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Fungal induced glycolysis in macrophage promotes colitis-associated colon cancer via regulating IL-22 production of ILC3

Yanan Zhu, Tao Shi, Xia Lu, Zhen Xu, Junxing Qu, Zhiyong Zhang, Guoping Shi, Sunan Shen, Yayi Hou, Yugen Chen, and Tingting Wang
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Corresponding author: Tingting Wang (wangtt@nju.edu.cn)

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 8th Jun 2020

Dear Dr Wang,

Thank you for your interest and for the submission of your manuscript (EMBOJ-2020-105320) to The EMBO Journal, as well as for your patience with our response, which got delayed due to detailed discussions in the team. Your study has been sent to three referees, and we have received reports from all of them, which I enclose below.

The referees acknowledge the potential interest of your results, although they also express major concerns. In particular, referee #1 raises substantial issues regarding the depth of mechanistic detail provided with respect to the links between macrophage metabolism and IL7 secretion as well as endogenous relevance of the proposed signaling axis (Ref#1, pt. 6,7). Referee #2 is concerned that the molecular characterisation of microbiota changes is too premature and requires additional experimentation (ref#2, pts.4,5,9). In addition, this referee points to inconsistent usage of mouse lines between various experimental settings and potential resultant confounding effects (ref#2, pt.3). Further, the referees raise a number of issues related to missing controls, methods annotation, statistics and data illustration, as well as wording and grammatical errors that would need to be conclusively addressed to achieve the level of robustness and clarity needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. I need to stress though that we do need strong support from the referees on a revised version of the study in order to move on to publication of the work and as to the open outcome of the revisional work suggest to keep EMBO Reports in mind for this work as an alternative venue.

In light of the extensive experimentation requested by the reviewers, I would appreciate if you could contact me during the next weeks via e.g. a video call to discuss your perspective on the comments and potential plan for revisions.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

I this context I also want to point to our adjusted GTA We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and have therefore extended our 'scooping protection policy' to cover the period required for a full revision to address the experimental issues highlighted in the editorial decision letter. Please contact us at any time to discuss an adapted revision plan for your manuscript should you need additional time, and also if you see a paper with related content published elsewhere.

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

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Editor
The EMBO Journal

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Instructions for preparing your revised manuscript:

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

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

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Method) that follows the model below (see also https://www.embopress.org/page/journal/14602075/authorguide#availabilityofpublishedmaterial). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

Our journal also encourages inclusion of *data citations in the reference list* to directly cite

datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14602075/authorguide#referencesformat

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (http://emboj.embopress.org/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

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

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 6th Sep 2020.

Link Not Available

Referee #1:

Zhu et al, "Fungal induced glycolysis in macrophage promotes colitis-associated colon cancer via regulating IL-22 production of ILC3". In this manuscript authors show the following findings: Dectin-3 KO mice have increased more tumors and more C.albicans in AOM-DSS model; Recognition of fungi by macrophages increases glycosis and IL-7 production; Macrophage-derived IL-7 led to IL-22 production in ILC3, that promotes CAC; Antifungal treatment ameliorates colitis-associated cancer in Dectin-3 KO mice.
Interestingly, co-housing abolished the difference between WT and Dectin 3 KO and transfer of

microbiota from Dectin 3 KO to germ free mice increased susceptibility to Aom/DSS tumors. Meanwhile, experiments with Candida detection, fluconazole and antibiotics show that Candida is important, while bacteria are not for the phenotype of Dectin 3 KO mice in AOM/DSS. Interestingly, among other inflammatory molecules, IL-22 and not IL-17A were differentially induced in Dectin 3 KO tumors/intestines, along with p-STAT3 presence/STAT3 activation. Convincingly, anti-IL-22 reduced tumor load especially in Dectin 3 KO mice. Specific determination of RORgT+ ILC3 as a main source of IL-22 is important and interesting, although partially predictable. Anti-CD90 data is nice and confirmatory, but authors should carefully discuss it because of course anti-CD90 should also deplete subsets of T cells. Mechanistic data linking IL-7, Ahr, STAT3 and IL-22 is strong. Human CRC data with fungal burden, prognosis, IL-22 expression and STAT3 activation is super exciting.
Several lines of criticism are noted and likely would need to be addressed in order to strengthen the paper:

1) Fig1 G,H- it would be very informative if flow and Q-RT-PCR data could be provided separately for LP cells from normal tissue and from excised tumor tissue. Otherwise changes may be just a characteristic of relative increase in tumor load over the normal tissue.
2) For Fig 1 and 2 levels of acute and/or chronic colitis with histology, inflammatory gene expression etc need to be characterized, as mere increases in severity of colitis may promote enhanced tumorigenesis. Alternatively, colitis may be the same, but fungi-Dectin3 axis may be mechanistically essential for the colitis-to-cancer step, i.e. for tumorigenesis itself.
3) Fig 2K- it is surprising that antibiotics did not decrease tumors in WT animals, as was previously described. Could author comment on that ?
4) Plots on Fig 4A appear to have possible compensation problems, especially for CD3+ fraction- please check and also provide fluorophores/names for each antibody used.
5) Experiments on Fig 5 may need some bacterial derived controls (LPS, CpG , things alike) to see how these will control metabolism, glycolysis and IL-17 and TNF production, because apparently microbial products do not influence IL-22 expression and activation of changes in macrophages is supposed to be a mechanistic link.
6) Also, data for glycolysis and changes in metabolism is not really mechanistically linked to IL-7 production and further activation of ILC and Il-22 production- it may be indeed a part of direct upstream mechanism or an independent accompanying phenomenon.
7) Mechanistic data on Fig 6 is interesting and strong, but exogenous IL-7 and macrophage derived IL-7 may be two different things in terms of amount and duration of the signal and at least some co-culture system with activated macrophages and ILC without exogenous IL-7 addition but with IL-7 blockade by neutralizing antibody may be warranted.

Minor:
1) Multiple typos throughout the manuscript- please run "spellcheck"
2) STAT3 KO mice are not well described at all in the text or in the Materials and methods. These mice are used for cell isolation. Of course, whole body KO is embryonically lethal. Is it some conditional knockout, like RORgtCre-STAT3?

Referee #2:

The study of Zhu et al. provides insights into how dectin-3 regulates anti-fungal immune responses and colorectal cancer development. The authors suggest a mechanism by which C. albicans activates macrophages to secrete IL-7, which subsequently activates IL-22 production by ILC-3's and drives CRC development. Although the study provides interesting findings, I have several comments and remarks:

1) The manuscript contains a lot of grammatical and spelling errors, too many to list.
2) In a previous study by Wang et al., Plos Pathog. 2016, it was shown that Dectin-3 deficient mice are more susceptible to DSS due to impaired innate antifungal defense in the gut and defective epithelial healing. Supplementation of C. tropicalis also aggravates DSS colitis. Since tumor development in the AOM/DSS model is inflammation dependent, and therefore related to colitis severity during DSS cycles, it is not surprising that Dectin-3 knockout mice develop more and larger tumors, so the novelty of this finding is limited. It is unfortunate that only end-stage analysis of the AOM/DSS experiments is shown. Evaluation of colitis intensity (stool scoring and weight loss) over the entire course of the experiment would have been interesting.
3) Mouse experiments are performed using different mouse lines from different origins (e.g. Dectin-/- mice and WT controls). In order to evaluate the genetic versus microbiota related effects, experiments using offspring from heterozygous breeding set-ups generating both KO and wild-type littermate controls are essential.
4) The manuscript would benefit from more detailed microbiota profiling (16S/18S or ITS sequencing) to evaluate the differences in microbiota and mycobiota structure and composition.
5) Microbiota/mycobiota profiling during co-housing and transplantation experiments would have been more informative than QPCR analysis.
6) Co-housing induced similar tumor development in WT and Dectin-3 knockout mice. Is colitis severity induced by DSS similar upon cohousing, in contrast to previous findings?
7) Experimental details of transplantation experiments are not fully provided, e.g. how long were mice colonized before starting AOM/DSS treatment?
8) Transplantation experiments in GF mice indicate that microbiota from tumor -bearing Dectin3-/- mice is driving pathology. Can also microbiota from non-tumor bearing Dectin3-/- mice induce this effect?
9) Transferring WT microbiota to Dectin-3 knockouts mice, protects from DSS/AOM induced CRC. Detailed microbiota analysis would be very interesting to associate microbiota components to disease
10) Germ-free mice are extremely susceptible to DSS induced toxicity. It is highly remarkable that germfree mice can cope with several rounds of DSS in a classic AOM/DSS experiment. How was the germfree status monitored throughout the experiment? What was the clinical colitis and body weight score throughout AOM/DSS experiments in germfree and Candida-monocolonized mice?
11) Anti-IL-22 and ILC3 depletion was done only during DSS treatment, and strongly impact on later tumor development. Does this treatment impact the colitis severity induced by DSS? Is the anti-tumor effect due to reduced colitis development?

Referee #3:

Description: The major findings of the work are: 1) Mice deficient in Dectin 3 have increased incidence of colitis associated colo-rectal cancer induced by administration of "AOM/DSS"
2) Feces from Dectin deficient mice transferred to germ free mice lead to increased tumor load in the AOM/DSS model and increased levels of C. albicans. C albicans gavage promotes increased

tumor load and fluconazole decreases tumor burden. 3) Cells from the lamnia propria of the gut of germ free mice treated with feces from Dectin 3 deficient mice produce increased levels of IL-22. Anti- IL-22 decreases tumor burden. 4) The sources of IL-22 are ILC3 lymphocytes. These cells have THY marker recognized by anti-CD90. anti-CD90 decreases the number of IL22+ cells and decreases tumor burden without altering fungal load. 5) Candida albicans exposure of Macrophages of germ free mice with fecal transfer from Dectin 3 deficient mice undergo anaerobic glycolysis and produce IL-7 . 6) IL-7 stimulates the synthesis of IL-22 in ILC3 cells via signaling that leads to STAT3 and Ahr transcription factors binding to the IL-22 promoter. 7) This is most important: several aspects of the pathogenesis of colitis associated colon cancer in the mice can be documented in patients with colon cancer. Patients with the more severe stages(III/IV) of colon cancer have lower levels of Dectin-3 and higher levels of fungi (?Candida) in their stools. Patients with high fungal burden have higher levels of STAT3 and IL-22.

Critique: This is an extensive study that is generally very well documented and produces an interesting model for pathogenesis involving cross talk between macrophages (exposed to C.albicans that produce IL-7 under conditions of anaerobic glycolysis) and innate immune lymphocytes that produce IL-22 in response to STAT3 signaling downstream of IL-7. IL-22 is a presumed proliferative stimulus for colonic epithelial cancer cells. The model has the potential for offering several possible sites for intervention in patients.

Specific points for minor revisions: The experiment illustrated in Figure 3 g is interpreted to show (using immunoblotting with antibodies to STAT3 and phosphorylated STAT3) that intestinal epithelial cells from germ free mice with adoptive transfer of colonic contents from Dectin 3 -/- mice show higher levels of STAT3 and relatively higher levels of phospho-STAT3 than GF mice given colon contents from WT mice. This data is not convincing. The immunoblots should be quantitated and the ratio of p-STAT3 to total STAT3 for each mouse (there were three in each group) calculated. The statistical differences of the immunoblot signals should be shown. The related experiment (Fig. 3f) based on counts of cells positive for p STAT3 does show a significant difference but this assay is subject to potential observer error and potential bias in selection of the area of the histologic section that was studied .

In general the paper needs two significant areas for editing. 1) It is saturated with abbreviations. There should be a glossary of abbreviations at the beginning of the paper. 2) It is badly in need of line editing to correct English, for example making subject and verb agree in number.

Point-to-point responses:

First, we would like to thank reviewers for their critical and constructive comments. Based on their critiques, we have performed additional experiments. As results, we have added new data in Figure 2E-G, Figure 3F, Figure 6B, Figure EV2E-G, Figure EV3E-G, and Figure EV5C. We also provide the following point-to-point responses to reviewers' comments and add our explanations in discussion section following the reviewer's suggestion. We have addressed all of reviewers' concerns, and hope this revised manuscript is acceptable for The EMBO Journal.

Referee #1:

Zhu et al, "Fungal induced glycolysis in macrophage promotes colitis-associated colon cancer via regulating IL-22 production of ILC3". In this manuscript authors show the following findings:

Dectin-3 KO mice have increased more tumors and more C.albicans in AOM-DSS model; Recognition of fungi by macrophages increases glycosis and IL-7 production; Macrophage-derived IL-7 led to IL-22 production in ILC3, that promotes CAC; Antifungal treatment ameliorates colitis-associated cancer in Dectin-3 KO mice.

Interestingly, co-housing abolished the difference between WT and Dectin 3 KO and transfer of microbiota from Dectin 3 KO to germ free mice increased susceptibility to Aom/DSS tumors. Meanwhile, experiments with Candida detection, fluconazole and antibiotics show that Candida is important, while bacteria are not for the phenotype of Dectin 3 KO mice in AOM/DSS. Interestingly, among other inflammatory molecules, IL-22 and not IL-17A were differentially induced in Dectin 3 KO tumors/intestines, along with p-STAT3 presence/STAT3 activation. Convincingly, anti-IL-22 reduced tumor load especially in Dectin 3 KO mice. Specific determination of RORgT+ ILC3 as a main source of IL-22 is important and interesting, although partially predictable. Anti-CD90 data is nice and confirmatory, but authors should carefully discuss it because of course anti-CD90 should also deplete subsets of T cells. Mechanistic data linking IL-7, Ahr, STAT3 and IL-22 is strong. Human CRC data with fungal burden, prognosis, IL-22 expression and STAT3 activation is super exciting.

Response: Thanks a lot for the comprehensive and positive feedback on our manuscript. As suggested, we add discussion about our anti-CD90 data in the revised manuscript since anti-CD90 also deplete subsets of T cells.

Our revised section of discussion:

In our study, by using anti-CD90 antibody and anti-IL22 antibody, we found specific determination of RORγt^{+} ILC3, as a main source of IL-22, reduced tumor load especially in Dectin-3$^{-/-}$ mice. Although anti-CD90 antibody also deplete subsets of T cells, experiments with detection of immune cells showed that T cells are not responsible for the phenotype of Dectin-3$^{-/-}$ mice in AOM/DSS.

1) Fig1 G,H- it would be very informative if flow and Q-RT-PCR data could be provided separately for LP cells from normal tissue and from excised tumor tissue. Otherwise changes may be just a characteristic of relative increase in tumor load over the normal tissue.

Response: We agree with the reviewer that to avoid the effect of tumor load, LP cells should be isolated from adjacent normal tissue. In Fig 1G,H, the flow and Q-RT-PCR data is provided from isolated LP cells and mLN tissues. The excised tumor tissues were also acquired and analyzed. We add the qPCR data of tumor tissues in the revised Figure EV1F.

2) For Fig 1 and 2 levels of acute and/or chronic colitis with histology, inflammatory gene expression etc need to be characterized, as mere increases in severity of colitis may promote enhanced tumorigenesis. Alternatively, colitis may be the same, but fungi-Dectin3 axis may be mechanistically essential for the colitis-to-cancer step, i.e. for tumorigenesis itself.

Response: We did acute colitis experiment in *Dectin-3*$^{-/-}$ and WT mice and this data was published in *Plos Pathogen* in 2016 (PMID: 27280399). In this previous study, we find *Dectin-3*$^{-/-}$ mice are susceptible to DSS-induced colitis, presenting by increased mucosal erosion, inflammatory cell infiltration, crypt destruction and loss of goblet cells in the colon of *Dectin-3*$^{-/-}$ mice, compared with WT mice. Although CAC is initialed by AOM and driven by the inflammation from DSS colitis, the development of CAC is still different with acute or chronic colitis, due to the complicated tumor microenvironment. For example, the role of ILC3 in colitis and colon cancer is different. Here, by using AOM/DSS-induced CAC mouse model, we find co-housing abolished the difference between WT and *Dectin-3*$^{-/-}$ mice and transfer of microbiota from *Dectin-3*$^{-/-}$ to germ free mice increased susceptibility to CAC. According to reviewer's suggestion, the clinical colitis scores were evaluated in the chronic colitis stage (day 56) during AOM-DSS treatment and were added in Figure EV2B, Figure EV2C, Figure EV3A and Figure EV5C.

3) Fig 2K- it is surprising that antibiotics did not decrease tumors in WT animals, as was previously described. Could author comment on that?

Response: In previous published papers, the effect of antibiotics on colon tumors depends on the time and stage of antibiotic treatment. Full-time antibiotic treatment significantly decreased the number and size of tumors, compared to the AOM/DSS group without antibiotic treatment. In contrast, the pretreatment antibiotic group (antibiotic administration from 3 weeks prior to AOM) did not exhibit decreased tumorigenesis. In our study, antibiotics were added only during DSS treatment, which

has no effect on tumor load in WT mice. Our data is in consistent with previous studies.

References:

Zackular JP, Baxter NT, Chen GY, Schloss PD. Manipulation of the gut microbiota reveals role in colon tumorigenesis. mSphere. 2015 Nov 4;1(1):e00001-15.

Jae Gon Lee, Chang Soo Eun, Su Vin Jo, A-Reum Lee, Chan Hyuk Park, Dong Soo Han. The impact of gut microbiota manipulation with antibiotics on colon tumorigenesis in a murine model. PLoS One. 2019 Dec 20;14(12):e0226907.

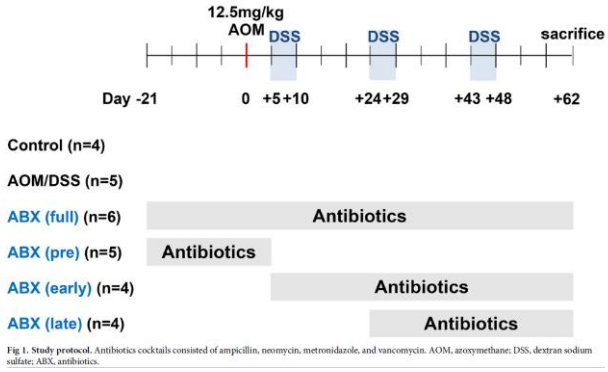
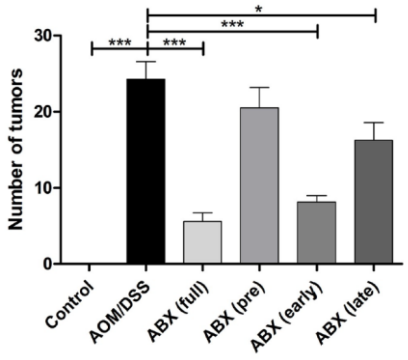


Fig 1. Study protocol. Antibiotics cocktails consisted of ampicillin, neomycin, metronidazole, and vancomycin. AOM, azoxymethane; DSS, dextran sodium sulfate; ABX, antibiotics.



Data from "The impact of gut microbiota manipulation with antibiotics on colon tumorigenesis in a murine model."

4) Plots on Fig 4A appear to have possible compensation problems, especially for CD3+ fraction- please check and also provide fluorophores/names for each antibody used.

Response: Thanks for pointing out this fault. We add names of fluorophores for each antibody used in the revised method section. We also provide a better representative image for Figure 4A.

5) Experiments on Fig 5 may need some bacterial derived controls (LPS, CpG, things alike) to see how these will control metabolism, glycolysis and IL-17 and TNF production, because apparently microbial products do not influence IL-22 expression and activation of changes in macrophages is supposed to be a mechanistic link.

Response: The effect of bacteria on cell metabolism especially on glycolysis has been reported by several studies. As reported, macrophages activated with LPS undergo metabolic changes toward glycolysis, whereas macrophages activated with IL-4 commit to oxidative phosphorylation, and both suggest that metabolic adaptation during macrophage activation is a key component of macrophage polarization, instrumental to their function in inflammation and tissue repair (Ip WKE, et al., *Science*, *2017*, Mills EV et al., *Cell*, *2016*). As to the cytokine production, previous work showed that inhibiting glycolysis with 2-deoxyglucose (2DG) prevented

activation of HIF-1a and induction of IL-1β in LPS-treated macrophages yet had no effect on TNF-a production (Tannahill et al., *Nature, 2013*).

We agree with the reviewer that microbial products do not influence IL-22 expression and activation of changes in macrophages is supposed to be the mechanistic link. In our study, experiments with Candida detection, fluconazole and antibiotics show that Candida is important, while bacteria are not for the phenotype of Dectin 3 KO mice in AOM/DSS. Therefore, we focused on the effect of fungi on the metabolism of macrophages.

References:

Ip WKE, et al. Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages. Science. 2017 May 5; 356 (6337):513-519.

Mills EV, et al. Succinate Dehydrogenase supports metabolic repurposing of mitochondria to drive inflammatory macrophages. Cell. 2016 Oct 6;167(2):457-470.e13.

Tannahill, G.M.,et al. (2013). Succinate is an inflammatory signal that induces IL-1b through HIF-1a. Nature. 2013; 496, 238–242.

6) Also, data for glycolysis and changes in metabolism is not really mechanistically linked to IL-7 production and further activation of ILC and Il-22 production- it may be indeed a part of direct upstream mechanism or an independent accompanying phenomenon.

Response: In our study, we find the phenomenon of increased IL-7 and increased glycolysis in macrophage after fungal stimulation. To confirm the link between glycolysis and IL-7 production, we use the inhibitor of glycolysis（2-DG）. As shown in Figure 5F, the IL-7 production was significantly decreased after inhibition of glycolysis. This data suggests the role of glycolysis in IL-7 production, although this effect maybe indirect and needs our further research.

7) Mechanistic data on Fig 6 is interesting and strong, but exogenous IL-7 and macrophage derived IL-7 may be two different things in terms of amount and duration of the signal and at least some co-culture system with activated macrophages and ILC without exogenous IL-7 addition but with IL-7 blockade by neutralizing antibody may be warranted.

Response: Thanks for this constructive suggestion. We agree with the referee that exogenous IL-7 and macrophage derived IL-7 is different in terms of amount and duration of the signal. Therefore, we use supernatant from macrophages stimulated with or without fungi, and detect and production of IL-22. The IL-7 blockade experiment is also added. This data is presented in the revised Figure 6B.

Minor:

1) Multiple typos throughout the manuscript- please run "spellcheck".

Response: We are very sorry for the grammatical and spelling errors. We have made an intense revision throughout the manuscript.

2) STAT3 KO mice are not well described at all in the text or in the Materials and methods. These mice are used for cell isolation. Of course, whole body KO is embryonically lethal. Is it some conditional knockout, like RORgtCre-STAT3?
Response: STAT3 KO mice used in our study is conditional (intestinal specific) knockout mice. All STAT3-cKO were obtained by crossing Villin-Cre mice with $STAT3^{flox/flox}$ mice on a C57BL/6 background. We added this important information in our revised Materials and Methods and modified the name of mice in our revised Figure 6E to make it clear.

Referee #2:

1) The manuscript contains a lot of grammatical and spelling errors, too many to list.
Response: We are very sorry for the grammatical and spelling errors. We have made an intense revision throughout the manuscript.

2) In a previous study by Wang et al., Plos Pathog. 2016, it was shown that Dectin-3 deficient mice are more susceptible to DSS due to impaired innate antifungal defense in the gut and defective epithelial healing. Supplementation of C. tropicalis also aggravates DSS colitis. Since tumor development in the AOM/DSS model is inflammation dependent, and therefore related to colitis severity during DSS cycles, it is not surprising that Dectin-3 knockout mice develop more and larger tumors, so the novelty of this finding is limited. It is unfortunate that only end-stage analysis of the AOM/DSS experiments is shown. Evaluation of colitis intensity (stool scoring and weight loss) over the entire course of the experiment would have been interesting.
Response: Thanks for this constructive comment. Although CAC is initialed by AOM and driven by the inflammation from DSS colitis, the development of CAC is still different with acute or chronic colitis, due to the complicated tumor microenvironment. For example, the role of Treg cells in colitis and colon cancer is totally different. According to reviewer's suggestion, we evaluate the clinical colitis scores in the revised manuscript. As shown is the revised Figure EV2B-C.

3) Mouse experiments are performed using different mouse lines from different origins (e.g. Dectin-$3^{-/-}$ mice and WT controls). In order to evaluate the genetic versus microbiota related effects, experiments using offspring from heterozygous breeding set-ups generating both KO and wild-type littermate controls are essential.
Response: We agree with the reviewer that the genetic background should be exactly same when comparing phenotype between two groups. We are sorry to make it confused in our method. The Dectin-$3^{-/-}$ mice and WT controls (Dectin-$3^{+/+}$) used in this study are from heterozygous Dectin-$3^{+/-}$ breeding set-ups, which generating both KO and WT littermate controls. We have revised our method section.

4) The manuscript would benefit from more detailed microbiota profiling (16S/18S or ITS sequencing) to evaluate the differences in microbiota and mycobiota structure and composition.

Response: We appreciate this useful suggestion. We send stool samples for detailed microbiota profiling analysis (16S/18S or ITS sequencing), including OTU number, mycobiota composition, alpha-diversity and beta-diversity. These data are now added in Figure 2 and Figure EV2. The original data is submitted to NCBI SRP database with database number provided in the manuscript.

5) Microbiota/mycobiota profiling during co-housing and transplantation experiments would have been more informative than QPCR analysis.

Response: The mycobiota profiling data of transplantation experiments is now added in Figure EV2G. The original data is submitted to NCBI SRP database with database number provided in the manuscript. The fungal burden, bacterial burden and specific fungal abundance for co-housing experiment is also added in Figure EV2I. Since the total fungal burden shows no difference in co-housing experiment, we did not send our sample for sequencing experiment.

6) Co-housing induced similar tumor development in WT and Dectin-3 knockout mice. Is colitis severity induced by DSS similar upon cohousing, in contrast to previous findings?

Response: We have specimen at the colitis stages in co-housing experiments and add this data in the revised Figure EV2B. We found co-housing induced similar colitis between WT and Dectin-3 knockout mice. This data is in consistent with our previous acute colitis study. In previous study, WT and Dectin-$3^{-/-}$ mice were separate at least three weeks before DSS treatment. This single-housed condition leads to severe colitis in Dectin-$3^{-/-}$ mice.

7) Experimental details of transplantation experiments are not fully provided, e.g. how long were mice colonized before starting AOM/DSS treatment?

Response: We are sorry for this defect. We add these experimental details of transplantation experiments in the revised “Method- mouse model” section.

8) Transplantation experiments in GF mice indicate that microbiota from tumor -bearing Dectin$3^{-/-}$ mice is driving pathology. Can also microbiota from non-tumor bearing Dectin$3^{-/-}$ mice induce this effect?

Response: Our previous study has proved that the basic fungal burden and bacterial burden is similar between non-tumor WT and *Dectin-*$3^{-/-}$ mice. Therefore, we did not test the tumor promotion effect of feces between non-tumor WT and Dectin-3 mice.

9) Transferring WT microbiota to Dectin-3 knockouts mice, protects from DSS/AOM induced CRC. Detailed microbiota analysis would be very interesting to associate microbiota components to disease.

Response: Thanks for this constructive suggestion. We agree with the reviewer that to make connection with microbiota components to disease, it is critical to do the detailed microbiota analysis. This data is now added in Figure EV3E-G. The original data is submitted to NCBI SRP database with database number provided in the manuscript.

10) Germ-free mice are extremely susceptible to DSS induced toxicity. It is highly remarkable that germfree mice can cope with several rounds of DSS in a classic AOM/DSS experiment. How was the germfree status monitored throughout the experiment? What was the clinical colitis and body weight score throughout AOM/DSS experiments in germfree and Candida-monocolonized mice?
Response: In our study, we find the germ-free mice are really susceptible to AOM/DSS induced toxicity. Due to the lack of mucus layer and impaired integrity of the epithelial barrier, about 30% germ free mice died within one week after AOM injection and one round of DSS treatment. The remaining 70% mice can survive till the end of experiments. During AOM/DSS experiments, the body weight was measured twice a week. We also observed the stool consistency and the occurrence rate of occult blood. The clinical colitis score and body weight score in germ-free and *Candida*-monocolonized mice is now added in Figure EV2C and Figure S3A.

11) Anti-IL-22 and ILC3 depletion was done only during DSS treatment, and strongly impact on later tumor development. Does this treatment impact the colitis severity induced by DSS? Is the anti-tumor effect due to reduced colitis development?
Response: Thanks for this constructive suggestion. We evaluate the colitis severity during anti-IL-22 and ILC3 depletion experiment. As shown in Figure EV4C and Figure EV5C, anti-IL22 and ILC depletion has no effect on DSS-induced colitis, suggesting the anti-tumor effect was not due to reduced colitis, but may due to fungi-ILC3-IL-22 axis.

Referee #3:
Specific points for minor revisions: The experiment illustrated in Figure 3 g is interpreted to show (using immunoblotting with antibodies to STAT3 and phosphorylated STAT3) that intestinal epithelial cells from germ free mice with adoptive transfer of colonic contents from Dectin 3 -/- mice show higher levels of STAT3 and relatively higher levels of phospho-STAT3 than GF mice given colon contents from WT mice. This data is not convincing. The immunoblots should be quantitated and the ratio of p-STAT3 to total STAT3 for each mouse (there were three in each group) calculated. The statistical differences of the immunoblot signals should be shown. The related experiment (Fig. 3f) based on counts of cells positive for p STAT3 does show a significant difference but this assay is subject to potential observer error and potential bias in selection of the area of the histologic section that was studied.
Response: We really appreciate the positive feedback from the referee and we will make minor revisions according to the suggestion. As shown in the revised Figure 3F,

the immunoblots are quantitated and the ratio of p-STAT3 to total STAT3 for each mouse was calculated. Since both immunoblotting and immunohistochemistry staining are used to detect protein levels of p-STAT3, we delete the previous Figure 3f in the revised figure.

In general, the paper needs two significant areas for editing. 1) It is saturated with abbreviations. There should be a glossary of abbreviations at the beginning of the paper. 2) It is badly in need of line editing to correct English, for example making subject and verb agree in number.

Response: Thanks for these helpful suggestions. A glossary of abbreviations is provided at the beginning of the manuscript. We also make an intense revision throughout the manuscript.

1st Revision - Editorial Decision 2nd Dec 2020

Dear Dr Wang,

Thank you for submitting your revised manuscript (EMBOJ-2020-105320R) to The EMBO Journal. My apologies for getting back to you with delay due to protracted reviewer input. Your amended study was sent back to the referees for re-evaluation, and we have received comments from referee #2, which I enclose below. Please note that while referees #1 and #3 were at this time not able to reassess the work, we have editorially evaluated your response to their concerns and found them to be convincingly addressed. As you will see, referee #2 stated that his-her issues have been sufficiently resolved and s/he is now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending the minor remaining issues related to formatting and data representation as detailed below are addressed at re-submission.

Further, I will share additional changes and comments from our production team during the next days to be considered.

Please contact me at any time if you have additional questions related to below points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Rename the current 'Declaration of Interests' section to 'Conflict of Interest'.

>> Limit the number of keywords to maximally five.

>> Change the format of the 'Data Availability' section to running text.

>> Provide figures and EV figures as individual, high-resolution .tiff files. The figure legends should stay in the manuscript.

>> Recheck callouts and their correct order in the main text for figures EV 4D-F, Fig EV 2D,G,I ; Fig EV 3B,C , Fig EV 4A, Fig EVD; EV 7A.

>> Adjust the reference format to EMBO Journal style, limiting to 10 authors et al. .

>> There is a file with 7 EV figures and 1 Supplemental Table. Up to 5 of the EV figures should be uploaded as individual, high-resolution figure files. Their legends should be added to the manuscript, after the main figure legends. The remaining two EV figures should be renamed "Appendix Figure S1" etc. and they should stay compiled in one 'Appendix' .pdf file, with their legends included. The table should be renamed "Appendix Table S1" and should also stay in that file and needs a title/short description. The appendix file needs a ToC on the first page.

>> Enter the complete funding information for your study into our online system.

>> Remove the list of abbreviations from the main text and instead introduce these in the running text at their first appearance.

>> Please adjust the compensation/processing of the FACS data shown in Figure 4A,C and EV5A to bring the data points off the x, y axes. Otherwise, percentages won't apply correctly.

>>Avoid redundance in wording to your 2018 Study (PMID: 30231984).

Instructions for preparing your revised manuscript:

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

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 2nd Mar 2021.

Link Not Available

Referee #2:

I am satisfied with the provided feedback and additional data.

2nd Authors' Response to Reviewers 8th Jan 2021

The authors performed the requested edits.

2nd Revision - Editorial Decision 8th Jan 2021

Dear Dr Wang,

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

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

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper.

Also in case you might NOT want the transparent process file published at all, you will also need to inform us via email immediately. More information is available here:
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On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work.
Please see the following link for a representative example:
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The videos are embedded in the respective article html page, see e.g.
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Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

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

Kind regards,

Daniel Klimmeck

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Senior Editor
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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: TINGTING WANG
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2020-105320

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

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 $\chi 2$ 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)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	In the mouse work, each group contains at least 8 mice.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Yes, we did mention all the sample size for animal study.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Animals died during experiment were excluded 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.	Mice were randomly divided into different groups.
For animal studies, include a statement about randomization even if no randomization was used.	In our study, randomization was used.
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.	Yes, during pathological evaluation, plthologist did not know the group.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Yes, we mentioned it in the method section.
5. For every figure, are statistical tests justified as appropriate?	Yes, we did for every figure.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	A two-tailed Student's t-test was employed to analyze statistical significance between two groups.
Is there an estimate of variation within each group of data?	Yes, we did in our study.

Is the variance similar between the groups that are being statistically compared?	Yes, the variance is similar between the groups in our study.

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).	Yes, all antibodies used in our study have a citation, catalog number and the supplementary information.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines used in our srudy have been tested for mycoplasam contamination.

* 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.	All these necessary information of each mouse is provided in our study.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	A statement of complicance with ethical regulations and the name of committee is provided in our study.
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.	All the relevant aspects of animal studies are adequately reported.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Yes, the name of committee approving this study is added.
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.	Written informed consent was obtained from all subjects.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	No patient photos published in our study.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	No restrictions on the availability of human data in our study.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	Yes, we provide a "data availability" section in our study.
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).	We have deposition in our study.
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).	NA
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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.	NA