nature portfolio

Peer Review File

Intestinal tuft cell subtypes represent successive stages of maturation driven by crypt-villus signaling gradients

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this article, Buissant des Amorie and colleagues adress the question of the heterogeneity, at a transcriptional level, of the intestinal tuft cell population, using approaches combining single cell RNAseq, appropriate bioinformatics analyses, reporter organoid culture and time lapse imaging. The major conclusion of their study is that the apparent transcriptional heterogeneity of this lineage does not reflect the existence of two (or even three) distinct sub-populations of tuft cells, but rather their state of maturation, under the influence of different environmental cues such as BMPs factors. This dynamic of the tuft cell transcriptome is conserved between mouse and human organoids, and occurs unidirectionally, depending on their respective position along the crypt/villus axis. Finally, using organoids culture conjugated to stimulation and calcium response assays, the authors illustrate how their study can allow the ex vivo study of tuft cell populations according to their degree of maturation.

Overall, the question and answers arising from this study are interesting, with regards to an article published in 2017, describing for the first time the heterogeneity of intestinal tuft cells. This could impact research in the fields of digestive physiology and mucosal immunity. However, certain points remain to be clarified/improved before publication. I would also like to point out here that my current skills in the analysis of single cell RNA-seq expression data do not allow me to establish any criticism or even to ensure that the methodology used is adequate.

Main points:
One of the most critical points, in my opinion, is the lack of validation of the conclusions reached by the authors in an integrated, in vivo system. This article would be strengthened by expression data for the various tuft cell markers obtained on mouse intestinal sections and, if possible, in humans. For example, the authors could consider triple-labeling by RNAscope analyses for the markers Nrep, Chat and Folr1 (transcript analysis being more precise than protein analysis, the latter being more or less stable) clearly illustrating the evolution of their expression along the crypt-villus axis?

Similarly, the authors report that treating organoids with type-2 cytokines (such as IL13 and IL4) results in massive differentiation of tuft cells towards the tuft-1 state. This finding should also be validated in vivo, by treating mice with this cytokine cocktail. Moreover, what is the impact of the experimental timing on this process? Would a tuft-2 population eventually emerge in experiments with longer exposure to type 2 cytokines?

One point remains difficult for me to interpret, in relation to the data in Figures 3C/D. How can we explain the fact that, following stimulation with type-2 cytokines such as IL4 and IL13, only a tiny fraction of Dclk1-expressing cells is positive for GFP (controlled by the Chat reporter transgene)? The co-expression rate reported by the authors seems to me abnormally low, and in contradiction with the data shown in figure 1b, where Dclk1 appears to be a stable marker of different tuft cell states. Would it be possible to illustrate this result with immunofluorescence images and discuss these data?

The notion of a tuft-p state has only been addressed in the mouse intestine, in vivo. Does such a state exist in murine organoid models, as well as in humans?

I don't understand why the authors of this study opted for a strictly identical fluorescent reporter system for the three genes of interest (i.e. Nrep, Gng13 and Folr1, all controlling nuclear Scarlet expression). Would it be possible to add, within a line of

Nrep-Scarlet-NLS and Chat-eGFP organoids, a Folr1 reporter system based on the expression of another fluorescent protein, making it possible to really track the maturation of tuft cells, in time lapse, on the basis of the sequential expression of these 3 markers?

Minor points: a few typos in the figures (e.g. in figure 3D “Possitive”).

Reviewer #2

(Remarks to the Author)
In the paper entitled « Mature tuft cell phenotypes are sequentially expressed along the intestinal crypt-villus axis following cytokine-induced tuft cell hyperplasia », the authors take advantage of a mouse strain in which tuft cells are GFP positive to carry out single-cell RNA Sequencing experiments. They also used a tuft cell-specific reporter knock-in approach in organoids to assess the transcriptional profile of two previously described tuft cell types. While IL-4 and IL-13 induce lineage specification of Nrep+ tuft-1 cells (i.e. tuft cells with a neuron-related transcriptional program), BMP and cholinergic signaling trigger the differentiation of immune-related ChAT+ tuft-2 cells. This study therefore provides new insights into mechanisms through which tuft cells differentiate in the intestine.

Tuft cells recently gained a lot of interest due to their chemosensory properties. The interest of this study is the fact that authors better link the activation of specific signaling pathways to tuft cell differentiation and also provide some insights into the sequential differentiation of both previously described tuft cell subtypes. The generation of three reporter lines with specific markers of each tuft cell subtype is a very elegant and novel approach as is the strategy to better mimicks signaling in the villus compartment in organoids. Yet, some statements sometimes appear contradictory or not clearly stated/explained, which bring some confusion. This may be due to some data obtained with organoids that do not perfectly match with what is seen in vivo (see Figure 4 for example). Here are my comments to improve this study :

Major comments

1. In the Introduction (lanes 77 and 78), the authors state that “cells mature from a tuft-1 to a tuft-2 transcriptomic state after stimulation with IL-4 and IL-13” while they state in the abstract (lanes 27 and 28) that “cytokines interleukin-4 and interleukin-13 only induce lineage specification to Nrep+ tuft-1 cells”. Both statements appear contradictory at first reading. If not, this would definitely deserves more explanation for clarity.

2. In the result section (lane 153 to lane 158), the authors observe that GFP+ cells in organoids express tuft-2 cell markers but only barely amplify upon IL-13 or IL-4 stimulation, unlike Dclk1+ cells. Why do not we see such nice amplification as for Dclk1+ cells? At this stage of reading, this is confusing. Mature tuft-2 cells are expected to be amplified by IL-4 or IL-13 (as stated by the authors themselves). Some information should be given on the precise location of Dclk1+ cells. I am assuming that they can be found in the crypts as well as in the villus. If we go back to the transcriptional signature of both tuft-1 and tuft-2 cells, where can we detect Dclk1 expression? Given the fact that Dclk1 is a widely used tuft cell marker, the authors should tell us more on the profile of Dclk1 expression in all tuft cell subtypes that they identified in the current study.

3. On result section (lanes 253-254), the authors state that tuft-2 cells originate from Nrep+ tuft-1 cells in a villus-inspired maturation medium in which levels of BPM members are higher. They also mention that BMP response genes (Id1 and Id3) are more expressed in tuft-2 than in tuft-1 cells. If I combine both observations together, I would rather believe that the enhanced number of tuft-2 cells in a villus-inspired medium is coming from an autocrine phenomenon in tuft-2 cells rather than from a maturation of tuft-1 into tuft-2 cells. Indeed, given the fact that these tuft-1 cells are (presumably…) poorly responsive to BMP signaling, how could they undergo further differentiation in response to BMP? The author should discuss this alternative scenario and tell us why it could not be relevant. By the way and still speaking about BMP signaling, this result is actually correlative and need further validation. For example, the authors should look at additional markers of BMP signaling (for example SMAD1 phosphorylation on S206) by IF. Where would we see such phosphorylation? We understand that BMP response genes Id1 and Id3 are higher in tuft-2 cells, which can be indeed be seen as the demonstration that BMP signaling if more effective there. Nevertheless, adding any demonstration that signaling proteins known to regulate the BMP cascade are phosphorylated is an added value.

4. The major challenge now is to try to integrate the current results with the recently published paper by Hans Clevers and colleagues in “Nature” in which 4 distinct tuft cell subtypes were defined (Tuft-1 to tuft-4). How can we link both studies? Do Tuft-1 to Tuft-4 subtypes share common markers with both tuft-1 and tuft-2 populations described here? If so, which ones? Where would we put tuft-p cells described here? Those issues should be experimentally addressed and discussed.

Minor points

1. In the Introduction (lanes 60 and 61), the authors mention that multiple studies reported that tuft cells differentiation is both Atoh-1-dependent or Atoh-1-independent and indeed mentioned key papers in which this issue was addressed. They may want to state that the current model supports the notion that tuft cell differentiation is Atoh1-independent in the small intestine while being Atoh1-dependent in the colon (even if the underlying mechanism is unknown).

2. For Figure 1a, I have not found the definition of “tuft-p” in the figure legend. The authors should add this information there.

Reviewer #3

(Remarks to the Author)
The study reported in the manuscript titled "Mature 1 tuft cell phenotypes are sequentially expressed along the intestinal crypt-villus axis following cytokine-induced tuft cell hyperplasia" by Buissant des Amorie et al. introduces an optimized organoid platform to model tuft cells in vitro, closely mirroring their in vivo counterparts. The authors utilize this system to explore the functional differences between two subpopulations of tuft cells, namely tuft-1 and tuft-2.

First, they characterized tuft cell heterogeneity in mice using single-cell RNA sequencing (scRNA-seq) by sorting Chat-eGFP+ epithelial cells. This analysis revealed three clusters: tuft precursors (tuft-p), which were previously described tuft1 cells, and tuft2 cells. The authors compared a human scRNA-seq dataset with their murine dataset and established that the gene signatures between tuft2 clusters were conserved, while tuft1 cells showed less conservation across species. Additionally, the authors conducted a computational analysis to predict the spatial location of tuft cells along the crypt-villus axis, concluding that tuft-1 cells are associated with crypts, while tuft2 cells are more abundant in the villi.

Following this analysis, the authors developed reporter organoid systems for each tuft cell population and validated their transcriptomic similarity to their in vivo counterparts. They observed that the organoid proportions of tuft1 and tuft2 favored tuft-1 cells compared to their abundance in vivo, suggesting that the organoid media induces a crypt-like environment. To investigate the effect of environmental signals on the proportions of tuft1 and tuft2, the researchers treated organoids with type 2 cytokines, IL-4 and IL-13. They found that this treatment expanded the tuft1 and tuft-p compartments but did not affect tuft-2 cells.

The authors then tracked the temporal expression of tuft-specific markers during IL-4/IL-13 treatment using time-lapse imaging. Their findings indicated that tuft1 cell specification precedes that of tuft2 cells, implying a linear differentiation from tuft1 to tuft2. In an effort to induce tuft2 expansion, the authors designed a villus enrichment media (BMP ligands) to mimic in vivo microenvironmental signals specific to the villi. The use of this villus media resulted in increased tuft2 expansion. Through time-lapse imaging, they demonstrated that Nrep+ tuft1 cells transitioned into Chat-eGFP+ cells, thus claiming tuft1 to tuft2 differentiation under villus media conditions.

Finally, calcium imaging was employed to measure tuft cell responses to organoid stimulation with various predicted tuft cell ligands. The authors showed tuft activation following treatment with succinate or propionate. They also highlighted the differential responsiveness of tuft1 and tuft2 cells to each ligand, emphasizing the importance of studying the unique functions of these tuft populations.

Overall, this study provides significant insight into the use of organoids as a platform for studying tuft subpopulations ex vivo and validates their relevance to in vivo conditions. The engineered organoids, especially concerning the rare tuft cells, will enhance our understanding of gut biology. Notably, this study serves as proof-of-concept for investigating GPCRs in rare cell states, tuft1 and tuft2, by combining calcium influx imaging with tuft cell reporters. However, some discrepancies in the study need to be addressed, and conclusions may extend beyond the data presented. Specifically, there is insufficient evidence to assert that tuft1 cells transition into tuft2 cells. The authors should either provide additional supporting evidence for this conclusion or rephrase it to more accurately reflect their results. Furthermore, the calcium influx results are not convincing enough to claim tuft subset-specific activation, nor do they clarify why other cell types (not tuft) are also activated. Lastly, and most importantly, a recent study (https://www.nature.com/articles/s41586-024-07952-6#Sec3), cited by the authors, showed a local expansion of human tuft cells upon stimulation with IL4/IL13 claiming for self-expansion of tuft cell and could imply for no linear differentiation of progenitors toward tuft cells under these conditions and second they claim for Wnt dependence for tuft cell differentiation while BMP reduces it – how these findings reconcile with the results shown in this study? Is it human vs mouse?

Comments:
1. The conclusion that type 2 cytokine treatment of organoids does not induce tuft2 expansion is not well established in this study. Organoids were treated with IL4/IL13 three days post-passaging, after the organoids' differentiation had already begun. It would be important to test the tuft1 and tuft2 proportions in organoids following treatment with cytokines immediately after passaging, and whether this induces more tuft2 cells, in agreement with in-vivo studies.
2. The intermediatory tuft cell state could be explained by a common progenitor for both subsets that have yet to commit to either tuft1 or tuft2 fate. Moreover, the tuft-p cells were shown to express markers of other lineages. Are tuft-p cells indeed committed to the tuft lineage, or are they secretory progenitors? More data should be presented to elucidate this cell population. In addition, are these cells proliferate?
3. The authors utilized differentiation media and showed BMP2+4 induces tuft cells; however, as mentioned above, a recent study (https://www.nature.com/articles/s41586-024-07952-6#Sec3) showed higher tuft cell differentiation upon wnt stimulation and a massive reduction of tuft cells when adding BMP2/4. Could the authors add Wnt/Rspo stimulation? Is the difference due to human vs mouse?
4. In the same context, could it be that BMP2+4 depletes stem cells and, as a consequence, other differentiated cells (See Fig.4g), elevating the tuft cell percentages? Absolute quantification of different tuft cell populations should be included in the FACS analysis in Fig. 4c.
5. The conclusions of the authors regarding Figure 4 are contradictory. On the one hand, they claim that type 2 cytokines induce expansion of tuft1 or intermediate tuft cells, but not of tuft2 cells. On the other hand, they claim to observe tuft1 cells transition into tuft2 cells and not in the opposite direction. There is no quantification of how many cells were counted to transition between tuft1 and tuft2. In addition, a longer time point should be conducted after the treatment to show tuft 1

differentiation to tuft 2 using the different reporter organoids. lastly, to provide genetic evidence supporting their claims, the author should consider a fate-mapping experiment or rephrase it to more accurately reflect their results.
6. The recent paper by Clevers' group showed local proliferation of tuft cells in the IL4/13 treatment. Could the authors comment on the proliferation state of the different tuft cell subsets? This could be measured by both IFA and looking at the single cell data. The authors should address this study in order to explain the differences between the two studies.
7. The calcium influx assay shows no specificity to tuft 1 cells in Fig. 6i and in Fig. 6l, I can't see the expression of the calcium reporter in tuft cells in the image, only in surrounding cells. Looking at the intensity plot in Fig. 6n, it seems that the activation of the FFAR3 is higher in other cells. What is the explanation for the non-specificity in these assays?

Minor comments:
1. Figure 3C shows an increase in Chat GFP+ cells under IL4/13 treatment, contrary to text description and conclusion.
2. Chat-GFP is used interchangeably as a general tuft cell marker, or as a tuft2 marker. The authors should be careful to use this marker in a consistent manner, to draw correct conclusions. For example, in the live imaging experiment fig. 4c and fig. 5d they use Chat as a marker for tuft2, however in flow cytometry, they use chat GFP+ cells as tuft cells consisting of all tuft subpopulations. In addition, in figure 5c most of the Flor1+ cells which are considered tuft2 are GFP negative.
3. Figure 5b-c – missing a quantification of PE+ cells percentage under crypt IL4+13 media vs. villus IL4+13 media
4. Figure 5c shows an increase of Nrep+ tuft1 cells in villus media, in addition to tuft2 cells. In general, according to the FACS analysis there seems to be an increase in all tuft cell populations.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have greatly improved their manuscript. The model of progressive maturation and differentiation of tuft cells, in response to signals from the stroma, will clearly help to clarify all the published data regarding the existence of tuft cell subpopulations, and will provide valuable information to the community interested in these cells.

There is one final point that I believe needs further clarification before publication. Could the authors explain the results of the lineage ablation experiments, via the DTR system, in the organoid cultures? The results are somewhat counter-intuitive, and some explanation would be welcome for the data showed in Figures 5 and Supplementary Figure 9. As an example, in the main figure 5J, I would have expected Folr1-mediated lineage ablation (thus, tuft 2 state-related) to induce a disappearance of tuft 2 cells, but not tuft 1. The data shown seem to suggest a total loss of lineage. Can the authors explain these data?

Reviewer #2

(Remarks to the Author)
The authors are now providing an improved version of their study in which all my comments/concerns were properly addressed. We now have a very elegant story that brings some new insights into mechanisms underlying tuft cell differentiation. Congratulations to all authors....

Reviewer #3

(Remarks to the Author)

The authors performed a substantial revision of the text and figures, providing new evidence to their claims, which strengthens the conclusions of the manuscript. Overall, this study provides significant insight into the use of organoids as a platform for studying tuft subpopulations ex vivo, with the potential to investigate their biological relevance.
The authors answered my comments in a satisfactory manner, and I do not have further comments.

Buissant des Amorie et al., Rebuttal letter

We thank the reviewers for the time dedicated to evaluate our manuscript and for their overall positive critique. Their comments and suggestions have been very valuable and have improved multiple aspects of our study. Our point-by-point responses can be found below. Noteworthy experimental additions are:

- We confirmed the *in vivo* localization/transitioning of tuft-1 to tuft-2 along the villi, by documenting the zonated expression patterns of our tuft subtype-specific markers *in vivo* using RNAscope (Fig. 3hij and Supplementary Fig. 4c).

- We now extensively studied the co-expression of our tuft subtype markers with pan-tuft cell marker *Dclk1* (Fig. 1fhim, Fig.3eg and Supplementary Fig. 4hi).

- We have intensively re-analyzed all our scRNA-seq datasets (from mouse, human, and mouse organoids) and now consistently find evidence for the presence of conserved tuft-p, tuft-1 and tuft-2 states (Fig. 1a, Fig.2b and Fig. 4n; Supplementary Fig. 1de, 2ab, 5bcd and 7).

- We cross-examined our data with the earlier published Nature paper from Clevers. We find extensive overlap between each other's tuft-1 and tuft-2 subtypes resp. Importantly, their tuft-3 exhibited proliferative potential and shows large overlap with our tuft-p population.

- We included several experiments to address the proliferative potential of our tuft subtypes. *First*, only a minor population of Dclk1+ cells, positioned deep in crypts prior the onset of tuft-1 marker expression, shows proliferative potential (EdU incorporation; Fig. 1l and Supplementary Fig. 1h). *Second*, extensive time-lapse imaging of organoids demonstrates that the last division virtually always occurs prior the emergence of Nrep expression (Supplementary Fig. 7). Nrep thus marks the first post-mitotic tuft stage, i.e. tuft-1. *Last*, in all our experiments with type 2 cytokines exposure, we have not seen tuft-1 or tuft-2 divisions, suggesting that tuft cell hyperplasia is fueled by tuft-p cells.

- We provide new experimental evidence that tuft-2 cells originate from tuft-1 cells, and that this differentiation trajectory is not facultative but obligatory. Our fluorescent marker knock-ins were accompanied with a Diphtheria Toxin Receptor to ablate cell types. Tuft-1 ablation (via Nrep) prevents any development of new Chat+ tuft-2 cells. Vice versa, late tuft-2 ablation (by Folr1) remains compatible with the generation of new Chat+ early tuft-2 cells (Fig. 5ijk).

Other textual and figure changes in the manuscript are mostly to improve clarity or to comply with Nature Communications formatting requirements, e.g. the shortened title. Major changes are highlighted in yellow.

Please note that figure and text references in our responses apply to the revised version of our manuscript, unless otherwise stated.

Reviewer #1

General: In this article, Buissant des Amorie and colleagues adress the question of the heterogeneity, at a transcriptional level, of the intestinal tuft cell population, using approaches combining single cell RNAseq, appropriate bioinformatics analyses, reporter organoid culture and time lapse imaging. The major conclusion of their study is that the apparent transcriptional heterogeneity of this lineage does not reflect the existence of two (or even three) distinct sub-populations of tuft cells, but rather their state of maturation, under the influence of different environmental cues such as BMPs factors. This dynamic of the tuft cell transcriptome is conserved between mouse and human organoids, and occurs unidirectionally, depending on their respective position along the crypt/villus axis. Finally, using organoids culture conjugated to stimulation and calcium response assays, the authors illustrate how their study can allow the ex vivo study of tuft cell populations according to their degree of maturation.

Overall, the question and answers arising from this study are interesting, with regards to an article published in 2017, describing for the first time the heterogeneity of intestinal tuft cells. This could impact research in the fields of digestive physiology and mucosal immunity. However, certain points remain to be clarified/improved before publication. I would also like to point out here that my current skills in the analysis of single cell RNA-seq expression data do not allow me to establish any criticism or even to ensure that the methodology used is adequate.

Response: We thank the reviewer for the detailed reading of the manuscript and the positive assessment regarding its impact to the field.

Main points:

1. One of the most critical points, in my opinion, is the lack of validation of the conclusions reached by the authors in an integrated, in vivo system. This article would be strengthened by expression data for the various tuft cell markers obtained on mouse intestinal sections and, if possible, in humans. For example, the authors could consider triple-labeling by RNAscope analyses for the markers Nrep, Chat and Folr1 (transcript analysis being more precise than protein analysis, the latter being more or less stable) clearly illustrating the evolution of their expression along the crypt-villus axis?

Response: We acknowledge the added value to document *in vivo* expression patterns for the various tuft cell markers along the crypt-villus axis.

RNAscope of tuft cell subtype markers

Along the suggestions of the reviewer, we have now included RNAscope analysis to document the expression patterns of *Nrep*, *Gng13*, *Chat* and *Folr1*, including the tuft cell position that co-expresses various combinations of these markers. This data is now included in main figure 3 (lines 190-201; Fig. 3hij and Supplementary Fig. 4c) and supports our main conclusion regarding the evolution of tuft cell states along the crypt-villus axis. In short, the transcript analysis demonstrates that *Nrep* shows the most confined expression pattern located to the flank of crypts. Next, *Gng13* becomes expressed in Nrep+ tuft cells, but remains present during the tuft cell's lifetime and migration to the villus tip. The moment that *Nrep* expression fades demarcates the position from where *Chat,* quickly followed by *Folr1,* start their gradual increase in expression level while the tuft cell migrates to the villus tip.

In addition, considering the confusion regarding the expression pattern of *Chat* (mentioned by reviewer 1 comment 3, reviewer 2 comment 2 and reviewer 3 minor comment 2), we have now included new analyses of scRNA-seq data (Fig. 1f), updated figure panels (Fig. 1m, *Dclk1* expression is annotated at the top) and immunofluorescence data (Fig. 1hi) in main figure 1 to correlate *Chat* expression in relation to general tuft cell marker *Dclk1*. In line with its transcript counts and our earlier findings, *Chat* becomes robustly expressed at the crypt-villus junction (Fig. 1hi) and is a solid marker for mature tuft cell states, but unlike *Dclk1*, it does not mark the earliest stages of the tuft cell lineage (Fig. 1gm and Fig. 5m). For a more elaborate comparison of *Dclk1*

and *Chat* in terms of their expression patterns within the intestinal tuft cell pool, please see our response to comment 3 of reviewer 1.

2. Similarly, the authors report that treating organoids with type-2 cytokines (such as IL13 and IL4) results in massive differentiation of tuft cells towards the tuft-1 state. This finding should also be validated in vivo, by treating mice with this cytokine cocktail. Moreover, what is the impact of the experimental timing on this process? Would a tuft-2 population eventually emerge in experiments with longer exposure to type 2 cytokines?
Response: The reviewer raises an important point about how our *in vitro* differentiation regimens relate to the *in vivo* situation.

The reviewer suggests an experiment where mice are treated with type 2 cytokines to see if this will result in a massive increase in tuft-1 cells *in vivo*, like we observed in our organoid experiments. We do not expect that cytokine-induced tuft cell hyperplasia solely yields tuft-1 cells *in vivo* (as mentioned in lines 243-246), since crypt-villus signaling gradients are intact in such an experiment and will therefore facilitate the maturation to mature tuft-2 phenotypes as the tuft-1 cells migrate up the crypt villus axis. The fact that we observed solely a tuft-1 increase in our initial organoid experiments with cytokines, is a result of the other medium components that keep the organoid cells in a crypt-like state (stem cell niche factors EGF, Noggin and R-spondin). Only when we change this crypt-mimicking environment to better represent the villus environment, where tuft-2 cells are found *in vivo*, do we observe a significant increase in tuft-2 cells in the organoids (Fig. 5abc). Below, we have summarized extensive previous findings with regards to the effects of type 2 cytokines on tuft cell differentiation *in vivo*.

Regarding the effect of type 2 cytokines *in vivo*
Type 2 cytokine treatments have been performed in mice *in vivo,* previously (Moltke *et al.*, Nature (2016)—fig. 2h). These experiments show a robust increase in DCLK1+ tuft cells, akin to the tuft cell hyperplasia observed in case of helminth infections (Moltke *et al.*, Nature (2016)—fig. 2a-e; Gerbe *et al.*, Nature (2016)—fig. 1), which is completely abrogated when the epithelium is rendered insensitive to type 2 cytokines, both *in vivo* (Moltke *et al.*, Nature (2016)—fig. 2e-f; Gerbe *et al.*, Nature (2016)—fig. 3e) or *in vitro* (Moltke *et al.*, Nature (2016)—fig. 3ab). Later, scRNA-seq of the small intestinal epithelium of helminth infected mice was performed (Haber *et al.*, Nature (2017)) and revealed that the tuft-2 subtype was strongest represented within the increased tuft cell population during infection (Haber *et al.*, Nature (2017)—fig. 6d). We found similar results when reanalyzing that data (Supplementary Fig. 6de) or later published bulk RNA-seq data of intestinal epithelial tissue of helminth infected mice (Supplementary Fig. 6c). The relative increase in tuft-2 cells could be a consequence of a higher concentration of tuft cell-secreted factors such as BMP2 and acetylcholine, which we show can drive maturation from tuft-1 to tuft-2 states (Fig. 5abc and Supplementary Fig. 8a).

In organoids, we initially observed an increase in tuft-1 cells and not tuft-2 cells when treating with type 2 cytokines (Fig. 4abc). The crypt-inspired growth factors (EGF, Noggin and R-spondin) in the standard organoid medium are very potent and keep the cells in this medium in a progenitor-like state. It is hard to recapitulate subtle signaling gradients *in vitro* and the choices with regards to what factors are in the medium are more binary. As a result, the stalling of tuft cell maturation at the tuft-1 state in crypt-mimicking medium until we expose the cells to villus-inspired medium reflects a binary switch in medium and is an organoid-intrinsic situation. This is further illustrated by a newly incorporated experiment (Supplementary Fig. 6ab), in which longer exposure to type 2 cytokines does not allow for maturation to tuft-2 states *in vitro* when the crypt growth factors (ENR) are present in the medium, clearly demonstrating that these crypt factors keep the cells in an immature state. *In vivo*, we expect that tuft-1 cells differentiate far more gradually into tuft-2 cells as they become continuously exposed to subtle differences in signaling pathway activities while they move up the crypt-villus axis.

3. One point remains difficult for me to interpret, in relation to the data in Figures 3C/D. How can we explain the fact that, following stimulation with type-2 cytokines such as IL4 and IL13, only a tiny fraction of Dclk1-expressing cells is positive for GFP (controlled by the Chat reporter transgene)? The co-expression rate reported by the authors seems to me abnormally low, and in contradiction with the data shown in figure 1b, where Dclk1 appears to be a stable marker of different tuft cell states. Would it be possible to illustrate this result with immunofluorescence images and discuss these data?

Response: We understand the confusion concerning the status of *Chat* as a general tuft cell marker and have thus incorporated new experiments and analyses clarifying the expression pattern of *Chat* and general tuft cell marker *Dclk1* in our revised manuscript (lines 110-119). Please find our direct response to the reviewer's comment below.

Regarding *Dclk1* and *Chat* expression in tuft cell subtypes

While we initially chose *Chat* for its reputation to label all tuft cells, we realized during our in-depth scRNA-seq analyses that *Chat,* in fact, does not mark the earliest stages after tuft cell specification, but becomes expressed during the tuft-1 state with increasing levels as the tuft cells mature to the tuft-2 state (Fig. 1fgm; also see the new RNAscope data (Fig. 3hij and Supplementary Fig. 4c) and our response to comment 1 of reviewer 1). To improve communication of this finding as early as possible in our manuscript, we now include immunofluorescence against tuft cell markers DCLK1 and ChAT in main figure 1 (Fig. 1hi) and added better comparisons of *Dclk1* and *Chat* in the scRNA-seq panels (Fig. 1fgm). *Dclk1* is a pan-tuft cell marker, labelling tuft cells in crypts and villi (Fig. 1h), in agreement with high transcript levels during all stages of post-mitotic tuft cell differentiation (Fig. 1cm; *Dclk1* expression is annotated at the top of the heatmap). In contrast, Chat-GFP is predominantly detected in Dclk1+ cells at the villus, with emerging levels found in the upper half of crypts (Fig. 1hij and Fig.3 hij) and is therefore more suitable to identify mature tuft cells. Similar observations that *Chat* does not label all Dclk1+ tuft cells have been reported before (Billip *et al*., Immunity 2024). To clearly communicate this to readers, we have changed the first subheading of the results section (lines 86-87).

In organoids, co-expression of *Dclk1* and *Chat* is low when cultured in ENR medium, because the tuft cells in these organoids are in a crypt-mimicking environment and therefore mostly resemble the immature Dclk1+ Nrep+ Gng13+ tuft cells (Fig. 4bc) that are found in crypts (Fig. 3hij and Fig. 1hi). Only a minor subfraction of Dclk1+ tuft cells with emerging Chat expression (Fig. 3cde), resembling tuft cells toward the upper regions of crypts (Fig. 1hi and Fig. 3i), can be observed. Upon changing the medium to mimic the villus environment, a significant fraction of tuft cells proceed maturation and start to express *Chat*, *Folr1* and other tuft-2 markers (Fig. 5abcd), which we know from our scRNA-seq data to be co-expressed with genes related to immunological (*Ptgs1*, *Ltc4s,* Fig. 1m) and chemosensory function of the tuft cell (*Sucnr1*, *Adgrg2, Ffar3,* Fig. 6f).

4. The notion of a tuft-p state has only been addressed in the mouse intestine, in vivo. Does such a state exist in murine organoid models, as well as in humans?

Response: The reviewer raises a valid point, also raised by other reviewers, that we should improve consistency in analyses and avoid unnecessary confusion concerning the tuft-p state.

Regarding tuft-p cells

While we had initially focused our analyses on post-mitotic tuft-1 and tuft-2 populations, we have now incorporated consistent and more thorough analyses on the scRNA-seq datasets presented throughout the manuscript, which now all document similar presence of a proliferative tuft precursor state (tuft-p). This tuft-p population is now observed in mice (Fig. 1ac and Supplementary Fig. 1cde), human (Fig. 2bc and Supplementary Fig. 2ab) and in organoids of mouse (Fig. 3n and Supplementary Fig. 5bd). The identified overlap between tuft-p phenotypes in mouse and human also helps to mitigate the confusion regarding the recently published data

(Huang, Bernink and Giladi *et al.,* Nature (2024)) concerning proliferative tuft cells in human organoids (see also point 4, reviewer 2; and point 6, reviewer 3).

We now validated the proliferative capacity and predicted location (Fig. 1k) of tuft-p cells in mice by assessing (recent) proliferative potential of Dclk1+ tuft cells through EdU incorporation following a 4-hour pulse (Fig. 1l and Supplementary Fig. 1h). In these experiments we detected a minor EdU+ fraction among the Dclk1+ tuft cell population in the crypt, which was predominantly Chat negative.

Furthermore, we have added extensive live-cell imaging experiments that show that the last cell division during differentiation to a mature tuft cell virtually always precedes the emergence of *Nrep* expression (lines 261-271; Supplementary Fig. 7). As such, *Nrep* reports the earliest stage (i.e. tuft-1) among post-mitotic tuft cells. Concordantly, we never observed cell divisions of more mature Gng13+, Chat+ or Folr1+ tuft cells, which are found higher on the crypt-villus axis (response to comment 1 of reviewer 1; Fig. 3hij), during all our organoid live imaging experiments.

Together, these lines of evidence illustrate the existence of a minor subpopulation of tuft cells that is proliferative, is conserved between mouse and human, and represents a progenitor state that precedes post-mitotic tuft-1 and 2 states.

5. I don't understand why the authors of this study opted for a strictly identical fluorescent reporter system for the three genes of interest (i.e. Nrep, Gng13 and Folr1, all controlling nuclear Scarlet expression). Would it be possible to add, within a line of Nrep-Scarlet-NLS and Chat-eGFP organoids, a Folr1 reporter system based on the expression of another fluorescent protein, making it possible to really track the maturation of tuft cells, in time lapse, on the basis of the sequential expression of these 3 markers?
Response: We fully agree with the reviewer and made multiple attempts to generate that triple reporter system**.** Unfortunately, we have not succeeded in that feat (pre or post manuscript submission), because the available selection cassettes that we need to use for the second knock-in are less stringent, severely complicating these attempts.

Nevertheless, to provide alternative evidence for the maturation of tuft cells, we made use of the fact that the knock-ins co-express Diphtheria Toxin Receptor along with the fluorescent Scarlet as a remnant of its original application in a different research topic in our lab (Fig. 3k). Consequently, exposure of the organoid lines to Diphtheria Toxin (DT) induces robust tuft cell ablation corresponding to the state that is marked by the knock-in. We have now included experiments which show that continuous depletion of the immature Nrep+ tuft-1 state prevents the emergence of mature Chat+ tuft cells, even in the villus-inspired medium (Fig. 5ijk and Supplementary Fig. 9c). On the other hand, depletion of the most mature tuft cell state marked by *Folr1* expression does still allow for the emergence of Chat+ tuft cells (Fig. 5ijk and Supplementary Fig. 9c). Together, these experiments indicate that there is no alternative differentiation trajectory by which mature Chat+ tuft cells can arise without going through a preceding tuft-1 stage marked by *Nrep*.

Minor points: a few typos in the figures (e.g. in figure 3D “Possitive”).
Response: We thank the reviewer for pointing out this error and have corrected it in the revised version of the manuscript.

Reviewer #2

General: In the paper entitled « Mature tuft cell phenotypes are sequentially expressed along the intestinal crypt-villus axis following cytokine-induced tuft cell hyperplasia », the authors take advantage of a mouse strain in which tuft cells are GFP positive to carry out single-cell RNA Sequencing experiments. They also used a tuft cell-specific reporter knock-in approach in organoids to assess the transcriptional profile of two previously described tuft cell types. While IL-4 and IL-13 induce lineage specification of Nrep+ tuft-1 cells (i.e. tuft cells with a neuron-related transcriptional program), BMP and cholinergic signaling trigger the differentiation of immune-related ChAT+ tuft-2 cells. This study therefore provides new insights into mechanisms through which tuft cells differentiate in the intestine.

Tuft cells recently gained a lot of interest due to their chemosensory properties. The interest of this study is the fact that authors better link the activation of specific signaling pathways to tuft cell differentiation and also provide some insights into the sequential differentiation of both previously described tuft cell subtypes. The generation of three reporter lines with specific markers of each tuft cell subtype is a very elegant and novel approach as is the strategy to better mimicks signaling in the villus compartment in organoids. Yet, some statements sometimes appear contradictory or not clearly stated/explained, which bring some confusion. This may be due to some data obtained with organoids that do not perfectly match with what is seen in vivo (see Figure 4 for example). Here are my comments to improve this study :

Response: We thank the reviewer for the compliments on our manuscript and the constructive remarks to improve our study.

Major comments

1. In the Introduction (lanes 77 and 78), the authors state that “cells mature from a tuft-1 to a tuft-2 transcriptomic state after stimulation with IL-4 and IL-13” while they state in the abstract (lanes 27 and 28) that “cytokines interleukin-4 and interleukin-13 only induce lineage specification to Nrep+ tuft-1 cells”. Both statements appear contradictory at first reading. If not, this would definitely deserves more explanation for clarity.

Response: The reviewer’s first sentiment about these statements creating unnecessary confusion was correct. Although some Nrep+ tuft-1 cells, which arise after stimulation with IL-4 and IL-13, transition to more mature Chat+ tuft-2 states (Fig. 4de), this is very inefficient in crypt-mimicking medium (Fig. 4bc) compared to villus-mimicking medium (Fig. 5bcd). Also see the last paragraph of our response to comment 2 of reviewer 1.

We have rephrased the statements mentioned by the reviewer to improve clarity:

Abstract lanes 27-28 of initial submission changed to: “*In vitro* stimulation with interleukin-4 and 13 is sufficient to fuel the generation of new Nrep+ tuft-1 cells, arising from tuft precursor (tuft-p) divisions. Subsequently, changes in crypt-villus signaling gradients, such as BMP signaling, are required to advance maturation towards Chat+ tuft-2 phenotypes.”

Introduction lanes 77-78 of initial submission changed to: “Moreover, new organoid models with tuft cell subtype-specific reporter knock-ins show that treatment with IL-4 and IL-13 leads to increased tuft cell numbers, but solely generates tuft-1 states. Adaptation of the crypt-like organoid medium to better mimic the villus environment, where tuft-2 cells reside *in vivo*, facilitates transitioning of post-mitotic immature tuft-1 states to mature tuft-2 states.”

2. In the result section (lane 153 to lane 158), the authors observe that GFP+ cells in organoids express tuft-2 cell markers but only barely amplify upon IL-13 or IL-4 stimulation, unlike Dclk1+ cells. Why do not we see such nice amplification as for Dclk1+ cells? At this stage of reading, this is confusing. Mature tuft-2 cells are expected to be amplified by IL-4 or IL-13 (as stated by the authors themselves). Some information should be given on the precise location of Dclk1+ cells. I am assuming that they can be found in the crypts as well as in the villus. If we go back to the

transcriptional signature of both tuft-1 and tuft-2 cells, where can we detect Dclk1 expression? Given the fact that Dclk1 is a widely used tuft cell marker, the authors should tell us more on the profile of Dclk1 expression in all tuft cell subtypes that they identified in the current study.
Response: The reviewer makes an excellent remark that we should improve on the analysis of the expression pattern of *Dclk1* in relation to the other tuft cell subtype-specific marker genes, as well as the proliferative capacity (amplification) along the various stages of tuft cell differentiation.

To address these points and improve clarity of the manuscript, we have substantially changed the text (lines 110-119; 127-132; 139-144; 178-183), subheadings (lines 86, 233 and 272), and incorporated new experiments (Fig. 1hil, Fig. 3ehij and Supplementary Fig. 4chi) and analyses (Fig. 1fm). In short, *Dclk1* is a true pan-tuft cell marker, with expression starting lower in the crypt, while *Chat* is expressed from late tuft-1 stage onwards, predominantly marking cells on the villus. For a more elaborate answer, please see our response to comment 3 of reviewer 1.

Regarding amplification/expansion capacity of tuft cells and the impact of type 2 cytokines:
- Our interpretation of the result section described in lines 153 to 158 of the initial submission, is that type 2 cytokines promote specification towards the tuft cell lineage that is always Dclk1+. However, since we are dealing with organoids in crypt-mimicking medium, the new Dclk1+ tuft cells do not progress beyond the immature crypt-like tuft-1 stage and become stalled in their maturation prior to the activation of *Chat-GFP* expression, until the organoid medium is changed to better mimic the villus environment (Figure 5).
- We have now included new data concerning the proliferative capacity of tuft cells. First, we have updated and improved consistency of our analyses of the scRNA-seq datasets presented throughout the manuscript, which now all document similar presence of a proliferative, Dclk1+ tuft precursor state (tuft-p) preceding tuft-1 and 2 states. This tuft-p population is now observed in mice *in vivo* (Fig. 1ac and Supplementary Fig. 1cde), human (Fig. 2bc and Supplementary Fig. 2ab) and in organoids of mouse (Fig. 3n and Supplementary Fig. 5bd). Second, we have added extensive live-cell imaging experiments that show that the last cell division during differentiation to a mature tuft cell virtually always precedes the start of *Nrep* expression (lines 261-271, Supplementary Fig. 7). As such, Nrep reports the earliest stage (i.e. tuft-1) among post-mitotic tuft cells. Concordantly, we never observed cell divisions of Gng13+, Chat+ or Folr1+ cells during all our live-cell imaging experiments. Third, we performed a 4-hour EdU incorporation in mice and detected a minor EdU+ fraction among the Dclk1+ tuft cell population in the crypt (Fig. 1l and Supplementary Fig. 1h).

Together, in all our organoid experiments where tuft cell numbers were boosted with IL-4 and IL-13, we have never observed mature tuft cells dividing. More specifically, the last division virtually always preceded the tuft-1 stage marked by Nrep expression. Thus, upon IL-4 and IL-13 exposure, the increase in mature, post-mitotic tuft cell numbers is fueled by early-stage tuft cells from the proliferative tuft stage (tuft-p). *In vivo*, the new tuft-1 cells will continue to migrate to the villus tip and will gradually mature into Chat+ tuft-2 cells under the influence of new signaling environments. In contrast, the new tuft-1 cells in organoids, cultured in crypt-like medium, will not transition to tuft-2 stages, creating a disbalance between Dclk1+/Nrep+ tuft-1 cells, over Dclk1+/Chat+ tuft-2 cells.

3. On result section (lanes 253-254), the authors state that tuft-2 cells originate from Nrep+ tuft-1 cells in a villus-inspired maturation medium in which levels of BPM members are higher. They also mention that BMP response genes (Id1 and Id3) are more expressed in tuft-2 than in tuft-1 cells. If I combine both observations together, I would rather believe that the enhanced number of tuft-2 cells in a villus-inspired medium is coming from an autocrine phenomenon in tuft-2 cells rather than from a maturation of tuft-1 into tuft-2 cells. Indeed, given the fact that these tuft-1 cells are (presumably…) poorly responsive to BMP signaling, how could they undergo further differentiation in response to BMP? The author should discuss this alternative scenario and tell

us why it could not be relevant. By the way and still speaking about BMP signaling, this result is actually correlative and need further validation. For example, the authors should look at additional markers of BMP signaling (for example SMAD1 phosphorylation on S206) by IF. Where would we see such phosphorylation? We understand that BMP response genes Id1 and Id3 are higher in tuft-2 cells, which can be indeed be seen as the demonstration that BMP signaling if more effective there. Nevertheless, adding any demonstration that signaling proteins known to regulate the BMP cascade are phosphorylated is an added value.

Response: The reviewer raises an important point about possible alternative scenarios leading to the increase in tuft-2 cells in villus-mimicking medium containing BMPs. We discuss the likelihood of alternative scenarios below. However, we also want to mention here, and explain below, that the revised manuscript contains tuft subtype-specific ablation experiments that show that a transient immature tuft-1 state is a prerequisite for the emergence of mature tuft-2 states in case of cytokine-induced tuft cell expansion, arguing against such alternative scenarios.

<u>The role of (autocrine) BMP signaling</u>

First, based on the data presented in Supplementary Fig. 8b and mentioned in lines 290-295 of our manuscript, we detect expression of BMP receptors in both tuft-1 and tuft-2 cells. Therefore, we do not see a reason to presume that the tuft-1 cells cannot respond to BMP ligands. Indeed, a role for autocrine BMP signaling in tuft cell differentiation is a possibility given the fact that the tuft cells themselves express BMP ligands and receptors. Such an autocrine loop could start in immature tuft-1 cells and reinforce the directionality of the differentiation towards mature tuft-2 cell states, since the latter express the highest level of BMP2 and experience the highest BMP stimulation based on expression of the BMP response genes Id1 and 3, as mentioned by the reviewer. All in all, we think that this autocrine BMP signaling could co-exist with strong BMP signals coming from the stroma in the villus region, together forming an important factor in tuft cell differentiation. It is for this reason that we mentioned the possibility of autocrine signaling in lines 284-287. Lastly, the reviewer is correct in stating that our findings are correlative. However, we have not succeeded in establishing reliable Smad phosphorylation stainings in our lab and have therefore changed the text accordingly (lines 292-293).

<u>The cellular source of tuft-2 cells and tuft subtype-specific ablation experiments</u>

The reviewer suggests the enhanced number of tuft-2 cells in villus-inspired medium to be a result of an autocrine phenomenon in tuft-2 cells instead of maturation from tuft-1 cells to tuft-2 cells. First, such a scenario is unlikely given that we observe no Chat+ tuft-2 cells 1 day after changing to villus-inspired medium (day 4, Supplementary Fig. 9a). In fact, we first observe an increase in Nrep+ tuft-1 cells and only later observe Chat+ and Chat+Folr1+ tuft-2 cells. This flow cytometry time course data is in line with the successive expression of tuft-1 and tuft-2 markers in our live microscopy experiments (Fig. 4d and Fig. 5f) and is further supported by new live cell imaging that shows induction of Folr1 expression only 2 days after changing to villus-inspired medium (Supplementary Fig. 9b).

To further test if a transient tuft-1 state is mandatory or facultative during cytokine-induced tuft cell hyperplasia, we now included tuft cell subtype-specific ablation experiments. For this, we made use of the fact that the knock-ins co-express Diphtheria Toxin Receptor along with the fluorescent Scarlet as a remnant of its original application in a different research topic in our lab (Fig. 3k). Consequently, exposure of the organoid lines to Diphtheria Toxin (DT) induces robust tuft cell ablation corresponding to the state that is marked by the knock-in. These experiments show that continuous depletion of the immature Nrep+ tuft-1 state prevents the emergence of more mature Chat+ tuft-2 cells, even in the villus-inspired medium (Fig. 5ijk and Supplementary Fig. 9c). On the other hand, depletion of the most mature tuft cell state marked by *Folr1* expression still allows for the emergence of Chat+ tuft cells (Fig. 5ijk and Supplementary Fig. 9c). Together, these experiments indicate that there is no alternative differentiation trajectory by which mature Chat+ tuft-2 cells can arise without going through a preceding tuft-1 stage marked by *Nrep*.

Last, our revised manuscript contains single-cell tracking analyses of live microscopy, showing that the last division during tuft cell differentiation almost always occurs prior to *Nrep* expression (Supplementary Fig. 7). *Nrep* reports the earliest stage (i.e. tuft-1) among post-mitotic tuft cells and during all our live-cell imaging experiments, we never observed divisions of more mature tuft cells (Gng13+, Chat+ or Folr1+), found predominantly on the villus (Fig. 3hij, please see our response to comment 1 of reviewer 1).

Together, these data are best explained by tuft-1 cells, originating either from cytokine-induced divisions of tuft precursors (tuft-p) or increased stem cell differentiation towards tuft-p cells, continuing their stalled differentiation towards mature tuft-2 cells when the medium is changed to better mimic the villus environment. Please also see our response to comment 2 of reviewer 1.

4. The major challenge now is to try to integrate the current results with the recently published paper by Hans Clevers and colleagues in "Nature" in which 4 distinct tuft cell subtypes were defined (Tuft-1 to tuft-4). How can we link both studies? Do Tuft-1 to Tuft-4 subtypes share common markers with both tuft-1 and tuft-2 populations described here? If so, which ones? Where would we put tuft-p cells described here? Those issues should be experimentally addressed and discussed.
Response: The reviewer brings up a very important point that was shared among other reviewers. We have now incorporated consistent analyses on the scRNA-seq datasets presented throughout the manuscript. All datasets now document the presence of three subtypes, including the presence of a proliferative tuft precursor state (tuft-p) preceding tuft-1 and 2 states. Importantly, the three subtypes are observed in *in vivo* datasets of both mouse (Fig. 1ac and Supplementary Fig. 1cde) and human intestine (Fig. 2bc and Supplementary Fig. 2ab), corroborating our organoid data (Fig. 3n and Supplementary Fig. 5bd).

Within the nomenclature of the 4 tuft cell subtypes identified in human organoids of the mentioned paper, our tuft-p shares a similar transcriptome with their cycling tuft-3 (e.g. Mki67, Pcna, Top2A). In the revised version of our manuscript, we now clarify the similarity between the tuft-3 cluster in the Nature paper and our tuft-p cells in lines 152 and 387-390. In addition, the tuft-1 and 2 subtypes described in our manuscript are similar between mouse and human (Fig. 1ckm, Fig. 2cdefg and Supplementary Fig. 1e and 2ab) and are also found, and referred to with the same names, by the authors of the Nature paper. In the revised version of our manuscript, we now mention the similarity between the tuft-1 and tuft-2 clusters found in both papers in lines 151-152.

The tuft-4 (regenerative) state described by the aforementioned paper was not found in any of our scRNA-seq analyses and the mentioned marker genes (PD-L1 (*CD274*), *EREG*, *HB-EGF* and *TACSTD2*) of this tuft subtype were lowly or not expressed in the tuft cells of the datasets we analyzed. Indeed, in the paper from Clevers and colleagues the tuft-4 subtype was mentioned to be absent in mice, mostly involved with regeneration and predominantly found in organoids only. Due to its current inconsistent and still elusive nature, we believe it was most prudent not to make any statements about this tuft-4 subtype in this manuscript.

Minor points
1. In the Introduction (lines 60 and 61), the authors mention that multiple studies reported that tuft cells differentiation is both Atoh-1-dependent or Atoh-1-independent and indeed mentioned key papers in which this issue was addressed. They may want to state that the current model supports the notion that tuft cell differentiation is Atoh1-independent in the small intestine while being Atoh1-dependent in the colon (even if the underlying mechanism is unknown).
Response: We thank for the suggestion, and we have incorporated it (lines 60-61).

2. For Figure 1a, I have not found the definition of "tuft-p" in the figure legend. The authors should add this information there.

Response: We apologize for the omission. We have now corrected the figure legend.

Reviewer #3

General: The study reported in the manuscript titled "Mature 1 tuft cell phenotypes are sequentially expressed along the intestinal crypt-villus axis following cytokine-induced tuft cell hyperplasia" by Buissant des Amorie et al. introduces an optimized organoid platform to model tuft cells in vitro, closely mirroring their in vivo counterparts. The authors utilize this system to explore the functional differences between two subpopulations of tuft cells, namely tuft-1 and tuft-2.

First, they characterized tuft cell heterogeneity in mice using single-cell RNA sequencing (scRNA-seq) by sorting Chat-eGFP+ epithelial cells. This analysis revealed three clusters: tuft precursors (tuft-p), which were previously described tuft1 cells, and tuft2 cells. The authors compared a human scRNA-seq dataset with their murine dataset and established that the gene signatures between tuft2 clusters were conserved, while tuft1 cells showed less conservation across species. Additionally, the authors conducted a computational analysis to predict the spatial location of tuft cells along the crypt-villus axis, concluding that tuft-1 cells are associated with crypts, while tuft2 cells are more abundant in the villi.

Following this analysis, the authors developed reporter organoid systems for each tuft cell population and validated their transcriptomic similarity to their in vivo counterparts. They observed that the organoid proportions of tuft1 and tuft2 favored tuft-1 cells compared to their abundance in vivo, suggesting that the organoid media induces a crypt-like environment. To investigate the effect of environmental signals on the proportions of tuft1 and tuft2, the researchers treated organoids with type 2 cytokines, IL-4 and IL-13. They found that this treatment expanded the tuft1 and tuft-p compartments but did not affect tuft-2 cells.

The authors then tracked the temporal expression of tuft-specific markers during IL-4/IL-13 treatment using time-lapse imaging. Their findings indicated that tuft1 cell specification precedes that of tuft2 cells, implying a linear differentiation from tuft1 to tuft2. In an effort to induce tuft2 expansion, the authors designed a villus enrichment media (BMP ligands) to mimic in vivo microenvironmental signals specific to the villi. The use of this villus media resulted in increased tuft2 expansion. Through time-lapse imaging, they demonstrated that Nrep+ tuft1 cells transitioned into Chat-eGFP+ cells, thus claiming tuft1 to tuft2 differentiation under villus media conditions.

Finally, calcium imaging was employed to measure tuft cell responses to organoid stimulation with various predicted tuft cell ligands. The authors showed tuft activation following treatment with succinate or propionate. They also highlighted the differential responsiveness of tuft1 and tuft2 cells to each ligand, emphasizing the importance of studying the unique functions of these tuft populations.

Overall, this study provides significant insight into the use of organoids as a platform for studying tuft subpopulations ex vivo and validates their relevance to in vivo conditions. The engineered organoids, especially concerning the rare tuft cells, will enhance our understanding of gut biology. Notably, this study serves as proof-of-concept for investigating GPCRs in rare cell states, tuft1 and tuft2, by combining calcium influx imaging with tuft cell reporters. However, some discrepancies in the study need to be addressed, and conclusions may extend beyond the data presented. Specifically, there is insufficient evidence to assert that tuft1 cells transition into tuft2 cells. The authors should either provide additional supporting evidence for this conclusion or rephrase it to more accurately reflect their results. Furthermore, the calcium influx results are not convincing enough to claim tuft subset-specific activation, nor do they clarify why other cell types (not tuft) are also activated. Lastly, and most importantly, a recent study (https://www.nature.com/articles/s41586-024-07952-6#Sec3), cited by the authors, showed a local expansion of human tuft cells upon stimulation with IL4/IL13 claiming for self-expansion of tuft cell and could imply for no linear differentiation of progenitors toward tuft cells under these

conditions and second they claim for Wnt dependence for tuft cell differentiation while BMP reduces it – how these findings reconcile with the results shown in this study? Is it human vs mouse?
Response: We thank the reviewer for acknowledging that our work will advance future studies investigating the functioning of tuft subpopulations using ex vivo organoid models. We have addressed the constructive remarks to improve our manuscript and reconciled our results with previous studies. The remarks summarized by the reviewer above are reiterated and elaborated on below. Therefore, we reply to them there.

Comments:
1. The conclusion that type 2 cytokine treatment of organoids does not induce tuft2 expansion is not well established in this study. Organoids were treated with IL4/IL13 three days post-passaging, after the organoids' differentiation had already begun. It would be important to test the tuft1 and tuft2 proportions in organoids following treatment with cytokines immediately after passaging, and whether this induces more tuft2 cells, in agreement with in-vivo studies.
Response: the reviewer highlights an important aspect for improvement, shared by the other reviewers.

Please see our response to comment 2 of reviewer 1 for an elaborate response with figure references. In short, along our current insights, cytokines will increase tuft cell specification in crypts after which newly generated tuft cells will naturally migrate along the crypt-villus axis through the transitory tuft-1 stage to eventually mature into tuft-2 cells under influence of changing crypt-villus signaling gradients. Accordingly, newly generated tuft-1 cells in organoids cultured in crypt-like medium with cytokines will not transition to villus residing tuft-2 cells (Fig. 4abc and Supplementary Fig. 6ab), until the organoid medium is changed to better represent the villus microenvironment (Fig. 5abcde).

<u>Regarding immediate/prolonged cytokine treatment of organoids</u>
We have performed the experiments as suggested, in which we treated organoids with Il-4 and Il-13 directly after passaging (Supplementary Fig. 6ab; manuscript text lines 241-243). Also during this prolonged cytokine exposure (5-7 days, directly after passaging), we did not observe more Chat+ tuft-2 cells than with short cytokine treatment or with normal culture medium. Only when we change the crypt-mimicking medium (ENR) to villus-inspired medium, we observe a significant increase in the numbers of Chat+ tuft-2 cells (Fig. 5abcd).

<u>Regarding the expansion of tuft cell populations in organoids following cytokine treatment</u>
Please also see our response to comment 2 of reviewer 2 with figure references and lines 261-275 of the revised manuscript regarding the proliferative capacity of tuft cells. In short, we never observed mature tuft-1 or tuft-2 cells dividing in organoids treated with IL-4 and IL-13. More specifically, the last division virtually always preceded the tuft-1 stage marked by emerging *Nrep* expression (Supplementary Fig. 7), in line with a minor proliferative population of Dclk1+ tuft-p cells in the crypt (Fig. 1l). Thus, upon IL-4 and IL-13 exposure, the increase in mature, post-mitotic tuft cell numbers is likely fueled by division of Dclk1+ early tuft cell precursors (tuft-p) or increased stem cell differentiation towards tuft-p cells, or a combination of these two.

2. The intermediatory tuft cell state could be explained by a common progenitor for both subsets that have yet to commit to either tuft1 or tuft2 fate. Moreover, the tuft-p cells were shown to express markers of other lineages. Are tuft-p cells indeed committed to the tuft lineage, or are they secretory progenitors? More data should be presented to elucidate this cell population. In addition, are these cells proliferate?
Response: the reviewer makes valid points that we should improve our manuscript regarding the alternative possibility of tuft-1 and tuft-2 cells originating from an intermediary precursor and the characterization of the tuft-p cells, which was also requested by other reviewers.

Regarding the intermediary tuft cells

The reviewer is correct that on first glance these intermediates may also represent a common precursor for tuft 1 and 2. However, we have multiple lines of evidence supporting a linear differentiation trajectory.

First, the live-cell imaging of our knock-in organoids always indicate the transitioning from early tuft-1 (Nrep+) cells to more mature (intermediary) stage which then also expresses *Chat* (Fig. 4de and Fig. 5f). Unlike a shared precursor for both tuft subtypes would predict, we never observed the other way round, i.e. an Nrep+Chat+ tuft cell losing *Chat* expression. Similarly, we did observe Chat+ tuft cells further differentiating to Chat+Folr1+ expression, but never the other way round (Fig. 4de). For prove of sequential expression of tuft-1 and tuft-2 markers *in vivo*, please also see our new RNAscope experiments (Fig. 3hij; discussed in manuscript lines 190-201 and our response to comment 1 of reviewer 1).

Second, if tuft-1 cells (Nrep+Chat-) arose from intermediary tuft cells (Nrep+Chat+) we should have observed this intermediary state at a much higher frequency in organoids treated with Il-4 and Il-13 (Fig. 4bc). In fact, we do observe substantial tuft-1 percentages under those conditions (as much as 25%), but almost no intermediary cells (0.03% Nrep+Chat+). Moreover, we now incorporated new flow cytometry time course experiments to monitor the emergence of tuft subtypes during treatment with our optimized villus-inspired differentiation protocol (Supplementary Fig. 9a). Over multiple consecutive days, we always see a dramatic rise in Nrep+ tuft-1 cells prior co-expression of Chat.

Third, we now added tuft subtype-specific depletion experiments to demonstrate that a transient tuft-1 state is a prerequisite for the emergence of tuft-2 states (manuscript lines 309-317). These experiments rely on the fact that our knock-ins also co-express Diphtheria Toxin Receptor (DTR; Fig. 3k and Supplementary Fig. 4d) along with the fluorescent mScarlet, as a remnant of its original application in a different research topic in our lab. Consequently, exposure of the organoid lines to Diphtheria Toxin (DT) induces robust tuft cell ablation corresponding to the state that is marked by the knock-in. In our new depletion experiments, continuous depletion of the immature Nrep+ tuft-1 state prevents the emergence of mature Chat+ tuft cells, even in the villus-inspired medium (Fig. 5ijk and Supplementary Fig. 9c). On the other hand, depletion of the most mature tuft cell state marked by Folr1 does still allow for the emergence of Chat+ tuft cells (Fig. 5ijk and Supplementary Fig. 9c). Together, these experiments indicate that there is no alternative differentiation trajectory by which mature Chat+ tuft cells can arise without going through a preceding tuft-1 stage marked by *Nrep,* in case of cytokine-induced tuft cell hyperplasia.

Regarding tuft-p cells

Please see our response to comment 4 of reviewer 1 for an explanation of the elaborate new analyses and experiments validating the existence of a tuft-p state in mice, humans and organoids. The reviewer is right to mention that the tuft-p cells express markers of other epithelial lineages (Supplementary Fig. 1d), which could mean that these cells are not fully committed to the tuft cell lineage. On the other hand, tuft-p cells of mice, humans and organoids do express well-known general tuft cell markers (Fig. 1c, Fig. 2c and Supplementary Fig. 5d: *Dclk1, Trpm5, Avil*) including the transcription factor that is referred to as master regulator of tuft cell fate *Pou2f3.* Moreover, in our live imaging data of cytokine-treated organoids, dividing tuft-p precursor cells (identified as *CD24+* shortly before division) give rise exclusively to pairs of tuft cell sisters, suggesting that lineage commitment is already present at the tuft-p stage under those conditions (Supplementary Fig. 7d).

Future experiments are warranted to explore how restricted their lineage commitment truly is, particularly concerning the raising number of reports documenting that fate plasticity is common in health and disease. However, we deem such explorations beyond the scope of the current

manuscript where we try to emphasize on the nature of post-mitotic and mature tuft cell stages involved with chemosensory and immunological tuft cell function.

3. The authors utilized differentiation media and showed BMP2+4 induces tuft cells; however, as mentioned above, a recent study (https://www.nature.com/articles/s41586-024-07952-6#Sec3) showed higher tuft cell differentiation upon wnt stimulation and a massive reduction of tuft cells when adding BMP2/4. Could the authors add Wnt/Rspo stimulation? Is the difference due to human vs mouse?
Response: Regarding the proposed experiment, we have added Wnt to our culture conditions and noticed no difference in terms of Nrep+ cells upon induction with type 2 cytokines (Supplementary Fig. 6ab). The difference with the results found in the paper mentioned by the reviewer could be related to a human vs mouse organoid difference. Unlike mouse organoids, where stem cell Wnt exposure follows natural production by Paneth cells in the organoids, human organoids require exogenous addition of WNT stimuli, and the integrity of the epithelial structure cannot be maintained without. Aberrant WNT levels could possibly have an effect on early tuft cell specification, which in the case of our mouse organoids was not a limiting factor due to the Paneth cell-derived WNT.

With respect to the effects of BMPs, reduction in tuft cell numbers upon BMP treatment is also mentioned in Lindholm *et al.*, Science Immunology 2022 that we already mentioned in the manuscript text (discussion lines 406-409). The timing of BMP exposure is important, since artificial BMP stimulation perturbs stem cell functioning including, differentiation into tuft cells. Physiologically, however, BMP signaling activity is found at the villus while being repressed at crypt bottoms where stem cells reside (e.g. by Gremlin *in vivo* and Noggin in the organoid medium). It is for this reason that we start our tuft cell differentiation regimen illustrated in Fig. 5a with a pretreatment in ENR with cytokines (day 3) to initiate tuft cell specification in the absence of BMP, prior to switching to the villus-mimicking medium (day 4) with BMP that facilitates maturation of tuft lineage-committed tuft-1 cells to mature to tuft-2 cells.

In sum, the results from our experiments and the previous publications indicate that the effects of BMPs depend on the cellular state of the cells receiving these signals. Stem cells and early progenitors may respond with decreased specification towards the tuft cell lineage (published), while post-mitotic cells committed to the tuft cell lineage will further differentiation to more mature tuft cell states (our data).

4. In the same context, could it be that BMP2+4 depletes stem cells and, as a consequence, other differentiated cells (See Fig.4g), elevating the tuft cell percentages? Absolute quantification of different tuft cell populations should be included in the FACS analysis in Fig. 4c.
Response: The reviewer makes a valid point about relative cell type frequencies being dependent on all cell types represented in a sample and not just the cell type of interest.

We have now added the absolute cell numbers featured in the plots of the FACS measurements in their respective figure legends (Fig. 4b, Fig. 5c and Supplementary Fig. 9ac) and included microscopy images to illustrate the increase in Chat+ tuft cells upon cytokines stimulation and villus-inspired medium differentiation (Fig. 5e).

Having said that, the point mentioned by the reviewer is probably true. Upon cellular differentiation in villus medium, we have less stem and progenitor cells skewing overall bias in relative cell numbers. However, perhaps the most important aspect of our advanced differentiation protocol is of practical origin. There are hardly any Chat+ tuft cells in regular organoid cultures, making it almost impossible to study them (not to mention the fact that they are still not the most mature tuft-2 cells). While type 2 cytokine stimulation generates a massive amount of tuft cells, these remain stalled at a tuft-1 stage that is unrelated to critical immune-related functioning of tuft cells. Combining cytokine-induced generation of tuft cells with villus-

inspired medium advanced the differentiation of significant numbers of tuft cells towards immune-relevant phenotypes, enabling functional interrogation of how various signals are processed by tuft cells to ignite an immune response.

5. The conclusions of the authors regarding Figure 4 are contradictory. On the one hand, they claim that type 2 cytokines induce expansion of tuft1 or intermediate tuft cells, but not of tuft2 cells. On the other hand, they claim to observe tuft1 cells transition into tuft2 cells and not in the opposite direction. There is no quantification of how many cells were counted to transition between tuft1 and tuft2. In addition, a longer time point should be conducted after the treatment to show tuft 1 differentiation to tuft 2 using the different reporter organoids. lastly, to provide genetic evidence supporting their claims, the author should consider a fate-mapping experiment or rephrase it to more accurately reflect their results.
Response: we apologize for the confusion in our original manuscript regarding the effect of type 2 cytokines on tuft cell differentiation. We have changed the subheadings in our manuscript (lines 233-234 and 276) to better convey our message.

Our phrasing regarding the effect of type 2 cytokines on tuft cell differentiation was also mentioned by reviewer 1 to be confusing. Please see our response to comment 2 of reviewer 1 for an elaborate response with figure references. In short, along our current insights, cytokines will increase tuft cell specification in crypts after which newly generated tuft cells will naturally migrate along the crypt-villus axis through the transitory tuft-1 stage to eventually mature into tuft-2 cells under influence of changing crypt-villus signaling gradients. Based on our new RNAscope experiments (explained in response to comment 1 of reviewer 1) *Nrep* is the earlies of our tuft subtype-specific markers and is expressed in the crypt. *Gng13* starts to be expressed in the crypt and remains expressed throughout the tuft cell's lifetime and journey along the crypt-villus axis. *Chat*, as early tuft-2 marker, is only expressed once the tuft cell reaches the higher part of the crypt (also see our new immunofluorescence experiments explained in our response to comment 3 of reviewer 1) and *Folr1* (late tuft-2) expression follows after that.

Accordingly, in organoids, newly generated tuft-1 cells in crypt-like medium with cytokines will not transition to villus residing tuft-2 cells (Fig. 4abc and Supplementary Fig. 6ab), until the organoid medium is changed to better represent the villus microenvironment (Fig. 5abcde). As mentioned in our response to the first comment of this reviewer, longer cytokine exposure in crypt-like medium did not result in an increase in tuft-2 cells and only yielded stalled Nrep+ tuft-1 cells (Supplementary Fig. 6ab), further demonstrating the importance of crypt-villus signaling gradients in the maturation of tuft cells to villus-residing tuft-2 phenotypes.

While we observe very low numbers of Chat+ tuft-2 cells in organoids treated with cytokines in the presence of the crypt factors (EGF, Noggin and R-spondin), we occasionally observed such cells emerging in the organoid villus region during our live imaging experiments (Fig. 4def). These experiments also showed that when these chat+ tuft-2 cells emerged, chat expression was preceded by tuft-1 marker expression (*Nrep* and *Gng13*) and followed by late tuft-2 marker (*Folr1*) expression. The quantification of these transitioning cells, requested by the reviewer, can be found in Fig. 4e and is referred to in the text (lines 252-255). To further illustrate the relative timing of tuft-1 and tuft-2 marker gene expression during tuft cell differentiation, we now incorporated new flow cytometry time course data that shows the number of Nrep+ cells to increase before Nrep+Chat+ cells arise (Supplementary Fig. 9a) during our optimized differentiation regimen (Fig. 5a). Regarding the cellular source of the Nrep+ tuft-1 cells, we kindly refer to our response to comment 2 of reviewer 2.

Lastly, we agree with the reviewer regarding the power of genetic lineage tracing experiments. As mentioned in one of our earlier responses to this reviewer, we now include tuft cell subtype ablation experiments as an alternative to lineage tracing experiments to demonstrate that Chat+ tuft-2 cells cannot emerge without transitioning through a Nrep+ tuft-1 stage (please see our

response to comment 2 of reviewer 3 for a more elaborate explanation) (Fig. 5ijk and Supplementary Fig. 9c). In contrast, depletion of the most mature tuft cell state marked by Folr1 does still allow for the emergence of Chat+ tuft cells. Together, these experiments indicate that all Chat+ cells were preceded by an Nrep+ state, but not by a Folr1+ state, further supporting a unidirectional sequential mode of expression (Nrep->Chat->Folr1).

6. The recent paper by Clevers' group showed local proliferation of tuft cells in the IL4/13 treatment. Could the authors comment on the proliferation state of the different tuft cell subsets? This could be measured by both IFA and looking at the single cell data. The authors should address this study in order to explain the differences between the two studies.
Response: We apologize for the confusion regarding the apparent mismatches between our analyses and the recent publication, a point which was also brought up by reviewer 2 (comment 4). Below we explain similarities between the tuft cell subtypes identified by our and their study and comment on the proliferative capacity of the different subtypes.

Regarding the tuft cell subtypes
We reanalyzed the scRNA-seq data presented in our manuscript and all datasets now consistently document the presence of three tuft cell subtypes, including a proliferative tuft precursor state (tuft-p) preceding tuft-1 and 2 states, which we validated experimentally (see below: regarding proliferative capacity of tuft cell subtypes). Importantly, the three subtypes are observed in *in vivo* datasets of both mouse (Fig. 1ac and Supplementary Fig. 1cde) and human intestine (Fig. 2bc and Supplementary Fig. 2ab), corroborating our organoid data (Fig. 3n and Supplementary Fig. 5bd).

Within the nomenclature of the 4 tuft cell subtypes identified in human organoids of the mentioned paper from the Clevers group, our tuft-p shares a similar transcriptome with their cycling tuft-3 (e.g. Mki67, Pcna, Top2A). In the revised version of our manuscript, we now clarify the similarity between the tuft-3 cluster in the Nature paper and our tuft-p cells in lines 152 and 387-390. In addition, the tuft-1 and 2 subtypes described in our manuscript are similar between mouse and human (Fig. 1ckm, Fig. 2cdefg and Supplementary Fig. 1e and 2ab) and are also found, and referred to with the same names, by the authors of the mentioned paper. In the revised version of our manuscript, we now mention the similarity between the tuft-1 and tuft-2 clusters found in both papers in lines 151-152.

The tuft-4 (regenerative) state described by the aforementioned paper was not found in any of our scRNA-seq analyses and the mentioned marker genes (PD-L1 (*CD274*), *EREG*, *HB-EGF* and *TACSTD2*) of this tuft subtype were lowly or not expressed in the tuft cells of the datasets we analyzed. Indeed, in the paper from Clevers and colleagues the tuft-4 subtype was mentioned to be absent in mice, mostly involved with regeneration and predominantly found in organoids only. Due to its current inconsistent and still elusive nature, we believe it was most prudent not to make any statements about this tuft-4 subtype in this manuscript.

Regarding proliferative capacity of tuft cell subtypes
As mentioned above, we have now incorporated consistent and more thorough analyses on the scRNA-seq datasets presented throughout the manuscript, which now all document similar presence of a proliferative tuft precursor state (tuft-p). We validated the proliferative capacity and predicted location (Fig. 1k) of tuft-p cells in mice by assessing (recent) proliferative potential of Dclk1+ tuft cells through EdU incorporation following a 4-hour pulse (Fig. 1l and Supplementary Fig. 1h). In these experiments we detected a minor EdU+ fraction among the Dclk1+ tuft cell population in the crypt, which was predominantly Chat negative.

Furthermore, we have added extensive live-cell imaging experiments that show that the last cell division during differentiation to a mature post-mitotic tuft cell virtually always precedes the emergence of *Nrep* expression (lines 261-275; Supplementary Fig. 7). As such, *Nrep* reports the earliest stage (i.e. tuft-1) among post-mitotic tuft cells. Concordantly, we never observed cell

divisions of more mature Gng13+, Chat+ or Folr1+ tuft cells, which are found higher on the crypt-villus axis (response to comment 1 of reviewer 1; Fig. 3hij), during all our organoid live imaging experiments. Moreover, the tuft-1 and tuft-2 clusters of our scRNA-seq analyses are post-mitotic based on cell cycle phase predictions (Supplementary Fig. 1e (mouse); Supplementary Fig. 1e (human)) and low expression of proliferation markers (Fig. 1c (mouse); Fig. 2c (human)).

Together, these lines of evidence illustrate the existence of a minor subpopulation of crypt-residing tuft cells that is proliferative, is conserved between mouse and human, and represents a progenitor state that precedes post-mitotic tuft-1 and 2 states.

7. The calcium influx assay shows no specificity to tuft 1 cells in Fig. 6i and in Fig. 6l, I can't see the expression of the calcium reporter in tuft cells in the image, only in surrounding cells. Looking at the intensity plot in Fig. 6n, it seems that the activation of the FFAR3 is higher in other cells. What is the explanation for the non-specificity in these assays?
Response: The Tq-Ca-FLITS reporter used in Fig. 6 is an intensity-based calcium reporter, which we stably integrated into our organoid lines by lentiviral transduction (Methods section of manuscript). As such, all cells express the sensor, but fluorescence intensity of the reporter will increase in case of high local calcium concentration. This is what is depicted by the color scale in Fig. 6im. While the reviewer is right about the sensor being highly activated in cells surrounding the tuft cells, the sensor is also clearly activated in the Nrep and Chat positive tuft cells as illustrated by the single-cell traces in Fig. 6j and Fig. 6n, respectively. These traces also show the sensor to be first activated in the tuft cells, followed by neighboring cells close to a tuft cell (Fig. 6o). We mention the response of the non-tuft cells in the manuscript text (lines 359-361) and refer to a recent paper finding acetylcholine-dependent paracrine spread of calcium pulses across tracheal monolayers following tuft cell stimulation, as a possible explanation for this (lines 361-362). Moreover, while the non-tuft cells in the example in Fig. 6mn respond with a stronger pulse than the Chat+ tuft cell, we mention the calcium spikes in response to stimuli to be overall similar in strength between the tuft and non-tuft cells (lines 361-362; Supplementary Fig. 12b).

In sum, we do not claim the calcium response to SUCNR1/FFAR3 activation to be tuft cell-specific in the manuscript, but do mention the tuft cells to be the first to respond and hypothesize that the concomitant calcium pulses in non-tuft cells are a result of secondary signals released from the stimulated tuft cells after ligand binding to the tuft-specific SUCNR1 or FFAR3 receptors. These we think are very interesting avenues to investigate further, but are in our opinion beyond the scope of our manuscript which aims to unlock organoid potential in cell biological research into tuft cell functioning.

Minor comments:

1. Figure 3C shows an increase in Chat GFP+ cells under IL4/13 treatment, contrary to text description and conclusion.
Response: Indeed, while there is an increase, this is minor (from 0.1% to 0.5% of the live cell population) and not significant (t-test P = 0.054). Moreover, the increase is far lower than the number of Dclk1+ tuft cells upon IL4/13 treatment in organoids (Fig. 3d). In lines 178-183 of the revised manuscript we describe the increase in GFP+ cells upon cytokine treatment to be minor and much smaller than that of Dclk1+ cells.

2. Chat-GFP is used interchangeably as a general tuft cell marker, or as a tuft2 marker. The authors should be careful to use this marker in a consistent manner, to draw correct conclusions. For example, in the live imaging experiment fig. 4c and fig. 5d they use Chat as a marker for tuft2, however in flow cytometry, they use chat GFP+ cells as tuft cells consisting of all tuft subpopulations. In addition, in figure 5c most of the Flor1+ cells which are considered tuft2 are GFP negative.

Response: This criticism is well taken. We have now extended the analysis of *Chat* expression in relation to the other marker genes including *Dclk1*, to provide clarity about the fact that *Chat* expression comes up much later than pan-tuft cell markers, like *Dclk1*, during tuft cell differentiation (Fig. 1hi and Fig. 3hij). Please see our response to comment 3 of reviewer 1 for an explanation of newly included data and analyses to address this point.

Regarding the Folr1+ cells that are GFP negative, we mentioned in the text of our original manuscript that single Folr1+ cells were not always identified to be tuft cells by scRNA-seq (lines 226-230 of the revised manuscript). This can be seen in Fig. 3q and we have now added Supplementary Fig. 5g to further illustrate this. Despite *Folr1* expression being less specific to tuft cells than we had anticipated based on scRNA-seq of the mouse small intestinal epithelium (Supplementary Fig. 4b), it can be used to distinguish the most mature tuft-2 cells within the Chat+ tuft cell population (Fig. 3o and Supplementary Fig. 5g). For this reason we also included a quantification of the Chat+Folr1+ cells in Fig. 5d.

3. Figure 5b-c – missing a quantification of PE+ cells percentage under crypt IL4+13 media vs. villus IL4+13 media
Response: Our apologies. We have now included quantification in Fig. 5d.

4. Figure 5c shows an increase of Nrep+ tuft1 cells in villus media, in addition to tuft2 cells. In general, according to the FACS analysis there seems to be an increase in all tuft cell populations.
Response: We have now included quantification in Fig. 5d. Moreover, comparing Nrep flow density on the FACS plot (Fig. 5c) in crypt versus villus medium, we see a downward shift indicating that the Nrep cells lose fluorescence (also in Fig. 5fg). The opposite happens for Gng13 expression (Fig. 5c), in line with advanced differentiation to more mature tuft-2 states (Fig. 3q).

Response to reviewers

We thank the reviewers for evaluating our revised manuscript and are happy to hear that they are satisfied with the experimental additions and overall improvements. Please find below our response to the final request by reviewer 1.

Reviewer #1 (Remarks to the Author):

The authors have greatly improved their manuscript. The model of progressive maturation and differentiation of tuft cells, in response to signals from the stroma, will clearly help to clarify all the published data regarding the existence of tuft cell subpopulations, and will provide valuable information to the community interested in these cells.

There is one final point that I believe needs further clarification before publication. Could the authors explain the results of the lineage ablation experiments, via the DTR system, in the organoid cultures? The results are somewhat counter-intuitive, and some explanation would be welcome for the data showed in Figures 5 and Supplementary Figure 9. As an example, in the main figure 5J, I would have expected Folr1-mediated lineage ablation (thus, tuft 2 state-related) to induce a disappearance of tuft 2 cells, but not tuft 1. The data shown seem to suggest a total loss of lineage. Can the authors explain these data?

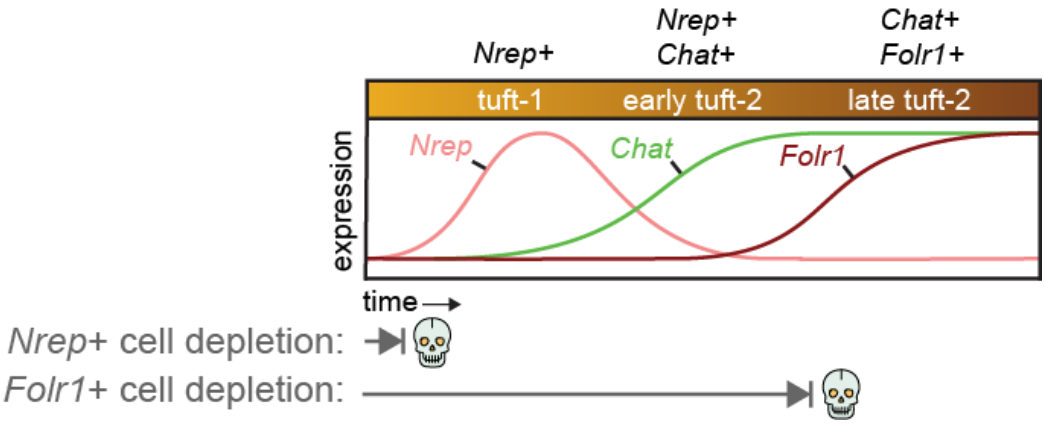
We acknowledge that the description of our lineage ablation experiment may have appeared counter intuitive. To avoid confusion among future readers of our manuscript, we now improved the explanation of the results. Foremost, the confusion relates to the successive onset and timing of expression of the various tuft subtype marker genes. This dynamic and successive expression within single cells undergoing tuft cell differentiation, enables not only to discriminate between tuft-1 (Nrep+) and tuft-2 cells (Chat+), but also subdivides tuft-2 cells into early and late stages based on gained expression of Folr1 in late tuft-2 cells.

In the text (lines 314-322), we now specify per tuft subtype the exact marker gene composition that in turn are utilized to trigger cell ablation:

"To evaluate if the transient tuft-1 state is mandatory or facultative during tuft cell differentiation we used diphtheria toxin (DT) mediated subtype ablation via the DT receptor that was simultaneously knocked-in with the mScarlet reporters (Fig. 3k, Supplementary Fig. 4d). Depletion of (*Nrep*+) tuft-1 cells with DT during our optimized differentiation regimen (Fig. 5i-j), blocked the generation of all tuft-2 cells (*Chat*^{BAC}-eGFP+) (Fig. 5k, Supplementary Fig. 9c-d). In contrast, under continuous depletion of late tuft-2 phenotypes (*Chat*^{BAC}-eGFP+ and Folr1+), early tuft-2 cells (*Chat*^{BAC}-eGFP+, but Folr1-) did arise (Fig. 5j-k, Supplementary Fig. 9c). These ablation experiments demonstrate that *Chat*+ tuft-2 cells cannot arise from an alternative cellular source other than *Nrep*+ tuft-1 cells, in the situation of cytokine-induced tuft cell hyperplasia."

In addition, we designed a schematic (Supplementary Fig. 9d, also reproduced below) to improve clarity on the successive timing of the marker gene expressions, based on the

RNAscope (Fig. 3i-j), live-imaging (Fig. 4d-f) and flow cytometry time course data (Supplementary Fig. 9a). This schematic should facilitate interpretation of the cell-type ablation results.



Reviewer #2 (Remarks to the Author):

The authors are now providing an improved version of their study in which all my comments/concerns were properly addressed. We now have a very elegant story that brings some new insights into mechanisms underlying tuft cell differentiation. Congratulations to all authors....

Reviewer #3 (Remarks to the Author):

The authors performed a substantial revision of the text and figures, providing new evidence to their claims, which strengthens the conclusions of the manuscript. Overall, this study provides significant insight into the use of organoids as a platform for studying tuft subpopulations ex vivo, with the potential to investigate their biological relevance.
The authors answered my comments in a satisfactory manner, and I do not have further comments.