nature research

Peer Review Information

Journal: Nature Immunology
Manuscript Title: Embryonic macrophages function during early life to determine iNKT cell levels at barrier surfaces
Corresponding author name(s): Richard S. Blumberg

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-LE29785

Message: 9th Jun 2020

Dear Dr Blumberg,

Your Letter, "Macrophages of embryonic origin function during early life to determine host iNKT cell levels at barrier surfaces" has now been seen by 3 referees. You will see from their comments copied below that while they find your work of considerable potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the manuscript for publication, but would be open to considering a revised version that addresses these serious concerns.

We hope you will find the referees' comments useful as you decide how to proceed. If you wish to submit a substantially revised manuscript, please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions.

The three Refs. raise numerous experimental and technical issues. Also, if you recall I was concerned there was insufficient mechanistic insights into what's so special about fetal macs that uniquely empowers them to establish NKT residency, the Refs. seemed to be similarly concerned about this issue so I'd say it's a critical one to address.

If you choose to revise your manuscript taking into account all reviewer and editor comments, please highlight all changes in the manuscript text file.

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

If revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed

each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

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

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

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If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere. Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

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

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

Thank you for the opportunity to review your work.

Sincerely,

Zoltan Fehervari, Ph.D.
Senior Editor

nature research

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Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Gensollen report here that macrophages of embryonic origin, rather than macrophages of bone marrow origin, control the number of iNKT cells in the colon, before the age of 12 days. This control, even limited to the neonatal period, has a persistent effect on the number of colonic iNKT cells into adulthood. As a consequence, the outcome of a pathological challenge in the adult, that is dependent on colonic iNKT cells, is determined by these early life events. Finally, the number of intestinal macrophages of embryonic origin is dependent on the microbiota, which therefore has an indirect but long lasting effect on the gene expression profile of colonic iNKT cells.

This study is very interesting, as it shows that a particular type of macrophage that is prevalent early in life determines the number of colonic iNKT cells into adulthood, and thus, phenotypes that are dependent on this subset of cells. On the other hand, a number of interpretations seem far-fetched, and most mechanisms in play remain to be uncovered.

Specific points:

1. Figure 1 shows that the neonatal colon is uniquely permissive to the expansion of thymus-derived iNKT cells. This experiment is key, but its implications largely unexplored. First, adult thymi were used for transfers, which is surprising as one may expect a time correlation between neonatal thymus output and colon-mediated recruitment of iNKT cells. Have the authors assessed the efficacy of colon colonization by the transfer of thymic cells of different ages? Second, thymus-derived iNKT cells appear to migrate or expand preferentially in the colon, as compared to the spleen. Are there specific adhesion molecules involved? As an example, former experiments on IELs suggested that thymic T cells expressing the integrin a4b7 were specifically recruited to the intestinal epithelium.

2. Figure 3 assumes a clear phenotypic definition between embryonic and bone marrow-derived macrophages, based on varying levels of F4/80 and CD11b. It is however my understanding that this definition is rather blurry, in particular as these markers are common macrophage markers. The authors should therefore show FACS plots, and re-assure the reader with additional markers that specific these two subsets. By the way, in figure 3F, the non-significance of the difference between wild-type and KO in F4/80low macrophages is rather difficult to appreciate.

3. In figure 3G, “conventional” Tab cells are also affected by the loss in Plvap, while these cells were never affected by any of the treatments affecting colon macrophages in the paper, so far. What does that tell us? Could this be relevant to the colonic function of

macrophages? Of note, no less than 4 strategies have been used in this study to deplete macrophages, which, on the one hand, makes it very robust. On the other hand, different strategies are used to makes different points. In particular, why is the administration of AFS98 antibody, in figure 4, used to assess the effect of microbiota, rather than, for example, the MM-DTR strategy?

4. The conclusion to figure 4A-F is that the microbiota is not necessary for the macrophages to control iNKT cell numbers in the colon. However, the authors do not directly compare the efficacy of macrophages to control iNKT cell number in GF, SPF and conventionalized GF mice. The only point that is clear, and that was already established in former studies, is that the number of embryo-derived macrophages in the intestine decreases with microbial colonization. It is expected that this effect modifies in turn the number of iNKT cells, but numbers reported in figures 4E and 4F indicate that they are not. The authors then turn to the examination of the gene expression profile of macrophages derived from GF and SPF mice. However, the analysis of these results does not contribute to an understanding of the mechanisms at play.

5. Figure 5 shows that embryonic macrophages lead to a higher number of Ki67+ iNKT cells in the colon. The authors conclude that these macrophages regulate extrathymic proliferation of iNKT cells. However, given the results shown in figure 1, it is possible that these iNKT cells proliferate at their site of production, and then, migrate to the colon. Note that the level of Ki67 in iNKT cells 8 days after birth in the absence of macrophages is similar to its level in the adult in the presence of macrophages, indicating that iNKT cells may only proliferate early in life, at least at the steady state. Finally, the in vitro experiments do not assess proliferation.

6. Figure 6 reports challenges in adult that depend on colonic iNKT cells. The authors show that decreased numbers in colonic iNKT cells, because of neonatal depletion in macrophages, leads to decreased severity in oxazolone-induced colitis. Even though this suggests that the neonatal events change an adult phenotype through iNKT cells, it is not shown that the decreased numbers in iNKT cells in the adult are the actual cause. It is possible that other changes concurrent to the neonatal depletion in macrophages lead to lower severity in colitis in the adult. Therefore, the causality of adult iNKT cells should be demonstrated.

7. The listeria model used here is unexpected. Listeria does not infect mice via the oral route unless the mice express the human E-cadherin. And therefore, an intravenous route of infection is often used in mice. Nonetheless, the authors use the oral route and find that the bacterial burden is increased in both the spleen and the colon when neonatal macrophages are depleted. Nevertheless, iNKT cell numbers only change in the colon, not in the spleen, in this depletion model, and finally, macrophages are also involved in the clearance of blood-borne listeria.

Reviewer #2:
Remarks to the Author:
This study deciphers the mechanism controlling the abundance of iNKT cells in the colon at early age in mice. iNKT cells are innate-like T cells that can exert protective or deleterious role in various immunopathology including autoimmunity and infection. Previous studies from the same laboratory had shown that microbiota in early life controlled iNKT cells

abundance in the colon of mice, which can aggravate colon inflammation and asthma.
In the present study the investigators analyze the mechanism linking the microbiota to colon iNKT cell abundance in early life. They first show that iNKT cells are resident in the colon using parabiosis experiments; iNKT cells migrate and expand in colon between 5 to 20 days of life. By using several mouse models, leading to all macrophages depletion, specific depletion of embryonic or bone marrow derived macrophages at different ages, they show that embryonic derived macrophages on day 8 to 10 of life determine the abundance of iNKT cells in colon and spleen. In adult mice, macrophages are involved in the abundance of spleen iNKT cells but not of colon iNKT cells.
Comparing GF, SPF and microbiota colonized SPF showed that microbiota decreases the abundance of embryonic macrophages in the colon at early life. RNAseq of macrophages from GF and SPF mice as well as iNKT cells from early and adult life showed gene expression differences.
The lower abundance of iNKT cells in colon of mice lacking macrophages in early life is associated with lower proliferation.
Further experiments with thymic T cell transfer and in vitro adult spleen explant culture show that iNKT cell expansion is controlled by macrophages independently of the thymus.
Lastly in adult mice, which have been depleted on macrophages on days 8-14, the number of macrophages is similar as control mice whereas the number of colon iNKT cells remains lower and these mice are less susceptible to oxazolone-induced colitis and more resistance to Listeria infection. Thus early life modification can influence iNKT cell mediated immune response in adults.

B. It is a very elegant study using multiple approaches to demonstrate the role of embryonic derived macrophages to control iNKT cell abundance in the colon. Previous study for this laboratory had shown the impact of early colonization of mice with microbiota to control colon iNKT cell abundance. This study reveals the key role of embryonic derived macrophages in regulating colon iNKT cells abundance and it shows the impact of the microbiota on early life colon macrophages.

However this studies raises several comments as mentioned below.

C. Major points:
- All the study is based on flow cytometry analysis of iNKT cells and macrophages but the raw data are not shown. There is only one figure (S10) showing some flow cytometry data. Example of each analysis for each set of experiments should be shown, at least in the supplementary data. It is critical to see example of staining to see the number of cells analyzed, the intensity of the staining with key markers in each experiments such as parabiosis, staining from different tissues skin, spleen and colon, upon the different types of macrophage depletion, Ki67 staining in spleen and colon, in the in vitro explants…

- In the previous article there was a very interesting parallel between colon and lung iNKT cell abundance (Olszak Science 2012). It would have been very interesting to see such data in this study at least for the key experiments (early life depletion, some specific macrophages depletion mouse models). Figure S2 A and C show the link between macrophages and iNKT cell abundance in the skin at early life based on all macrophages depletion. Further analyses of skin iNKT cells would strengthen the study, particularly in the models with specific depletion of embryonic derived macrophages.

- The data in Fig 3F show that embryonic derived macrophages (Plvap-/- mice) also control total abT cells level and not only iNKT cells in the colon, which is different from the

data shown in Fig 2C and G with early life macrophages depletion in MMDTR mice. These different results in different mouse models should be discussed.

- Figure 4E and F: the absolute number of iNKT cells is similar in the colon of GF and GFCV mice at day 21. Are these data in agreement with the previous study by Olszak et al. Science 2012, which is the basis for this present study?

Minor comments:
- Page 10 lines 213-214: sentence to be checked: microbial cues are (not) necessary for the ability of macrophages to supervise iNKT cells in the colon.

-Figure 5B: it summarizes the data on Ki67 MFI. Would be also interesting to have the percentage of iNKT cells positive for Ki67. Again, it would be important to see the flow cytometry staining.

D. No comments on the statistics.

E. The conclusions are robust since many experimental approaches are used to demonstrate the link between colon iNKT cell, embryonic derived macrophages and microbiota colonization.

F. Suggested improvements.
- It would be interesting to have more insight in the molecular mechanisms involved in the link between colon embryonic derived macrophages and iNKT cells. Further comparison by RNAseq of colon embryonic derived macrophages and colon bone marrow derived macrophages would be of high interest since they are in the same tissue they might exhibit only moderate transcriptomic differences, which could be key in local iNKT cell expansion.
Similarly comparison of colon embryonic derived macrophages versus adult spleen macrophages could provide more insight in the molecule(s) involved since spleen adult macrophages are also required for spleen iNKT cell expansion.
Multiple comparisons should help to identify the molecular differences between the repressive and non repressive macrophage subsets.
In the same line, It would be important to see on colon embryonic derived macrophages (versus other macrophages mentioned above) whether they are higher expression level of critical proteins relevant to iNKT cells such as CD1d (recognized by iNKT cell TCR), SAP and SLAM molecules (involved in thymic and peripheral iNKT cell activation/expansion) and CXCL16 (association of mucosal CXCL16 and iNKT cell levels reported by the same laboratory, Olszak et al. Science 2012).

G. References of previous work are appropriate.

H. The manuscript is very well written although it is a complex study. In each figures, many graphs illustrate the different experimental set up.

Reviewer #3:
Remarks to the Author:
This manuscript reports that embryonic-derived macrophages, together with the microbiota, regulate the number of natural killer T cells (NKT cells) in the colon lamina

propria of mice; in the absence of these embryonic macrophages, the number of colonic NKT cells was decreased. They were important, however, only during a window up to 2 weeks of age. Surprisingly, systemic, DTR-mediated depletion of macrophages had effects that lasted into adulthood, as 7-week old mice were more susceptible to two models in which NKT cell function has been implicated: they were more susceptible to oxazolone-induced colitis and less able to clear Listeria.

Here the authors have attacked a difficult but important question: how does the neonatal immune system imprint changes that last later in life? They imply, based on their findings, that colonic NKT cells are seeded in the colon early in life and are mostly self-renewing—suggested by the parabiotic mouse experiments—and that the maintenance of these cells long-term is dependent on the early presence of embryonic macrophages. This is an interesting hypothesis. The authors show that NKT cell proliferation is in fact reduced in day 8-old mice after depletion of macrophages, but adult depletion does not have this effect—however, it would be important to know if NKT cell proliferation (BrdU or Ki67) is affected by early depletion when the mice are analyzed at 7 weeks age.

The functional analysis of NKT cells should be improved. Were iNKT cell-mediated inflammatory responses in fact altered later life (line 272)? There was less Ifng mRNA produced in the colon after antigen stimulation of NKT cells (Fig. S9D), but probably not on a per cell basis, as the number of colonic NKT cells was similarly reduced. Is the representation of NKT cell subsets different? The effects of systemic macrophage depletion on the oxazalone model were not shown to be NKT cell-dependent, although NKT cells play an important role in this model. The same is true for Listeria, and the authors should explain why Ifng mRNA was increased rather than reduced in the macrophage depleted mice that have a higher CFU (likely immunopathology).

The data are fascinating, but this reader was left hungry for details or even a speculative hypothesis as to how do the embryonic macrophages change NKT cells later in life? Cytokines such as IL-15 (or IL-7 for NKT cells that make IL-17) have been shown to be important for NKT cell homeostasis and could be responsible for their findings regarding spleen NKT cells—but there must be something that the embryonic macrophages do in the colon that affects NKT cells weeks later when they are reduced or no longer effective. Even at day 25 age there were still plenty of colonic embryonic macrophages (Fig. 3A), but depletion at two weeks of age or later had little effect, so the early embryonic macrophages must be critical. The RNA-seq results of colonic macrophages from day 8 and 14 (Fig. S7A) were not very revealing regarding mechanism. (note that lines 222-224 of the manuscript seems to indicate this figure compares GF to SPF mice, which disagrees with the figure legend). Did the authors analyze IL-15 levels in the colon?

Additional points:
Tissues. Lamina propria and not epithelium was analyzed, but this was never mentioned in the text, only in the Methods. Skin was analyzed in a few experiments, but the skin data were incomplete and can be omitted. Was small intestine ever analyzed?

Experimental rigor. Were there experimental repeats for fig 1E and 1F with only 3 mice analyzed? Same is true for some other experiments such as 4E. Student's t test may not be appropriate with only 3 mice and probably not for the data in 6H which do not appear to be normally distributed. Some representative flow data for the Ki67 staining should be shown. Were mice of both sexes analyzed in the experiments? Note that for the

experiments carried out in Turku the ratios of NKT cells to macrophages were entirely different—the Turku wild type mice have a much higher number of NKT cells than the US mice, but fewer macrophages, showing the importance of other factors (microbiome?) in regulating colon NKT cell numbers. This complexity should be acknowledged, but nevertheless, the lack of embryonic macrophages was shown in their experiments as well (Fig. 3G).

Author Rebuttal to Initial comments

We are grateful to the efforts by the reviewers and all of the important points that they raised which have been very helpful to us in revising and significantly improving the manuscript. We have taken their comments seriously and performed a large number of additional experiments as outlined below and summarized in the point-by-point reponses to each reviewer. Please note that the reviewer's comments are in bold. In the revised manuscript, the inclusion of these new data and modifications in response to the reviewer's concerns are indicated by an underline in the text.

New experiments/analyses included in the manuscript :

- Representative FACS plots were included for every main figures of the manuscript.
- Increase of the number of experimental replicates (**Fig. 1E-F, Fig. 4D-G**).
- Analysis of the capacity of CD45.1$^+$ iNKT and TCR-$\alpha\beta^+$ T cells transferred during early life to persist in the CD45.2$^+$ adult hosts colon and spleen (**Fig. S1C-D**)
- Analysis of iNKT and TCR-$\alpha\beta^+$ T cell levels in the lung and small intestine following early life macrophage depletion (**Fig. S2D-E**).
- Analysis of colonic iNKT cell numbers in SPF, GF and GFCV over time in C57BL/6 mice (**Fig. 4A, S6C**).
- Reanalysis of the transcriptional data (**Fig. 5, Table S1-5**).
- Analysis of Ki67 expression in transferred CD45.1$^+$ iNKT and TCR-$\alpha\beta^+$ T cells following early life macrophage depletion in the colon (**Fig. S7F,G**).
- Analysis of gene expression by quantitative PCR of the whole colon following macrophage depletion during early life (**Fig. S6E**).
- Analysis of CD45.1$^+$ iNKT cell subsets in the colon of 8 day old CD45.2$^+$ host mice following macrophage depletion and adoptive transfer during early life (**Fig. S7H**).
- Analysis of iNKT cell subsets in the spleen and colon of adult mice following macrophage depletion during early life (**Fig. S10 A-B**)
- Analysis of Ki67 and CD69 expression by iNKT and TCR-$\alpha\beta^+$ T cell in the spleen and colon of adult mice following macrophage depletion during early life (**Fig. S10C-H**).

New experiments/analysis not included in the manuscript :

- Analysis of the capacity of CD45.1$^+$ iNKT and TCR-$\alpha\beta^+$ T cells of different ages to engraft into the colon of CD45.2$^+$ adult hosts colon and spleen (**Rebuttal Fig. 1A-C**)
- Analysis of the expression of $\alpha4\beta7$ in CD45.1$^+$ and CD45.2$^+$ iNKT cells in the spleen and colon following early life transfer of thymic T cells (**Rebuttal Fig. 2**).
- Analysis of F4/80 and CD11b expression in CD45.1$^+$ macrophages following bone marrow derived monocytes adoptive transfer during early life in the colon (**Rebuttal Fig. 3A-B**)
- Analysis of iNKT and TCR-$\alpha\beta^+$ T cell levels in the thymus of PLVAP deficient mice (**Rebuttal Fig. 4**)
- Analysis of *Il15* and *Il7* mRNA expression in the colon following macrophage depletion during early life (**Rebuttal Fig. 5**)
- Analysis of the normal distribution of the data from Figure 7H by "D'Agostino-Pearson omnibus normality test" (**Rebuttal Fig. 6**).

Reviewer 1

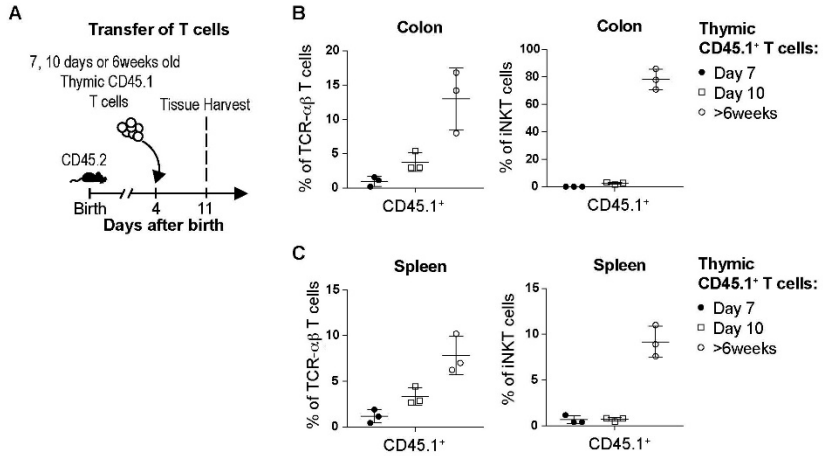
Gensollen report here that macrophages of embryonic origin, rather than macrophages of bone marrow origin, control the number of iNKT cells in the colon, before the age of 12 days. This control, even limited to the neonatal period, has a persistent effect on the number of colonic iNKT cells into adulthood. As a consequence, the outcome of a pathological challenge in the adult, that is dependent on colonic iNKT cells, is determined by these early life events. Finally, the number of intestinal macrophages of embryonic origin is dependent on the microbiota, which therefore has an indirect but long lasting effect on the gene expression profile of colonic iNKT cells. This study is very interesting, as it shows that a particular type of macrophage that is prevalent early in life determines the number of colonic iNKT cells into adulthood, and thus, phenotypes that are dependent on this subset of cells. On the other hand, a number of interpretations seem far-fetched, and most mechanisms in play remain to be uncovered.

We are grateful that you took great interest in reading our study and believe that the additional supporting data we provide in the revised manuscript strengthen our conclusions.

Specific points:

1. Figure 1 shows that the neonatal colon is uniquely permissive to the expansion of thymus-derived iNKT cells. This experiment is key, but its implications largely unexplored. First, adult thymi were used for transfers, which is surprising as one may expect a time correlation between neonatal thymus output and colon-mediated recruitment of iNKT cells. Have the authors assessed the efficacy of colon colonization by the transfer of thymic cells of different ages?

To answer this interesting question, we first transferred similar numbers of CD45.1$^+$ thymic T cells of different ages into 4 day old CD45.2$^+$ pups and examined their levels at day 11 (**Rebuttal Fig.1A**). We observed that the efficacy of the CD45.1$^+$ iNKT and TCR-$\alpha\beta^+$ T cell to seed the colon (**Rebuttal Fig.1B**) and spleen (**Rebuttal Fig.1C**) is decreased when the transferred T cells originate from an early compared to an adult thymus. This result can be explained by the low numbers of differentiated iNKT cells that can be found in the thymus before weaning (*1*) and suggests that the number of iNKT cells and TCR-$\alpha\beta^+$ T cells that seed and then engraft in the colon during early life strongly depends upon the availability of differentiated cells in the thymus. As such, taken together, we suspect that the quantities we transfer of early life thymocytes does not adequately capture the flux of iNKT cells that are presumably migrating out of the thymus during the neonatal period which is likely to be substantively greater than the amounts administered experimentally but able to be achieved with the iNKT cell enrichment in later life. Secondly, we newly observed that early life transferred CD45.1$^+$ iNKT cells but not TCR-$\alpha\beta^+$ T cells had the capacity to persist long term in the recipient CD45.2$^+$ adult host's colon but not in the spleen (**Fig. S1C-D**). These results globally confirm that the colon compared to the spleen is uniquely permissive to the migration and expansion of thymically-derived iNKT cells during early life to establish long term residency.



Rebuttal Fig. 1:
A) Schematic of adoptive transfer strategy. Adoptive transfer of thymic T cells from 7, 10 days or 6 weeks old CD45.1 mice in 4 day old CD45.2 mice (n=3) followed by quantitative analysis of TCR-αβ+ T (left) and iNKT (right) cells in the colon (B) and spleen (C) on day 11.

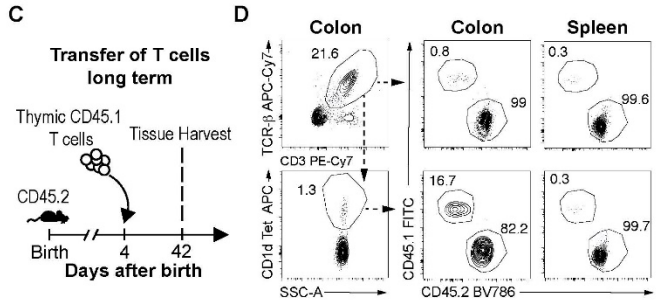
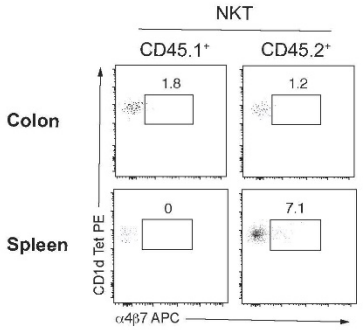


Fig. S1.
C) Schematic of adoptive transfer strategy. D) Adoptive transfer of CD45.1 adult thymic cells into a 4 day old CD45.2 host followed by quantitative analysis of colonic CD45.1 or CD45.2 TCR-αβ+ T and iNKT cells by flow cytometry on day 42.

Are there specific adhesion molecules involved? As an example, former experiments on IELs suggested that thymic T cells expressing the integrin α4β7 were specifically recruited to the intestinal epithelium.

RNAseq analysis of iNKT cells and macrophages did not allow us to identify any specific adhesion molecules involved in this process (see **Fig. 5**, below and **Table S1/3/4**). We therefore also performed adoptive transfer of enriched CD45.1+ thymic T cells into 4 day old CD45.2+ hosts and harvested the colon and spleen at day 11. We assessed the expression of α4β7 in the CD45.1+ and CD45.2+ iNKT cells but did not identify differential or high expression in the colon or spleen (**Rebuttal Fig. 2**).



Rebuttal Fig. 2:
Adoptive transfer of CD45.1 adult thymic cells into a 4 day old CD45.2 host followed by quantitative analysis of splenic and colonic CD45.1 or CD45.2 iNKT cells by flow cytometry on day 11.

2. Figure 3 assumes a clear phenotypic definition between embryonic and bone marrow-derived macrophages, based on varying levels of F4/80 and CD11b. It is however my understanding that this definition is rather blurry, in particular as these markers are common macrophage markers. The authors should therefore show FACS plots, and re-assure the reader with additional markers that specific these two subsets. By the way, in figure 3F, the non-significance of the difference between wild-type and KO in F4/80low macrophages is rather difficult to appreciate.

The rationale of using F4/80 and CD11b levels to distinguish embryonic from bone marrow macrophages in the colon was previously demonstrated by fate mapping (*2*). We agree with the reviewer that there is not a specific marker defined to date for those of embryonic origin such that the levels of F4/80 and CD11b are the ones that we used as we now explicitly state in the manuscript to read as below (Line 168-171).

"We therefore monitored the kinetics of these populations using surface markers whose levels differ on macrophages of embryonic (F4/80^{hi}CD11b^{lo}) or bone marrow (F4/80^{lo}CD11b^{hi}) origin (*12*)."

Therefore, as suggested, we also include FACS plots of F4/80 and CD11b staining in the manuscript (**Fig. 3B-F**). To further reassure the reviewer, we performed a transfer experiment of adult bone marrow monocytes into mice during early life which shows that bone marrow macrophages in the colon belong to the F4/80^{lo} CD11b^{hi} population (**Rebuttal Fig. 3A-B**). Thus, in Figure 3F, the non-significance of the difference between WT and Plvap$^{-/-}$ mice in F4/80^{lo} CD11b^{hi} macrophage population levels is only indicative that Plvap$^{-/-}$ mice exhibit embryonic but not bone marrow derived macrophage deficiency as previously described (*3*).

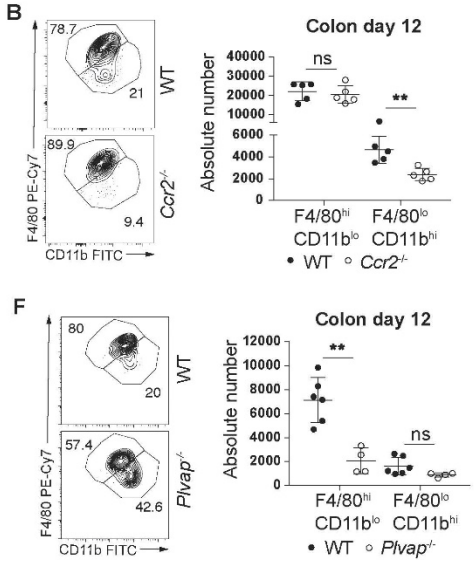
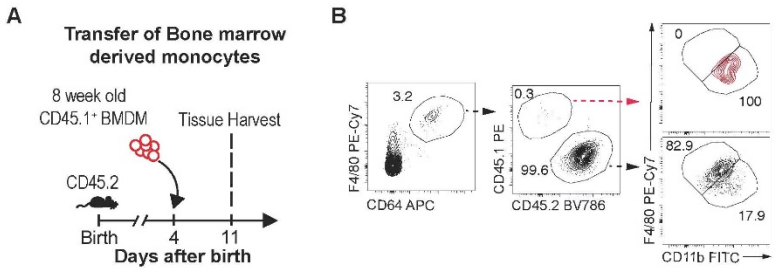


Fig. 3.
B) Representative plot (left) and absolute count (right) of F4/80^{hi}/CD11b^{lo} and F4/80^{lo}/CD11b^{hi} macrophages in the colon of wild type littermates (WT) or *Ccr2*$^{-/-}$ animals at day 12 after birth. F) Representative plot (left) and absolute count (right) of F4/80^{hi}/CD11b^{lo} and F4/80^{lo}/CD11b^{hi} macrophages in the colon of WT littermates or *Plvap*$^{-/-}$ animals at day 12 after birth. Numbers in the representative plots indicate cell frequency. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. *P* values were calculated by unpaired Student's t-test. $^{**}P < 0.01$, ns: not-significant.

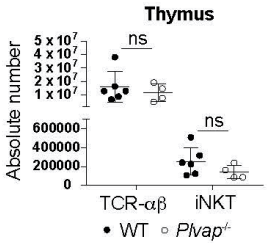


Rebuttal Fig. 3:
A) Schematic of adoptive transfer strategy. B) Adoptive transfer of CD45.1 adult bone marrow derived monocytes in a 4 day old CD45.2 host followed by quantitative analysis of macrophages by flow cytometry on day 11.

3. In figure 3G, "conventional" Tαβ cells are also affected by the loss in Plvap, while these cells were never affected by any of the treatments affecting colon macrophages in the paper, so far. What does that tell us? Could this be relevant to the colonic function of macrophages?

We thank the reviewer for this question. We performed analysis of the thymus from PLVAP deficient mice but did not observe difference in TCR-αβ$^+$ T or iNKT cells (**Rebuttal Fig. 4**). We conclude that the conventional TCR-αβ$^+$ T cells defect we observed in PLVAP deficient mice was not systemic and could be relevant to the colonic function of macrophages and/or PLVAP specific function in T cells. This suggests that other subsets of resident T cells may be affected by a defect in embryonic macrophages, which is interesting for future studies. We now discuss this specific point in the manuscript as below (Line 405-409).

"Based upon our results in other organ systems and with *Plvap*$^{-/-}$ mice that specifically disable fetal-derived macrophage emigration into peripheral tissues, our results likely extend to iNKT cell affiliation with other organs such as the small intestine, skin and lung and potentially the development of other types of resident cells such as subsets of TCR-αβ T cells."



Rebuttal Fig. 4:
Absolute count of iNKT and TCR-αβ$^+$ T cells in the thymus of WT littermates or *Plvap*$^{-/-}$ animals at day 12 after birth. ns: not-significant.

4. Of note, no less than 4 strategies have been used in this study to deplete macrophages, which, on the one hand, makes it very robust. On the other hand, different strategies are used to makes different points. In particular, why is the administration of AFS98 antibody, in figure 4, used to assess the effect of microbiota, rather than, for example, the MM-DTR strategy?

The main reason is technical. It would have been complicated and costly to rederive MM^{DTR} mice as germ free. Therefore, we opted for using the AFS98 antibody that could be injected in a sterile fashion.

5. The conclusion to figure 4A-F is that the microbiota is not necessary for the macrophages to control iNKT cell numbers in the colon. However, the authors do not directly compare the efficacy of macrophages to control iNKT cell number in GF, SPF and conventionalized GF mice. The only point that is clear, and that was already established in former studies, is that the number of embryo-derived macrophages in the intestine decreases with microbial colonization. It is expected that this effect modifies in turn the number of iNKT cells, but numbers reported in figures 4E and 4F indicate that they are not. The authors then turn to the examination of the gene expression profile of macrophages derived from GF and SPF mice. However, the analysis of these results does not contribute to an understanding of the mechanisms at play.

The reviewer makes very good points. The effects of macrophage depletion and the GF state on iNKT cell numbers exhibit different kinetics as the reviewer notes. At day 11, when iNKT cell levels

are decreased in mice depleted of macrophages for example, we never observed colonic iNKT cell differences in GF compared to SPF and GFCV mice at this age (*4*, *5*). Previously reported studies by us and others were performed in Swiss-Webster (SW) mice which have shown that iNKT cells are increased in GF relative to SPF mice and GFCV mice from day 21 after birth (*4*, *5*). We therefore performed a similar experiment in mice on a C57BL/6 (B6) background which were used in the current study to determine when colonic iNKT cell numbers start to diverge in response to (or lack of) early life microbial signals. We observed elevated levels of colonic iNKT cells in GF mice compared to SPF from day 28-35 after birth indicating a 7-14 day delay in observing this phenotype in GF B6 relative to SW mice (**Fig. 4A**). This was confirmed by us wherein we now show that GF mice that were conventionalized in early life exhibit repression of iNKT cell number relative to that observed in GF mice at day 35 of life (**Fig. S6C**). Therefore, the consequences of increased quantities of embryonic macrophages in GF mice (and other effects of the microbiota which impinge on iNKT colonization and/or expansion) are more gradual in their onset in B6 relative to SW mice and thus accumulate over time within early life. Thus, loss of microbial repression on the number of embryonic macrophages during early life correlates with a decrease in iNKT cell levels at a later stage in the colon (day 21 in SW and days 28-35 in B6). The fact that abrupt deletion of early life macrophages has a more rapid phenotype relative to that observed in GF mice suggests that both the quantity and quality (namely functional attributes) of the macrophages are involved in the phenotype observed.

To better comprehend the possible mechanisms involved, we performed new transcriptional analyses of macrophages at day 8 compared to day 14 (**Fig. 5A-B**) and between GF and SPF conditions at day 9 of life (**Fig. 5C-D**). In addition, we compared the transcriptional profile of iNKT cells in early life (day 14) and adult life (day 56) (**Fig. 5E-F**). The reason we chose day 14 for the iNKT cell analysis is to allow for enough iNKT cells to be analyzed (**Fig. 5**). These studies showed that macrophages during the first week of life in SPF mice and under GF conditions are enriched in genes associated with pathways that indicate a commitment to producing factors that determine the nature of the extracellular niche (*Col6a2*, *Lum*, *Mfap5*, *Angptl1*, *Dcn*) and factors involved in the growth and/or recruitment (*Cxcl12*) of iNKT cells. Further, iNKT cells in early life are enriched in genes and associated pathways indicative of increased cell division consistent with the proliferation we observed and reported herein that requires macrophages as we show. Together, this suggests that during early life, macrophages primarily of embryonic origin are establishing the niche and directing the expansion, differentiation and development of colonic iNKT cells. We have added the following to the manuscript to make these points on line 225 to 296.

"To understand the potential mechanisms by which macrophages may regulate iNKT cells in early life, we performed bulk RNA-Seq of colonic macrophages and iNKT cells. We investigated the transcriptional signatures of macrophages defined as $CD64^{+}F4/80^{+}$ at day 8 and day 14 after birth to identify transcripts specifically upregulated or downregulated during the time embryonic macrophages control iNKT cell levels in early life within the colon. We observed 325 transcripts with elevated abundance and 378 transcripts with decreased abundance in colonic macrophages purified at day 8 after birth compared to day 14 after birth (**Fig. 5A**, **Table S1**) suggesting a major switch in macrophage function during this period. Part of the differentially expressed genes we identified encoded secreted immune factors such as interleukins (IL) and chemokines (CXCL) which suggest an important role of macrophages in determining differentiation, proliferation and/or migration of iNKT cells. Transcripts encoding CXCL12 (*Cxcl12*) were enriched at day 8 while transcripts encoding IL12b (*Il12b*) and IL27 (*Il27*) were enriched at day 14 (**Fig. 5A**). Consistent with a potential role of one or several of these immune factors in colonic iNKT cell regulation during early life, CXCL12 has been shown to regulate iNKT cell migration *in vitro* (*22*). Further, colonic macrophages at day 8 after birth were highly enriched in transcripts encoding proteins generally associated with the extracellular matrix (ECM) such as Decorin (*Dcn*), Lumican (*Lum*),

Microfibrillar-associated protein 5 (*Mfap5*), Collagen 6a2 (*Col6a2*) as well as proteins associated with angiogenesis such as Angiopoietin-related protein 1 (*Angptl1*) (**Fig. 5A**). The upregulation of genes associated with ECM formation suggest colonic macrophages at day 8 compared to day 14 may have an important role in the structural development of the colon that is unique to early life and thus involved in creating a niche conducive to iNKT cell seeding and development. In agreement with this, the Gene Ontology (GO) term analysis showed that ECM organization was the most significantly enriched biological process followed by blood vessel development in day 8 macrophages (**Fig. 5B, Table S2**).

We also examined macrophages from SPF and GF mice at day 9 after birth. We observed 259 transcripts with elevated abundance and 27 transcripts with decreased abundance at day 9 after birth in macrophages from GF compared to SPF mice in the colon (**Fig. 5C**, **Table S3**), which advocate for a specific role of microbiota in regulating macrophages likely of embryonic origin during early life when the establishment of iNKT cell residency is taking place. We intersected the transcripts differentially expressed in colonic macrophages at day 8 compared to day 14 after birth (**Fig. 5A**) with transcripts differentially expressed in GF compared to SPF mice at day 9 after birth (**Fig. 5C**) and found 19 transcripts to be commonly dysregulated in these two datasets among which 13 were enriched in GF versus SPF and day 8 versus day 14 after birth (**Fig. 5D**). These similarities (>2.5%) between the differentially expressed genes in both datasets predicted a coincidence in their mechanisms of action. Indeed, the 13 genes commonly identified in macrophages as enriched in GF versus SPF and day 8 versus day 14 after birth included at least 3 transcripts that encoded proteins associated with ECM organization (*Dcn*, *Lum*, *Mfap5*), suggesting early life macrophages may participate in this process under the influence of the microbiota.

Interestingly, some of the genes we identified as differentially expressed in sorted macrophages during early life and participating to ECM organization (*Dcn*, *Lum*, *Mfap5, Col6a2*) or angiogenesis (*Angptl1, Cxcl12*) are more generally associated with non-hematopoietic cells (*23*, *24*). This makes it possible that their enrichment in macrophages during early life were derived from recent phagocytosis of neighboring non-hematopoietic cells (*25*) or doublets due to strong anatomical association (*26*). However, we observed a significant decrease in the expression of these genes in the whole colon following specific macrophage depletion at day 8 (**Fig. S6E**). This supports the notion that early life embryonic macrophages may be an alternative source of these transcripts. We did not observe differences in CXCL16 or CD1d expression by transcriptional analysis of macrophages (data not shown) or by quantitative PCR of the whole colon following macrophage depletion during early life (**Fig. S6E**) suggesting alternative cellular sources of these factors in the regulation of iNKT cells such as the intestinal epithelium as previously shown (*7*, *27*). Our studies thus suggest that the control of iNKT cell levels and differentiation by early life embryonic macrophages was most likely derived from a multifactorial process involving their role in sculpting the structure of the colon to create the proper niche for iNKT cell establishment and providing factors that affect iNKT cell expansion and/or differentiation.

To further investigate this, we next turned our attention to the transcriptional profiles of iNKT cells during early (day 14) and later (day 56) life by RNA-Seq and identified 61 differentially expressed transcripts (**Fig. 5E**, **Table S4**). Of these, 32 transcripts exhibited elevated abundance in the adult relative to day 14 of life and 29 transcripts were uniquely increased in neonatal iNKT cells. From GO term analysis, the early life iNKT cells were especially enriched in pathways associated with cell division (**Fig. 5F**, **Table S5**). This supports the notion that iNKT cells adopt a distinct transcriptional program during early compared to adult life consistent with cells in the process of establishing residency and point toward a potential role for proliferation of iNKT cells during this time period."

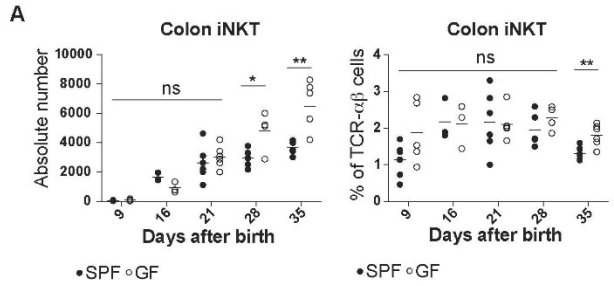


Fig. 4.

(A) iNKT (CD45$^+$ CD3ε$^+$ TCRβ$^+$ CD1d Tetramer$^+$) absolute counts (left) and cell percentage (right) in the colon of specific pathogen free (SPF) and germ free (GF) mice over time. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. *P* values were calculated by unpaired Student's t-test. **P* < 0.05, ***P* < 0.01, ns: not-significant.

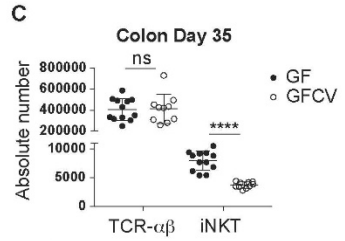


Fig. S6.

C) Absolute count of iNKT and TCR-αβ$^+$ T cells in the colon of germ-free (GF) and GF conventionalized with specific pathogen free (SPF) microbiota prior to birth (GFCV) animals at 35 days of life. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired Student's t-test. ****P<0.0001. ns: not-significant.

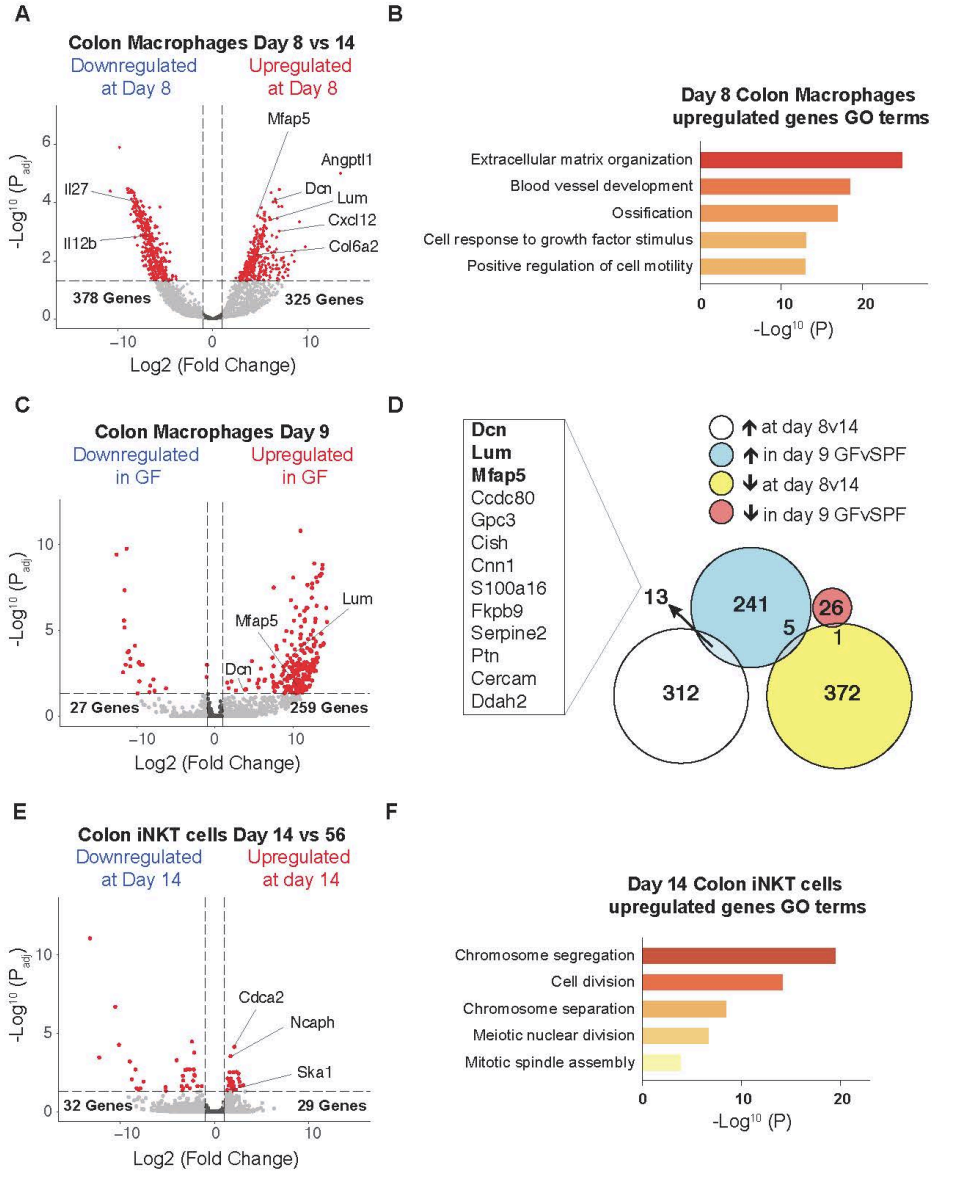


Fig. 5. Macrophage and iNKT cell transcriptional signature during early life.
A) Transcriptome analysis of colonic macrophages ($CD45^+$ Lin^- $F4/80^+$ $CD64^+$ cells) from 8 and 14 day old specific pathogen free (SPF) animals (n=4). B) Pathway analysis by gene ontology enrichment of transcripts increased in colonic macrophages at day 8 compared to day 14 after birth in SPF animals. C) Transcriptome analysis of colonic macrophages from day 9 SPF (n=2) and GF (germ free, n=4) animals. D) Intersection

of the differentially expressed genes identified by the transcriptome analysis of colonic macrophages from 8 and 14 day old SPF animals and the transcriptome analysis of colonic macrophages from day 9 SPF and GF animals. E) Transcriptome analysis of colonic iNKT (CD45$^+$ CD3ε^+ TCRβ^+ CD1d Tetramer$^+$) cells from 14 day old (n=3) and 56 day old (n=4) animals raised under SPF conditions. F) Pathway analysis by gene ontology enrichment of transcripts differentially expressed in colon iNKT cell populations at day 14 compared to the adult in SPF animals. Transcripts differentially expressed between groups were identified by DESeq2 analyses ($log_2|FC|>1$, $p_{adj}<0.05$, red).

6. Figure 5 shows that embryonic macrophages lead to a higher number of Ki67+ iNKT cells in the colon. The authors conclude that these macrophages regulate extrathymic proliferation of iNKT cells. However, given the results shown in figure 1, it is possible that these iNKT cells proliferate at their site of production, and then, migrate to the colon. Note that the level of Ki67 in iNKT cells 8 days after birth in the absence of macrophages is similar to its level in the adult in the presence of macrophages, indicating that iNKT cells may only proliferate early in life, at least at the steady state. Finally, the in vitro experiments do not assess proliferation.

We agree with the reviewer. We now include an experiment showing that transferred iNKT cells in the colon have decreased Ki67 expression following macrophage depletion (**Fig. S7F,G**). This directly demonstrates that macrophages regulate the extrathymic proliferation of iNKT cells in situ and consistent with the transcriptional studies in the revised **Fig. 5**. It was previously demonstrated that iNKT cells mainly proliferate during early life in the steady state colon of Swiss Webster mice (*5*) which is consistent with iNKT cell being resident cells in the adult. Our results globally suggest that the colonic iNKT cell population may emerge from a few thymus derived progenitors with proliferative capacities that are at least in part regulated by embryonic macrophages during early life. This may also explain why the abrupt elimination of embryonic macrophages has dramatic effects on iNKT cell quantities compared to the more gradual changes in iNKT cells observed in GF mice that do not quantitatively reveal themselves for 2-3 weeks.

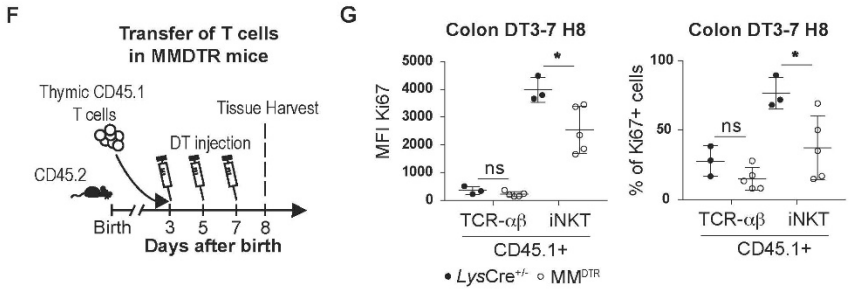


Fig. S7.
F) Schematic of adoptive transfer and macrophage depletion model. G) Adoptive transfer of CD45.1 adult thymic cells into 3 day old CD45.2 control littermates *Lys*Cre$^{+/-}$ or MM^{DTR} animals followed by DT administration from day 3 to 7 (DT3-7) after birth and quantitative analyses on day 8 (H8) of the Ki67 MFI (left) and percentage (right) of CD45.1 expressing TCR-$\alpha\beta^+$ T and iNKT cells in the colon. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired Student's t-test. *$P < 0.05$, ns: not-significant.

7. Figure 6 reports challenges in adult that depend on colonic iNKT cells. The authors show that decreased numbers in colonic iNKT cells, because of neonatal depletion in macrophages, leads to decreased severity in oxazolone-induced colitis. Even though this suggests that the neonatal events change an adult phenotype through iNKT cells, it is not shown that the decreased numbers in iNKT cells in the adult are the actual cause. It is possible that other changes concurrent to the neonatal depletion in macrophages lead to lower severity in colitis in the adult. Therefore, the causality of adult iNKT cells should be demonstrated.

We agree with the reviewers. The best experiment to prove causality would be to breed the MM^{DTR} ($LysCre^{+/-}$ and $CSF1R^{DTR}$) with CD1d or Jα18 knock out mice strains which are deficient for iNKT cells. Such an experiment would be costly and take a long time to establish. Further, there is good evidence in the literature that NKT cells are essential for induction of oxazolone induced colitis suggesting that they are at least one, if not the main, factor involved (*6*). That said, we agree that there are conceivably other factors downstream of macrophage depletion in addition to iNKT cells and therefore have downplayed the conclusion of these experiments to read as below (Line 31-34, 374-390, 414-417).

"Consequently, early life perturbations of fetal-derived macrophages result in persistent decreases of mucosal iNKT cells and is associated with later life susceptibility or resistance to iNKT cell associated mucosal disorders."

"In association with persistent reductions in colonic iNKT cells, MM^{DTR} mice depleted of macrophages in early life were protected from oxazolone-induced colitis in later life as shown by significantly diminished weight loss (**Fig. 7F**), mortality (**Fig. 7G**) and pathology (**Fig. 7H**) relative to control mice. As oxazolone-induced colitis is known to depend upon iNKT cells (*10*), these observations are consistent with the protection from colitis observed. Conversely, when MM^{DTR} and littermate control mice were treated with DT between day 8 and day 14 after birth and orally inoculated on day 49 with a modified form of *Listeria monocytogenes* able to infect mice (**Fig. 7I**), the number of *L. monocytogenes* colony forming units (CFU) recovered from the colon and spleen were significantly greater in MM^{DTR} compared to littermate control mice (**Fig. 7J**), in association with increased levels of *Ifnγ* and *Il12p40* mRNA expression in the colon (**Fig. S11C-D**). *L. monocytogenes* infection in *Cd1d* deficient mice, that lack iNKT cells, exhibit similar findings in the colon and spleen (*31*) suggesting the decreased colonic iNKT cell levels observed in MM^{DTR} mice may result in increased susceptibility to such an infection. Together, these results reveal that the embryonic macrophage-mediated control of mucosal iNKT cell proliferation during early life is associated with durable functional consequences."

"Consequently, early life perturbations of colonic macrophages of embryonic origin are associated with differential host susceptibility to iNKT cell-dependent responses to enteropathogens and environmental stimuli that induce inflammation which models inflammatory bowel disease."

8. The listeria model used here is unexpected. Listeria does not infect mice via the oral route unless the mice express the human E-cadherin. And therefore, an intravenous route of infection is often used in mice. Nonetheless, the authors use the oral route and find that the bacterial burden is increased in both the spleen and the colon when neonatal macrophages are depleted.

We apologize for the confusion. Indeed, we used a specific strain of *L. monocytogenes*, which is known as "murinized" strain in collaboration with Matthew Waldor's laboratory at Brigham and Women's Hospital. In this strain, two amino acids in the major invasion protein Internalin A were

substituted, so that this strain has a higher binding affinity to the mouse orthologue of E-cadherin (*7*). With this strain of *L. monocytogenes*, we were able to infect the mice via oral route and study the dissemination. This is now clearly documented in the method of the manuscript as below (Line 856-859).

"*L. monocytogenes* infection was performed as previously described (*38*). Briefly, 7 weeks old C57BL/6 male mice were inoculated by gavage with 3 x 10^9 CFUs of a mutated strain of *L. monocytogenes* with high binding affinity to mouse E-cadherin that is able to infect mice via the oral route (*39*)."

Nevertheless, iNKT cell numbers only change in the colon, not in the spleen, in this depletion model, and finally, macrophages are also involved in the clearance of blood-borne listeria.

Firstly, we do not observe differences in macrophages at day 49 following their depletion during early life in either the colon or spleen as they are replenished by bone marrow derived monocytes (**Fig. 7B**). Previous publications have shown that *Cd1d* knock out mice which lack iNKT cells, were more susceptible to *L. monocytogenes* infection with higher CFU in the spleen as well as higher IFNγ expression in the colon (*8*) which phenocopy what we observed in the MM^{DTR} mice (**Fig.7 I-J, Fig. S11C-D**). In light of the replenishment of splenic macrophages observed, we hypothesize there is more translocation in the setting of decreased iNKT cells. In other published work, we have observed that *Cd1d* knock out mice exhibit increased bacterial translocation from the lumen (*9*). We have modified our conclusions from this experiment and refer in light of the reviewer's suggestions (Line 31-34, 374-390, 414-417).

"Consequently, early life perturbations of fetal-derived macrophages result in persistent decreases of mucosal iNKT cells and is associated with later life susceptibility or resistance to iNKT cell associated mucosal disorders."

"In association with persistent reductions in colonic iNKT cells, MM^{DTR} mice depleted of macrophages in early life were protected from oxazolone-induced colitis in later life as shown by significantly diminished weight loss (**Fig. 7F**), mortality (**Fig. 7G**) and pathology (**Fig. 7H**) relative to control mice. As oxazolone-induced colitis is known to depend upon iNKT cells (*10*), these observations are consistent with the protection from colitis observed. Conversely, when MM^{DTR} and littermate control mice were treated with DT between day 8 and day 14 after birth and orally inoculated on day 49 with a modified form of *Listeria monocytogenes* able to infect mice (**Fig. 7I**), the number of *L. monocytogenes* colony forming units (CFU) recovered from the colon and spleen were significantly greater in MM^{DTR} compared to littermate control mice (**Fig. 7J**), in association with increased levels of *Ifnγ* and *Il12p40* mRNA expression in the colon (**Fig. S11C-D**). *L. monocytogenes* infection in *Cd1d* deficient mice, that lack iNKT cells, exhibit similar findings in the colon and spleen (*31*) suggesting the decreased colonic iNKT cell levels observed in MM^{DTR} mice may result in increased susceptibility to such an infection. Together, these results reveal that the embryonic macrophage-mediated control of mucosal iNKT cell proliferation during early life is associated with durable functional consequences."

"Consequently, early life perturbations of colonic macrophages of embryonic origin are associated with differential host susceptibility to iNKT cell-dependent responses to enteropathogens and environmental stimuli that induce inflammation which models inflammatory bowel disease."

Reviewer 2:

This study deciphers the mechanism controlling the abundance of iNKT cells in the colon at early age in mice. iNKT cells are innate-like T cells that can exert protective or deleterious role in various immunopathology including autoimmunity and infection. Previous studies from the same laboratory had shown that microbiota in early life controlled iNKT cells abundance in the colon of mice, which can aggravate colon inflammation and asthma. In the present study the investigators analyze the mechanism linking the microbiota to colon iNKT cell abundance in early life. They first show that iNKT cells are resident in the colon using parabiosis experiments; iNKT cells migrate and expand in colon between 5 to 20 days of life. By using several mouse models, leading to all macrophages depletion, specific depletion of embryonic or bone marrow derived macrophages at different ages, they show that embryonic derived macrophages on day 8 to 10 of life determine the abundance of iNKT cells in colon and spleen. In adult mice, macrophages are involved in the abundance of spleen iNKT cells but not of colon iNKT cells. Comparing GF, SPF and microbiota colonized SPF showed that microbiota decreases the abundance of embryonic macrophages in the colon at early life. RNAseq of macrophages from GF and SPF mice as well as iNKT cells from early and adult life showed gene expression differences. The lower abundance of iNKT cells in colon of mice lacking macrophages in early life is associated with lower proliferation. Further experiments with thymic T cell transfer and in vitro adult spleen explant culture show that iNKT cell expansion is controlled by macrophages independently of the thymus. Lastly in adult mice, which have been depleted on macrophages on days 8-14, the number of macrophages is similar as control mice whereas the number of colon iNKT cells remains lower and these mice are less susceptible to oxazolone-induced colitis and more resistance to Listeria infection. Thus early life modification can influence iNKT cell mediated immune response in adults. It is a very elegant study using multiple approaches to demonstrate the role of embryonic derived macrophages to control iNKT cell abundance in the colon. Previous study for this laboratory had shown the impact of early colonization of mice with microbiota to control colon iNKT cell abundance. This study reveals the key role of embryonic derived macrophages in regulating colon iNKT cells abundance and it shows the impact of the microbiota on early life colon macrophages.

We appreciate the accurate description of our work with emphasis on its elegance and have performed the suggested experiments as described below.

Specific points:

Major:

All the study is based on flow cytometry analysis of iNKT cells and macrophages but the raw data are not shown. There is only one figure (S10) showing some flow cytometry data. Example of each analysis for each set of experiments should be shown, at least in the supplementary data. It is critical to see example of staining to see the number of cells analyzed, the intensity of the staining with key markers in each experiments such as parabiosis, staining from different tissues skin, spleen and colon, upon the different types of macrophage depletion, Ki67 staining in spleen and colon, in the in vitro explants.

We thank the reviewer for this comment. Representative FACS plots are now shown for each set of experiments (**Fig. 1F/H, Fig. 2B/C/E/L/M/O, Fig. 3B/C/F/G, Fig. 4D/E/F/G, Fig. 6B/D, Fig S1B/D, Fig. S6D, Fig S7D/E/H, Fig. S9A/D/E, Fig S10A/B**).

In the previous article there was a very interesting parallel between colon and lung iNKT cell abundance (Olszak Science 2012). It would have been very interesting to see such data in this study at least for the key experiments (early life depletion, some specific macrophages depletion mouse models).

As suggested, we performed macrophage depletion during early life and observed a decrease in iNKT and TCR-αβ$^+$ cell levels in the lung (**Fig. S2E**). This is quite interesting as we also see some decreases in conventional T cells, albeit not as great as we see with iNKT cells. This is similar to observations with *Plvap*$^{-/-}$ mice suggesting that embryonic macrophages may regulate other types of resident cells in different tissue beds or contexts. Thank-you for this very good suggestion.

E

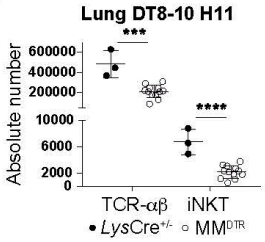


Fig. S2.
E) DT administered from day 8 to 10 (DT8-10) after birth followed by quantitative analyses on day 11 (H11) of the absolute count of iNKT and TCR-αβ$^+$ T cells in the lung. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired Student's t-test. ***$P < 0.001$, ****$P < 0.0001$.

Figure S2 A and C show the link between macrophages and iNKT cell abundance in the skin at early life based on all macrophages depletion. Further analyses of skin iNKT cells would strengthen the study, particularly in the models with specific depletion of embryonic derived macrophages.

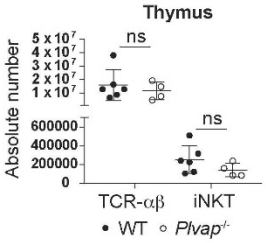
We agree this would have been interesting to investigate the skin phenotype further. Unfortunately, PLVAP deficient mice show a high rate of lethality at birth (15% of the expected frequency of homozygous *Plvap*$^{-/-}$ pups from heterozygous × heterozygous breedings survive to early adulthood) (*3*) which makes it difficult to perform such experiments in the current situation. Therefore, we were not able to extend our skin analyses.

The data in Fig 3F show that embryonic derived macrophages (Plvap-/- mice) also control total abT cells level and not only iNKT cells in the colon, which is different from the data shown in Fig 2C and G with early life macrophages depletion in MMDTR mice. These different results in different mouse models should be discussed.

We thank the reviewer for this comment. As requested, we now discuss in the manuscript how TCR-αβ$^+$ T cell levels being decreased in PLVAP mice could be relevant to the colonic function of macrophages or PLVAP specific function in TCR-αβ$^+$ T cells as below (Line 405-409).

"Based upon our results in other organ systems and with *Plvap*$^{-/-}$ mice that specifically disable fetal-derived macrophage emigration into peripheral tissues, our results likely extend to iNKT cell affiliation with other organs such as the small intestine, skin and lung and potentially the development of other types of resident cells such as subsets of TCR-αβ T cells."

Of note, we performed an analysis of the thymus in PLVAP-deficient mice but did not observe differences in TCR-αβ^{+} T cell or iNKT cells during early life demonstrating the decrease is not systemic (**Rebuttal Fig. 4**).



Rebuttal Figure 4:
Absolute counts of iNKT and TCR-αβ^{+} T cells in the thymus of WT littermates or *Plvap*$^{-/-}$ animals at day 12 after birth. ns: not-significant.

Figure 4E and F: the absolute number of iNKT cells is similar in the colon of GF and GFCV mice at day 21. Are these data in agreement with the previous study by Olszak et al. Science 2012, which is the basis for this present study?

We apologize to the reviewer for the confusion. Previous studies were performed in Swiss-Webster mice. These have shown that iNKT cells are increased in GF relative to SPF mice and GFCV mice from day 21 after birth (*4*, *5*). In mice of a C57BL/6 background as used in this study, we observed elevated levels of colonic iNKT cells in GF mice compared to SPF and GFCV from starting at day 28 and quantitatively different at day 35 after birth as shown in new experiments performed to address this question (**Fig. 4A, S6C**). This confirms that microbiota repress the number of embryonic macrophages and iNKT cells during early life as we describe and that the kinetics of the divergence between GF and SPF animals is delayed in B6 mice by 7-14 days of life. We further show in **Fig. S6C** that iNKT cells are elevated at day 35 in GF mice relative to that observed at day 35 in mice which were conventionalized with microbiota in early life.

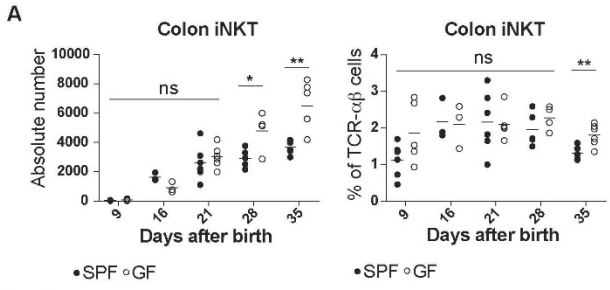


Fig. 4.

(A) iNKT (CD45$^+$ CD3ε^+ TCRβ^+ CD1d Tetramer$^+$) absolute counts (left) and cell percentage (right) in the colon of specific pathogen free (SPF) and germ free (GF) mice over time. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. *P* values were calculated by unpaired Student's t-test. **P* < 0.05, ***P* < 0.01, ns: not-significant.

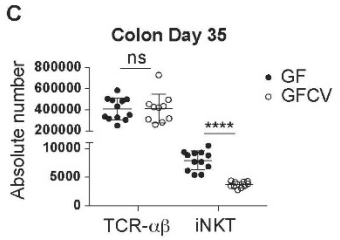


Fig. S6.
C) Absolute count of iNKT and TCR-$\alpha\beta^+$ T cells in the colon of germ-free (GF) and GF conventionalized with specific pathogen free (SPF) microbiota prior to birth (GFCV) animals at 35 days of life. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired Student's t-test. ****P<0.0001. ns: not-significant.

Minor:

Page 10 lines 213-214: sentence to be checked: microbial cues are (not) necessary for the ability of macrophages to supervise iNKT cells in the colon.

Microbial cues are not necessary for the ability of macrophages to supervise iNKT cell levels in the colon. We are sorry for the confusion. What we are saying is that although macrophages are regulated by the microbiota, they do not require microbiota to influence iNKT cells as their deletion in GF mice leads to decreased iNKT cells. We clarified our conclusions in the manuscript as below (Line 219-223).

"These results demonstrate that microbiota repress colonic macrophage levels during early life which is associated with decreased iNKT cell levels in the adult relative to that observed in GF mice. However, a microbial signal is not required for the ability of macrophages to supervise iNKT cell levels in the colon as depletion of macrophages in GF animals results in decreased iNKT cells."

Figure 5B: it summarizes the data on Ki67 MFI. Would be also interesting to have the percentage of iNKT cells positive for Ki67. Again, it would be important to see the flow cytometry staining.

As requested, we provide the percentage of iNKT cells positive for Ki67 as well as the flow cytometry staining (**Fig. 6B/D**, **Fig. S7A/C**).

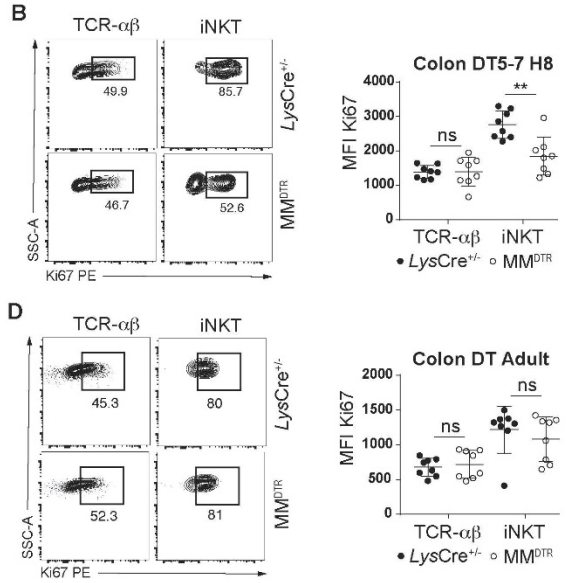


Fig. 6.

B) Representative plot (left) and Ki67 mean fluorescent intensity (MFI) (right) of TCR-αβ⁺ T (CD45⁺ CD3ε⁺ TCRβ⁺) and iNKT (CD45⁺ CD3ε⁺ TCRβ⁺ CD1d Tetramer⁺) cells on day 8 (H8) in the colon of control littermates *Lys*Cre$^{+/-}$ or MM^{DTR} animals treated with diphtheria toxin (DT) from day 5 to 7 (DT5-7) after birth. D) Representative plot (left) and Ki67 mean fluorescent intensity (MFI) (right) of TCR-αβ⁺ T and iNKT cells on day 63 in the colon of control littermates *Lys*Cre$^{+/-}$ or MM^{DTR} animals treated DT from day 56 to 62 (DT Adult) after birth. SSC-A, side scatter. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired Student's t-test. $^{**}P < 0.01$, ns: not-significant.

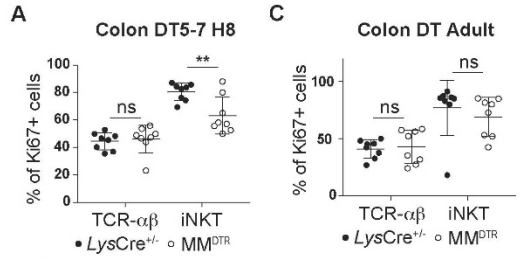


Fig. S7.

A) Percentage of Ki67 positive TCR-αβ⁺ T (CD45⁺ CD3ε⁺ TCRβ⁺) and iNKT (CD45⁺ CD3ε⁺ TCRβ⁺ CD1d Tetramer⁺) cells on day 8 (H8) in the colon of control littermates *Lys*Cre$^{+/-}$ or MM^{DTR} animals treated with diphtheria toxin (DT) from day 5 to 7 (DT5-7) after birth. C) Percentage of Ki67 positive TCR-αβ⁺ T and iNKT cells on day 63 in the colon of control littermates *Lys*Cre$^{+/-}$ or MM^{DTR} animals treated DT from day 56 to 62 (DT Adult) after birth. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. *P* values were calculated by unpaired Student's t-test. $^{**}P < 0.01$, ns: not-significant.

Suggested improvements:

It would be interesting to have more insight in the molecular mechanisms involved in the link between colon embryonic derived macrophages and iNKT cells. Further comparison by RNAseq of colon embryonic derived macrophages and colon bone marrow derived macrophages would be of high interest since they are in the same tissue they might exhibit only moderate transcriptomic differences, which could be key in local iNKT cell expansion.
Similarly comparison of colon embryonic derived macrophages versus adult spleen macrophages could provide more insight in the molecule(s) involved since spleen adult macrophages are also required for spleen iNKT cell expansion. Multiple comparisons should help to identify the molecular differences between the repressive and non-repressive macrophage subsets.

We performed new transcriptional analysis summarizing the potential mechanisms that are involved between early life macrophages and iNKT cells (**Fig. 5**).To do so we have significantly extended our RNA sequencing analyses of macrophages and iNKT cells as summarized below. These studies suggest a multifactorial involvement with two overarching broad mechanisms. First, early life macrophages may have an important role in the structural development of the colon during early life that is essential to creation of the iNKT cell niche. Secondly, early macrophages control the proliferation, expansion and differentiation of iNKT cells in a temporal fashion during early life. Our analysis and discussion of these new transcriptional studies are included in the manuscript as below (Line 225-296, 398-404).

"To understand the potential mechanisms by which macrophages may regulate iNKT cells in early life, we performed bulk RNA-Seq of colonic macrophages and iNKT cells. We investigated the transcriptional signatures of macrophages defined as $CD64^{+}F4/80^{+}$ at day 8 and day 14 after birth to identify transcripts specifically upregulated or downregulated during the time embryonic macrophages control iNKT cell levels in early life within the colon. We observed 325 transcripts with elevated abundance and 378 transcripts with decreased abundance in colonic macrophages purified at day 8 after birth compared to day 14 after birth (**Fig. 5A**, **Table S1**) suggesting a major switch in macrophage function during this period. Part of the differentially expressed genes we identified encoded secreted immune factors such as interleukins (IL) and chemokines (CXCL) which suggest an important role of macrophages in determining differentiation, proliferation and/or migration of iNKT cells. Transcripts encoding CXCL12 (*Cxcl12*) were enriched at day 8 while transcripts encoding IL12b (*Il12b*) and IL27 (*Il27*) were enriched at day 14 (**Fig. 5A**). Consistent with a potential role of one or several of these immune factors in colonic iNKT cell regulation during early life, CXCL12 has been shown to regulate iNKT cell migration *in vitro* (*22*). Further, colonic macrophages at day 8 after birth were highly enriched in transcripts encoding proteins generally associated with the extracellular matrix (ECM) such as Decorin (*Dcn*), Lumican (*Lum*), Microfibrillar-associated protein 5 (*Mfap5*), Collagen 6a2 (*Col6a2*) as well as proteins associated with angiogenesis such as Angiopoietin-related protein 1 (*Angptl1*) (**Fig. 5A**). The upregulation of genes associated with ECM formation suggest colonic macrophages at day 8 compared to day 14 may have an important role in the structural development of the colon that is unique to early life and thus involved in creating a niche conducive to iNKT cell seeding and development. In agreement with this, the Gene Ontology (GO) term analysis showed that ECM organization was the most significantly enriched biological process followed by blood vessel development in day 8 macrophages (**Fig. 5B, Table S2**).

We also examined macrophages from SPF and GF mice at day 9 after birth. We observed 259 transcripts with elevated abundance and 27 transcripts with decreased abundance at day 9 after

birth in macrophages from GF compared to SPF mice in the colon (**Fig. 5C**, **Table S3**), which advocate for a specific role of microbiota in regulating macrophages likely of embryonic origin during early life when the establishment of iNKT cell residency is taking place. We intersected the transcripts differentially expressed in colonic macrophages at day 8 compared to day 14 after birth (**Fig. 5A**) with transcripts differentially expressed in GF compared to SPF mice at day 9 after birth (**Fig. 5C**) and found 19 transcripts to be commonly dysregulated in these two datasets among which 13 were enriched in GF versus SPF and day 8 versus day 14 after birth (**Fig. 5D**). These similarities (>2.5%) between the differentially expressed genes in both datasets predicted a coincidence in their mechanisms of action. Indeed, the 13 genes commonly identified in macrophages as enriched in GF versus SPF and day 8 versus day 14 after birth included at least 3 transcripts that encoded proteins associated with ECM organization (*Dcn*, *Lum*, *Mfap5*), suggesting early life macrophages may participate in this process under the influence of the microbiota.

Interestingly, some of the genes we identified as differentially expressed in sorted macrophages during early life and participating to ECM organization (*Dcn*, *Lum*, *Mfap5, Col6a2*) or angiogenesis (*Angptl1, Cxcl12*) are more generally associated with non-hematopoietic cells (*23*, *24*). This makes it possible that their enrichment in macrophages during early life were derived from recent phagocytosis of neighboring non-hematopoietic cells (*25*) or doublets due to strong anatomical association (*26*). However, we observed a significant decrease in the expression of these genes in the whole colon following specific macrophage depletion at day 8 (**Fig. S6E**). This supports the notion that early life embryonic macrophages may be an alternative source of these transcripts. We did not observe differences in CXCL16 or CD1d expression by transcriptional analysis of macrophages (data not shown) or by quantitative PCR of the whole colon following macrophage depletion during early life (**Fig. S6E**) suggesting alternative cellular sources of these factors in the regulation of iNKT cells such as the intestinal epithelium as previously shown (*7*, *27*). Our studies thus suggest that the control of iNKT cell levels and differentiation by early life embryonic macrophages was most likely derived from a multifactorial process involving their role in sculpting the structure of the colon to create the proper niche for iNKT cell establishment and providing factors that affect iNKT cell expansion and/or differentiation.

To further investigate this, we next turned our attention to the transcriptional profiles of iNKT cells during early (day 14) and later (day 56) life by RNA-Seq and identified 61 differentially expressed transcripts (**Fig. 5E**, **Table S4**). Of these, 32 transcripts exhibited elevated abundance in the adult relative to day 14 of life and 29 transcripts were uniquely increased in neonatal iNKT cells. From GO term analysis, the early life iNKT cells were especially enriched in pathways associated with cell division (**Fig. 5F**, **Table S5**). This supports the notion that iNKT cells adopt a distinct transcriptional program during early compared to adult life consistent with cells in the process of establishing residency and point toward a potential role for proliferation of iNKT cells during this time period."

"Transcriptional profiling coupled with phenotypic and functional analyses suggest that early life macrophages are critical to the development of a niche within the colon that preferentially authorize the immigration and settlement of thymically-derived iNKT cells including those that determine the structure of the ECM. At the same time, embryonic macrophages promote the intense proliferative expansion of iNKT cells during early life and their ultimate differentiation into iNKT cell subsets which together culminate in establishing iNKT cell residency and its quantitative and functional set-point."

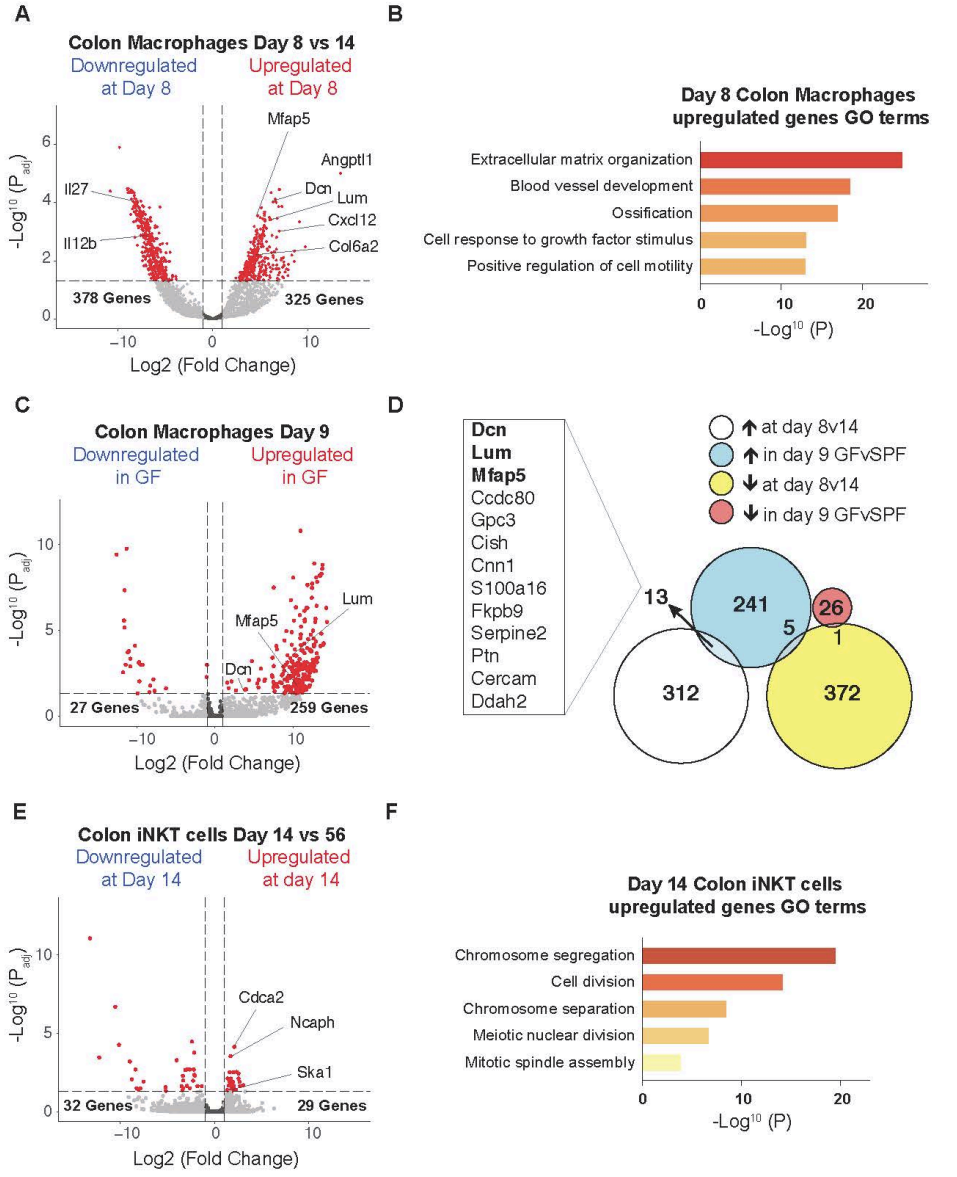


Fig. 5. Macrophage and iNKT cell transcriptional signature during early life.
A) Transcriptome analysis of colonic macrophages (CD45$^+$ Lin$^-$ F4/80$^+$ CD64$^+$ cells) from 8 and 14 day old specific pathogen free (SPF) animals (n=4). B) Pathway analysis by gene ontology enrichment of transcripts increased in colonic macrophages at day 8 compared to day 14 after birth in SPF animals. C) Transcriptome analysis of colonic macrophages from day 9 SPF (n=2) and GF (germ free, n=4) animals. D) Intersection

of the differentially expressed genes identified by the transcriptome analysis of colonic macrophages from 8 and 14 day old SPF animals and the transcriptome analysis of colonic macrophages from day 9 SPF and GF animals. E) Transcriptome analysis of colonic iNKT ($CD45^+$ $CD3\varepsilon^+$ $TCR\beta^+$ CD1d $Tetramer^+$) cells from 14 day old (n=3) and 56 day old (n=4) animals raised under SPF conditions. F) Pathway analysis by gene ontology enrichment of transcripts differentially expressed in colon iNKT cell populations at day 14 compared to the adult in SPF animals. Transcripts differentially expressed between groups were identified by DESeq2 analyses ($log_2|FC|>1$, $p_{adj}<0.05$, red).

In the same line, It would be important to see on colon embryonic derived macrophages (versus other macrophages mentioned above) whether they are higher expression level of critical proteins relevant to iNKT cells such as CD1d (recognized by iNKT cell TCR), SAP and SLAM molecules (involved in thymic and peripheral iNKT cell activation/expansion) and CXCL16 (association of mucosal CXCL16 and iNKT cell levels reported by the same laboratory, Olszak et al. Science 2012).

Cd1d or *Cxcl16* gene transcripts did not appear as differentially expressed in the transcriptional analysis performed on macrophages (**Table S1 and S3**). Moreover, we did not observe differences in CD1d or CXCL16 expression by quantitative PCR of the whole colon following macrophage depletion during early life (**Fig. S6E**), suggesting that early macrophage regulate iNKT cell number through a previously undefined mechanism that will necessitate further studies. We have added the following to the manuscript to make these points on line 241 to 245.

"We did not observe differences in CXCL16 or CD1d expression by transcriptional analysis of macrophages (data not shown) or by quantitative PCR of the whole colon following macrophage depletion during early life (**Fig. S6E**) suggesting alternative cellular sources of these factors in the regulation of iNKT cells such as the intestinal epithelium as previously shown (*7*, *27*)."

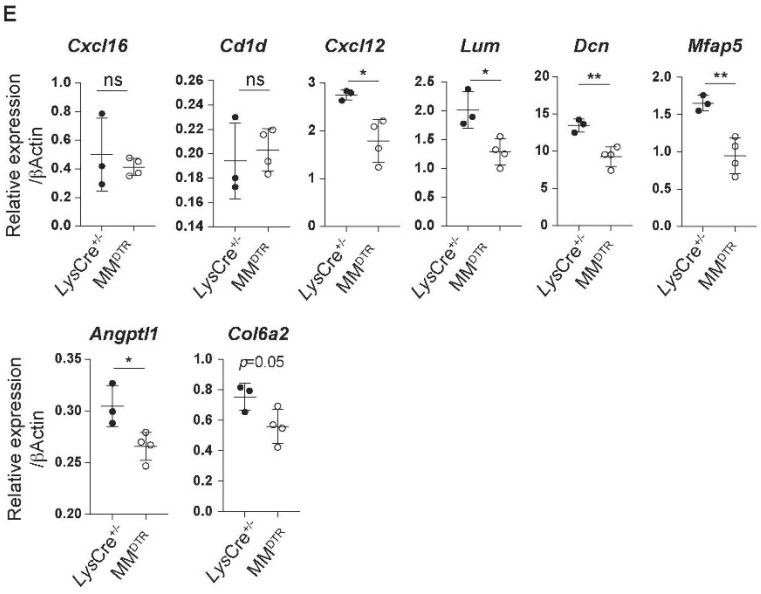


Fig. S6.
E) DT administered from day 5 to 7 after birth followed by the analysis of *Cxcl16, Cd1d, Cxcl12, Lum, Dcn, Mfap5, Angptl1 and Col6a2* transcript expression by quantitative polymerase chain reaction analysis of the colon. Numbers in the representative plots indicate cell frequency. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. *P* values were calculated by unpaired Student's t-test. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$. ns: not-significant.

Reviewer 3:

This manuscript reports that embryonic-derived macrophages, together with the microbiota, regulate the number of natural killer T cells (NKT cells) in the colon lamina propria of mice; in the absence of these embryonic macrophages, the number of colonic NKT cells was decreased. They were important, however, only during a window up to 2 weeks of age. Surprisingly, systemic, DTR-mediated depletion of macrophages had effects that lasted into adulthood, as 7-week old mice were more susceptible to two models in which NKT cell function has been implicated: they were more susceptible to oxazolone-induced colitis and less able to clear Listeria. Here the authors have attacked a difficult but important question: how does the neonatal immune system imprint changes that last later in life? They imply, based on their findings, that colonic NKT cells are seeded in the colon early in life and are mostly self-renewing—suggested by the parabiotic mouse experiments—and that the maintenance of these cells long-term is dependent on the early presence of embryonic macrophages. This is an interesting hypothesis.

We thank the reviewer for the positive comments and suggestions. We have performed the suggested experiments that we believe strengthens the significance of the manuscript as described below.

The authors show that NKT cell proliferation is in fact reduced in day 8-old mice after depletion of macrophages, but adult depletion does not have this effect—however, it would be important to know if NKT cell proliferation (BrdU or Ki67) is affected by early depletion when the mice are analyzed at 7 weeks age.

We now demonstrate that Ki67 expression in iNKT cell is unchanged in adult MM^{DTR} versus littermate control mice treated with DT during early life (**Fig. S10C-F**). Therefore, macrophages do not affect the long term capacity of iNKT cells to proliferate. This observation is in accordance with the decreased capacity of iNKT cells to proliferate in the adult compared to early life as shown by RNA sequencing which compares iNKT cells at day 14 and 56 of life (**Fig. 5E-F**) (*5*). These RNA sequencing studies provide transcriptional evidence for activation of cellular proliferation signatures in early relative to later life. Thank-you for these important suggestions.

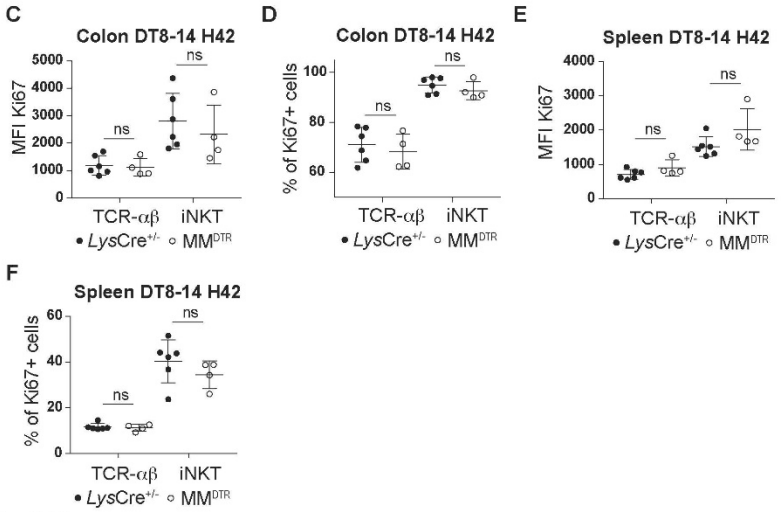


Fig. S10.
Ki67 mean fluorescent intensity (MFI) or cell percentage of TCR-αβ⁺ T and iNKT cells on day 42 (H42) in the colon (C,D) or spleen (E,F) of control littermates *Lys*Cre$^{+/-}$ or MM^{DTR} animals treated with diphtheria toxin (DT) from day 8 to 14 after birth. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. *P* values were calculated by unpaired Student's t-test. ns: not-significant.

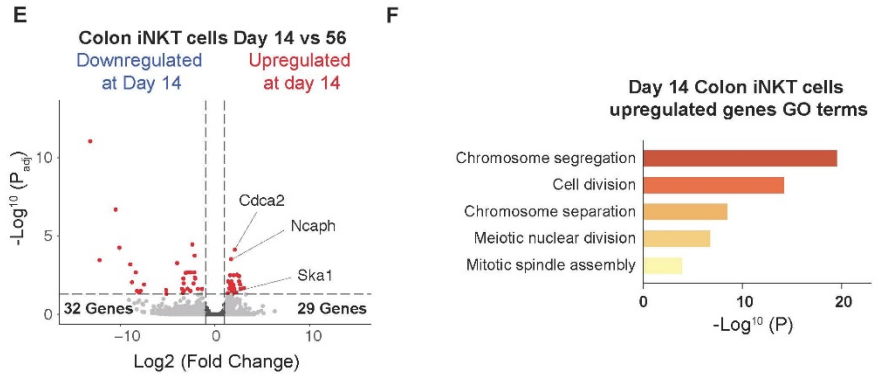


Fig. 5.
E) Transcriptome analyses of colonic iNKT cells from 14 day old (n=3) and 56 day old (n=4) animals raised under SPF conditions. F) Pathway analysis by gene ontology enrichment of transcripts differentially expressed in colon iNKT cell populations at day 14 compared to the adult in SPF animals. Transcripts differentially expressed between groups were identified by DESeq2 analyses ($log_2|FC|>1$, $p_{adj}<0.05$, red).

The functional analysis of NKT cells should be improved. Were iNKT cell-mediated inflammatory responses in fact altered later life (line 272)? There was less Ifng mRNA

produced in the colon after antigen stimulation of NKT cells (Fig. S9D), but probably not on a per cell basis, as the number of colonic NKT cells was similarly reduced.

We agree with the reviewer and have performed additional experimentation. We now demonstrate that expression of the activation marker CD69 on iNKT cells was unchanged in adult MM^{DTR} versus littermate control mice treated with DT during early life suggesting per cell activation is not impaired (**Fig. S10G-H**). We introduce this point in the revised manuscript as below (Line 352-357).

"However, the iNKT cells which remain within the colon in later life after early life depletion of macrophages in MM^{DTR} mice exhibit similar levels of proliferation and activation relative to that observed in littermate controls based upon Ki67 or CD69 expression, respectively (**Fig. S10C-H**)."

The decreased IFNγ production observed in the adult MM^{DTR} mice that had been treated with DT during early life and were exposed to αGal was aimed to test the general inflammatory state of the tissue. We predicted that if iNKT cells were decreased in adult life as a consequence of early life macrophage depletion, inflammatory responses to CD1d-mediated triggers would be diminished. We now clarify this point in the manuscript to read as below (Line 361-367).

"Therefore, 49-day old MM^{DTR} mice that had been treated with DT between days 8 and 14 of life (**Fig. S11A**) were exposed with α-galactosylceramide (αGal), the prototypical antigen for stimulation of iNKT cells. Although NKT1 subsets were relatively enriched as observed in **Fig. S10A**, we observed that interferon (IFN)-γ production in the adult colon was lower in mice depleted of macrophages during early life (**Fig. S11B**) despite the fact that the levels of macrophages had recovered (**Fig 7B**). These results are consistent with a persistent reduction in iNKT cells."

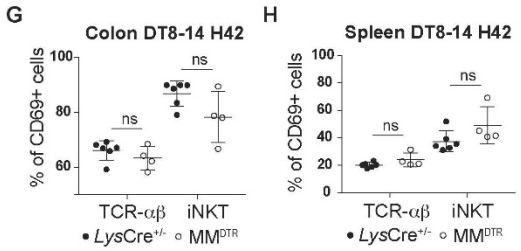


Fig. S10.
Percentage of $CD69^+$ TCR-$\alpha\beta^+$ T and iNKT cells on day 42 (H42) in the colon (G) or spleen (H) of control littermates *Lys*$Cre^{+/-}$ or MM^{DTR} animals treated with diphtheria toxin (DT) from day 8 to 14 after birth.. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. *P* values were calculated by unpaired Student's t-test. ns: not-significant.

Is the representation of NKT cell subsets different?

We now investigate this specific point in the manuscript. We observed a decrease in the proportion of NKT17 cells and an increase in NKT1 iNKT cell subsets in the colon but not spleen (**Fig. S10 A-B**) in adult MM^{DTR} versus littermate control mice treated with DT during early life. In contrast, we observed a similar proportion of congenic $CD45.1^+$ iNKT cell subsets in the colon of the recipient MM^{DTR} $CD45.2^+$ mice compared to littermate controls at day 8 after birth following

DT treatment (**Fig. S7H**). This suggests that early life macrophage depletion not only affects the quantity of iNKT cells but also their differentiation in later life. We discuss this point in the manuscript as below (Line 345-357).

"We first examined iNKT cell differentiation. iNKT cells can be subdivided into 3 main differentiated subsets in tissues: NKT1, NKT2 and NKT17 (*28*). Although we observed similar proportions of CD45.1^{+} iNKT cell subsets in the colon of the MM^{DTR} CD45.2^{+} recipients at day 8 of life after having received DT treatment from days 3-7 mice (**Fig. S7H**), we found that the iNKT cells which survived macrophage deletion during early life exhibited a decreased proportion of NKT17 cells and a relative increase in NKT1 iNKT cell subsets in the colon but not spleen in later life (**Fig. S10A-B**). However, the iNKT cells which remain within the colon in later life after early life depletion of macrophages in MM^{DTR} mice exhibit similar levels of proliferation and activation relative to that observed in littermate controls based upon Ki67 or CD69 expression, respectively (**Fig. S10C-H**). These results demonstrate that early life embryonic macrophages in the colon provide cues that determine the state of iNKT cell differentiation but not the proliferation or activation state in later life."

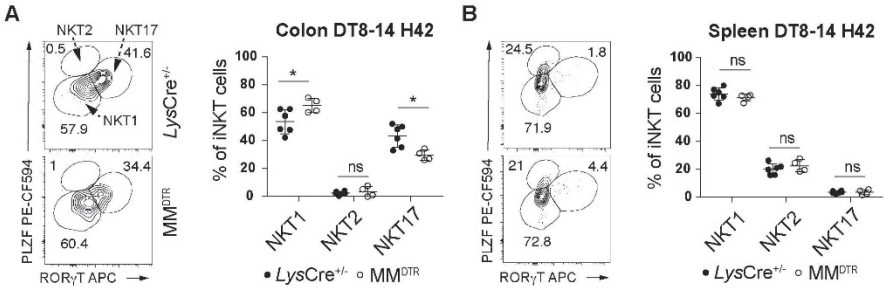


Fig. S10.
Representative plot (left) and cell percentage (right) of iNKT cell subsets (NKT1, NKT2, NKT 17) from 42 day old control littermates *Lys*Cre$^{+/-}$ or MM^{DTR} animals treated with DT from day 8 to 14 (DT8-14) after birth in the colon (A) or spleen (B). Numbers in the representative plots indicate cell frequency. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired Student's t-test. *$P < 0.05$, ns: not-significant.

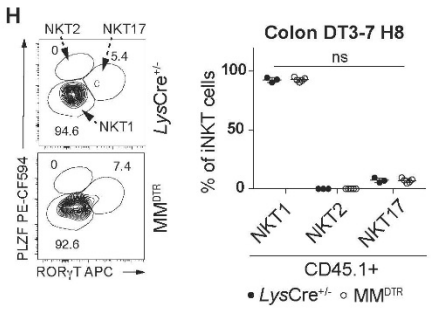


Fig. S7.

H) Representative plot (left) and cell percentage (right) of iNKT cell subsets (NKT1, NKT2, NKT 17) from 8 day old CD45.2 control littermates *Lys*Cre$^{+/-}$ or MM^{DTR} animals adoptively transferred with CD45.1 adult thymic cells at 3 days old and treated with DT from day 3 to 7 (DT3-7) after birth in the colon. Numbers in the representative plots indicate cell frequency. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. *P* values were calculated by unpaired Student's t-test. ns: not-significant.

The effects of systemic macrophage depletion on the oxazolone model were not shown to be NKT cell-dependent, although NKT cells play an important role in this model. The same is true for Listeria, and the authors should explain why Ifng mRNA was increased rather than reduced in the macrophage depleted mice that have a higher CFU (likely immunopathology).

We agree with the reviewers. The best experiment to prove causality would be to breed the MM^{DTR} (LysCre$^{+/-}$ and CSF1R^{DTR}) with CD1d or Jα18 knock out mice strains which are deficient for iNKT cells. Such an experiment would be costly and take a long time to establish. Further, there is good evidence in the literature that NKT cells are essential for induction of oxazolone induced colitis suggesting that they are at least one, if not the main, factor involved (*6*). That said, we agree that there are conceivably other factors downstream of macrophage depletion in addition to iNKT cells. Previous publications have shown that *Cd1d* knock out mice which lack iNKT cells, were more susceptible to *L. monocytogenes* infection with higher CFUs in the spleen as well as higher IFNγ expression in the colon (*8*), suggesting the decreased iNKT cell levels observed in MM^{DTR} mice following early life depletion of macrophages results in increased susceptibility to such infection. These points are now clearly explained in the manuscript as below (Line 374-394) and therefore have downplayed the conclusion of these experiments to read as below (Line 31-34, 414-417).

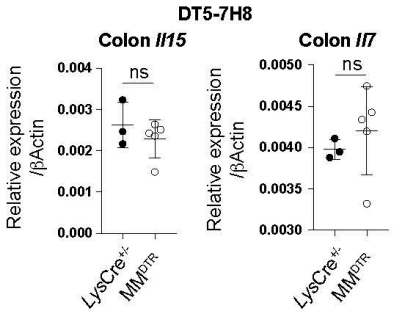
"In association with persistent reductions in colonic iNKT cells, MM^{DTR} mice depleted of macrophages in early life were protected from oxazolone-induced colitis in later life as shown by significantly diminished weight loss (**Fig. 7F**), mortality (**Fig. 7G**) and pathology (**Fig. 7H**) relative to control mice. As oxazolone-induced colitis is known to depend upon iNKT cells (*10*), these observations are consistent with the protection from colitis observed. Conversely, when MM^{DTR} and littermate control mice were treated with DT between day 8 and day 14 after birth and orally inoculated on day 49 with a modified form of *Listeria monocytogenes* able to infect mice (**Fig. 7I**), the number of *L. monocytogenes* colony forming units (CFU) recovered from the colon and spleen were significantly greater in MM^{DTR} compared to littermate control mice (**Fig. 7J**), in association with increased levels of *Ifnγ* and *Il12p40* mRNA expression in the colon (**Fig. S11C-D**). *L. monocytogenes* infection in *Cd1d* deficient mice, that lack iNKT cells, exhibit similar findings in the colon and spleen (*31*) suggesting the decreased colonic iNKT cell levels observed in MM^{DTR} mice may result in increased susceptibility to such an infection. Together, these results reveal that the embryonic macrophage-mediated control of mucosal iNKT cell proliferation during early life is associated with durable functional consequences."

"Consequently, early life perturbations of fetal-derived macrophages result in persistent decreases of mucosal iNKT cells and is associated with later life susceptibility or resistance to iNKT cell associated mucosal disorders."

"Consequently, early life perturbations of colonic macrophages of embryonic origin are associated with differential host susceptibility to iNKT cell-dependent responses to enteropathogens and environmental stimuli that induce inflammation which models inflammatory bowel disease."

The data are fascinating, but this reader was left hungry for details or even a speculative hypothesis as to how do the embryonic macrophages change NKT cells later in life? Cytokines such as IL-15 (or IL-7 for NKT cells that make IL-17) have been shown to be important for NKT cell homeostasis and could be responsible for their findings regarding spleen NKT cells—but there must be something that the embryonic macrophages do in the colon that affects NKT cells weeks later when they are reduced or no longer effective. Even at day 25 age there were still plenty of colonic embryonic macrophages (Fig. 3A), but depletion at two weeks of age or later had little effect, so the early embryonic macrophages must be critical. The RNA-seq results of colonic macrophages from day 8 and 14 (Fig. S7A) were not very revealing regarding mechanism. (note that lines 222-224 of the manuscript seems to indicate this figure compares GF to SPF mice, which disagrees with the figure legend). Did the authors analyze IL-15 levels in the colon?

We thank the reviewer for this comment. We assessed *Il15* and *Il7* mRNA expression levels in the colon of MM^{DTR} mice and control littermates treated with DT from day 5 to 7 after birth and observed no significant differences (**Rebuttal Fig. 5**).



Rebuttal Figure 5:
DT administered from day 5 to 7 (DT5-7) after birth followed on day 8 (H8) by the analysis of *Il15* and *Il7* transcript expression by quantitative PCR in the colon. ns: not-significant.

We performed new transcriptional analysis summarizing the potential mechanisms that are involved between early life macrophages and iNKT cells (**Fig. 5**).To do so we have significantly extended our RNA sequencing analyses of macrophages and iNKT cells as summarized below. These studies suggest a multifactorial involvement with two overarching broad mechanisms. First, early life macrophages may have an important role in the structural development of the colon during early life that is essential to creation of the iNKT cell niche. Secondly, early macrophages control the proliferation, expansion and differentiation of iNKT cells in a temporal fashion during early life. Our analysis and discussion of these new transcriptional studies are included in the manuscript as below (Line 225-296, 398-404).

"To understand the potential mechanisms by which macrophages may regulate iNKT cells in early life, we performed bulk RNA-Seq of colonic macrophages and iNKT cells. We investigated the transcriptional signatures of macrophages defined as $CD64^{+}F4/80^{+}$ at day 8 and day 14 after birth to identify transcripts specifically upregulated or downregulated during the time embryonic macrophages control iNKT cell levels in early life within the colon. We observed 325 transcripts with elevated abundance and 378 transcripts with decreased abundance in colonic macrophages purified at day 8 after birth compared to day 14 after birth (**Fig. 5A**, **Table S1**) suggesting a major switch in macrophage function during this period. Part of the differentially expressed genes we

identified encoded secreted immune factors such as interleukins (IL) and chemokines (CXCL) which suggest an important role of macrophages in determining differentiation, proliferation and/or migration of iNKT cells. Transcripts encoding CXCL12 (*Cxcl12*) were enriched at day 8 while transcripts encoding IL12b (*Il12b*) and IL27 (*Il27*) were enriched at day 14 (**Fig. 5A**). Consistent with a potential role of one or several of these immune factors in colonic iNKT cell regulation during early life, CXCL12 has been shown to regulate iNKT cell migration *in vitro* (*22*). Further, colonic macrophages at day 8 after birth were highly enriched in transcripts encoding proteins generally associated with the extracellular matrix (ECM) such as Decorin (*Dcn*), Lumican (*Lum*), Microfibrillar-associated protein 5 (*Mfap5*), Collagen 6a2 (*Col6a2*) as well as proteins associated with angiogenesis such as Angiopoietin-related protein 1 (*Angptl1*) (**Fig. 5A**). The upregulation of genes associated with ECM formation suggest colonic macrophages at day 8 compared to day 14 may have an important role in the structural development of the colon that is unique to early life and thus involved in creating a niche conducive to iNKT cell seeding and development. In agreement with this, the Gene Ontology (GO) term analysis showed that ECM organization was the most significantly enriched biological process followed by blood vessel development in day 8 macrophages (**Fig. 5B, Table S2**).

We also examined macrophages from SPF and GF mice at day 9 after birth. We observed 259 transcripts with elevated abundance and 27 transcripts with decreased abundance at day 9 after birth in macrophages from GF compared to SPF mice in the colon (**Fig. 5C**, **Table S3**), which advocate for a specific role of microbiota in regulating macrophages likely of embryonic origin during early life when the establishment of iNKT cell residency is taking place. We intersected the transcripts differentially expressed in colonic macrophages at day 8 compared to day 14 after birth (**Fig. 5A**) with transcripts differentially expressed in GF compared to SPF mice at day 9 after birth (**Fig. 5C**) and found 19 transcripts to be commonly dysregulated in these two datasets among which 13 were enriched in GF versus SPF and day 8 versus day 14 after birth (**Fig. 5D**). These similarities (>2.5%) between the differentially expressed genes in both datasets predicted a coincidence in their mechanisms of action. Indeed, the 13 genes commonly identified in macrophages as enriched in GF versus SPF and day 8 versus day 14 after birth included at least 3 transcripts that encoded proteins associated with ECM organization (*Dcn*, *Lum*, *Mfap5*), suggesting early life macrophages may participate in this process under the influence of the microbiota.

Interestingly, some of the genes we identified as differentially expressed in sorted macrophages during early life and participating to ECM organization (*Dcn*, *Lum*, *Mfap5, Col6a2*) or angiogenesis (*Angptl1, Cxcl12*) are more generally associated with non-hematopoietic cells (*23*, *24*). This makes it possible that their enrichment in macrophages during early life were derived from recent phagocytosis of neighboring non-hematopoietic cells (*25*) or doublets due to strong anatomical association (*26*). However, we observed a significant decrease in the expression of these genes in the whole colon following specific macrophage depletion at day 8 (**Fig. S6E**). This supports the notion that early life embryonic macrophages may be an alternative source of these transcripts. We did not observe differences in CXCL16 or CD1d expression by transcriptional analysis of macrophages (data not shown) or by quantitative PCR of the whole colon following macrophage depletion during early life (**Fig. S6E**) suggesting alternative cellular sources of these factors in the regulation of iNKT cells such as the intestinal epithelium as previously shown (*7*, *27*). Our studies thus suggest that the control of iNKT cell levels and differentiation by early life embryonic macrophages was most likely derived from a multifactorial process involving their role in sculpting the structure of the colon to create the proper niche for iNKT cell establishment and providing factors that affect iNKT cell expansion and/or differentiation.

To further investigate this, we next turned our attention to the transcriptional profiles of iNKT cells during early (day 14) and later (day 56) life by RNA-Seq and identified 61 differentially expressed transcripts (**Fig. 5E**, **Table S4**). Of these, 32 transcripts exhibited elevated abundance in the adult relative to day 14 of life and 29 transcripts were uniquely increased in neonatal iNKT cells. From GO term analysis, the early life iNKT cells were especially enriched in pathways associated with cell division (**Fig. 5F**, **Table S5**). This supports the notion that iNKT cells adopt a distinct transcriptional program during early compared to adult life consistent with cells in the process of establishing residency and point toward a potential role for proliferation of iNKT cells during this time period."

"Transcriptional profiling coupled with phenotypic and functional analyses suggest that early life macrophages are critical to the development of a niche within the colon that preferentially authorize the immigration and settlement of thymically-derived iNKT cells including those that determine the structure of the ECM. At the same time, embryonic macrophages promote the intense proliferative expansion of iNKT cells during early life and their ultimate differentiation into iNKT cell subsets which together culminate in establishing iNKT cell residency and its quantitative and functional set-point."

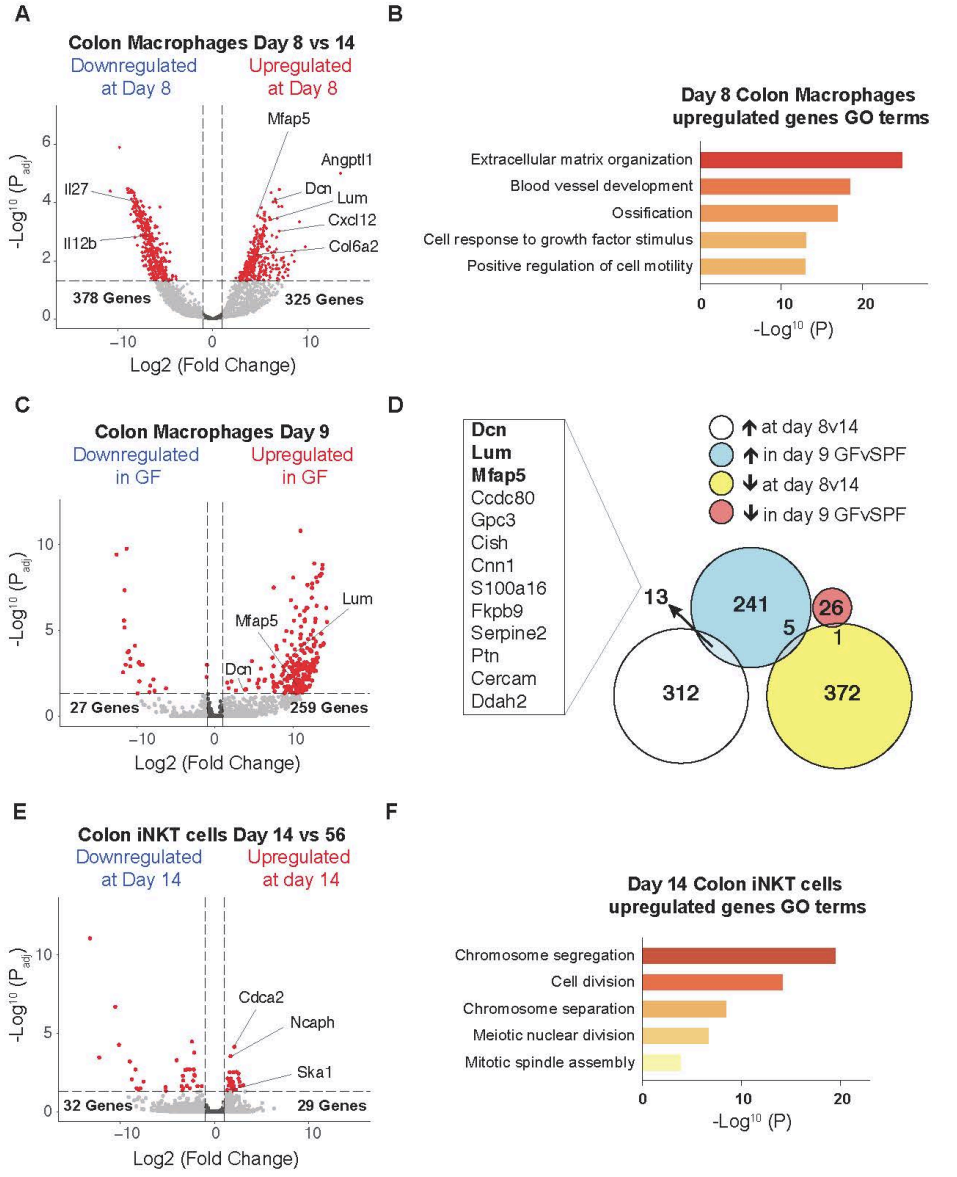


Fig. 5. Macrophage and iNKT cell transcriptional signature during early life.
A) Transcriptome analysis of colonic macrophages ($CD45^+$ Lin^- $F4/80^+$ $CD64^+$ cells) from 8 and 14 day old specific pathogen free (SPF) animals (n=4). B) Pathway analysis by gene ontology enrichment of transcripts increased in colonic macrophages at day 8 compared to day 14 after birth in SPF animals. C) Transcriptome analysis of colonic macrophages from day 9 SPF (n=2) and GF (germ free, n=4) animals. D) Intersection

of the differentially expressed genes identified by the transcriptome analysis of colonic macrophages from 8 and 14 day old SPF animals and the transcriptome analysis of colonic macrophages from day 9 SPF and GF animals. E) Transcriptome analysis of colonic iNKT ($CD45^+$ $CD3\varepsilon^+$ $TCR\beta^+$ CD1d $Tetramer^+$) cells from 14 day old (n=3) and 56 day old (n=4) animals raised under SPF conditions. F) Pathway analysis by gene ontology enrichment of transcripts differentially expressed in colon iNKT cell populations at day 14 compared to the adult in SPF animals. Transcripts differentially expressed between groups were identified by DESeq2 analyses ($log_2|FC|>1$, $p_{adj}<0.05$, red).

Additional points:

Tissues. Lamina propria and not epithelium was analyzed, but this was never mentioned in the text, only in the Methods. Skin was analyzed in a few experiments, but the skin data were incomplete and can be omitted. Was small intestine ever analyzed?

We now mention we analyzed colonic lamina propria in the main text (Line 66, 79, 117, 202, 395). We added data showing that early life macrophage depletion results in decreased iNKT cell levels in the small intestine (**Fig. S2D**).

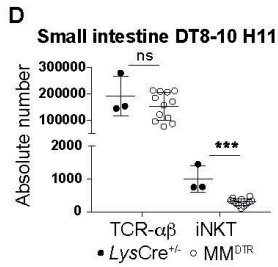


Fig. S2.
D) DT administered from day 8 to 10 (DT8-10) after birth followed by quantitative analyses on day 11 (H11) of the absolute count of iNKT and TCR-$\alpha\beta^+$ T cells in the small intestine. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired Student's t-test. ***$P < 0.001$, ns: not-significant.

Experimental rigor:

Were there experimental repeats for fig 1E and 1F with only 3 mice analyzed? Same is true for some other experiments such as 4E. Student's t test may not be appropriate with only 3 mice and probably not for the data in 6H which do not appear to be normally distributed.

As suggested, we increased the number of experimental replicates for Figures 1E, F, G, H (now n=6) as well as Figure, 4C, D, E, F, G (now n>6).

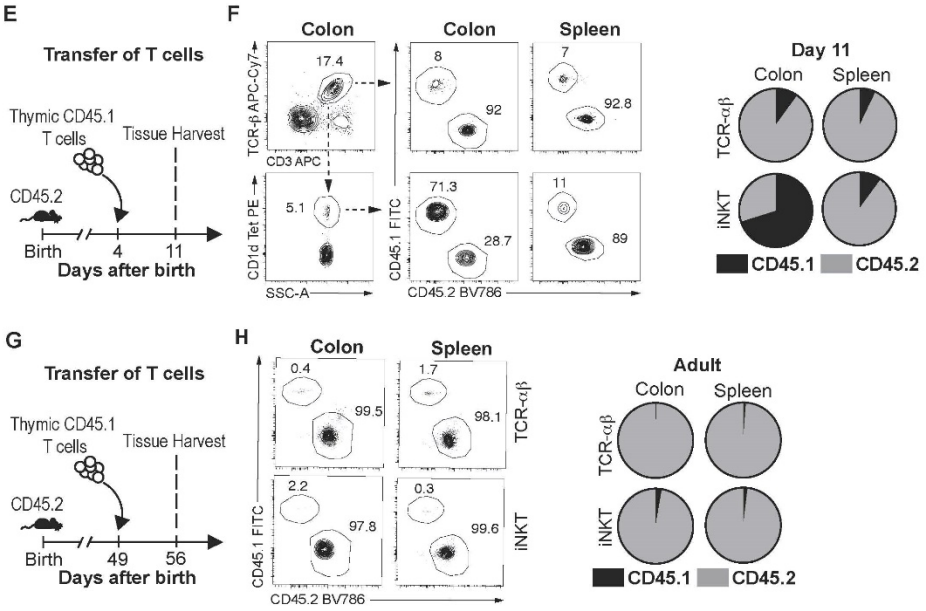


Fig. 1.

E) Schematic of adoptive transfer strategy. (F) Adoptive transfer of CD45.1 adult thymic cells into a 4 day old CD45.2 (n=6) host followed by quantitative analyses of splenic and colonic CD45.1 (black) or CD45.2 (grey) TCR-$\alpha\beta^+$ T and iNKT cells by flow cytometry on day 11. Representative plots (left). Circles are representative of average cell frequency (right). G) Schematic of adoptive transfer strategy. (H) Adoptive transfer of CD45.1 adult thymic cells into a 49 day old CD45.2 host (n=6) followed by quantitative analyses of splenic and colonic CD45.1 (black) or CD45.2 (grey) TCR-$\alpha\beta^+$ T and iNKT cells by flow cytometry on day 56. Representative plots (left). Circles are representative of average cell frequency (right). Tet, Tetramer. SSC-A, Side scatter.

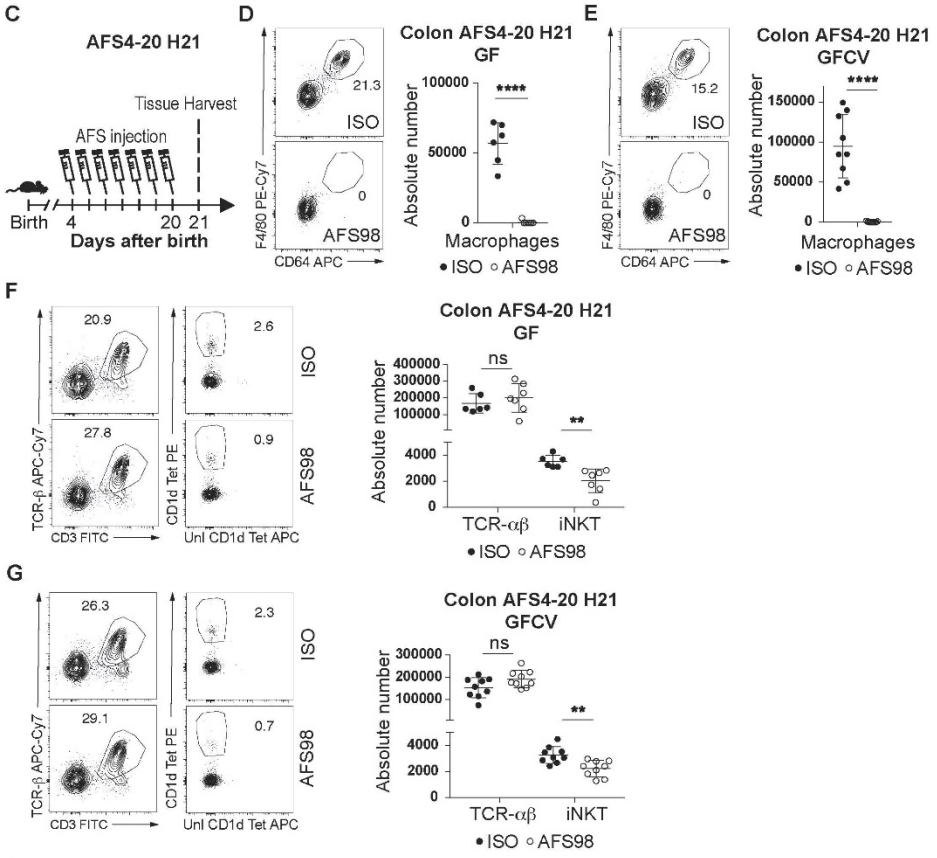


Fig. 4.

C) Schematic of macrophage depletion model with AFS98 antibody. AFS98 or Isotype control antibody administered from day 4 to 20 (AFS4-20) after birth followed by quantitative analyses on day 21 (H21) of the absolute count of macrophages (D,E), and the absolute count of iNKT and TCR-$\alpha\beta^+$ T ($CD45^+$ $CD3\varepsilon^+$ $TCR\beta^+$) cells (F,G) in the colon of GF or GFCV animals. Unl, Unloaded. Tet, Tetramer. Numbers in the representative plots indicate cell frequency. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired Student's t-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns: not-significant.

We confirmed the normal distribution for the data from Figure 6H by performing the "D'Agostino-Pearson omnibus normality test" (Rebuttal Fig. 6).

D'Agostino & Pearson normality test	*LysCre* +/- Ox	MMDTR Ox
K2	1.942	5.566
P value	0.3787	0.0619
Passed normality test (alpha=0.05)?	Yes	Yes
P value summary	ns	ns

Rebuttal Figure 6:

D'Agostino and Pearson normality test for LysCre$^{+/-}$ Ox and MMDTR Ox (cf. manuscript **Fig. 7H**)

Some representative flow data for the Ki67 staining should be shown.

We are now showing FACS plots for the Ki67 staining (**Fig. 6B/D**) as well as every main experiment in the manuscript.

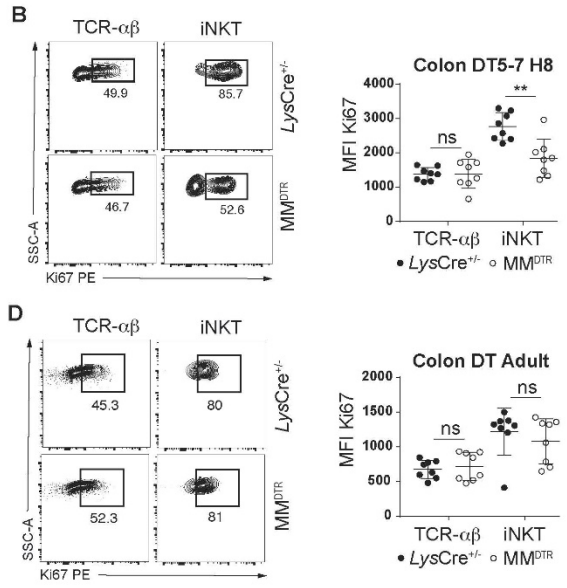


Fig. 6.
B) Representative plot (left) and Ki67 mean fluorescent intensity (MFI) (right) of TCR-αβ$^+$ T (CD45$^+$ CD3ε$^+$ TCRβ$^+$) and iNKT (CD45$^+$ CD3ε$^+$ TCRβ$^+$ CD1d Tetramer$^+$) cells on day 8 (H8) in the colon of control littermates *Lys*Cre$^{+/-}$ or MM^{DTR} animals treated with diphtheria toxin (DT) from day 5 to 7 (DT5-7) after birth. D) Representative plot (left) and Ki67 mean fluorescent intensity (MFI) (right) of TCR-αβ$^+$ T and iNKT cells on day 63 in the colon of control littermates *Lys*Cre$^{+/-}$ or MM^{DTR} animals treated DT from day 56 to 62 (DT Adult) after birth. SSC-A, side scatter. Error bars indicate standard error of mean. Each dot is representative of an individual mouse. P values were calculated by unpaired Student's t-test. $^{**}P < 0.01$, ns: not-significant.

Were mice of both sexes analyzed in the experiments?

Mice were from both sexes except for the oxazolone colitis experiment which was performed in females. We added this information in the methods as below (Line 727-728).

"Mice analyzed were from both sexes except for oxazolone colitis experiment which was performed in females."

Note that for the experiments carried out in Turku the ratios of NKT cells to macrophages were entirely different—the Turku wild type mice have a much higher number of NKT cells than the US mice, but fewer macrophages, showing the importance of other factors (microbiome?) in regulating colon NKT cell numbers. This complexity should be

acknowledged, but nevertheless, the lack of embryonic macrophages was shown in their experiments as well (Fig. 3G).

To decrease the mortality levels at birth, the PLVAP deficient mice and control littermates are hybrids containing a mixture of BALB/c, C57BL/6N and NMRI backgrounds (*3*) which could explain why the number of cells are different compared to mice with a C57BL/6N background. This specificity is now included in the methods as below (Line 733-736).

"PLVAP deficient mice and control littermates were hybrids containing a mixture of BALB/c, C57BL/6N and NMRI backgrounds obtained from heterozygous × heterozygous breedings."

Rebuttal references

1. D. G. Pellicci *et al.*, A natural killer T (NKT) cell developmental pathway iInvolving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage. *J. Exp. Med.* **195**, 835–44 (2002).
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3. P. Rantakari *et al.*, Fetal liver endothelium regulates the seeding of tissue-resident macrophages. *Nature*. **538**, 392–396 (2016).
4. T. Olszak *et al.*, Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function. *Science (80-.).* **336**, 489–493 (2012).
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6. F. Heller, I. J. Fuss, E. E. Nieuwenhuis, R. S. Blumberg, W. Strober, Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. *Immunity*. **17**, 629–638 (2002).
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9. E. E. E. E. S. S. Nieuwenhuis *et al.*, Cd1d-dependent regulation of bacterial colonization in the intestine of mice. *J. Clin. Invest.* **119**, 1241–1250 (2009).

Decision Letter, first revision:

Subject: Your manuscript, NI-LE29785A

Message: Our ref: NI-LE29785A

23rd Mar 2021

Dear Dr. Blumberg,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "Macrophages of embryonic origin function during early life to determine host iNKT cell levels at barrier surfaces" (NI-LE29785A). Please carefully follow the step-by-step instructions provided in the personalised checklist attached, to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments and please make sure to upload your checklist.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Immunology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Macrophages of embryonic origin function during early life to determine host iNKT cell levels at barrier surfaces". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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If you have any further questions, please feel free to contact me.

Best regards,

Elle Morris
Editorial Assistant
Nature Immunology
Phone: 212 726 9207
Fax: 212 696 9752
E-mail: immunology@us.nature.com

On behalf of

Zoltan Fehervari, Ph.D.
Senior Editor

Nature Immunology

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Fax: 212-696-9752
z.fehervari@nature.com

Reviewer #1:
Remarks to the Author:
The authors did a great work addressing my criticisms, with experiments and arguments. I have no more points.

Reviewer #2:
Remarks to the Author:
The authors have addressed all the concerns of the reviewer and their new transcriptomic data are very interesting.
It is a superb study demonstrating the role of embryonic origin macrophages in the control of mucosal iNKT cells.

Final Decision Letter:

Subject: Decision on Nature Immunology submission NI-A29785B

Message: In reply please quote: NI-A29785B

Dear Dr. Blumberg,

I am delighted to accept your manuscript entitled "Embryonic macrophages function during early life to determine iNKT cell levels at barrier surfaces" for publication in an upcoming issue of Nature Immunology.

The manuscript will now be copy-edited and prepared for the printer. Please check your calendar: if you will be unavailable to check the galley for some portion of the next month, we need the contact information of whom will be making corrections in your stead. When you receive your galleys, please examine them carefully to ensure that we have not inadvertently altered the sense of your text.

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Sincerely,

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Senior Editor
Nature Immunology

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