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Gut microbiota regulate lacteal integrity by inducing VEGF-C in intestinal villus macrophages

Sang Heon Suh, Kibaek Choe, Seon Pyo Hong, Seung-hwan Jeong, Taija Mäkinen, Kwang Soon Kim, Kari Alitalo, Charles Surh, Gou Young Koh and Joo-Hye Song

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 14 September 2018

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think the manuscript is of interest, but requires a major revision to allow publication in EMBO reports. All three referees have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript, and/or in a detailed rebuttal letter. As the reports are below, I will not detail them here. As EMBO reports emphasizes novel functional over detailed mechanistic insight, we will not require to address points regarding more refined mechanistic details (as e.g. point 5 of referee #3).

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The manuscript by Suh and coworkers deals with a highly interesting aspect of microbiota-host interaction, the microbiota-driven pathways that shape mucosal architecture and lymphatic development. In general this manuscript is well-written and the microscopy is nice, but unfortunately very often not representative and contradictory to previous publications and well-established morphologic features of the germ-free gut mucosa. The authors use a set of cell-specific knock-out models, which is in principle interesting, but could be chosen more specific. The canonical signaling pathways were studied in many aspects, but they did not at all consider any non-canonical signaling pathways that might explain the described microbiota-dependent phenotype. In

fact, many of the conclusions are overstated and not coherent with the presented data. Most imporant, the manuscript lacks any important innovative aspects, which should be one of the primary requirements for considering publication in EMBO Reports. For example, the implication of VEGF-C in lymphangiogenesis is meanwhile well-understood and the senior authors are most likely aware of that (Nurmi H, EMBO Mol Med., 2015). Also the impact of the microbiota on lymphatic development of the intestine is not really new and this aspect is not convincingly addressed by the results shown. Therefore, this manuscript clearly needs further experiments that deal with more innovative mechanisms based primarily on germ-free mouse technology and not on antibiotics.

Major Comments:

1. The fact that VEGF3 supports lacteal development is not new and the authors even quote relevant literature (Becker, 2016; Harvey & Gordon, 2012; Nurmi, 2015). It is hence questionalble if they really provide a deeper mechanistic insight. Also the analyses on VEGF signaling are somewhat uncomplete. For example the performed ELISA measurements on VEGF-C in antibiotics treated mice, but did not at all determine VEGF-C protein levels in the more relevant germ-free model.

2. The main conclusions of this manuscript are based on image analyes and the authors state in the methods that the investigators were unblinded to group allocation and outcome when analyzing the stainings. There might be differences in the preparation and stainings of tissues and therefore it is certainly necessary to include additional methods to corroborate the main conclusions as the authors did not take into consideration the entire villus structure. Also the analysis of lacteal length should not be normalized tot he capillary length, but to total villus area. In Fig. 7F, why did they not normalize to villus lenght or even better to villus area?

3. Many claims made in the text do not correspond to the representative images shown. For instance, the authors claim that villus morophlogy is not changed, but many other studies have shown that the colonization status impacts villus morphology and epithelial renewal (Abrams GD et al, 1963). For example in Fig. 4C and D they show wider villi in the GF mice. In fact, based on the literature, on would expect thinner and elongated villus structures in GF mice compared with SPF or CONV mice. In Figure 8I the imaging analysis appears biased.

4. As a cell culture model, the authors used primary cultured human dermal lymphatic endothelial cells. This is not an appropriate system that can be compared to mouse models. Why were no mouse endothelial cells used here?

5. Antibiotic treatent is not appropriate to study the role of the microbiota in fatty acid uptake as it may have many systemic effects. It would be more conclusive to quantify BODYBY-FA uptake in the germ-free mouse model. Also it would be better to collect lymphatic fluid and perform a direct measurement rather than to draw conclusions from the fluorescence intensity of the surrounding tissue.

6. At the end of the first paragraph (Fig. 3), the authors state that the expansion of the gut microbiota between P14 and P28 promotes maturation of lacteals. This is not documented by their data as the authors did not analyze the abundance and quantity of the microbiota in their experimental system, e.g. by bacterial 16S rDNA sequencing.

7. The finding that the macrophage pool is regulated by the gut microbiota is also not new. In fact, this was intensively studied for Ly6C+MHCII+ macrophages (Bain CC, Nat Immunol., 2014). Why did the authors not FACS-sort the MHCII+ F4/80+ CXCR1+ macrophage population to comparatively analyze VEGF-C levels and other factors promoting lymphangiogenesis? This would be very interesting. Also, how would the WT behave if it would be treated with DT? This essential control is missing in Fig. 7.

8. In Figure 8 a number of specificity controls are missing. It would be more conclusive if the authors would have studied tissue-specific Trif-/- mice along with tissue-specific Tlr4-/- mice. Of note, MyD88 is also an adaptor protein of IL-R signaling. Therefore the experiments shown are not specific for TLR signaling. It was not at all reslolved which TLR is critically influnecning microbiota-induced lacteal development. Further, how can the authors be sure that TLR signaling is

weaker in the macrophages in the germ-free setting? Administering the TLR4 agonist LPS to germ-free mice in the drinking water and subsequent analysis of the macrophages could give an answer.

Minor Comments:

1. The error range of the triglyceride measurements are quite huge. Therefore, the analytics needs to be improved or additional mice have to be included in order to get more conclusive results.

2. Did the authors record the weight curves of the mice treated with antibiotics? Very often the treatment with antibiotics results in a reduction in body weight which may influence tissue homeostasis.

3. The analysis on the SMCs on Fig. 6 is not very convincing. Why were the germ-free mice not analyzed here?

Referee #2:

This manuscript from Suh and colleagues presents a comprehensive body of beautifully illustrated work demonstrating that gut microbiota are important for regulating the development and maintenance of lacteals, a specialised component of the intestinal lymphatic vasculature responsible for lipid absorption. Suh and co demonstrate here that the encounter of macrophages with microbiota stimulates macrophage production of VEGF-C via a mechanism dependent at least in part on signal transduction via TLRs/MyD88. This manuscript builds upon work done by others previously demonstrating that, in contrast to the lymphatics of other tissue beds, VEGF-C is continuously required to maintain lacteal structure during adulthood and that downstream of VEGF-C mediated activation of VEGFR3, Dll4/Notch signalling in the lymphatic endothelium is important for lacteal regeneration. From this perspective, it would be interesting to assess the levels of Dll4 in LECs of microbiota deficient mice, to determine whether VEGF-C produced by macrophages is responsible for lacteal Dll4 expression and Notch signalling. Addressing the following points will further strengthen the paper:

Figure 2G: What is that impact of the delay in elevation of TG/FFA levels following lipid administration in ABX treated mice? Is the weight of ABX treated/germ free mice reduced compared to controls?

Figure EV3/4: The quality of LN images depicted in this figure is poor compared to other tissues, could higher resolution images be included?

Why are the jejunum and ileum the most affected with respect to the decrease in lacteal/villus length in ABX and germ free mice, while VEGFR3 deletion has impact in the duodenum as well? Is the effect on lipid absorption more severe in the VEGFR3 deleted mice?

Figure 6 A-C: Please clarify in this figure whether you are measuring VEGF-C mRNA or protein levels and how this was done (in which tissue component).

What underlies the selectivity of ABX treatment for macrophages in the jejunum and ileum compared to the duodenum?

The distinction and relevance of the marker used to examine macrophages in the intestine as illustrated in Figure 6E and F should be explained. How do MHCII+ F4/80+ macrophages compare to F4/80+, CX3CR1 positive macrophages?

Page 12: "transcription level of VEGF-C" should read "Vegfc mRNA" and the way in which this was measured should be reported here, eg, in macrophages/whole villi/whole intestine/duodenum/jejunum/ileum.

Why is the number of macrophages reduced in LysMCre;MyD88 mice? How are you discriminating between the reduction in macrophages and the total level of VEGF-C compared with the level of

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VEGF-C produced by MyD88 deficient macrophages? This should be assessed experimentally to conclude that MyD88 mediated signal transduction of microbiota is responsible for macrophage-produced VEGF-C.

Page 13, Figure EV6: The 15% lacteal shortening in the jejunum of vancomycin treated mice is not as substantial as that observed in ABX treated mice, so it is difficult to conclude that Gram-positive microbes are responsible for VEGF-C production. Moreover, there is no dissection of the relative amounts of VEGF-C produced in distinct populations of macrophages to support this claim. Either the work should be done to address this experimentally, or the claim should be toned down.

Page 13: "LECs barely associated with lacteal integrity" needs to be re-written, do you mean that MyD88 signal transduction in LECs does not contribute to production of VEGF-C in the intestine?

The part of the discussion comparing macrophage versus SMC-derived VEGF-C production needs to be revised; to date there has not been a study that has dissected the roles and relative contribution of macrophage versus SMC-derived VEGF-C (though this would be fascinating to do). The Nurmi study investigated mice deficient in VEGF-C in all tissues, but demonstrated that SMC express Vegfc mRNA.

Referee #3:

The manuscript of Suh et al investigated the role of intestinal microbiota in the maintenance of intestinal lacteals using antibiotic-treated and germ free mice. Depletion of microbiota both in ABX-treated and GF animals resulted in decreased lacteal length and modification of cell-cell junctions in lymphatic capillaries. Functionally, ABX-treated mice show delayed appearance of blood TGs and decreased peak value of FFA after gavage with the vegetable oil. In vivo imaging on intestinal lacteal function revealed delayed clearance of BODIPY-FA from intestinal lamina propria, indicating impaired defective function of intestinal lymphatics. Levels of VEGF-C were found to be reduced by 35% in the gut of ABX-treated animals, suggesting that reduced VEGF-C/VEGFR-3 signaling underlies the degeneration of lacteals. F4/80+ macrophages were found to be express VEGF-C. VEGF-C expression and the total number of macrophages were strongly reduced in the absence of microbiota. Further depletion of CX3CR1+ immune cells using DT system also resulted in decreased levels of macrophages and VEGF-C in small intestine and the similar effect was observed following depletion of Myd88 in myeloid cells. In vitro experiment further showed that VEGF-C xecretion from macrophages could be only elicited after stimulation with TLR1/2 but not other agonists. Overall, these data suggest that gut microbiota stimulates intestinal macrophages to produce VEGF-C and promote maintenance of small intestinal lymphatics. The results are well described, using appropriate models and show that, intestinal lymphatic vasculature relies on signals from gut microbiota for its maintenance. I have the following questions:

Main points:

1. The data suggest that either complete inactivation of VEGFR-3 (Vegfr3flox/flox:Prox1-CreERT2 model) or less than 50% reduction of VEGF- C (35% on the protein levels) are sufficient to reduce lacteal length to the same extent - how do the authors explain this discrepancy? The ultimate proof of the proposed mechanism would have been the inactivation of VEGF-C in macrophages using LysM-Cre, however I am not sure how feasible these experiments are. The authors could analyse VEGF-C heterozygous mice to demonstrated that 50% reduction in VEGF-C levels is sufficient to impair lacteal maintenance. Also, it would be interesting to test whether depletion of gut microbiota affect other factors, necessary for lymphangiogenesis, such as Ccbe1 and Adamts3 either in macrophages or in stromal cells.
2. The functional defects of lymphatic vessels in ABX- treated animals is convincing, it will be important to study whether lymphatic vessel function also impaired in germ-free mice.
3. Figure 2F How do the authors explain that the peak of FFA in blood remains at 2 h both in the control and ABX treated mice, whereas TG peak is shifted to 4h?
4. Figure 2E: how representative are these EM pictures? The authors state that both control and ABX-treated lacteals harbor zipper and button-like junctions - please provide a quantification of the observation shown in 2E.

5. P16 - the statement that " no clear evidence has been presented to show how digested lipid particles are transported into a lacteal" is not entirely correct. Please check "The identification of chylomicra and lipoproteins in tissue sections and their passage into jejunal lacteals" by Casley-Smith (1962), it has clear TEM images showing chylomicron passage via flap valves.

Minor points:

1. It is not clear from the materials and methods how gut microbiota depletion was achieved in pups.
2. The specific response of macrophages to TLR1/2 agonists in terms of induction of VEGF-C is interesting, please provide evidence that other agonists worked as expected in these conditions.

1st Revision - authors' response 28 November 2018

Detailed Point-by-Point Response to Reviewers' Comments

We deeply appreciate the editor and reviewers for their thoughtful, critical and constructive comments, which have undoubtedly provided us with valuable opportunities to improve our work. We have performed additional experiments and revised the manuscript to address the issues raised by the reviewers.

Referee #1:
The manuscript by Suh and coworkers deals with a highly interesting aspect of microbiota-host interaction, the microbiota-driven pathways that shape mucosal architecture and lymphatic development. In general this manuscript is well-written and the microscopy is nice, but unfortunately very often not representative and contradictory to previous publications and well-established morphologic features of the germ-free gut mucosa. The authors use a set of cell-specific knock-out models, which is in principle interesting, but could be chosen more specific. The canonical signaling pathways were studied in many aspects, but they did not at all consider any non-canonical signaling pathways that might explain the described microbiota-dependent phenotype. In fact, many of the conclusions are overstated and not coherent with the presented data. Most important, the manuscript lacks any important innovative aspects, which should be one of the primary requirements for considering publication in EMBO Reports. For example, the implication of VEGF-C in lymphangiogenesis is meanwhile well-understood and the senior authors are most likely aware of that (Nurmi H, EMBO Mol Med., 2015). Also the impact of the microbiota on lymphatic development of the intestine is not really new and this aspect is not convincingly addressed by the results shown. Therefore, this manuscript clearly needs further experiments that deal with more innovative mechanisms based primarily on germ-free mouse technology and not on antibiotics.

Major Comments:
Comment 1: The fact that VEGF3 supports lacteal development is not new and the authors even quote relevant literature (Becker, 2016; Harvey & Gordon, 2012; Nurmi, 2015). It is hence questionable if they really provide a deeper mechanistic insight. Also the analyses on VEGF signaling are somewhat uncomplete. For example the performed ELISA measurements on VEGF-C in antibiotics treated mice, but did not at all determine VEGF-C protein levels in the more relevant germ-free model.

Response: We appreciate this constructive comment. We performed an additional experiment and included a new data on VEGF-C protein levels in germ-free (GF) mice, as well as in conventionalized (CONV) ex-GF mice (Fig 5J and K). Tissue VEGF-C protein level was reduced in GF mice, which was restored by conventionalization (page 9-10).

Main text p9-10: The reduction of tissue VEGF-C protein level was also observed in GF mice, compared to SPF mice (Fig 5J). Conventionalization of GF mice showed increased both VEGF-C mRNA and protein levels in the tissue lysates, which is equivalent to the level of SPF mice (Fig 5J and K).

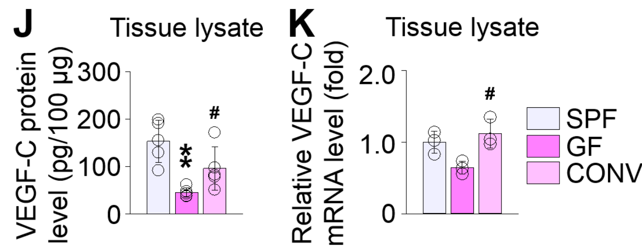

Figure 5. (J, K) Comparison of protein (J) and mRNA (K) levels of VEGF-C in the whole tissue of jejunum and ileum from SPF, GF, and CONV mice (n = 3-5 mice/group).

<u>Comment 2</u>: The main conclusions of this manuscript are based on image analyes and the authors state in the methods that the investigators were unblinded to group allocation and outcome when analyzing the stainings. There might be differences in the preparation and stainings of tissues and therefore it is certainly necessary to include additional methods to corroborate the main conclusions as the authors did not take into consideration the entire villus structure. Also the analysis of lacteal length should not be normalized to the capillary length, but to total villus area. In Fig. 7F, why did they not normalize to villus lenght or even better to villus area?

<u>Response</u>: We appreciate these critical comments. We wrote 'unblinded' in the methods as the first author was always involved in sample preparation and data analyses. However, the first author was always accompanied by at least one of the co-authors to ensure transparency and unbiased conclusions. Moreover, to minimize the chance of bias, we measured as many numbers of villi as we could, as described in the method and figure legends. Each dot in the graphs indicates the mean of 10 villi in a mouse, on average. Moreover, compared to the traditional sectional imaging or previously introduced whole mount methods, the technique we used in this study provided the much superior visualization of villi structure (Bernier-Latmani et al, 2015; Bernier-Latmani et al, 2016). This method minimizes the chance of undesired damage during sectioning that inevitably leads to the exclusion of damaged villi from analyses. Therefore, our method enables the most unbiased observation of villus structure from tip to base.
We obtained the same measurements regarding the villus structure (Figure 1E), and concluded that the length of villi is not affected by the presence of gut microbiota. To clearly state our observations on the villus morphology, we included a part of this explanation and rephrased the text in the revised manuscript (page 5)
Main text p5: Quantitative analyses revealed that absolute and relative lacteal lengths reduced by 15–17% in jejunum and ileum, but no change was detected in duodenum of ABX-treated mice compared to vehicle-treated mice, while the villus lengths were not different between the two groups along the entire length of intestine.

Due to the intrinsic limitation of the method, to avoid the inaccurate quantification, we adopted the villus lengths for the normalization of lacteal lengths, instead of villus area. Transversely sectioned villi do not always appear as round, but often do also as oval, oblong, or polygonal shapes (Abbas et al, 1989). Thus, depending on the angle with Z-axis the villus is forming, the measurement regarding the width and area of a villus could be extremely variable in the microscopic field, while the values related to lengths are precisely obtained. In Fig. 7F, for the uniformity of data presentation, we normalized the lacteal length to the villus length, as the reviewer recommended.

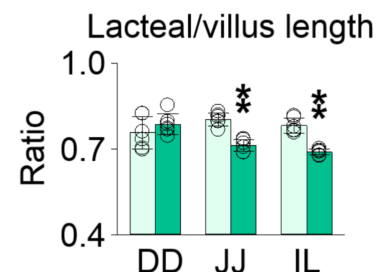

Figure 7. (F) Images and comparisons of absolute and relative lacteal lengths in duodenum (DD), jejunum (JJ) and ileum (IL) of WT and DTR mice. Each dot indicates mean value of 5-10 villi in a mouse (n = 6 mice/group). Scale bars, 100 µm.

<u>Comment 3:</u> Many claims made in the text do not correspond to the representative images shown. For instance, the authors claim that villus morphology is not changed, but many other studies have shown that the colonization status impacts villus morphology and epithelial renewal (Abrams GD et al, 1963). For example in Fig. 4C and D they show wider villi in the GF mice. In fact, based on the literature, on would

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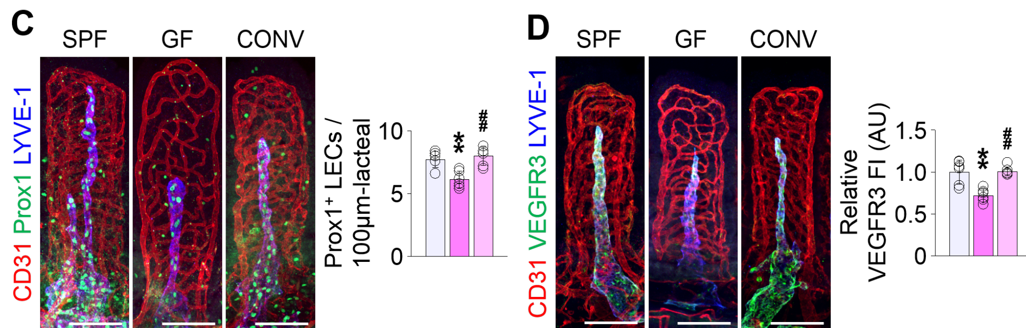
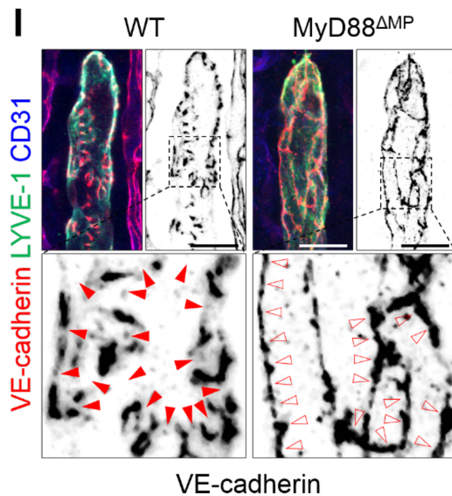
expect thinner and elongated villus structures in GF mice compared with SPF or CONV mice. In Figure 8I the imaging analysis appears biased.

Response: We adopted a whole mount technique to obtain a precise measurement, and believe that this presents more accurate data on the villus morphology, compared to the traditional sectional image. Even though, the lateral morphology is dependent on the position of the villi, because transversely sectioned villi do not always appear as round, but often do also as oval, oblong, or polygonal shapes (Abbas et al, 1989). Moreover, although thinner and elongated shape of villi in GF mice has been reported, it's been also reported the length of villi was the same in GF and conventional mice (Thompson et al, 1971), as we stated in the manuscript.
As far as we observed, the overall shape of the villi in GF mice was not significantly different compared with those of SPF or CONV mice. Instead, we replaced the figures (Fig 4C and D) with more representative ones.



We also replaced the images in Fig 8I with ones that show more distinct button- and zipper-like junctions.

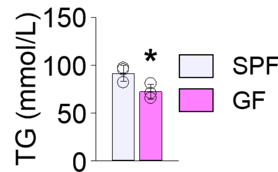


Comment 4: As a cell culture model, the authors used primary cultured human dermal lymphatic endothelial cells. This is not an appropriate system that can be compared to mouse models. Why were no mouse endothelial cells used here?
Response: We chose human dermal lymphatic endothelial cells (HDLECs), because this system has been widely accepted and used in *in vitro* experiments to validate the characteristics of lymphatic endothelial cells *in vivo.* On the other hand, it is still technically challenging to obtain enough primary cultured LECs from mouse. We wish the reviewer favorably consider this situation.

Comment 5: Antibiotic treatment is not appropriate to study the role of the microbiota in fatty acid uptake as it may have many systemic effects. It would be more conclusive to quantify BODYBY-FA uptake in the germ-free mouse model. Also it would be better to collect lymphatic fluid and perform a direct measurement rather than to draw conclusions from the fluorescence intensity of the surrounding tissue.

Response: We appreciate this constructive comment. Following the reviewer's suggestion, we performed an additional experiment to directly measure and compare TG levels in the lymph from thoracic ducts of SPF and GF mice (Appendix Fig S5). Compared to SPF mice, TG level in the lymph of thoracic duct was reduced in GF mice, indicating the compromised function of lipid drainage from lacteals. We included this new result and its description into the revised manuscript (page 8).

Main text p8: Compared to SPF mice, TG level in the lymph of thoracic duct was reduced in GF mice, indicating the compromise in the lipid drainage function of lacteals (Appendix Fig S5).

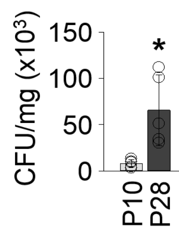


Appendix Figure S5. Triglyceride level in the lymph from thoracic duct is reduced in GF mice. Comparisons of triglyceride (TG) in in the lymph from thoracic duct from SPF and GF mice (n = 3 mice/group). The lymph was collected 1 hour after oral lipid loading. Data are represented as means ± SD. *P < 0.05 vs. SPF mice by two-tailed unpaired Student's t-test.

Comment 6: At the end of the first paragraph (Fig. 3), the authors state that the expansion of the gut microbiota between P14 and P28 promotes maturation of lacteals. This is not documented by their data as the authors did not analyze the abundance and quantity of the microbiota in their experimental system, e.g. by bacterial 16S rDNA sequencing.

Response: We appreciate this critical comment. To provide the evidence that the abundance and quantity of the microbiota expand during the given period, colony forming units were measured in feces cultures (Appendix Fig S4). We observed about 10-fold increase in quantity of gut microbiota between P10 and P28 (page 7)

Main text p7:…as we confirmed the quantitative expansion of microbiota between P10 and P28 (Appendix Fig S4).



Appendix Figure S4. Gut microbiota quantitatively expands during weaning from lactation. Comparison of bacterial colony forming unit (CFU) in feces from P10 and P28 mice (n = 5 mice/group) Data are represented as means ± SD. *P < 0.05 vs. P10 mice by two-tailed unpaired Student's t-test.

Comment 7: The finding that the macrophage pool is regulated by the gut microbiota is also not new. In fact, this was intensively studied for Ly6C+MHCII+ macrophages (Bain CC, Nat Immunol., 2014). Why did the authors not FACS-sort the MHCII+ F4/80+ CXCR1+ macrophage population to comparatively analyze VEGF-C levels and other factors promoting lymphangiogenesis? This would be very interesting. Also, how would the WT behave if it would be treated with DT? This essential control is missing in Fig. 7.
Response: We appreciate this insightful comment. We demonstrated that the macrophage pool responding to microbiota is able to produce VEGF-C to maintain lacteal integrity. As shown in Fig 6F, $MHCII^{+}F4/80^{+}$ population mostly expresses CX3CR1. Our sorting strategy, $MHCII^{+}F4/80^{+}$ was sufficient to analyze VEGF-C in intestinal macrophage responding microbiota.
The wild type (WT) group in Fig 7 was treated with diphtheria toxin (DT). To avoid misunderstanding, we additionally explained the detail in Method section (page 19). According to the review's comments, we additionally compared the tissue VEGF-C mRNA levels including WT treated with only PBS (Appendix Fig S7). We found no significant difference in the tissue VEGF-C mRNA levels between WTs treated with PBS and treated with DT (page 10-11).

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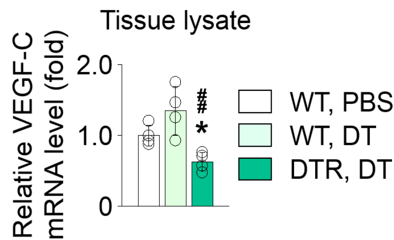
Main text p19: DT was also administered to the WT mice, as a control group.
Main text p10-11: Intraperitoneal administration vehicle or diphtheria toxin in WT did not affect the tissue VEGF-C mRNA level (Appendix Fig S7).



Appendix Figure S7. Intraperitoneal administration of diphtheria toxin does not increase tissue VEGF-C level in the intestine of wild type mice. Comparison of VEGF-C mRNA levels in the jejunum and ileum of PBS-treated WT (WT, PBS), diphtheria toxin (DT)-treated WT (WT, DT), and DT-treated CX3CR1-DTR (DTR, DT) mice (n = 4 mice/group). Data are represented as means ± SD. *P < 0.05 vs WT, PBS; ## P < 0.01 vs. WT, DT by one-way ANOVA with Bonferroni's multiple comparison test.

Comment 8-1: In Figure 8 a number of specificity controls are missing. It would be more conclusive if the authors would have studied tissue-specific Trif-/- mice along with tissue-specific Tlr4-/- mice. Of note, MyD88 is also an adaptor protein of IL-R signaling. Therefore the experiments shown are not specific for TLR signaling. It was not at all resolved which TLR is critically influencing microbiota-induced lacteal development.

Response: We appreciate this important comment. We have already shown that TLR1/2, but not TLR4 or its agonist LPS, in macrophages are most likely relevant to VEGF-C production (Fig EV6A and B).

Comment 8-2: Further, how can the authors be sure that TLR signaling is weaker in the macrophages in the germ-free setting? Administering the TLR4 agonist LPS to germ-free mice in the drinking water and subsequent analysis of the macrophages could give an answer.

Response: This study is highlighting which subset of gut microbiota and its corresponding TLR subsets are responsible for VEGF-C production in the macrophages of steady state. So, our findings do not suggest that the overall TLR signals are weaker in antibiotics cocktail (ABX)-treated or GF mice than in the control mice. Actually, there's a report that intestinal macrophages from GF mice showed less production of IL-10 and increased levels of LPS-induced TNF-α and IL-6 production (Ueda et al, 2010).

Minor Comments:

Comment 1:The error range of the triglyceride measurements are quite huge. Therefore, the analytics needs to be improved or additional mice have to be included in order to get more conclusive results.

Response: To present more reliable data, we re-evaluated the serum triglyceride level after lipid loading (page 7), with some modification in the method (page 24).

Main text p24: TG and FFA analysis was performed on FUJI DRI-CHEM 7000i (Fuji Film) and VetTest Chemistry analyzer (IDEXX Lab), respectively.

The result shows much less variability among the individual mice, which is included as Fig 2H.

Main text p7: Compared to vehicle-treated mice, ABX-treated mice had 39% and 24% reduction in peak TG and FFA levels at 2 h after the corn-oil administration, respectively (Fig 2H).

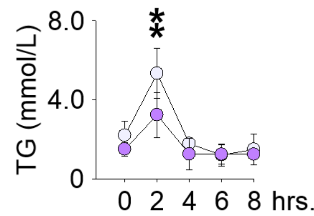
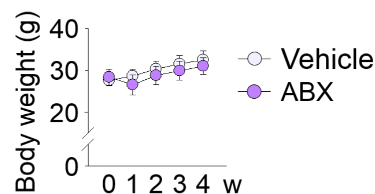


Figure 2. (H) Comparisons of serum triglyceride (TG) and free fatty acids (FFA) at indicated time points in vehicle- and ABX- treated mice (n = 5-7 mice/group).

Comment 2:Did the authors record the weight curves of the mice treated with antibiotics? Very often the treatment with antibiotics results in a reduction in body weight which may influence tissue homeostasis.

Response: Following the reviewer's comment, we included the body weight curves of vehicle- or ABX-treated mice in the revised manuscript (Appendix Fig S1). Body weight did not differ significantly between the groups during ABX treatment (page 5).

Main text p5: Body weight was not different between vehicle- and ABX-treated mice (Appendix Fig S1)



Appendix Figure S1. The effect of gut microbiota depletion is insignificant on the body weight during regular chow feeding. Body weight curve of vehicle- and ABX-treated mice. w, weeks after vehicle or ABX treatment (n = 8 mice/group). Data information: Data are represented as means ± SD.

Comment 3:The analysis on the SMCs on Fig. 6 is not very convincing. Why were the germ-free mice not analyzed here?

Response: Following the reviewer's comments, we re-analyzed the density of SMCs in vehicle- or ABX-treated mice from another set of experiments (page 10). We also additionally analyzed the SMCs in GF and CONV mice. The results are included in the Fig EV3.

Main text p10: Moreover, immunostaining revealed that alignment and density of the SMCs along the entire length of the small intestine was not affected by germ depletion by ABX treatment, GF condition or conventionalization (Fig EV3).

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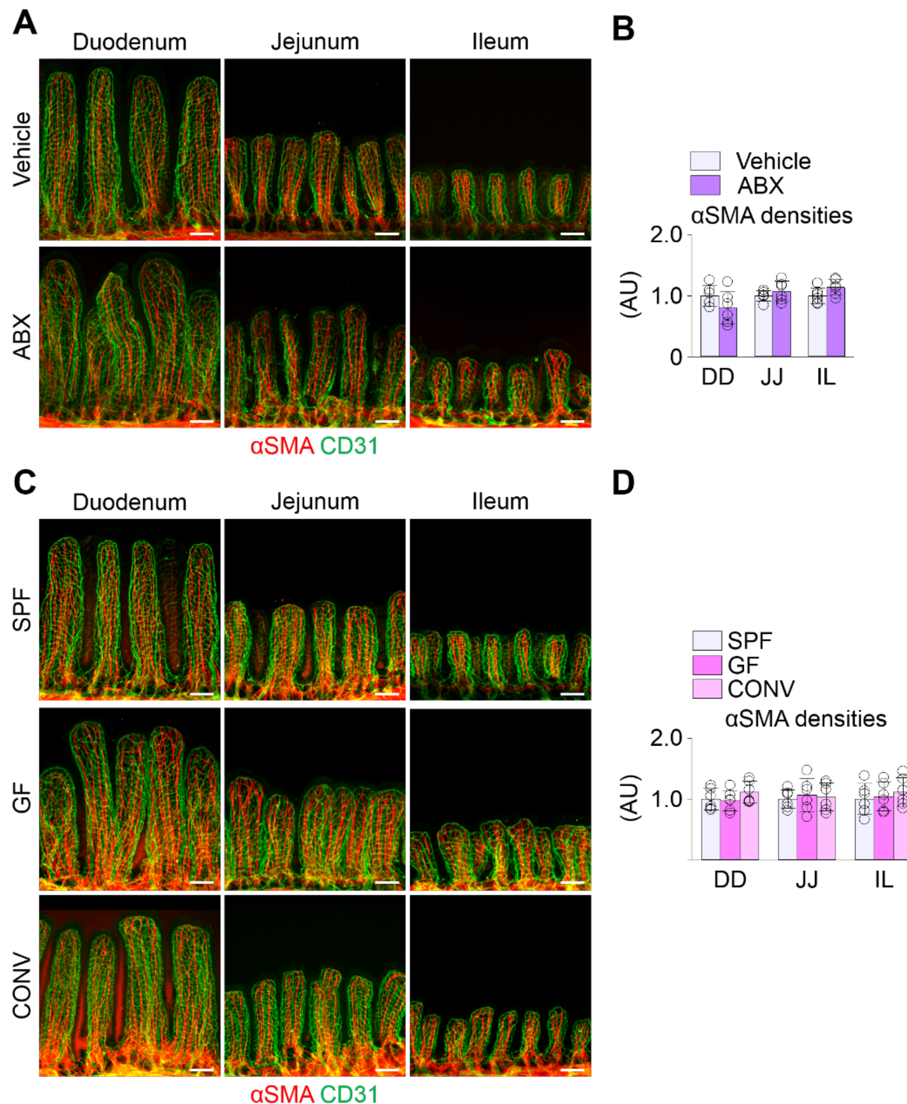


Figure EV3. The density of SMCs in the villi is independent of gut microbiota. (A-D) Images and comparison of αSMA$^+$ SMCs in the villi of duodenum (DD), jejunum (JJ), and ileum (IL) from vehicle- or ABX-treated mice (A and B) and from SPF, GF, and CONV mice (C and D) . Each dot indicates mean value of 5 sites in a mouse (n = 6 mice/group). AU, arbitrary unit. Scale bars, 100 µm. Data are represented as means ± SD.

Reference for the reviewer 1.

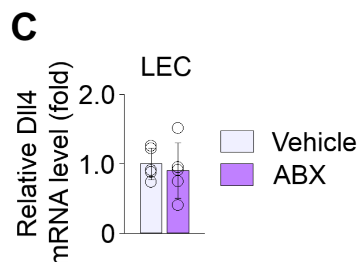
1. Abbas BH, T. L..; Wilson, D. J..; Carr, K. E. (1989) Internal structure of the intestinal villus: morphological and morphometric observations at different levels of the mouse villus. *J Anat* **162:** 263-273
2. Bernier-Latmani J, Cisarovsky C, Demir CS, Bruand M, Jaquet M, Davanture S, Ragusa S, Siegert S, Dormond O, Benedito R*, et al.* (2015) DLL4 promotes continuous adult intestinal lacteal regeneration and dietary fat transport. *J Clin Invest* **125:** 4572-4586
3. Bernier-Latmani J, Petrova TV (2016) High-resolution 3D analysis of mouse small-intestinal stroma. *Nat Protoc* **11:** 1617-1629
4. Thompson GR, Trexler PC (1971) Gastrointestinal structure and function in germ-free or gnotobiotic animals. *Gut* **12:** 230-235
5. Ueda Y, Kayama H, Jeon SG, Kusu T, Isaka Y, Rakugi H, Yamamoto M, Takeda K (2010) Commensal microbiota induce LPS hyporesponsiveness in colonic macrophages via the production of IL-10. *Int Immunol* **22:** 953-962

Referee #2:

This manuscript from Suh and colleagues presents a comprehensive body of beautifully illustrated work demonstrating that gut microbiota are important for regulating the development and maintenance of lacteals, a specialised component of the intestinal lymphatic vasculature responsible for lipid absorption. Suh and co demonstrate here that the encounter of macrophages with microbiota stimulates macrophage production of VEGF-C via a mechanism dependent at least in part on signal transduction via TLRs/MyD88. This manuscript builds upon work done by others previously demonstrating that, in contrast to the lymphatics of other tissue beds, VEGF-C is continuously required to maintain lacteal structure during adulthood and that downstream of VEGF-C mediated activation of VEGFR3, Dll4/Notch signalling in the lymphatic endothelium is important for lacteal regeneration. From this perspective, it would be interesting to assess the levels of Dll4 in LECs of microbiota deficient mice, to determine whether VEGF-C produced by macrophages is responsible for lacteal Dll4 expression and Notch signalling. Addressing the following points will further strengthen the paper:

Response: We appreciate this constructive comment. As the reviewer recommended, we evaluated the transcription level of Dll4 in intestinal LECs of vehicle- and ABX-treated mice (Appendix Fig S6C), using LEC-specifically RiboTag-expressing mice (page 26). We found that expression of Dll4 mRNA was not different between the two groups (page 10).

Main text p26: mRNA isolation Using RiboTag method
RiboTag mouse was crossed to Prox1-CreER^{T2} to isolate the LEC-specific mRNA. To induce Cre activity in CreERT2 mice, tamoxifen (100 mg/kg mouse, Sigma-Aldrich) in corn oil was subcutaneously injected in 8 week-old mice every other day for three times. 2 weeks after induction of Cre recombinase, the mice were sacrifced to isolate polysome-bound mRNAs of LECs in the intestine, with minor modification from previously described method. Briefly, intestine was harvested, opened longitudinally, and cut into 2-cm pieces. Tissues were incubated for 20 min at 37°C in DMEM containing 10 mM EDTA under gentle agitation (200 rpm). Tissue pieces were washed by vortexing quickly with PBS until obtaining a clear supernatant devoid of epithelial cells. The samples were immediately snap freezed. Then, polysome buffer (50 mM Tris, pH 7.5, 100 mM KCl, 12 mM MgCl2, 1% Nonidet P-40, 1 mM DTT, 200 U/ml RNasin, 1 mg/ml heparin, 100 μg/ml cyclohexamide, and 1× protease inhibitor mixture) were added to each sample and homogenized using Precellys lysis kit (Bertin). For immunoprecipitation against hemaggultinin, anti-hemagglutinin antibody-conjugated magnetic beads (MBL, M180-11) were added to the supernatant after centrifugation for 10 min at 12000rpm 4 °C, and incubated on a rotating shaker at 4 °C overnight. Beads were washed for four times with high-salt buffer (50 mM Tris, pH 7.5, 300 mM KCl, 12 mM MgCl2, 1% Nonidet P-40, 1 mM DTT, and 100 μg/ml cyclohexamide) and resuspended in 350 μl of RLT plus buffer with β-mercaptoethanol. Total RNAs were extracted using the RNA isolation mentioned in methods.
Main text p10: The mRNA level of Dll4, which is also known to organ-specifically regulate the lacteal maintenance (Bernier-Latmani et al, 2015), was not different in the intestinal LECs between the two groups.

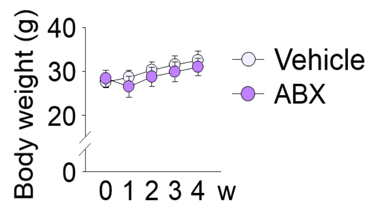


Appendix Figure S6. (C) Comparison of mRNA levels of Dll4 in LECs of jejunum and ileum from vehicle- and ABX-treated mice (n = 5 mice/group). Data are represented as means ± SD.

Figure 2G: What is that impact of the delay in elevation of TG/FFA levels following lipid administration in ABX treated mice? Is the weight of ABX treated/germ free mice reduced compared to controls?

Response: We appreciate this constructive comment. To reveal the functional impact of delay lipid absorption, we monitored the body weight of ABX-treated mice (page 5). Although the body weight was not different significantly between vehicle- and ABX-treated mice on regular chow diet (Appendix Fig S1), this does not necessarily mean that the lacteal absorption function is normal. Body weight gain significantly differed between conventional SPF mice and GF mice only when high fat diet (HFD) was provided (Fleissner et al, 2010), although protection of GF mice from HFD-induced obesity has been reported. Moreover, despite the defects in lipid absorption from lacteals of Dll4 knout-out mice, body weight did not differ to that of WT mice on regular chow diet (Bernier-Latmani et al, 2015). Therefore, our interpretation that delay in elevation of TG/FFA levels following lipid administration in ABX-treated mice as a functional defect in lacteals seems still valid.

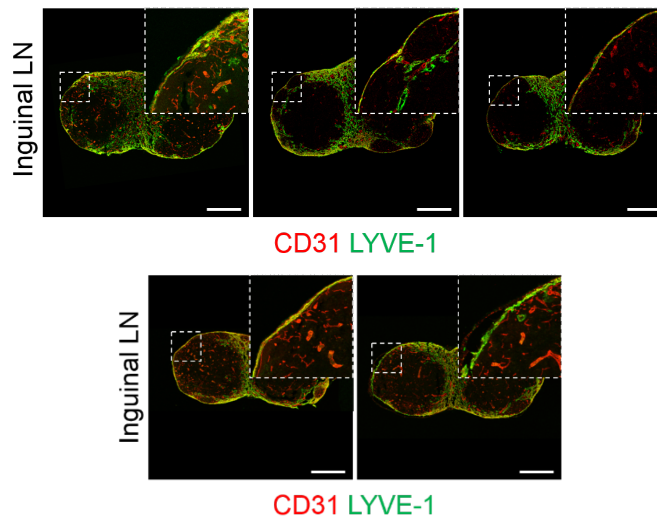
Main text p5: Body weight was not different between vehicle- and ABX-treated mice (Appendix Fig S1)



Appendix Figure S1. The effect of gut microbiota depletion is insignificant on the body weight during regular chow feeding. Body weight curve of vehicle- and ABX-treated mice. w, weeks after vehicle or ABX treatment (n = 8 mice/group). Data information: Data are represented as means ± SD.

Figure EV3/4: The quality of LN images depicted in this figure is poor compared to other tissues, could higher resolution images be included?

Response: We appreciate this constructive comment. The image quality issue arise from the size of scanning area, because the scanning area of lymph node images shown here is much wider than that of other tissues. We included the magnified images of indicated areas in the original images, so that the reader could clearly identify the lymphatic vessels of inguinal lymph nodes (Fig EV2 and Appendix Fig S3).



Why are the jejunum and ileum the most affected with respect to the decrease in lacteal/villus length in ABX and germ free mice, while VEGFR3 deletion has impact in the duodenum as well? Is the effect on lipid absorption more severe in the VEGFR3 deleted mice?

Response: This is a valid point that needs to be carefully addressed. Based on the observation from VEGFR3-deleted mice, we believe that VEGF-C – VEGFR3 axis crucially regulates the lacteal integrity along the entire length of intestinal tract, including the duodenum. We speculate that while gut microbiota is one of the major regulator for tissue VEGF-C level in the distal part of intestine (e.g., jejunum and ileum), the other factors might work as a regulator of tissue VEGF-C level in more proximal part of intestine (e.g., duodenum), since the abundance of microbiota is exponentially less in this segment of intestine than in the jejunum and ileum, and depletion of gut microbiota would only minimally alter the microenvironment in the duodenum. We included a part of this explanation and rephrased the text in the revised manuscript (page 15).

Main text p15: Based on the observation from VEGFR3$^{i\Delta LEC}$ mice, we believe that VEGF-C – VEGFR3 axis crucially regulates the lacteal integrity along the entire length of intestinal tract, including the duodenum. We speculate that other factors, rather than gut microbiota, might regulate tissue VEGF-C level in more proximal part of intestine (e.g., duodenum), since the abundance of microbiota is exponentially less in this segment of intestine than in the jejunum and ileum, and depletion of gut microbiota would only minimally alter the microenvironment in the duodenum.

We performed an additional experiment to evaluate the effect of VEGFR3 deletion on the lipid absorption (Fig 5I). We found that deletion of VEGFR3 in the lacteal delays the lipid absorption (page 9). The degree of reduction in the peak level was comparable in the germ-depleted and VEGFR3$^{i\Delta LEC}$ mice (39% vs. 32%).

Main text p9: Accordingly, compared to WT mice, VEGFR3$^{i\Delta LEC}$ mice showed defected dietary lipid absorption (Fig 5I).

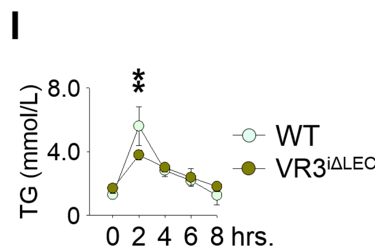


Figure 5. (I) Comparisons of serum triglyceride (TG) at indicated time points in WT and VR3$^{i\Delta LEC}$ mice (n = 6 mice/group). Data are represented as means ± SD. **P < 0.01 vs. WT by two-way ANOVA with Bonferroni post-hoc analysis.

Figure 6 A-C: Please clarify in this figure whether you are measuring VEGF-C mRNA or protein levels and how this was done (in which tissue component).

Response: Following the review's recommendation, we more clearly revised that those data are comparing relative VEGF-C mRNA levels from the indicated tissue components of jejunum and ileum.

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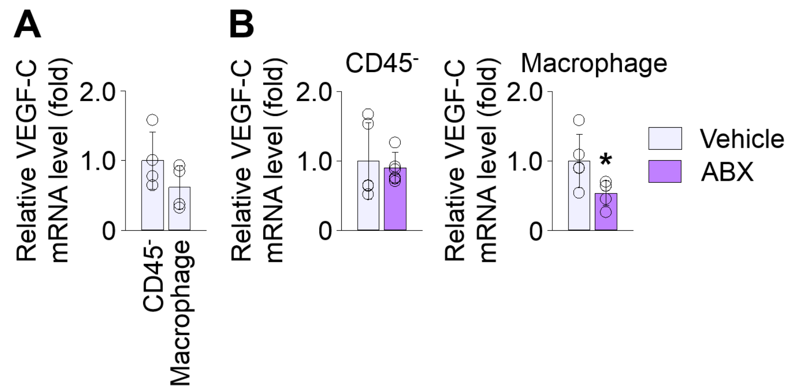

Figure 6. (A) Comparison of mRNA levels of VEGF-C in sorted CD45$^-$ stromal cells and CD45$^+$ MHCII$^+$ F4/80$^+$ macrophages from jejunum and ileum of vehicle-treated mice. The mean of transcription levels of VEGF-C in CD45$^-$ stromal cells was normalized to 1, and the relative levels in macrophages were presented as fold change (n = 4 mice/group). **(B)** Comparisons of VEGF-C transcription level in CD45$^-$ stromal cells (left) and CD45$^+$ MHCII$^+$ F4/80$^+$ macrophages (right) in jejunum and ileum of vehicle- and ABX-treated mice. The mean of transcription levels of VEGF-C in vehicle-treated mice was normalized to 1, and the relative levels in ABX-treated mice were presented as fold change (n = 5 -6 mice/group). Data are represented as means ± SD. *P < 0.05 vs. vehicle-treated mice by two-tailed unpaired Student's t-test.

What underlies the selectivity of ABX treatment for macrophages in the jejunum and ileum compared to the duodenum?

Response: We again appreciate the reviewer's constructive comment. We speculate that the selectivity of ABX treatment for macrophages of different intestinal regions lies on the distinct abundance of gut microbiota. Since the abundance of microbiota exponentially increase in the jejunum and ileum than in the duodenum, the depletion of gut microbiota would exert more potent effect on the microenvironment of jejunum and ileum. We included a part of this explanation and rephrased the text in the revised manuscript (page 15).

Main text p15: …since the abundance of microbiota is exponentially less in this segment of intestine than in the jejunum and ileum, and depletion of gut microbiota would only minimally alter the microenvironment in the duodenum.

The distinction and relevance of the marker used to examine macrophages in the intestine as illustrated in Figure 6E and F should be explained. How do MHCII+ F4/80+ macrophages compare to F4/80+, CX3CR1 positive macrophages?

Response: We appreciate the constructive comment. At Figure 6E, F4/80$^+$ cells are divided into MHC II$^+$ macrophage and MHC II$^-$ monocytes, of which only MHCII$^+$ F4/80$^+$ intestinal macrophage population decreased in the intestine of ABX-treated mice. Further characterization of the macrophage phenotypes by analyzing F4/80 and CX3CR1 revealed that the F4/80$^+$ intestinal macrophage responding to gut microbiota express CX3CR1 (Figure 6F). Indeed, the rates of intestinal macrophage reduction by ABX treatment shown in Figure 6E and 6F are similar. The reason why the percentage of macrophages shown in Figure 6E and Figure 6F is different (15% vs. 24%) is the different pre-gating strategy (CD45$^+$ hematopoietic cells or CD45$^+$ MHCII$^+$ mononuclear phagocytes). We included a part of this explanation and rephrased the text in the revised manuscript (page 10-11).

Main text p10-11: Flow cytometric analysis revealed that the number of MHCII$^+$ F4/80$^+$ villi macrophages isolated from jejunum and ileum decreased by 49% in ABX-treated mice (Fig 6E). Further characterization of the macrophage phenotypes revealed that MHCII$^+$ F4/80+ villi macrophages also express CX3CR1. MHCII$^+$ F4/80$^+$ CX3CR1$^+$ villi macrophages isolated from jejunum and ileum decreased by 41% in ABX-treated mice (Fig 6F).

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Page 12: "transcription level of VEGF-C" should read "Vegfc mRNA" and the way in which this was measured should be reported here, eg, in macrophages / whole villi / whole intestine / duodenum / jejunum / ileum.

Response: Following the review's comments, we revised the manuscript as the following (page 11).

Main text p11: VEGF-C mRNA level in the whole tissue lysate of jejunum and ileum was decreased by 57% in CX3CR1-DTR mice compared with the WT mice.

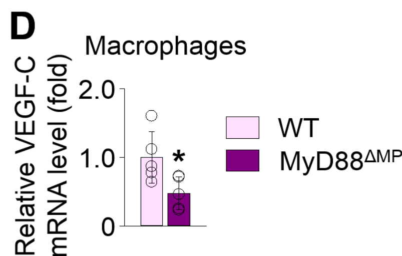
Why is the number of macrophages reduced in LysMCre;MyD88 mice?

Response: We appreciate this constructive comment. Sensing of commensal microbes via MyD88 in the macrophages leads to the immune homeostasis in the intestine (Mortha et al, 2014). Macrophages lack of MyD88 or microbial signals fail to stimulate a subset of innate lymphoid cells, which produce colony-stimulating factor 2 that is critical for the maintenance of the number and function of mononuclear phagocytes in the intestine. We included a part of this explanation and rephrased the text in the revised manuscript (page 16).

Main text p16: For instance, the reduction in the number of $CD45^{+}$ $MHCII^{+}$ $F4/80^{+}$ macrophages and VEGF-C mRNA levels in sorted villi macrophages of $MyD88^{\Delta MP}$ mice was in line with the previous report (Mortha et al, 2014). Sensing of commensal microbes via MyD88 in the macrophages leads to the immune homeostasis in the intestine by stimulating a subset of innate lymphoid cells, which in turn regulates the number and function of mononuclear phagocytes in the intestine.

How are you discriminating between the reduction in macrophages and the total level of VEGF-C compared with the level of VEGF-C produced by MyD88 deficient macrophages? This should be assessed experimentally to conclude that MyD88 mediated signal transduction of microbiota is responsible for macrophage-produced VEGF-C.

Response: We absolutely agree with your point about the relation between MyD88-mediated signal transduction of microbiota and VEGF-C produced by macrophages. The data presented in Fig 8D is the result from qPCR of sorted intestinal macrophages. We modified figures so that the readers could more easily recognize the meaning of data.



Page 13, Figure EV6: The 15% lacteal shortening in the jejunum of vancomycin treated mice is not as substantial as that observed in ABX treated mice, so it is difficult to conclude that Gram-positive microbes are responsible for VEGF-C production. Moreover, there is no dissection of the relative amounts of VEGF-C produced in distinct populations of macrophages to support this claim. Either the work should be done to address this experimentally, or the claim should be toned down.

Response: We appreciate the reviewer's critical comment. To avoid the over-interpretation, we revised the related part of manuscript (page 12).

Main text p12: These data imply that the narrow spectrum of gut microbiota sensitive to vancomycin, at least in part, contribute to the lacteal integrity.

Page 13: "LECs barely associated with lacteal integrity" needs to be re-written, do you mean that MyD88 signal transduction in LECs does not contribute to production of VEGF-C in the intestine?

Response: As the reviewer pointed out, we original meant that, while MyD88-transduced signal in macrophages is critical for VEGF-C production and for lacteal integrity, MyD88 in LECs is not essential for the lacteal maintenance. Following the reviewer's recommendation, we revised the related part of manuscript (page 13)

Main text p13: …direct stimulation of the microbial component on LECs via MyD88 is barely associated with lacteal integrity.

The part of the discussion comparing macrophage versus SMC-derived VEGF-C production needs to be revised; to date there has not been a study that has dissected the roles and relative contribution of macrophage versus SMC-derived VEGF-C (though this would be fascinating to do). The Nurmi study investigated mice deficient in VEGF-C in all tissues, but demonstrated that SMC express Vegfc mRNA.

Response: Following the reviewer's comment, we revised the manuscript (page 15-16).

Main text p15-16: While the contribution of VEGF-C originated from SMCs is indispensable throughout the entire length of intestine, SMCs seem to have regulatory factors other than gut microbiota regarding to VEGF-C production. To define the differential regulatory mechanisms of VEGF-C production and the relative contribution of VEGF-C from different sources to the lacteal integrity would also be interesting questions further to be dissected.

Reference for the reviewer 2.

1. Bernier-Latmani J, Cisarovsky C, Demir CS, Bruand M, Jaquet M, Davanture S, Ragusa S, Siegert S, Dormond O, Benedito R*, et al.* (2015) DLL4 promotes continuous adult intestinal lacteal regeneration and dietary fat transport. *J Clin Invest* **125:** 4572-4586
2. Fleissner CK, Huebel N, Abd El-Bary MM, Loh G, Klaus S, Blaut M (2010) Absence of intestinal microbiota does not protect mice from diet-induced obesity. *Br J Nutr* **104:** 919-929
3. Mortha A, Chudnovskiy A, Hashimoto D, Bogunovic M, Spencer SP, Belkaid Y, Merad M (2014) Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. *Science* **343:** 1249288

Referee #3:

The manuscript of Suh et al investigated the role of intestinal microbiota in the maintenance of intestinal lacteals using antibiotic-treated and germ free mice. Depletion of microbiota both in ABX-treated and GF animals resulted in decreased lacteal length and modification of cell-cell junctions in lymphatic capillaries. Functionally, ABX-treated mice show delayed appearance of blood TGs and decreased peak value of FFA after gavage with the vegetable oil. In vivo imaging on intestinal lacteal function revealed delayed clearance of BODIPY-FA from intestinal lamina propria, indicating impaired defective function of intestinal lymphatics. Levels of VEGF-C were found to be reduced by 35% in the gut of ABX-treated animals, suggesting that reduced VEGF-C/VEGFR-3 signaling underlies the degeneration of lacteals. F4/80+ macrophages were found to be express VEGF-C. VEGF-C expression and the total number of macrophages were strongly reduced in the absence of microbiota. Further depletion of CX3CR1+ immune cells using DT system also resulted in decreased levels of macrophages and VEGF-C in small intestine and the similar effect was observed following depletion of Myd88 in myeloid cells. In vitro experiment further showed that VEGF-C xecretion from macrophages could be only elicited after stimulation with TLR1/2 but not other agonists. Overall, these data suggest that gut microbiota stimulates intestinal macrophages to produce VEGF-C and promote maintenance of small intestinal lymphatics.

The results are well described, using appropriate models and show that, intestinal lymphatic vasculature relies on signals from gut microbiota for its maintenance. I have the following questions:

Main points:

<u>Comment 1-1:</u>**The data suggest that either complete inactivation of VEGFR-3 (Vegfr3flox/flox:Prox1-CreERT2 model) or less than 50% reduction of VEGF- C (35% on the protein levels) are sufficient to reduce lacteal length to the same extent - how do the authors explain this discrepancy? The ultimate proof of the proposed mechanism would have been the inactivation of VEGF-C in macrophages using LysM-Cre, however I am not sure how feasible these experiments are. The authors could analyse VEGF-C heterozygous mice to demonstrated that 50% reduction in VEGF-C levels is sufficient to impair lacteal maintenance.**

<u>Response:</u> We appreciate these constructive comments. We understood that the reviewer raised the issue on the discrepancies between the magnitude of reduction in VEGF-C or VEGFR3 and the phenotypes. However, considering the complex mode of action of VEGF-C and its cognate receptors, this discrepancy could be explained. While the VEGF-C retains its dose-dependent on the lymphangiogenesis after birth (Baluk et al, 2017), haploinsufficiency of VEGFR3 did not show distinct phenotypes, compare to the WT mice (Tammela et al, 2011). Therefore, 50% reduction of VEGF-C mRNA level should not be equal to 50% reduction of VEGFR3 mRNA level. Moreover, although VEGFR3 is a major receptor of VEGF-C, VEGF-C also binds to VEGFR2 to regulate lymphangiogenesis (Goldman et al, 2007), eliciting functional redundancy with VEGFR3. We included a part of this explanation and rephrased the text in the revised manuscript (page 15).

Main text p15: In the present study, either complete inactivation of VEGFR3 in VEGFR3$^{i\Delta LEC}$ mice or less than 50% reduction of VEGF- C in ABX-treated mice was sufficient to reduce lacteal length to the same extent. While the VEGF-C retains its dose-dependent on the lymphangiogenesis after birth (Baluk et al, 2017) haploinsufficiency of VEGFR3 did not show distinct phenotypes, compare to the WT mice (Tammela et al, 2011) Therefore, 50% reduction of VEGF-C mRNA level should not be equal to 50% reduction of VEGFR3 mRNA level. Moreover, although VEGFR3 is a major receptor of VEGF-C, VEGF-C also binds to VEGFR2 to regulate lymphangiogenesis (Goldman et al, 2007), eliciting functional redundancy with VEGFR3. Considering this complex mode of action of VEGF-C and its cognate receptors, the lacteal phenotypes in ABX-treated and VEGFR3$^{i\Delta LEC}$ mice seem quite reasonable.

In addition, although VEGF-C heterozygous mouse (VEGF-C LacZ mouse) truly bears 50% reduction in VEGF-C allele, this is not considered to be an appropriate model for the lacteal maintenance, because it is not working as an inducible manner and the subject is affected even during the developmental periods.

<u>Comment 1-2:</u>**Also, it would be interesting to test whether depletion of gut microbiota affect other factors, necessary for lymphangiogenesis, such as Ccbe1 and Adamts3 either in macrophages or in stromal cells.**

<u>Response:</u> Following the reviewer's comments, we evaluated the Ccbe1 and Adamts3 mRNA levels in the whole tissue lysates from vehicle- and ABX-treated mice (Appendix Fig S6B). Tissue mRNA levels between vehicle- and ABX-treated groups were not different (page 10).

Main text p10: Although angiopoietin 1(Kajiya et al, 2012), angiopoietin 2 (Zheng et al, 2014), transforming growth factor β1 (Clavin et al, 2008), collagen- and calcium-binding EGF domains 1 (Bos et al, 2011) and a disintegrin and metalloproteinase with thrombospondin motifs 3 (Bui, Enis et al., 2016) have been known to regulate lymphangiogenesis and lymphatic remodeling, tissue mRNA levels between vehicle- and ABX-treated groups were not different (Appendix Fig S6B).

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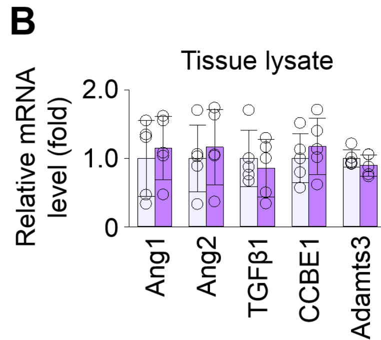
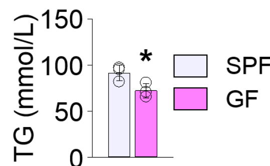


Appendix Figure S6. Tissue VEGF-C level depends on gut microbiota. (B) Comparison of mRNA levels of angiopoietin 1 (Ang1), Ang2, transforming growth factor β1 (TGFβ1), collagen- and calcium-binding EGF domains 1 (CCBE1) and a disintegrin and metalloproteinase with thrombospondin motifs 3 (Adamts3) in the whole tissue of jejunum and ileum from vehicle- and ABX-treated mice (n = 5 mice/group). Data are represented as means ± SD.

Comment 2:The functional defects of lymphatic vessels in ABX- treated animals is convincing, it will be important to study whether lymphatic vessel function also impaired in germ-free mice.

Response: We appreciate this constructive comment. Following the reviewer's suggestion, we performed an additional experiment to directly measure and compare TG levels in the lymph from thoracic ducts of SPF and GF mice (Appendix Fig S5). Compared to SPF mice, TG level in the lymph of thoracic duct was reduced in GF mice, indicating the compromised function of lipid drainage from lacteals. We included this new result and its description into the revised manuscript (page 8).

Main text p8: Compared to SPF mice, TG level in the lymph of thoracic duct was reduced in GF mice, indicating the compromise in the lipid drainage function of lacteals (Appendix Fig S5).



Appendix Figure S5. Triglyceride level in the lymph from thoracic duct is reduced in GF mice. Comparisons of triglyceride (TG) in in the lymph from thoracic duct from SPF and GF mice (n = 3 mice/group). The lymph was collected 1 hour after oral lipid loading. Data are represented as means ± SD. *P < 0.05 vs. SPF mice by two-tailed unpaired Student's t-test.

Comment 3: Figure 2F How do the authors explain that the peak of FFA in blood remains at 2 h both in the control and ABX treated mice, whereas TG peak is shifted to 4h?

Response: The original data showed a considerable range of variation. To present more reliable data, we repeated the measurement of the serum triglyceride level after lipid loading (page 7), with some modification in the method (page 24).

Main text p24: TG and FFA analysis was performed on FUJI DRI-CHEM 7000i (Fuji Film) and VetTest Chemistry analyzer (IDEXX Lab), respectively.

The result shows the coincidence of peak time point between triglyceride and free fatty acid at 2 hours after lipid loading, which is included in the Fig 2H.

Main text p7: Compared to vehicle-treated mice, ABX-treated mice had 39% and 24% reduction in peak TG and FFA levels at 2 h after the corn-oil administration, respectively (Fig 2H).

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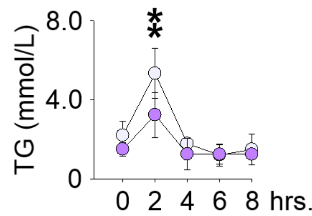

Figure 2. (H) Comparisons of serum triglyceride (TG) and free fatty acids (FFA) at indicated time points in vehicle- and ABX- treated mice (n = 5-7 mice/group). Data are represented as means ± SD. **P < 0.01 vs. vehicle-treated mice by two-way ANOVA with Bonferroni post-hoc analysis.

Comment 4: Figure 2E: how representative are these EM pictures? The authors state that both control and ABX-treated lacteals harbor zipper and button-like junctions - please provide a quantification of the observation shown in 2E.

Response: Following the reviewer's recommendation, we included the quantification data of EM findings from vehicle- and ABX-treated mice (Fig 2F). We found that ultrastructurally open junctions in lacteals were significantly reduced by germ depletion.

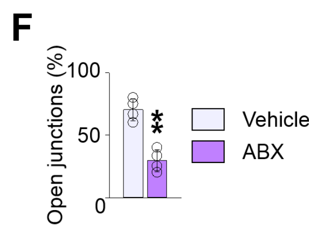

Figure 2. (E, F) Representative images from transmission electron microscopic examination of lacteals and quantification of frequency of open junctions (n = 4 mice/group, 4-6 images/mouse). Jejunum of vehicle- and ABX-treated mice were harvested 2 hours after oral lipid loading. Note that the junction between LECs (red colored bidirectional arrows) is open in vehicle-treated mice, but not in ABX-treated mice. CM, chylomicron; IS, interstitium of lamina propria; LC, large caveola; Lu, lacteal lumen; Ve, vesicle containing lipoproteins. Scale bars, 1 μm. Data are represented as means ± SD. **P < 0.01 vs. vehicle-treated mice by two-tailed unpaired Student's t-test.

Comment 5: P16 - the statement that "no clear evidence has been presented to show how digested lipid particles are transported into a lacteal" is not entirely correct. Please check "The identification of chylomicra and lipoproteins in tissue sections and their passage into jejunal lacteals" by Casley-Smith (1962), it has clear TEM images showing chylomicron passage via flap valves.

Response: We appreciate this constructive comment. Although the researchers have reported the presence of button-like junctions in initial lymphatics confers functional superiority, no clear mechanism based on the ultrastructural observations explained how button-like junctions in lacteals could facilitate the transport of digested lipid particles. Since the vast majority of ultrastructural observations (Casley-Smith, 1962; Dixon et al, 2010) on the lipid transport via lacteals has been reported before the characterization of junctional patterns in lacteals (Bernier-Latmani et al, 2015), we speculate that the ultrastructural findings were not translated into the appearance of VE-cadherin junction in the lacteal. In this study, we observed both VE-cadherin junctional pattern by confocal microscopy and open or closed flap of LECs by transmission electron microscopy, and proved that button-like junctions are closed correlated to the ultrastructurally open flaps by statistical quantification. In this respect, we believe that our findings present a mechanistic insight how button-like junctions could facilitate the lipid transport via ultrastructurally open junctions between LECs in healthy lacteals. We included a part of this explanation and rephrased the text in the revised manuscript (page 17).

Main text p17: It is widely accepted that the ease of access of large molecules to the lymphatic vasculature is primarily due to the specialized button-like junctions in initial lymphatics, and that zipper-like junctions are less penetrable (Baluk et al, 2007). However, no clear ultrastructural evidence explained how button-like junctions in lacteals could facilitate the transport of digested

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lipid particles. Because of the huge time gap between the ultrastructural study (Casley-Smith et al, 1962) and the characterization of junctional patterns in lacteals (Bernier-Latmani et al, 2015), the functional significance of ultrastructurally open flaps was not translated into the appearance of VE-cadherin junction in the lacteal.

Minor points:

Comment 1: It is not clear from the materials and methods how gut microbiota depletion was achieved in pups.

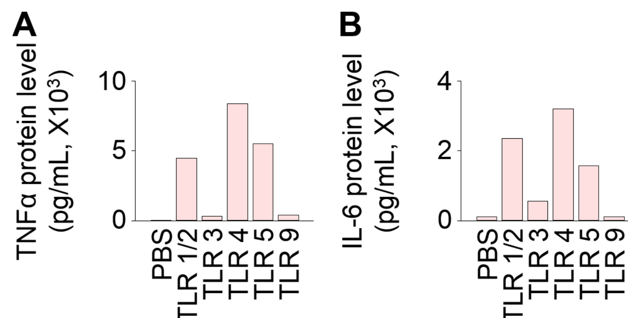
Response: Following the reviewer's recommendation, we revised the related manuscript to more clearly explain how gut microbiota depletion was achieved in pups (page 19).

Main text p19: To investigate the effect of postnatal microbial expansion on the lacteal maturation, ABX was given to WT mice from 4 weeks before mating. ABX administration was maintained after the mating and birth of pups and was continued until the sacrifice of their pups on P7, P14 and P28.

Comment 2: The specific response of macrophages to TLR1/2 agonists in terms of induction of VEGF-C is interesting, please provide evidence that other agonists worked as expected in these conditions.

Response: We appreciate the reviewer's constructive comment. To validate that the TLR receptors are expectedly stimulated by specific agonists, we also analyzed the inflammatory cytokines that are known to be released from the macrophages in response to the stimulation (Appendix Fig S8). We found that tumor necrosis factor α and interleukin-6 production from the macrophages was increased by stimulation with specific TLR agonists, including TLR 1/2 (page 12).

Main text p12: …although TNFα and IL-6 mRNA levels were also increased by the other specific TLR agonists (Appendix Fig S8).



Appendix Figure S8. The stimulation of TLRs with specific agonists induces the production of inflammatory cytokines by macrophages. (A, B) Measurement of TNFα (A) and IL-6 (B) protein level in the culture media after stimulation of primary intestinal macrophages with specific TLR agonists (n = 3). The following TLR agonists at indicated concentrations were treated: palmitoyl-3-cysteineserine-lysine-4 (TLR1/2 agonist, 1 μg/ml), poly(I-C) (TLR3 agonist, 10 μg/ml), lipopolysaccharide (TLR4 agonist, 5 μg/ml), flagellin (TLR5 agonist, 0.1 mg/ml), or bacterial DNA (TLR9 agonist, 10 μg/ml). Data are representative of three independent experiments.

Reference for the reviewer 3.

1. Baluk P, Yao LC, Flores JC, Choi D, Hong YK, McDonald DM (2017) Rapamycin reversal of VEGF-C-driven lymphatic anomalies in the respiratory tract. *JCI Insight* **2:**
2. Bernier-Latmani J, Cisarovsky C, Demir CS, Bruand M, Jaquet M, Davanture S, Ragusa S, Siegert S, Dormond O, Benedito R*, et al.* (2015) DLL4 promotes continuous adult intestinal lacteal regeneration and dietary fat transport. *J Clin Invest* **125:** 4572-4586
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4. Dixon JB (2010) Mechanisms of chylomicron uptake into lacteals. *Ann N Y Acad Sci* **1207 Suppl 1:** E52-57

5. Goldman J, Rutkowski JM, Shields JD, Pasquier MC, Cui Y, Schmokel HG, Willey S, Hicklin DJ, Pytowski B, Swartz MA (2007) Cooperative and redundant roles of VEGFR-2 and VEGFR-3 signaling in adult lymphangiogenesis. *FASEB J* **21:** 1003-1012
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2nd Editorial Decision 8 January 2018

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, referees #2 and #3 now support the publication of your manuscript in EMBO reports. Referee #1 has some remaining concerns, we ask you to address in a final revised version, and a detailed point-by-point response.

Further, I have these editorial requests:

- Please remove the 'the' from the title:
Gut microbiota regulate lacteal integrity by inducing VEGF-C in intestinal villus macrophages

- Please provide the abstract written in present tense.

- In the Appendix, please provide a legend for Appendix Table S1. Please also add the S tot the title of this table.

- It seems author Seung-hwan Jeong (S.H.J.) is missing in the author contributions. Please provide this information. Please also change Kb.C. to K.C..

- Please provide an ORCID for the co-corresponding author Joo-Hye Song, and link it to her EMBO reports profile.

- Please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

When submitting your revised manuscript, we will require:

- a letter detailing your responses to the final referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the final revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures) of those with changes
- the revised Appendix file

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

REFEREE REPORTS

Referee #1:

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Suh and coworkers provide a substantially revised manuscript. They now added some of the necessary controls. Unfortunately, this manuscript still lacks imprtant references on microbiota-induced vascular remodeling, the main theme of their manuscript, which the authors did not discuss (i.e. Moghadamrad S, Hepatol., 2015; Reinhardt C, Nature, 2012). It is unclear to the reviewer why they do not cite literature on the influence of microbiota and minimal microbial consortia on intestinal vascularization.

Importantly, based on the literature and own studies, this reviewer does not agree with the authors' conclusion that villus length is not influenced by the presence of the gut microbiota. There is evidence in the literature that shows the microbiota's impact on villus length. This effect oft he microbiota was unambiguously described by Abrams GD in Lab Invest., 1963. Again, this is work that is clearly relevant for their study and the authors did not mention this reference. Instead the authors did not consider that Thompson et al. described germ-free dogs in their overview article. Most likely, a number of 6 mice per group is not sufficient to determine these differences. Abrams and coworkers analyzed more than 20 mice per group. Therefore, the results on villus morphometry are doubtful.

The authors did not provide the specificity controls, which I asked for in my comment 8-1. They only have included macrophage-specific deletion of Myd88, which is unsufficient to explain a complex cellular mechanism. The study still lacks the analysis of a tissue-specific Trif control and the analysis of Tlr4-deficient mice. Also the analysis of germ-free mice subsituted with TLR agonists is missing. The authors did not perform the LPS substituion experiment of germfree mice via the drinking water, suggested in comment 8-2. Instead of showing causality with additional experiments they now start to cite the existing literature.

Suh and coworkers did not satisfactorily address my comments during the revision of their manuscript and there are many open questions that the authors did not answer.

Referee #2:

With regard to this revised submission, the authors have satisfactorily responded to all of the concerns that I had raised.

Referee #3:

The authors responded adequately, no additional comments.

2nd Revision - authors' response 11 January 2018

Referee #1:

Comment 1: Suh and coworkers provide a substantially revised manuscript. They now added some of the necessary controls. Unfortunately, this manuscript still lacks important references on microbiota-induced vascular remodeling, the main theme of their manuscript, which the authors did not discuss (i.e. Moghadamrad S, Hepatol., 2015; Reinhardt C, Nature, 2012). It is unclear to the reviewer why they do not cite literature on the influence of microbiota and minimal microbial consortia on intestinal vascularization.

Response: We appreciate this additional and constructive comment. The role of gut microbiota in the postnatal development of intestinal vasculature has been previously reported (Stappenbeck et al, 2002). The following study revealed detailed mechanisms how the gut microbiota drives the remodeling of intestinal vasculature (Reinhardt et al, 2012). Regarding the intestinal lymphatics, the morphological analysis in germ-free (GF) mice reported decreased LYVE-1^{+} lymphatic vessel, mainly focusing on the crypt region of villi densities (Moghadamrad et al, 2015). Following those publications, we explored the effect of depletion of gut microbiota/GF condition on the lacteal

maintenance/development and the detailed action mechanism of gut microbiota on lacteal integrity, which has not been investigated yet. Following the reviewer's suggestion, we included a part of this explanation and rephrased the text in the revised manuscript, with additionally cited references (page 4).
Main text p4: The postnatal development of intestinal vasculature is driven by gut microbiota (Stappenbeck et al, 2002), where tissue factor and protease-activated receptor promote vascular remodeling (Reinhardt et al, 2012). While the diverse organ-specific roles of gut microbiota have been extensively studied, their role in lacteals is so far only limitedly understood (Moghadamrad et al, 2015).

<u>Comment 2:</u> **Importantly, based on the literature and own studies, this reviewer does not agree with the authors' conclusion that villus length is not influenced by the presence of the gut microbiota. There is evidence in the literature that shows the microbiota's impact on villus length. This effect of the microbiota was unambiguously described by Abrams GD in Lab Invest., 1963. Again, this is work that is clearly relevant for their study and the authors did not mention this reference. Instead the authors did not consider that Thompson et al. described germ-free dogs in their overview article. Most likely, a number of 6 mice per group is not sufficient to determine these differences. Abrams and coworkers analyzed more than 20 mice per group. Therefore, the results on villus morphometry are doubtful.**

<u>Response:</u> We appreciate this constructive comment. As the reviewer pointed out, we stated that the depletion of gut microbiota by oral antibiotics cocktail (ABX) administration primarily affect the lacteal length in the jejunum and ileum of adult mice, but not the villus length (Fig 1). To address the issue on villus length raised by the reviewer, we sacrificed additional sets of SPF/GF/CONV mice and analyzed the villus morphology with a larger sample size (9 mice per group). On top of lacteal shortening in the jejunum and ileum of GF mice, we found that the villus length were increased in the corresponding region of GF mice (Fig 4B), which results are comparable to the previous report (Abrams et al, 1963). The conventionalization of GF mice normalized the villus length in the ileum, but not in the jejunum. We speculate that the impact of absence of gut microbiota on the villus length appears more dramatic in GF mice than in ABX-treated mice, because the villi in GF mice are not exposed to gut flora even during the postnatal periods. We included a part of this explanation and rephrased the text in the revised manuscript, with additionally cited references (page 8-9).

Main text p8-9: Compared to mice bred in specific pathogen-free (SPF) conditions [SPF mice], absolute and relative lacteal lengths decreased by 20–24% in jejunum and ileum but did not change in duodenum, while the villus length increased by 5-7% (Fig 4A and B), as previously reported (Abrams et al, 1963). … Conventionalization normalized the villus lengths in the ileum, but not in the jejunum (Fig 4A and B).

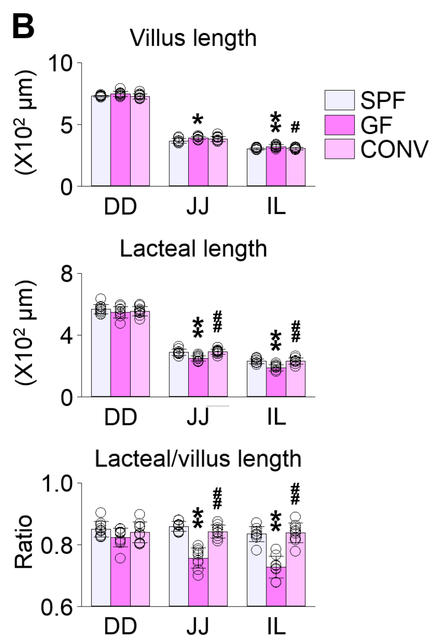

Figure 4. (A, B) Images and comparisons of villus lengths and absolute and relative lacteal lengths in duodenum (DD), jejunum (JJ) and ileum (IL) of 8-week old SPF, GF or CONV mice. Each dot indicates mean value of 5-10 villi in a mouse (n = 9 mice/group).

<u>Comment 3</u>: **The authors did not provide the specificity controls, which I asked for in my comment 8-1. They only have included macrophage-specific deletion of Myd88, which is unsufficient to explain a complex cellular mechanism. The study still lacks the analysis of a**

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tissue-specific Trif control and the analysis of Tlr4-deficient mice. Also the analysis of germ-free mice subsituted with TLR agonists is missing. The authors did not perform the LPS substituion experiment of germ-free mice via the drinking water, suggested in comment 8-2. Instead of showing causality with additional experiments they now start to cite the existing literature.

Response: We appreciate the reviewer's comment and apologize for our insufficient explanation. The present study aimed to define how gut microbiota stimulate the villus macrophages to produce VEGF-C and suggested the interaction between gut microbiota and a subtype of TLRs as one of possible mechanisms.
We used macrophage-specifically MyD88 deleted mice ($MyD88^{\Delta MP}$), to define whether the microbe recognition is a direct stimulation for VEGF-C production in villi macrophages. For our purpose, the choice of $MyD88^{\Delta MP}$ seems reasonable, because the deletion of MyD88 has been adopted for interference of gut bacterial sensing by various cell types (Hoshi et al, 2012).
It would be an intriguing story to look into the TRIF knock-out (KO) and TLR4 KO mice, if we had the evidence that stimulation of TLR4 with its agonist LPS induced VEGF-C production in macrophages. Indeed, the LPS stimulation is closely related to lymphangiogenesis directly or indirectly (Filster et al, 2010; Kang et al, 2009). We reported, however, that the stimulation of TLR1/2, but not TLR4 by its agonist LPS, in macrophages are most likely relevant to VEGF-C production (Fig EV4A and B), and that elimination of vancomycin-sensitive microbes, which are closely linked to TLR1/2, showed disrupted lacteal integrity in vivo (Figure EV4C and D). In this context, we assumed that it is not logically justified to additionally dissect TLR4 KO and TRIF KO mice (TRIF-mediated signal is not linked to TLR2) as well as GF mice substituted with LPS to define which TLR subtype is critically influencing microbiota-induced lacteal development.
We also considered the previous reports of ours and others that the stimulation with LPS induced VEGF-C production in dermal (Kataru et al, 2009), peritoneal (Kim et al, 2009) or bone marrow-derived macrophages (Zhang et al, 2014). Those somewhat contradictory results should be interpreted based on the special adaptation of intestinal macrophages to their environment (Bain et al, 2011), as seen in the refractoriness of intestinal macrophages to release inflammatory cytokine in response to LPS stimulation (Ueda et al, 2010), where the bone marrow-derived macrophages robustly released IL-6 and TNFα. Therefore, we assume that the unique mode of VEGF-C regulation we proposed also results from the organ-specific characteristics of intestinal macrophages.

References for the referee 1:

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3. Moghadamrad S, McCoy KD, Geuking MB, Sagesser H, Kirundi J, Macpherson AJ, De Gottardi A (2015) Attenuated portal hypertension in germ-free mice: Function of bacterial flora on the development of mesenteric lymphatic and blood vessels. *Hepatology* **61:** 1685-1695
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Journal Submitted to: EMOB Reports

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A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical method was used to predetermine the sample size as we used all littermates without exclusion.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No power analyses were performed to determine sample size prior to experiments as we used all littermates and could not control the number of littermates that the breeding pair gives birth to.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All the animals or samples of the littermates of the appropriate gender were analyzed without exclusion from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Animals or samples were not randomized during experiments as we did not randomize the mice but instead used all littermates without exclusion.
For animal studies, include a statement about randomization even if no randomization was used.	We did not randomize the mice, but instead used all littermates without exclusion of animals for analyses.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The first author was not blinded to group allocation during experiments and outcome analyses, as the first author was always involved in sample preparation and data analyses. However, the first author was always accompanied by at least one of the co-authors who was blinded to ensure transparency and unbiased conclusions.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The first author was not blinded to group allocation during experiments and outcome analyses, as the first author was always involved in sample preparation and data analyses. However, the first author was always accompanied by at least one of the co-authors who was blinded to ensure transparency and unbiased conclusions.
5. For every figure, are statistical tests justified as appropriate?	Statistical tests are justified as appropriate for every figure.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Two-tailed unpaired Student's t-test, one-way ANOVA with Tukey's multiple comparison test, Dunnett's multiple comparison test or Bonferroni's multiple comparison test, two-way ANOVA with Bonferroni post-hoc analyses
Is there an estimate of variation within each group of data?	Yes, the data are represented as mean +/- SD or SEM as indicated in the respective figure legends.
Is the variance similar between the groups that are being statistically compared?	Yes, both groups behave according to gaussian distribution. The datasets include independent biological replicates. A statemet including replicate numbers and variance between the groups is included when relevant.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The following primary antibodies were used in the immunostaining: anti-CD31 (hamster monoclonal, clone 2H8, Millipore, #MAB1398Z); anti-CD31 (rat monoclonal, clone MEC 13.3, BD Bioscience, #550274); Cy3-conjugated anti-αSMA (mouse monoclonal, clone 1A4, Sigma-Aldrich, #C6198); anti-F4/80 (rat monoclonal, clone BM8, Biolegend, #123101); anti-Prox1 (rabbit polyclonal, ReliaTech, #102-PA32AG); anti-VEGFR2 (goat polyclonal, R&D, #AF644); anti-VEGFR3 (goat polyclonal, R&D, #AF743); anti-VE-cadherin (goat polyclonal, R&D, #AF1002); anti-LYVE-1 (rabbit polyclonal, AngioBio); anti-LYVE-1 (rat monoclonal, clone ALY7, eBioscience, #11-034). Alexa 488-, Alexa 594-, Alexa647-conjugated secondary antibodies were purchased from Jackson ImmunoResearch. These antibodies have been validated either by manufacturer or previous published literatures in our group.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Primary human derma lymphatic endothelial cells (HDLECs) were isolated and cultured with the approval of the University of Southern California Institutional Review Board (Y.K. Hong). HDLECs were authentificated based on their morphology, growth condition and specific gene expression. HDLECs were tested with a MycoAlert Myplasma Detection Kit (LT07-318) and no mycoplasma contamination was found.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	WT, RiboTag (Sanz et al, 2009) (Jackson stock number 011029), LysM-Cre (Clausen et al, 1999) (Jackson stock number 004781), and Myd88fl/fl (Hou et al, 2008) (Jackson stock number 009108) mice on C57BL/6J background were purchased from Jackson Laboratory. Prox1-CreERT2 (Bazigou et al, 2011), VEGFR3fl/fl (Haiko et al, 2008) and CX3CR1-DTR mice (Longman et al, 2014) were transferred and bred in our SPF animal facilities at KAIST. GF C57BL/6 mice (Kim et al, 2016a) were raised in sterile flexible film isolators (Class Biological Clean Ltd, USA) and maintained in the animal facility of POSTECH Biotech Center. Male and female mice were not distinguished in pup study, while only male mice were used in adult experiments. All mice were fed with free access to a standard diet (PMI Lab diet) and water.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal care and experimental procedures were performed under the approval from the Institutional Animal Care and Use Committee of KAIST (IACUC-17-51) and POSTECH (POSTECH-2014-0030-C1).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Results are reported in accordance with the ARRIVE guidelines. Compliance has been confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	All critical data sets are provided in the manuscript either as main figures, expanded view figures or appendix figures.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A