cell subsets that were population specific preclude any strong statements about association of subsets with gene expression studies since most genes are in fact expressed to some extent in most cell types.

Supplementary figures are cited out of order (e.g., Supp. Fig. 6 before Supp. Fig. 3; Supp. Fig. 5 before Supp. Fig. 1).

Sorry, this is now corrected with many additional figures.

Overall, the aforementioned edits and revisions in response to the thoughtful and invaluable reviewer feedback have substantially improved our manuscript.

Below please find the responses to your and the reviewer's comments. For reference, we have included the comment in italics with our responses included in plain type dark blue. Insertions into the text as well as line numbers are provided in quotes and underlined.



Dear Dr Hancock,

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The addition of the flow cytometry data strengthens the manuscript substantially. However, some key information on this method is missing in the method section. It doesn't describe how cells were preserved for flow cytometry, which is important especially given that the panel contains live neutrophil staining, unless a fixable viability dye was used, either way this needs explanation. The full list of antibodies used is required.

The following additional information has been included in the Methods section. "For flow cytometry samples, EDTA was added to 225µL of fresh blood within 4 hours of collection. Cells were stained with fixable visibility dye prior to lysis and storage at -80°C in Smart Tube reagents (Smart Tube Inc., San Carlos, California)." and "Upon thawing, flow cytometry samples were washed in staining buffer (PBSAN; 0.5% BSA, 0.1% sodium azide) and stained at room temperature in PBSAN with a cocktail of markers to determine the frequency of target immune cell populations as previously described."

When this information is provided, I would suggest to make a stronger point out of this interesting dataset: Please consider putting a shortened version of figure S11 to the main manuscript (it is much more informative than current figure 3).

Thank you for this suggestion. We have included selected panels from Figure S11 to in Figure 3 that highlight some key cell types, namely (a) CD11b⁻ CD16⁻, Immature neutrophils 1; (b) CD56⁻ CD16⁺, Natural Killer Cells; (c) CD64⁺ myeloid lineage cells (e.g. monocytes, macrophages, and dendritic cells). Early marker for immune activation and inflammation; (d) Non classical monocytes, (e) HLADR⁺ CD14⁺ monocytes, and (f) plasmacytoid dendritic cells (pDC).

I don't think "pathway rewiring" is the right term for what we see in figure 3. Instead, what it shows is that the DE gene set in GAM vs PNG implicates different interacting modules/pathway engagement. There is no "re" involved here.

We appreciate the reviewer's feedback on this particular terminology used to describe this phenomenon. In the context of protein-protein interaction (PPI) networks, this is a previously well-established term referring to a situation where the set of protein-protein interactions or pathways change between two set of circumstances (in this case population). The use of "re-" is because two things are being compared and indicates that one of the two is likely wired differently. Thus, we have decided to leave the language as-is, but have provided a definition of the term for clarity.

In figure 4C-D, are all green nodes upregulated? If yes, please state so in the legend, if not, please add this info somehow to the figure.

Thank you for pointing this out. We've added a note in the legend to clarify. These green nodes were highlighted as the nodes in the PPI network that were from the "Interferon signaling" pathway specifically, and were either up- or down- regulated.

Fig. S15 is not mentioned in the result section. In fact this is potentially interesting data. I would encourage the authors to also perform a direct sex-comparison (either merged for all locations or as a gene-wise 2x2 linear model considering sex and location). Even if this reveals only few genes, it is worth exploring and mentioning.

Thank you for this suggestion. We have included an additional section in the results to discuss these data, however, because some of the groups were quite small (n=4), interpretation of this data should be taken with caution. We have provided some high-level indications that should be evaluated further using a larger sample size.

The following papers should be included in the discussion: Ulas et al. 2017, PMID: 28459433; Olin et al. 2018, PMID: 30142345; Ariyakumar et al. 2022, PMID: 35711432; McDavid et al. 2022, PMID: 35310935; Dzanibe et al. 2024, PMID: 38744812

Thank you for bringing these additional studies to our attention. They are all relevant and provide additional context for the discussion of the findings from this study and we have cited and discussed them within the manuscript text.

Minor:

line 407 should point to fig. S11

Fixed now. Thank you.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you for your valuable contributions.

Reviewer #5 (Remarks to the Author):

General comments

Structure and flow of the analysis

Although the manuscript has substantially improved following revision, the overall narrative remains somewhat difficult to follow due to the order in which the analyses are presented. The readability might benefit from a restructuring in which the authors first describe the general features of postnatal immune ontogeny, followed by a more focused analysis of population-specific differences. Such an organization could help guide the reader more clearly from global developmental patterns to the study's comparative aspects.

Our original submission was organized in this fashion, but 3 previous reviewer's comments indicated that we should highlight population-specific differences first to showcase the novelty of the findings in this study. It was noted that the novelty was getting lost in discussion of similarities between populations, since this had been previously reported (with somewhat less detail but similar overall trends) in our pilot study. We revised the text in order to improve highlighting the novelty first and hope that it is OK for us to maintain the current organization of the manuscript for these important reasons.

Positioning and impact of the study

While the manuscript presents interesting and carefully generated data, it remains somewhat unclear what specifically elevates the work to the level expected for publishing in this highly ranked journal. This concern may be addressed if the authors further clarify and more explicitly highlight the conceptual advances arising from their findings — in particular, the biological implications and broader relevance of the observed differences between the two studied populations. Strengthening the interpretation and contextualization of these comparative results would help emphasize the manuscript's overall impact.

Thank you for this comment. In response we have tried to further emphasize the novelty, which we submit lies in the key population-specific differences in the efficiency of neonatal immune responses, including cell cycle, kinesins, and DAP12 signaling in Papua New Guinea, and antigen presentation, clathrin-mediated endocytosis and alpha-defensins in The Gambia despite tremendous overall conservation of ontogeny (88-96%). While comparisons have been performed between species indicating that immunity evolves due e.g. to contact with pathogens, this topic has been barely addressed in different populations of humans.

Minor comments

Overall, the authors have done an excellent job revising the manuscript and addressing the major concerns raised previously. I only have a few remaining minor points:

Line 216: The reference to the supplementary figure appears inconsistent (“Supplementary Figure SX??”). Please verify whether this should refer to Figure S3.

Amended as suggested. Thank you for noticing this error.

Line 228: Please confirm whether the correct reference is Supplementary Figure S4.

Correct. Fixed now.

Lines 239 onwards (environmental factors):

While I agree that genetic factors are controlled for in this context, I do not fully agree with the statement that variation in nutrition, environment, microbiota composition, and pathogen exposure is eliminated. These factors are unlikely to be fully established or in a steady state at day 0 and cannot be assumed to remain constant over time within an individual. The wording of this conclusion should therefore be moderated or clarified.

We appreciate the reviewer's critical lens on this comment and have edited this comment to “Use of a paired-design strategy in which each infant was compared to themselves at birth, largely eliminated sources of variation such as differences in genetics and epigenetics, but might be less comprehensive in accounting for other external factors such as environment, nutrition, microbial

load, exposure to pathogens, which could vary somewhat over the first week of life in any given infant”.

FACS cell counts:

Please clarify whether cell numbers are reported as counts per μL of blood. Values below ~ 20 T cells/ μL would indicate severe T-cell lymphopenia (within the range observed in SCID), which would require explanation or contextualization.

Yes, cell numbers are reported as counts per μL of blood, but they were adjusted based on bead count and normalized by an additional factor of 200 for computational scaling. We have provided additional clarification in the Methods and in the captions of the Figures describing these data and the computational scaling. The axis should be multiplied by this factor of 200 to get the true counts per μL of blood. So in the case of T cells, the median presented was around ~ 10 , which after multiplying by 200, gives us the true count which is $\sim 2,000$ cells per μL of blood.

Line 518 (PCA interpretation): Later time points appear to cluster more toward the lower right region of the PCA plot. Given this distribution, the stated conclusion may be somewhat strong and could benefit from more cautious wording.

We have revised the wording as follows to be more cautious: “This may in part explain some of the clustering observed in the principal component analysis (Figure 1A) whereby PNG DOL1 samples clustered more closely with all DOL0 samples, indicating that the gene expression data may have been influenced by the predominant neutrophil population.”

Figure S9: Figure S9 is intuitive and convincing. The authors may consider moving these data to the main figures (e.g., replacing or complementing Figure 3), as they substantially support the central message.

Thank you for your feedback. We agree that Figure S9 is very helpful for interpretation of the population-specific differences, however the panels were quite large when put together. Therefore, we opted to include it in the Supplementary Figures where the reader can easily expand portions of the Figure. We have provided additional interpretation of these data in the Results of the main paper to further draw attention to and strengthen these interesting findings.