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Peer Review Information

Journal: Nature Immunology
Manuscript Title: Global and cell type-specific immunological hallmarks of severe dengue progression identified via a systems immunology approach
Corresponding author name(s): Shirit Einav, Fabio Zanini

Reviewer Comments & Decisions:

Decision Letter, initial version:

27th Mar 2023

Dear Shirit,

We have now finished reviewing your manuscript entitled "Global and cell type-specific immunological hallmarks preceding severe dengue progression", reference number NI-A35401. Although the editors thought that the manuscript was interesting enough to send out for in-depth review, the reviewers were not in favor of publishing the paper in Nature Immunology. Referees #1 and #2 both express some conceptual novelty concerns, in particular with your previously published studies cited as references #9,10,11. Referee #3 voices a number of technical concerns with their data analysis and donor cohort comparisons. The referees do acknowledge some novel findings involving the B cell and NK cells, but expressed that both aspects appear to be currently preliminary. While we might be interested in a substantially revised manuscript, a number of unanswered questions would need to be addressed. Given these comments on the current manuscript version, we are returning the reviews to you with the hope that you find them useful when you prepare the paper for another journal.

You might want to consider our sister journal Nature Communications as a potential venue for the publication of these results. Nature Communications publishes high quality and influential research and across the full spectrum of the natural sciences. More information on the journal, the potential benefits of transfer and a link to transfer your paper, can be found at the bottom of this email. Please note that the editorial team at Nature Communications will consider your manuscript independently of our suggestion to transfer.

We realize that this is disappointing. I hope that you continue to consider Nature Immunology for your results most significant for the immunology community and wish you well in your future investigations.

Sincerely,

Laurie A. Dempsey, Ph.D.
Senior Editor
Nature Immunology
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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

To study the pathogenesis of severe dengue virus (DENV) infection, here the authors study PBMCs from 19 Dengue infected children –ages 4-17 years– (and 4 healthy subjects) as follow up to the authors' previous work (Biorxiv and in press at Science Advances, Ref. 11) comparing the immune features and temporal response of PBMCs from children versus adults during progression to severe Dengue (SD) infection. In this study, PBMCs were collected early 1-7 days from symptom onset and half of the children developed SD. Unlike the previous work which utilized CyTOF, the authors rely here on an optimized virus-inclusive single cell RNA-seq (viscRNA-Seq2) which they had previously developed to concurrently detect host and viral transcripts (2019, PMID 30699342).

viscRNA-Seq2 surprisingly revealed that most of the vRNA was present in 40% of the B cells when it was thought that myeloid cells are the primary DENV targets in the blood. Compared to myeloid cells, B cells isolated from DENV infected patients were best at infecting a human hepatoma cell line upon coculture. Furthermore, the most DENV reads originating from the negative DENV strand were detected primarily in B cells indicating that the most active replication occurred in B cells. viscRNA-Seq2 also allowed the authors to directly compare the transcriptome of viral RNA harboring cells (VHCs) to corresponding bystander cells. This elegant approach revealed that the transcriptome of DENV-harboring B cells, monocytes, and NK cells was indicative of elevated oxidative phosphorylation. DENV-harboring B cells showed decreased transcripts involved in BCR signaling, antigen presentation, and interferon response.

The authors then switch to studying the transcriptome of total cells (presumably both VHCs and bystander) and find precent increases in the fractions of classical monocytes, signaling NK cells, proliferating plasmablasts, Tregs, memory CD4 T cells and exhausted CD8 T cells in severe dengue progression (SDp) compared to healthy subjects and symptomatic non-progressors (D/DWS). They note decreased HLA-DR expression in APC types –monocytes, conventional DC (cDC), and B cells (both transcript and protein) and suggest impaired antigen presentation in these APC types. They also note identify differentially expressed genes in monocytes, cDC and B cells that have to do with cell adhesion and migration, FcgR signaling, scavenging function, and viral translation. All these observations were concordant with the authors previous observations using CyTOF (Ref. 11). The authors also identify a dysregulated type I IFN response in this study such that monocytes but not DC and B cells showed decreased ISG expression even while all these cells show increased transcripts such as IRF1 and STAT1 associated with IFN signaling.

In contrast to APC types, HLA-DR expression levels on a small percentage of NK cells were elevated in DENV-infected patients vs healthy control subjects, and NK cells from SDp patients showed increased levels of cytotoxicity related genes but also exhaustion markers. Exhausted CD8 T cells and Treg cells

were abundant in SDp vs D while multiple ISGs were downregulated as well as the T cell activation marker IL-32. The authors also identified the largest number of cell-cell communications in SDp vs D patients involving monocytes and cDCs and lowest in B cells.

Major comments:
o The authors state that biomarkers for the early prediction of progression of Dengue infection to severe Dengue (SD) are urgently needed (lines 70-71). Yet work from the authors (Ref. 9) has identified a 20-gene signature predictive of SD. What does the analysis here add over the previous study and is there a biomarker that emerges from this study that could be used to predict progressive severe dengue infection?
o While the focus of the analyses on SDp vs D is understandable, much of the particulars of the immune response had already been reported in Ref. 11 as indicated above. The authors have a unique opportunity to examine evolution of the immune response at the transcriptional level in specific VHCs over the course of disease and identify the specific responses in VHCs that are altered in SDp. However, the comparison of VHCs to bystander cells is dropped from Figure 3 onwards where the study offers little over what the authors had already conducted previously. For example, the authors could analyze whether the levels of viral transcripts/cell and percentages of virus harboring B cells increase upon disease progression from D/DWS to SDp.
o Virus harboring B cells expressed moderate levels of the envelope protein compared to high levels in myeloid cells and the authors attribute this to modulation by subgenomic flavivirus RNA. However, while only <5% of monocytes and NK cells harbor viral transcripts, flow cytometry shows DENV E protein expression by the majority of the monocytes and CD56bright NK cells. What is the reason for this discrepancy between detection of viral transcripts and intracellular staining for viral E protein? Is this related to the sensitivity of detecting viral transcripts in the scRNA-seq dataset? Do the authors find this concerning?
o Unlike B cells, antigen presentation related DEGs between virus harboring monocytes compared to bystander cells show an increase and not a decrease in HLA-DR and HLA-DP. Are these differences a result of DENV altering the transcriptome of VHCs or activation of the monocytes by DENV? How do the HLA-DR levels compare in virus harboring monocytes in SDp?
o The authors do not directly validate lowered HLA-DR levels as a result of viral infection. Importantly, viscRNA-seq showed decreased levels of antigen processing related genes in virus harboring B cells suggesting that the decrease in HLA-DR may be due to DENV infection.
- To understand whether the lowered HLA-DR levels are a result of infection and not a different indirect mechanism, the authors should compare the levels of HLA-DR on DENV E protein positive versus negative B cells, inflammatory monocytes, DC, and NK cells.
- Is DENV known to impair specific steps in antigen processing or presentation?
o The authors conclude that expansion of HLA-DR expressing possibly adaptive-like (i.e. able to activate T cells) NK cells characterizes SDp. How are these cells related to the virus harboring NK cells which have lower expression of HLA-DR (Fig. 2g)? The authors speculate that NK cells may adopt antigen presentation roles in DENV infection particularly in SDp patients. However, these conclusions are based on a small percentage of cells showing 4-fold elevated HLA-DR transcripts in patients vs healthy subjects (Fig. 5b), and 20% NK cells with higher HLA-DR protein in SDp vs D (Extended Data Fig. 5b). They are also discrepant with the data in Fig. 2g.
- Side-by-side flow cytometry for HLA-DR on NK cells and APC types would best show how the NK cell HLA-DR levels compare to those on monocytes, DC and B cells including healthy controls to show baseline HLA-DR levels. It would be a plus if they separated the cells into E protein positive versus negative to address the role of DENV on the levels of HLA-DR.
- Can the authors explain how HLA-DR levels increase on a subset of NK cells when both APC types

and NK cells were collected from the blood and presumably in the same milieu?
- Percentage of NK cells with elevated HLA-DR do not correlate with the <5% cells harboring viral transcripts or the discrepant majority NK cells that are positive for intracellular DENV E protein by flow cytometry.
o The authors suggest that APCs have impaired antigen presentation. This suggestion is also based solely on the decreased transcript and protein levels of HLA-DR in these cells. Impairment in antigen presentation could be due to a decrease in surface HLA-DR expression or a decrease in antigen processing capacity of the cells or both. Are there differences in the expression of genes involved in antigen processing, cathepsins, aminopeptidases, lysosome function associated transcripts in VHCs compared to bystander cells? The authors could also consider using antibodies to measure components of the antigen processing machinery in human cells by flow cytometry.
o The authors conclude that antigen uptake is greater in all APC subsets from DENV infected patients compared to healthy controls. However, it is surprising that the healthy APC types show little to no uptake of pHrodo S. aureus bioparticles to begin with. Why do the majority of healthy APC harbor 0 bioparticles (Fig. 4f)? Are the healthy data points shown in this graph at 4 oC and if so, please show the healthy APC uptake at 37oC. Is the 4oC control in Fig. 4g paired for each DENV-infected patient or healthy subject?

Minor comments:
o What is the physiological significance of inversely regulated interacting differentially expressed genes (iDEGs) noted in NK cells from SDp vs D.
o Luminex assay serum concentrations of cytokines/chemokines (Fig. 6e) show some differences not commented on by the authors, e.g. MIF, CCL26, FAS, IL-27, RETN, SERPINE1 while TNF levels are not different contrary to what the authors state (line 420).

Reviewer #2 (Remarks to the Author):

Summary of the key results
This manuscript entitled "Global and cell type-specific immunological hallmarks preceding severe dengue
Progression" by Ghita et al. is well written and has a broad appeal. The manuscript profiles PBMCs from 19 DENV-infected children and 4 healthy controls using an optimized viscRNA-Seq 2 approach. Within the DENV-infected cohort, 7 progressed to severe DENV(SD). The aims of the manuscript are to investigate the cellular and molecular determinants preceding SD progression to identify markers that could be used to predict SD. The manuscript identifies several phenotypic differences in cell populations present in SD including the types of monocytes and NK cells present. Additionally noting difference in HLA-DR expression levels on APC in SD cohort.

Originality and significance: The analysis was done at the time of hospitalization and therefore retrospective of precursors to SD has rarely been done in this amount of detail. However, as noted in the manuscript this group had completed a similar study, albeit, with an older cohort using Cytof with some individuals having already progressed to SD. As noted in the study many of the findings observed in this study were previously identified in the authors earlier published work.

Data & methodology and statistical treatment: validity of approach, quality of data, quality of presentation
The data as presented was of high quality. The authors noted the limitations of the study with the

number of subjects being limited and the variability within the population as in regards to level of symptoms in the cohort that did not progress to SD creating confounding issues with the data.

Conclusions: The conclusions drawn are valid based on the data provided. As noted below, the results of this study while providing a highly detailed characterization and comparison of SD progressors and non-progressors does not provide a practical solution that can be implemented to predict who will progress to SD. Additionally due to the small cohort (19), unique population (adolescents), and high variability it will be interesting to see if larger studies using less costly techniques support the differences in cell populations and HLA-DR expression noted.

Suggested improvements:
Within the manuscript, there are several minor areas that should be improved. It is not clear if attempts were made to determine if any or all of the 19 subjects had a prior DENV exposure. Given that an aim of the manuscript was to determine indicators of progression to SD it would be important to know what the prior history of dengue was in the subjects. Additionally, it is unclear why adolescents were used in the study. It would be interesting to determine if there are differences between adolescent and adult progressors to SD.
Within the section about dengue replicating in B cells lines 124... how many of the 6 PBMC samples were from SD progressors.
The entire section on dengue replicating in B cells is very important and rarely discussed in the field and should be published. However, it does not currently fit with the aims of the manuscript as it is written it does not appear to have much to do with progression to SD in the cohort analyzed. Some effort should be made to integrate this section into the manuscript if it is to remain as part of the study.

References: Appropriate credit is given to previous work The literature on this subject is vast so while the author's reference list is not comprehensive, it reasonably covers the subject area.

Clarity and context
Given that the conclusion of the study could not be directly used as a prognostic screen to identify progressors to SD the language in the abstract, introduction and discussion should be toned down. Additionally, the claim that this study identified a molecular mechanism that predicts SD should be removed as the study did more to characterize differences in markers between the cohort and did not directly show that those marker differences could contribute to the progression to SD.

Reviewer #3 (Remarks to the Author):

Summary:

Ghita, Yao, Xie, and Duran et al. have comprehensively characterized the transcriptional profiles of acute phase samples from patients with dengue, dengue with warning signs, and severe dengue. They perform single cell RNA sequencing, incorporating additional primers to improve detection of dengue viral RNA, to characterize the major cell populations involved in viral replication as well as transcriptional signatures associated with disease. They also perform validation assays to confirm their transcriptional findings. Overall, this work provides novel insights into the factors driving dengue disease severity at finer resolution than has been previously described. The manuscript would be

improved by being more transparent about the limitations of the study including sample size and how samples were selected for each sub study, and including an analysis comparing primary versus secondary dengue.

Major comments:

1) The authors make statements about the relative contribution of antibody-dependent enhancement to severity but do not perform an analysis comparing primary vs. secondary dengue, although they have these data. This analysis should be added to the manuscript especially when talking about antibody-dependent enhancement hypothesis.
2) Table 1 is overly large. It would be helpful if the authors could provide a table with basic demographic and severity data as well as which samples were used in which sub-analysis.
3) The participants ranged in age from 4 to 17 years. Were there any differences noted across age in transcriptional profiles? And was age accounted for in any way in the analyses? It would be helpful if the authors at least commented on this point and provided an analysis of differences across age, if possible.
4) The authors state on Line 95 that the samples were collected early in the course of disease. However, many of the samples were collected on days 5-7 after fever onset. Does that really count as early?
5) For their single-cell analyses, the authors measure the pairwise differences between all individuals. To our knowledge, this is an unconventional method. Generally, comparisons are performed between groups. The authors should cite literature using the pairwise method or describe their approach in greater detail, if it is a novel approach. The authors should also consider analyzing data using conventional approach looking between groups.
6) Single-cell RNA experiments are expensive, and identification of severe dengue patients is difficult, making it challenging to have large sample sizes. However, the authors should still provide greater explanation for how samples were selected for each analysis. For instance, different assays were performed on different participants: Fig. 1G – uses data from 3 participants, Fig. 2A from 6. It would be helpful if the authors justify the sample size used for each analysis. As another example, Fig. 4G is not significant. Is this because a small number of samples was used and thus the effect was not observed? It would be helpful for the authors to comment on how confident they are that the effect was not present, or the sample size may have been too small and thus the analysis was underpowered.
7) In the yellow box in Figure 7, the authors could mention B cells as distinct from antigen presenting cells, as this is a major finding of their study. Also in this box, the authors should mention the reduced antiviral response in infected cells.
8) In the methods (line 570), it is explained that CD3 depletion was used to enrich for B cells, monocytes, and dendritic cells. Then these rare populations were re-pooled with undepleted fractions. We assume this is why none of the figures show the relative prevalence of infected cells. The method used seems to be justified, but the authors should make it clearer in the main text that this approach was used and why that prevents direct comparison of frequencies.

Minor comments:

- It is not clear in text that the study included only DENV1 and DENV3 cases. This makes it confusing why primers were only included for DENV1 and DENV3. This should be stated in the manuscript.
- One of the severe dengue patients was pregnant. Given that pregnancy is expected to modulate the transcriptional profile, it would be helpful to state if analyses were conducted to check if this person

was an outlier.
- The manuscript includes a lot of acronyms for immune cell populations, disease outcomes, and analysis methods. In some instances, acronyms are never defined. In other places, the term is spelled out (e.g. line 211, interferon signaling genes, and then line 212 uses ISGs). Keeping some acronyms as fine, but where terms are only used a few times, it would be helpful if the authors could just write it out to make the manuscript easier to read.
- Line 145: The authors mention using HLA-DR+ cells to look at other antigen presenting cells besides B cells. It would be helpful to explain that these are the other antigen presenting cells, to make it easier to follow.
- Line 102: "viscRNA-Seq 2's improvements we introduced included…". This is awkward phrasing. Consider rewording to "Improvements introduced to viscRNA-Seq 2 include … "
- Figure 5: T cells and plasmablasts are mislabeled in the figure compared to the legend.
- Line 377: consider saying healthy individuals?
- Line 412-415: The wording here is confusing. Consider rephrasing.

Author Rebuttal to Initial comments

Response to reviewers

Manuscript title: "Global and cell type-specific hallmarks preceding severe dengue progression via a systems immunology" (NI-A35401).

Dear Dr. Dempsey,

We thank the Editors and the Reviewers for their thoughtful and helpful comments. Below we provide a detailed response to each of the items mentioned by the reviewers and indicate how we have incorporated these suggestions into our revised manuscript. Please, note that all page locations and figure numbers referred to below are with respect to the revised manuscript.

Editor's comments:

Referees #1 and #2 both express some conceptual novelty concerns, in particular with your previously published studies cited as references #9,10,11.

--We would like to outline important differences between the current work and our former studies and to highlight its conceptual novelty. The work we published in references #9 and #10 focused on the discovery of 8- and 20-gene sets predictive of progression to severe dengue

(SD) in existing bulk-sample transcriptomic datasets and their validation in our dengue Colombia cohort. While it has exciting translational implications, its contribution to better understanding SD pathogenesis is limited.

Indeed, as commented by the Reviewers and discussed in our manuscript, some overlap exists between the current work and our former CyTOF work (Ref #11). Nevertheless, please note that the previous study was based solely on CyTOF analysis using a pre-selected and limited panel of 36 markers, of which only 15 were functional (vs. markers for cell identification). In contrast, here, we use an unbiased, genome-wide approach to compare between SD and dengue patients. This unbiased approach coupled with cellular granularity enabled us to not only confirm the prior observations in an unbiased manner and to understand modulation of gene expression in a broader context of pathways, but also – crucially – to discover novel immunological hallmarks associated with SD progression different from those previously identified.

Even more importantly, beyond mere identification of expression markers, in the current study we utilized a system immunology approach to comprehensively analyze the immune response to dengue virus (DENV) infection. By integrating findings from two single cell technologies that capture both cellular and viral elements (which our and others' CyTOF work failed in doing) and bulk sample studies and examining cell-cell communication networks, the current study discovers and functionally validates several key novel findings. **Specific areas of novelty,** several of which were introduced in this revised version in response to the Reviewers' comments, include:

i. This study reveals that SD progression in natural infection is associated with impaired antigen processing and presentation in antigen presenting cells, despite intact uptake activity.

ii. The novel cell-cell communication analysis uncovers the complex interaction networks of the immune system preceding SD progression at a single cell resolution, beyond the available knowledge limited to a few cell types. Integrating this analysis with measurement of soluble factors in the serum of the same patients revealed novel cytokine/chemokine pathways involving candidate interactions between specific cell subtypes (e.g. IL-10 secretion by Tregs with IL10 receptors on myeloid cells), providing novel intriguing avenues for further validation.

iii. This study reveals dysregulation of type I IFN response on myeloid cells in children with SD, which to the best of our knowledge has not been described previously.

iv. This study reveals dysregulation of effector immune responses preceding SD progression, including increased exhaustion (beyond the regulation we reported in out CyTOF paper) in T cells and uncoordinated NK cell interactions. It also reveals association of SD progression with modulation of specific NK cell receptors (CD2 and KLRB1) for further validation.

v. The identification of the specific cell subtypes in the human blood in which DENV replicates has been a major challenge in the field, due to technical difficulties in detecting DENV (non-polyadenylated) RNA and protein in patient-derived single cells. Indeed, we and others (personal communications) have failed in identifying DENV-infected cells via CyTOF. In the revised manuscript:

- We identify the specific cell subtypes that harbor viral RNA and protein by two distinct single cell approaches.

- We reveal infectious DENV production in natural infection in circulating B cells, in addition to monocytes and cDCs, using three orthogonal approaches.

- We discover that downregulation of HLA-DR is driven, at least in part, by DENV and is associated with downregulation of additional factors mediating antigen processing, loading and presentation. These findings provide new insight into one mechanism involved in downregulation of HLA-DR, a hallmark of severe dengue, and a potential link between viral-mediated immunomodulation and disease severity.

vi. This study reveals that whereas pathways implicated in antibody-dependent enhancement (ADE) are associated with prior DENV exposure, other pathways including impaired antigen processing and presentation are only partially associated or not associated with prior exposure but rather with SD progression.

Collectively, we therefore believe that this study provides valuable novel insights into the immunopathogenesis of dengue and formulates intriguing new specific hypotheses for multiple future basic as well as translational investigations.

A more detailed discussion of our conceptual novelties is outlined in the response to comment # of Reviewer 1.

Referee #3 voices a number of technical concerns with their data analysis and donor cohort comparisons.

--The primary technical concern raised by Reviewer 3 (comment #5) is about the pairwise comparison we used in our differential gene expression analysis.

We recognize that the pair matching employed for several comparisons in the manuscript is not often explained in detail and would benefit from a more detailed explanation to avoid confusing the readers. Nevertheless, this analysis is an elementary statistical device that serves a clear purpose: decoupling effect size and noise level in our analyses.

First, it is correctly noted that "Generally, comparisons are performed between groups." Like other methods, the pairwise analysis is a group comparison, or comparison across groups, because we never pair samples from the same group (say, SD progressors or dengue patients). Indeed, comparing the groups is the aim of those analyses, not comparing each single patient to the others. Second, we would like to emphasize that the results of the pairwise comparisons are highly consistent with a traditional non-parametric Kolmogorov-Smirnov (KS) test between groups: we now include a table reporting the median fold change presented in the main figures next to the KS statistic (see Table 7). As shown, the two methods agree in terms of describing the effect direction (or sign) and the effect size, which are the key metrics in any statistical framework. Third, we would like to clarify why despite this high agreement, we chose to show pairwise comparisons in the main figures. Rigorous statistical tests rely on solid assumptions (e.g. about the shape or tails of the data empirical distribution) and, provided those assumptions are correct, deliver an estimate of statistical significance (e.g. P-value) that compounds two elements into one: effect size and noise level. If the effect size is small, P-values will be close to 1 (with a small number of samples). Similarly, if the effect size is large but the data is noisy, the P-value will also be close to 1, but for a very different reason. Given the unknown shape of our empirical distribution and the small sample size of our data - it is still the largest such dataset on

dengue generated – we decided to avoid this mixing by visualizing separately the effect size (median of pairwise comparisons) and the noise level (standard deviation or quartiles of pairwise comparisons). The most important aspect of our analysis is to use patient-level averages before computing any differential measurement, which is entirely consistent with current recommendations from statistical single cell literature (Squair et al., 2021) Given the technical soundness of the computations, the choice to separate effect size from noise level visually is merely designed to help readers assess those metrics separately, which we believe provides additional insight into the interpatient variability of dengue virus infection.

In response to the Reviewer's comment, in the revised manuscript we include Table 7: Kolmogorov-Smirnov (KS) test and pairwise comparison largely agree on the sign and effect size for DEGs in SDp vs. D, and clarify the rationale behind using this method. See lines 134-136 and 670-681.

The other technical comment raised by Reviewer 3 ("it will be helpful if the authors justify the sample size used for each analysis") has also been addressed (see response to comment # 6 of Reviewer 3).

The referees do acknowledge some novel findings involving the B cell and NK cells, but expressed that both aspects appear to be currently preliminary.

--In response to several insightful comments made by the Reviewers, in the revised manuscript, we have expanded the data analysis with respect to B cells and NK cells. First, we investigated the expression profiles of multiple elements in the machineries that mediate antigen processing and presentation on both MHC-I and MHC-II molecules. This analysis reveals modulation of multiple genes involved in antigen unfolding, degradation, transport, loading and presentation specifically in viral-RNA harboring B cells relative to corresponding bystander B cells (see Fig. 4i and Fig. R3 below). Second, we studied HLA-DR surface expression on distinct cell subtypes stained positive or negative for intracellular DENV envelope protein via flow cytometry analysis on patient derived samples, validating that the modulation of antigen presentation we observed is induced by DENV (Fig.4j and Fig R1 below). Third, we have compared immune responses in

B and NK (and other cell types) between patients with primary and secondary infections (See Extended Data Fig. 4a and Extended Data Fig. 5e). Collectively, these new studies propose that whereas in NK cells, upregulation of MHC II is not DENV-mediated, in B cells, there is viral-induced downregulation of antigen processing capacity in addition to presentation that is not associated with DENV exposure but rather with disease progression. These findings thus establish a link between viral-mediated immunomodulation in B cells and disease severity.

--Moreover, we realize that lack of clarity about which samples were used for various analyses caused confusion with respect to some of the NK cell data. Thus, we have now addressed Reviewer 1's comments regarding NK cells by revising the text to simplify the interpretation of our data.

We believe that the clearer presentation of our former findings combined with the addition of key new findings provide a deeper understanding of the DENV-induced responses in B, NK (and myeloid) cells.

While we might be interested in a substantially revised manuscript, a number of unanswered questions would need to be addressed.

--We thank the editor for their potential interest in a substantially revised manuscript. In the attached revised manuscript, we have addressed all the Editor's and Reviewers' unanswered questions, and restructured the entire manuscript to better highlight the novelty of the work.

Reviewers' comments:

Reviewer #1:

To study the pathogenesis of severe dengue virus (DENV) infection, here the authors study PBMCs from 19 Dengue infected children –ages 4-17 years– (and 4 healthy subjects) as follow up to the authors' previous work (Biorxiv and in press at Science Advances, Ref. 11) comparing the immune features and temporal response of PBMCs from children versus adults during progression to severe Dengue (SD) infection. In this study, PBMCs were collected early 1-7

days from symptom onset and half of the children developed SD. Unlike the previous work which utilized CyTOF, the authors rely here on an optimized virus-inclusive single cell RNA-seq (viscRNA-Seq2) which they had previously developed to concurrently detect host and viral transcripts (2019, PMID 30699342).

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The authors then switch to studying the transcriptome of total cells (presumably both VHCs and bystander) and find precent increases in the fractions of classical monocytes, signaling NK cells, proliferating plasmablasts, Tregs, memory CD4 T cells and exhausted CD8 T cells in severe dengue progression (SDp) compared to healthy subjects and symptomatic non-progressors (D/DWS). They note decreased HLA-DR expression in APC types –monocytes, conventional DC (cDC), and B cells (both transcript and protein) and suggest impaired antigen presentation in these APC types. They also note identify differentially expressed genes in monocytes, cDC and B cells that have to do with cell adhesion and migration, FcgR signaling, scavenging function, and viral translation. All these observations were concordant with the authors previous observations using CyTOF (Ref. 11). *The authors also identify a dysregulated type I IFN response in this study such that monocytes but not DC and B cells showed decreased ISG expression even while all these cells show increased transcripts such as IRF1 and STAT1 associated with IFN signaling.*

In contrast to APC types, HLA-DR expression levels on a small percentage of NK cells were elevated in DENV-infected patients vs healthy control subjects, and NK cells from SDp patients showed increased levels of cytotoxicity related genes but also exhaustion markers. Exhausted CD8 T cells and Treg cells were abundant in SDp vs D while multiple ISGs were downregulated as well as the T cell activation marker IL-32. *The authors also identified the largest number of cell-cell communications in SDp vs D patients involving monocytes and cDCs and lowest in B cells.*

Major comments:

1. The authors state that biomarkers for the early prediction of progression of Dengue infection to severe Dengue (SD) are urgently needed (lines 70-71). Yet work from the authors (Ref. 9) has identified a 20-gene signature predictive of SD. *What does the analysis here add over the previous study and is there a biomarker that emerges from this study that could be used to predict progressive severe dengue infection?*

--Our prior work the Reviewer is referring to aimed at identifying clinically usable biomarkers of severe dengue (SD) progression. The work involved leveraging existing transcriptomic bulk sample datasets from multiple cohorts to discover a 20-gene set, and more recently an 8-gene set, which are predictive of SD progression, and validation of the findings in our Colombia dengue cohort. While the discovery of the 8-gene set has exciting translational implications, focused on a low-resolution, bulk sample transcriptomic analysis, the contribution of the work to better understanding SD pathogenesis has been overall limited.

In contrast, the primary goal of the current work was to contribute to the understanding of the pathogenesis of SD progression. Unlike the former work, here, the goal was to capture tissue heterogeneity in patients PBMC samples to enable high-resolution monitoring of cell type-specific responses preceding SD development. Using a system immunology approach that integrates virus-inclusive single cell RNAseq (viscRNAseq), spectral flow cytometry, functional assays and serum cytokine analysis, we discovered immunological signatures preceding SD development in multiple cell subtypes and in virally-infected vs. bystander cells.

The secondary, future goal of the work we present here is to identify cell-type specific determinants and/or alterations in cell-type abundance as candidate alternative predictive biomarkers. Focusing on measurement of a smaller number of markers in a specific cell type could potentially reduce the cost involved in developing/using prognostic assays (which is currently prohibiting the development of an assay based on the 8-gene set), and it may thus provide easier access to such a test in poor-resource countries, as many of those where dengue is endemic. Sample-to-answer technologies that could achieve rapid cell isolation and transcript measurement already exist (see example: https://pubmed.ncbi.nlm.nih.gov/26009151/, https://cdn.stemcell.com/easysep-mouse-cell-isolation). While we provide a proof-of-concept that changes in abundance of three cell subtypes could distinguish SDp from D (Extended Fig. 1e), the identification of cell-type specific predictive biomarkers among the hits we discovered via machine learning approaches and their validation will be pursued in the future.

In response to the Reviewer's comment, we now better highlight the aim and scope of the current work. See line 92-95 Moreover, we have toned down sentences mentioning predictive biomarkers in the introduction and discussion sections. See lines 69-70

2. While the focus of the analyses on SDp vs D is understandable, much of the particulars of the immune response had already been reported in Ref. 11 as indicated above.

--Please, see response to the first comment of the Editor above outlining the conceptual novelties of the revised study.

3. The authors have a unique opportunity to examine evolution of the immune response at the transcriptional level in specific VHCs over the course of disease and identify the specific responses in VHCs that are altered in SDp. However, the comparison of VHCs to bystander cells is dropped from Figure 3 onwards where the study offers little over what the authors had already conducted previously. For example, the authors could analyze whether the levels of

viral transcripts/cell and percentages of virus harboring B cells increase upon disease progression from D/DWS to SDp.

--We agree with the Reviewer that analysis of transcriptional changes in VHCs between SDp and D patients would have been interesting. Unfortunately, however, while we put a lot of effort in optimizing our capture oligo, detection of the non-polyadenylated RNA of DENV in single cells is not easy (and to date, outside of our work, was shown in a single patient (Sanborn et al., 2020)). To overcome this challenge, in order to maximize our chances to identify VHCs, we selected 4 DWS patients with very high viremia (Table 1). As shown in Table 3 (included below Table R1), the majority of VHCs detected were from three of these DWS samples, with very small numbers of VHCs in SDp and D patients. The first analysis proposed by the Reviewer, i.e. to identify the specific responses in VHCs that are altered in SDp, is thus not feasible with this dataset. Nevertheless, we have now addressed the second analysis the Reviewer proposed, revealing that dengue severity in our dataset does not correlate with the number of viral transcripts/cell or the percentage of VHCs.

--In response to the Reviewer's comment, we have now:

i. included Table 3 showing VHC numbers per disease category (see below Table R1).

ii. showed in Fig. 4a that the presence of vRNA correlates with viral load in serum but not with disease severity (see line 234-235).

iii. provided an explanation how the DWS patients were selected (see lines 230-234).

iv. added a sentence about the inability to monitor VHCs across disease categories as a limitation of the study (see lines 490-492).

v. compared HLA-DR protein expression in DENV E+ and bystander cells revealing that modulation of HLA-DR, a hallmark of SD we identified, is induced by DENV in natural infection. See Fig. 4j and response to comment #6 below.

Condition	DWS	Healthy	S_dengue	dengue
cell_subtype_new				
activated B cells	30	0	0	1
classical monocytes	49	0	0	1
megakaryocytes	0	0	0	0
memory B cells	27	0	0	6
naive B cells	797	0	4	56
CD4+ naive T cells	5	0	0	1
CD4+ memory T cells	8	0	0	2
CD8+ naive T cells	2	0	0	0
CD8+ effector memory T cells	9	0	0	0
MAIT	3	0	0	0
Tregs	0	0	0	0
CD8+ exhausted T cells	0	0	0	0
proliferating plasmablasts	13	0	0	2
non-proliferating plasmablasts	22	0	0	2
non-classical monocytes	36	0	0	2
intermediate monocytes	20	0	0	1
cytotoxic NK cells	86	0	0	2
signaling NK cells	2	0	0	0
cDC1	2	0	0	0
cDC2	0	0	0	0
pDCs	8	0	0	1

Table R1: Total number of vRNA harboring cells (VHCs) detected via viscRNA-Seq 2 across all 23 samples sequenced. The table shows that the large majority of VHCs was detected in DWS patients (who had high viremia), with only small number of VHCs in D and SDp, thus limiting the analysis of VHCs across disease severity.

4. Virus harboring B cells expressed moderate levels of the envelope protein compared to high levels in myeloid cells and the authors attribute this to modulation by subgenomic flavivirus RNA. However, while only <5% of monocytes and NK cells harbor viral transcripts, flow cytometry shows DENV E protein expression by the majority of the monocytes and CD56bright NK cells. What is the reason for this discrepancy between detection of viral transcripts and intracellular staining for viral E protein? Is this related to the sensitivity of detecting viral transcripts in the scRNA-seq dataset? Do the authors find this concerning?

--We thank the Reviewer for this comment. As shown in Fig.4d, there is a large patient-to-patient variability in the number of VHCs and E+ cells. The discrepancy between the size of the

fractions of monocytes and NK cells harboring vRNA and the respective fractions harboring intracellular DENV E protein likely reflects this variability, as the two measurements were done on samples derived from different patients whose viremia levels were variable. Differences in the sensitivity of the methods, as proposed by the Reviewer, might have also played a role.

Regardless, however, this discrepancy is not concerning to us. While the magnitude may be different, the two platforms largely agree about the specific cell subtypes that harbor vRNA and protein. Furthermore, we have validated these findings via co-culture experiments.

In response to the Reviewer's comment, we have now added the following sentence to the manuscript: *"Patient-to-patient variability and differences in assay sensitivity may account for the discrepancy in magnitude between VHCs and E+ monocytes and NK cells."* (See line 386-388).

5. Unlike B cells, antigen presentation related DEGs between virus harboring monocytes compared to bystander cells show an increase and not a decrease in HLA-DR and HLA-DP. Are these differences a result of DENV altering the transcriptome of VHCs or activation of the monocytes by DENV? How do the HLA-DR levels compare in virus harboring monocytes in SDp?

--These are interesting questions that we have now addressed. As discussed above, the low number of VHCs in SDp precluded our ability to compare HLA-DR in VHCs in SDp vs. D.

Moreover, whereas the large number of vRNA reads in B cells enabled meaningful comparison between VHCs and bystander cells (Fig. 7h), noise resulting from the small vRNA reads in monocytes (and NK cells) limited our ability to conduct such a comparison in these cell subtypes (see Extended Fig.7c).

To overcome this limitation, we have now analyzed the expression of HLA-DR protein on distinct cell subtype fractions stained positive or negative for intracellular DENV envelope protein via spectral flow cytometry (see Fig. R1). In agreement with the transcriptomic data, the expression of HLA-DR was significantly reduced on DENV E+ vs. bystander B cells (Fig. 4j). Interestingly, HLA-DR protein expression was also significantly lower on DENV E+ than DENV-

non-classical monocytes and cDC2s (Fig. 4j)—the myeloid cell subtypes in which we measured the highest mean percentage of E+ cells (Fig. 4d).

Our collective data thus provide evidence that DENV induces downregulation of antigen presentation in infected B cells and myeloid cells.

We have now added these data to the manuscript (see lines 284-298 and 403-411).

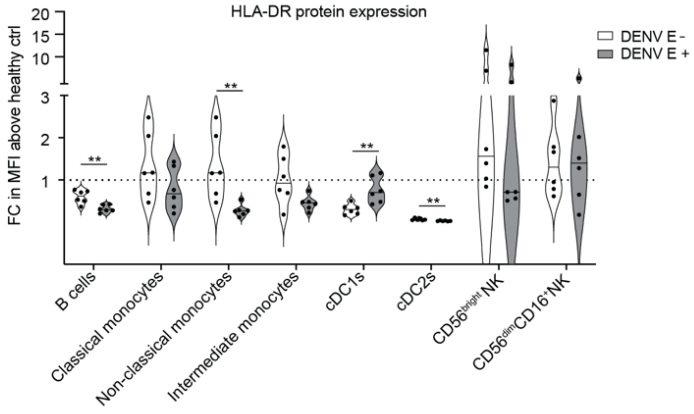

Figure R1: Violin plots showing cell surface expression of HLA-DR in DENV envelope (E)+ and DENV E- B cell, monocyte, cDC and NK cell subtypes measured via spectral flow cytometry and displayed as fold change (FC) of mean fluorescence intensity (MFI) above healthy control (N=2, n=6 combined data)

6. The authors do not directly validate lowered HLA-DR levels as a result of viral infection. Importantly, viscRNA-seq showed decreased levels of antigen processing related genes in virus harboring B cells suggesting that the decrease in HLA-DR may be due to DENV infection.
- To understand whether the lowered HLA-DR levels are a result of infection and not a different indirect mechanism, the authors should compare the levels of HLA-DR on DENV E protein positive versus negative B cells, inflammatory monocytes, DC, and NK cells.
- Is DENV known to impair specific steps in antigen processing or presentation?

--This is an excellent comment that has now been addressed. As mentioned above (comment #5), we have now measured HLA-DR protein expression on distinct cell subtype fractions

stained positive or negative for intracellular DENV envelope protein via flow cytometry detecting significant HLA-DR reduction in DENV E+ vs. bystander B cells, non-classical monocytes and cDC2s (see figure Fig. R1) (Fig. 4d). Contrastingly, no such drop in HLA-DR expression was detected in DENV E+ vs. bystander NK cells (see figure R1 above), supporting a hypothesis that the viral elements detected in NK cells represent uptake of infected cells rather than active replication. See Fig. 4j and text lines 411- 414.

--It was previously reported that infecting blood donor derived DCs with DENV ex vivo inhibits their maturation and reduces HLA-DR expression(Palmer et al., 2005), however, modulation of specific elements required for effective antigen processing, presentation and cross presentation has not been characterized. Our results validate this finding in natural dengue infection in several DENV target cell subtypes and propose that this viral-mediated immune modulation may contribute to the reduced antigen presentation signatures associated with SD progression.

--In response to the Reviewer's comment we have now added these sentences to the text. See lines 403- 414. Please, also see response to comment #8 below.

7. The authors conclude that expansion of HLA-DR expressing possibly adaptive-like (i.e. able to activate T cells) NK cells characterizes SDp. How are these cells related to the virus harboring NK cells which have lower expression of HLA-DR (Exteded figure 7c)? The authors speculate that NK cells may adopt antigen presentation roles in DENV infection particularly in SDp patients. However, these conclusions are based on a small percentage of cells showing 4-fold elevated HLA-DR transcripts in patients vs healthy subjects (Fig. 3b), and 20% NK cells with higher HLA-DR protein in SDp vs D (Extended Data Fig. 5b). They are also discrepant with the data in Extended Fig. 7c.

a- Side-by-side flow cytometry for HLA-DR on NK cells and APC types would best show how the NK cell HLA-DR levels compare to those on monocytes, DC and B cells including healthy controls to show baseline HLA-DR levels. It would be a plus if they separated the cells into E protein positive versus negative to address the role of DENV on the levels of HLA-DR.

--We apologize for the unclarity in presenting and discussing these findings and have now revised the manuscript to increase clarity and address the Reviewer's questions.

--The data shown in Figs. 2d and 3b was generated side-by-side via flow cytometry, as suggested by the Reviewer (please, see Fig 2R pasted below). Since we discuss APCs and NK cell responses in separate sections of the manuscript, we split it into to two panels.

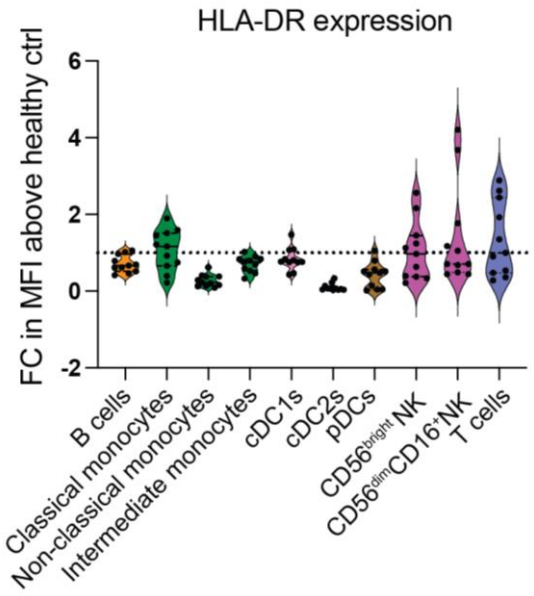

Figure R2: Violin plots showing HLA-DR cell surface expression measured via spectral flow cytometry in patient-derived PBMCs expressed as fold change (FC) of mean fluorescence intensity (MFI) above healthy control (n=11, N>2). Dotted line represents mean value of healthy controls normalized at value 1, horizontal lines in violin plots represent median.

-- The dotted line represents mean value of healthy controls normalized at value 1.

HLA-DR cell surface expression is expressed as fold change (FC) of mean fluorescence intensity (MFI) above healthy control.

In response to the Reviewer's comment, we have now added a comment in the legend of Fig. 3b, that the experiment was conducted side-by-side with that shown in Fig. 2d. See line 806.

Moreover, as suggested by the Reviewer, we have now separated the cells into E+ and bystander cells (see response to comment #6 above). No significant alteration in HLA-DR expression was detected in DENV E+ vs. bystander NK cells (see Figure R1 above), supporting a hypothesis that the viral elements detected in NK cells represent uptake of infected cells rather than active replication. See Fig. 4j and text lines 411-414.

b- Can the authors explain how HLA-DR levels increase on a subset of NK cells when both APC types and NK cells were collected from the blood and presumably in the same milieu?

We agree with the Reviewer that the blood milieu is the same for APCs and NK cells; however, the immune system as a whole is extremely complex and characterized by cell type-specific immune responses that can be triggered by the same ligand receptor-interaction yet induce a diverse cascade of events depending on the immune cells. One example is the regulation of cell death induced by TNF-α in different cell types. TNF-α can induce two different immune responses in different cell types: a protective pro-inflammatory response that promotes apoptosis and the elimination of infected or damaged cells in some immune cells, vs. a negative response that can lead to necroptosis and the release of damage-associated molecular patterns (DAMPs) in other non-immune cells (van Loo and Bertrand, 2023).

--The spectral flow cytometry analysis shown in figures 3b was conducted in whole PBMC samples, rather than in isolated cell fractions. As shown in supp Extended data Figure 6c we used a large panel of antibodies to define specific cell subtype populations, thus achieving single cell resolution for this proteomic analysis. We have now clarified that these single cell analyses were done in whole PBMC samples: "*Except for CD14^{+} monocytes that showed large patient-to-patient variability, lower expression of HLA-DR protein was also detected on APCs by flow cytometry of patient-derived PBMC samples from 6 DWS patients…* " See lines 156-158 And, "*the expression of HLA-DR protein measured by flow cytometry in PBMC samples was higher in DENV-infected, patient-derived CD56bright and CD56dimCD16+ NK cells than healthy controls*." (see lines 196-198).

c- Percentage of NK cells with elevated HLA-DR do not correlate with the <5% cells harboring viral transcripts or the discrepant majority NK cells that are positive for intracellular DENV E protein by flow cytometry.

--We apologize for the lack of clarity. These two populations are entirely distinct. Extended Fig. 7c compares vRNA harboring and bystander NK cells from 3 DWS patients with the highest viremia, whereas Fig. 3a analyzes DEGs in NK cells between SDp (7 patients) and uncomplicated D (8 patients). DWS patients were not included in this analysis.

Nevertheless, please note that HLA-DR is upregulated in NK cells from SDp vs. D (Fig. 3a) and shows a trend towards upregulation in NK cells harboring vRNA vs. bystander cells (albeit the latter analysis is limited by noise due to small number of viral reads in NK cells) (Extended Fig. 7c). Moreover, while variable across patients, HLA-DR protein expression measured by flow cytometry also trended towards an increase in DENV E+ vs. healthy controls (fig. 3b). There is thus no discrepancy in the data.

In response to the Reviewer's comment we have now clarified better the samples used for both analyses. For examples, see line: 235. "Focusing on three DWS samples with the highest number of vRNA harboring cells (VHCs) (and viremia), …".

8. The authors suggest that APCs have impaired antigen presentation. This suggestion is also based solely on the decreased transcript and protein levels of HLA-DR in these cells. Impairment in antigen presentation could be due to a decrease in surface HLA-DR expression or a decrease in antigen processing capacity of the cells or both. Are there differences in the expression of genes involved in antigen processing, cathepsins, aminopeptidases, lysosome function associated transcripts in VHCs compared to bystander cells? The authors could also consider using antibodies to measure components of the antigen processing machinery in human cells by flow cytometry.

--This is a thoughtful comment that we now address. As suggested by the Reviewer, we have now analyzed the expression levels of multiple genes involved in antigen processing, transport, loading on MHC molecules, presentation, and cross presentation (Figure R3 and Fi.4i).

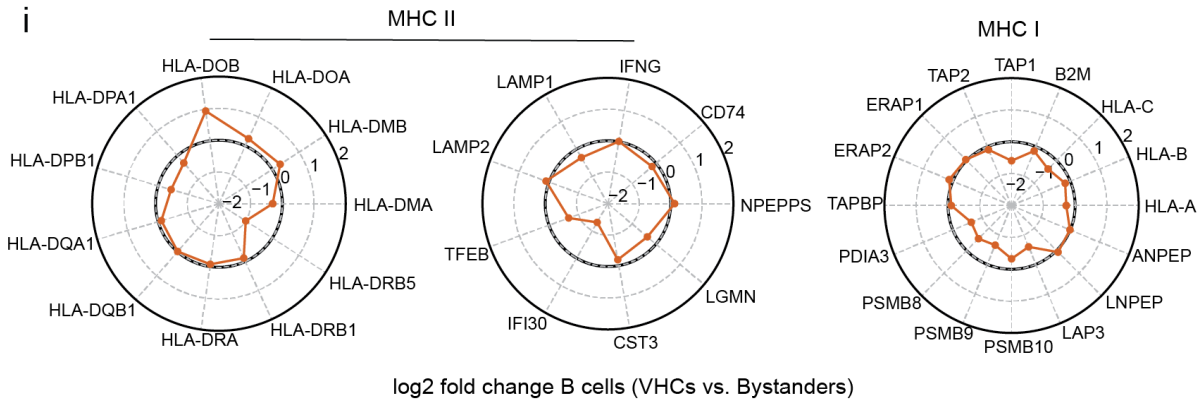

Figure R3: Radar plots showing log2 fold change of selected genes associated with MHC II and MHC I pathways between vRNA-harboring and corresponding bystander B cells.

This analysis revealed several interesting and novel findings for further validation:

- Prominent downregulation of IFN-gamma induced lysosomal thiol reductase (*IFI30*/GILT), a key regulator of unfolding of antigens with disulfide bridges required for effective peptidome generation in VHC B vs. bystander cells. While validation is required, it is tempting to hypothesize that since DENV E, Pre M and NS1 proteins harbor six disulfide bridges each (Lai et al., 2008), the downregulation of *IFI30* (GILT)--critical for unfolding antigens with disulfide bridges (Pishesha et al., 2022) --could impact effective peptidome generation. While Epstein-Barr viral microRNA downregulates *IFI30* ex vivo in primary B cells, no such modulation has been previously reported in natural infection(Tagawa et al., 2016).

- Upregulation of HLA-DOB in virus-harboring vs. bystander B cells– this unconventional (accessory) MHC molecule has been shown in various disease models to negatively regulate HLA-DM-mediated antigen uploading on conventional MHC-II.

- While MHC-I transcripts were not altered, TAP1 (MHC-I transporter) and *PSMB8/9/10* and *LAP3* (cytoplasmic peptidases) were mildly downregulated in vRNA-harboring vs. bystander B cells (FIG), revealing potential alterations in cross presentation signatures previously described in DNA viruses only (Pishesha *et al.*, 2022).

We have added this information to the text. See lines 278-284, 415-425, and Fig. 4i.

9. The authors conclude that antigen uptake is greater in all APC subsets from DENV infected patients compared to healthy controls. However, it is surprising that the healthy APC types show little to no uptake of pHrodo S. aureus bioparticles to begin with. Why do the majority of healthy APC harbor 0 bioparticles (Fig. 2f)? Are the healthy data points shown in this graph at 4 ^{o}C and if so, please show the healthy APC uptake at 37 ^{o}C. Is the 4 ^{o}C control in Fig. 2g paired for each DENV-infected patient or healthy subject?

--We apologize for this error and have now fixed it. The legend to Fig.2f had an error that was now fixed. The gray histograms show a negative (no bioparticles) control Bioparticle uptake quantification in the indicated cell subtypes from dengue patients and healthy controls is shown in Fig.2g as fold change of mean MFI above the corresponding 4 °C control. As shown, the healthy control cells do show uptake (which is lower in several cell subtypes than dengue patients). We have now modified the legend to correct this error. See lines 788-792.

Minor comments:

1. What is the physiological significance of inversely regulated interacting differentially expressed genes (iDEGs) noted in NK cells from SDp vs D.

--Inverse regulation can happen for a variety of reasons, including a regulatory mechanism in an attempt to dampen a certain immune response or vice versa, a compensatory mechanism in an attempt to reverse an immune impairment. In all those instances, however, it signifies that separate branches of the immune system are triggering distinct downstream functions and that rebalancing of this conflict via paracrine communications is required by the system as a whole. We have now added this speculation to the text, see line 324.

2. Luminex assay serum concentrations of cytokines/chemokines (Fig. 5e) show some differences not commented on by the authors, e.g. MIF, CCL26, FAS, IL-27, RETN, SERPINE1 while TNF levels are not different contrary to what the authors state (line 334).

--We thank the Reviewer for this comment. To comply with the word limit, we chose to focus on the most prominent cytokines and the ones for which we identified interesting expression patterns in our viscRNA-seq cell-cell communication dataset. The color-coding scheme we used for the original heat map shown in Fig. 5e made it difficult to observe differences in secretion of TNF-a (and some other cytokines), however, as shown in Fig. 5f, the concentration of TNF-a measured in the plasma of SDp is dramatically different from that in D and healthy controls.

--In response to the Reviewer's comment, we have now changed the color-coding scheme of the heat map to better capture differences in cytokine concentrations (see Fig. 5e). Moreover, we indicate in the text that these are examples. "*Among the differentially secreted cytokines*, …" See line 334.

Reviewer #2:

Summary of the key results: This manuscript entitled "Global and cell type-specific immunological hallmarks preceding severe dengue Progression" by Ghita et al. is well written and has a broad appeal. The manuscript profiles PBMCs from 19 DENV-infected children and 4 healthy controls using an optimized viscRNA-Seq 2 approach. Within the DENV-infected cohort, 7 progressed to severe DENV(SD). The aims of the manuscript are to investigate the cellular and molecular determinants preceding SD progression to identify markers that could be used to predict SD. The manuscript identifies several phenotypic differences in cell populations present in SD including the types of monocytes and NK cells present. Additionally, noting difference in HLA-DR expression levels on APC in SD cohort.

Originality and significance: The analysis was done at the time of hospitalization and therefore retrospective of precursors to *SD has rarely been done in this amount of detail*. However, as noted in the manuscript this group had completed a similar study, albeit, with an older cohort using Cytof with some individuals having already progressed to SD. *As noted in the study many of the findings observed in this study were previously identified in the authors earlier published work.*

Data & methodology and statistical treatment: validity of approach, quality of data, quality of presentation: The data as presented *was of high quality*. The authors noted the limitations of the study with the number of subjects being limited and the variability within the population as in regards to level of symptoms in the cohort that did not progress to SD creating confounding issues with the data.

Conclusions: The conclusions drawn *are valid based on the* data provided. As noted below, the results of this study while providing a highly detailed characterization and comparison of SD progressors and non-progressors *does not provide a practical solution that can be implemented to predict who will progress to SD.* Additionally due to the small cohort (19), unique population (adolescents), and high variability it will be interesting to see if larger studies using less costly techniques support the differences in cell populations and HLA-DR expression noted.

--Please, see response to the first comment of the Editor above outlining the conceptual novelties of the revised study. Moreover, please see response to comment #1 of Reviewer 1 regarding potential practical translational implications.

Suggested improvements:

1. Within the manuscript, there are several minor areas that should be improved.

a. It is not clear if attempts were made to determine if any or all of the 19 subjects had a prior DENV exposure. Given that an aim of the manuscript was to determine indicators of progression to SD it would be important to know what the prior history of dengue was in the subjects.

--We apologize for this unclarity. The requested information is included in the manuscript in Table 1. To improve clarity we have now highlighted this information in the results section (see lines 123 and Supplementary text line 23).

--Moreover, we have now added additional analyses aimed at determining whether alterations in cell subtype abundance and differential gene expression are associated with prior exposure to DENV. Please see response to comment #1 of Reviewer 3.

b. Additionally, it is unclear why adolescents were used in the study. It would be interesting to determine if there are differences between adolescent and adult progressors to SD.

--We agree with the Reviewer that it would have been interesting to focus the study on younger children. Nevertheless, while over 400 patients were enrolled in our cohort, as reported in other cohorts, only 5% of the patients progressed to SD, thus precluding our ability to focus on tighter age groups. We are, however, motivated to compare the host response in various pediatric age groups and between adolescent and adults in the future, once enough samples are available. In response to the Reviewer's comment, we have now added this as a limitation of our study. "*Inclusion of younger children and adolescents is another limitation*." See line 489-490.

c. Within the section about dengue replicating in B cells lines 262... how many of the 6 PBMC samples were from SD progressors.

--The number of SDp PBMC samples available to us and the number of cells in these pediatric samples is limited and not sufficient for conducting multiple analyses. Therefore, the quantification of DENV E protein via flow cytometry was conducted in DWS patients.

In response to the Reviewer's comment, this information is now stated in line 262 and is better highlighted in table 1 and Figure 4 legend (line 823).

2. The entire section on dengue replicating in B cells is very important and rarely discussed in the field and should be published. However, it does not currently fit with the aims of the manuscript as it is written it does not appear to have much to do with progression to SD in the cohort analyzed. Some effort should be made to integrate this section into the manuscript if it is to remain as part of the study.

--We thank the Reviewer for this insightful comment. To better integrate this important component with the rest of the manuscript, we have changed the structure of the manuscript. The relevant figure now comes after the analysis of DEGs in various cell subtypes, and we highlight how it fits with the overall aims. See lines 227-230.

--Moreover, we have expanded our analysis of DEGs between VHCs and bystander cells to include additional genes involved in antigen processing and presentation. See response to comment #8 by Reviewer 1.

-- We have also probed the observed HLA-DR phenotype at the protein level by measuring HLA-DR protein expression on distinct cell subtype fractions stained positive or negative for intracellular DENV envelope protein via flow cytometry. The expression of HLA-DR was significantly reduced on DENV E+ vs. bystander B cells and on DENV E+ non-classical monocytes and cDC2s—the myeloid cell subtypes in which we measured the highest mean percentage of E+ cells. See response to comment #6 by Reviewer 1 and Figs. 4d and 4j.

--Collectively, our findings provide evidence that DENV replication is involved in inducing alterations in antigen processing and presentation signatures, thereby likely contributing at least in part to a hallmark of SD progression. This information is now included in the text. See lines 293-298.

References: Appropriate credit is given to previous work. The literature on this subject is vast so while the author's reference list is not comprehensive, it reasonably covers the subject area.

--We thank the Reviewer for the supportive comment.

Clarity and context: Given that the conclusion of the study could not be directly used as a prognostic screen to identify progressors to SD the language in the abstract, introduction and discussion should be toned down. Additionally, the claim that this study identified a molecular mechanism that predicts SD should be removed as the study did more to characterize differences in markers between the cohort and did not directly show that those marker differences could contribute to the progression to SD.

--We apologize for these overstatements and have now addressed them in the abstract, introduction and discussion.

Lines 69-70: "*Better understanding SD pathogenesis may aid with the discovery of candidate biomarkers and preventive therapies".*

Lines 86-87*: "Our findings reveal DENV target cells in the human blood and point to a coordinated but aberrant immune response preceding SD progression."*

Reviewer #3:

Summary:

Ghita, Yao, Xie, and Duran et al. *have comprehensively* characterized the transcriptional profiles of acute phase samples from patients with dengue, dengue with warning signs, and severe dengue. They perform single cell RNA sequencing, incorporating additional primers to improve detection of dengue viral RNA, to characterize the major cell populations involved in viral replication as well as transcriptional signatures associated with disease. They also perform validation assays to confirm their transcriptional findings. ***Overall, this work provides novel insights into the factors driving dengue disease severity at finer resolution than has been previously described.*** The manuscript would be improved by being more transparent about the limitations of the study including sample size and how samples were selected for each sub study, and including an analysis comparing primary versus secondary dengue.

--We thank the Reviewer for their supportive comments. As detailed below, we have now addressed all their comments.

Major comments:

1) The authors make statements about the relative contribution of antibody-dependent enhancement to severity but do not perform an analysis comparing primary vs. secondary dengue, although they have these data. This analysis should be added to the manuscript especially when talking about antibody-dependent enhancement hypothesis.

--This is an important comment that has now been addressed. To determine whether the alterations we detected in cell subtype abundance and gene expression in SD progressors are associated with prior exposure to DENV, we analyzed cell subtype fractions and DEGs in primary vs. secondary DENV-infected patients across disease outcomes (Table 1). Activated B cells and exhausted CD8+ T cell fractions were expanded in secondary relative to primary dengue patients regardless of disease outcome, suggesting association with prior DENV

exposure but not disease severity (Extended Data Fig. 2c). In contrast, memory B cell and Treg fractions were expanded only in SDp with secondary infection, confirming that some alterations are unique to secondary SDp. The expansion of proliferating plasmablasts and memory CD4+ T cell fractions was comparable in primary and secondary SDp relative to uncomplicated dengue (D/DWS), thus more likely associated with disease progression rather than prior exposure to DENV. Lastly, reduction of non-classical monocyte and cytotoxic NK cell fractions was observed in all patient categories except for primary uncomplicated dengue (D/DWS), suggesting association with both prior exposure and progression.

Next, we compared the expression of DEGs we identified between SD progressors and D patients in APCs (Fig. 2a-c) and effector cells (Fig. 3a-c) in primary vs. secondary dengue infection in D and SDp patients. Several interesting observations were made; however, these require further validation given the small number of patients for each disease category: primary (total n=4: D (n=2), SDp (n=2)); secondary (total n=10: D (n=5), and SDp (n=5)).

Genes involved in antigen uptake (*CD163, FCGR1A and FCGR1B*) were mildly upregulated in secondary dengue infections in APCs (Extended Data Fig. 4a), and *IgG* genes and genes involved in antibody secretion were upregulated in secondary dengue infections in plasmablasts (Extended Data Fig. 5f). Since similar patterns were observed for these genes in SDp vs. D, these findings highlight association of signatures linked with ADE with prior DENV exposure, as previously reported(Katzelnick et al., 2017; Wang et al., 2017).

In contrast, MHC-II genes were upregulated in secondary vs. primary infection in APCs (Extended Data Fig. 4a), suggesting that their downregulation in SDp vs. D (Fig. 2a) is not associated with prior exposure to DENV, but rather with disease progression. Variable patterns were observed upon comparison of IFN response genes and genes involved in inflammation, migration and adhesion between secondary vs. primary infections (Extended Data Fig. 4a) and SDp vs. D (Fig. 2a-c), suggesting that these APCs' responses are only partially associated with DENV exposure. Similarly, whereas genes associated with exhaustion were upregulated in T and NK cells in secondary vs. primary infections as in SDp vs. D, variable expression patterns were observed for genes involved in cell activation (e.g. IL32 in T cells and KLRB1 in NK cells) and IFN response (e.g. IFIT3 in T cells), suggesting only partial association of altered responses

observed in these effector cells in SDp with prior DENV exposure (Extended Data Fig. 5f, Fig. 3a-c).

Taken together, these findings support that prior DENV exposure is associated with pathways involved in ADE, as previously reported, and possibly with effector cell exhaustion. Importantly, these findings reveal that other hallmarks of SD progression, such as downregulation of MHC-II genes, may be only partially or not associated with DENV exposure. Yet, these findings require validation in larger cohorts given the limited number of samples.

We have now added this information and figures to the manuscript (See text lines: 123-124; 175-179; 218-221; 479-483 and Extended Data Text and Extended Data Fig. 2c, 4, 5f).

2) Table 1 is overly large. It would be helpful if the authors could provide a table with basic demographic and severity data as well as which samples were used in which sub-analysis.

--In response to the Reviewer's comment, we have modified table 1 to reduce the amount of information and improve readability. The information about which sample was used for each sub-analysis is also included.

3) The participants ranged in age from 4 to 17 years. Were there any differences noted across age in transcriptional profiles? And was age accounted for in any way in the analyses? It would be helpful if the authors at least commented on this point and provided an analysis of differences across age, if possible.

--This is an important question that has now been addressed. In response to the Reviewer's comment, we have now color coded the viscRNA-seq dataset's UMAP based on age groups. As shown below, age did not impact the clustering or embedding (i.e. graph neighborhood) of cell populations. Moreover, age did not impact the observed changes in cell subtype abundance (Figure R4). We have now added these figures and sentence to the text: "*Differences in cell subtype abundance did not correlate with serum viral load (Extended Data Fig. 1e), were independent of age (<10, 10-13 and 14-17 years) or pregnancy status (1 SDp patient was*

pregnant) (Extended Data Fig. 2a,b)…" (see Extended Data Fig. 2a,b and lines 121-124 and supplementary text).

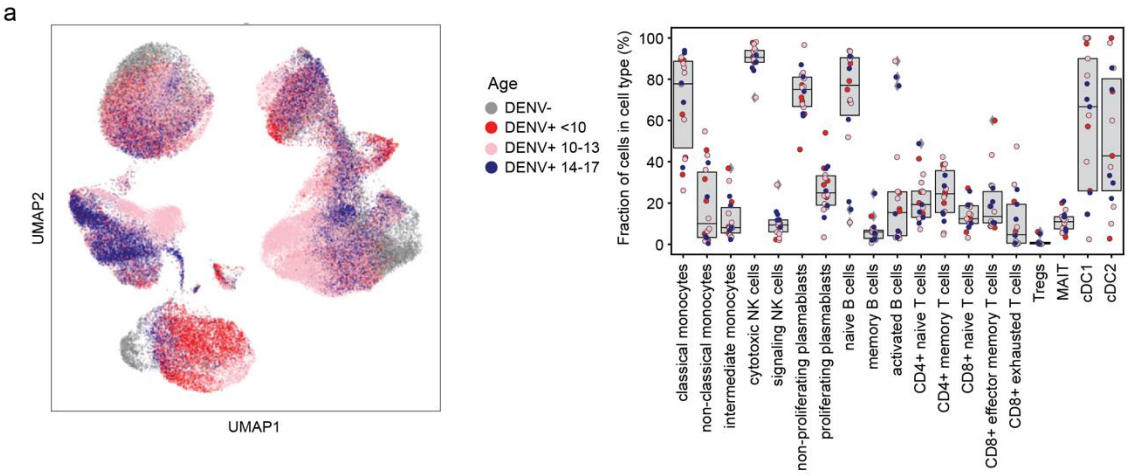

Figure R4: UMAP of the entire dataset (left panel) and box plots showing the fractions (%) of cell subtypes within each major immune cell type (right panel) color-coded by patients' age (in years): <10 =red; 10-14 =pink; 14-17 =blue years old. Box plots' horizontal lines indicate the first, second (median) and third quartiles, each dot Each dot represents an individual.

4) The authors state on Line 94 that the samples were collected early in the course of disease. However, many of the samples were collected on days 5-7 after fever onset. Does that really count as early?

--We agree with the Reviewer and have now changed this sentence to clarify that we meant that samples were collected prior to progression to severe infection. "*To characterize the immune response prior to SD progression during natural infection in children, we first profiled the transcriptome of PBMCs collected from individuals presenting within 1 to 7 days from symptom onset and testing positive for DENV who were enrolled in our Colombia dengue cohort*." (see lines 92-94).

5) For their single-cell analyses, the authors measure the pairwise differences between all individuals. To our knowledge, this is an unconventional method. Generally, comparisons are performed between groups. The authors should cite literature using the pairwise method or describe their approach in greater detail, if it is a novel approach. The authors should also consider analyzing data using conventional approach looking between groups.

As outlined in the response to the Editor's comment, the type of pairwise matching used in some panels throughout the manuscript is a statistical and data visualization device aimed to assess the effect size of the change (between SDp and D) and gain a sense of the noise in the data separately, which is especially useful with relatively small n since estimating P-values is naturally very inaccurate. As shown in Table 7 that we have now added, there is a high degree of agreement between the median log2 fold change for pairwise comparison of patients versus the KS statistic of a two-tailed test at the patient level. P-values are not computed explicitly for the reasons explained in the response to the Editor's comment (third point) – essentially it is not really possible to get accurate nonparametric P-value estimates from empirical data of this size.

6) Single-cell RNA experiments are expensive, and identification of severe dengue patients is difficult, making it challenging to have large sample sizes. However, the authors should still provide greater explanation for how samples were selected for each analysis. For instance, different assays were performed on different participants: Fig. 1G – uses data from 3 participants, Fig. 2A from 6. It would be helpful if the authors justify the sample size used for each analysis. As another example, Fig. 2G is not significant. Is this because a small number of samples was used and thus the effect was not observed? It would be helpful for the authors to comment on how confident they are that the effect was not present, or the sample size may have been too small and thus the analysis was underpowered

--In response to the Reviewer's comment, we have now clarified better how samples were selected for each analysis.

In terms of sample size, as correctly noted by the reviewer, scRNA-Seq and similar technologies are expensive and represent the sharp end of hypothesis generation in

biomedicine upon which hypothesis-driven follow-up studies with much larger N can be designed. In addition, this is the largest scRNA-Seq cohort of dengue patients ever collected, and we are actively expanding the data collection further in the next years to further improve the robustness of our conclusions.

For inter-patient group analyses, around 5-6 patients per category can give a rough estimate of the basic statistical properties of the data, e.g. whether outliers are common i.e. the empirical distribution of data measurement is fat-tailed. This number of samples can therefore be considered sufficient as long as parametric tests (e.g. t-tests) are avoided as much as possible and the level of noise in the comparisons is showcased very clearly (as we have done via our pairwise matching). Of course, higher N in future studies will improve the overall robustness of the current analyses. For intra-patient analyses (e.g. VHC versus non-VHC cells in 3 DWS patients), the more relevant factor is the number of cells in the least represented group (in our case, VHCs) within each patient and cell type. The same argument as above applies (around 6 cells per group minimum, to get a rough idea). For this reason, certain analyses cannot be performed, e.g. comparison of virus-harboring cells outside of the 3 DWS patients, because very few VHCs of a cell type were detected in a single patient, making the comparison brittle.

Flow cytometry analysis for protein expression and uptake assay were conducted in patients with DWS, given the overall limited number of samples derived from SDp and the small number of cells per sample in this pediatric populations. This is now mentioned as a limitation of the study.

--In terms of statistical significance in Fig. 2g, while the difference in uptake between DENV-infected patients and healthy controls is statistically nonsignificant for some cell subtypes, it is significant for classical monocytes and cDC2. We agree with the Reviewer that with a larger sample size the observed trend towards increased uptake in B cells might have become statistically significant. In response to the Reviewer's comment we have now modified the sentence to say: "….*in classical (CD14+CD16-) monocytes, cDC2s (CD1c+), and B cells (CD19+CD20+), albeit the latter was not statistically significant in this sample size*." See lines 173-174.

7) In the yellow box in Figure 6, the authors could mention B cells as distinct from antigen presenting cells, as this is a major finding of their study. Also in this box, the authors should mention the reduced antiviral response in infected cells.

--We thank the Reviewer for this comment and have now addressed it. Please see the revised Figure 6.

8) In the methods (line 620), it is explained that CD3 depletion was used to enrich for B cells, monocytes, and dendritic cells. Then these rare populations were re-pooled with undepleted fractions. We assume this is why none of the figures show the relative prevalence of infected cells. The method used seems to be justified, but the authors should make it clearer in the main text that this approach was used and why that prevents direct comparison of frequencies.

--The Reviewer is correct. In response to the Reviewer's comments, we have now added the following sentence for clarity: "*First, we assessed whether SD progression is preceded by alterations in cell abundance. Since samples were enriched for more rare cell populations (see Methods), we compared the composition of cell subtypes within the major immune cell populations in SDp, symptomatic non-progressors (D, DWS), and healthy controls (rather than cell type frequencies) (Fig. 1d)*". (see lines 110-113).

Minor comments:

1. It is not clear in text that the study included only DENV1 and DENV3 cases. This makes it confusing why primers were only included for DENV1 and DENV3. This should be stated in the manuscript.

--We now state: "We used oligonucleotides complementary to the positive strand of the viral genome that matched the patients' serotypes (in this work, serotypes 1 and 3)." See lines 228-232 and 628.

2. One of the severe dengue patients was pregnant. Given that pregnancy is expected to

modulate the transcriptional profile, it would be helpful to state if analyses were conducted to check if this person was an outlier.

--This is an important comment that has now been addressed. As shown in the figure below, the pregnancy status of the single SDp patient, did not impact cell clustering or embedding, which indicates those cells are tightly enmeshed in the cell similarity graph just like any other patient's. Also, cell subtype abundance for this patient looks unremarkable compared to all other patients, indicating there is no obvious reason to believe the immune response to dengue was peculiar in any way.

We have now added this figure and sentence to the text: "*Differences in cell subtype abundance…. did not correlate with serum viral load (Extended Data Fig. 1e), were independent of age (<10, 10-13 and 14-17 years) or pregnancy status (1 SDp patient was pregnant) (Extended Data Fig. 2a,b)……"* (see Extended Data Fig. 2a,b and lines 119-124 and supplementary text).

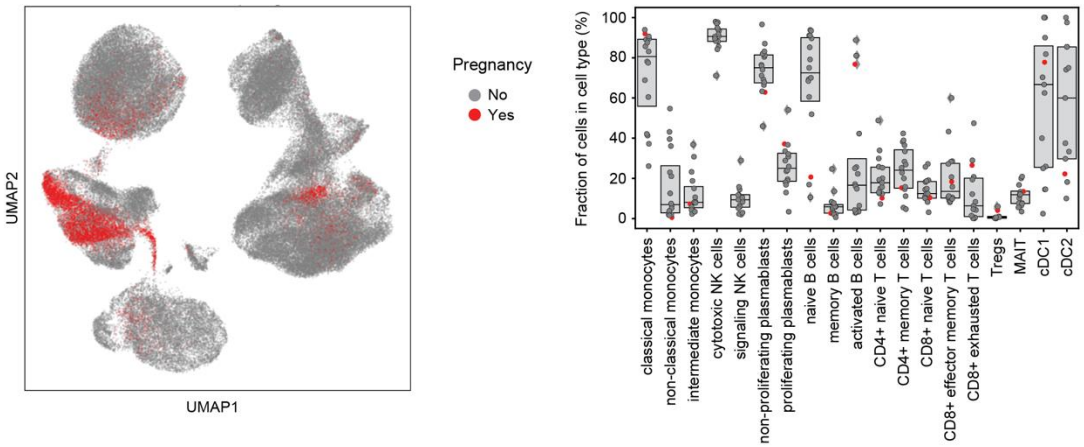

Figure R5: UMAP (left panel) and box plots (right panel) showing distribution and fraction of cells (%) color-coded by pregnancy status, pregnant= red, non-pregnant= gray.

3. The manuscript includes a lot of acronyms for immune cell populations, disease outcomes, and analysis methods. In some instances, acronyms are never defined. In other places, the

term is spelled out (e.g. line 211, interferon signaling genes, and then line 212 uses ISGs). Keeping some acronyms as fine, but where terms are only used a few times, it would be helpful if the authors could just write it out to make the manuscript easier to read.

--We apologize for this, and have now defined all acronyms and eliminated their use in cases where they infrequently appeared in the manuscript.

--In using the term interferon signaling genes we mean to refer to genes that signal upstream of interferon stimulated genes (= ISGs), such as IRF and STAT.

4. Line 263: The authors mention using HLA-DR+ cells to look at other antigen presenting cells besides B cells. It would be helpful to explain that these are the other antigen presenting cells, to make it easier to follow.

--We modified the text to state: “HLA-DR+ non-B cells (CD45+CD3-CD19-CD20-HLA-DR+). See line 263

5. Line 100: “viscRNA-Seq 2’s improvements we introduced included…”. This is awkward phrasing. Consider rewording to “Improvements introduced to viscRNA-Seq 2 include … “

--This sentence has now been reworded: “Improvements incorporated in the viscRNA-Seq platform 2 include magnetic.” See lines 100-103

6. Figure 3: T cells and plasmablasts are mislabeled in the figure compared to the legend.

--This has now been corrected. See line 798.

7. Line 158: consider saying healthy individuals?

--We have reworded this sentence.

8. Line 468-…: The wording here is confusing. Consider rephrasing.

--This sentence has now been rephrased.

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Decision Letter, first revision:

27th Jul 2023

Dear Shirit,

Thank you for supplying your point-by-point responses to the referees comments on your revised

manuscript entitled "Global and cell type-specific immunological hallmarks preceding severe dengue progression". I think the additional clarifications on the patient cohorts and the sampling times until progress of severe disease can address those concerns. Regarding the validation of the findings that patient B cells can be infected by virus and can serve as a virus reservoir, the orthogonal data presented - especially the E protein staining of
infected B cells should suffice. Fine if you have already started protein validation staining for the PrM protein, but given the orthogonal methodology supports this conclusion, I don't think it's mandatory for these additional experiments.

We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Please highlight all changes in the manuscript text file in Microsoft Word format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your manuscript:

* Include a “Response to referees” document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/ni/authors/index.html. Refer also to any guidelines provided in this letter.

* Please include a revised version of any required reporting checklist. It will be available to referees to aid in their evaluation of the manuscript goes back for peer review. They are available here:

Reporting summary:
https://www.nature.com/documents/nr-reporting-summary.pdf

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-portfolio/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Please use the link below to submit your revised manuscript and related files:

[REDACTED]

We hope to receive your revised manuscript within four weeks. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Kind regards,

Laurie

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Referee expertise:

Referee #1: APCs/Antigen presentation

Referee #2: Dengue infection

Referee #3: Dengue infection

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have made considerable effort in satisfactorily addressing the concerns raised.

A couple of points to address:
1. One reminder to not equate transcript/HLA-DR levels with antigen presentation per se. Antigen specific T cells are sensitive to even low levels of cognate peptide-MHC complexes. Given that direct measures of DENV antigen processing and/or antigen presentation have not been conducted, the authors should rather limit their statements/conclusions throughout the manuscript including lines 45-46 (abstract) to the effect of DENV on 'expression' of molecules associated with antigen processing/presentation or a gene signature thereof (as they have done on lines 132-133), rather than 'antigen presentation'.
2. To explain why unlike myeloid cells, E+ and bystander NK cells have similar HLA-DR levels, the authors hypothesize that 'viral elements detected in NK cells represent uptake of infected cells rather than active replication' (lines 412-414). However, NK cells are not known to be phagocytic and unlikely to internalize infected dying cells. The authors should revise the explanation they offer in lines 412-414.

Reviewer #4:
Remarks to the Author:
"Global and cell type-specific immunological hallmarks preceding severe dengue progression"

The work presented in this manuscript aimed to investigate the cellular and molecular determinants preceding progression to severe dengue (SD) in children. To do so, single-cell transcriptomics of PBMCs isolated from dengue virus (DENV)-infected children was performed, identifying genes differentially expressed between children with dengue (D) or those who progressed to SD, and the results were compared with previously published data from the same group. Cell-to-cell communication networks were inferred based on expression of receptors and ligands using data from the same transcriptomic analysis and measurement of cytokines. In separate sample sets, the authors investigated whether identified transcriptomic signatures were induced by infection of cells, as assessed by viral inclusive single cell RNA-seq (viscRNA-Seq), co-culture of primary cells with human hepatoma (Huh7) cells, and spectral flow-cytometry. The main strengths of the study are the use of a systems immunology approach for interrogating features of the immune response including single-cell transcriptomics, re-analysis of previous published CyTOF data, co-culture of primary cells obtained ex vivo, spectral flow-cytometry, and measurement of cytokines. However, there are a number of concerns that need to be addressed. First, this appears to be two separate stories deriving from 3 different samples sets -- one about potential transcriptomic predictors of severe dengue and another about DENV replication in B cells in 4 DWS patients that is separate from the initial story. A major concern is the sample set used for the transcriptomic analysis, in that more detail about timing of sample collection in terms of days since symptom onset and days before evolution to severity is required in order to claim that the signatures revealed are truly "preceding" SD, as they were collected late in disease progression. Regarding the second story about B cells as a target of DENV infection, additional samples and validation is required to support the assertions made.

Major Comments

Regarding the sample sets used:

1. Table 1 needs to be condensed from the Excel spreadsheet into a table that is included in the text

of the manuscript as the first item to orient the readers as to the sample sets being analyzed. I am concerned by several aspects (if in fact I am interpreting this correctly, as the labeling in the Excel spreadsheet provided is not clear nor defined) as detailed below.

2. The study is based on a convenience sampling strategy in children infected with DENV in Colombia. The inclusion and exclusion criteria for enrollment in the overall study are presented, but the criteria for the selection of a subset of 20 children infected with DENV and 4 healthy controls for viscRNA-Seq and cytokine analysis within the overall study are not presented. These criteria should be included. The sample set is composed of seven individuals that progressed from dengue (D) to severe dengue (SD) (n=1) or from dengue with warning signs (DWS) to SD (n=6) and a group of D that did not progress (n=8) and a group of 4 children with DWS at discharge (3 of whom had a diagnosis of DWS at admission as well), in which the transcriptomic analysis was performed with the aim of identifying hallmarks preceding SD progression. In addition, the group of 4 children with DWS were used for another study on viral replication and infectious virus production in B cells. Further, a separate group of 11 individuals in which flow cytometry was performed are reported in Table 1 (7 of which list "day of fever" as 4-7 days); how this group relates to the other groups is unclear. The inclusion and exclusion criteria of participants in each group needs further clarification, as well as the rationale for performing different assays in different subsets.

3. Overall, it is not clear nor consistent how the authors selected the individuals for their analyses. The authors state that 20 children were selected. However, Table 1 presents information about 19 children for the "viscRNAseq" study, and there is no information about the characteristics of the healthy controls. Similarly, in Table 2, data is presented for only 23 children. Please clarify and include the additional data. Further, Table 1 shows that 11 individuals were tested using flow-cytometry but Figure 4 panels d and j show results of "N=2,n=6 combined data", which is not clear. .

Regarding the transcriptomic analysis for the identification of hallmarks preceding severe dengue progression:

4. It is critical to demonstrate that each individual's sample for analysis was collected prior to the onset of SD manifestations in that patient, where the timing of the key clinical signs and symptoms in each patient is needed. It is very concerning that all the individuals in the SD progressor group have listed days 4-7 as "day of fever", and 6/7 already had DWS at the moment of admission. Thus, although the "dx_admission" is listed as DWS and "dx_discharge" is listed as SD, the "day of fever" (presumably when the patient presented and when the sample was collected?) is on day 4-7 since symptom/fever onset. Since the "critical phase" in dengue is generally considered to be days 4-6 since onset of symptoms, it appears that ALL of the SD progressors had samples collected DURING this period rather than PRIOR (e.g., days 1-3); collection prior would be necessary to claim predictive value or "hallmarks preceding severe dengue progression" as stated in the title of the manuscript. If samples were not collected on days 1-3, then it is necessary to show by reviewing the medical records that the sample for each individual was collected a reasonable amount of time before the onset of the manifestations that led to their SD diagnosis.

5. Further, although D and SD groups are balanced (though late in the course of disease progression), the DWS group for viscRNA-Seq analysis is not balanced, with fewer days since fever onset, younger children, and 75% primary infections, which limits comparability with the other groups. This should be noted in the limitations.

6. Enrichment of PBMC in samples: PBMCs were enriched for rare populations using magnetic bead separation of CD3+ cells, followed by mixing the remaining CD3- cells with an aliquot of PBMCs not subjected to this process. This process allowed the authors to characterize rare populations of immune cells in the samples. However, Figure 1d shows a wide variability in the relative abundance of cells among individuals, even within the same clinical group. The authors do not present evidence to support that this variation is due to biological variability and not due to inter-assay variation in the enrichment process. Evidence of inter-assay variation in the enrichment process should be presented, as this affects the interpretation of the results of the experiment.

Regarding the claim of B cells as source of infectious virus during infection:

7. The authors claim that circulating B cells are a source of infectious DENV production in natural infection (Lines 293-294). This is an important finding, and the transcriptomic analysis of infected versus bystander B cells is interesting. However, this is disconnected from the rest of the paper on disease progression, as it is based on a different sample set that is driven by 4 non-progressing DWS patients (see Figure 4a and other panels). While consistent with some literature, DENV replication in B cells should be confirmed using standard methods (see point 8) in larger cell populations (numbers of cells) and in larger numbers of patients, in order to support the authors' claim that they "identify… B cells as the major DENV target in the bloodstream" (lines 498-499). Also, since the sample set analyzed for DENV replication in B cells are not in the group that progressed to SD (3 DWS who did not progress and 1 D who progressed to DWS), it is not valid to claim that "By inducing these alterations, DENV replication in B cells and monocytes may thus contribute, at least in part, to the aberrant immune response that accompanies SD progression" (296-298).

8. It is important to confirm the DENV replication in B cells at a population level, given that the evidence derives from 419 reads in 224 cells, in only four individuals with high viral load. Can the authors provide confirmation using an orthogonal, accepted method such as detection of a nonstructural protein (e.g., NS3) in the cells of interest in the population of PBMCs and in more patients?

9. The results of RNAseq (Figure 4c) and flow-cytometry (Figure 4d) are contradictory in terms of the relative abundance of infected cells. How do the authors explain that the majority of B cells are very low in E detection? Given that the authors believe B cells have replicating virus, it would be expected that they should have high levels of intracellular E protein as well, but most do not (Figure 4d).

10. Regarding the high level of E detected in monocytes in Figure 4d, although all E-positive cells do not necessarily have replicating virus, other publications have shown that ~50-60% of E-positive PBMCs in DENV and ZIKV-infected patients are also NS3-positive (e.g., replicating virus); however, Figure 4c shows very low levels of vRNA in monocytes. It is odd that these data are discrepant with multiple publications showing active replication in monocytes, why do the authors think this is the case?

11. The levels of expression of antigen presentation genes are contradictory between panels h and i in Figure 4, particularly HLA-DRB5. Please explain.

Cell-to-cell interaction networks:

12. The inferences of interaction networks were based on the level of gene expression at the transcriptomic level, but there is no confirmation at the protein level and previous publications have reported lack of correlation between levels of RNA transcripts and proteins. Please note this in the limitations.

13. The transcriptomic analysis revealed markers associated with NK cell exhaustion in the SD progressor group. Nevertheless, the role of effector exhaustion is not evident in the final proposed model (Figure 6). Moreover, did the cell-to-cell interaction network analysis and the profile of cytokines provide information about the possible mechanism of effector exhaustion?

Minor comments:

1. In Figure 2a-c, the authors should consider performing random permutations of the pairwise analysis and bootstrapping of the log2 change of expression to provide confidence intervals on this parameter.

2. According to Figure 4a, the correlation of reads and viral load is apparently driven by four DWS cases that the authors included in this analysis. Because of this, the 95% confidence interval of rho should be calculated, for example by bootstrapping, even if the p-value is lower than 0.05.

3. Since only one pregnant patient was recruited, is not possible to claim that cell subtype abundance and serum viral load was independent of pregnancy status (lines 120-121).

4. The authors should clarify in which instances multiple comparison analysis was performed and using which approach.

5. Did the authors evaluate the quantity of each cell subtype as a function of age without stratifying by outcome?

6. In the methods section on PBMC isolation, please include the volume of blood from which PBMCs were isolated (line 602) and the concentration at which the cells were stored (line 607).

7. It is very difficult to distinguish the two light green colors used for classical and nonclassical monocytes in Figure 1c, would it be possible to use colors that can be distinguished more easily?

Reviewer #5:
Remarks to the Author:
A. Summary of the key results

Ghita et al. use single cell approaches and functional assays to examine genetic markers at hospital admission that are associated with progression to severe dengue vs. dengue. Using virus-inclusive single cell RNAseq and co-culturing techniques they demonstrate that B cells harbor the majority of replicating DENV and these patient-derived cells are infectious to others in co-culture. Additionally, they report that severe dengue is associated with impaired interferon responses and antigen processing and presentation and expansion of HLA-DR-expressing NK cells. Overall, this manuscript

provides novel insights into the molecular changes associated with severe dengue and contribute to the detailed immunological understanding facilitated by single cell sequencing.

B. Originality and significance: if not novel, please include reference
In lines, 77-79 the authors explicitly state the limitations of their previous work and describe how this manuscript advances the field. Appreciate the addition of lines describing the limitations of previous work and where this work fits in. Additionally, they describe improvements to the viscRNA-Seq platform (lines 101-103), which allowed them to identify B cells as the primary location of replicating DENV. I agree with the authors' perspective that this work is novel and presents significant additions to the field.

C. Data & methodology: validity of approach, quality of data, quality of presentation

#Overall, this is a value dataset and a valid approach for exploring the cell populations harboring replicating DENV and the sequencing changes associated with progression to severe dengue. I just have the following few additional points/questions:

#Figure 1A: Per table 1 column G, most participants seemed to have blood collected between day 5 and 7. As such, the location of the sampling arrow is a bit misleading, making it seem like sampling happened around day 1 of symptom onset. Am I misreading table 1 or should the arrow be moved closer to day 5-7 mark on the image? Overall, I think the arrow should be placed on the average day of sampling among the participants.

#The authors note that the samples are collected on admission and precede progression to severe dengue. From a clinical standpoint, it would be helpful to know, on average, how many days before progression was sampling performed. That way, clinicians and researchers could look for the reported findings x number of days prior to progression, and this and future work could potentially be used prognostically. If the authors have access to the number of days between sampling and documented progression, I think that could be a helpful addition.

#Figure 1D: were the Tregs significantly expanded in SDp population as noted in line 114? I don't see a star indicating significance on the figure.

D. Appropriate use of statistics and treatment of uncertainties
Overall, authors use valid statistical techniques, and they do a good job explaining these methods. My only addition is as follows:

Authors use pairwise differential expression analyses to compare individual SDp and D patients. In lines 134-135, they note that this "enables the visualization of both the effect size and the noise level in the data). In lines 670 – 681, they explain the method of pairwise comparisons, which is very helpful. However, since not all readers are familiar with this analysis and may want to replicate it in their own work, I think it would be helpful for the authors to add their justification for this method in the manuscript. Specifically, in the rebuttal, they note that they chose this method because of the unknown shape of the empirical distribution and the small sample size of the data, and this could be added to the manuscript.

E. Conclusions: robustness, validity, reliability

The conclusions are valid, the appropriate statistical tests were performed to ensure reliability and robustness for this cohort, and the limitations are helpfully outlined in lines 489-495. Authors may clarify that they included only younger children and adolescents. The way it is currently worded, it does not quite seem a limitation to include both these groups. The limitation is that they excluded other ages (although this is understandable).

F. Suggested improvements: experiments, data for possible revision
Improvements as above, all are minor.
G. References: appropriate credit to previous work?
Appropriate citations
H. Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions
Abstract, introduction and conclusion are clear and helpful.

Author Rebuttal, first revision:

Response to reviewers

Manuscript title: "**Global and cell type-specific immunological hallmarks of severe dengue progression identified via a systems immunology approach**" (NI-A35401B).

Dear Dr. Dempsey,

We thank the Editor and the Reviewers for their thoughtful and helpful comments. Below we provide a detailed response to each of the items mentioned by the reviewers and indicate how we have incorporated these suggestions into our revised manuscript. Please, note that all page locations and figure numbers referred to below are with respect to the revised manuscript.

Response to the Editor's comments:

Thank you for supplying your point-by-point responses to the referees comments on your revised manuscript entitled "Global and cell type-specific immunological hallmarks preceding severe dengue progression". I think the additional clarifications on the patient cohorts and the sampling times until progress of severe disease can address those concerns. Regarding the validation of the findings that patient B cells can be infected by virus and can serve as a virus reservoir, the orthogonal data presented - especially the E protein staining of infected B cells should suffice. Fine if you have already started protein validation staining for the PrM protein, but given the orthogonal methodology supports this conclusion, I don't think it's mandatory for these additional experiments.

We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Please highlight all changes in the manuscript text file in Microsoft Word format.

--We thank the Editor for being supportive of this manuscript.

Response to the Reviewers' comments:

Reviewer #1

(Remarks to the Author)
The authors have made considerable effort in satisfactorily addressing the concerns raised.

--We thank the Reviewer for their supportive comment.

A couple of points to address:
1. One reminder to not equate transcript/HLA-DR levels with antigen presentation per se. Antigen specific T cells are sensitive to even low levels of cognate peptide-MHC complexes. Given that direct measures of DENV antigen processing and/or antigen presentation have not been conducted, the authors should rather limit their statements/conclusions throughout the manuscript including lines 46-48 (abstract) to the effect of DENV on 'expression' of molecules associated with antigen processing/presentation or a gene signature thereof (as they have done on lines 132-133), rather than 'antigen presentation'.

--We apologize for this overstatement and have now modified the relevant sentences to reflect that antigen presentation signatures are altered rather than antigen presentation. Please see lines 48, 184, 442, 480, and 498.

2. To explain why unlike myeloid cells, E+ and bystander NK cells have similar HLA-DR levels, the authors hypothesize that 'viral elements detected in NK cells represent uptake of infected cells rather than active replication' (lines 421-425). However, NK cells are not known to be phagocytic and unlikely to internalize infected dying cells. The authors should revise the explanation they offer in lines 421-425.

--Indeed, phagocytosis by NK cell has not been broadly studied, yet reported. Please see an example in the following manuscript (https://pubmed.ncbi.nlm.nih.gov/24163416). We hypothesized that in the context of dengue, where antibody-dependent enhancement of infection involving FcG receptors is well-documented, it is conceivable that NK cells may exhibit temporal phagocytic capacity. Alternatively, NK cells might directly engage with other potentially-infected cells, such as monocytes, to promote their inflammatory signals and eventual apoptosis, as described in this manuscript (https://pubmed.ncbi.nlm.nih.gov/15528382/). This could result in the adhesion of cell debris and potentially leftover viral proteins to the cell membrane of NK cells.

In response to the Reviewer's comment we have now provided the alternative explanation. Please see lines 424-428: "…supporting a hypothesis that the viral elements detected in NK cells represent debris of infected cells (directly) engaged by NK cells adhering to the NK cell membrane[1] rather than active replication."

Reviewer #4

(Remarks to the Author)
"Global and cell type-specific immunological hallmarks preceding severe dengue progression"

The work presented in this manuscript aimed to investigate the cellular and molecular determinants preceding progression to severe dengue (SD) in children. To do so, single-cell transcriptomics of PBMCs isolated from dengue virus (DENV)-infected children was performed, identifying genes differentially expressed between children with dengue (D) or those who progressed to SD, and the results were compared with previously published data from the same group. Cell-to-cell communication networks were inferred based on expression of receptors and ligands using data from the same transcriptomic analysis and measurement of cytokines. In separate sample sets, the authors investigated whether identified transcriptomic signatures were induced by infection of cells, as assessed by viral inclusive single cell RNA-seq (viscRNA-Seq), co-culture of primary cells with human hepatoma (Huh7) cells, and spectral flow-cytometry. The main strengths of the study are the use of a systems immunology approach for interrogating features of the immune response including single-cell transcriptomics, re-analysis of previous published CyTOF data, co-culture of primary cells obtained ex vivo, spectral flow-cytometry, and measurement of cytokines. However, there are a number of concerns that need to be addressed. First, this appears to be two separate stories deriving from 3 different samples sets -- one about potential transcriptomic predictors of severe dengue and another about DENV replication in B cells in 4 DWS patients that is separate from the initial story. A major concern is the sample set used for the transcriptomic analysis, in that more detail about timing of sample collection in terms of days since symptom onset and days before evolution to severity is required in order to claim that the signatures revealed are truly "preceding" SD, as they were collected late in disease progression. Regarding the second story about B cells as a target of DENV infection, additional samples and validation is required to support the assertions made.

Major Comments

Regarding the sample sets used:

1. Table 1 needs to be condensed from the Excel spreadsheet into a table that is included in the

text of the manuscript as the first item to orient the readers as to the sample sets being analyzed. I am concerned by several aspects (if in fact I am interpreting this correctly, as the labeling in the Excel spreadsheet provided is not clear nor defined) as detailed below.

--We apologize for this unclarity and have now included a more condensed and clearer version of Table 1 (see more details below).

2. The study is based on a convenience sampling strategy in children infected with DENV in Colombia. The inclusion and exclusion criteria for enrollment in the overall study are presented, but the criteria for the selection of a subset of 20 children infected with DENV and 4 healthy controls for viscRNA-Seq and cytokine analysis within the overall study are not presented. These criteria should be included.
The sample set is composed of seven individuals that progressed from dengue (D) to severe dengue (SD) (n=1) or from dengue with warning signs (DWS) to SD (n=6) and a group of D that did not progress (n=8) and a group of 4 children with DWS at discharge (3 of whom had a diagnosis of DWS at admission as well), in which the transcriptomic analysis was performed with the aim of identifying hallmarks preceding SD progression. In addition, the group of 4 children with DWS were used for another study on viral replication and infectious virus production in B cells. Further, a separate group of 11 individuals in which flow cytometry was performed are reported in Supplementary Table 1 (7 of which list “day of fever” as 4-7 days); how this group relates to the other groups is unclear. The inclusion and exclusion criteria of participants in each group needs further clarification, as well as the rationale for performing different assays in different subsets.

--We apologize for not outlining the criteria for selecting patient samples clearly. At the time the work was conducted, we have enrolled approximately 200 children to our cohort, of whom 9 progressed to severe dengue (this 4.5% progression rate is in line with other cohorts). Of these 9 patients, one did not have sufficient cells for the viscRNA-seq pipeline and the other already presented with SD upon enrollment. All the remaining 7 patients were included. The 8 dengue patients were selected to largely match the age, gender, and prior exposure status of the SD patients.

While in our initial analysis with the samples above we detected viral reads, the read numbers were small. To improve this, we selected DWS patients with high viremia. Thus, these patients indeed represent a separate group. This is stated in line 236:” samples from 4 DWS patients with high serum viral load were included among the 19 processed by viscRNA-Seq 2”. It is important to note that in the DEG analysis in distinct cell subtypes, the cell-cell communications analysis, and the cytokine analysis we only compare SD and D patients. The DWS samples were included only for the cell type abundance analysis and identification of target cells.

In an ideal situation, we would have used PBMC samples from the same SD patients for the flow cytometry-based experiments. However, following WHO and IRB guidelines, the blood volume obtained from children was not sufficient to conduct the functional assays on samples from the same patients. Since the majority of the patients in our (and other) cohorts present with DWS, we selected DWS patients for the functional studies. For the flow cytometry analysis of DENV target cells and the uptake assay we therefore chose 11 patients with DWS. For the co-culture assays, we chose DWS patients with various levels of viremia (ranging from 10^5 to 10^9 viral copies/ml).

The healthy controls samples used in the viscRNA-seq analysis were derived from children presenting to the clinic in Colombia for healthy checkups or prior to elective procedures. We have now provided information about their age and gender. The healthy controls samples used for some of the functional assays were derived from adults from the blood bank. See Table 1 and lines 587-588.

To address the Reviewer's comment, we have now included clearer explanations in the main text and a new section in the methods outlining how samples were selected and included more information in Table 1 regarding the specific analysis samples were used for. See lines: 97-100, 234-237, 589-604.

Moreover, we have added the use of DWS samples (vs. SD samples) as a limitation of the study. See lines 512-513: "Moreover, due the low number of PBMCs obtained from children, DWS samples were used for functional experiments."

3. Overall, it is not clear nor consistent how the authors selected the individuals for their analyses. The authors state that 20 children were selected. However, Supplementary Table 1 presents information about 19 children for the "viscRNAseq" study, and there is no information about the characteristics of the healthy controls. Similarly, in Supplementary Table 2, data is presented for only 23 children. Please clarify and include the additional data.
Further, Supplementary Table 1 shows that 11 individuals were tested using flow-cytometry but Figure 4 panels d and j show results of "N=2,n=6 combined data", which is not clear.

--We apologize for this error and have now corrected the method section to reflect that 19 samples were studied, as indicated in Supplementary Table 2 and the remaining of the manuscript.

--Supplementary Table 2 includes only the samples used for viscRNA-seq analysis since it shows the number of cells sequenced and those with viral reads.

--As per the response to point 2 above, Table 1 now clarifies better which samples were used specifically for each flow cytometry-based study (i.e. co-culture experiments (Figure 4g, 5

samples); target cell identification (Figure 4d 6 samples); and uptake assays (Figure 2g, 5 patient and 5 healthy control samples).

Regarding the transcriptomic analysis for the identification of hallmarks preceding severe dengue progression:

4. It is critical to demonstrate that each individual's sample for analysis was collected prior to the onset of SD manifestations in that patient, where the timing of the key clinical signs and symptoms in each patient is needed. It is very concerning that all the individuals in the SD progressor group have listed days 4-7 as "day of fever", and 6/7 already had DWS at the moment of admission. Thus, although the "dx_admission" is listed as DWS and "dx_discharge" is listed as SD, the "day of fever" (presumably when the patient presented and when the sample was collected?) is on day 4-7 since symptom/fever onset. Since the "critical phase" in dengue is generally considered to be days 4-6 since onset of symptoms, it appears that ALL of the SD progressors had samples collected DURING this period rather than PRIOR (e.g., days 1-3); collection prior would be necessary to claim predictive value or "hallmarks preceding severe dengue progression" as stated in the title of the manuscript. If samples were not collected on days 1-3, then it is necessary to show by reviewing the medical records that the sample for each individual was collected a reasonable amount of time before the onset of the manifestations that led to their SD diagnosis.

--We agree with the Reviewer that ideally patients should be captured during the first 3 days of disease onset. While this is achievable in a challenge study involving healthy volunteers, it is extremely difficult to achieve in a natural infection cohort, since patients do not typically present to the hospital during this time window. The definition of "preceding" we used meant to say that the patients studied did not meet criteria of SD when they were enrolled (i.e. when their blood sample was obtained). We do have the clinical information regarding the number of days between patients' enrollment and progression to SD and have now included it in Table 1. The range is between less than a day to 2 days.

--It's important to note that we are not claiming ANY predictive value for the hallmarks we identified in this manuscript. The word prediction does not appear in the manuscript. Moreover, we refer to biomarkers only with a general statement in line 70 generally stating that: "Better understanding SD pathogenesis may aid with the discovery of candidate biomarkers and preventive therapies." It is also important to state that while enrolling patients between 0-3 days is of academic interest, from a prediction stand point this would be less relevant in a clinical setting, since at least in Colombia based on our experience, patients uncommonly present within this time window.

--In response to the Reviewer's comment we have deleted the word "preceding" in the title of the manuscript and changed it in some of the subtitles and text to "accompanying" (see examples in lines 2, 79, 87, 90, 92, 110, 130, 136, 517, 807).

--Moreover, we have added information about the number of days between enrollment and diagnosis of SD Please see Table 1 and line 98: "…of whom seven met criteria of SD within less than a day to two days following enrollment (SDp, n=7).

--Lastly, we have added this as a limitation in the discussion. See lines 504-506: "Among the limitations of this study is that while SDp were enrolled prior to meeting criteria of SD, presentation on days 4-7 following symptom onset precluded capture of the earliest events in the disease course."

5. Further, although D and SD groups are balanced (though late in the course of disease progression), the DWS group for viscRNA-Seq analysis is not balanced, with fewer days since fever onset, younger children, and 75% primary infections, which limits comparability with the other groups. This should be noted in the limitations.

--As stated in the response to comment #2 above, DWS samples were not included in the DEG analyses, cell-cell communications analysis, and the cytokine analysis. The only place where we include them (except for DENV target identification) is in the cell type abundance analysis. Per the Reviewer's request we have now added the following sentence: "Incomplete balance in age, day of presentation and DENV exposure between the DWS group and the D and SDp groups limits comparability of cell subtype abundance in DWS to the other two groups." (lines 508-510).

6. Enrichment of PBMC in samples: PBMCs were enriched for rare populations using magnetic bead separation of CD3+ cells, followed by mixing the remaining CD3- cells with an aliquot of PBMCs not subjected to this process. This process allowed the authors to characterize rare populations of immune cells in the samples. However, Figure 1d shows a wide variability in the relative abundance of cells among individuals, even within the same clinical group. The authors do not present evidence to support that this variation is due to biological variability and not due to inter-assay variation in the enrichment process. Evidence of inter-assay variation in the enrichment process should be presented, as this affects the interpretation of the results of the experiment.

--We apologize for this unclarity. Because we excluded some of the T cells, we indeed could not compute relative abundance (i.e. fraction of cells relative to the entire PBMC populations). Instead, we computed the abundance relative to the respective specific cell populations, which

is not impacted by reducing the T cell population. The title of the Y axis in Figure 1d is thus misleading. In response to the Reviewer's comment, we have now changed the title of the Y axis in Figure 1d to state: "Fraction of cells relative to major cell type (%)."

--Moreover, our CyTOF data (see supplementary Fig. 1C), which was generated without such an enrichment procedure, reveals a large variability across cell subtypes between individuals even within the same clinical group, indicating that the variability is biological rather than technical. Importantly, despite of this variability, it is possible to draw conclusions regarding alterations in cell subtype abundance between progressor and non-progressors, and the results of the viscRNA-seq are in agreement with those generated via CyTOF. The Y axis title of this figure was now also changed to "Fraction of cells relative to major cell type (%)."

Regarding the claim of B cells as source of infectious virus during infection:

7. The authors claim that circulating B cells are a source of infectious DENV production in natural infection (Lines 295-296). This is an important finding, and the transcriptomic analysis of infected versus bystander B cells is interesting. However, this is disconnected from the rest of the paper on disease progression, as it is based on a different sample set that is driven by 4 non-progressing DWS patients (see Figure 4a and other panels). While consistent with some literature, DENV replication in B cells should be confirmed using standard methods (see point 8) in larger cell populations (numbers of cells) and in larger numbers of patients, in order to support the authors' claim that they "identify… B cells as the major DENV target in the bloodstream" (lines 515-516).
Also, since the sample set analyzed for DENV replication in B cells are not in the group that progressed to SD (3 DWS who did not progress and 1 D who progressed to DWS), it is not valid to claim that "By inducing these alterations, DENV replication in B cells and monocytes may thus contribute, at least in part, to the aberrant immune response that accompanies SD progression" (298-299).

--Beyond showing that B cells harbor the majority of positive-strand vRNA in children's blood, we measured active replication in myeloid and B cells via three orthogonal experimental methods: negative-strand vRNA detection in patient cells, E protein expression measurement via flow cytometry, and viral spread detection upon co-culture with permissive cells.

--We have attempted to stain patient-derived PBMCs for NS3 (and NS1) using several antibodies both via CyTOF and via spectral flow cytometry, however, high background in the range of the isotype control was observed. It should be noted that the few papers reporting NS3 staining do not include the isotype controls data, and thus the interpretation of the presented data is somewhat limited. We previously reported this challenge in a recent publication: please see section titled "Challenges in identifying DENV-infected cells in patient-derived PBMCs via

mass cytometry" in PMID: 36961888. In light of these results and following the Editor's comment ("Regarding the validation of the findings that patient B cells can be infected by virus and can serve as a virus reservoir, the orthogonal data presented - especially the E protein staining of infected B cells should suffice."), we have not pursued with a fourth orthogonal method.

--The Reviewer's comment about the claim stated is important and has now been addressed. We have rephrased and weakened this claim to better adhere to the results by highlighting that some of the phenotypes observed in SDp vs. D patients (e.g. reduced expression of MHC molecules) are also observed specifically in cells harboring vRNA vs. bystanders. See lines 301-302: "Some of the phenotypes observed specifically in cells harboring vRNA vs. bystanders are also observed in SDp vs. D patients."

8. It is important to confirm the DENV replication in B cells at a population level, given that the evidence derives from 419 reads in 224 cells, in only four individuals with high viral load. Can the authors provide confirmation using an orthogonal, accepted method such as detection of a nonstructural protein (e.g., NS3) in the cells of interest in the population of PBMCs and in more patients?

----Please see response to comment 7 above.

9. The results of RNAseq (Figure 4c) and flow-cytometry (Figure 4d) are contradictory in terms of the relative abundance of infected cells. How do the authors explain that the majority of B cells are very low in E detection? Given that the authors believe B cells have replicating virus, it would be expected that they should have high levels of intracellular E protein as well, but most do not (Figure 4d).

--In lines 392-396 in the discussion we speculate about this discrepancy stating: "Subgenomic flavivirus RNAs (sfRNAs) might account for the greater vRNA abundance in B cells yet greater E protein expression in myeloid cells since sfRNAs can (i) suppress antiviral IFN response[2, 3] consistent with our finding that vRNA-harboring B cells downregulate IFN response genes relative to bystander cells; and (ii) compete for packaging with DENV genomic RNA[4], increasing intracellular vRNA."

We also see quite large patient-to-patient variability in the vRNA and E protein abundance in B cells which can account for this difference. And, differences in assay sensitivity may be contributing as well. We have now added the latter explanation as well. See lines 396-398.

10. Regarding the high level of E detected in monocytes in Figure 4d, although all E-positive

cells do not necessarily have replicating virus, other publications have shown that ~50-60% of E-positive PBMCs in DENV and ZIKV-infected patients are also NS3-positive (e.g., replicating virus); however, Figure 4c shows very low levels of vRNA in monocytes. It is odd that these data are discrepant with multiple publications showing active replication in monocytes, why do the authors think this is the case?

--In agreement with former studies, our results also show consistent high level of replication in monocytes based on two orthogonal assays: E protein staining via flow cytometry and co-culture experiments. The number of vRNA molecules detected in monocytes is low compared to B cells. Since it is unknown what fraction of these vRNA molecules, which are mostly derived from the positive strand, are related to active replication versus passive internalization and/or adsorption onto the cell surface, the comparison across cell types is hardly statistically unbiased. It is likely that while B cells in some patients can harbor a large amount of vRNA, monocytes could still provide a more efficient environment for active replication.

--Moreover, in absolute terms, it is not technically correct that we observed “very low levels of vRNA” because detecting non-polyadenylated vRNA molecules at the scale of 100,000s of cells is extremely challenging in the first place, due to limited capture efficiency (sensitivity). Even for polyadenylated host RNA molecules, it has been shown many times that droplet-based protocols operate well below the saturation limit at which most or all RNA molecules are captured and sequenced.

--In response to the Reviewer’s comment we have now added the following sentences to the discussion:
Lines 379-382: “In agreement with these studies, our results show consistent high level of replication in monocytes based on two orthogonal assays: E protein staining via flow cytometry and co-culture experiments, yet lower number of vRNA molecules relative to B cells.”

Lines 389-392: “Nevertheless, since the fraction of detected vRNA molecules, largely derived from the positive viral strand, representing active replication versus passive internalization and/or adsorption onto the cell surface is unknown, the comparison across cell types is hardly statistically unbiased.”

Lines 399-400: “It is possible and, in the light of current data, likely that while B cells in some patients harbor a large amount of vRNA, monocytes still provide a more efficient environment for active replication.”

11. The levels of expression of antigen presentation genes are contradictory between panels h and i in Figure 4, particularly HLA-DRB5. Please explain.

--We thank the Reviewer for noting this. This was an error in labeling of a few of the genes in figure 4h that was now fixed. See corrected Figure 4h.

Cell-to-cell interaction networks:

12. The inferences of interaction networks were based on the level of gene expression at the transcriptomic level, but there is no confirmation at the protein level and previous publications have reported lack of correlation between levels of RNA transcripts and proteins. Please note this in the limitations.

--This has been added as a limitation. See lines 511-512: "….and the lack of validation of cell-cell communications are additional limitations."

13. The transcriptomic analysis revealed markers associated with NK cell exhaustion in the SD progressor group. Nevertheless, the role of effector exhaustion is not evident in the final proposed model (Figure 6). Moreover, did the cell-to-cell interaction network analysis and the profile of cytokines provide information about the possible mechanism of effector exhaustion?

--We thank the Reviewer for pointing this out and have now added effector exhaustion to the model.
The cell-to-cell interaction network and the cytokine analysis identified signatures of exhaustion, such as the upregulation of LAG3 on NK cells that is associated with activation, cytokine production and exhaustion, yet its function on NK cells exhaustion in the context of infection is not clear yet (PMID: 33122640). Similarly, the increased concentration of IL-10, TNFa and IFNg can be theoretically linked to effector cell exhaustion, however, since these cytokines are also involved in multiple effector functions and their effect on exhaustion is often delayed, to fully address their role in exhaustion, longitudinal analysis should be perfumed in the future.

Minor comments:
1. In Figure 2a-c, the authors should consider performing random permutations of the pairwise analysis and bootstrapping of the log2 change of expression to provide confidence intervals on this parameter.

The main advantage of the pairwise method over a straight statistical test is that it enables visualization of the rough noise level, which we report in Figures 2a-c as error bars. A clean statistical characterization including P-values and confidence intervals has been avoided on purpose because there are too few samples in each category to make accurate estimates of those metrics (of course, estimating a CI requires more data than estimating the effect size only). In agreement with the Reviewer's request, we performed not just randomized but all

pairwise combinations since they are in the order of ~60 (number varies slightly by cell type).

2. According to Figure 4a, the correlation of reads and viral load is apparently driven by four DWS cases that the authors included in this analysis. Because of this, the 95% confidence interval of rho should be calculated, for example by bootstrapping, even if the p-value is lower than 0.05.

--In response to the Reviewer's comment, we have computed the 95% CI for the Spearman rho using standard pairwise bootstrapping and added it to figure 4a.

3. Since only one pregnant patient was recruited, is not possible to claim that cell subtype abundance and serum viral load was independent of pregnancy status (lines 121-124).

--The Reviewer's comment is correct. We were trying to be concise and ended up being imprecise. We have rephrased it in a weaker manner now as "not affected by pregnancy status", line 124.

4. The authors should clarify in which instances multiple comparison analysis was performed and using which approach.

--For most analyses in the manuscript, P-values were not estimated and we relied instead on thresholds on effect size and biological knowledge when necessary. We performed Bonferroni correction on p-values from KS test on Extended Fig. 6b (Cumulative distributions of DENV reads/million reads per cell in B cell subtypes). We also added Bonferroni correction on the comparison in serum cytokine concentrations between SDp and D in Supplementary Table 6.

5. Did the authors evaluate the quantity of each cell subtype as a function of age without stratifying by outcome?

--We have not performed that analysis specifically as the focus of the paper was on determining the effect of dengue virus on the immune system rather than on the developmental changes in immunity itself.

6. In the methods section on PBMC isolation, please include the volume of blood from which PBMCs were isolated (line 602) and the concentration at which the cells were stored (line 631).

--We have now added the following sentence: “PBMCs were isolated using SepMate tubes (Stemcell Technologies) from 18 mL (>10 years), 9 mL (5-10 years) and 5 mL (2-5 years) of blood and stored at a cell concentration ranging from 1x10^6 to 5x10^6 cells/ml depending on availability.” (see lines 636-638).

7. It is very difficult to distinguish the two light green colors used for classical and nonclassical monocytes in Figure 1c, would it be possible to use colors that can be distinguished more easily?

We have changed the shades of green in Figure 1c to make the two types of monocytes more distinguishable.

Reviewer #5

(Remarks to the Author)
A. Summary of the key results

Ghita et al. use single cell approaches and functional assays to examine genetic markers at hospital admission that are associated with progression to severe dengue vs. dengue. Using virus-inclusive single cell RNAseq and co-culturing techniques they demonstrate that B cells harbor the majority of replicating DENV and these patient-derived cells are infectious to others in co-culture. Additionally, they report that severe dengue is associated with impaired interferon responses and antigen processing and presentation and expansion of HLA-DR-expressing NK cells. Overall, this manuscript provides novel insights into the molecular changes associated with severe dengue and contribute to the detailed immunological understanding facilitated by single cell sequencing.

--We thank the Reviewer for their supportive comments.

B. Originality and significance: if not novel, please include reference
In lines, 77-79 the authors explicitly state the limitations of their previous work and describe how this manuscript advances the field. Appreciate the addition of lines describing the limitations of previous work and where this work fits in. Additionally, they describe improvements to the viscRNA-Seq platform (lines 101-103), which allowed them to identify B cells as the primary location of replicating DENV. I agree with the authors’ perspective that this work is novel and presents significant additions to the field.

--We thank the Reviewer for their supportive comments.

C. Data & methodology: validity of approach, quality of data, quality of presentation

#Overall, this is a value dataset and a valid approach for exploring the cell populations harboring replicating DENV and the sequencing changes associated with progression to severe dengue. I just have the following few additional points/questions:

#Figure 1A: Per supplementary table 1 column G, most participants seemed to have blood collected between day 5 and 7. As such, the location of the sampling arrow is a bit misleading, making it seem like sampling happened around day 1 of symptom onset. Am I misreading supplementary table 1 or should the arrow be moved closer to day 5-7 mark on the image? Overall, I think the arrow should be placed on the average day of sampling among the participants.

--The Reviewer is correct; most patients were enrolled closer to day 5-7. We apologize for this inaccuracy and have now moved the arrow in the schematic (see revised Fig 1a).

#The authors note that the samples are collected on admission and precede progression to severe dengue. From a clinical standpoint, it would be helpful to know, on average, how many days before progression was sampling performed. That way, clinicians and researchers could look for the reported findings x number of days prior to progression, and this and future work could potentially be used prognostically. If the authors have access to the number of days between sampling and documented progression, I think that could be a helpful addition.

--The information regarding number of days prior to meeting the criteria of SD is now included in a new table (Table 1) and in text (lines 98-100): “of whom seven met criteria of SD within less than a day to two days following enrollment (SDp, n=7),…”. Please, also see response to comment #4 of Reviewer #4.

#Figure 1D: were the Tregs significantly expanded in SDp population as noted in line 115? I don’t see a star indicating significance on the figure.

--The reviewer is correct that the change in T regs in Figure 1D is not significant. The statistical framework of the paper is very conservative (almost all nonparametric, distribution-level tests), thereby making it difficult by design to call out small effect sizes such as this one. If more assumptions could be made about the statistical property (noise) of Treg abundance in healthy and sick humans, a more powerful statistical test could perhaps be adopted. At the limit of current knowledge, this specific result is more a trend more than a certainty: in our view, a very interesting trend. In response to the Reviewer’s comment we have now changed this sentence to state: “The fractions of classical monocytes, signaling NK cells, proliferating plasmablasts and

regulatory T cells (Tregs) were expanded in SDp relative to non-progressors, albeit the Treg expansion did not meet statistical significance." (see line 116).

--Please, see extended data figure 1B which more clearly illustrates this trend.

--Moreover, as shown in supplementary figure S1C and D, when identified based on protein markers, the expansion of this cell subpopulation did meet statistical significance.

D. Appropriate use of statistics and treatment of uncertainties
Overall, authors use valid statistical techniques, and they do a good job explaining these methods. My only addition is as follows:

Authors use pairwise differential expression analyses to compare individual SDp and D patients. In lines 134-135, they note that this "enables the visualization of both the effect size and the noise level in the data). In lines 700-713, they explain the method of pairwise comparisons, which is very helpful. However, since not all readers are familiar with this analysis and may want to replicate it in their own work, I think it would be helpful for the authors to add their justification for this method in the manuscript. Specifically, in the rebuttal, they note that they chose this method because of the unknown shape of the empirical distribution and the small sample size of the data, and this could be added to the manuscript.

--The justification has now been included in the manuscript (in addition to the method section). See lines 136-139: "We profiled the transcriptional landscape of APCs accompanying SD progression via pairwise differential expression analysis between individual SDp and D patients, which enables visualization of both effect size and noise levels in the data, thereby compensating for the unknown empirical distribution of gene expression and the small sample size."

E. Conclusions: robustness, validity, reliability

The conclusions are valid, the appropriate statistical tests were performed to ensure reliability and robustness for this cohort, and the limitations are helpfully outlined in lines 501-512. Authors may clarify that they included only younger children and adolescents. The way it is currently worded, it does not quite seem a limitation to include both these groups. The limitation is that they excluded other ages (although this is understandable).

--The limitation was now restated to: "Other limitations are the lack of longitudinal monitoring, the exclusion of age groups other than children and adolescents,…" (see line 506-507).

F. Suggested improvements: experiments, data for possible revision
Improvements as above, all are minor.

--We thank the Reviewer for their supportive comments.

G. References: appropriate credit to previous work?
Appropriate citations

H. Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions
Abstract, introduction and conclusion are clear and helpful.

Decision Letter, second revision:

22nd Aug 2023

Dear Shirit,

Thank you for submitting your revised manuscript "Global and cell type-specific immunological hallmarks of severe dengue progression identified via a systems immunology approach" (NI-A35401B). It has now been seen by the original referees and their comments are below. I discussed the revisions with the other editors, noting the textual revisions that you made along the lines that we had previously discussed. Thus, we'll be happy in principle to publish it in Nature Immunology, pending minor revisions to comply with our editorial and formatting guidelines.

We will now perform detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

If you had not uploaded a Word file for the current version of the manuscript, we will need one before beginning the editing process; please email that to immunology@us.nature.com at your earliest convenience.

Thank you again for your interest in Nature Immunology Please do not hesitate to contact me if you have any questions.

Kind regards,

Laurie

Laurie A. Dempsey, Ph.D.
Senior Editor
Nature Immunology

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Final Decision Letter:

Dear Shirit,

I am delighted to accept your manuscript entitled "Global and cell type-specific immunological hallmarks of severe dengue progression identified via a systems immunology approach" for publication in an upcoming issue of Nature Immunology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Immunology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

After the grant of rights is completed, you will receive a link to your electronic proof via email with a request to make any corrections within 48 hours. If, when you receive your proof, you cannot meet this deadline, please inform us at rjsproduction@springernature.com immediately.

You will not receive your proofs until the publishing agreement has been received through our system.

Due to the importance of these deadlines, we ask that you please let us know now whether you will be difficult to contact over the next month. If this is the case, we ask you provide us with the contact information (email, phone and fax) of someone who will be able to check the proofs on your behalf, and who will be available to address any last-minute problems.

Acceptance is conditional on the data in the manuscript not being published elsewhere, or announced in the print or electronic media, until the embargo/publication date. These restrictions are not intended to deter you from presenting your data at academic meetings and conferences, but any enquiries from the media about papers not yet scheduled for publication should be referred to us.

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About one week before your paper is published online, we shall be distributing a press release to news organizations worldwide, which may very well include details of your work. We are happy for your institution or funding agency to prepare its own press release, but it must mention the embargo date and Nature Immunology. Our Press Office will contact you closer to the time of publication, but if you or your Press Office have any enquiries in the meantime, please contact press@nature.com.

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