Peer Review File

Manuscript Title: Human intestinal organoids with an autologous tissue-resident immune compartment

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

A. Summary of the key results

The authors detail a new methodology to generate human intestinal immuno-organoids (IIOs) from adult intestinal tissue, which they have used to study intestinal inflammation generated by a novel cancer therapeutic. They have developed a novel enzyme-free lymphocyte method to isolate intestinal immune cells from tissue, allowing for the isolation of tissue-resident memory T-cells (TRMs), which is a cell population that has been previously difficult to study in-vitro. Importantly, the study collects donor-match peripheral blood mononuclear cells (PBMCs) as a control to assess TRM-specific properties, such as those related to IIO assembly. Microscopy reveals these autologous TRMs self-integrate with epithelial organoids when co-cultured, and single cell RNA sequencing (scRNA-seq) data indicates this assembly is driven by an enrichment of transcriptomic programmes related to cell motility and cytoskeletal rearrangement in TRMs (compared to PBMCs). The applicability of the IIOs to model tissue–specific host physiology was then demonstrated through the replication of colitis induced by a T-cell bispecific (TCB) cancer therapeutic in phase I clinical trials in vitro with TRM-organoid co-cultures (but not PBMC-organoid co-cultures). scRNA-seq data of this process at 4 h and 48 h after drug introduction show inflammation is initiated by the activation of a CD4+ Th1 population, which is subsequently followed by the emergence of an activated CD8+ IEL population. Finally, the paper investigates known and novel inflammation mitigation strategies in the model through antibody neutralisation of TNF-alpha and Rock ½ inhibition, respectively.

B. Originality and significance: if not novel, please include reference

The incorporation of immune cells in intestinal organoids is an active area of research, with many invested in improving these models to better represent host physiology in vitro. Since there are a limited number of studies that have developed such a model, this paper would be of significant interest to the community. Specifically, the self-integration of TRMs into the IIOs is not something that has been previously shown (although other papers have co-cultured organoids with autologous immune cells, see section 7). The scaffold-based crawl-out isolation method developed by the authors has the potential to isolate other lymphocytes (besides TRMs) that can be co-cultured in organoid models, expanding the field's capacity to generate diverse IIOs. Furthermore, the ability to demonstrate cancer therapeutic-

induced colitis in the model is compelling, demonstrating the applicability of IIOs for a wide range of perturbations. However, as the IIOs have only been tested in one specific setting, further testing would be required to truly assess their utility,

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

The authors use flow cytometry in figure 1 and scRNA-seq in figure 2 to profile the lymphocytes present in the immune-organoid models. However, different markers are used with the two methods. Particularly, the widely-used TRM markers CD69 and CD39 are used in figure 1 but not figure 2. It would be useful to have consistent markers or provide justification if this for some reason is not feasible.

There is information missing about data generation, especially with regards to figure 2 and 3, that would improve the reader's understanding of the paper. First, it is unclear how many and which donors are used to generate data in each figure. It appears that one donor was used in figure 2 and a different one in figure 3 based on supplementary table 1, but this is information that should be moved to the main text. It would significantly add to the value of the paper to describe the interindividual variation in immune and epithelial cell cluster proportions and phenotypes, for example by integrating the data from a larger number of donors together. Additionally, there is no information about which region(s) of intestinal tissue was used to derive IIOs and generate scRNA-seq data. Studies have shown interregional differences across the intestinal tract in terms of gene expression, abundance of different cell type populations, areas/mechanism of inflammation, etc. so this information is necessary for contextualising the paper's findings. Finally, there is no experimental evidence or reasoning given for why scRNA-seq data was generated at the 24 h timepoint for figure 2. Is epithelial-TRM integration optimal after 24 hours?

The relevance of the EpCAM-targeting bispecific antibody (solitomab) chosen as the model of cancer immunotherapy is somewhat questionable as this is an experimental treatment currently in phase I trials. Using a more relevant treatment such as immune checkpoint blockade would better enable the assessment of how the proposed organoid models recapitulate cancer immunotherapy-related toxicities.

Related to Figures 4G-J, "Predicted TNF loss", the authors state that they investigated the role of TNF in "promoting differentiation and activation profiles". However, the experiment shows that TNF blockade prevents apoptosis of epithelial cells (which is quite expected based on TNF being a well-known mechanism of T cell cytotoxicity), but doesn't address the predicted effects on the differentiation and activation profiles. It would be useful to test whether the model predicts the effects on differentiation and activation profiles as claimed by for example doing scRNA-seq after TNF blockade.

D. Appropriate use of statistics and treatment of uncertainties

In general, the study would benefit from using a sufficient number of donors to be able to statistically assess the inter-donor variation in scRNA-seq-based immune states and responses.

Minor Comments:
Figure 1b-c: It is unclear how many samples/organoids these data are from. The differences between PBMCs and TRMs could also be statistically evaluated.
Figure 1h: A t-test is used to assess significance but the distribution does not appear normal?
Figure 3b-c: The dots and grey shading are not defined. How many experimental replicates were used?
Figure 4j: What are the biological replicates that the statistical test is performed on? Additionally, the grey shading is not defined.
Extended Data Figure 1b: Error bars are not defined.

E. Conclusions: robustness, validity, reliability

As mentioned in section 3, the robustness of the model and resulting data is not fully known due to the limited number of tested organoids/donors and cancer therapeutics.
In lines 272-273, the authors comment on the identification of the Rho-pathway as a target for reducing T-cell induced inflammation from TCB administration. However, several papers have also investigated the use of Rock inhibitors as cancer therapeutics due to the association of Rock1/2 expression with cancer progression. Because its use as a cancer therapeutic remains somewhat controversial due to off-target effects, limiting the number of drugs that have actually made it to clinical trials, it would be interesting for the authors to comment on the feasibility of targeting the Rho-pathway for reducing side effects of cancer therapeutics.

F. Suggested improvements: experiments, data for possible revision

IHC staining of TRMs in IIOs for conventional TRM markers, such as CD69, ITGA1, and ITGAE, would confirm TRM identity is maintained in co-culture.

Although the scaffold-based crawl-out method for isolating lymphocytes is noted as cytokine-independent, which the authors indicate is important for preserving “tissue-like physiological properties of intestine-derived lymphocytes,” the efficacy of this method for actually doing so is not tested or quantified. Lymphocytes isolated with cytokine- dependent and independent pathways should be compared at the transcriptomic and/or protein level, such as with scRNA-seq and/or IHC staining, respectively. This data should then be compared to those from primary, undissociated tissue to confirm cytokine-independent isolation methods are indeed an improvement over cytokine-dependent methods.
Inclusion of data from tissue and organoids derived from additional donors would bolster the reliability and reproducibility of the data.

Extended data figure 1e indicates the ratio of immune cells to epithelial cells decreases over time, suggesting that immune cells may be losing their TRM identity without appropriate signalling (either from the organoid or from external sources, such as cytokine support). Therefore, it would be insightful to do IHC staining and/or scRNA-seq on TRMs in IIOs that have been in co-culture longer than the data

generated in this paper (e.g., >48 hours), such as 1-2 weeks after IIO generation, to test the robustity of the TRM identity over time.

To investigate further the inflammation mitigation strategy, it would be interesting to test the predicted effects of in silico perturbations (TNF loss) on differentiation and activation profiles using techniques such as scRNA-seq.
In order to better assess the applicability of the model for recapitulating colitis induced by cancer therapeutics, the model could be tested on a more relevant cancer immunotherapeutic(s) that is in wider clinical use, such as immune checkpoint inhibitors (e.g., PD-1 blockade).

G. References: appropriate credit to previous work?

In lines 49-55, studies that have previously generated intestinal organoids containing immune cells are cited. However, two other papers of relevance include:

A 2019 Immunity paper (with an associated 2021 Star Protocols paper: https://www.sciencedirect.com/science/article/pii/S2666166721002264?via=ihub) has described the generation of fetal-tissue derived intestinal organoids containing autologous CD4+ T-cells from the lamina propria: https://www.sciencedirect.com/science/article/pii/S1074761318305363

A 2022 Inflammatory Bowel Diseases paper has described the generation of iPSC-derived intestinal organoids containing tissue-resident immune cells (macrophages): https://academic.oup.com/ibdjournal/article/28/Supplement_1/S57/6514119

H. Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions

Abstract is clear and focuses on the main takeaways of the paper. However, the authors claim their “system recapitulates clinical outcomes”. This is a rather strong statement given that no correlation to clinical outcomes is performed in the study but rather it is speculated how the observations in the organoids may correspond to events occurring in patients.

Introduction provides sufficient background to understand the paper’s contents, but could expand upon the development of intestinal organoid models that have immune components (see section 7).
Conclusion effectively highlights the key findings of the article and contextualises them in the broader literature.

Minor Comments:
Several microscope images are missing scale bars (figure 1d, 1e-g, 1j, 3a, etc.)

Referee #2 (Remarks to the Author):

Testing human-relevant model to validate any discoveries or treatment strategies obtained from using mouse models is essential in the autoimmune disease or cancer biology field. Currently, there are two approaches in the field to address this challenge: using humanized mouse model by transplanting human bone marrow or umbilical cord blood into immunodeficient recipient mice or conductiong in vitro tests with patient-derived organoids (PDO) and patient derived peripheral blood (PPB). However, it remains largely unclear whether PPB behaviors similar to resident immune cells. In this study, Gjorevski's group introduces a novel system in which human Intestinal organoids were co-cultured with autologous Tissue-resident Immune cells (IIO was called) comparing to PB cells. Unexpectedly they discovered that, unlike PB cells that don't actively interact with intestinal organoid epithelial cells, resident immune cells actively engage with epithelial cells within the intestinal organoids. Molecularly, there is a substantial difference between resident immune cells and PB cells, with the former showing enrichment with molecular programs for chemotaxis and migration. Morphologically, resident immune cells are elongated and larger in size than that of PB cells. The IIO system enables to test immune cell response in a setting of clinical related cancer biology. Indeed CD8+ T cells can be activated using the IIO system. This system provides an alternative model to validate any discoveries or treatment strategies obtained from using mouse models. I have the following concerns.

Major concerns

1. It is well known that myeloid cell including macrophages are an active intestinal component that affect both epithelial cells as well as T cells. The IIO system does not include the study for the myeloid derived cells.
2. Though PB cells in this study does not reveal an active interaction with intestinal epithelial cells, resident immune cells including T cells can be exhausted. In this context, it is well documented that recruiting immune cells from circulation is essential. How can authors address this issue?

Minor issues.

Tissue-resident lymphocytes were called as TRMs seems

Referee #3 (Remarks to the Author):

Recaldin et al. have established a co-culture system for human intestinal organoids and tissue-resident memory lymphocytes (TRMs). Previous studies reported similar co-culture systems, but those studies have been difficult to reproduce by others due to availability (scalability and the storability) of immune cells. In contrast, the authors have demonstrated the frozen preservation of TRMs and their subsequent successful co-culture with organoids. This co-culture method may greatly facilitate investigations into

the human mucosal immune system and pave the way for a better understanding of inflammatory bowel diseases. However, there are some technical concerns that need to be addressed before publication.

1. Figure 1d requires control experiments involving co-culture with PBMCs. Additionally, the authors should quantify the distance between each lymphocyte and organoid and calculate statistical differences.
2. The manuscript suggests that some of the TRMs that migrated near the organoids were IELs, but this needs to be confirmed through immune staining for IEL markers. Furthermore, it would be valuable if the authors first sorted IELs from TRMs and then performed co-culture with organoids to confirm whether IELs alone are sufficient to reproduce the co-culture phenotypes, including EpCAM-TCB cytotoxicity. Alternatively, the co-culture phenotypes may require complex interactions among diverse TRMs.
3. In Figure 1e-g, the structures of intestinal organoids appear unusual. They seem stratified. Did the intestinal epithelium form a monolayer with apicobasal polarity? If the epithelium produces laminin-5, the authors may distinguish epithelium-derived laminin from Matrigel. If so, it would be interesting to show that IELs reside between the epithelium and basal membrane, where IELs reside in vivo. The multi-color immunostaining is well-executed, but some colors, such as FABP1 and ECAD signals, are difficult to distinguish in the figure images.
4. In Figure 2a-c, it is unclear to what extent fresh TRMs and co-cultured TRMs are similar.
5. Figure 2h needs a control (PBMC co-culture) for comparison.
6. The authors mentioned "at least three passages" (line 96-97, Extended Data Fig 1e). Passageability is crucial when researchers aim to examine long-term immune responses. However, the number of TRMs seemed scarce in weeks 2-3. The authors need to quantify the rate of TRMs at each passage. Additionally, it's important to demonstrate whether co-cultured organoids retain EpCAM-TCB response after passage. Although IL2 and IL15 were included to prolong the co-culture, it's unclear whether this condition was fully optimized. There is no data showing that IL2+IL15 treatment is superior to the control.
7. In Figure 2a, scRNA-seq data from tissue-resident and blood-derived immune cells alone or co-cultured with organoids is presented. In the extended data figure, it would be helpful to include plots based on the origins (with or without co-culture).
8. There is no detailed information about donors, such as sex, age, or disease status. It is unclear how many donor patients were involved in this study.
9. To fairly assess the data in Figure 4i,j, some control experiments are required: (1) Determine whether TNF-alpha alone can directly kill organoids within 30 minutes or if other factors from TRMs are required. This possibility can be easily addressed by treating organoids with TNF-alpha or organoids+TRMs. (2) Investigate whether ROCKi treatment may suppress TNF-alpha-induced apoptosis independently of TRMs. If this experiment yields positive results, the authors should examine organoids + TNFa vs. organoids + TNFa + ROCKi. (3) Related to (2), quantify the speed of TRM migration with or without ROCKi. (4) Exclude the possibility that ROCKi reduces TNFa production from TRMs.
10. Biological replication details are missing throughout the manuscript. Results in Figure 2h, Figure 3a-c, Figure 4i-j, and Extended Data Fig 1e should be reproduced using at least three independent donors.
11. Before co-culture, organoids were differentiated. Is this step essential? Did TRMs fail to migrate to

organoids when cultured in stem cell conditions? These data should be included.
Minor points:
1. The nomenclature for the same molecules varies depending on the context (gene name and protein). For example, CD49a and ITGA1, CD117 and KIT. It would be helpful to provide explanations for these terms, especially for readers unfamiliar with immunology.
2. Some abbreviations, such as TEM, TCM, and TEMRA, are not explained in the manuscript and should be defined for clarity.

Referee #4 (Remarks to the Author):

In this study Recaldin et al describe human intestinal immuno-organoids (IIOs) that form through self-organization of epithelial organoids and autologous tissue-resident immune cells. Using this culture system and single-cell transcriptomics, the authors model intestinal inflammation triggered by cancer-targeting biologics in patients and propose that the system recapitulates clinical outcomes and the underlying cellular mechanisms. They test the Rho pathway as a putative novel target for mitigating immunotherapy-associated intestinal inflammation and propose that IIOs can be used to broadly study tissue-resident immune responses in the context of tumorigenesis, infectious and autoimmune diseases.
This study is interesting as it reports a novel system to generate organoids with an integrated immune cells compartment. Such a system could be useful to study the precise roles of immune cells in non-lymphoid tissue development, homeostasis or function.
However, these questions were not really addressed by the authors. Although they performed a series of experiments aimed at testing the interplay between immune cells and organoids, the relevance of their findings remains unclear and several of the author's claims remain unsupported.

Major points

In the abstract, the authors propose that "IIO formation was driven by TRM migration and interaction with epithelial cells".
I cannot see any evidence for this claim. Indeed, as far as I can see the authors do not show if or how immune cells impact organoid formation, integrity or function. This was simply not tested. Do tissue-resident lymphocytes impact any of these features? This would be an important starting point for further studies.
Similarly, I cannot see any evidence for the claim that 'IIO formation … was orchestrated by TRM-enriched transcriptomic programs". While the authors show some transcriptomic data consistent with TRM signatures, it remains unclear what precisely was done, and certainly it was not tested how these transcriptional programs impacts IIO formation.

Related to this point, can the authors please explain precisely what was done in experiments related to figure 2. Based on the scheme, it appears that IIO were generated either with tissue-resident

lymphocytes or PBMC. After formation of these IIO, they were disintegrated, and lymphocytes were subjected to scRNAseq. This would suggest two conditions in their analysis. In the text of the manuscript, however, they state “we used single-cell RNA sequencing (scRNA-seq) to analyze donor-matched tissue resident and blood-derived immune cells alone, or cocultured with organoids”. This would suggest a four-way comparison. Please clarify.

Independent from the precise experimental approach, it remains unclear what the purpose of this experiment is. The authors present an analysis that reports that TRM-derived samples were enriched for an features consistent with TRM. That is self-evident; everything else would be a surprise. So, is this all just a confirmation, meant to confirm that the authors are indeed dealing with tissue-resident lymphocytes? At this stage I find it difficult to understand the relevance of figure 2.

Throughout the text, the authors refer to TRM (which mean tissue resident memory T cells). In their abstract they introduce TRM as “tissue-resident lymphocytes”, while later they call them “tissue-resident memory T cells (TRMs)”. Thus, this reviewer was unclear about the precise nature of the authors experiments. While initially, it sounded like the authors were specifically culturing organoids with T cells, it later becomes clear that they use whole immune cells (either from blood or intestine). This should be made clear early on. “TRM” should not be used to refer to “tissue-resident lymphocytes”.

Overall, it remains unclear which tissue-resident immune cell population(s) migrate into the organoid, how they impact the organoid, which cells survive long-term in these cultures and if tissue-resident lymphocytes maintain their identity over longer periods of time. These are critical questions that need to be answered before the study can be fully assessed.

In the second half of their manuscript, the authors test if and how their IIO culture system can be used to address clinically relevant questions. They show that in vitro organoid destruction by an EpCAM-targeting T-cell bispecific (TCB) molecule is tissue immune cells specific and associated with the emergence of an activated population of CD8+ T cells, which progressively acquired intraepithelial and cytotoxic features.

These experiments are interesting but difficult to interpret at this stage as they are lacking some important details and controls.
For example, when were IIO cultures treated? When were immune cells added?

The authors state that they “assessed T cell behaviour at early (5h), mid (24h) and late (48h) timepoints by digesting and staining IIOs for surface and intracellular markers of T-cell activation and cytotoxicity” How was this done? How long were the organoids established before treatment? Were the lymphocytes added just before treatment or much earlier?

These experiments should also contain controls such as circulating memory T cells, which would provide a fair comparison to TRM, rather than PBMC which contain largely naïve cells.
An even better control would be organoids populated with specific immune cell populations, T cell only,

B cell only, myeloid cells, NK cells …

The authors state: “We used scRNA-seq to interrogate the transcriptomic dynamics underlying TCB-dependent TRM activity at the onset (4h) and peak (48h) of epithelial cell targeting. Lymphocyte populations within the integrated dataset were annotated using differential gene expression together with previously published signatures and surface markers revealing diverse T cell, macrophage and B cell populations” Again, here it become clear that the authors use cultures that contain not only TRM, not even only tissue-resident lymphocytes, but indeed all immune cells, even macrophages. This makes it very difficult to assess what precisely is going on. Indeed, it remains unclear if TRM cells are really the ones that lead to organoid destruction. It could be any of the other lymphoid or myeloid cells that have been used to populate the organoid.

In their single cell transcriptomics related to this experiment, the authors focus on two tome points, 4h and 48h after TCB treatment. This seems problematic, given that 48h after treatment the organoid is destroyed. Even after 24h more or less all cells are apoptotic as shown in Fig 3A.

Finally, the authors use a ROCK1/2 inhibitor to demonstrate that the IIO cultures can be used to identify clinically relevant treatments or drugs. While I like the idea, the experiments related to this approach are very underdeveloped. It remains unclear why a ROCK1/2 inhibitor was chosen in the first place. Similarly, it was not tested what the ROCK1/2 inhibitor does to T cells per se. Does it impact T cell activation in general? Survival? Function? Motility? Differentiation? This should be tested in cultures containing isolated naïve T cells, TRM and other lymphocytes or myeloid cells involved in the culture system.

Overall, in the experiments related to the second half of the manuscript, it re4mains unclear which cell type precisely is involved in organoid destruction and how any of the drugs act that were used.

Author Rebuttals to Initial Comments:

We would like to thank all four reviewers for their thorough review and constructive comments. We believe that we have addressed their suggestions, thus increasing the clarity and potential for impact of our manuscript in the field.

Reviewer 1:

1. ***"The incorporation of immune cells in intestinal organoids is an active area of research, with many invested in improving these models to better represent host physiology in vitro. Since there are a limited number of studies that have developed such a model, this paper would be of significant interest to the community. Specifically, the self-integration of TRMs into the IIOs is not something that has been previously shown (although other papers have co-cultured organoids with autologous immune cells, see section 7). The scaffold-based crawl-out isolation method developed by the authors has the potential to isolate other lymphocytes (besides TRMs) that can be co-cultured in organoid models, expanding the field's capacity to generate diverse IIOs. Furthermore, the ability to demonstrate cancer therapeutic-induced colitis in the model is compelling, demonstrating the applicability of IIOs for a wide range of perturbations."***

   We thank the reviewer for recognizing the novelty and potential impact of our manuscript.

2. **"*The authors use flow cytometry in figure 1 and scRNA-seq in figure 2 to profile the lymphocytes present in the immune-organoid models. However, different markers are used with the two methods. Particularly, the widely-used TRM markers CD69 and CD39 are used in figure 1 but not figure 2. It would be useful to have consistent markers or provide justification if this for some reason is not feasible.*"**

   We thank the reviewer for raising this important point. The classical surface markers associated with TRM gut-residence are the C-lectin receptor CD69 and the collagen receptor CD49a (gene name *ITGA1*) (Okla et al, *J Exp Med* 2021; Park et al, *Trends Immunol* 2019). Expression of CD103 (gene name *ITGAE*) is used to delineate cells that sit in the epithelium, so-called intraepithelial lymphocytes (IELs), from those in the lamina propria (Mayassi and Jabri, *Mucosal Immunol* 2018). Although CD39 is detectable to varying degrees on TRM cells, particularly $CD8^+$ TRMs (see Figure 1c and Extended Data Fig. 1d), we do not consider it a classic TRM marker. Rather, it is an indicator of actively responding T cells, and accordingly has been used to identify tumor-antigen reactive T cells in primary and metastatic tumors (Duhen et al, *Nat Commun* 2018).

   Figure 1c is a surface protein analysis of donor-matched blood and gut lymphocytes performed using flow cytometry, with a pre-selected 15-colour panel based on known T cell markers of naivety, memory and tissue residence. The data from Figure 2 is an unbiased assessment of global transcriptomic profiles from matched blood and gut T cells. As is well recognised, stable lineage-defining surface proteins are often poorly detected at the transcript level due to low mRNA turnover. The RNA encoding CD4 for example, a fundamental identifier of helper T cells, is often detected at higher levels in monocytes than T cells (see RNA-seq data in the human protein atlas). It is presumably for these reasons that *CD69* and *ITGAE* were not detected among the most differentially expressed TRM transcripts (*ITGA1* was), despite the "black and white" nature of their expression at the protein level, relative to blood T cells. Nonetheless, all of these markers are now included in the immune cell cluster annotation in Figure 2c (rightmost box).

3. **"*There is information missing about data generation, especially with regards to figure 2 and 3, that would improve the reader's understanding of the paper. First, it is unclear how many and which donors are used to generate data in each figure. It appears that one donor was used in figure 2 and a different one in figure 3 based on supplementary table 1, but this is information that should be moved to the main text. It would significantly add to the value of the paper to*

***describe the interindividual variation in immune and epithelial cell cluster proportions and phenotypes, for example by integrating the data from a larger number of donors together.*"**

In the original submission, the scRNA seq data in Figure 2 comparing matched blood and gut T cells was derived from one biological replicate. We have now added two further replicates to the scRNA-seq comparison. We have also added flow cytometry analysis of critical tissue residency, memory, and activation markers from 10 biological intestinal donors after crawl out isolation, 6 of which have matched blood T cells as a comparator (Extended Data Fig. 1d). Collectively, these data indicate that the phenotype of TRM cells isolated from healthy tissue is remarkably stable, particularly the residency and memory markers that define their identity.

The assessment of response to the EpCAM TCB in Figure 3 was based on one donor (different from that used for the analysis in Figure 2). We have supplemented these observations with additional kinetic flow cytometry analysis of 5 EpCAM TCB-treated cocultures (originating from 5 different donors) (Extend Data Fig. 3d), quantifying critical cytokines and granules (IFNγ, TNF-α and Granzyme B) as well as indicators of activation and proliferation (CD38, HLA-DR and Ki67) across multiple timepoints covering the initiation, peak and end of the response (5h, 24h, 48h). Each of the 5 replicates includes a matched blood immune cell control, demonstrating the heightened sensitivity and rapidity of the TRM response across a range of different donors. The overall outcome, kinetics and the molecular signatures associated with response were strikingly similar across donors, which highlights the robustness of the model, and is also consistent with the clinical observations of nearly every patient treated with EpCAM TCB undergoing intestinal adverse events.

4. **"*Additionally, there is no information about which region(s) of intestinal tissue was used to derive IIOs and generate scRNA-seq data. Studies have shown interregional differences across the intestinal tract in terms of gene expression, abundance of different cell type populations, areas/mechanism of inflammation, etc. so this information is necessary for contextualising the paper's findings*"**

Given our focus on the intestinal pathology associated with an EpCAM bispecific T cell engager, we concentrated on the small intestine, where the severe toxicities in patients strongly manifested. In total, IIO cultures were generated from 24 different biological samples. 20 from the small intestine (7 duodenum, 8 jejunum, 4 ileum, 1 undefined) and 4 from the colon (2 ascending, 2 sigmoid). We acknowledge that there are of course interregional differences in overall immune cell make up, particularly in the likely TCR antigen specificities of the T cells that inhabit the different intestinal regions, and the abundance of permanent and transient lymphoid follicles. However, our focus on extrafollicular, antigen-experienced tissue-resident T cells, enabled us to hone in on the general features that facilitate residence and function within the small intestine, regardless of the exact location. Indeed, we have now performed scRNA-seq analysis on 3 untreated IIO cultures: 1 originating from a duodenal sample, 1 from a jejunal sample and one of undefined small intestine origin (we have added this information in the text for the readers benefit). In these experiments we saw that the signatures that define TRM identity and function relative to circulating T cells were remarkably consistent across all three replicates (Fig. 2d-f). Similarly, response to EpCAM TCB stimulation led to comparable profiles of activation, cytokine expression and proliferation (Extended Data Fig. 3d) across five biological replicates. Furthermore, the impact of TNF-α neutralization or ROCK inhibition on inflammatory responses was also very similar across three replicates from slightly different regions of the intestine (Extended Data Fig. 6a-b and e-g). Overall, we believe these data demonstrate that, while there are likely nuanced interregional differences in TRM behaviour, the broad biological phenomena (motility, epithelial cell surveillance and integration, response to inflammatory triggers and neutralizing therapeutics) are similar.

5. ***"Finally, there is no experimental evidence or reasoning given for why scRNA-seq data was generated at the 24 h timepoint for figure 2. Is epithelial-TRM integration optimal after 24 hours?"***

The integration of TRMs within organoids begins immediately after the start of the co-culture, owing to the high motility of the immune cells, which rapidly encounter and engage with epithelial cells. We consider the integration of TRMs to be complete and stable (rather than optimal) at the 24h time point. Longitudinal flow cytometry experiments have revealed no substantial differences in the TRM numbers, composition and activation profiles within the first four days of co-culture, in the absence of any treatment. Therefore, we consider their transcriptional profile at 24 h to be representative of the entire early culture period.

We have now provided our experimental rationale for performing scRNAseq analysis at the 24-h time point in the main text (lines 127-128).

6. **"*The relevance of the EpCAM-targeting bispecific antibody (solitomab) chosen as the model of cancer immunotherapy is somewhat questionable as this is an experimental treatment currently in phase I trials. Using a more relevant treatment such as immune checkpoint blockade would better enable the assessment of how the proposed organoid models recapitulate cancer immunotherapy-related toxicities.*"**

When initiating the project and assessing suitable use-cases, solitomab was attractive because intestinal symptoms were reported in 100% of patients (Kobenko et al, *Oncoimmunology* 2018). This allowed us to avoid the donor-to-donor variability that complicates model establishment. While the exact mechanism that immune checkpoint inhibitors (ICIs) can induce colitis-like symptoms in around 15% of patients receiving the combination of anti-PD-1 and anti-CTLA-4 is still poorly understood, it is very likely that these molecules exacerbate a contemporaneous immune response to gut microflora (Dougan, *Front Immunol* 2017; Beck et al, *J Clin Oncol* 2006; Luoma et al, *Cell* 2020; Lo et al, *Science* 2024). As our IIO system is a sterile organoid-immune cell coculture lacking bacterial, fungal and food matter, where we do not have *de novo* immune responses to gut antigens, we believe ICIs are not the most appropriate immuno-therapeutic for our model. Nonetheless, we introduced experimental modifications that indicate our system is adaptable to such investigations. Specifically, by including allogeneic dendritic cells in our IIO cultures, which recapitulate the role on APCs presenting antigen to cognate T cells, we made some interesting observations when applying anti-CTLA-4 (ipilimumab) and anti-PD-1 (Nivolumab). Although our standard caspase assay, where the window is relatively small, did not detect a change in overall caspase signal, the more sensitive LDH release-based assay of epithelial damage showed a statistically significant increase following CPI treatment in one of the two biological replicates (Extended Data Fig. 7a-b). This correlated with a significant increase in inflammatory $CD8^+$ TRM cells specifically in this donor relative to the control (Extended Data Fig. 7c-d). These cells are equivalent to the pathogenic $CD8^+$ T cell populations described in gut biopsies of patients actively undergoing ICI-induced colitis (Luoma et al, *Cell* 2020). The donor-matched PBMC condition showed no difference in LDH signal or pathogenic $CD8^+$ T cell numbers. This intriguing observation potentially indicates that the increased inflammatory response of T cells to ICIs only leads to detectable epithelial cell damage when it manifests in the $CD8^+$ TRM cells that embed within the epithelium and express inflammatory factors locally, in the proximity of epithelial cells.

We have included these new data in Ext. Data Fig. 7 of the revised manuscript and discussed the feasibility of applying the model for ICI safety assessment in the main text (lines 330-353).

7. **"*Related to Figures 4G-J, "Predicted TNF loss", the authors state that they investigated the role of TNF in "promoting differentiation and activation profiles". However, the experiment shows that TNF blockade prevents apoptosis of epithelial cells (which is quite expected based on TNF being a well-known mechanism of T cell cytotoxicity), but doesn't address the predicted effects on the differentiation and activation profiles. It would be useful to test whether the model predicts the effects on differentiation and activation profiles as claimed by for example doing scRNA-seq after TNF blockade.*"**

We thank the reviewer for this interesting question. To address this point, we generated IIOs from three different donors, and treated them with EpCAM TCB, in the presence or absence of the TNF-α inhibitor Adalimumab (Ext. Data Fig. 6a-b), and analyzed changes in activation states by flow cytometry. In addition to a reduction in apoptosis, as observed before, we found that TNF-α blockade led to a significant reduction in key activation markers predicted by the original *in silico* perturbation analysis (Extended Data Fig. 5j) in both $CD4^+$ and $CD8^+$ TRMs, including ICAM-1, 4-1BB (*TNFRSF9*), CD25 (*IL2RA*) and Granzyme B, which is likely the factor most responsible for organoid apoptosis. We also observed an attenuated expression of the CCL2 chemokine, which drives additional recruitment of immune cells to the site of inflammation.

It is worth mentioning that we do not believe that the organoid apoptosis observed in these experiments is the single and direct outcome of TNF-α activity. We rather believe that TNF-α is a key mediator of inter-population interaction that ultimately culminates in the formation of highly activated, cytotoxic populations of $CD4^+$ and $CD8^+$ T cells, as shown in Fig. 3 h and i. To verify this, we treated organoids with TNF-α only and found that it was insufficient to drive extensive epithelial apoptosis (Ext. Data Fig. 6d). These data support a role for TNF-α as a key mediator of immune cell activation, rather than a direct agent of cytotoxicity.

8. **"*In general, the study would benefit from using a sufficient number of donors to be able to statistically assess the inter-donor variation in scRNA-seq-based immune states and responses.*"**

The issue of reproducibility is a critical concern for our study and we thank the reviewer for raising this question. In the revised manuscript we extended the donor base for the scRNAseq data probing the differences between PBMCs and TRMs. In Figure 2 and Extended Data Fig. 2 we now provide characterization of the diverse immune cell types and states that characterize our models across three biologically-dependent small intestine donors. We highlight proportional differences in different donor samples as well as reporting critical commonalities that underlay PBMC and TRM transcriptional states and that are robust across donors.

Overall we found that donors from the representative conditions contributed to each annotated T cell population (Fig. 2d), with the exception of cluster 15, a very small population of resting $CD8^+$ TRMs, confirming the reproducibility of the different immune cell states at baseline. The signatures that distinguished gut and blood T cells, namely the enriched migratory, motility and cytoskeletal signatures, remained consistent with our initial observations after incorporation of the new replicates (Fig. 2e-f).

With regards to immune cell states following inflammatory trigger, we reinforced our kinetic scRNA-seq analysis of EpCAM-treated IIOs (Fig 3-4) with flow cytometry analysis of 5 biologically-independent cultures. This demonstrated the response to EpCAM TCB stimulation led to highly reproducible profiles of activation, cytokine expression and proliferation (Extended Data Fig. 3d). Furthermore, the impact of TNF-α neutralization or ROCK inhibition on inflammatory responses was very similar across three further biological replicates (Extended Data Fig. 6a-b and e-g). Overall, these data demonstrate that the original observations are reproducible phenomena that can be consistently modeled to understand immune-epithelial interactions in the context of both homeostasis and inflammation.

9. **"*Figure 1b-c: It is unclear how many samples/organoids these data are from. The differences between PBMCs and TRMs could also be statistically evaluated.*"**

The flow cytometry-derived tSNE analysis presented in Fig. 1b-c is from 1 representative biological replicate. We have now added collated flow cytometry analysis of critical tissue residency, memory, and activation markers from 10 biological gut donors after crawl out isolation, 6 of which have matched blood T cells as a comparator (Extended Data Fig. 1d). Statistical differences have been evaluated and included.

10. **"*Figure 1h: A t-test is used to assess significance but the distribution does not appear normal?*"**

We thank the reviewer for their suggestion. As a result, we have subjected the data set to normality tests, and can indeed confirm it does not follow a Gaussian distribution. Accordingly we ran a Mann-Whitney test on the data set. The difference remains highly statistically significant. The figure legend has been updated to reflect the different statistical test.

11. ***"Figure 3b-c: The dots and grey shading are not defined. How many experimental replicates were used?"***

We apologize for the omission and lack of clarity. The dots in Fig. 3b represent three independent IIO cultures from one biological replicate. The experiment was repeated with three different donors, all yielding similar results.

The solid line and grey shading in Fig. 3c represent an average caspase signal and a standard deviation of duplicate IIO co-cultures from the same donor, treated with increasing concentrations of EpCAM TCB. Given that the dose response experiment shown in Fig. 3b yielded extremely consistent and reproducible results across multiple donors, we did not believe that repeating the granular concentration ramp-up experiment in Fig. 3c would bring much value (while requiring high quantities of starting material - IIOs). We have adjusted the figure legend to clarify what the plot in Fig. 3c represents.

12. **"*Figure 4j: What are the biological replicates that the statistical test is performed on? Additionally, the grey shading is not defined.*"**

We have now replaced Fig. 4j with separated graphs analysing the effect of TNF-α neutralization and ROCK inhibition on epithelial apoptosis. In each case, the statistical test has been performed on the collation of three biological replicates, each of which is the average of three technical triplicates. The grey shading indicates the standard deviation. This information has now been added to the figure legend.

13. **"*Extended Data Figure 1b: Error bars are not defined.*"**

We thank the reviewer for their keen eye and apologize for the omission. The error bars reflect the standard deviation of the values from the six biological replicates displayed. This information has now been added to the figure legend.

14. **"*IHC staining of TRMs in IIOs for conventional TRM markers, such as CD69, ITGA1, and ITGAE, would confirm TRM identity is maintained in co-culture.*"**

We thank the reviewer for their suggestion. We have now stained the TRMs for classical markers CD69 and CD103 (*ITGAE*) at multiple timepoints across a 14-day co-culture via multispectral immunofluorescence (Extended Data Fig. 1f,h-i). These data demonstrate that the gut T cells clearly maintain their identity over the course of the culture. Unfortunately, we were not able to find and validate a reliable IHC antibody for CD49a (*ITGA1*).

15. **"*Although the scaffold-based crawl-out method for isolating lymphocytes is noted as cytokine-independent, which the authors indicate is important for preserving "tissue-like physiological properties of intestine-derived lymphocytes," the efficacy of this method for actually doing so is not tested or quantified. Lymphocytes isolated with cytokine- dependent and independent pathways should be compared at the transcriptomic and/or protein level, such as with scRNA-seq and/or IHC staining, respectively. This data should then be compared to those from primary,*

***undissociated tissue to confirm cytokine-independent isolation methods are indeed an improvement over cytokine-dependent methods. Inclusion of data from tissue and organoids derived from additional donors would bolster the reliability and reproducibility of the data.*"**

We thank the reviewer for their comment. At project conception, we undertook a thorough characterisation of cytokine-dependent versus cytokine-independent crawl out methodologies across multiple biological replicates. We observed that the inclusion of classic T cell/TRM cytokines IL-2 and IL-15 modulated expression of several activation and exhaustion markers, including PD-1, LAG-3, TIGIT and CD25 (which were upregulated by cytokine supplementation) and CD127 (which was downregulated by cytokine supplementation). We have included this data below for your reference. We reasoned that changing expression of these key markers outweighed the benefit of the modest increase in cell number, given the ultimate goal of the project was to generate an *in vitro* system that could model and predict physiological responses to immunotherapeutics modulating exactly these types of protein targets. Although we compared cell type abundance between enzymatically digested *ex vivo* intestinal samples and crawl-out derived immune cells, we did not extend our analysis to activation and exhaustion markers, given the difficulty in accurately quantifying expression following enzymatic isolation protocols, which leads to surface marker cleavage.

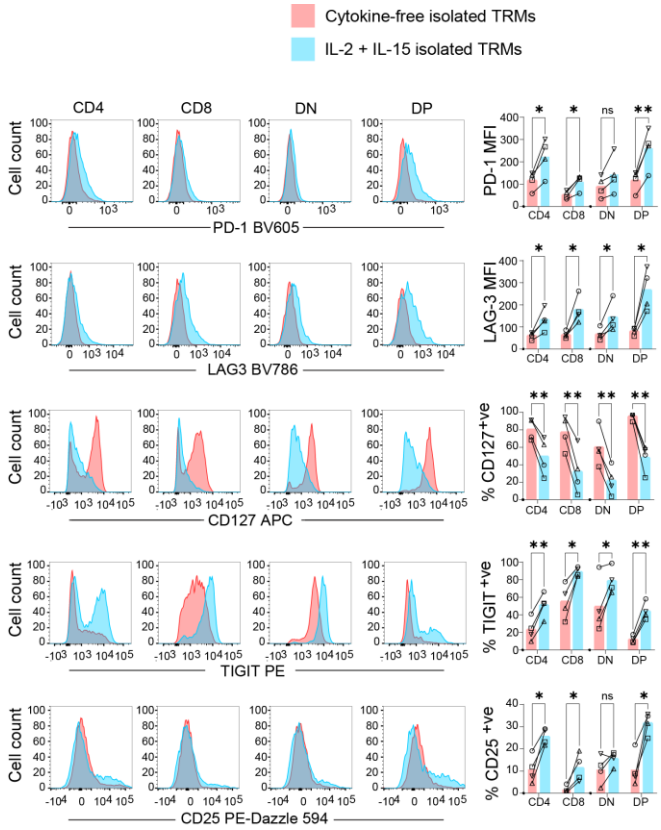

Figure legend: Flow cytometry histograms comparing expression of key activation markers in gut-derived T cells isolated via crawl out in cytokine-free or cytokine-supplemented (10IU/ml IL-2, 2ng/ml IL-15) media. T cell subtype is indicated above the histogram columns. Bar graphs displaying the collated data from 4 biological replicates, with statistics (paired T-test, *P<0.05, **P<0.01) are included. DN: double negative T cells (CD3+ve CD4-ve CD8-ve), DP: double positive T cells (CD3+ve CD4+ve CD8+ve)

16. **"*Extended data figure 1e indicates the ratio of immune cells to epithelial cells decreases over time, suggesting that immune cells may be losing their TRM identity without appropriate signalling (either from the organoid or from external sources, such as cytokine support).***

***Therefore, it would be insightful to do IHC staining and/or scRNA-seq on TRMs in IIOs that have been in co-culture longer than the data generated in this paper (e.g., >48 hours), such as 1-2 weeks after IIO generation, to test the robustity of the TRM identity over time.*"**

We thank the reviewer for this suggestion. To address it, we cultured IIOs from three different donors for two weeks, supplemented with low levels of IL-2 and IL-15, which are required for TRM maintenance. After each passage, we generated sections of the IIOs, which we then used to visualize and quantify the presence of T cells (CD3) and the expression of residency markers (CD69 and CD103) Ext. Data Fig. 1f-i).

We found that IIOs from each of the three donors preserved substantial numbers of both $CD4^+$ and $CD8^+$ TRMs, even after two weeks in culture. Likewise, the tissue-resident nature of the TRMs was preserved, as supported by the sustained expression of CD69 and CD103. We should note that, whereas the number of TRMs and their residency status within the IIOs were preserved, there appeared to be a tendency towards a slight decrease in CD103 expression and an increase in CD69 expression, indicative of increased baseline activation. These data suggest that sustained maintenance in an unaltered state over more than two weeks will likely require optimization of the culture conditions. Nonetheless, we are confident that the system already provides a valuable experimental window of several weeks, allowing us to monitor both acute and longer-term immune responses.

Furthermore, we verified the responsiveness of the IIOs to activation after prolonged culture (10 days). Treatment with EpCAM TCB led to organoid apoptosis, whereby caspase was induced with kinetics similar to those observed upon treatment of freshly formed IIOs (Ext. Data Fig. 3i).

17. "*To investigate further the inflammation mitigation strategy, it would be interesting to test the predicted effects of in silico perturbations (TNF loss) on differentiation and activation profiles using techniques such as scRNA-seq*"

We thank the reviewer for this suggestion. *In silico* removal of TNF-α from the system resulted in loss of both the $CD8^+$ cytotoxic lymphocytes and late-appearing population of cytotoxic $CD4^+$ Th1 cells (Fig. 4g). To confirm this prediction experimentally, we used flow cytometry to assess the effect of TNF-α blockade on TCB-mediated T-cell activation at the protein level. We found that treatment with the anti-TNF-α antibody Adalimumab led to a significant decrease in the population of $CD4^+$ and $CD8^+$ T cells producing the cytotoxic molecule Granzyme B. We also observed a decrease in the expression of activation markers ICAM-1 (mediating adhesion to endothelium and infiltration into tissue), 4-1BB (potent co-stimulatory receptor on T cells) and CD25 (IL-2 receptor). Using ELISA, we also detected that TCB-treated IIOs produced significantly lower amounts of the chemokine CCL2 upon TNF-α blockade. Thus, TNF-α blockade led to a reduction in both T-cell activation, cytotoxicity and cytokine production, which is in line with the computationally predicted effects of TNF-α removal from the system.

18. "*In order to better assess the applicability of the model for recapitulating colitis induced by cancer therapeutics, the model could be tested on a more relevant cancer immunotherapeutic(s) that is in wider clinical use, such as immune checkpoint inhibitors (e.g., PD-1 blockade).*"

We acknowledge that immune checkpoint inhibitors such as anti-PD-1 antibodies are in wide clinical use, and the intestinal side effects associated with checkpoint blockade is something that we have explored (see our answer to point 6 and Extended Data Fig. 7a-e).

We believe nonetheless, that the EpCAM TCB is a highly relevant tool that can be exploited to interrogate intestinal inflammation triggered by tissue-resident immune cells. Here we used it to mechanistically dissect the side effects reported during its trial as a cancer immunotherapeutic. However, when used at lower sub-optimal concentrations, it can be implemented as a useful tool for triggering and localizing low-level T cell inflammation towards the epithelium in a chronic setting, akin

to intestinal autoimmune conditions such as colitis, Crohn's and celiac disease. This approach bypasses the requirement for complicated incorporation of gut microflora of luminal pathogens in order to promote T-cell inflammatory activity towards the epithelium. Such convenience should facilitate widespread adoption, in order to understand aspects of inflammatory disease pathology and test therapeutic mitigation strategies.

As our work illustrates (Figure 4j and Extended Data Figure 6), the inflammation generated in the IIO model is sensitive to both established (Adalimumab) and novel (ROCK inhibitor) therapeutic concepts. Critically, these represent distinct large molecule and small molecule compounds, demonstrating the amenability of the IIO setup to different therapeutic modalities, and its suitability as a drug discovery tool for validating and understanding a broad range of new biological targets and compounds. Thus, we believe it is of significant interest to the wider scientific community.

19. **"*In lines 49-55, studies that have previously generated intestinal organoids containing immune cells are cited. However, two other papers of relevance include:***

A 2019 Immunity paper (with an associated 2021 Star Protocols paper: https://www.sciencedirect.com/science/article/pii/S2666166721002264?via=ihub) has described the generation of fetal-tissue derived intestinal organoids containing autologous CD4+ T-cells from the lamina propria: https://www.sciencedirect.com/science/article/pii/S1074761318305363

A 2022 Inflammatory Bowel Diseases paper has described the generation of iPSC-derived intestinal organoids containing tissue-resident immune cells (macrophages): https://academic.oup.com/ibdjournal/article/28/Supplement_1/S57/6514119"

We apologize for the omission of these important and interesting studies and we thank the reviewer for pointing them out to us. We have now cited them in the Introduction of the revised manuscript (lines 52-61).

20. **"*Abstract is clear and focuses on the main takeaways of the paper. However, the authors claim their "system recapitulates clinical outcomes". This is a rather strong statement given that no correlation to clinical outcomes is performed in the study but rather it is speculated how the observations in the organoids may correspond to events occurring in patients.*"**

We agree with the reviewer and have now toned down the language in the abstract.

21. **"*Introduction provides sufficient background to understand the paper's contents, but could expand upon the development of intestinal organoid models that have immune components (see section 7). Conclusion effectively highlights the key findings of the article and contextualises them in the broader literature*."**

We have followed the reviewer's recommendation, and have expanded the discussion of existing organoid models that incorporate an immune component in the Introduction (lines 52-61).

22. **"Several microscope images are missing scale bars (figure 1d, 1e-g, 1j, 3a, etc.)"**

We have now added scale bars to all images throughout the manuscript.

Reviewer 2:

1. **"*It is well known that myeloid cell including macrophages are an active intestinal component that affect both epithelial cells as well as T cells. The IIO system does not include the study for the myeloid derived cells*"**

   "*Though PB cells in this study does not reveal an active interaction with intestinal epithelial cells, resident immune cells including T cells can be exhausted. In this context, it is well documented that recruiting immune cells from circulation is essential. How can authors address this issue?*"

   We thank the reviewer for these insightful questions. Our system was indeed focused on tissue-resident immune cells (and lymphocytes in particular), given the scarcity of *in vitro* models that contain them and allow studies related to them. Nonetheless, we found the reviewer's point to be important and devised an experiment in which the contribution of both resident and peripheral immune cells, as well as their mutual interactions, are captured. In addition, we believe that the same experiment addresses the reviewer's first question, related to myeloid cells, given that PBMCs contain a monocyte population.

   In particular, we co-cultured IIOs (which contain TRMs) with PBMCs derived from the same donor and treated them with EpCAM TCB. IIOs alone and organoid-PBMC co-cultures served as controls. In the tri-culture experiment, TRMs and PBMCs were pre-labeled with different live dyes to track their interactions and spatial behaviors during the course of the experiment. We made two interesting observations:

   - Whereas at the start of the culture PBMCs were excluded from the organoid epithelium, they infiltrated the organoids upon EpCAM TCB treatment only in the tri-culture condition. This suggests that PBMCs sense inflammation generated by TRMs within IIOs and are recruited to the inflammation site, much like they would *in vivo*, and as the reviewer suggests (Ext. Data Fig. 4a-b).
   - To investigate the contribution of myeloid cells in the system, we quantified the production and secretion of myeloid cell-associated cytokines IL-8, IL-6 and IL-1β. The levels of these cytokines were low in the conditions where organoids were separately co-cultured with either TRMs or PBMCs and treated with EpCAM TCB. However, TCB treatment of the tri-culture led to an increase in the concentration of IL-8, IL-6 and IL-1β in a manner that surpassed those recorded in the separate co-cultures, suggesting a synergistic effect, whereby TRM-initiated inflammation induces an activation within the myeloid cell population within PBMCs (Ext. Data Fig. 4d. We verified the presence of myeloid cells within the system by staining for CD14 (Ext. Data Fig. 4c).

   These data indicate that our system captures not only tissue-resident immune responses, but also the interactions between the resident and peripheral immune compartment, which opens exciting inroads for exploring immunity at different scales (local vs. systemic, acute vs. prolonged).

2. **"*Tissue-resident lymphocytes were called as TRMs seems*"**

   We apologize for the confusion. Although the scaffold-based isolation method occasionally yields a small proportion of B cells and myeloid cells, the overwhelming majority of the cells isolated from human intestinal specimens and included in the IIO system are tissue-resident memory T cells (mean 83.5%). We have now verified that the harvested cells are nearly exclusively composed of T cells using samples from 6 different donors (Ext. Data Fig. 1b). Given the T-cell dominance, we refer to the tissue-resident immune cells isolated as TRMs throughout the manuscript, unless we specifically refer to those integrated within the epithelium, in which case we describe them as IELs. We have now clearly communicated the nomenclature we use early on in the manuscript and have explained our reasoning behind it (lines 89-94).

Reviewer 3:

1. "***Recaldin et al. have established a co-culture system for human intestinal organoids and tissue-resident memory lymphocytes (TRMs). Previous studies reported similar co-culture systems, but those studies have been difficult to reproduce by others due to availability (scalability and the storability) of immune cells. In contrast, the authors have demonstrated the frozen preservation of TRMs and their subsequent successful co-culture with organoids. This co-culture method may greatly facilitate investigations into the human mucosal immune system and pave the way for a better understanding of inflammatory bowel diseases.***"

   We thank the reviewer for recognizing the novelty and potential impact of our manuscript.

2. **"*Figure 1d requires control experiments involving co-culture with PBMCs. Additionally, the authors should quantify the distance between each lymphocyte and organoid and calculate statistical differences.*"**

   The control experiment for Fig. 1d (organoids co-cultured with autologous PBMCs) is presented in Fig. 1g, which depicts that PBMCs are fully excluded/absent from the epithelial barrier and present only in the surrounding extracellular matrix. Owing to the highly dynamic movement of TRMs, we do not find that measuring distances between them and organoids at a given snapshot would be very informative. For example, within a single hour, TRMs frequently cover the distance between neighboring organoids (Ext. Data Video 2, 3). We compared the spatial distribution of PBMCs and TRMs and their relative positions to organoids by using histological sections to quantify the number of immune cells within the organoid epithelium. We found that a substantial portion of TRMs were directly contacting epithelial cells, whereas PBMCs were almost exclusively located outside the organoid, lacking direct contact (Fig. 1h).

3. **"*The manuscript suggests that some of the TRMs that migrated near the organoids were IELs, but this needs to be confirmed through immune staining for IEL markers. Furthermore, it would be valuable if the authors first sorted IELs from TRMs and then performed co-culture with organoids to confirm whether IELs alone are sufficient to reproduce the co-culture phenotypes, including EpCAM-TCB cytotoxicity. Alternatively, the co-culture phenotypes may require complex interactions among diverse TRMs.*"**

   We thank the reviewer for this suggestion. The scRNA-seq data presented in Figure 2 and 3 identified a clear IEL population in IIOs, at baseline and upon EpCAM TCB treatment, which actually increased the intraepithelial character of TRMs (Ext. Data Fig. 5d). To confirm this at the protein level and *in situ*, we analyzed the expression of CD103 (receptor for E-cadherin) within IIOs using immunohistochemistry (image included below). We observed striking expression of CD103 by TRMs integrated within the epithelial barrier, confirming their IEL identity.

   The reviewer's question about which population drives the response was very interesting to us. As suggested, we isolated tissue-resident immune cells and organoids from three different donors, and sorted $CD103^-$ cells (lamina propria lymphocytes) from $CD103^+$ cells (intraepithelial lymphocytes). We then co-cultured the two different populations with organoids and treated them with EpCAM TCB. We found that both populations were equally responsive, as evidenced by comparable induction of inflammatory cytokines (TNF-α, IFNγ and Granzyme B) and organoid apoptosis (Ext. Data Fig. 3g-h).

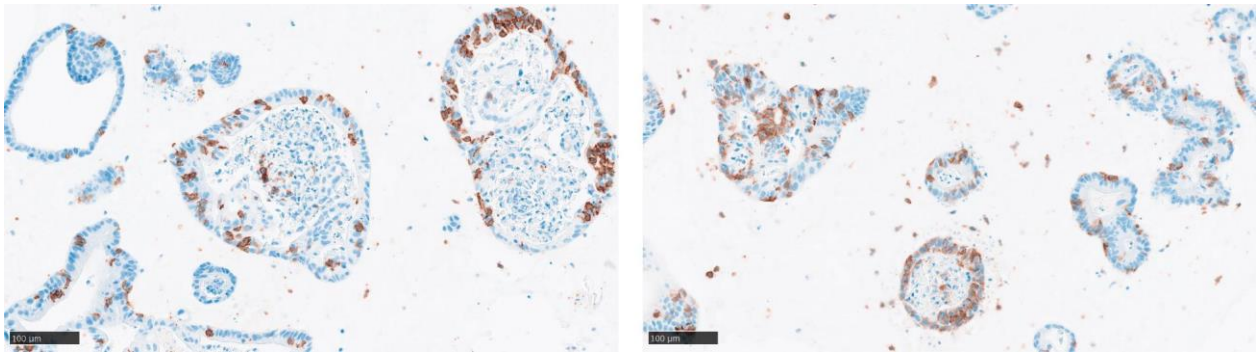

Figure Legend: IHC staining for tissue resident markers CD103 (left image) and CD69 (right image), in IIO cultures, 14 days after immune cell introduction.

4. **"*In Figure 1e-g, the structures of intestinal organoids appear unusual. They seem stratified. Did the intestinal epithelium form a monolayer with apicobasal polarity? If the epithelium produces laminin-5, the authors may distinguish epithelium-derived laminin from Matrigel. If so, it would be interesting to show that IELs reside between the epithelium and basal membrane, where IELs reside in vivo. The multi-color immunostaining is well-executed, but some colors, such as FABP1 and ECAD signals, are difficult to distinguish in the figure images.*"**

We apologize for the misleading images. The organoids appear stratified at places because the images represent 3D reconstructions of confocal stacks spanning 20 µm. These, in turn, were generated by fully imaging thick histological sections. We chose to show thick optical sections because we wished to highlight the shapes of the IELs in full (not captured by a single optical section). Fig. 1e, in particular, includes the side "walls" but also the top surface epithelium of an organoid. We found the number and shapes of IELs present to be striking and wanted to share that with the reader. To the reviewer's question, we have stained for the intestinal epithelial apical marker CEA (Harter et al, *Nat Biomed Eng* 2023) in thinner sections to confirm that the organoids comprise simple columnar epithelium that is polarized (please see below).

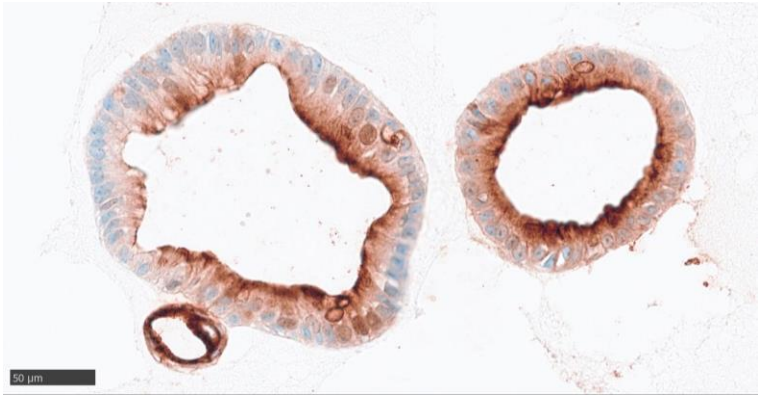

Figure legend: histological sections of small intestinal organoids, stained for carcinoembryonic antigen (CEA, brown)

However, based on our observations using static stained sections and live imaging of IELs, we would not be comfortable claiming that IELs in this system are preferentially located on the basal side of the epithelium. We have actually noted that IELs occupy a range of locations within the barrier and frequently exhibit "flossing" behavior by being stretched out along the junctions between the epithelial cells (Fig. 1j; Ext. Data Fig. 1e). We do not fully understand why the positioning relative to epithelial cells is different from that accepted for IELs *in vivo*, but we believe that a number of spatial cues that might govern it in the native context are missing, including microbiota on the luminal side, tissue-resident macrophages on the basel side etc.

We also appreciate the reviewer's point on the multi-color immunostaining. We chose that particular color scheme with the aim to accentuate the IELs, whereas we found the other markers to be less important. However, we agree that this is rather visually confusing and does not add much value to the figure. Therefore, we have removed the E-cadherin stain, and only kept the FABP1 stain, because we felt that it was more important to highlight the differentiated enterocytes (in addition to the immune cells present).

5. **"*In Figure 2a-c, it is unclear to what extent fresh TRMs and co-cultured TRMs are similar.*"**

This is an interesting question that we have sought to address in the revised version of the manuscript (Ext. Data Fig. 2g-j). While TRMs cultured in matrigel alone were invariably similar to TRMs derived from 24h IIO culture, we observed IIO-TRMs had a stronger "intraepithelial" signature, including but not limited to, increased expression of motility genes such as *ANXA1, ANXA2, S100A6, S100A10*, and *S100A11*, as well as *CCL5*, a critical chemokine that recruits immune cells into the epithelium (Ext. Data Fig. 2j). Conversely, genes required to navigate into the gut and around the ECM (*JAML, SOS1, CD96, ITGA1*), but not integrate into the epithelium, were more abundantly expressed in immune cells from the TRM alone condition (Ext. Data Fig. 2j). That is to say, epithelial cells appear to reinforce TRM intraepithelial identity, likely through the rapid and frequent cell-to-cell contact we observed with our imaging experiments. Although not the focus of this project, long term IIO coculture would presumably amplify these differences.

6. **"*Figure 2h needs a control (PBMC co-culture) for comparison.*"**

We apologize for this omission. We repeated the experiment, this time also tracking the movement of autologous PBMCs co-cultured with organoids. There were striking differences in the migratory behaviors of resident and circulating immune cells. First, the fraction of moving (vs. static) cells was substantially higher in the TRM compared with the PBMC population (54±13.9% vs. 5±3.4%). Furthermore, the fraction of motile cells within the PBMC population moving at significantly lower speeds compared with TRMs. We also analyzed the shape distribution of PBMCs and TRMs over time and found that the former were significantly more round, while the latter exhibited irregular, eccentric shapes. We have now added the new data in Fig. 2 of the revised manuscript (Fig. 2h).

7. **"*The authors mentioned "at least three passages" (line 96-97, Extended Data Fig 1e). Passageability is crucial when researchers aim to examine long-term immune responses. However, the number of TRMs seemed scarce in weeks 2-3. The authors need to quantify the rate of TRMs at each passage. Additionally, it's important to demonstrate whether co-cultured organoids retain EpCAM-TCB response after passage. Although IL2 and IL15 were included to prolong the co-culture, it's unclear whether this condition was fully optimized. There is no data showing that IL2+IL15 treatment is superior to the control.*"**

Acute responses to immunomodulatory drugs or pathological factors were the focus of our work, rather than chronic TRM-organoid cultures. However, we performed initial experiments to prove in principle that the system is adaptable to different experimental questions. Accordingly, we performed longer-term co-culture experiments with three biological replicates, quantifying expression of key TRM markers CD103 and CD69 via immunofluorescent IHC at regular intervals, as well as the immune cell integration ratio. We found that the ratio of epithelial cells to immune cells actually decreased (indicative of increased immune cell integration) upon longer-term culture (Extended Data Fig.1 f-i). We also applied the EpCAM TCB after 10 days of coculture and observed extremely rapid organoid targeting. Collectively, this demonstrated the TRMs readily retained their organ-specific character and their EpCAM TCB response after passage (Extended Data Fig. 3i).

With this experimental setup, we could reproducibly maintain the coculture for approximately 2 weeks. After around 14 days of coculture we saw that some TRM donors triggered increased epithelial

expulsion into the organoid lumen preventing healthy coculture for longer. This epithelial "shedding" reflects the physiological function of intraepithelial lymphocytes. We did not think it was manageable to optimize the conditions required for truly long-term culture (beyond two weeks) within the timeframe of this revision. We agree with the reviewer that relatively minimal optimization could establish conditions that can facilitate co-culture over several months, should it be desired by a particular experimental question or study. Nonetheless, we are confident that the system already provides a valuable experimental window of two weeks, allowing us to monitor both acute and longer-term immune responses.

8. **"*In Figure 2a, scRNA-seq data from tissue-resident and blood-derived immune cells alone or co-cultured with organoids is presented. In the extended data figure, it would be helpful to include plots based on the origins (with or without co-culture).*"**

   We thank the reviewer for their recommendation and accordingly, have included this data in the updated version of the manuscript (Fig. 2c, right tab, Extended Data Fig. 2e-j).

9. **"*There is no detailed information about donors, such as sex, age, or disease status. It is unclear how many donor patients were involved in this study.*"**

   We are limited in the metadata we can share due to the ethical agreement we have with the foundation supplying the intestinal tissue. As a source of tissue, we collaborate with the non-profit HTCR foundation (https://www.htcr.de/) as stated in the Material & Methods section. Its framework, which includes written informed consent from all donors, has been approved by the ethics commission of the Faculty of Medicine in the LMU (Number 025-12) and the Bavarian State Medical Association (Number 11142).

   For this study, we received pseudonymized non-disease-affected small intestine and colon tissues from 24 consenting patients (20 small intestine, 4 colon) undergoing planned surgeries, all for oncology indications and mostly resection of pancreatic adenocarcinoma or colorectal carcinoma. Patient selection was opportunistic by availability and size of tissue, and timing of procedure vs experimental resources. Resectates were classified as non-diseased by the operating surgeon and/or pathologist of the hospital in the HTCR framework (mostly University of Munich, Germany). E.g., we leveraged as "healthy" tissue duodenal resectates in PDAC Whipple pancreaticojejunostomy, or colon tissue between microscopically confirmed tumor-free margin and vascular supply resection boundaries.

   We have now added into the Material & Methods age statistics (age: mean 67.9y, median 70y, Stdev 11.3) and ICD-10 code examples (e.g. pancreaticojejunostomy due to pancreatic adenocarcinoma (examples ICD10 codes C18.x, C24.x, C25.x)), the location of the intestinal samples, and clarified that we only used confirmed tumor-free parts of the specimens.

10. "***To fairly assess the data in Figure 4i,j, some control experiments are required: (1) Determine whether TNF-alpha alone can directly kill organoids within 30 minutes or if other factors from TRMs are required. This possibility can be easily addressed by treating organoids with TNF-alpha or organoids+TRMs. (2) Investigate whether ROCKi treatment may suppress TNF-alpha-induced apoptosis independently of TRMs. If this experiment yields positive results, the authors should examine organoids + TNFa vs. organoids + TNFa + ROCKi. (3) Related to (2), quantify the speed of TRM migration with or without ROCKi. (4) Exclude the possibility that ROCKi reduces TNFa production from TRMs.*"**

    We thank the reviewer for this interesting question. We have addressed the reviewer's points as follows:

    1) We performed the experiment they suggested and treated the IIOs with a wide range of TNF-α concentrations (0.01 - 156 ng/ml). To our surprise, TNF-α treatment alone did not lead to any organoid damage, even after prolonged incubation (40h) (Ext. Data Fig. 6d). This finding suggests

that TNF-α does not induce organoid apoptosis through a direct mechanism, but plays an indirect (albeit crucial) role in the system by orchestrating the appearance of the two cytotoxic, Granzyme B-producing $CD4^+$ and $CD8^+$ T cells. In line with this indirect role, *in silico* removal of TNF-α from the system resulted in loss of both the $CD8^+$ cytotoxic lymphocytes and late-appearing population of cytotoxic $CD4^+$ Th1 cells (Fig. 4g). To confirm this prediction experimentally, we used flow cytometry to assess the effect of TNF-α blockade on TCB-mediated T-cell activation. We found that treatment with the anti-TNF-α antibody Adalimumab led to a significant decrease in the population of activated $CD4^+$ and $CD8^+$ T cells producing Granzyme B (Ext. Data Fig. 6a). Altogether, these data strongly suggest that the effect of TNF-α within the system unfolds through its effect on TRMs, rather than a direct effect on the organoid epithelium.

2) Given the lack of effect of TNF-α in the absence of TRMs, we did not find it informative to additionally inhibit ROCK.
3) To the reviewer's third point, we performed a live imaging experiment in which we monitored and quantified the motility of TRMs upon inhibition of ROCK. We observed a striking reduction in the proportion of motile TRMs, as well as the average speed of movement and distance traveled (Ext. Data Fig. 6e; Ext. Data Video 4).
4) Quantifying TNF-α levels by flow cytometry, we did indeed find that ROCKi leads to attenuated TNF-α production (Ext. Data Fig. 6g). This is not a surprise to us, because we did suspect that the treatment results in an overall decrease in TRM activation. Nonetheless, we consider the effects of ROCKi to be interesting and to merit a closer investigation, because we believe that, in addition to a *bona fide* inactivation, the treatment mitigates the destruction of organoids through lowering TRM migration and physical engagement with epithelial cells, as confirmed by the results of the motility analysis (Ext. Data Fig. 6e). Indeed, the rescue effect of ROCKi was greater than that of TNF-α blockade itself (Fig. 4j).

11. "*Biological replication details are missing throughout the manuscript. Results in Figure 2h, Figure 3a-c, Figure 4i-j, and Extended Data Fig 1e should be reproduced using at least three independent donors.*"

These figures have been reproduced in a minimum of 3 biological replicates and the data either reflects the collated results from the different replicates, averaged from the technical triplicates or quadruplicates and then combined, or 1 representative replicate to aid visualization. These details have been included in the appropriate figure legends.

12. "*Before co-culture, organoids were differentiated. Is this step essential? Did TRMs fail to migrate to organoids when cultured in stem cell conditions? These data should be included.*"

For our acute IIO cultures (<96h), intestinal organoids were first differentiated for 72h prior to addition of the gut immune cells. This information is detailed in the methods section. For the longer-term cocultures where passaging was required, organoids were retained in stem cell growth media. This experimental detail has been clarified in the methods under "Preparation and culture of intestinal immune-organoids, including treatment". Immune cell integration appeared completely independent of epithelial differentiation status, and integration rate was in the physiological range (see Fig. 1e-f (integration into differentiated organoids) and Extended Data Fig. 1f-g (integration into stem cell organoids)).

13. "*The nomenclature for the same molecules varies depending on the context (gene name and protein). For example, CD49a and ITGA1, CD117 and KIT. It would be helpful to provide explanations for these terms, especially for readers unfamiliar with immunology.*"

We thank the reviewer for their comment and have updated the manuscript accordingly. Upon first reference to CD117, CD103 and CD49a we have added parentheses containing the gene name that

encodes the protein (*KIT*, *ITGAE* and *ITGA1*). Later references to 4-1BB (encoded by *TNFRSF9*), CD25 (encoded by *IL2RA*) are also clarified in this manner.

14. **"*Some abbreviations, such as TEM, TCM, and TEMRA, are not explained in the manuscript and should be defined for clarity*."**

    These abbreviations are no longer used in the updated version of the manuscript.

Reviewer 4:

1. **"*This study is interesting as it reports a novel system to generate organoids with an integrated immune cells compartment. Such a system could be useful to study the precise roles of immune cells in non-lymphoid tissue development, homeostasis or function.*"**

   We thank the reviewer for recognizing the novelty and potential of our work.

2. **"*However, these questions were not really addressed by the authors. Although they performed a series of experiments aimed at testing the interplay between immune cells and organoids, the relevance of their findings remains unclear and several of the author's claims remain unsupported.*"**

   We hope to have addressed the reviewer's concerns in the revised manuscript.

3. **"*In the abstract, the authors propose that 'IIO formation was driven by TRM migration and interaction with epithelial cells'. I cannot see any evidence for this claim. Indeed, as far as I can see the authors do not show if or how immune cells impact organoid formation, integrity or function. This was simply not tested. Do tissue-resident lymphocytes impact any of these features? This would be an important starting point for further studies.*"**

   We believe that some of the new terminology we introduced created some confusion, for which we apologize. Throughout the manuscript, we use the term 'intestinal immuno-organoids' (IIOs) to refer to the structure composed of the epithelial organoid and integrated intraepithelial lymphocytes. 'IIO formation' thus refers to the process of lymphocyte infiltration into the epithelial organoid to form the combined epithelial-immune structure. Live imaging data (e.g. Ext. Data Video 2) highlights the striking motility of TRMs, which move directionally toward the epithelium, as evidenced by the ray-like tracks emanating from the organoids, and integrate within the latter over prolonged periods.

4. **"*Similarly, I cannot see any evidence for the claim that 'IIO formation … was orchestrated by TRM-enriched transcriptomic programs'. While the authors show some transcriptomic data consistent with TRM signatures, it remains unclear what precisely was done, and certainly it was not tested how these transcriptional programs impacts IIO formation.*"**

   As the reviewer pointed out, it stands to reason that TRM-specific transcriptomic programs would aid their primary functions and behaviors, including their integration within intestinal epithelia. The data presented in Fig. 2 aimed at not only demonstrating that TRMs exhibit these *in vivo*-relevant behaviors, but also shedding light on the mechanisms that underlie them, with a particular focus on intraepithelial infiltration.

   The ability of the IEL subset of TRMs to interact with epithelial cells via the CD103 receptor is well established (Cepek et al, *Nature* 1994; Edelblum et al, *Gastroenterol* 2015). However, within our system (and likely within the native intestine) CD103 expression would explain the integration of TRMs that are

directly adjacent or in direct contact with organoids, but not those that are initially distant. In other words, TRMs need to somehow reach organoids before they can leverage CD103 expression to engage with epithelial cells. We use the transcriptomic data to infer that TRM-specific programs of enhanced motility may enable these cells to encounter organoids, even from distant sites. We use live imaging to verify that these transcriptomic programs translate directly into enhanced motility and directional persistence (Fig. 2h; Ext. Data Video 2). Consistently, PBMCs, which are far less motile (Ext. Data Video 3) (and exhibit low expression of motility-related genes) fail to integrate within organoids.

To address the reviewer's question more directly, we blocked TRM motility by treating IIOs with the ROCK inhibitor Y-27632. As expected, the reduction in motility significantly attenuated the integration of TRMs within epithelial organoids (Ext. Data Video 4, Ext. Data Fig. 6e), ascribing a functional importance to motility as one the most striking transcriptomic features of TRMs.

To further strengthen our conclusions and ensure that they are reproducible across individuals, we extended the analysis described above to samples from two additional donors. Figure 2 of the revised manuscript summarizes the transcriptomic features of all three donors analyzed, yielding results in line with those of the original submission: TRMs *in vitro* preserve *in vivo*-like gene expression profiles and exhibit striking upregulation of motility and adhesion genes compared with blood-derived T cells.

5. **"*Related to this point, can the authors please explain precisely what was done in experiments related to figure 2. Based on the scheme, it appears that IIO were generated either with tissue-resident lymphocytes or PBMC. After formation of these IIO, they were disintegrated, and lymphocytes were subjected to scRNAseq. This would suggest two conditions in their analysis. In the text of the manuscript, however, they state "we used single-cell RNA sequencing (scRNA-seq) to analyze donor-matched tissue resident and blood-derived immune cells alone, or cocultured with organoids". This would suggest a four-way comparison. Please clarify.*"**

We apologize for the lack of clarity and thank the reviewer for suggesting we clarify. In the analysis described in the first submission, we indeed performed a four-way comparison (matched TRMs alone, IIO-derived TRMs, organoids alone, organoids + PBMCs, PBMCs alone), which, briefly, yielded the following observations: 1) Limited interaction between PBMCs and organoids, as the gene expression profiles of both epithelial and immune cells were unchanged between mono- and co-culture. 2) An interaction between TRMs and organoids, as co-culture led to gene expression changes in both compartments, although the epithelial transcriptome was relatively stable across the conditions. 3) Striking differences in TRM and PBMC gene expression profiles upon organoid co-culture, and given our focus on understanding how TRMs are capable of migrating into and interacting with the epithelium, these formed the focus of our discussion in the original manuscript.

As described above, we repeated the scRNAseq analysis with two additional donors. Given the size of the experiment and analysis, we had to prioritize conditions, and analyzed: TRMs in mono-culture, TRMs within IIOs as well as PBMCs in co-culture with intestinal epithelial organoids. Guided by the results from the first experiment (lack of interaction between PBMCs and organoids), we considered the PBMC-organoid co-culture a suitable representation of either PBMCs alone or organoids alone. In addition to comparing TRMs to PBMCs, we explored the following:

<u>Effect of TRMs on organoids</u>: Epithelial cell proportions and gene expression were stable across the different conditions (Ext. Data Fig. 2e-f). Intriguingly however, we observed that TRM presence led to a significant upregulation of *ID3* and *HES1*, both of which are associated with intestinal stem cell maintenance (Hu et al, *Front Cell Dev Biol* 2021; Pellegrinet et al, *Gastroenterol* 2005) and microbial defense (Guo et al, *Mucosal Immunol* 2018). Although it was not the focus of our experiments, it is likely that longer-term culture in the presence of luminal antigens would be required for TRM effects on intestinal fate decisions to manifest more substantially.

Effect of organoids on TRMs: While TRMs cultured in matrigel alone were invariably similar to TRMs derived from 24h IIO culture, we observed IIO-TRMs had a stronger "intraepithelial" signature, including but not limited to, increased expression of motility genes such as *ANXA1, ANXA2, S100A6, S100A10*, and *S100A11*, as well as *CCL5*, a critical chemokine that recruits immune cells into the epithelium (Ext. Data Fig. 2j). Conversely, genes required to navigate into the gut and around the ECM (*JAML, SOS1, CD96, ITGA1*), but not integrate into the epithelium, were more abundantly expressed in immune cells from the TRM alone condition. That is to say, epithelial cells appear to reinforce and amplify TRM intraepithelial identity, presumably through the rapid and frequent cell-to-cell contact we observed with our imaging experiments. We have updated Extended Data Fig. 2 to capture this.

6. **"*Independent from the precise experimental approach, it remains unclear what the purpose of this experiment is. The authors present an analysis that reports that TRM-derived samples were enriched for an features consistent with TRM. That is self-evident; everything else would be a surprise. So, is this all just a confirmation, meant to confirm that the authors are indeed dealing with tissue-resident lymphocytes? At this stage I find it difficult to understand the relevance of figure 2.*"**

We apologize for not communicating the rationale behind the data in Figure 2 effectively. In addition to confirming that we have indeed isolated tissue-resident lymphocytes, we deemed it important to verify that TRM-specific features are preserved in *in vitro* co-culture with organoids. We would like to remind the reviewer that the scRNAseq analysis was not performed directly after isolation of TRMs, but after isolation, freezing, thawing and 24-hour culture. It is not guaranteed that TRM-specific gene expression and functional behaviors are preserved after this process.

As discussed in our answer to point 5 above, aside from verifying that the TRM character of the cells is preserved, a main objective behind the single-cell transcriptomic data in Figure 2 was to also understand the mechanisms that facilitate interactions between organoids and TRMs and formation of IIOs.

We have explained our rationale behind the transcriptomic analysis presented in Fig. 2 in lines 119-125 of the revised manuscript.

7. **"*Throughout the text, the authors refer to TRM (which mean tissue resident memory T cells). In their abstract they introduce TRM as "tissue-resident lymphocytes", while later they call them "tissue-resident memory T cells (TRMs)". Thus, this reviewer was unclear about the precise nature of the authors experiments. While initially, it sounded like the authors were specifically culturing organoids with T cells, it later becomes clear that they use whole immune cells (either from blood or intestine). This should be made clear early on. "TRM" should not be used to refer to "tissue-resident lymphocytes".*"**

We apologize for the confusion and the inconsistency. Although the scaffold-based isolation method occasionally yields a small proportion of B cells and myeloid cells, the overwhelming majority of the cells isolated from human intestinal specimens and included in the IIO system are tissue-resident memory T cells (>90%). We have now verified that the harvested cells are nearly exclusively composed of T cells using samples from 6 different donors (Ext. Data Fig. 1b). Given the T-cell dominance, we refer to the tissue-resident immune cells isolated as TRMs throughout the manuscript, unless we specifically refer to those integrated within the epithelium, in which case we describe them as IELs. We have now clearly communicated the nomenclature we use early on in the manuscript and have explained our reasoning behind it (lines 89-94). We have also changed the mention of 'tissue-resident lymphocytes' in the abstract, to 'tissue-resident memory T cells'.

8. ***"Overall, it remains unclear which tissue-resident immune cell population(s) migrate into the organoid, how they impact the organoid, which cells survive long-term in these cultures and if tissue-resident lymphocytes maintain their identity over longer periods of time. These are critical questions that need to be answered before the study can be fully assessed."***

   The immune cell populations that migrate into the organoid are with near totality, T cells. Our multispectral immunofluorescent (mIF) staining of the IIO cultures demonstrated that all E-cadherin-negative cells found within the organoids were $CD3^+$ (Extended Data Fig. 1f). We did not observe $DAPI^+$ cells negative for both epithelial lineage or T cell lineage markers within organoid regions. This is unsurprising as the intraepithelial lymphocyte populations that integrate within the epithelium *in vivo* are also T cells (Cheroutre et al, *Nat Rev Immunol* 2011; Ma et al, *J Leukoc Biol* 2020). To facilitate these activities physiologically, mucosal T cells express a number of surface receptors that define their identity and support their residence within the epithelium. Specifically, they express CD103 (E-cadherin receptor, necessary for epithelial cell tethering, Cepek et al, *Nature* 1994), CD49a (collagen receptor necessary for ECM navigation, Bank et al, *Cell Immunol* 1994), and the tissue egress inhibitor CD69 (Kumar et al, *Cell Rep* 2017). IHC staining of CD103 and CD69 two weeks after introduction of the immune cells into our organoid cultures, showed that the $CD3^+$ T cells remained heavily integrated within the epithelium, and maintained expression of their tissue-resident defining markers (Extended Data Fig. 1f-i). Below we have attached single colour IHC images of CD103 (left) and CD69 (right) from IIO culture 14 days after immune cell introduction. We generated these images for your reference, to emphatically confirm the retention of these tissue residence features on the immune cells throughout the culture.

   The impact of the immune cells on the epithelium within homeostatic, non-inflamed conditions was not something we focused on during the project. Rather, we were interested in recapitulating physiologically-relevant pathogenic epithelial damage using the genuine immune cell populations reported to orchestrate inflammatory disease in patients. We would not anticipate a notable impact of homeostatic mucosal T cell behaviour on epithelial cell differentiation (although would not entirely rule it out), but we would expect there to be significant crosstalk between the two populations. Based on the scientific literature, this crosstalk likely contributes host defense against luminal pathogens, epithelial barrier functions and end of lifecycle epithelial cell expulsion. These phenomena could all be explored further with our model.

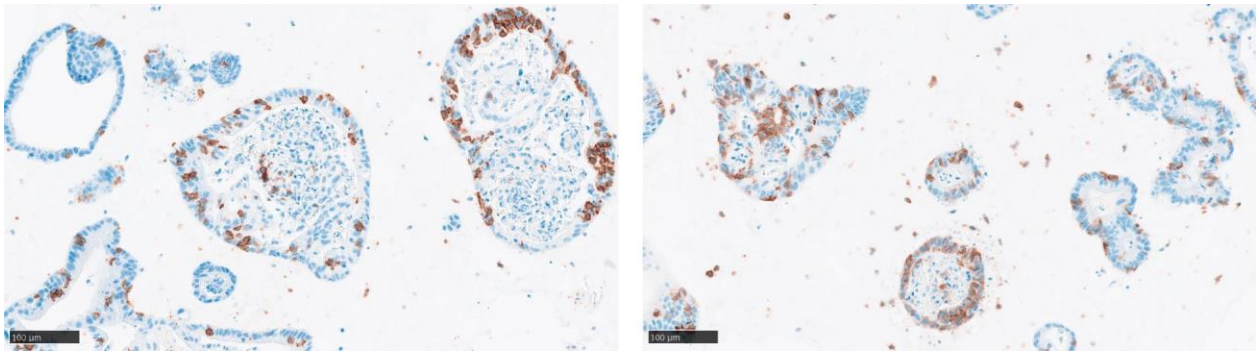

Figure Legend: IHC staining for tissue resident markers CD103 (left image) and CD69 (right image), in IIO cultures, 14 days after immune cell introduction.

9. **"*These experiments are interesting but difficult to interpret at this stage as they are lacking some important details and controls. For example, when were IIO cultures treated? When were immune cells added?*"**

We apologize for the lack of clarity. We have tried to address the reviewer's concerns and questions in our response to point 10 below.

10. **"*The authors state that they "assessed T cell behaviour at early (5h), mid (24h) and late (48h) timepoints by digesting and staining IIOs for surface and intracellular markers of T-cell activation and cytotoxicity" How was this done? How long were the organoids established before treatment? Were the lymphocytes added just before treatment or much earlier?*"**

After conventional organoid establishment (typically 2-4 weeks after crypt isolation), organoids were switched from growth media into differentiation media for 72h. After this 72h differentiation period, organoids were liberated without digestion from the extracellular matrix, combined with their matched immune cells, and resuspended in matrigel. It is this combination of organoids and immune cells that we termed intestinal immuno-organoids (IIOs). Treatment was added into the media supernatant immediately after coculture setup, thus initiating the inflammatory reaction. Sacrificial wells were digested into a single cell suspension to facilitate flow cytometry staining and analysis at the indicated timepoints after treatment addition. These timepoints allowed us to characterize immune cell changes right before detection of epithelial apoptosis (5h), during the height of response (24h), and at plateau (48). We used this data to build a picture of the kinetics and trajectory of the T cell inflammatory response in the context of EpCAM TCB treatment that we describe in detail in Extended Data Fig. 3, and further reinforce with scRNA-seq analysis in Figure 3 and Figure 4. The information relating to the experimental setup is also described in depth in the Methods section of the manuscript.

11. **"*These experiments should also contain controls such as circulating memory T cells, which would provide a fair comparison to TRM, rather than PBMC which contain largely naïve cells. An even better control would be organoids populated with specific immune cell populations, T cell only, B cell only, myeloid cells, NK cells …*"**

We very much agree with the reviewer's point that the memory rather than the tissue-resident character of TRMs might be responsible for the differences in responsiveness compared with PBMCs. To address this possibility, we separately co-cultured organoids with 1) TRMs, 2) isolated circulating memory T cells and 3) isolated circulating naive T cells and monitored their response to EpCAM TCB treatment. We found that circulating memory T cells were more effective in damaging organoids upon TCB treatment, compared with circulating naive T cells. However, they were still drastically less effective than TRMs: whereas TRMs began inducing organoid apoptosis after 10 h of co-culture, circulating memory T cells began inducing apoptosis after more than 40 h. Therefore, we conclude that memory T cells isolated from peripheral blood would not provide a faithful representation of T-cell-mediated responses within the intestinal tissue (Extended Data Fig. 3f).

To the reviewer's second point, we have definitively identified T cells to be the primary responder and effector population upon TCB treatment, using both flow cytometry (Ext. Data Fig. 3a-d) and transcriptomic analysis (Fig. 3 and 4). When present, monocytes and B cells likely potentiate the response by sensing the initial T-cell-drive inflammation and secreting additional cytokines. However, we do not expect them to have any effect in the absence of T cells, which are the primary targets of these drugs via their CD3 receptor (Bacac et al, *Clin Canc Res* 2016).

12. **"*The authors state: "We used scRNA-seq to interrogate the transcriptomic dynamics underlying TCB-dependent TRM activity at the onset (4h) and peak (48h) of epithelial cell targeting. Lymphocyte populations within the integrated dataset were annotated using differential gene expression together with previously published signatures and surface markers revealing diverse T cell, macrophage and B cell populations" Again, here it become clear that the authors use cultures that contain not only TRM, not even only tissue-resident lymphocytes, but indeed all immune cells, even macrophages. This makes it very difficult to assess what precisely is*

***going on. Indeed, it remains unclear if TRM cells are really the ones that lead to organoid destruction. It could be any of the other lymphoid or myeloid cells that have been used to populate the organoid.*"**

The reviewer is correct in pointing out that the IIO model (organoids co-cultured with intestine-resident immune cells) contains diverse immune cell types, including B-cell and myeloid cell populations. As illustrated in Ext. Data Fig. 1b, there is some donor-to-donor variability in the immune cell populations we isolate from the intestinal specimens, likely related to technical variability of the method, but also biological variability in the immunological state of the patient. It is true that we frequently isolate B cells (between 0 and 20%) and myeloid cells (between 0 and 10%) in addition to T cells. However, the overwhelming majority (between 80 and 100%) of the isolated population are T cells, which is why we chose to use the term TRMs to describe them collectively, as mentioned above.

Despite the slight variability in the immune cell populations isolated from the tissue samples and ultimately included in the IIO model, we respectfully disagree with the reviewer that the system is intractable or that the events and mechanisms unfolding within the system are not clear. Multiple independent readouts and methods reveal a clear and consistent picture of how EpCAM TCB treatment affects the system and the mechanisms whereby organoids are ultimately damaged. In particular:

- As shown in Fig. 4a-b, PBMCs do not undergo activation and inflict damage to organoids upon TCB treatment, whereas intestinal TRM-enriched isolates target organoids rapidly and extensively. This difference strongly implicates TRMs in particular as the driver of the response and suggests that B cells, monocytes and peripheral-blood T cells are far less responsive or not responsive at all. Comparing TRMs and blood-derived T-cell populations, we found that the former formed the majority of the responder population (defined by the induction of TNF-α, IFNγ or Granzyme B upon TCB treatment) (Ext. Data Fig 3b-c).
- Single-cell transcriptomic analysis demonstrated that the tissue-resident T cells (both $CD4^+$ and $CD8^+$) undergo the greatest changes upon TCB treatment, even in the presence of a B-cell and a myeloid cell population (Fig. 3f, h). Moreover, these changes entail the adoption of an activated and cytotoxic state, characterized by the expression of IFNγ and Granzyme B. Even though myeloid cells and B cells likely potentiate and shape the system through soluble cytokines acting on T-cell populations (as demonstrated by the ligand-receptor analysis in Fig. 4f), it would be very unlikely that the final outcome of organoid damage is mediated by any population other than activated, cytotoxic T cells.
- Repeating TCB treatment experiments with IIOs containing sorted populations of TRMs ($CD103^-$ and $CD103^+$) yielded identical levels of organoid apoptosis (Extended Data Fig. 4g), once again implicating TRMs as the main drivers of the response.

13. "*In their single cell transcriptomics related to this experiment, the authors focus on two tome points, 4h and 48h after TCB treatment. This seems problematic, given that 48h after treatment the organoid is destroyed. Even after 24h more or less all cells are apoptotic as shown in Fig 3A.*"

We now realize that the rationale behind the time points chosen may not be clear, and we thank the reviewer for pointing that out. The reviewer is correct - apoptosis induction began within 8 h of TCB treatment, and peaked around 48h (Ext. Data Fig. 3a). Beyond the 48h time point, the caspase signal decreased, likely indicating that the organoids were completely destroyed. The goal of the sequencing experiment was to analyze the TCB-mediated molecular and cellular changes that occur specifically within the immune compartment, and which are poorly understood. We found the epithelial changes to be less interesting, because it is clear from the experiments that organoids undergo apoptosis, as the reviewer also pointed out.

Focusing on the TRMs, we chose the 4 h time point because we were interested in documenting the early transcriptomic events that occur before any epithelial damage is recorded. Unsurprisingly, these

turned out to entail the differentiation of $CD4^+$ T cells into a cytokine-producing Th1-like state. We chose 48h as the second time point, because we wished to analyze how the TRMs had evolved to the point when epithelial damage was most extensive or even complete. Indeed, we found that both $CD4^+$ and $CD8^+$ T cells had acquired cytotoxic characteristics, including Granzyme B production, which explain the organoid destruction.

We have now modified the text to better communicate the rationale behind the experimental design (lines 189-191).

14. "*Finally, the authors use a ROCK1/2 inhibitor to demonstrate that the IIO cultures can be used to identify clinically relevant treatments or drugs. While I like the idea, the experiments related to this approach are very underdeveloped. It remains unclear why a ROCK1/2 inhibitor was chosen in the first place. Similarly, it was not tested what the ROCK1/2 inhibitor does to T cells per se. Does it impact T cell activation in general? Survival? Function? Motility? Differentiation? This should be tested in cultures containing isolated naïve T cells, TRM and other lymphocytes or myeloid cells involved in the culture system.*"

Our scRNA-seq analysis demonstrated that the transcriptomic signature of gut-derived T cells, relative to blood-derived T cells, was enriched in genes governing migration, motility and cytoskeletal rearrangements (Fig. 2e, f). We believe that this facilitates the epithelial surveillance activities that TRMs undertake as part of their inherent biological function, in particular the rapid locomotion and epithelial cell integration that we describe in figures 1-2. Given that ROCK proteins are well described mediators of adhesion, protrusion and cell movement, we reasoned that adding ROCK inhibitors to our cultures would cripple TRM homeostatic behavior and their response to immunotherapeutic drugs.

Indeed, we observed that in the presence of the inhibitor, homeostatic TRM motility was near completely lost. (Ext. Data Video 4, Ext. Data Fig. 6e). In the context of EpCAM TCB treatment, ROCK inhibition significantly impaired induction of key activation and effector markers CD25, Granzyme B and perforin (Extended Data Fig. 6f). Epithelial targeting was significantly attenuated (Figure 4j). T cell numbers and viability was equivalent between the ROCK inhibitor condition and the vehicle control, demonstrating no detrimental effect on T cell survival (Extended Data Fig. 6h). We posit that the crippling of TRM motility via ROCK inhibition reduces the ability of T cells to contact the epithelium in the presence of the EpCAM TCB. The absence of T-cell activation and effector functionality, as seen via the failure to robustly induce CD25, Granzyme B and perforin, reflects that the T cells are no longer able to contact the epithelium; this contact is required for inflammation in response to TCB treatment.

15. "*Overall, in the experiments related to the second half of the manuscript, it remains unclear which cell type precisely is involved in organoid destruction and how any of the drugs act that were used.*"

We apologize for the lack of clarity. We believe that the combined data from the initial transcriptomic analysis, the accompanying flow cytometry analysis and the additional revision experiments we performed all convey a clear and consistent picture of the mechanisms whereby the organoids are destroyed and the main players involved. In particular:

- The scRNAseq data revealed that early on (4 h) after TCB treatment, $CD4^+$ helper T cells differentiate into a Th1-like population that produces inflammatory cytokines, including TNF-α (Fig. 3h). According to the results of the receptor-ligand interaction analysis we performed, this population drives the differentiation of $CD8^+$ TRMs into a population that is increasingly cytotoxic (Granzyme B^+), proliferative ($Ki67^+$) and intraepithelial (Fig. 3h; Fig. 4a, e, f; Ext. Data Fig. 5d). In addition, the cytokine-producing $CD4^+$ population itself adopts a cytotoxic and proliferative state later on (Fig. 3h; Fig. 4b). *It is these two populations of TRMs that drive the organoid destruction*. Indeed, blocking TNF-α or motility leads to a

decrease in Granzyme B production in both $CD4^+$ and $CD8^+$ TRMs (Ext. Data. 6a, f) and a concomitant reduction in organoid destruction (Fig. 4i, j).

- We have verified the results from the transcriptomic analysis at the protein level by flow cytometry, finding a striking correlation: we observed early induction of TNF-α within T-cell populations at 4h, whereas the cytotoxicity marker Granzyme B and the proliferation marker Ki67 were upregulated at the 48h time point (Fig. 3i).

Following the reviewer's suggestion, we have now more clearly communicated the findings related to the mechanisms behind organoid destruction in the Discussion paragraphs of the revised manuscript Lines 370-375).

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have addressed most of the comments and significantly improved the manuscript and its presentation. However, the following essential points would need further clarification:

The authors have clarified the numbers of patients/samples profiled in the study, but it is still unclear from how many samples IIOs were successfully derived and which analyses were performed on which samples. Could the authors add a supplementary table (preferably within an extended data figure where it is easily accessible for readers) where for each of the 24 donors they received tissue from, from which were IIOs successfully established (including tissue site of origin) and which ones they used for which analyses (scRNAseq, flow, IHC/IF stainings, EpCAM treatment)? Additionally, it would be great to provide sample IDs in figures/figure legends to know which data are derived from the same samples.

The authors have added new donors to the scRNA-seq experiment and although 3 donors is not extensive, the concordance across donors looks sufficient, suggesting reproducibility of the approach. In the revised data, there is a substantial MAIT cell population in the IIOs which appears to be the most enriched population in TRM/IIO compared to PBMC. Were MAIT cells not detected in the IIOs in the previous version of the manuscript or were they not annotated correctly? Given the large fraction of MAIT cells in the IIOs, they would warrant discussion in the manuscript in addition to TRMs.

Could the authors unify the cell type annotations between Figures 2b and 3d? The annotations in Figure 3d are now completely different from Figure 2b, making it challenging to interpret the changes induced by the EpCAM antibody treatment. Can the authors integrate the EpCAM experiment data with the bigger scRNA-seq data to demonstrate how the identified populations in Figure 2b respond to stimulation? Additionally, there is a significant proportion of B cells in the EpCAM treatment data, is this something specific to this individual?

In the response to comment 5 to justify the used 24 h timepoint the authors state: ‘Longitudinal flow cytometry experiments have revealed no substantial differences in the TRM numbers, composition and activation profiles within the first four days of co-culture’. Can the authors provide the longitudinal flow cytometry data from the 1st four days of co-culture as supplementary data and briefly comment on the chosen timepoint in the text?

The authors have successfully provided additional experimental data suggesting potential of the model to assess immune checkpoint blockade-induced effects in the intestine and provided experimental validation of the predicted effects of TNF blockade.

In the methods section, the authors describe the tissue processing as “removing the underlying

muscularis, serosa, and fat from the basal side of the tissue," which suggests the mucosa (epithelium, lamina propria) and submucosa are used for IIO derivation. Could the authors comment on the source of the TRMs and whether they are coming from the lamina propria or lymphoid follicles, like Peyer's patches? Additionally, can the authors clarify how they are defining IELs? Were there specific markers they stained for or identified in sequencing data?

In response to comment 15 about the benefits of the cytokine-independent versus cytokine-dependent isolation method, can the authors include the provided flow cytometry data in an extended figure? Extended figures A-D show that similar proportions of immune cells are isolated with enzymatic and crawl out methods, but do not provide functional information about the effect of cytokines on marker expression (which seems to be the primary benefit of the crawl out method).

Referee #2 (Remarks to the Author):

The authors largely addressed my concerns. Regarding tissue-resident memory lymphocytes, I don't think my comment is about cell identity but simply the choice of an acronym. Because these cells are T-cells, 'TRM' may be confused due to the 'M' component standing for myeloid in the field. I suggest using TRMLs for tissue-resident memory lymphocytes.

Referee #3 (Remarks to the Author):

The authors have appropriately addressed the points I raised in the first round of review. Their findings will serve as a catalyst to advance organoid-based medicine. Toshiro Sato

Referee #4 (Remarks to the Author):

The authors demonstrate convincingly that TRM cells integrate into in vitro formed organoids. However, they do not show if or how immune cells impact organoid formation, integrity or function. I had requested this to be addressed but it was simply not tested. Do tissue-resident lymphocytes impact any of these features?

"Similarly, I cannot see any evidence for the claim that 'IIO formation … was orchestrated by TRM-enriched transcriptomic programs'. Again, this point remains unaddressed.

Admittedly, I am under the impression that the authors misunderstood the intention of my questions. I am interested in understanding how TRM cells impact on organoid formation or function. Not how TRM cells integrate into these organs.

The authors state: “An interaction between TRMs and organoids, as co-culture led to gene expression changes in both compartments, although the epithelial transcriptome was relatively stable across the conditions … Epithelial cell proportions and gene expression were stable across the different conditions (Ext. Data Fig. 2e-f). Intriguingly however, we observed that TRM presence led to a significant upregulation of ID3 and HES1, both of which are associated with intestinal stem cell maintenance (Hu et al, Front Cell Dev Biol 2021; Pellegrinet et al, Gastroenterol 2005) and microbial defense (Guo et al, Mucosal Immunol 2018). Although it was not the focus of our experiments, it is likely that longer-term culture in the presence of luminal antigens would be required for TRM effects on intestinal fate decisions to manifest more substantially."

This is beginning to address my question and should be expanded experimentally.

I would also like to restate my original question: “Overall, it remains unclear which tissue-resident immune cell population(s) migrate into the organoid, how they impact the organoid, which cells survive long-term in these cultures and if tissue-resident lymphocytes maintain their identity over longer periods of time. These are critical questions that need to be answered before the study can be fully assessed.”

The authors reply that “The impact of the immune cells on the epithelium within homeostatic, non-inflamed conditions was not something we focused on during the project.” However, as I stated, I think this is an important point that needs to be considered to make this an impactful study.

If the authors opt not to address this point further, I would request to substantially change their conclusion. The abstract states “IIO formation was driven by TRM migration and interaction with epithelial cells, as orchestrated by TRM-enriched transcriptomic programs governing cell motility and epithelial inspection” I understand this as a statement that organoid formation is altered/driven by immune cells and TRM-specific transcriptional programs impact on the state/quality/function of the organoid. That is not shown. If the authors intend their conclusion to convey a different message, please adjust the abstract and the conclusions throughout the text.

A final point, in their disease model, the authors conclude that “CD4+ helper T cells differentiate into a Th1-like population that produces inflammatory cytokines, including TNF-α (Fig. 3h) which then drives the differentiation of CD8+ TRMs into a population that is increasingly cytotoxic (Granzyme B+), proliferative (Ki67+) and intraepithelial (Fig. 3h; Fig. 4a, e, f; Ext. Data Fig. 5d). In addition, the cytokine-producing CD4+ population itself adopts a cytotoxic and proliferative state later on (Fig. 3h; Fig. 4b). It is these two populations of TRMs that drive the organoid destruction.” This is a complicated model, that may or may not be correct. Can the authors show direct involvement of CD4 T cells by depleting them from their setup?

Author Rebuttals to First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

"The authors have addressed most of the comments and significantly improved the manuscript and its presentation. However, the following essential points would need further clarification:

1. **The authors have clarified the numbers of patients/samples profiled in the study, but it is still unclear from how many samples IIOs were successfully derived and which analyses were performed on which samples. Could the authors add a supplementary table (preferably within an extended data figure where it is easily accessible for readers) where for each of the 24 donors they received tissue from, from which were IIOs successfully established (including tissue site of origin) and which ones they used for which analyses (scRNAseq, flow, IHC/IF stainings, EpCAM treatment)? Additionally, it would be great to provide sample IDs in figures/figure legends to know which data are derived from the same samples."**

   We have now provided Supplementary Information Table 4 detailing all the biological material contributing to data plotted in the manuscript. Within this table, we have included information on the exact region of the intestine from which the biological material was derived, and exactly which data (figure and tab) this material contributed to.

   With the additional donors included for the new Extended Data Figure 1d tab, which the reviewer requested we include in the manuscript (see comment 10 below), this list now totals 27 different donations. IIOs were always established, even if the data included in the manuscript focused specifically on the epithelial or immune compartment. Due to the space constraints within the figure legends, we are not able to include sample IDs for every experiment there - however this information is very easily retrievable within the provided table, should it be of interest to the reader.

2. **"The authors have added new donors to the scRNA-seq experiment and although 3 donors is not extensive, the concordance across donors looks sufficient, suggesting reproducibility of the approach. In the revised data, there is a substantial MAIT cell population in the IIOs which appears to be the most enriched population in TRM/IIO compared to PBMC. Were MAIT cells not detected in the IIOs in the previous version of the manuscript or were they not annotated correctly? Given**

the large fraction of MAIT cells in the IIOs, they would warrant discussion in the manuscript in addition to TRMs"

Indeed, the more granular cluster annotation we were able to run as part of the newly expanded figure 2 enabled identification of a small but notable population of MAIT cells, expressing high levels of *KLRB1* (protein name: CD161), *SLC4A10* and *MAF.* Although we did not specifically reference this population in the text, we did refer to expression of CD161 within our intestine-derived immune cells (line 76). MAIT cells are also an annotated population in Ext Fig. 1c (based on expression of CD161 and Vα7.2), and CD161 expression levels are documented across multiple donors in Ext Fig. 1e.

However, the reviewer raises an excellent point with regards to the heat map. Unfortunately, the heat map was incorrectly aligned with the bubble plots in 2c during the figure editing process. The most enriched gut populations, relative to PBMCs, are CD4+ TRMs and CD8+ TRMs, not MAIT cells. We have now corrected the ordering mistake in the figure, and express our gratitude to the reviewer for their keen eye and alerting us to this!

3. **"Could the authors unify the cell type annotations between Figures 2b and 3d? The annotations in Figure 3d are now completely different from Figure 2b, making it challenging to interpret the changes induced by the EpCAM antibody treatment. Can the authors integrate the EpCAM experiment data with the bigger scRNA-seq data to demonstrate how the identified populations in Figure 2b respond to stimulation?"**

We agree with the reviewer's suggestion, and have made the following adaptations to the cluster annotations in Fig 3d to the harmonize the two data sets:

- Changed the "CD8+ Res. IEL" name from Fig 3d to the all encompassing "CD8+ TRM" name used in Fig 2b. Both names are equally scientifically valid, as CD8 IELs are also tissue-resident memory cells
- Changed the drug-induced cluster name "CD8+ Act. IEL" from Fig 3d to "Effector CD8+ TRM" to align with the new name of its progenitor population
- Reordered the wording of the "CCL5- CD4+ TRMs" and "CCL5+ CD4+ TRMs" names from Fig 3d to "CD4+ TRM 1 (CCL5-)" and "CD4+ TRM 2 (CCL5+)" to align with the "CD4+ TRM" cluster from Fig 2b.

With these changes, all T cell clusters derived from control-treated IIO samples in Fig 3d are matched to the clusters described in the homeostatic IIOs in Fig 2b. We believe this should make interpretation of the data between figures more intuitive for a broad readership.

4. **"Additionally, there is a significant proportion of B cells in the EpCAM treatment data, is this something specific to this individual?"**

We observed B cells at varying proportions across different donors. As can be seen in the cumulative data in Ext. Fig 1b, the tSNE plot in Ext. Fig 1c and in the scRNA-seq cell type annotations in Ext. Fig 2a, B cells could comprise up to 30% of the cultures, but averaged 9%. Thus, although there was a substantial proportion of B cells in the EpCAM-TCB-treated scRNA-seq data set, this was not unusual.

5. **"In the response to comment 5 to justify the used 24 h timepoint the authors state: 'Longitudinal flow cytometry experiments have revealed no substantial differences in the TRM numbers, composition and activation profiles within the first four days of co-culture'. Can the authors provide the longitudinal flow cytometry data from the 1st four days of co-culture as supplementary data and briefly comment on the chosen timepoint in the text?"**

This data has now been included as a new tab in Extended Data Figure 2a and demonstrates the stability of CD8 TRM composition and activation status during the first 4 days of coculture. We have also added a line to the main text to comment on the chosen timepoint, as requested.

6. **"In the methods section, the authors describe the tissue processing as "removing the underlying muscularis, serosa, and fat from the basal side of the tissue," which suggests the mucosa (epithelium, lamina propria) and submucosa are used for IIO derivation. Could the authors comment on the source of the TRMs and whether they are coming from the lamina propria or lymphoid follicles, like Peyer's patches?"**

We did not separate lamina propria from permanent or temporary lymphoid follicles during our crawl out isolation, however, given the near complete absence of lymph node homing receptors such as CCR7 (Ext. Fig 1d, Fig 2c) and *SELL* (Fig 2c) on the TRM cells, in combination with ubiquitous expression of ECM markers such as CD49a (encoded by *ITGA1*, Ext. Fig 1d, Fig 2c) we would speculate with high confidence that these cells are derived from the lamina propria. We have added a line to the methods to confirm the lamina propria and lymphoid follicles were not separated prior to crawl out.

7. **“Additionally, can the authors clarify how they are defining IELs? Were there specific markers they stained for or identified in sequencing data?”**

The methodology for identifying intraepithelial lymphocytes at the protein level is detailed in the example gating strategy, included in the supplemental information. Here, we defined IELs using the classical and lineage-defining IEL surface molecules CD69 and CD103 (Mayassi and Jabri, Mucosal Immunol 2018). Close to 90% of CD8+ve cells isolated from intestine samples were CD103+ve (Ext. Fig 1d) and thus IELs. Accordingly, nearly all CD8+ve T cells isolated using our crawl out protocol are IELs.

As discussed in our first revision, the gene encoding CD103 (*ITGAE*) is lowly expressed transcriptomically. Therefore, scRNA-seq-based identification of IEL clusters within broader gut-derived TRM populations were based on similarity to annotated IEL populations from previously published intestinal data sets (Luoma et al., Cell 2020). This, amongst others, included elevated expression of *ITGAE, ITGA1* and *KLRD1,* as well as reduced expression of *ANXA1* and *KLRB1,* when compared to other TRM populations.

In order to harmonize the data presented from the separate scRNA-seq experiments, we have adapted the “CD8+ Res. IEL” name from Fig 3d to the all encompassing “CD8+ TRM” name used in Fig 2b. Both names are equally scientifically valid, as CD8 IELs are also tissue-resident memory cells, but we believe this should make interpretation of the data between figures more intuitive for a broad readership.

8. **“In response to comment 15 about the benefits of the cytokine-independent versus cytokine-dependent isolation method, can the authors include the provided flow cytometry data in an extended figure? Extended figures A-D show that similar proportions of immune cells are isolated with enzymatic and crawl out methods, but do not provide functional information about the effect of cytokines on marker expression (which seems to be the primary benefit of the crawl out method).”**

We have now included these data in the manuscript, as an additional tab in Extended Data Figure 1 (Ext. Fig 1d).

Referee #2 (Remarks to the Author):

1. **"The authors largely addressed my concerns. Regarding tissue-resident memory lymphocytes, I don't think my comment is about cell identity but simply the choice of an acronym. Because these cells are T-cells, 'TRM' may be confused due to the 'M' component standing for myeloid in the field. I suggest using TRMLs for tissue-resident memory lymphocytes."**

   We agree with the reviewer that the field's use of the abbreviation is confusing. However, we wanted to avoid introducing a new abbreviation and decided to adhere to the standard one, which is widely used and accepted (e.g. Schenkel and Masopust, Immunity 2014; Szabo et al, Sci Immunol 2019). The myeloid field uses the abbreviation 'RTMs' for tissue-resident macrophages (e.g. recent review by Florent Ginhoux - Bleriot et al, Cancer Cell 2024).

Referee #3 (Remarks to the Author):

"The authors have appropriately addressed the points I raised in the first round of review. Their findings will serve as a catalyst to advance organoid-based medicine. Toshiro Sato"

Dear Dr. Sato - thank you for your thoughtful review and constructive comments. We admire your pioneering body of work on organoids, and are glad that we could build on it.

Referee #4 (Remarks to the Author):

1. **"The authors demonstrate convincingly that TRM cells integrate into in vitro formed organoids. However, they do not show if or how immune cells impact organoid formation, integrity or function. I had requested this to be addressed but it was simply not tested. Do tissue-resident lymphocytes impact any of these features?"**

We apologize for not fully addressing the reviewer's question and suggestion. Our approach to assessing the effect of TRMs on organoids was the scRNAseq experiment, whereby we defined the gene expression of epithelial organoids in the presence and absence of TRMs (Fig. 2; Ext Data Fig. 2). Although we observed upregulation of genes related to stem cell self-renewal (e.g. *HES1* and *ID3*; Ext. Data Fig. 2f), we concluded that TRMs did not have a striking effect on organoid formation, growth and differentiation. We stated our findings in the revised manuscript.

We agree with the reviewer that TRMs and IELs might have far-reaching effects on organoid/epithelial features related to pathogen defense, barrier integrity, function and regeneration (Lockhart et al., 2024, Annu. Rev. Immunol. Olivares-Villagómez and Van Kaer, 2018, Trends Immunol). We believe that these would manifest under conditions of challenge, however, whether it is pathogenic infection or injury. Therefore, to capture the processes effectively the system needs to be further developed in terms of biological complexity and technical features. Most urgently, the IIOs would need to be grown in a barrier format, with independently accessible luminal and basal surfaces, which would permit both introducing luminal pathogens and assessing changes in barrier function, defense and healing. Indeed, we have previously developed methods for growing intestinal organoids in barrier formats that also feature crypt-villus architecture (Gjorevski et al, *Science* 2022). We are highly interested in pursuing the directions pointed out by the reviewer in a follow up study, but find them to be too ambitious for the timeframe of this revision.

2. **"Similarly, I cannot see any evidence for the claim that 'IIO formation … was orchestrated by TRM-enriched transcriptomic programs'. Again, this point remains unaddressed. Admittedly, I am under the impression that the authors misunderstood the intention of my questions. I am interested in understanding how TRM cells impact on organoid formation or function. Not how TRM cells integrate into these organs."**

The authors state: 'An interaction between TRMs and organoids, as co-culture led to gene expression changes in both compartments, although the epithelial transcriptome was relatively stable across the conditions … Epithelial cell proportions and gene expression were stable across the different conditions (Ext. Data Fig. 2e-f). Intriguingly however, we observed that TRM presence led to a significant upregulation of ID3 and HES1, both of which are associated with intestinal stem cell maintenance (Hu et al, Front Cell Dev Biol 2021; Pellegrinet et al, Gastroenterol 2005) and microbial defense (Guo et al, Mucosal Immunol 2018). Although it was not the focus of our experiments, it is likely that longer-term culture in the presence of luminal antigens would be required for TRM effects on intestinal fate decisions to manifest more substantially.'

This is beginning to address my question and should be expanded experimentally."

We acknowledge that our use of the 'IIO/IIO formation' terminology has created some misunderstanding and confusion. To avoid confusion on the reader's part, we will simply describe the process as TRM integration within organoids (instead of 'IIO formation'). As we discussed above, we observed modest TRM-mediated transcriptomic effects on intestinal organoids (Ext. Data Fig. 2g), whereas modeling and studying functional effects effectively would require further adaptations of the system.

3. **"I would also like to restate my original question: "Overall, it remains unclear which tissue-resident immune cell population(s) migrate into the organoid, how they impact the organoid, which cells survive long-term in these cultures and if tissue-resident lymphocytes maintain their identity over longer periods of time. These are critical questions that need to be answered before the study can be fully assessed.**

The authors reply that "The impact of the immune cells on the epithelium within homeostatic, non-inflamed conditions was not something we focused on during the project." However, as I stated, I think this is an important point that needs to be considered to make this an impactful study.

If the authors opt not to address this point further, I would request to substantially change their conclusion. The abstract states "IIO formation was driven by TRM migration and interaction with epithelial cells, as orchestrated by TRM-enriched transcriptomic programs governing cell motility and epithelial inspection" I understand this as a statement that organoid formation is altered/driven by immune cells and TRM-specific transcriptional programs impact on the state/quality/function of the organoid. That is not shown. If the authors intend their conclusion to convey a different message, please adjust the abstract and the conclusions throughout the text."

The reviewer's point resonates with us and we find studying the effects of TRMs on the epithelium *per se* to be very exciting. Given the biological and technological developments that are required for enabling such investigations, however, we believe that these questions would be answered most thoroughly and rigorously in a follow-up study.

We will follow the reviewer's suggestion and modify our conclusions in the abstract to communicate that our study focused on defining 1) how TRMs interact and integrate within intestinal organoids and 2) how they impact organoids upon exposure to inflammatory stimuli like cancer immunotherapy

drugs. We have also now commented in the discussion as to how further improvements to the model could be utilised to further explore the relationship between TRMs and epithelial cells (lines 309-313).

4. **"A final point, in their disease model, the authors conclude that "CD4+ helper T cells differentiate into a Th1-like population that produces inflammatory cytokines, including TNF-α (Fig. 3h) which then drives the differentiation of CD8+ TRMs into a population that is increasingly cytotoxic (Granzyme B+), proliferative (Ki67+) and intraepithelial (Fig. 3h; Fig. 4a, e, f; Ext. Data Fig. 5d). In addition, the cytokine-producing CD4+ population itself adopts a cytotoxic and proliferative state later on (Fig. 3h; Fig. 4b). It is these two populations of TRMs that drive the organoid destruction." This is a complicated model, that may or may not be correct. Can the authors show direct involvement of CD4 T cells by depleting them from their setup?"**

We have used flow cytometry analysis to confirm some of the key steps of the model proposed based on the transcriptomic data (Fig. 3i): 1) both $CD4^+$ and $CD8^+$ TRMs ultimately adopt a cytotoxic ($GzmB^+$), cytokine-producting ($TNF\alpha^+$, $IFN\gamma^+$) and proliferative ($Ki67^+$) character, but 2) $CD4^+$ switch on cytokine production very early on, mere hours after TCB treatment. Admittedly, we have not causatively shown the direct influence of $CD4^+$ on $CD8^+$ TRM differentiation and activation. We did however, show that blocking TNFα – one of the major and early cytokine products of the $CD4^+$ population – dampens the activation of the entire system (Ext. Data Fig. 6), which is consistent with the computational predictions and supports a strong role for $CD4^+$ TRMs.

Nonetheless, we should emphasize that we believe any population of $CD3^+$ T cells would be capable of differentiation, activation and ultimately epithelial damage, in response to TCB treatment, given the mode of activation of these molecules (Goebeler & Bargou, 2020, Nat. Rev. Clin. Oncol.), even a pure population of $CD4^+$ or $CD8^+$ TRMs. Whereas the final outcome may be unaltered, we expect the exact composition of T-cell subtypes to influence the kinetics of the process.