THE EMBO JOURNAL

Interferon γ selectively promotes survival of alveolar progenitor cells in a human lung organoid model

Antonella Dost, Katarína Balážová, Carla Pou Casellas, Lisanne van Rooijen, Wisse Epskamp, Gijs van Son, Willine van de Wetering, Carmen Lopez-Iglesias, Harry Begthel, Peter Peters, Niels Smakman, Johan van Es, and Hans Clevers

Corresponding authors: Hans Clevers (h.clevers@hubrecht.eu) , Antonella Dost (a.dost@hubrecht.eu)

Review Timeline:

Submission Date:	2nd Dec 25
Editorial Decision:	4th Dec 25
Revision Received:	21st Jan 26
Editorial Decision:	24th Feb 26
Revision Received:	11th Mar 26
Accepted:	26th Mar 26

Editor: Daniel Klimmeck

Transaction Report:

This manuscript was previously reviewed at another journal. As EMBO Press has a transfer agreement (including the identities of the referees) with that journal, revision was invited based on the reports from that previous external submission.

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Reviewer #1 (Remarks to the Author):

This manuscript described a modified protocol of generating and maintaining alveolar organoids from isolated epithelial cells derived from primary human lobectomy material. The authors use this model (that was improved with regard to a differentiation step (LATS-i) and with regard to prior expansion in ALVO-EM) to demonstrate a role of recombinant IFNy on organoid cell population behaviour, and extrapolate a role of IFNy in human COPD. While some aspects of the presented data are interesting, I have concerns regarding data interpretation and conclusions drawn.

The authors use lobectomy material derived from lung cancer surgeries. No information is provided as to patient history regarding age, gender, type of lung cancer, smoking history, COPD stage, or steroid medication history, which might impact the composition and cellular responses of the organoids, particularly in light of the findings on IFNy and a putative role in COPD.
Given that the authors identify quite some heterogeneity in the different cultures (e.g., Fig 1C, S1H), and that such criteria might significantly affect alveolar stem cell behaviour, such information should be provided to better interpret the results.
On a minor note, might the inflammatory tumor microenvironment/signature within the lobectomy material additionally impact the cells of origin used for organoids (how distant from tumors was the tissue used for preps)?
For ethical and practical reasons, we work with surgical “rest material” that is fully anonymized, so patient-specific data (age, sex, smoking history, etc.) are unavailable. However, we do know that none of the patients were diagnosed with COPD or other lung diseases beyond lung cancer, for which the surgery was performed. Furthermore, they did not receive any form of cancer-therapy before the surgery. We wil add this information to the methods section.
Regarding heterogeneity: variation between human donors is expected and intrinsic to primary organoid systems. It is not possible to get tissue from a “normal, healthy” lung. Material either comes from lungs that were rejected for transplantation, or from lobectomies, like in our case. We consistently use at least three biological replicates to ensure reproducibility of observed responses. We also choose to show donor identifiers for full transparency. The distal lung tissue that we receive is at least 5cm from the tumor and we will add this information to the methods section.

The data on establishment of the new protocol seem to lack consistency, and clarity regarding information on experimental setup, which makes interpretation difficult, and it remains unclear what message the authors want to convey about their new protocol.
We tried to be as clear and transparent with the protocol as possible. We will make some clarifications regarding which media was used to establish the organoid lines, and add information regarding passage numbers, as suggested by other reviewers. Current approaches depend on HTII-280, a widely used AT2-specific membrane antigen whose corresponding gene remains unknown. It is known that HTII-280-negative cells can generate SFTPC+ organoids (Basil, Nature, 2022; figure 3) and that not all AT2 cells stain positive for HTII-280 (PMID: 32272001). This suggests that progenitors capable of forming AT2-like cells are excluded when HTII-280-based sorting is used. We therefore see a conceptual advantage in our more inclusive approach. Morevoer, we achieve long-term expansion of AT2 progenitor cells AND secretory AT2 progenitor cells, which has not been demonstrated before. Another novel aspect of our system is that the sole variable between our progenitor expansion media and our differentiation media is LATS-inhibitor, making precise mechanistic studies possible. We have extensive characterization of our organoids, including scRNA-Seq,

which is not the case for existing protocols (Katsura, CSC, 2020, Salahudeen, CSC, 2020). Together, we think that our system presents an advancement to currently available protocols.

For example, Fig 1C right panel lacks Lu44 that was referred to before to be the most long-lasting line (21 passages). Fig 1C and 1F again show different donors. Figure 1H shows data from donor 37, 53 and 54, and Figure 1I only from 53 and 54.
Due to practical reasons, it is not feasible to use the exact same three donors for every experiment. We view the diversity of donors as a strength rather than a weakness, and we are fully transparent which lines were used for which experiment. We did not continue using Lu44 because it represented an outlyer when it came to long-term expansion, and we chose to continue with more representative lines.

The scRNASeq experiments (from donors 37-39) lack information about passage numbers and how long these organoids have been in culture. Can the authors explain why there is such low amount of proliferating cells, within the cells with progenitor phenotype that should reveal higher proliferation rates compared to AT2 cells ("secAT2", C6+; "RAS-like")? The scRNA-Seq was done at passage 5 with each passage being 14 days apart; we will add this information to the manuscript. We would like to emphasize that our goal is not to model the alveolus at homeostasis, where epithelial cells are quiescent, but rather a regenerative state. This is why organoid-derived cells form the majority of proliferating cells when integrated with lung atlas data (Figure 4A). Under regenerative conditions, we do not expect many mature, and therefore quiescent, AT2 cells in expansion media. As Reviewer 2 pointed out, the field increasingly recognizes that alveolar progenitors are heterogeneous. Our organoid conditions promote stemness and proliferation, enriching two AT2-like progenitor subpopulations, one of which resembles the secretory alveolar phenotype described in recent Nature papers (Murthy, Nature, 2022 and Basil, Nature, 2022). These "secAT2" cells share features with AT0, TRB-SC, and AT2-3A2 populations, cells thought to lie along a continuum between secretory and alveolar lineages.

In Figure 2D, there are organoids that are HT2-280 negative after AT2-MM culture, but Nile Red positive. What are these organoids, and can the authors comment on the heterogeneity of their cultures? To answer this question, we will do FACS sorting on nile red high / low populations in AT2-MM with subsequent gene expression analysis. Regarding HTII-280, it has been noted that "HTII-280 is a less useful marker for subculture of AT2 cells due to loss of expression during culture." (PMID: 32272001). This is why we show maturation with functional dyes such as lysotracker and nile red instead.

It is difficult to assess the ultimate advantage of the presented over previously published models (e.g., PMID: 33128895), also in light of the results of Fig 3F and 3I indicating separation between C6+ and C6- cells seems dispensable. – Please refer to page lines 37+ for the advantage of our system to the published protocols. Moreover, figure 3F and 3I show that both AT2 and secAT2 populations can give rise to AT1-like cells, but that does not make their separation dispensable.

Also, it remains open how well the ALVOs are suited to model the alveolus containing AT1 cells. Although AT1 markers are increasingly expressed (particularly in AT1/AT2-M, no matter if used directly or after ALVO-EM expansion), there is little contribution from organoids compared to tissue to the AT1 pool when looking at identity contribution (Fig 4E). Given that only very few AT1 cells are contained in the organoids, the abstract statement that "Unlike previous models, this system supports long-term expansion of newly identified human-specific alveolar progenitor cells and serum-free differentiation into alveolar type 1 (AT1)

cells." seems not to be supported by data. – The cells in our organoids have high expression of AT1 markers such as AGER on protein level, which is generally accepted by the lung field to indicate AT1 identity. We have acknowledged and discussed the low contribution of fully differentiated AT1 cells in the scRNA-Seq data in the discussion as follows: *"However, the majority of AT1-like cells have a gene expression profile resembling the AT2-to-AT1 transitional state rather than that of fully differentiated AT1 cells. It is possible that organoid-derived differentiated AT1 cells are underrepresented in the scRNA-Seq dataset due to technical reasons. The typical shape of AT1 cells makes this cell type especially sensitive to dissociation methods necessary to achieve single-cell suspensions. A biological explanation for the underrepresentation is that additional signals or prolonged culture periods might be required for terminal AT1 differentiation. Nevertheless, we posit that the presence of transitional states in our organoids better reflects regenerative conditions and provides a valuable platform for optimizing AT1 differentiation in therapeutic contexts."*

The culture type-specific effects of IFNy are an interesting finding (ALVO-EM vs AT1/2 medium). The conclusion that in particular, AT1-like cells are "negatively" affected would benefit from a more detailed analysis (i.e. type/pathways of cell death induced by the cytokine, etc). Are the IFNy effects observed on "AT1-like" cells, or trans_AT2-AT1 cells (Fig 4), similarly observed in truly differentiated AT1 cells? –We clearly demonstrate a reduction in cell numbers (5A), an increase in cytotoxicity (5B), and an upregulation of genes associated with cell death (5H). We also demonstrate reduced AT1 marker expression (5C), indicating a selective loss of AT1 marker-expressing cells. To further address this question, we will perform a more detailed analysis of the cells that survive after IFN-gamma exposure, including stainings of the organoids. The focus of this publication is not to achieve terminal AT1 differentiation in vivo but to model alveolar regeneration.

In light of the different actions of IFNy on secAT2/AT2 vs "AT1-like" cells, what is the expected "net outcome" of IFNy signaling in COPD according to the authors data, and how can this be interpreted with regard to COPD (emphysema) lung phenotype, given that such lungs do not contain more or more proliferating AT2/progenitor cells? – There is in fact an accumulation of secAT2s in COPD and smoker lungs (Basil et al, Nature), and we do not know the reason for this yet. However, the inflammatory landscape of COPD lungs is too complex to make a prediction regarding a net outcome of the effect of IFN-gamma.
How much "COPD phenotype" is imprinted in the organoids (given that likely, at least some of the donors were smokers/COPD patients, see above)? – As mentioned above, none of the donors had a lung disease diagnosis apart from lung cancer. The question of an imprinted smoking or COPD phenotype – even after months in organoid culture conditions – is very interesting but beyond the scope of this manuscript.

On a minor note, why do the authors use PBMC-derived macrophage-like cells for co-cultures, given that T cells are major sources of IFNy? – This is for practical reasons: T cells need to be patient matched, which is not the case for macrophages. While T cells are a major source for IFN-y, macrophages also secrete the cytokine.

All figure legends should contain information on n numbers, number of independent experiments, p values and statistics applied. - We will add this information to the figure legends.

Other:
• Fig 1A: which protocol ("published methods") do the authors exactly refer to? – The protocols corresponding to ref 20 Katsura et al., 2020, and 21 Youk et al., 2020. We will add this information to the

figure.

• P4: what do the authors mean by “appeared healthier”? (line 114). – the organoids appear bigger and with less shedding cells. We will clarify this in the manuscript.

• How were the stats calculated in Fig 3I and 5G (for datapoints normalized to 1)? – this is described in the methods: “For comparisons between two groups, an unpaired t-test was used, or a paired t-test if the data were normalized to the control group. For comparisons involving multiple groups against a control group, or among multiple groups, a two-way ANOVA followed by Dunnett’s post-hoc test was employed. When data were normalized to the control group, a matched two-way ANOVA with Dunnett’s post-hoc test was performed on log-transformed values to account for baseline differences”

• Fig S5F does not show cell death (line 348) – These images show excessive shedding of cells and cell debris from the organoids, which is an indication of dying cells. We will rephrase this in the manuscript.

Reviewer #2 (Remarks to the Author):

In this manuscript entitled “Interferon-γ selectively promotes survival of alveolar progenitor 1 cells in a human 2 organoid model” the authors describe the development of a novel approach to adult human alveolar epithelial culture modelling with particular emphasis on an inclusive approach to cell isolation to ensure the capture of all potential alveolar progenitors. They then define media components required for AT2 maturation and AT2-AT1 differentiation from their mixed population. Having undertaken detailed characterisation, they then use this model to identify the differing role of interferon-γ on the proliferation and survival of different alveolar epithelial population, finding that interferon-γ is cytotoxic to AT1 cells but promotes survival of AT2 cells.

The manuscript is well written and easy to follow. The figures are carefully prepared and they present their data clearly with appropriate analysis. Their transparency in presenting negative and variable data is appreciated.

Their novel organoid model is potentially of great interest to the field. By taking a more unbiased approach to sorting lung epithelial cells from fresh adult lung tissue than current protocols, they find different AT2 subtypes (AT2, secretory AT2) and then push them toward different fates (AT2-like or AT1-like) by manipulation of media components. As we increasingly recognise that alveolar progenitors are not simply one entity, the ability to generate a manipulable model containing several AT2- and AT1-like subtypes is of real significance both in disease modelling and understanding AT2 transdifferentiation in health and disease.

To convince the reader of their conclusions about the true originality of the model and the ability to use it for the disease modelling described in the second part of the manuscript, I would suggest the following:

Major comments

1. The authors state in the introduction that using HTII-280 sorting strategies may exclude important alveolar progenitors; indeed, they then use this argument for creating a novel culture strategy which does not rely on this poorly characterised transmembrane protein. Though I agree with their concerns about HTII-280, the absence of any data to support their statement is a concern. I would suggest:

• They should provide evidence that HTII-280 is absent from non-standard AT2 cells (e.g. by citing existing scRNAseq datasets) to support the logic of their experimental approach.
Because the corresponding gene to HT2-280 is not known, it cannot be looked up in scRNA-Seq data. However, it is known that HTII-280-negative cells can generate AT2 organoids (Basil, Nature, 2022, figure 3). This suggests that progenitors capable of forming AT2 cells are excluded when HTII-280-based sorting is used. Furthermore, it has been observed that "(…) although the majority of AT2 cells express HTII-280, HTII-280− AT2 cells are observed even in healthy human lungs" (PMID: 32272001).

• They should show whether HTII-280-based AT2 isolation generates a similarly heterogeneous AT2-like population, and whether the same manipulations of the culture media (i.e. use of AT2-MM / AT1/2-M) allow maturation of AT2 and AT1-like cells from HTII-280-isolated organoids. This will likely highlight the key advantages of this new model.
- To satisfy multiple reviewer requests, we will sort p0 organoids at day 14 for EPCAM+/NGFR-/HT2-280+ and EPCAM+/NGFR-/HT2-280- populations and plate them in media containing either HRG1 or EGF. This experiment will tell us whether the observed alveolar progenitor heterogeneity comes from the sorting strategy and therefore from the starting cell populations, or whether it comes from the media conditions.

2. Figures 5 and 6 describe the differential response of AT2-like and AT1-like cells to interferon-γ. By necessity, the culture media for each cell type is different with AT1-like cells maintained in LATS-i. Bearing in mind the crosstalk between LATS and IFN pathways, how are the authors sure that the observed effects on cell proliferation and survival due to cell type and not the effect of LATS-i? – We have experiments ongoing to address this question. We will differentiate the organoids first, withdraw LATS-i, then add IFN to see if you still see increased cytotoxicity.

The following would be reassuring:
• To demonstrate that LATS-i does itself not affect cell proliferation and survival.
- LATS-i in fact increases the proliferation rate of our organoids, data we can add to the manuscript. Additionally, in figure 5F we compare the IFN-treated cultures to their own controls and therefore normalize for difference in cell growth between the ALVO-EM and AT1/2-M conditions.
• To check that the combination of LATS-i and interferon-γ does not affect the phenotype of the AT1-like cells grown in AT1/2-M.
- We will add better characterization (RNA and stainings) of the cells that survive long-term IFN-g exposure in the AT1/2-condition apart from the RNA analysis we did (figure 5C). However, our cytotoxicity assays are conducted in already grown and differentiated cultures that were exposed to IFN-g for only 3 days (5B) and we do not expect drastic changes in cell identity in this time frame.

Other comments

1. The introduction opens with a brief discussion of COPD and the need for better models of the effect of inflammatory insults in the lung. While I agree, this really isn't the focus of the manuscript which has far broader reach in modelling the alveolar epithelium in health and disease. Indeed, I would suggest the models they describe would be more immediately relevant to modelling diseases of the alveolus e.g. ILD. To this end, I would suggest the introduction (and discussion) are reworded to downplay the emphasis on COPD.
- We agree with this comment and will rephrase the introduction.

2. Fig S1E&F: AGER is used in S1E then CAV1 in S1F as AT1 markers. Subsequently both are used. For transparency and continuity, I would suggest including both in Fig S1.
- We will add this data.

3. Fig 2B/Fig S2A&B: There is an assumption here that higher lysotracker = more AT2-like. It would be nice to have a) freshly isolated AT2 cells (e.g by HTII-280 sorting) as a control to define physiological levels of lysotracker, and b) a control line which does not have LBs as a readout for physiological lysosome-resident lysotracker staining.
- We will try to add this data pending availability of fresh patient material. However, the correlation between lysotracker staining and AT2 maturity has been described in the literature (https://doi.org/10.1186/1465-9921-14-123).

4. Fig 2C: The EM of secreted surfactant is beautiful – thank you for including this. The contrast and zoom in the middle panel could be improved to make the LBs more convincing, however.
- We are also very excited about these images! We will improve the quality as suggested.

5. Line 196: It has been shown that LATS manipulation alone can drive an AT1 phenotype in organoids derived from precursors (e.g. Burgess et al PMID 38642558; Lim et al PMID 39815007). This should be mentioned.
- Both publications are already cited, but we will additionally cite them at relevant parts in the manuscript. However, in both publications, LATS-i is used in combination with Wnt withdrawal, and not by itself. We do show in our own manuscript that Wnt withdrawal can cause differentiation, even without LATS-I addition (Figure S3A, base media "BM").

6. In Fig 5, ruxolitinib is used at concentrations of 100nM-1uM then at 1uM in Fig 6. Can the authors justify this choice? The IC50 appears to vary widely according to cell type, but these seem to be high concentrations. Might there be off-target effects?
- We tested the drug at 0.1uM, 1uM, and 10uM (figure S5B) and saw no cytotoxicity at 0.1uM and 1uM. We therefore tested 0.1uM and 1uM in our rescue studies (figure 5D, 5E, S5, S5C, S5D) and saw a dose-dependent effect, with a partial rescue of viability, AT1 gene expression, and suppression of IFN target genes at 0.1uM and a full rescue at 1uM. Because we wanted a full rescue, we chose 1uM in our macrophage studies in figure 6. Ruxolitinib is used widely, and the dose-dependent repression of IFN target genes indicates that the drug is on-target.

7. In Fig 5A&F, different symbols for each donor would help understanding of the individual responses to interferon-γ.
- We will add this to the figure.

8. In Fig 5C, SFTPA is used as an AT2 marker rather than SFTPC (which is used everywhere else). Why is this?
- We will add the data for SFTPC as well.

9. Figure 5J demonstrates a cytotoxic effect of BIRC-i in interferon-γ cells grown in ALVO-EM. This experiment should also be undertaken in in AT1/2-M-cultured cells, despite there being no upregulation of BIRC3 in this population.

- We will do this experiment and add it to the manuscript.

10. In figure 6, activated macrophages are used as a source of interferon-γ, but there are no data to confirm that this is the case. Data should be included to confirm interferon-γ production, and ideally that (as stated in line 394-395) that a consistent supply of interferon-γ is indeed being provided when cells are replaced every 3-4 days.
- There is a lot of literature showing that LPS and IFN-g stimulation of macrophages leads to a pro-inflammatory ("M1-like") phenotype and IFN-g secretion (doi: 10.3389/fimmu.2019.01084, PMID: 29921905, https://doi.org/10.1093/intimm/5.11.1383, PMID: 24680476). Furthermore, the fact that we get a rescue of the phenotype (figure 6E) with the JAK/STAT inhibitor ruxolitinib indicates that it is indeed IFN-g causing the phenotype.

11. In figure 3G, the authors show a significant difference in AT-1 like phenotypes emerging dependent on the ALI transwell culture coating. This is not explored further in this manuscript, but is important. I would suggest this is expanded on in the discussion.
- We will expand on this in the discussion

Minor points
Fig S1A: The SFTPC signal is very hard to see in row 3, column 2 (I assume this is supposed to represent positive staining) – we will improve the image
Fig 2C legend: Spelling (DIC) – we will correct this
Fig 4E (legend on right), Fig S4A,B,F,H: The text is too small to read – we will improve this
Line 102: A reference, and ideally a brief justification, is required to justify negative sorting of NGFR – NGFR is a well-established surface molecule used to sort lung basal cells, the stem cells of the airway epithelium. We will add a reference for this (https://doi.org/10.1073/pnas.0906850106 and https://doi.org/10.1165/rcmb.2020-0464OC)
Line 563: I assume this should read 10 million cells/ml – indeed, we will change this
Line 710: Spelling (Quiagen) – we will correct this

Reviewer #2 (Remarks on Protocol(s)):

Very well written and comprehensive

Reviewer #3 (Remarks to the Author):

In this manuscript, Dost et al. present a new set of protocols for deriving human distal lung organoids with varying differentiation statuses. Using these culture systems, the authors examine how IFN-γ treatment differentially affects organoids depending on the epithelial cell types present. While the study provides improved methods for expanding organoids from primary human lung epithelial cells and includes detailed characterization, several critical concerns remain unresolved, as outlined below.

Major comments:

1. Overall, although the sorting-free approach for the initial culture of lung single-cell suspensions and the inclusion of potential distal progenitor cells capable of generating alveolar lineages may enhance cell survival and expansion, the manuscript does not fully substantiate its conclusions, partly due to the heterogeneity of the starting cell population that gives rise to organoids. Specifically,

a. The authors should contextualize better the rationale for using NGFR to enrich “progenitor cells” for alveolar organoid generation. What does NGFR mark within P0 organoids? The authors should demonstrate what populations exist in EpCAM+NGFR- cells. Flow cytometry for EpCAM, NGFR and HTII-280 require to show cellular profiling. scRNA-seq of EpCAM+NGFR- vs EpCAM+HTII-280+ cell comparison is required to determine the initial populations in their P0 organoids. Interpretation is further hindered by the lack of scRNA-seq or other profiling data at the critical timepoint between P0 and the subsequent EpCAM+NGFR- sorting, when cellular heterogeneity would be manifested. The authors should include such data to clarify which cell types are present and potentially contribute to the observed progenitor diversity.

NGFR is a well-established surface molecule used to sort lung basal cells, the stem cells of the airway epithelium (https://doi.org/10.1073/pnas.0906850106 and https://doi.org/10.1165/rcmb.2020-0464OC). Our rational is to exclude basal cells from our alveolar organoid cultures. To satisfy multiple reviewer requests, we will sort p0 organoids at day 14 for EPCAM+/NGFR-/HT2-280+ and EPCAM+/NGFR-/HT2-280- populations and plate them in media containing either HRG1 or EGF. This experiment will tell us whether the observed alveolar progenitor heterogeneity comes from the sorting strategy and therefore from the starting cell populations, or whether it comes from the media conditions.

b. What does CEACAM6 specifically mark in this culture? Although the authors state that CEACAM6 is a marker for “RAS” cells based on Basil et al. (2022), CEACAM6 expression is known to span a broad range of distal epithelial populations, including AT0, TRB-SC/RAS, club, goblet, and AT1 cells. Figure 1D also showed heterogeneity of CEACAM6-expressing cells in organoids.

- As shown in figure 1D and 1G, CEACAM6 expression correlates with reduced, albeit still high, expression of SFTPC and increased expression of the secretory markers SCGB3A1 and SCGB3A2. Figure 1E shows varying degrees of co-expression of CEACAM6 and SFTPC on protein level. The club and RAS cell marker SCGB1A1 is not expressed. Moreover, our CEACAM6+ cells in ALVO-EM do not express higher levels of AT1 markers, which is the case for AT0 cells. The expression pattern of the organoid secAT2s most closely aligns with the expression profile of TRB-SCs (Murthy et al, Nature, 2022) or AT2/3A2 cells (Basil et al, Nature, 2022).

The mentioned populations (AT0, TRB-SC, RAS, AT2/3A2) are very recent cell type/cell state discoveries that are not well described or understood yet. Because the populations were described by two separate labs independently, there are different nomenclatures that likely describe overlapping populations. It is likely that these cell types lie on a spectrum from secretory to alveolar cells, with varying levels of secretory and alveolar gene co-expression. We do not claim that our organoids contain one specific cell population/state that corresponds to the populations described in vivo. Instead, we chose the term secAT2 cells, because they co-express secretory and alveolar genes, one of the common themes of the newly described populations.

c. What are secAT2 cells? Based on the presented data, these cells appear to resemble AT0-like population, reported by Murthy et al. (2022). However, Murthy et al. demonstrated that AT0 cells exhibit markedly reduced SFTPC expression compared to AT2 cells, whereas the so-called secAT2 cells in this study show SFTPC and LAMP3 levels comparable to AT2 cells (Figure 1G, J,). Does this imply that secAT2 cells are an in vitro artifact, a culture-specific intermediate state, or a transitional population bridging AT2 and AT0 states? Critically, Murthy et al. further demonstrated that AT2 cells transition into AT0 cells and subsequently into TRB-SC (SFTPB+SCGB3A2+SFTPC-) cells upon EGF withdrawal. In contrast, in the current study (Figure S1E),

EGF withdrawal appears to increase both SFTPC and SCGB3A2 expression. The authors also claim that ALVO-EM culture promotes CEACAM6 and SCGB3A2 expression in "AT2" cells (CEACAM6-neg AT2) – is this effect simply attributable to the absence of EGF in ALVO-EM? Moreover, Murthy et al. reported unidirectional differentiation of AT2 toward AT1 or TRB-SC lineages through AT0 states. Given this, the rationale for including secAT2 cells to enrich AT2 cells or to improve alveolar organoid formation requires clearer justification. Are secAT2 and AT2 cells interconvertible under this culture condition? To substantiate these findings, it would be important to determine whether HTII-280+AT2 cells (EpCAM+NGFR-HTII-280+ vs EpCAM+NGFR-HTII-280-) can give rise to secAT2-like or AT0-like cells – or vice versa – when cultured with EGF or HRG1.

- As mentioned in the above comment, we do not claim that our organoids contain one specific cell population/state that corresponds to the populations described in vivo. Our rational for including secAT2 cell is that such a cell type/state likely contributes to alveolar regeneration. In mice, club cells and bronchioalveolar stem cells (BASCs, co-expressing the secretory club cell marker SCGB1A1 and the AT2 marker SFTPC) contribute to alveolar repair. In humans, the respiratory bronchioles contain multiple populations that likewise have secretory-alveolar gene co-expression (AT0, TRB-SC, RAS, AT2/3A2). If and how these cells contribute to alveolar regeneration in humans is not known yet, but it has been demonstrated that iPSC-derived RAS cells can give rise to AT2 cells (Basil, 2022, Nature and doi: 10.1038/s41587-025-02569-0.). This data suggests a trajectory from secretory to alveolar cells.
While it has been shown that withdrawal of EGF can cause transdifferentiation of AT2s to TRB-SC through an AT0 state, our organoid cultures grow without EGF long-term and still contain an AT2 population, indicating that we have two stable progenitor populations in our organoid cultures.
To address the effect of HRG1/EGF in our cultures, we will sort for EpCAM+NGFR-HTII-280+ and EpCAM+NGFR-HTII-280- as suggested, and grow these two populations in media containing either EGF or HRG1 subsequently.

2. Although substituting EGF with the alternative EGFR family ligand HRG1 in alveolar organoid cultures is an interesting modification, it appears to only modestly improve organoid propagation. Previous study using SFFF medium containing EGF (Katsura et al. 2020) already demonstrated long-term subculture beyond 10 passages. In the current study, while the authors report somewhat improved expansion, SFTPC and SFTPA1 expression levels decline after passage 10 (Figure S1G), restricting organoid use to the first 10 passages. Therefore, the practical benefit of this protocol appears limited. Furthermore, a similar sorting-free approach was previously employed by Salahudeen et al. (2020) to generate human distal lung organoids from primary lung tissues. They used EpCAM+Lysotracker+ cells from P0 organoids, which likely contain AT2 and secAT2 (or AT0 cells) based on the current study. So, it raises an important question – would this strategy provide a more effective means to enrich AT2 and/or potential progenitor-like populations than the current use of EpCAM+NGFR- cells, given the non-specificity of NGFR and the resulting cellular heterogeneity?

- One important practical benefit is that the FACS/MACS sorting is not done on the day of the tissue processing. In practicality, receiving tissue from the hospital, processing it to single cell suspensions, then sorting and plating the cells is a long and harsh process that reduces cell viability. There are other practical considerations rarely talked about in scientific publications, like the availability of a FACS machine on the day the tissue is received, which can be unpredictable. For further explanations of advantages of the system, please refer to page 1 line 37+.
Regarding the EpCAM+Lysotracker+ sorting strategy from Salahudeen et al. (2020): Lysotracker signal corresponds to the maturity level of AT2 cells. However, it has been shown that AT2 "stem/signaling" cells (PMID: 33208946), and also the newly described progenitor populations residing in the respiratory

bronchioles (AT0, TRB-SC, RAS) have lower levels of canonical AT2 markers. It makes sense that proliferating AT2 progenitor cells would not have the same level of maturity as quiescent AT2 cells that produce high levels of surfactant. AT2 progenitor cells would therefore likely have lower levels of lamellar bodies and lysotracker staining, which is why we think that lysotracker sorting is a less inclusive approach than our sorting strategy. We see the heterogeneity of our ALVO-EM cultures as an advantage because it gives us the opportunity to start studying the secretory-alveolar populations in vitro.

3. Regarding the use of HRG1, the signaling aspects related to this alteration require further exploration. Since HRG1 does not bind to EGFR but rather ERBB3 and ERBB4, what are the levels of expression of each of these receptors in secAT2 and AT2 cells during organoid culture? Ideally, this should be assessed at the timepoint following the unsorted culture of lung single cell suspensions at day 14 prior to sorting for further organoid culture. Are there differences in downstream signaling between these populations when cultured with EGF vs. HRG1? Does HRG1 specifically drive the generation of secAT2 cells from AT2 cells, or is this effect simply due to the absence of EGF, as noted above? Finally, what are the cellular sources of HRG1 in lung tissues, and what is the expression pattern of its receptors within these tissues?
- We have data showing that ERBB3 and ERBB4 is expressed in AT2 cells in lung tissue, while HRG1 (NRG1) is expressed by multiple cell types, including AT1 cells. We can also show that ERBB3 and ERBB4 are expressed in our organoid cultures, both in the AT2 and secAT2 populations.
To satisfy multiple reviewer requests, we will sort p0 organoids at day 14 for EPCAM+/NGFR-/HT2-280+ and EPCAM+/NGFR-/HT2-280- populations and plate them in media containing either HRG1 or EGF. This experiment will tell us whether the observed alveolar progenitor heterogeneity comes from the sorting strategy and therefore from the starting cell populations, or whether it comes from the media conditions.

4. In Figure S1D and E, organoids cultured without HRG1 and EGF revealed poor organoid formation but higher expression levels of SFTPC and AGER. The authors should provide an explanation for this observation.
– Removal of these factors decreases proliferation of the progenitor cells. In the absence of these pro-proliferative and pro-stemness cues, the cells likely mature/differentiate to either AT2 or AT1 cells. However, this effect is very small compared to our AT1 differentiation protocol where AGER is upregulated almost 100 fold (figure 3I).

5. Single cell RNA-seq data from three separate patients of organoids from initial single cell culture in ALVO-EM (Figure 1D/S1H-I), and after switching to ALVO-EM, AT1/2-M, and AT2-MM (Figure 4C-E/S4A-C), clearly show that one patient (Lu39) is responsible for the majority of 3 separate clusters in Fig. 1/S1, and furthermore is responsible for the vast majority of neuroendocrine-like cells from the different culturing conditions in Figure 4/S4 (see most pertinently Figure S4C). However, the authors do not refer to this clear demarcation in the Results section related to each of these figures. The authors should update the Results section to communicate to the readers that this is the case, probably attributable to heterogeneity in human samples taken for organoid culture. Furthermore, the authors may want to soften their interpretation of these results as representative of general principles of neuroendocrine phenotype emergence in DCI-containing organoid cultures (see Discussion, line 425-430), given that 2/3 patient samples do not seem to appreciably show this conversion. – We will add qPCR data showing that we also get neuroendocrine-gene upregulation in the other organoid lines. While this is not the focus of the paper, we think that it is an interesting observation to point out in the discussion, and this observation is not only based on our own data but also data from the referenced publication (ref 53) and another recent publication that we will add to the discussion (PMID: 41075787).

6. In Figure 2A, what was the initial cell population used for this experiment? Did the authors test whether AT2 and secAT2 cells differ in their capacity to differentiate into mature AT2 cells? – The initial cell population are all cells present in our ALVO-EM conditions, so both AT2-like cells and secAT2 cells. To answer this question, we will sort out these two populations and expose them to our AT2-MM separately.

Given the heterogeneity of this culture, it is surprising that minimal differences were observed in response to the screening factors. Furthermore, in Figure 2D, the majority of cells in the ALVO-EM condition do not express HTII-280 despite of their high expression of AT2 markers shown in Figure 1. Additionally, in the AT2-MM condition, one organoid contains Nile red+ lipids but is negative for HTII-280, whereas another organoid shows lower lipid content but high HTII-280 expression. How do the authors explain this discrepancy? –To answer the question regarding the Nile red-high cells, we will do FACS and subsequent gene expression analysis on the Nile red-high/low populations. Regarding HT2-280, it has been pointed out that this surface molecule might be downregulated in in vitro cultures and might therefore not be a reliable marker: "However, it is important to note that although the majority of AT2 cells express HTII-280, HTII-280– AT2 cells are observed even in healthy human lungs (Figure 2B). Furthermore, HTII-280 is a less useful marker for subculture of AT2 cells due to loss of expression during culture." (PMID: 32272001)

Since DCI is known to promote alveolar maturation in both iPSC-derived and fetal lung organoids, a similar effect is likely in primary alveolar organoids. Does HRG1 modulate this maturation process differently than EGF? – Both iPSC- and fetal-derived organoids are in a more developmental-like state compared to primary adult cells, so it is not obvious that adult alveolar cells would respond the same. To answer the question regarding HRG1/EGF, we will test the AT2-MM in the presence of either EGF or HRG1.

7. LATS-i has previously been reported to induce AT1 differentiation in both iPSC- and primary lung tissue-derived alveolar organoids (Burgess et al. 2024). In this current study (Figure 3), adding LATS-i to ALVO-EM induces upregulation of AT1 markers, but the cells do not show AT1 phenotypes; some AGER$^+$ or CAV1$^+$ cells remain proliferative, suggesting that AT1-like cells can divide – an event that does not occur in lung tissue. Moreover, the majority of cells co-express AT2 markers and/or SCGB3A1 and SCGB3A2, indicative of transitional or immature state. In Figure S3D, the claim that AT1/2-M organoids retain a mixture of AT1 and AT2 cells is likely misleading; the data suggest that many cells co-express AT1 and AT2 markers, consistent with immature or transitioning cells rather than a true mixture of two distinct cell types. Critically, in Figure 3I, LAMP3 expression remains stable under the AT1/2-M condition. Given these observations, the current culture condition favors the maintenance of transitional or immature cells, despite LATS-i stimulation, and still requires additional cues such as ECM components or mechanical signals, similar to previous methods. It would be important to clarify what advantages and novel insights this protocol offers over prior approaches.
- In Burgess et al (PMID: 38642558), the authors induce differentiation of primary AT2 cells with their L+DCI medium and compare it to SFFF medium (PMID: 35355018). These two mediums are very different, most notably the absence of CHIR in the differentiation medium. Other published strategies include the addition of human serum to induce differentiation (PMID: 33128895). The strength of our approach is that the sole variable between our expansion and our differentiation medium is LATS-inhibitor. This minimizes confounding factors when comparing progenitor cells to differentiated cells and it enables precise mechanistic studies. Furthermore, we do acknowledge the presence of immature or transitioning cells in the manuscript, e.g. in the discussion "*A biological explanation for the underrepresentation is that additional signals or prolonged culture periods might be required for terminal AT1 differentiation. Nevertheless, we posit that the presence of transitional states in our organoids better reflects regenerative conditions and provides a valuable platform for optimizing AT1 differentiation in therapeutic contexts.*"

8. What is the rationale for adding IL-1β to all organoid culture conditions during the first 0–3 days? Could this early exposure precondition the cells and thereby confound their responsiveness to the additional cytokines tested in Figure 5, making it difficult to interpret the screening results?
- We only add IL-1β at p0, as it has been reported to increase organoid outgrowth (PMID: 33128895). The screening of the cytokines was done at passage 6/7 after several months of in vitro expansion. We do not think that the cells and their daughter cells have such a long-lived IL-1β memory. Even if they did, coming from human lungs, the cells would most certainly have been exposed to IL-1β in vivo at some point due to infections such as the common cold, which is something we cannot control for.

9. The findings related to differential effects of IFN-γ on cells from different organoid conditions are intriguing, but overall analysis is limited. Given the presence of multiple cell types and states within these organoids (Figure 4) and the lack of a cell-tracking system, it remains unclear which specific populations are actually responding to IFN-γ, especially since the analysis relies on qPCR of only one or two genes and viability assays on bulk organoids. Furthermore, AT1/2-M organoids seem to retain immature or transitional cells instead of "AT1" cells.
- figure 5G shows that both AT2 and secAT2s respond with increased proliferation to IFN. Additionally, we will further characterize the cells that survive IFN exposure in AT1/2-M conditions using stainings.

10. 10. In Major et al. (PMID: 32527928), although IFN-γ is not investigated (rather Type I and III interferons), it is shown that interferon signaling is deleterious for lung epithelial regeneration in vivo during influenza A models. In Lin et al. (PMID: 39352385), the authors show that IFN-γ signaling can lead to dysplastic lung epithelial regeneration during an in vivo influenza A model, including differentiation of dysplastic-like cells from AT2 cells in organoid culture. The authors show in Figure 5/S5 that IFN-γ exposure up to day 10 leads to ALVO-EM expansion. Does this exposure to IFN-γ have consequences for later differentiation characteristics of these populations? Experiments to address this may involve the short-term exposure to IFN-γ of ALVO-EM organoids, (up to day 10, since longer exposure seems to have deleterious effects – see Figure S5F), with subsequent passaging to AT1/2-M to see if their differentiation ability is altered compared to controls.
- We think that this is a good suggestion. We will differentiate organoids after IFN exposure to see if the exposure impaired their differentiation capacity.

11. In Figure 6, how do macrophages influence the cellular phenotypes of ALVO-EM and AT1/2-M organoids beyond their effects on organoid growth? Do they also impact differentiation? What specific evidence supports the author's conclusion that pre-treated macrophages selectively modulate secAT2/AT2 proliferation and AT1 cell death in these experiments?
- Our goal with these experiments is to show that we see the same effect on our progenitor cultures (increased growth) and differentiated cultures (decreased growth) with macrophage-secreted IFNg as we see with the addition of recombinant IFNg, adding more physiological relevance to the model. Since our focus is not on macrophage-epithelial interactions, we feel that further characterization is beyond the scope of this manuscript.

Minor comments:

1. Please update your diagram in Figure 1A and your methods to clarify your final culture conditions for initial investigation of organoid growth characteristics in Figure 1/S1 – it seems that ultimately SFFF was used for

the initial unsorted 14 day culture, and ALVO-EM and other types were used upon passaging for p1 and further, but this could use clarification. – This is indeed not clearly communicated and we will change the phrasing and add explanations. In fact, we only used SFFF media (containing EGF) until we had optimized the ALVO-EM conditions (replacing EGF with HRG1). To establish the organoid lines that we used in this publication, we used both EGF and HRG1 in p0 and then switched to ALVO-EM from p1 onwards.

2. The UMAPs in Figure 1D combined with the text explanations are laborious to interpret with the cluster assignments not given anywhere in Figure 1D. Furthermore, related to Major Point 4 above, it is critical for the interpretation of this scRNA-seq data that certain clusters seem to be derived almost entirely from the cells of one patient (Lu39). Thus, the data in Figure S1H would be better served alongside the UMAPs in Figure 1D. – We will adjust the figures accordingly.

3. Related to the data in Figure 1 – for the secAT2 sorting and clustering, please clarify the details of the passage number this was done at – for the scRNA-seq, presumably this was after passage 1 (~14 days after sorting/plating of Epcam+ NGFR- cells and culture in ALVO-EM), and for the secAT2 organoids, these were presumably sorted at p1 and the data displayed in Figure 1H-I are from p2 after this? These important details are not currently included in the figure legends or Methods. – We will add passage information as requested. The scRNA-Seq was done at p5. The data from figure 1H-I are at p4. Because we can expand, freeze, and thaw our organoid lines as needed, there is no "linear time line", but all experiments are done before passage 10, as stated in the manuscript.

4. Line 119: what is the rationale saying a 'more progenitor-like state' based on the higher expression of SCGB3A2? – we will change the phrasing to "more secretory-like state", as SCGB3A2 is a marker for secretory cells

5. Line 160: Should there be a reference to Figure 1H here? Figure 1H is seemingly not currently referenced anywhere in the paper as of now. – indeed, thank you for pointing this out.

6. Line 546: "canonical tube" – the authors likely meant to say "conical tube" - indeed

7. In the Methods section "Making single cell suspensions and passaging organoids", please clarify what types of suspension culture plates were used and how many "drops" of cells/BME were included on each plate (line 555), as these are important details for recapitulation of these methods. – we will add these details

Reviewer #3 (Remarks on Protocol(s)):

Overall, the authors provide the detailed methods for this study. Please also see our comments to the authors.

1st Editorial Decision **4th Dec 2025**

Dear Hans, dear Antonella,

Thank you for transferring your manuscript for consideration by the EMBO Journal, as well as sharing a preliminary point-by-point response on the concerns raised by experts during peer-review at another journal. I now went through your rebuttal response in detail and also discussed the matter with my editorial colleagues. I am pleased to share that we found your arguments sensible as such and the complementary experiments potentially suitable to address the major critique by the referees.

We can accordingly invite you to complete a revised version of your work for swift reconsideration by the existing referees for the EMBO Journal.

Related, I enclose formatting requirements for our venue at resubmission,
Please let me know if there are any questions related.

Thank you for the opportunity to consider your work for publication. I look forward to receiving your revision.

Best regards,
Daniel

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Read our guidance for manuscript revisions and related editorial policies: https://link.springer.com/journal/44318/submission-guidelines#cms-Revised-submissions

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://media.springernature.com/original/springer-cms/rest/v1/content/27825798/data/v1

At EMBO Press we ask authors to provide source data for the main manuscript figures. You will receive a separate email with instructions for providing source data with your revised manuscript, including how to upload and organize the files.

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist
- Expanded View files (replacing Supplementary Information)
- a Reagents and Tools Table as part of the Methods section

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (4th Mar 2026). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Reviewer #1 (Remarks to the Author):

This manuscript described a modified protocol of generating and maintaining alveolar organoids from isolated epithelial cells derived from primary human lobectomy material. The authors use this model (that was improved with regard to a differentiation step (LATS-i) and with regard to prior expansion in ALVO-EM) to demonstrate a role of recombinant IFNy on organoid cell population behaviour, and extrapolate a role of IFNy in human COPD. While some aspects of the presented data are interesting, I have concerns regarding data interpretation and conclusions drawn.

The authors use lobectomy material derived from lung cancer surgeries. No information is provided as to patient history regarding age, gender, type of lung cancer, smoking history, COPD stage, or steroid medication history, which might impact the composition and cellular responses of the organoids, particularly in light of the findings on IFNy and a putative role in COPD.
Given that the authors identify quite some heterogeneity in the different cultures (e.g., Fig 1C, S1H), and that such criteria might significantly affect alveolar stem cell behaviour, such information should be provided to better interpret the results.
On a minor note, might the inflammatory tumor microenvironment/signature within the lobectomy material additionally impact the cells of origin used for organoids (how distant from tumors was the tissue used for preps)?
A: For ethical and practical reasons, we work with surgical "rest material" that is fully anonymized, so patient-specific data (age, sex, smoking history, etc.) are unavailable. However, we do know that none of the patients were diagnosed with COPD or other lung diseases beyond lung cancer, and that they did not receive cancer treatment before surgery. We added this information to the material section "Samples were collected at least 5 cm from the tumor. Patients had no diagnosed lung diseases other than lung cancer and had received no cancer treatment before surgery."
Regarding heterogeneity: variation between human donors is expected and intrinsic to primary organoid systems. It is not possible to get tissue from a "normal, healthy" lung. Material either comes from lungs that were rejected for transplantation, or from lobectomies, like in our case. We consistently use at least three biological replicates to ensure reproducibility of observed responses and we show donor identifiers for full transparency.

The data on establishment of the new protocol seem to lack consistency, and clarity regarding information on experimental setup, which makes interpretation difficult, and it remains unclear what message the authors want to convey about their new protocol.
A: *We apologize for the confusion. We now reworked this whole part of the manuscript and added the final media information to figure 1A and also adjusted table EV1.*
As for the message, we see multiple advantages of our protocol over existing protocols:
1) Current approaches depend on HTII-280, a widely used AT2-specific membrane antigen whose corresponding gene remains unknown. It is known that HTII-280-negative cells can generate SFTPC+ organoids (Basil, Nature, 2022) and that not all AT2 cells stain positive for HTII-280 (Evans et al., 2020, PMID: 32272001). This suggests that progenitors capable of forming AT2-like cells are excluded when HTII-280-based sorting is used. We therefore see a conceptual advantage in our more inclusive approach.
2) We show that HRG1(replacing EGF) leads to better growth and higher expression of alveolar markers.
3) We achieve long-term expansion of AT2 progenitor cells AND secretory AT2 progenitor cells, which has not been demonstrated before.
4) The sole variable between our progenitor expansion media and our differentiation media is LATS-inhibitor, making precise mechanistic studies possible. This is in contrast to existing protocols, where differentiation is achieved by the addition of human serum, or the addition of LATS-i in combination with Wnt-withrawal.
5) We did extensive characterization of our organoids, including scRNA-Seq, which is not the case for existing protocols (Katsura et al., CSC, 2020, Youk et al., CSC, 2020).
Together, we think that our system presents an advance to currently available protocols.

For example, Fig 1C right panel lacks Lu44 that was referred to before to be the most long-lasting line (21

passages). Fig 1C and 1F again show different donors. Figure 1H shows data from donor 37, 53 and 54, and Figure 1I only from 53 and 54.
A: Due to practical reasons, it is not feasible to use the exact same three donors for every experiment. We view the diversity of donors as a strength rather than a weakness, and we are fully transparent which lines were used for which experiment. We did not continue using Lu44 because it represented an outlyer when it came to long-term expansion, and we chose to continue with more representative lines.

The scRNASeq experiments (from donors 37-39) lack information about passage numbers and how long these organoids have been in culture. Can the authors explain why there is such low amount of proliferating cells, within the cells with progenitor phenotype that should reveal higher proliferation rates compared to AT2 cells ("secAT2", C6+; "RAS-like")?
A: The scRNA-Seq was done at passage 5 with each passage being 14 days apart, as outlined in the methods section "Making single-cell suspensions and passaging organoids"; we added the passage information to the "10x Genomics scRNA-Seq" methods section of the manuscript and to the figure legend.
We would like to emphasize that our goal is not to model the alveolus at homeostasis, where epithelial cells are quiescent, but rather a regenerative state. This is why organoid-derived cells form the majority of proliferating cells when integrated with lung atlas data (Figure 4A). Under regenerative conditions (i.e. in expansion media), we do not expect many mature, and therefore quiescent, AT2 cells. As Reviewer 2 pointed out, the field increasingly recognizes that alveolar progenitors are heterogeneous. Our organoid conditions promote stemness and proliferation, enriching two AT2-like progenitor subpopulations, one of which resembles the secretory alveolar phenotype described in recent Nature papers (Murthy et al., Nature 2022, and Basil et al., Nature 2022). These "secAT2" cells share features with AT0, TRB-SC, and AT2-3A2 populations, cells thought to lie along a continuum between secretory and alveolar lineages.

In Figure 2D, there are organoids that are HT2-280 negative after AT2-MM culture, but Nile Red positive. What are these organoids, and can the authors comment on the heterogeneity of their cultures?
A: It has been stated elsewhere: "HTII-280 is a less useful marker for subculture of AT2 cells due to loss of expression during culture." (Evans et al., 2020, PMID: 32272001). This is why we show maturation with functional dyes such as lysotracker and nile red instead. We agree that the presented image may be confusing so we removed it from the figure. Instead, we added qPCR analysis of FACS-sorted nile red low and nile red high cells in AT2-MM to show that nile red intensity indeed correlates with AT2-marker expression (figure EV2D and S2E).

It is difficult to assess the ultimate advantage of the presented over previously published models (e.g., PMID: 33128895), also in light of the results of Fig 3F and 3I indicating separation between C6+ and C6- cells seems dispensable.
A: Please see one of our previous answers (page 1; lines 37+) for the advantage of our system to the published protocols. Figure 3F and 3I show that both AT2 and secAT2 populations can give rise to AT1-like cells, but that does not make their separation dispensable. While we agree that in this manuscript we mostly show transcriptional rather than functional differences between the two populations, we believe this system to be useful to probe the differences in more detail in the future.

Also, it remains open how well the ALVOs are suited to model the alveolus containing AT1 cells. Although AT1 markers are increasingly expressed (particularly in AT1/AT2-M, no matter if used directly or after ALVO-EM expansion), there is little contribution from organoids compared to tissue to the AT1 pool when looking at identity contribution (Fig 4E). Given that only very few AT1 cells are contained in the organoids, the abstract statement that "Unlike previous models, this system supports long-term expansion of newly identified human-specific alveolar progenitor cells and serum-free differentiation into alveolar type 1 (AT1) cells." seems not to be supported by data.
A: The cells in our organoids show high expression of AT1 markers such as AGER at the protein level, which is generally accepted by the lung field to indicate AT1 identity. We have acknowledged and discussed the modest contribution of fully differentiated AT1 cells in the scRNA-Seq data in the discussion as follows:
"However, the majority of AT1-like cells have a gene expression profile resembling the AT2-to-AT1 transitional

state rather than that of fully differentiated AT1 cells. It is possible that organoid-derived differentiated AT1 cells are underrepresented in the scRNA-Seq dataset due to technical reasons. The typical shape of AT1 cells makes this cell type especially sensitive to dissociation methods necessary to achieve single-cell suspensions. A biological explanation for the underrepresentation is that additional signals or prolonged culture periods might be required for terminal AT1 differentiation. Nevertheless, we posit that the presence of transitional states in our organoids better reflects regenerative conditions and provides a valuable platform for optimizing AT1 differentiation in therapeutic contexts." Moreover, we changed the term AT1 to AT1-like cell in the abstract and in the summary figures of figure 5 and 6.

The culture type-specific effects of IFNy are an interesting finding (ALVO-EM vs AT1/2 medium). The conclusion that in particular, AT1-like cells are "negatively" affected would benefit from a more detailed analysis (i.e. type/pathways of cell death induced by the cytokine, etc). Are the IFNy effects observed on "AT1-like" cells, or trans_AT2-AT1 cells (Fig 4), similarly observed in truly differentiated AT1 cells?
A: We demonstrate a reduction in cell numbers (5A), an increase in cytotoxicity (5B), and an upregulation of genes associated with cell death (5H). We also demonstrate reduced AT1 marker expression (5C), indicating a selective loss of AT1 marker-expressing cells. It is difficult to define "truly differentiated AT1 cells". Some reports indicate that mechanical cues from breathing might be necessary to maintain "true" AT1 identity (Shiraishi et al, 2024, doi: 10.1016/j.cell.2023.02.010). However, the goal of our organoid model is not to achieve terminal AT1 differentiation in vitro but to model alveolar regeneration. We think that the observation that IFN-g affects AT1-like or transitioning AT1 cells is highly relevant to acute injury, infection, and chronic disease.

In light of the different actions of IFNy on secAT2/AT2 vs "AT1-like" cells, what is the expected "net outcome" of IFNy signaling in COPD according to the authors data, and how can this be interpreted with regard to COPD (emphysema) lung phenotype, given that such lungs do not contain more or more proliferating AT2/progenitor cells?
A: An accumulation of secAT2s is reported in COPD lungs (Basil et al, Nature), the reason for which remains unknown. However, the inflammatory landscape of COPD lungs is too complex to make a prediction regarding a net outcome of the effect of IFN-g.

How much "COPD phenotype" is imprinted in the organoids (given that likely, at least some of the donors were smokers/COPD patients, see above)?
A: As now added to the methods, none of the donors had a lung disease diagnosis apart from lung cancer. The question of an imprinted smoking or COPD phenotype – even after months in organoid culture – is very interesting but beyond the scope of this manuscript.

On a minor note, why do the authors use PBMC-derived macrophage-like cells for co-cultures, given that T cells are major sources of IFNy?
A: This is for practical reasons: T cells need to be patient matched, which is not the case for macrophages. While T cells are a major source for IFN-y, macrophages also secrete the cytokine.

All figure legends should contain information on n numbers, number of independent experiments, p values and statistics applied.
A: Our n numbers are always specified by the number of dots/symbols in the figures. Each symbol represents a biological replicate, which is specified in the methods section. We added the information regarding the statistical test to the figure legends and we now show the exact p values in the figures.

Other:
• Fig 1A: which protocol ("published methods") do the authors exactly refer to?
A: We added this information to the figure. We specifically referred to Youk et al. and Katsura et al.

• P4: what do the authors mean by "appeared healthier"? (line 114).

A: We added the following sentence to the manuscript as a better explanation: "(…) the organoids grown in HRG1-containing media appeared larger and more numerous compared to EGF (figure EV1D)."

• How were the stats calculated in Fig 3I and 5G (for datapoints normalized to 1)?

A: We now added all information regarding statistical testing to the figure legends and also updated the methods section. All statistical testing was done on raw values and not on values that were normalized to 1. The normalized data is only used for visualization purposes.

• Fig S5F does not show cell death (line 348)

A: The image of IFN-treated organoids at day 16 shows excessive shedding of cells and cell debris from the organoids, which is an indication of dying cells. We increased the magnification of the images to show this more clearly, and also added an explanation to the manuscript.

Reviewer #2 (Remarks to the Author):

In this manuscript entitled "Interferon-γ selectively promotes survival of alveolar progenitor 1 cells in a human 2 organoid model" the authors describe the development of a novel approach to adult human alveolar epithelial culture modelling with particular emphasis on an inclusive approach to cell isolation to ensure the capture of all potential alveolar progenitors. They then define media components required for AT2 maturation and AT2-AT1 differentiation from their mixed population. Having undertaken detailed characterisation, they then use this model to identify the differing role of interferon-γ on the proliferation and survival of different alveolar epithelial population, finding that interferon-γ is cytotoxic to AT1 cells but promotes survival of AT2 cells.

The manuscript is well written and easy to follow. The figures are carefully prepared and they present their data clearly with appropriate analysis. Their transparency in presenting negative and variable data is appreciated.

Their novel organoid model is potentially of great interest to the field. By taking a more unbiased approach to sorting lung epithelial cells from fresh adult lung tissue than current protocols, they find different AT2 subtypes (AT2, secretory AT2) and then push them toward different fates (AT2-like or AT1-like) by manipulation of media components. As we increasingly recognise that alveolar progenitors are not simply one entity, the ability to generate a manipulable model containing several AT2- and AT1-like subtypes is of real significance both in disease modelling and understanding AT2 transdifferentiation in health and disease.

To convince the reader of their conclusions about the true originality of the model and the ability to use it for the disease modelling described in the second part of the manuscript, I would suggest the following:

Major comments

1. The authors state in the introduction that using HTII-280 sorting strategies may exclude important alveolar progenitors; indeed, they then use this argument for creating a novel culture strategy which does not rely on this poorly characterised transmembrane protein. Though I agree with their concerns about HTII-280, the absence of any data to support their statement is a concern. I would suggest:

• They should provide evidence that HTII-280 is absent from non-standard AT2 cells (e.g. by citing existing scRNAseq datasets) to support the logic of their experimental approach.

A: Because the corresponding gene to HT2-280 is not known, it cannot be looked up in scRNA-Seq data. However, it is known that HTII-280-negative cells can generate AT2 organoids (Basil, Nature 2022, figure 3). This suggests that progenitors capable of forming AT2 cells are excluded when HTII-280-based sorting is used. Furthermore, it has been observed that "(…) although the majority of AT2 cells express HTII-280, HTII-280− AT2 cells are observed even in healthy human lungs" (Evans et al., 2020, PMID: 32272001). We now added this information and the additional references to the introduction.

• They should show whether HTII-280-based AT2 isolation generates a similarly heterogeneous AT2-like

population, and whether the same manipulations of the culture media (i.e. use of AT2-MM / AT1/2-M) allow maturation of AT2 and AT1-like cells from HTII-280-isolated organoids. This will likely highlight the key advantages of this new model.

A: To respond to this point (also raised elsewhere), we sorted p0 organoids at day 14 for EPCAM+/NGFR-/HT2-280+ (HT2+) and EPCAM+/NGFR-/HT2-280- (HT2-) populations. Because this experiment required us to get new lung tissue and the total cell numbers we obtain per tissue and for each of the populations varies between donors, we could not do all requested experiments with three biological replicates within a reasonable time frame. We were able to show that both HT2+ and HT2- cells lead to organoid outgrowth in HRG1-containing media (figure EV1C) and that both populations lead to cultures that have high SFTPC and SCGB3A2 expression (figure EV1E). SFTPC expression levels of HT2- derived cells were lower compared to HT2+ derived cells, indicating that the starting cell population does make a difference in subsequent gene expression of cells. Due to cell number restraints, we were only able to derive flow analysis of one lung at p1 day 14. We found that HT2+ derived organoids had more HTII-280 signal and less CEACAM6 signal than HT2- derived organoids (in HRG1-containing cultures) (reviewer figure C). However, the presence of HTII-280+ cells that were derived from HT2- cells indicates that indeed HT2- cells can give rise to HTII-280+ cells. This supports our reasoning of not relying on HTII-280 sorting for our organoid cultures.

2. Figures 5 and 6 describe the differential response of AT2-like and AT1-like cells to interferon-γ. By necessity, the culture media for each cell type is different with AT1-like cells maintained in LATS-i. Bearing in mind the crosstalk between LATS and IFN pathways, how are the authors sure that the observed effects on cell proliferation and survival due to cell type and not the effect of LATS-i?
The following would be reassuring:
• To demonstrate that LATS-i does itself not affect cell proliferation and survival.

A: LATS-i in fact increases the proliferation rate of our organoids. We added this data to the manuscript (figure EV3F). Of note, in figure 5F we compare the IFN-treated cultures to their own controls and therefore normalize for difference in cell growth between the ALVO-EM and AT1/2-M conditions.

• To check that the combination of LATS-i and interferon-γ does not affect the phenotype of the AT1-like cells grown in AT1/2-M.

A: The cytotoxicity assays are conducted in differentiated cultures that were exposed to IFN-g for only 3 days (5B) and we do not expect drastic changes in cell identity within this time frame.

Other comments

1. The introduction opens with a brief discussion of COPD and the need for better models of the effect of inflammatory insults in the lung. While I agree, this really isn't the focus of the manuscript which has far broader reach in modelling the alveolar epithelium in health and disease. Indeed, I would suggest the models they describe would be more immediately relevant to modelling diseases of the alveolus e.g. ILD. To this end, I would suggest the introduction (and discussion) are reworded to downplay the emphasis on COPD.

A: Well taken. We rephrased the introduction to lessen the focus on COPD.

2. Fig S1E&F: AGER is used in S1E then CAV1 in S1F as AT1 markers. Subsequently both are used. For transparency and continuity, I would suggest including both in Fig S1.

A: Thank you. We added the requested data.

3. Fig 2B/Fig S2A&B: There is an assumption here that higher lysotracker = more AT2-like. It would be nice to have a) freshly isolated AT2 cells (e.g by HTII-280 sorting) as a control to define physiological levels of lysotracker, and b) a control line which does not have LBs as a readout for physiological lysosome-resident lysotracker staining.

A: We added this data for both lysotracker and Nile red staining, using airway organoid-derived cells as a negative control and tissue sorted HT2-280+ cells as a positive control. Our data confirms that our organoid-derived cells exhibit lysotracker- and Nile red-signals comparable to tissue AT2s (figure 2E).

4. Fig 2C: The EM of secreted surfactant is beautiful – thank you for including this. The contrast and zoom in the middle panel could be improved to make the LBs more convincing, however.
A: We are also very excited about these images! We improved the zoom and contrast as suggested.

5. Line 196: It has been shown that LATS manipulation alone can drive an AT1 phenotype in organoids derived from precursors (e.g. Burgess et al PMID 38642558; Lim et al PMID 39815007). This should be mentioned.
A: In both mentioned publications, LATS-i is used in combination with Wnt withdrawal to induce AT1 differentiation, and not by itself. We do show in our own manuscript that Wnt withdrawal alone can cause AT1 differentiation, even without the addition of LATS-i (Figure EV3A, base media "BM"). Our observation that LATS-i alone can drive AT1-differentiation even in the presence of Wnt-activating agents was to our knowledge not described previously. We added this distinction now more specifically to our manuscript.

6. In Fig 5, ruxolitinib is used at concentrations of 100nM-1uM then at 1uM in Fig 6. Can the authors justify this choice? The IC50 appears to vary widely according to cell type, but these seem to be high concentrations. Might there be off-target effects?
A: We tested the drug at 0.1uM, 1uM, and 10uM (figure EV5B) and saw no cytotoxicity at 0.1uM and 1uM. We therefore tested 0.1uM and 1uM in our rescue studies (figure 5D, 5E, S5, S5C, S5D) and saw a dose-dependent effect, with a partial rescue of viability, AT1 gene expression, and suppression of IFN target genes at 0.1uM and a full rescue at 1uM. Because we aimed for a full rescue, we chose 1uM in our macrophage studies in figure 6. Ruxolitinib is used widely, and the dose-dependent repression of IFN target genes indicates that the drug is on-target. We found several studies that use Ruxolitinib at concentrations between 1-10uM in organoids (Katsura et al, CSC, 2020 doi: 10.1016/j.stem.2020.10.005; Kolawole et al., 2019, doi: 10.1371/journal.ppat.1008057)

7. In Fig 5A&F, different symbols for each donor would help understanding of the individual responses to interferon-γ.
A: We added the different symbols to the figure.

8. In Fig 5C, SFTPA is used as an AT2 marker rather than SFTPC (which is used everywhere else). Why is this?
A: We apologize for the inconsistency. We now also did the analysis for SFTPC and added this to the figure.

9. Figure 5J demonstrates a cytotoxic effect of BIRC-i in interferon-γ cells grown in ALVO-EM. This experiment should also be undertaken in in AT1/2-M-cultured cells, despite there being no upregulation of BIRC3 in this population.
A: We undertook this experiment and added it to the manuscript. "In AT1/2-M, both the addition of LCL161 alone and in combination with IFN-γ caused an increase in cytotoxicity (S5H). This indicates that even though BIRC3 is not upregulated upon IFN-γ in AT1-like cells, it might still play a protective role in these cells in general."

10. In figure 6, activated macrophages are used as a source of interferon-γ, but there are no data to confirm that this is the case. Data should be included to confirm interferon-γ production, and ideally that (as stated in line 394-395) that a consistent supply of interferon-γ is indeed being provided when cells are replaced every 3-4 days.
A: Previous studies show that LPS and IFN-g stimulation of macrophages leads to a pro-inflammatory ("M1-like") phenotype and IFN-g secretion (doi: 10.3389/fimmu.2019.01084, PMID: 29921905, https://doi.org/10.1093/intimm/5.11.1383, PMID: 24680476). Furthermore, the fact that we get a rescue of the phenotype (figure 6E) with the JAK/STAT inhibitor ruxolitinib indicates that it is indeed IFN-g causing the phenotype.

11. In figure 3G, the authors show a significant difference in AT-1 like phenotypes emerging dependent on the

ALI transwell culture coating. This is not explored further in this manuscript, but is important. I would suggest this is expanded on in the discussion.
A: We now expanded on this in the discussion: "Moreover, 2D cultures may be better suited to capture the characteristic morphology of AT1 cells. AT1 cells are tightly associated with the basement membrane, a relationship that is central to their function. We show that AT1 cell shape varies markedly depending on the ECM coating of the transwells. Although this was not the primary focus of the present study, further optimization of ECM composition to enhance AT1 attachment and differentiation may enable in vitro systems that better support functional testing of AT1 cells."

Minor points
Fig S1A: The SFTPC signal is very hard to see in row 3, column 2 (I assume this is supposed to represent positive staining)
A: We added magnifications to the figure EVo that the stainings are easier to see.

Fig 2C legend: Spelling (DIC)
A: We corrected this.

Fig 4E (legend on right), Fig S4A,B,F,H: The text is too small to read
A: We improved the readability of the mentioned figures.

Line 102: A reference, and ideally a brief justification, is required to justify negative sorting of NGFR
A: NGFR is a well-established surface molecule used to sort lung basal cells, the stem cells of the airway epithelium. We added references for this to the manuscript (https://doi.org/10.1073/pnas.0906850106 and https://doi.org/10.1165/rcmb.2020-0464OC)

Line 563: I assume this should read 10 million cells/ml
A: Good catch!

Line 710: Spelling (Quiagen)
A: Thanks

Reviewer #2 (Remarks on Protocol(s)):

Very well written and comprehensive

Reviewer #3 (Remarks to the Author):

In this manuscript, Dost et al. present a new set of protocols for deriving human distal lung organoids with varying differentiation statuses. Using these culture systems, the authors examine how IFN-γ treatment differentially affects organoids depending on the epithelial cell types present. While the study provides improved methods for expanding organoids from primary human lung epithelial cells and includes detailed characterization, several critical concerns remain unresolved, as outlined below.

Major comments:

1. Overall, although the sorting-free approach for the initial culture of lung single-cell suspensions and the inclusion of potential distal progenitor cells capable of generating alveolar lineages may enhance cell survival and expansion, the manuscript does not fully substantiate its conclusions, partly due to the heterogeneity of the starting cell population that gives rise to organoids. Specifically,
a. The authors should contextualize better the rationale for using NGFR to enrich "progenitor cells" for alveolar organoid generation. What does NGFR mark within P0 organoids? The authors should demonstrate what

populations exist in EpCAM+NGFR- cells. Flow cytometry for EpCAM, NGFR and HTII-280 require to show cellular profiling. scRNA-seq of EpCAM+NGFR- vs EpCAM+HTII-280+ cell comparison is required to determine the initial populations in their P0 organoids. Interpretation is further hindered by the lack of scRNA-seq or other profiling data at the critical timepoint between P0 and the subsequent EpCAM+NGFR- sorting, when cellular heterogeneity would be manifested. The authors should include such data to clarify which cell types are present and potentially contribute to the observed progenitor diversity.

A: NGFR is a well-established surface molecule used to sort lung basal cells, the stem cells of the airway epithelium (https://doi.org/10.1073/pnas.0906850106 and https://doi.org/10.1165/rcmb.2020-0464OC). We added these references to the manuscript. Our rational is to exclude basal cells from our alveolar organoid cultures. To satisfy this request, we obtained new lung tissues and sorted p0 organoids at day 14 for EPCAM+/ a) NGFR-/HT2-280+ (HT2+), b) NGFR-/HT2-280- (HT2-), and c) NGFR+ populations for subsequent qPCR profiling (reviewer figure A+B). Because total cell numbers and population sizes vary widely between human lung samples, we were only able to obtain this profiling for 2 lungs. As expected, NGFR+ cells have high expression of basal cell markers KRT5 and TP63 and low expression of Club (SCGB1A1) and AT2 (SFTPC, LAMP3) markers. NGFR-/HT2-280+ cells have high expression of AT2 markers and low expression of basal cell markers. NGFR-/HT2-280- cells fall in between. They express lower levels of AT2 markers than HT2+ cells but higher than NGFR+ cells. They express lower levels of basal cell markers than NGFR+ cells but higher than HT2+ cells. They also express some of the secretoglobins (SCGB3A1/3A2/1A1) but the expression varies between samples and no clear trend is visible. In summary, NGFR-/HT2-280- co-express alveolar and airway (basal and secretory) markers, similar to what can be found in the respiratory bronchioles.

b. What does CEACAM6 specifically mark in this culture? Although the authors state that CEACAM6 is a marker for “RAS” cells based on Basil et al. (2022), CEACAM6 expression is known to span a broad range of distal epithelial populations, including AT0, TRB-SC/RAS, club, goblet, and AT1 cells. Figure 1D also showed heterogeneity of CEACAM6-expressing cells in organoids.

A: As shown in figure 1D and 1G, CEACAM6 expression correlates with reduced, albeit still high, expression of SFTPC and increased expression of the secretory markers SCGB3A1 and SCGB3A2. Figure 1E shows varying degrees of co-expression of CEACAM6 and SFTPC on protein level. The club cell marker SCGB1A1 is not expressed. Moreover, our CEACAM6+ cells in ALVO-EM do not express higher levels of AT1 markers, which is the case for AT0 cells. The expression pattern of the organoid secAT2s most closely aligns with the expression profile of TRB-SCs (Murthy et al, Nature, 2022) or AT2/3A2 cells (Basil et al, Nature, 2022).
The mentioned populations (AT0, TRB-SC, RAS, AT2/3A2) are very recent cell type/cell state discoveries that are not well described or understood yet. Because the populations were described by two separate labs independently, there are different nomenclatures that likely describe overlapping populations. It is likely that these cell types represent a spectrum from secretory to alveolar cells, with varying levels of secretory and alveolar gene co-expression. We do not claim that our organoids contain one specific cell population/state that corresponds to the populations described in vivo. Instead, we chose the term secAT2 cells, because they co-express secretory and alveolar genes, one of the common themes of the newly described populations.

c. What are secAT2 cells? Based on the presented data, these cells appear to resemble AT0-like population, reported by Murthy et al. (2022). However, Murthy et al. demonstrated that AT0 cells exhibit markedly reduced SFTPC expression compared to AT2 cells, whereas the so-called secAT2 cells in this study show SFTPC and LAMP3 levels comparable to AT2 cells (Figure 1G, J,). Does this imply that secAT2 cells are an in vitro artifact, a culture-specific intermediate state, or a transitional population bridging AT2 and AT0 states? Critically, Murthy et al. further demonstrated that AT2 cells transition into AT0 cells and subsequently into TRB-SC (SFTPB+SCGB3A2+SFTPC-) cells upon EGF withdrawal. In contrast, in the current study (Figure EV1E), EGF withdrawal appears to increase both SFTPC and SCGB3A2 expression. The authors also claim that ALVO-EM culture promotes CEACAM6 and SCGB3A2 expression in “AT2” cells (CEACAM6-neg AT2) – is this effect simply attributable to the absence of EGF in ALVO-EM? Moreover, Murthy et al. reported unidirectional differentiation of AT2 toward AT1 or TRB-SC lineages through AT0 states. Given this, the rationale for including secAT2 cells to enrich AT2 cells or to improve alveolar organoid formation requires clearer justification. Are secAT2 and AT2 cells interconvertible under this culture condition? To substantiate these findings, it would be important to determine whether HTII-280+AT2 cells (EpCAM+NGFR-HTII-280+ vs

EpCAM+NGFR-HTII-280-) can give rise to secAT2-like or AT0-like cells – or vice versa – when cultured with EGF or HRG1.

A: We do not claim that our organoids contain one specific cell population/state that corresponds to the populations described in vivo. Our rational for including secAT2 cell is that such a cell type/state likely contributes to alveolar regeneration. In mice, club cells and bronchioalveolar stem cells (BASCs, co-expressing the secretory club cell marker SCGB1A1 and the AT2 marker SFTPC) contribute to alveolar repair. In humans, the respiratory bronchioles contain multiple populations that likewise show secretory-alveolar gene co-expression (AT0, TRB-SC, RAS, AT2/3A2). If and how these cells contribute to alveolar regeneration in humans is not known, but it has been demonstrated that iPSC-derived RAS and primary human RAS cells (HT2-280-/CEACAM6+) can give rise to AT2 cells (Pezet et al, 2025 (doi: 10.1038/s41587-025-02569-0), and Basil, 2022, Nature). This data implies a trajectory from secretory to alveolar cells.

While it has been shown that withdrawal of EGF can cause transdifferentiation of AT2s to TRB-SC through an AT0 state, our organoids are cultured without EGF over a long time period and still contain an AT2 population, indicating that we have two stable progenitor populations in our organoid cultures.

To address the effect of HRG1/EGF in our cultures, we sorted for EpCAM+/NGFR-/HTII-280+ and EpCAM+/NGFR-/HTII-280- as suggested and cultured these two populations in media containing either EGF or HRG1. We show that organoids grown in HRG1 grow better and express higher levels of the AT2 marker SFTPC and the RAS/progenitor marker SCGB3A2 (new figure EV1C+E). We also show that HRG1 (NRG1) and its receptors ERBB3/4 are expressed in alveolar cells (figure EV1D), adding physiological relevance to the culture conditions.

2. Although substituting EGF with the alternative EGFR family ligand HRG1 in alveolar organoid cultures is an interesting modification, it appears to only modestly improve organoid propagation. Previous study using SFFF medium containing EGF (Katsura et al. 2020) already demonstrated long-term subculture beyond 10 passages. In the current study, while the authors report somewhat improved expansion, SFTPC and SFTPA1 expression levels decline after passage 10 (Figure EV1G), restricting organoid use to the first 10 passages. Therefore, the practical benefit of this protocol appears limited. Furthermore, a similar sorting-free approach was previously employed by Salahudeen et al. (2020) to generate human distal lung organoids from primary lung tissues. They used EpCAM+Lysotracker+ cells from P0 organoids, which likely contain AT2 and secAT2 (or AT0 cells) based on the current study. So, it raises an important question – would this strategy provide a more effective means to enrich AT2 and/or potential progenitor-like populations than the current use of EpCAM+NGFR- cells, given the non-specificity of NGFR and the resulting cellular heterogeneity?

A: We refer the referee to page 1 line 37+ of this rebuttalk letter for a detailed explanation regarding the advantages of our system. Another practical benefit is that the FACS/MACS sorting is not done on the day of the tissue processing. In practicality, receiving tissue from the hospital, processing it to single cell suspensions, then sorting and plating the cells is a long and harsh process that reduces cell viability. There are other practical considerations rarely talked about in scientific publications, like the availability of a FACS machine on the day the tissue is received, which can be unpredictable.

Regarding the EpCAM+/Lysotracker+ sorting strategy from Salahudeen et al. (2020): Lysotracker signal corresponds to the maturity level of AT2 cells. However, it has been shown that AT2 "stem/signaling" cells (PMID: 33208946), and also the newly described progenitor populations residing in the respiratory bronchioles (AT0, TRB-SC, RAS) show lower levels of canonical AT2 markers. It makes sense that proliferating AT2 progenitor cells would not be at the same level of maturity as quiescent AT2 cells that produce high levels of surfactant. AT2 progenitor cells would therefore likely have lower levels of lamellar bodies and lysotracker staining, which is why we think that lysotracker sorting is a less inclusive approach than our sorting strategy. We see the heterogeneity of our ALVO-EM cultures as an advantage because it gives us the opportunity to start studying the secretory-alveolar populations in vitro.

3. Regarding the use of HRG1, the signaling aspects related to this alteration require further exploration. Since HRG1 does not bind to EGFR but rather ERBB3 and ERBB4, what are the levels of expression of each of these receptors in secAT2 and AT2 cells during organoid culture? Ideally, this should be assessed at the timepoint following the unsorted culture of lung single cell suspensions at day 14 prior to sorting for further organoid culture. Are there differences in downstream signaling between these populations when cultured with

EGF vs. HRG1? Does HRG1 specifically drive the generation of secAT2 cells from AT2 cells, or is this effect simply due to the absence of EGF, as noted above? Finally, what are the cellular sources of HRG1 in lung tissues, and what is the expression pattern of its receptors within these tissues?

A: We added lung tissue data showing that HRG1 (NRG1) and its receptors ERBB3/4 are expressed in alveolar cells (figure EV1D), adding physiological relevance to the culture conditions. Moreover, ERBB3 and ERBB4 are expressed in our organoid cultures, both in the AT2 and secAT2 populations (reviewer figure D). To address the effect of HRG1/EGF in our cultures, we sorted for EpCAM+/NGFR-/HTII-280+ and EpCAM+/NGFR-/HTII-280- and cultured these two populations in media containing either EGF or HRG1. We show that organoids cultured in HRG1 grow better and express higher levels of the AT2 marker SFTPC and the RAS/progenitor marker SCGB3A2 (new figure EV1C+E). We also show that HT2- derived organoid cultures still express high levels of SFTPC, but lower than HT2+ derived organoids. This indicates that both the initial population (HT2+/-) and the culture conditions (HRG1/EGF) affect the identify of outgrowing cells.

4. In Figure EV1D and E, organoids cultured without HRG1 and EGF revealed poor organoid formation but higher expression levels of SFTPC and AGER. The authors should provide an explanation for this observation.

A: Removal of these factors decreases proliferation of the progenitor cells. In the absence of these pro-proliferative and pro-stemness cues, the cells likely mature/differentiate to either AT2 or AT1 cells. However, this effect is very small compared to our AT1 differentiation protocol where AGER is upregulated almost 100 fold (figure 3I). We now restructured figure 1, following reviewers' requests, and removed the data referred to in this comment. In the end, the viability and growth of cultures without EGF or HRG1 was so poor that gene expression analysis on these cells did not add any valuable information to the manuscript.

5. Single cell RNA-seq data from three separate patients of organoids from initial single cell culture in ALVO-EM (Figure 1D/S1H-I), and after switching to ALVO-EM, AT1/2-M, and AT2-MM (Figure 4C-E/S4A-C), clearly show that one patient (Lu39) is responsible for the majority of 3 separate clusters in Fig. 1/S1, and furthermore is responsible for the vast majority of neuroendocrine-like cells from the different culturing conditions in Figure 4/S4 (see most pertinently Figure EV4C). However, the authors do not refer to this clear demarcation in the Results section related to each of these figures. The authors should update the Results section to communicate to the readers that this is the case, probably attributable to heterogeneity in human samples taken for organoid culture. Furthermore, the authors may want to soften their interpretation of these results as representative of general principles of neuroendocrine phenotype emergence in DCI-containing organoid cultures (see Discussion, line 425-430), given that 2/3 patient samples do not seem to appreciably show this conversion.

A: We added qPCR data showing that we also observe upregulation of ASCL1, an important transcription factor of neuroendocrine identity, in Lu37 and Lu38 (figure EV4D). The qPCR shows that the baseline expression of ASCL1 is highest in Lu39, which might be the reason why the scRNA-Seq mainly picked it up in this line. While the neuroendocrine phenotype is not the focus of the paper, we think that it is an interesting observation to point out in the discussion, and this observation is not only based on our own data but also data from the referenced publication (Lim et al, 2025).

6. In Figure 2A, what was the initial cell population used for this experiment? Did the authors test whether AT2 and secAT2 cells differ in their capacity to differentiate into mature AT2 cells?

A: Unless stated otherwise, the initial cell population is ALVO-EM cultured organoid-derived cells. To answer the question whether AT2 (C6+) and secAT2 (C6-) cells differ in their AT2 maturation capacity, we sorted these two populations and exposed them to our AT2-MM conditions. After the maturation period, both populations showed high levels of Nile red staining compared to AO-derived cells, with no clear trend among the three tested lines (reviewer figure E).

Given the heterogeneity of this culture, it is surprising that minimal differences were observed in response to the screening factors. Furthermore, in Figure 2D, the majority of cells in the ALVO-EM condition do not express HTII-280 despite of their high expression of AT2 markers shown in Figure 1. Additionally, in the AT2-

MM condition, one organoid contains Nile red+ lipids but is negative for HTII-280, whereas another organoid shows lower lipid content but high HTII-280 expression. How do the authors explain this discrepancy?
A: It has been observed that "HTII-280 is a less useful marker for subculture of AT2 cells due to loss of expression during culture." (PMID: 32272001). This is why we show maturation with functional dyes such as lysotracker and nile red instead. We agree that the presented image was not optimal so we removed it from the figure. Instead, we added a flow histogram and qPCR analysis of FACS-sorted Nile red-low and Nile red-high cells in AT2-MM to show that Nile red intensity indeed correlated with AT2-marker expression (figure EV2D and S2E). It is also noteworthy that by flow analysis, all AT2-MM-derived cells have high levels of Nile red staining, comparable to primary tissue AT2 cells and much higher than airway organoid (AO)-derived cells (figure EV2D). We therefore do not think that there is noteworthy heterogeneity in the AT2-MM organoids.

Since DCI is known to promote alveolar maturation in both iPSC-derived and fetal lung organoids, a similar effect is likely in primary alveolar organoids. Does HRG1 modulate this maturation process differently than EGF?
A: Both iPSC- and fetal-derived organoids are in a much earlier developmental state compared to primary adult cells, and increased maturation with DCI has to our knowledge not been described in adult AT2s. To answer the question regarding HRG1/EGF, we compared the maturation of C6- and C6+ cells in AT2-MM (which contains HRG1) and AT2-MM without HRG1 with the addition of EGF. We found that HRG1-containing media led to slightly higher Nile red signal in the C6- cells compared to EGF-containing media, while there was no difference in C6+ cells (reviewer figure F). Therefore, HRG1 has a slight advantage over EGF when it comes to AT2 maturation.

7. LATS-i has previously been reported to induce AT1 differentiation in both iPSC- and primary lung tissue-derived alveolar organoids (Burgess et al. 2024). In this current study (Figure 3), adding LATS-i to ALVO-EM induces upregulation of AT1 markers, but the cells do not show AT1 phenotypes; some $AGER^{+}$ or $CAV1^{+}$ cells remain proliferative, suggesting that AT1-like cells can divide – an event that does not occur in lung tissue. Moreover, the majority of cells co-express AT2 markers and/or SCGB3A1 and SCGB3A2, indicative of transitional or immature state. In Figure EV3D, the claim that AT1/2-M organoids retain a mixture of AT1 and AT2 cells is likely misleading; the data suggest that many cells co-express AT1 and AT2 markers, consistent with immature or transitioning cells rather than a true mixture of two distinct cell types. Critically, in Figure 3I, LAMP3 expression remains stable under the AT1/2-M condition. Given these observations, the current culture condition favors the maintenance of transitional or immature cells, despite LATS-i stimulation, and still requires additional cues such as ECM components or mechanical signals, similar to previous methods. It would be important to clarify what advantages and novel insights this protocol offers over prior approaches.
A: In Burgess et al (PMID: 38642558), the authors induce differentiation of primary AT2 cells with their L+DCI medium and compare it to SFFF medium (PMID: 35355018). These two mediums are very different, most notably in the absence of CHIR in the differentiation medium. Other published strategies include the addition of human serum to induce differentiation (PMID: 33128895). The strength of our approach is that the sole variable between our expansion and our differentiation medium is LATS-inhibitor. This minimizes confounding factors when comparing progenitor cells to differentiated cells and it enables precise mechanistic studies. Furthermore, we do acknowledge the presence of immature or transitioning cells in the manuscript, e.g. in the discussion "A biological explanation for the underrepresentation is that additional signals or prolonged culture periods might be required for terminal AT1 differentiation. Nevertheless, we posit that the presence of transitional states in our organoids better reflects regenerative conditions and provides a valuable platform for optimizing AT1 differentiation in therapeutic contexts."

8. What is the rationale for adding IL-1β to all organoid culture conditions during the first 0–3 days? Could this early exposure precondition the cells and thereby confound their responsiveness to the additional cytokines tested in Figure 5, making it difficult to interpret the screening results?
A: We only add IL-1β at p0, as it has been reported to increase organoid outgrowth (PMID: 33128895). The screening of the cytokines was done at passage 6/7 after several months of in vitro expansion. We do not think that the cells and their daughter cells have such a long-lived IL-1β memory. Even if they did, coming from human lungs, the cells would most certainly have been exposed to IL-1β in vivo at some point due to

infections such as the common cold, which is something we cannot control for.

9. The findings related to differential effects of IFN-γ on cells from different organoid conditions are intriguing, but overall analysis is limited. Given the presence of multiple cell types and states within these organoids (Figure 4) and the lack of a cell-tracking system, it remains unclear which specific populations are actually responding to IFN-γ, especially since the analysis relies on qPCR of only one or two genes and viability assays on bulk organoids. Furthermore, AT1/2-M organoids seem to retain immature or transitional cells instead of "AT1" cells.
A: Figure 5G shows that both AT2 and secAT2s respond with increased proliferation to IFN. Additionally, we changed "AT1" to "AT1-like" throughout the text and in our summary figure 5K.

10. 10. In Major et al. (PMID: 32527928), although IFN-γ is not investigated (rather Type I and III interferons), it is shown that interferon signaling is deleterious for lung epithelial regeneration in vivo during influenza A models. In Lin et al. (PMID: 39352385), the authors show that IFN-γ signaling can lead to dysplastic lung epithelial regeneration during an in vivo influenza A model, including differentiation of dysplastic-like cells from AT2 cells in organoid culture. The authors show in Figure 5/S5 that IFN-γ exposure up to day 10 leads to ALVO-EM expansion. Does this exposure to IFN-γ have consequences for later differentiation characteristics of these populations? Experiments to address this may involve the short-term exposure to IFN-γ of ALVO-EM organoids, (up to day 10, since longer exposure seems to have deleterious effects – see Figure EV5F), with subsequent passaging to AT1/2-M to see if their differentiation ability is altered compared to controls.
A: To answer this question, we performed the following experiment: We exposed organoids in ALVO-EM to 1 ng/ml IFN-g for 7 days, removed the cytokine for 3 days, then switched the organoids to AT1/2-M differentiation condition for an additional 7 days. We performed gene expression analysis on day 10 (start of differentiation) and day 17 (end of differentiation). We found that the exposure to IFN-g did not influence subsequent AT1 differentiation capacity. The previously exposed cells showed upregulation of AT1 markers CAV1 and AGER to the same level as the control cells (figure EV5I).

11. In Figure 6, how do macrophages influence the cellular phenotypes of ALVO-EM and AT1/2-M organoids beyond their effects on organoid growth? Do they also impact differentiation? What specific evidence supports the author's conclusion that pre-treated macrophages selectively modulate secAT2/AT2 proliferation and AT1 cell death in these experiments?
A: Our goal with these experiments is to show that we see the same effect on our progenitor cultures (increased growth) and differentiated cultures (decreased growth) with macrophage-secreted IFNg as we see with the addition of recombinant IFNg, adding more physiological relevance to the model. Since our focus is not on macrophage-epithelial interactions, we feel that further characterization is beyond the scope of this manuscript.

Minor comments:

1. Please update your diagram in Figure 1A and your methods to clarify your final culture conditions for initial investigation of organoid growth characteristics in Figure 1/S1 – it seems that ultimately SFFF was used for the initial unsorted 14 day culture, and ALVO-EM and other types were used upon passaging for p1 and further, but this could use clarification.
A: We apologize that this was not clearly presented. We have now rewritten this part of the manuscript and added the final media information to figure 1A and also adjusted table EV1. To establish the organoid lines that we used in this publication, we used both EGF and HRG1 in p0 (which would correspond to SFFF media with the addition of HRG1). For clarification, we now termed this media ALVO-p0. From p1 onwards, we switched to ALVO-EM.

2. The UMAPs in Figure 1D combined with the text explanations are laborious to interpret with the cluster assignments not given anywhere in Figure 1D. Furthermore, related to Major Point 4 above, it is critical for the interpretation of this scRNA-seq data that certain clusters seem to be derived almost entirely from the cells of one patient (Lu39). Thus, the data in Figure EV1H would be better served alongside the UMAPs in Figure 1D.

A: We added the cluster and donor ID annotations to the main figure 1D for easier interpretation. For further clarification, we focus our DEG dotplot on the three large main clusters that are made up of all three donors (figure EV1I). We also added a rescaling step that was previously missing, which is the reason why the UMAP shape looks different than before. This does not change the data or the interpretation thereof.

3. Related to the data in Figure 1 – for the secAT2 sorting and clustering, please clarify the details of the passage number this was done at – for the scRNA-seq, presumably this was after passage 1 (~14 days after sorting/plating of Epcam+ NGFR- cells and culture in ALVO-EM), and for the secAT2 organoids, these were presumably sorted at p1 and the data displayed in Figure 1H-I are from p2 after this? These important details are not currently included in the figure legends or Methods.

A: We added the passage information to the figure legends as requested. The scRNA-Seq was done at p5. The data from figure 1H-I are at p4. Because we can expand, freeze, and thaw our organoid lines as needed, there is no "linear time line", but all experiments are done before passage 10, as stated in the manuscript.

4. Line 119: what is the rationale saying a 'more progenitor-like state' based on the higher expression of SCGB3A2?

A: We change the phrasing to "(…) the organoids expressed higher levels of the (…)secretory gene SCGB3A2 (figure EV1E), recently described as a marker for multiple alveolar progenitor populations"

5. Line 160: Should there be a reference to Figure 1H here? Figure 1H is seemingly not currently referenced anywhere in the paper as of now.

A: Indeed, thank you for pointing this out. This is now corrected

6. Line 546: "canonical tube" – the authors likely meant to say "conical tube"

A: Good catch! Corrected

7. In the Methods section "Making single cell suspensions and passaging organoids", please clarify what types of suspension culture plates were used and how many "drops" of cells/BME were included on each plate (line 555), as these are important details for recapitulation of these methods.

A: We added these details to the methods section.

Reviewer #3 (Remarks on Protocol(s)):

Overall, the authors provide the detailed methods for this study. Please also see our comments to the authors.

1st Revision - Editorial Decision **24th Feb 2026**

Dear Hans, dear Antonella,

Thank you again for transferring your amended manuscript (EMBOJ-2025-123201-T) to The EMBO Journal, together with a point-by-point response to the issues raised during peer-review at the previous venue. Please accept my apologies for the unusual protraction in getting back to you due to delayed expert input and detailed discussion in the editorial team. Your revised study was sent back to the referees for their scientific reassessment, and we have received detailed re-reports from all of them, which I enclose below. As you will see, the reviewers state that the work has been reasonably enhanced by the revisions and they can now support publication at the EMBO Journal, pending minor revision.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Please carefully consider the remaining minor points raised by referees #2 and #3 by adding complementary results and-or amending the manuscript text - discussion of the results and related literature - where appropriate.

Also, we now need you to take care of a number of minor issues related to formatting and data annotation, as detailed below.

Please submit a revised version of the manuscript using the link enclosed below.

As you might remember from previous experience, every paper at the EMBO Journal includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Finally, we have noted that the submitted version of your article is also posted on the preprint platform bioRxiv. We would appreciate if you could alert bioRxiv on the acceptance of this manuscript at The EMBO Journal in order to allow for an update of the entry status. Thank you in advance!

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to hearing from you and receiving your final revised version of the manuscript.

Best regards,

Daniel

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Please add up to five keywords to your study.

>> Author Contributions: Remove the author contributions information from the manuscript text. Note that CRediT has replaced the traditional author contributions section as of now because it offers a systematic machine-readable author contributions format that allows for more effective research assessment. and use the free text boxes beneath each contributing author's name to add specific details on the author's contribution.

More information is available in our guide to authors.
https://www.embopress.org/page/journal/14602075/authorguide

>> Please correct the order of the manuscript sections to: Abstract / Introduction / Results / Discussion / Methods / Data Availability / Acknowledgements / Disclosure and Competing Interests Statement / References / Figure Legends

>> Adjust the title of the 'Summary' section to 'Abstract'.

>> Figure captions are to be included in main manuscript file (a separate file is not needed).

>> References: please adjust reference format to EMBO Journal format, 10 authors et al. DOIs should only be used for preprints and datasets that have not been published yet

>> Funding: check the following duplicate grant entries in our online system: Dutch Research Counsel (NWO) and Longfonds (Lung Foundation Netherlands).

>> Figure callouts: currently missing for Figure 3I; there are callouts for Fig. S2D and S2E, S4A/C, S5C, however no such figures. (supplementary figures are not allowed; only EV figures or Appendix figures).

>> Dataset EV legends: datasets are complex Excel tables that have multiple rows, columns and sheets but currently cannot be properly converted to PDF; the nomenclature should be adjusted to 'Dataset EV1', etc. (Tables EV1-EV4 to be uploaded as Datasets, callouts to be adjusted in manuscript. Check Table EV5 as well). Captions are to be included in the file itself as a separate sheet.

>> 'Disclosure and Competing Interests Statement'. Please add a statement: 'H.C. is a member of the EMBO Journal editorial advisory board.'.

>> Data availability section: remove the referee token and make sure the GEO dataset is made publicly accessible.

>> Consider additional changes and comments from our production team as indicated below:

Figure Legends - Comments

- Please note that the exact p values are not provided in the legend of figure 3I"
- Please note that information related to n is missing in the legends of figures 1B, G, H, J; 2B, D, E, F; 3I, 4F, 5A-G, I, J
- Please note that the error bars are not defined in the legend of figure 2E "

Further information is available in our Guide For Authors: https://link.springer.com/journal/44318/submission-guidelines

---

Referee #1:

The manuscript highlights a modified protocol of generating and maintaining alveolar organoids from isolated epithelial cells that does not rely on previous Ats cell definition (HTII-280) which has likely excluded defined progenitor cell subsets, derived from primary human lobectomy material. The authors use this model to demonstrate a role of recombinant IFNy on organoid cell population behaviour, revealing different actions toward AT2 vs AT1, which might be relevant for lung regeneration.

The manuscript has now been improved by different aspects, some mentioned here:

In the revised version of the manuscript, the authors now add data to better highlight the novelty over previously published protocols that were based on HTII-280 AT2 cell selection. It is also stated now more clearly what the further improvements over published models are.

Also, the introduction is no longer focused on COPD, but in regeneration in general, which is appreciated as the organoid model is not a COPD model per se. In addition, information on donors (particularly regarding preexisting chronic lung disease/COPD) has been added, and information on statistics in the figure legends.

As to the heterogeneity of their cultures, the authors state that this is a strength of their model, however, when used to model defined aspects of lung regeneration, it might also pose difficulties. In a final version of their ms, the authors might at least state this limitation in comparison to iPSC-derived models.

Referee #2:

I was pleased to receive the resubmitted version of this manuscript entitled "Interferon-γ selectively promotes survival of alveolar progenitor cells in a human organoid model" which is now being considered for publication in EMBO J.

The authors describe the development of a novel approach to adult human alveolar epithelial culture modelling with particular emphasis on an inclusive approach to cell isolation to ensure the capture of all potential alveolar progenitors and defining media components required for AT2-AT1 differentiation. Having undertaken detailed characterisation, they then use this model to identify the differing role of Interferon-γ on AT1 and AT2 survival. Their findings provide a transcriptional analysis-focused dataset which will nonetheless provide a useful platform for further refinement and functional analysis of the cell types that are / can be derived using this approach.

The resubmitted version has been significantly amended to reflect the reviewers' comments and, in my view, is strengthened. This is particularly the case for the first part of the results section in which they provide further experimental evidence to support their stance that using HTII-280 sorting from primary tissue excludes a significant AT2 population which would otherwise be able to form viable alveolar organoids. The rewording of this section also improves readability for the non-expert.

I just have a few minor observations, then would support acceptance of the manuscript once addressed:

1. The ability to define AT2 (C6-) and secAT2 (C6+) transcriptionally and then sort them using a cell surface marker is a promising development. However, in this manuscript at least, these two populations behave in a very similar manner when placed in differentiation medium and/or challenged with IFNγ. The discussion would benefit from a comment on this to highlight to the reader the potential importance of being able to separate the populations despite there being no significant functional differences demonstrated in this work.
2. There are several places in which the formatting and font of the additional references need aligning with the rest of the manuscript.
3. On page 8, line 279 and 281 I think the figure reference should be EV3G, not EV3F.
4. Typo page 11 line 389 (figure EVV5E).

Referee #3:

Summary

The authors present an improved protocol for generating human primary distal lung organoids, along with detailed phenotypic analyses that add an additional layer to our understanding of heterogeneity within the human distal lung epithelium. They use negative selection for NGFR to exclude airway basal cells while retaining distal airway populations that may contribute to, or serve as progenitors for, alveolar epithelial cells. The authors then substitute EGF with HRG1, which enhances organoid expansion. Using this system, they show that IFN-γ is cytotoxic to AT1 cells while promoting the survival and proliferation of AT2 cells. Overall, the manuscript provides careful characterization and would be of interest to the field.

In general, the revised manuscript and the authors' responses address the major concerns raised previously, including issues related to the initial sorting strategy, starting cell populations, and organoid heterogeneity, particularly with respect to secAT2 cells. However, a few remaining points merit further consideration.

Major comments:

1. Reviewer Figures A-B are critical for readers to understand the initial culture inputs at the p0->p1 transition and should be included in the supplemental material, particularly in the absence of more comprehensive profiling (e.g., scRNA-seq of NGFR- cells from unsorted/plated distal lung versus HTII-280+ sorted populations after 14 days of culture under these conditions), which would provide a clearer assessment of starting cell heterogeneity but may not be available. In addition, the qPCR data show that in one of the two donors screened, KRT5 and TP63 are expressed at relatively high levels in the NGFR-HTII-280- population, suggesting that a subset of basal-like cells not marked by NGFR may persist in the starting population. This could potentially explain the presence of KRT5-SCGB1A1-SFTPC- organoids in these cultures (lines 137-138), possibly reflecting basal cell-derived outgrowths that have downregulated KRT5 and are restrained by the media conditions. While the optimized organoid system benefits from incorporating diverse cell types and states that may contribute to alveolar differentiation, the presence of heterogeneous airway populations in the initial cultures complicates interpretation. Without single-cell-level tracking or lineage-resolved analyses, some of the authors' conclusions are difficult to fully support. The authors should more clearly acknowledge this critical limitation in the Discussion and, where appropriate, temper their conclusions accordingly.

2. Further aiding reader understanding of this heterogeneity as it relates to SFTPC+ organoids compared to the KRT5-SCGB1A1-SFTPC- organoids (lines 137-138), it would be highly useful to include some quantification of the ratio of these types of organoids and its stability over early vs. late passages.

3. Fig. EV1D: The manuscript text describing the reanalyzed scRNA-seq data from Sikkema et al. 2023 (lines 121-123) states that "HRG/NRG1 and its receptors ERBB3 and ERBB4 are more highly expressed in lung alveolar cells than their family members EGF and EGFR (Fig. EV1D) and HRG1 has a positive effect on the growth and survival of AOs." However, unless the dot plot is mislabeled, this does not appear to be supported by the figure. In particular, ERBB4 shows low "Percent Expressed" in AT1 and AT2 cells (with possibly higher "Average Expression" in AT2 cells), and ERBB4 also appears low in the "Secretory" population that the authors may argue contains distal airway progenitors relevant to their culture strategy. In addition, the wording is confusing and potentially inaccurate in implying that EGF and EGFR are "family members" of ERBB3 and ERBB4; it would be important to clearly distinguish ligands (e.g., HRG/NRG1, EGF) from receptors (ERBB family members, including EGFR/ERBB1, ERBB2, ERBB3, ERBB4). The authors should revise this statement to accurately reflect the EV1D data, clarify which "lung alveolar cells" they are referring to (e.g., AT2 only vs. broader alveolar populations), and more precisely describe receptor versus ligand expression. It may also be preferable to emphasize the presence of ERBB3/ERBB4 (even if ERBB4 is low) as rationale for investigating HRG1, rather than overstating relative expression levels, ideally supported by ERBB3/ERBB4 expression in the authors' own organoid scRNA-seq data (e.g., Reviewer Fig. D).

4. The data presented in Reviewer Fig. E, concerning the differentiation potential of CEACAM6+ versus CEACAM6- organoids, are important for readers to fully appreciate the distinct behaviors of these cell populations in culture. These results should therefore be included in the Supplemental Figs.

5. Line 125, this appears to reference the wrong figure - should be Fig. EV1C.

A: We thank the reviewers for their comments and their approval of the revised version of the manuscript. We addressed any remaining concerns below.

Referee #1:

The manuscript highlights a modified protocol of generating and maintaining alveolar organoids from isolated epithelial cells that does not rely on previous Ats cell definition (HTII-280) which has likely excluded defined progenitor cell subsets, derived from primary human lobectomy material. The authors use this model to demonstrate a role of recombinant IFNy on organoid cell population behaviour, revealing different actions toward AT2 vs AT1, which might be relevant for lung regeneration.

The manuscript has now been improved by different aspects, some mentioned here:

In the revised version of the manuscript, the authors now add data to better highlight the novelty over previously published protocols that were based on HTII-280 AT2 cell selection. It is also stated now more clearly what the further improvements over published models are.

Also, the introduction is no longer focused on COPD, but in regeneration in general, which is appreciated as the organoid model is not a COPD model per se. In addition, information on donors (particularly regarding preexisting chronic lung disease/COPD) has been added, and information on statistics in the figure legends.

As to the heterogeneity of their cultures, the authors state that this is a strength of their model, however, when used to model defined aspects of lung regeneration, it might also pose difficulties. In a final version of their ms, the authors might at least state this limitation in comparison to iPSC-derived models.

A: We thank the reviewer for their suggestions and are pleased to hear that we have improved the manuscript to be accepted for publication. To satisfy the remaining comment of the reviewer, we have added the following paragraph to the discussion: “It should be noted that we observed marked heterogeneity between organoid lines in both marker expression and the magnitude of responses to stimuli such as IFN-γ. This variability likely reflects differences between donors and is therefore expected when working with primary human material. Organoid models derived from adult primary cells, which represent a more mature cellular state than fetal or iPSC-derived lung cells, are particularly valuable for modeling age-related lung diseases and for identifying therapeutic strategies to enhance alveolar regeneration in adults. At the same time, the intrinsic heterogeneity between primary cells from different donors can pose challenges and represents a limitation of the system. Elucidating the sources of these heterogeneous responses may provide insight into patient-to-patient variability and ultimately help inform patient stratification in the future.”

Referee #2:

I was pleased to receive the resubmitted version of this manuscript entitled "Interferon-γ selectively promotes survival of alveolar progenitor cells in a human organoid model" which is now being considered for publication in EMBO J.

The authors describe the development of a novel approach to adult human alveolar epithelial culture modelling with particular emphasis on an inclusive approach to cell isolation to ensure the capture of all

potential alveolar progenitors and defining media components required for AT2-AT1 differentiation. Having undertaken detailed characterisation, they then use this model to identify the differing role of Interferon-γ on AT1 and AT2 survival. Their findings provide a transcriptional analysis-focused dataset which will nonetheless provide a useful platform for further refinement and functional analysis of the cell types that are / can be derived using this approach.

The resubmitted version has been significantly amended to reflect the reviewers' comments and, in my view, is strengthened. This is particularly the case for the first part of the results section in which they provide further experimental evidence to support their stance that using HTII-280 sorting from primary tissue excludes a significant AT2 population which would otherwise be able to form viable alveolar organoids. The rewording of this section also improves readability for the non-expert.

A: We are grateful for the reviewer's suggestions which helped us improved the manuscript.

I just have a few minor observations, then would support acceptance of the manuscript once addressed:

1. The ability to define AT2 (C6-) and secAT2 (C6+) transcriptionally and then sort them using a cell surface marker is a promising development. However, in this manuscript at least, these two populations behave in a very similar manner when placed in differentiation medium and/or challenged with IFNγ. The discussion would benefit from a comment on this to highlight to the reader the potential importance of being able to separate the populations despite there being no significant functional differences demonstrated in this work.

A: We agree with this comment and added the following paragraph to the discussion: "Although we identified transcriptional differences between the AT2 and secAT2 populations and showed that they can be enriched using a C6-based sorting strategy, we have not yet observed clear functional differences between the two populations. Future studies should therefore assess their long-term self-renewal capacity, engraftment potential, and ability to transdifferentiate toward airway epithelial fates. Notably, the co-expression of multiple lineage markers may signify enhanced cellular plasticity and a greater potential to differentiate into diverse lung epithelial cell types."

2. There are several places in which the formatting and font of the additional references need aligning with the rest of the manuscript.

A: We have corrected the formatting.

3. On page 8, line 279 and 281 I think the figure reference should be EV3G, not EV3F.

A: We have made this correction.

4. Typo page 11 line 389 (figure EVV5E).
A: We fixed this typo.

Referee #3:

Summary

The authors present an improved protocol for generating human primary distal lung organoids, along with detailed phenotypic analyses that add an additional layer to our understanding of heterogeneity within the human distal lung epithelium. They use negative selection for NGFR to exclude airway basal cells while retaining distal airway populations that may contribute to, or serve as progenitors for, alveolar epithelial cells. The authors then substitute EGF with HRG1, which enhances organoid expansion. Using this system, they show that IFN-γ is cytotoxic to AT1 cells while promoting the survival and proliferation of AT2 cells. Overall, the manuscript provides careful characterization and would be of interest to the field.

In general, the revised manuscript and the authors' responses address the major concerns raised previously, including issues related to the initial sorting strategy, starting cell populations, and organoid heterogeneity, particularly with respect to secAT2 cells. However, a few remaining points merit further consideration.

Major comments:

1. Reviewer Figures A-B are critical for readers to understand the initial culture inputs at the p0->p1 transition and should be included in the supplemental material, particularly in the absence of more comprehensive profiling (e.g., scRNA-seq of NGFR- cells from unsorted/plated distal lung versus HTII-280+ sorted populations after 14 days of culture under these conditions), which would provide a clearer assessment of starting cell heterogeneity but may not be available. In addition, the qPCR data show that in one of the two donors screened, KRT5 and TP63 are expressed at relatively high levels in the NGFR-HTII-280- population, suggesting that a subset of basal-like cells not marked by NGFR may persist in the starting population. This could potentially explain the presence of KRT5-SCGB1A1-SFTPC- organoids in these cultures (lines 137-138), possibly reflecting basal cell-derived outgrowths that have downregulated KRT5 and are restrained by the media conditions. While the optimized organoid system benefits from incorporating diverse cell types and states that may contribute to alveolar differentiation, the presence of heterogeneous airway populations in the initial cultures complicates interpretation. Without single-cell-level tracking or lineage-resolved analyses, some of theauthors' conclusions are difficult to fully support. The authors should more clearly acknowledge this critical limitation in the Discussion and, where appropriate, temper their conclusions accordingly.

A: We thank the reviewer for this comment. We do not feel comfortable adding reviewer figure B to the manuscript, as we only have data of 2 lungs for this analysis. Moreover, we do acknowledge that the starting population for organoids is heterogenous, but our scRNA Seq shows that there are no basal-like cells in our system. We do not think that we overstated any interpretations.

2. Further aiding reader understanding of this heterogeneity as it relates to SFTPC+ organoids compared to the KRT5-SCGB1A1-SFTPC- organoids (lines 137-138), it would be highly useful to include some quantification of the ratio of these types of organoids and its stability over early vs. late passages.

A: We agree that this information could be useful for some research questions, but this data would not directly add to the conclusions presented in this manuscript.

3. Fig. EV1D: The manuscript text describing the reanalyzed scRNA-seq data from Sikkema et al. 2023 (lines 121-123) states that "HRG/NRG1 and its receptors ERBB3 and ERBB4 are more highly expressed in lung alveolar cells than their family members EGF and EGFR (Fig. EV1D) and HRG1 has a positive effect on the growth and survival of AOs." However, unless the dot plot is mislabeled, this does not

appear to be supported by the figure. In particular, ERBB4 shows low "Percent Expressed" in AT1 and AT2 cells (with possibly higher "Average Expression" in AT2 cells), and ERBB4 also appears low in the "Secretory" population that the authors may argue contains distal airway progenitors relevant to their culture strategy. In addition, the wording is confusing and potentially inaccurate in implying that EGF and EGFR are "family members" of ERBB3 and ERBB4; it would be important to clearly distinguish ligands (e.g., HRG/NRG1, EGF) from receptors (ERBB family members, including EGFR/ERBB1, ERBB2, ERBB3, ERBB4). The authors should revise thisstatement to accurately reflect the EV1D data, clarify which "lung alveolar cells" they are referring to (e.g., AT2 only vs. broader alveolar populations), and more precisely describe receptor versus ligand expression. It may also be preferable to emphasize the presence of ERBB3/ERBB4 (even if ERBB4 is low) as rationale for investigating HRG1, rather than overstating relative expression levels, ideally supported by ERBB3/ERBB4 expression in the authors' own organoid scRNA-seq data (e.g., Reviewer Fig. D).

A: We now rephrased this sections according to the reviewer's suggestions to: "HRG1/NRG1 and its receptors ERBB3 and ERBB4 are expressed in lung alveolar cells, similar to EGF and its receptor EGFR/ERBB1 (figure EV1D)."

4. The data presented in Reviewer Fig. E, concerning the differentiation potential of CEACAM6+ versus CEACAM6- organoids, are important for readers to fully appreciate the distinct behaviors of these cell populations in culture. These results should therefore be included in the Supplemental Figs.

A: We now added reviewer figure E as figure EV2F to the manuscript.

5. Line 125, this appears to reference the wrong figure - should be Fig. EV1C.

A: We corrected this mistake.

Figure for reviewers removed

Dear Hans, dear Antonella,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

I am thus pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Your manuscript will be processed for publication by EMBO Press. It will be copy edited and you will receive page proofs prior to publication. Please note that you will be contacted by Springer Nature Author Services to complete licensing and payment information.

Please note that it is The EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://www.embopress.org/transparent-process#Review_Process

You may qualify for financial assistance for your publication charges - either via a Springer Nature fully open access agreement or an EMBO initiative. Check your eligibility: https://www.embopress.org/page/journal/14602075/authorguide#chargesguide

Should you be planning a Press Release on your article, please get in contact with embo_production@springernature.com as early as possible in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to The EMBO Journal.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Best regards,

Daniel

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal
EMBO
Postfach 1022-40
Meyerhofstrasse 1
D-69117 Heidelberg
contact@embojournal.org

*****

Please note that it is The EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letters) to be published as an online supplement to each paper. If you should prefer removal of any referee-only figures included in the point-by-point response(s), e.g. because they may still be used for future publication or because they have been reproduced from published work by others, please do let us know immediately via response email.
More information is available here: https://link.springer.com/partners/embo-press/editorial-policies#Peer%20review

EMBO Press Author Checklist

Corresponding Author Name: Hans Clevers
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2025-123201

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures
1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Reagents and Tools table

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Table EV5, referenced in the Reagents and Tools Table

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Methods section
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Methods section

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends, methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	methods
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	methods

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	methods
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Yes	Methods
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Yes	Methods
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	