THE EMBO JOURNAL

A tryptophan metabolite made by a gut microbiome eukaryote induces pro-inflammatory T cells

Nicholas Gascoigne, Lukasz Wojciech, Chin Wen Png, Eileen Koh, Dorinda Yan Qin Kioh, Lei Deng, Ziteng Wang, Liang-zhe Wu, Maryam Hamidinia, Desmond Tung, Wei Zhang, Sven Pettersson, Eric Chun Yong Chan, Yongliang Zhang, and Kevin Tan
DOI: 10.15252/embj.2022112963

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Review Timeline:

Submission Date:	31st Oct 22
Editorial Decision:	9th Dec 22
Appeal Received:	19th Dec 22
Editorial Decision:	22nd Dec 22
Appeal Received:	30th Jun 23
Editorial Decision:	3rd Jul 23
Revision Received:	9th Jul 23
Editorial Decision:	4th Aug 23
Revision Received:	11th Aug 23
Accepted:	6th Sep 23

Editor: Karin Dumstrei

Transaction Report:

(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.)

Dear Prof. Gascoigne,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and I am afraid that the overall conclusion is not a positive one.

While they both appreciate that the analysis provides new insight they also find that the analysis doesn't go far enough to consider publication here. We would need more molecular insight into how the Blastocystis tryptophan metabolite modulates downstream cellular processes and regulate the intestinal balance between Treg and T cells.

Given these comments from good experts in the field, I am afraid that I can't offer to consider publication in The EMBO Journal.

I thank you for the opportunity to consider your manuscript for publication here and I am sorry that I can't be more positive on this occasion.

Yours sincerely,

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

Summary:
A protist member of the gut microbiome, Blastocystis was found to generate indole derivatives to influence host immunity. The authors observed that in a Blastocystis-induced (using subtype ST7) colonic inflammation, there was an overall reduction in Tregs with a skew towards Helioshi Tregs, accompanied by an increase in Th17-like cells in the colonic LP. ST7 infection induced this alteration in CD4+ T cell differentation via ST7 metabolites, and more specifically, I3AA. I3AA abrogated TGF signaling in CD4+ T cells, reducing Treg polarization and CD103 expression. Furthermore, pMHC-TCR stimulation in conjunction with ST7 metabolites/I3AA-enhanced CD69 expression but not PD-1, indicating that I3AA induces hyperactivation, but not exhaustion of CD4+ T cells. The authors show that protist-derived I3AA impaired Treg generation, resulting in a loss of mucosal tolerance.

Overall Comments: The experimental approach taken to elucidate the contribution of ST7 on intestinal mucosal immunity was thorough and well-executed. Identification of the protist metabolite and amino acid transferase involved in this shift in T cell polarization provides strong mechanistic support for the observed phenomenon. Regarding the presentation of the data, it is difficult to follow the figures as multiple panels are all labeled under the same letter and in several instances, the figures are incorrectly referenced (i.e. Fig 4D, 7E) or described out of order in the text. Clearer delineation of individual panels would greatly improve the readability of the manuscript.

From the reading the introduction/discussion, it is unclear as to whether Blastocystis infection is perceived as a pathogen or as a member of the stable gut microbiome. Moreover, the perceived relationship between the protist and microbiota in relation to the production of indole derivatives was not clearly stated or directly tested. The described effect of the protist as a commensal and how it affects mucosal tolerance conflicts with the experimental design of using Blastocystis as an infection model. The discussion is limited and does not clearly elaborate on how I3AA production by this protist fits into the context of mucosal homeostasis and/or disease development. While the data are sound, it was difficult to capture the significance of the study without the appropriate context.

Major concerns:

1. Figure 1A shows the proportion, not the number of CD4+ T cells.
2. Although the authors suggest that clonal T cell expansion may contribute to the observed effect following colonization with ST7, this was not directly tested. Moreover, several places within the manuscript explicitly state that the altered CD4+ T cell differentiation results in "enhanced responsiveness against self-flora". To support this statement, Blastocystis infections should be performed in mice pretreated with antibiotics to clearly demonstrate that these shifts in CD4+ populations are in response to self-flora induced by ST7 metabolites.
3. The rationale for the DSS experiments shown in Fig S1 is unclear.
4. It is unclear what data is shown in the heatmap in Figure 2E (right).
5. The experiment shown in Fig 3A should include a control treated with I3AA and no TCR stimulation to demonstrate whether

I3AA in the absence of stimulation alters CD69 expression.
6. In Fig 4A, a previous study showed that 2-NBDG is a substrate for the GLUT2 receptor, which is not expressed by lymphocytes; therefore, 2-NBDG is not a reliable tool to assess glucose uptake by T cells (Sinclair et. al., Immunometabolism, 2020). This experiment should be repeated with 6-NBDG, which has affinity for the GLUT1 receptor.
7. In Fig 4B-C, exposure to increasing concentrations of ST7 metabolites/ I3AA elevates the frequency of dysfunctional mitochondria in CD4+ T cells; however, in Fig 3A, 10 ug/ml I3AA significantly reduces cell viability at 1:10 bead:cells. Moreover, the text states that "the negative effect on cell viability... depended largely on the strength of the TCR stimulus". This statement was confusing based on the data presented in Fig 4C where mitochondria were dysfunction at either 1:10 or 1:1 ratio bead stimulation + 10 ug/ml I3AA. Further, the frequency of dysfunctional mitochondria observed may be due to the high levels of cellular stress at this concentration of I3AA. It would be beneficial to show the viability of the cells when treated with 20 ug/ml ST7B metabolites to assess if the increase in dysfunctional mitochondria is due to the metabolites or the viability of the cells.

Minor concerns:
1. Next to last sentence of the introduction: "I3AA-directed remodeling...caused misinterpretation of TCR signaling upon recognition of pMHC" is a subjective interpretation of the data and was not directly demonstrated in the studies.
2. In many places, acronyms need to be defined upon first use.
3. Fig 6B, the x-axis label is unclear and also incompletely described in the figure legend.

Referee #2:

In the manuscript entitled "A tryptophan metabolite made by a microbiome eukaryote induces pro-inflammatory T cells" by Lukasz Wojciech and co-workers, the authors have investigated the changes occurring at the level of regulators and responder intestinal CD4+ T cells upon infection with the protist Blastocystis ST7 (the only Stramenopile that can also infect the human gut), in order to identify the potential endogenous factors implicated in the ulcerative colitis-like modification of the large intestine after infection with that microbe. Importantly, they found that the reduction of anti-inflammatory Treg cells and simultaneous expansion of pro- inflammatory Th17 responders in the CD4+ T cell population may depend on the local presence of one metabolite produced by the infective eukaryote, i.e. indole-3-acetaldehyde (I3AA), an indole derivative produced by the Blastocystis during the metabolism of tryptophan. The study achieved to unveil how the reduction of tissue resident Treg cell subset upon I3AA sensing was associated with modified TGFβ response by those cells and concomitant over-exuberant TCR signaling, with increased CD69 expression and down-regulation of co-inhibitor PD-1 in conventional CD4+ T cells, which may affect their reactivity toward self-flora antigens. The paper aims at shedding light on the microbiome-derived modulators of the adaptive immunity in the context of gut inflammatory disorders; in this regard, the work is relevant, since it advances knowledge about the complex beneficial or detrimental outcomes of host-microbiome coexistence, which plays a major part in host physiology.

However, in this reviewer's view, the authors should improve the characterization of the cellular modification by the protist metabolite and the dissection of the actual molecular mechanism at work in the modulation of the intestinal balance between Treg and Tconv cells by a further experimental effort.

1. By analyzing the colon of mice colonized by the Blastocystis, the authors noted that the infection hallmark was a substantial decrease of CD4+ T cells expressing IL2 receptor α-chain (CD25), which, as the authors report, is a population comprised by an overwhelming majority of Foxp3-expressing Treg cells. Indeed, the analysis of Foxp3 status confirmed that the intestinal CD4+ T cell compartment of infected animals showed impaired Treg development, with a significant decline of the "intersect" Th17-Treg population, defined as Foxp3+ RORγt+, and a notable expansion of the CD4+ Foxp3- RORγt+ compartment, representing the majority of the Th17 effectors. The authors, though, should show the co-expression of these two sub-populations and CD25, in order to elucidate the activation status of the Th17 subpopulation in the infected intestine, which is a relevant parameter to validate their hypothesis.

2. The authors went on and tested the effect of filtered supernatants from cultures of Blastocystis (both ST7B and ST7H) on Tconv cells under in vitro iTreg and Th17 polarization conditions, which led to a substantial decrease in iTreg number and a detectable higher polarization towards the Th17 phenotype. In details, supernatants from Blastocystis cultures were first pelleted at 13,000xg and then passed through 10kDa Filter Units. Although these passages are surely important to avoid any potential microbial contamination (as actually confirmed by authors through bacterial-specific 16S and 18S PCR quantification), nonetheless, they completely eliminate small extracellular vesicles (exosomes) recently shown to be indeed released by the protist (exosome size = 100 nm, Front Med (Lausanne). 2022 Aug 11;9:940332. doi: 10.3389/fmed.2022.940332) and potentially concurring to affect T cells. Since we must recognize that, in vivo, the protist is possibly producing both the metabolites and the exosomes, with unknown resultant biological function, authors should evaluate the effect of either I3AA by itself (pure compound) or I3AA in combination with protist-derived exosomes (which can be pelleted at 110000xg after the 13,000xg centrifugation). Authors indeed evaluated the negative effect of this compound, either alone or in the complex mixture (Blastocystis culture-derived supernatant) in Fig.S2E and Fig.S2F, but it is not clear from the figure legend what type of supernatant they used for these experiments. The ones suggested by this reviewer may show synergistic effects of I3AA with exosomes, leading to a better definition of the Blastocystis whole secretome function.

3. In the gut milieu of the large intestine from ST7B and ST7H infected animals, authors found significantly higher concentrations of the I3AA than their non-infected counterparts. The data are shown in Figure 2C, but it is not obvious to which Log2 Fold change authors are referring, since the controls are not equal to 0 (linear value =1). The appreciation in the figure of the actual metabolite quantities (not fold changes) would be important to appreciate the baseline concentration of this specific molecule, which seems not to be absent in non-infected animals. If this is the case, which other microbes produce I3AA? Is it known? May it be relevant to infer its physiological fluctuations, which may anyway modulate local immunity? The story about some Clostridium species, which produce IPA, but are negatively affected by Blastocystis, is telling us that the resultant level of specific metabolites depends on the interactive community of microbes and their reciprocal continuous reshaping, not only one. In summary, this reviewer thinks that the experimental data shown in Figure 2 do not fully support the notion that Blastocystis is a major source of this tryptophan metabolite.

4. Authors have shown that the blastocystis-derived I3AA increased the number of T cells with dysfunctional mitochondria in a dose-dependent manner; moreover, functional mitochondria negatively correlated with CD69 expression and glucose uptake. The obvious conclusion was not made though: do these cells rely on an anaerobic glycolysis? It is not clear, also, the connection between the metabolite and the mitochondrial dysfunction. Do AhR deficient cells become insensible to respiring mitochondrial decrease upon I3AA treatment? Do authors hypothesize any potential mechanism, which they may discuss? Moreover, the description of the results concerning T cell exhaustion and naïve T cell plasticity is convoluted; authors should try and explain the connection between the actual experimental results and the originated hypothesis in a more plane manner. Moreover, they should try to reconcile the effect of I3AA on Tconv cells, which seems intrinsic, with the hypothesis that Tconv cells are hyper-activated upon losing Treg-cell dependent suppression when latter cells decrease in number and differentiation upon the encounter with the metabolite.

5. Authors make a beautiful job in describing the complex effects upon T cell exposure to I3AA, including Treg inhibition, antigen recognition, CD69 upregulation and TCR signal interpretation. However, the involvement of the AhR pathway is a crucial point here, and the use of AhR deficient cells should not be relegated in one panel of one supplementary figure (Figure S5D). Authors should investigate in more details whether the metabolite actually makes its entrance in T cells. They do show differential nuclear translocation, but what about AhR-dependent down-stream events? Are they indeed modified upon I3AA treatment. Moreover, AhR is the node which may dictate the differential action of I3AA metabolite on Treg compared with Tconv cells, and so authors may try to explain by which mechanism CH-22319, the synthetic AhR inhibitor, may exert differential activity of the different T cell subpopulation fitness. May this same compound used in vivo to evaluate the participation of AhR in the tissue response to I3AA?

Minor points:
- "The efficiency of in vitro Treg polarization (Fig.5B,C) and the decreased CD103 expression after exposure to CH-223191 (Fig.7D)". Should be Fig. 5D
- In figures, adding more experimental details next to the panels may help the reader following.
- In several figures, there is not an obvious sequence from A to B to C. There is space for some improvements in spatial distribution of the panels.

Referee #1:

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A protist member of the gut microbiome, Blastocystis was found to generate indole derivatives to influence host immunity. The authors observed that in a Blastocystis-induced (using subtype ST7) colonic inflammation, there was an overall reduction in Tregs with a skew towards Helioshi Tregs, accompanied by an increase in Th17-like cells in the colonic LP. ST7 infection induced this alteration in CD4+ T cell differentation via ST7 metabolites, and more specifically, I3AA. I3AA abrogated TGFβ signaling in CD4+ T cells, reducing Treg polarization and CD103 expression. Furthermore, pMHC-TCR stimulation in conjunction with ST7 metabolites/I3AA-enhanced CD69 expression but not PD-1, indicating that I3AA induces hyperactivation, but not exhaustion of CD4+ T cells. The authors show that protist-derived I3AA impaired Treg generation, resulting in a loss of mucosal tolerance.

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Response:

From the reading the introduction/discussion, it is unclear as to whether Blastocystis infection is perceived as a pathogen or as a member of the stable gut microbiome. Moreover, the perceived relationship between the protist and microbiota in relation to the production of indole derivatives was not clearly stated or directly tested. The described effect of the protist as a commensal and how it affects mucosal tolerance conflicts with the experimental design of using Blastocystis as an infection model.

Response:

Blastocystis constitutes a very diverse group of organisms divided into many subtypes. Importantly, subtype ST7's presence in the human gut flora correlates with pathological states and in the experimental infection model induces pathological changes in the gut tissue. Other subtypes like ST1 to ST4 are indeed considered as commensal.

We agree with the reviewer that the aspect of the host-parasite interaction in the context of different *Blastocystis* subtypes should have been more exhaustedly elaborated in the manuscript. Therefore, the relevant section will be added to the Discussion in a revised manuscript.

The discussion is limited and does not clearly elaborate on how I3AA production by this protist fits into the context of mucosal homeostasis and/or disease development. While the data are sound, it was difficult to capture the significance of the study without the appropriate context.

Response:

We appreciate this constructive comment. The discussion of the revised manuscript will include all the matters raised by the reviewer.

Major concerns:

1. Figure 1A shows the proportion, not the number of CD4+ T cells.

Response:

This is a good point. Information regarding total number of isolated T cells will be added to the revised version of the manuscript.

2. Although the authors suggest that clonal T cell expansion may contribute to the observed effect following colonization with ST7, this was not directly tested. Moreover, several places within the manuscript explicitly state that the altered CD4+ T cell differentiation results in "enhanced responsiveness against self-flora". To support this statement, Blastocystis infections should be performed in mice pretreated with antibiotics to clearly demonstrate that these shifts in CD4+ populations are in response to self-flora induced by ST7 metabolites.

Response:

The conclusion that *Blastocystis*-derived I3AA "enhanced responsiveness against self-flora" was made as a result of our experiments with *Lactobacillus*-specific hybridomas exposed to *Blastocystis*-derived I3AA. Additionally, we have shown that I3AA negatively affects PD-1 expression on the activated T cells and positively affects Th17 development. Importantly, it has been shown that expression of the PD-1 exhaustion marker impairs polarization towards Th1/17 responders (PMID: 32048861; DOI: 10.1165/rcmb.2019-0234OC). Additionally, our experimental panel includes the well-defined antigen OVA and MHC class II-restricted CD4+, OVA-specific hybridomas (sFig 5A,B). All these data strongly indicate that the described phenomenon of TCR signal misinterpretation upon I3AA exposure which leads to the skewed T cell polarization, is not restricted to the bacterial-derived antigens. Thus, by antibiotic treatment, we would only change the antigenic landscape in the gut milieu, but we would not be able to exclude TCR-MHC interaction as the remaining antigens in this scenario will have a food origin. The statement "enhanced responsiveness against self-flora" refers to the physiological state of the gut environment, which to a large extent, is enriched in bacterial-derived antigens and in T cells expressing bacterial-specific TCR. However, we agree with the reviewer that the statement sounds too restrictive. Thus, for the sake of clarity, we modified this sentence to "enhanced recognition against gut-derived antigens that leads to over responsiveness to self-gut-flora".

3. The rationale for the DSS experiments shown in Fig S1 is unclear.

Response:

The analysis of the CD4+ lamina propria lymphocytes (LPL) just after DSS treatment reflects the time zero point of the *Blastocystis* infection. The main goal of this experiment was to investigate the distribution of the Foxp3+ (Treg) and Rorγt+ (Th17) cells just after DSS treatment and prior to parasite infection. It is worth noting that DSS treatment *per se* does not induce the Th17 compartment and has no effect on the Treg subset (SFig 1C) (comparing the phenotype from these experiments with the control group data in Fig. 1D). The shift towards Th17 in lamina propria was only observed when animals were infected with *Blastocystis* ST7 or orally treated with I3AA (Fig1D and Fig6E). These data give strong evidence that Th17 cells during *Blastocystis* infection polarize and expand in response to the *Blastocystis*-derived I3AA.

4. It is unclear what data is shown in the heatmap in Figure 2E (right).

Response:

To estimate the concentration of tryptophan metabolites, we used a colorimetric method based on a modified version of Ehlrich's and Kovac's reagents, as described in the Materials and Methods section (Indole Assay Kit (Sigma-Aldrich)). The heat map represents the intensity of the produced coloured compound from the supernatant (left) or the compound standard (right), which is proportional to the indole derivative in the sample.

5. The experiment shown in Fig 3A should include a control treated with I3AA and no TCR stimulation to demonstrate whether I3AA in the absence of stimulation alters CD69 expression.

Response:

The experiment depicted in Fig3A was carried out on isolated T cells. Analysis of the T cell phenotype was done 48h after isolation. Importantly, for the *in vitro* culture, T cells (in particular those isolated from mice) require TCR stimulation for survival. Unfortunately, without TCR stimulation, CD4+ T cells will die after 48h.

6. In Fig 4A, a previous study showed that 2-NBDG is a substrate for the GLUT2 receptor, which is not expressed by lymphocytes; therefore, 2-NBDG is not a reliable tool to assess glucose uptake by T cells (Sinclair et. al., Immunometabolism, 2020). This experiment should be repeated with 6-NBDG, which has affinity for the GLUT1 receptor.

Response:

In the revised version of manuscript, we will include the data with 6-NBDG.

7. In Fig 4B-C, exposure to increasing concentrations of ST7 metabolites/ I3AA elevates the frequency of dysfunctional mitochondria in CD4+ T cells; however, in Fig 3A, 10 ug/ml I3AA significantly reduces cell viability at 1:10 bead:cells. Moreover, the text states that "the negative effect on cell viability... depended largely on the strength of the TCR stimulus". This statement was confusing based on the data presented in Fig 4C where mitochondria were dysfunction at either 1:10 or 1:1 ratio bead stimulation + 10 ug/ml I3AA.

Response:

The negative effect on cell viability was visible at a concentration of 10 ug/ml I3AA when cells were stimulated with either 1:1 or 1:10 cell/bead ratio. However, at the lower 5 ug/ml concentration the negative effect of I3AA on cell viability and mitochondria physiology was only observed in the case of strong stimulation (1:1 cells/beads ratio)(Fig.3C) but not when saturation of the TCR signal was reduced (1:10 cell/bead ratio). These results indicate that I3AA modulates TCR signal strength interpretation.

Further, the frequency of dysfunctional mitochondria observed may be due to the high levels of cellular stress at this concentration of I3AA. It would be beneficial to show the viability of the cells when treated with 20 ug/ml ST7B metabolites to assess if the increase in dysfunctional mitochondria is due to the metabolites or the viability of the cells.

Response:

This is a very good point. We would add that, to exclude dead cells from analysis, we used LIVE/DEAD™ Fixable Near-IR staining (Molecular Probes). Hence, the viability was calculated based on the gated live and dead cells percentage upon flow cytometric analysis. Most importantly, the mitochondria status was estimated only on gated events representing only live (viable) cells.

We agree with the last comment. Therefore, the viability of the cells, when treated with 20 ug/ml ST7B metabolites, is added to the revised manuscript.

Minor concerns:
1. Next to last sentence of the introduction: "I3AA-directed remodeling...caused misinterpretation of TCR signaling upon recognition of pMHC" is a subjective interpretation of the data and was not directly demonstrated in the studies.

Response:

We would like to point out that role of I3AA in the context of Th polarization and TCR signal interpretation was investigated using:

a. aCD3/aCD28 stimulation and isolated naïve polyclonal T cells. We investigated plasticity of T cells towards Treg, Th17 subsets and TCR signal interpretation upon I3AA exposure.
b. bacterial derived antigens and bacterial-specific T cell clones.
c. well defined OVA antigen and OVA-specific T clones.
d. *in vivo* colitis model with oral administration of I3AA.

2. In many places, acronyms need to be defined upon first use.

Response:

This will be corrected in the amended manuscript.

3. Fig 6B, the x-axis label is unclear and also incompletely described in the figure legend.
Response:

Response:

Figure is corrected in the revised version of manuscript.

Referee #2:

In the manuscript entitled "A tryptophan metabolite made by a microbiome eukaryote induces pro-inflammatory T cells" by Lukasz Wojciech and co-workers, the authors have investigated the changes occurring at the level of regulators and responder intestinal CD4+ T cells upon infection with the protist Blastocystis ST7 (the only Stramenopile that can also infect the human gut), in order to identify the potential endogenous factors implicated in the ulcerative colitis-like modification of the large intestine after infection with that microbe. Importantly, they found that the reduction of anti-inflammatory Treg cells and simultaneous expansion of pro- inflammatory Th17 responders in the CD4+ T cell population may depend on the local presence of one metabolite produced by the

infective eukaryote, i.e. indole-3-acetaldehyde (I3AA), an indole derivative produced by the Blastocystis during the metabolism of tryptophan. The study achieved to unveil how the reduction of tissue resident Treg cell subset upon I3AA sensing was associated with modified TGFβ response by those cells and concomitant over-exuberant TCR signaling, with increased CD69 expression and down-regulation of co-inhibitor PD-1 in conventional CD4+ T cells, which may affect their reactivity toward self-flora antigens. The paper aims at shedding light on the microbiome-derived modulators of the adaptive immunity in the context of gut inflammatory disorders; in this regard, the work is relevant, since it advances knowledge about the complex beneficial or detrimental outcomes of host-microbiome coexistence, which plays a major part in host physiology.

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1. By analyzing the colon of mice colonized by the Blastocystis, the authors noted that the infection hallmark was a substantial decrease of CD4+ T cells expressing IL2 receptor α-chain (CD25), which, as the authors report, is a population comprised by an overwhelming majority of Foxp3-expressing Treg cells. Indeed, the analysis of Foxp3 status confirmed that the intestinal CD4+ T cell compartment of infected animals showed impaired Treg development, with a significant decline of the "intersect" Th17-Treg population, defined as Foxp3+ RORγt+, and a notable expansion of the CD4+ Foxp3- RORγt+ compartment, representing the majority of the Th17 effectors. The authors, though, should show the co-expression of these two sub-populations and CD25, in order to elucidate the activation status of the Th17 subpopulation in the infected intestine, which is a relevant parameter to validate their hypothesis.

Response:

CD25 cannot be used in this context as the majority of peripheral CD4+CD25+ cells are Foxp3+ Treg. Importantly, we have shown a decrease in the generation of Treg *in vitro* when cells were exposed to I3AA. This correlated with a drop in CD25 and Foxp3 expressing cells in the *in vivo* experiments (Fig1 and Fig6). Instead, we used CD69 as a marker of T cell activation. CD69 expression on *in vitro* activated cells correlated with data in the *in vivo* infection experiment (Fig 1B).

2. The authors went on and tested the effect of filtered supernatants from cultures of Blastocystis (both ST7B and ST7H) on Tconv cells under in vitro iTreg and Th17 polarization conditions, which led to a substantial decrease in iTreg number and a detectable higher polarization towards the Th17 phenotype. In details, supernatants from Blastocystis cultures were first pelleted at 13,000xg and then passed through 10kDa Filter Units. Although these passages are surely important to avoid any potential microbial contamination (as actually confirmed by authors through bacterial-specific 16S and 18S PCR quantification), nonetheless, they completely eliminate small extracellular vesicles (exosomes) recently shown to be indeed released by the protist (exosome size = 100 nm, Front Med (Lausanne). 2022 Aug 11;9:940332. doi: 10.3389/fmed.2022.940332) and potentially concurring to affect T cells.

Response:

With respect, we cannot agree with these comments. One of the main goals of this work was to address an unexpected biological activity of the *Blastocystis*-derived tryptophan metabolites. The exosome, among many other *Blastocystis*-derived factors that were not addressed in this work, might or might not play a role in the context of the adaptive immune system that resides in the lamina propria of the intestines. It should also be noted that *Blastocystis*-produced exosomes are secreted into the gut lumen. We would need proof that these complex particles can cross the gut epithelium border and directly interact with lamina propria lymphocytes (we consider this highly unlikely due to the complex nature of the exosome). Tryptophan metabolites, on the other hand, penetrate tissue barriers easily. Such tryptophan metabolites produced by the gut flora affect T cells in the lamina propria (PMID: 33086747; DOI: 10.3390/ijms21207740). The experiments that are presented in the paper pointed out by the reviewer (PMID: 36035429**;** doi: 10.3389/fmed.2022.940332) were done *in vitro* on cultured THP-1 cell line (human monocyte cell line) and pertain to the innate response. The data cited by the reviewer paper might be, to some extent, relevant to the gut epithelial compartments' innate response (not addressed in our work). However, the *in vitro* experiments presented in that work do not recapitulate the interaction of the *Blastocystis*-derived exosomes with host adoptive immune components *in vivo*.

The main goal of supernatant preparation was to narrow down the number of potential *Blastocystis*-derived metabolites that could trigger changes within the CD4+ T cell compartment. That is why we are highly confident that flowthrough fractions of the supernatants contain only small and EtOH soluble molecules produced exclusively by *Blastocystis*. Our experimental setup therefore excludes any potential effect on the phenotype of T cells exerted by *Blastocystis*-derived exosomes. The induction of specific phenotypes *in vitro* and *in vivo* by I3AA alone is obvious. Thus, we generally agree with the reviewer that *Blastocystis*-derived exosomes may affect the innate response of gut epithelial compartments. However, in our opinion these comments are not relevant in the context of *Blastocystis*-derived metabolite isolation or in the context of the outcomes of T cell fate decisions resulting from exposure to *Blastocystis*-derived indole derivatives.

Since we must recognize that, in vivo, the protist is possibly producing both the metabolites and the exosomes, with unknown resultant biological function, authors should evaluate the effect of either I3AA by itself (pure compound) or I3AA in combination with protist-derived exosomes (which can be pelleted at 110000xg after the 13,000xg centrifugation).

Response:

We must stress that the biological effect of the pure compound on T cells was evaluated *in vitro* and *in vivo*. The data from the *in vivo* experiment are described in the result section "I3AA induces colitis by inducing Th17 and functionally skewing Tregs" and depicted in Figure 6. Importantly I3AA alone induced changes within the CD4 compartment similar to those observed after *Blastocystis* infection. In our view, these data strongly support the notion that the compound itself exhibits potent immune system modulatory activity independently from the *Blastocystis*-derived exosomes.

Authors indeed evaluated the negative effect of this compound, either alone or in the complex mixture (Blastocystis culture-derived supernatant) in Fig.S2E and Fig.S2F, but it is not clear from the figure legend what type of supernatant they used for these experiments. The ones suggested by this reviewer may show synergistic effects of I3AA with exosomes, leading to a better definition of the Blastocystis whole secretome function.

Response:

The preparation of the supernatants is exhaustedly described in the Materials and Methods section. All the experiments regarding T cell polarization were carried out using the supernatant prepared and standardized as described in the manuscript method or the commercially available I3AA compound. In addition to the *Blastocystis*-derived supernatants and the commercially available I3AA compound, we also investigated the effect of I3AA produced by a human cell line transfected with cDNA of aminotransferase isolated from *Blastocystis* ST7. The scope of the experiments presented in this work very strongly supports the notion that I3AA produced by *Blastocystis* directly interacts with the host adaptive immune compartment. As such, I3AA produced by this organism constitutes a substantial (yet not sole) element that orchestrates the pro-inflammatory reshaping of gut-resident CD4+ T cells.

3. In the gut milieu of the large intestine from ST7B and ST7H infected animals, authors found significantly higher concentrations of the I3AA than their non-infected counterparts. The data are shown in Figure 2C, but it is not obvious to which Log2 Fold change authors are referring, since the controls are not equal to 0 (linear value =1). The appreciation in the figure of the actual metabolite quantities (not fold changes) would be important to appreciate the baseline concentration of this specific molecule, which seems not to be absent in non-infected animals. If this is the case, which other microbes produce I3AA? Is it known? May it be relevant to infer its physiological fluctuations, which may anyway modulate local immunity?

Response:

It is correct. The concentration of the I3AA in the control group was not equal when we compared individual animals. As a baseline (linear value equal to 1, and when converted -log2=0), we chose the mouse that exhibited the highest residual concentration of this compound from the control group. For the sake of clarity, we are providing the table with absolute values (ng/ml):

I3AA ng/ml		
ST7B	ST7H	control
2633.385637	2991.439	1693.065
2277.169555	5484.272	1137.209
2291.237767	2406.697	1664.958
2808.129149	2546.01	1962.516 -log2=0

Importantly, the concentration of the I3AA in total gut content reached a biologically active dose of 2.5ug/ml (see Fig 2D, 2F) only in the *Blastocystis*-infected animals (2.5ug/ml is the minimal dose that negatively affects iTreg generation *in vitro*). However, it is worth noting that the natural *Blastocystis* niche is not the gut lumen but rather the apical side of the gut epithelium. Therefore, it is highly likely that the local concentration of I3AA close to the epithelial layer would be much higher than the values estimated from the entire gut content.

Enterobacter cloacae is the only known, potentially gut microbiome member capable of producing I3AA. However, the isolate of *E. cloacae* that was investigated in the context of I3AA synthesis was isolated not from the gut (human or mouse) but from cucumber. Hence, we do not have *bone fide* proof that any known gut-dwelling residents can produce I3AA. The whole-16S sequencing data and *in silico* analysis (sFig 4) revealed a lack of candidates that can produce this compound in mouse intestines. However, I3AA can be generated with a very low efficiency directly from tryptophan by a non-enzymatic pathway upon exposure to H_2O_2 (PMID: 26686552; DOI: 10.1021/acs.chemrestox.5b00416). H_2O_2 is known to be produced by gut-derived bacteria, and

residual I3AA in the control group can therefore be explained by such non-enzymatic synthesis. Hence, a strong positive correlation of I3AA concentration in the gut milieu with *Blastocystis* infection indicates that only I3AA produced by this protist reached a biologically active concentration after gut colonization. This notion was further supported by the *in vivo* experiments where we administered I3AA orally and induced changes in CD4+ LPL that mirrored those after *Blastocystis* infection.

The story about some Clostridium species, which produce IPA, but are negatively affected by Blastocystis, is telling us that the resultant level of specific metabolites depends on the interactive community of microbes and their reciprocal continuous reshaping, not only one. In summary, this reviewer thinks that the experimental data shown in Figure 2 do not fully support the notion that Blastocystis is a major source of this tryptophan metabolite.

Response:

We are not arguing with the fact that *Blastocystis* infection can induce changes in the gut-dwelling microbiome. However, as pointed out above, the residual presence of I3AA in control animals may be a consequence of tryptophan conversion by H_2O_2, but the increased I3AA in infected animals is clearly a result of I3AA production by *Blastocystis*. To prove this we:

a. Analyzed the microbiome at a species level resolution. This revealed that the mouse gut-derived flora lacked any potential candidate that could produce I3AA by any enzymatic reaction known to date.
b. We showed very solid evidence that *Blastocystis* itself can efficiently produce I3AA from tryptophan and demonstrated the enzyme that can mediate this.

4. Authors have shown that the blastocystis-derived I3AA increased the number of T cells with dysfunctional mitochondria in a dose-dependent manner; moreover, functional mitochondria negatively correlated with CD69 expression and glucose uptake. The obvious conclusion was not made though: do these cells rely on an anaerobic glycolysis?

Response:

The fact that activated T cells rely on an anaerobic glycolysis is widely accepted and well documented (e.g. PMID: 22889213; doi: 10.1111/j.1600-065X.2012.01150.x., PMID: 29032101; https://doi.org/10.1016/j.mito.2017.10.006). We will be happy to mention this explicitly.

It is not clear, also, the connection between the metabolite and the mitochondrial dysfunction.

Response:

The T cells, upon activation, switch off oxidative phosphorylation and use primary aerobic glycolysis to obtain ATP. The non-respiring mitochondria are an indicator of the T cells' activation. We demonstrate that upon I3AA exposure, T cells misinterpreted the TCR signal and became hyperactivated (CD69 expression, glucose uptake, and non-respiring mitochondria). Hence, hyperactivation of T cells induced by I3AA leads to an increased number of non-respiring mitochondria.

Do AhR deficient cells become insensible to respiring mitochondrial decrease upon I3AA treatment? Do authors hypothesize any potential mechanism, which they may discuss? Moreover, the description of the results concerning T cell exhaustion and naïve T cell plasticity is convoluted; authors should try and explain the connection between the actual experimental results and the originated hypothesis in a more plane manner.

Response:

As noted in the response to Reviewer 1, expression of PD-1 exhaustion marker impairs polarization towards Th responders (PMID: 32048861; DOI: 10.1165/rcmb.2019-0234OC). The relevant information – hopefully more clearly described – will be added to the discussion section of the revised manuscript.

Moreover, they should try to reconcile the effect of I3AA on Tconv cells, which seems intrinsic, with the hypothesis that Tconv cells are hyper-activated upon losing Treg-cell dependent suppression when latter cells decrease in number and differentiation upon the encounter with the metabolite.

Response:

This is a very interesting point. However, impaired Treg generation was associated with impaired TGFβ signal transduction of T cells exposed to I3AA. In our view, both phenomena (reduced Treg generation and Tconv cell hyperactivation) rely on intrinsic changes orchestrated by the I3AA. Thus, polarization of CD4+ T cells towards Th17 responders, as shown *in vivo* and *in vitro*, is a consequence of I3AA-induced T cell reprogramming rather than loss of Treg-dependent suppression resulting from the skewed proportion of Treg/Tnaive cells.

5. Authors make a beautiful job in describing the complex effects upon T cell exposure to I3AA, including Treg inhibition, antigen recognition, CD69 upregulation and TCR signal interpretation. However, the involvement of the AhR pathway is a crucial point here, and the use of AhR deficient cells should not be relegated in one panel of one supplementary figure (Figure S5D). Authors should investigate in more details whether the metabolite actually makes its entrance in T cells.

Response:

We appreciate this positive comment. However the context of intrinsic interaction of I3AA with AhR is depicted in Figure 5A and described in the results section entitled “I3AA inhibition of Tregs mimics biological activity of a synthetic AhR inhibitor”. According to these data we are confident that I3AA penetrates the cell membrane.

They do show differential nuclear translocation, but what about AhR-dependent down-stream events? Are they indeed modified upon I3AA treatment. Moreover, AhR is the node which may dictate the differential action of I3AA metabolite on Treg compared with Tconv cells, and so authors may try to explain by which mechanism CH-22319, the synthetic AhR inhibitor, may exert differential activity of the different T cell subpopulation fitness.

May this same compound used in vivo to evaluate the participation of AhR in the tissue response to I3AA?

Response:

This is a very good point. However, CH-22319 is rather toxic and as such cannot be used in the *in vivo* experiments (like other synthetic AhR ligands). To address some of the questions raised by the reviewer we will include data generated in T cells isolated from germline AhR KO mice.

Minor points:
- "The efficiency of in vitro Treg polarization (Fig.5B,C) and the decreased CD103 expression after exposure to CH-223191 (Fig.7D)". Should be Fig. 5D

Response:

This was corrected.

- In figures, adding more experimental details next to the panels may help the reader following.

Response:

We added more information to figure legends.

- In several figures, there is not an obvious sequence from A to B to C. There is space for some improvements in spatial distribution of the panels.

Response:

We did our best to correct this issue.

Hi Nick,

Thanks for your email regarding the decision taken on your MS.

I have looked at your point-by-point response and I don't find that it goes far enough to address the concerns raised. Your response relies to a large degree on previous work and better clarifications.

I am not asking you to do experiments that don't make sense, but I have two good referees that raise valid points and are asking for more causal data to link the effects seen.

I do find the study interesting and to use T cells from AhR KO mice is a good start, but then it is hard from your response to see exactly why type of experiments you plan to do. We also gain limited insight into how IAA affects TGFβ signalling.

So based on your response as is I would have to say no. If you can provide some meaningful data to address the concerns raised and also get some more insight into the cellular mechanisms of IAA - we don't need the full mechanism but some more - then I can offer to take another look at a point-by-point response.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Appeal - Editorial Decision **3rd Jul 2023**

Dear Nick,

Thank you for sending me your point-by-point response to the concerns raised by the referees. I have now had a chance to take a careful look at it.

If you can extend the findings as indicated in your response, then I am open to consider a revised version. I should add that I do need strong support from the referees to move forward with the manuscript and that I can't provide any guarantees regarding the outcome of the review process.

However, if you are open to significantly revised the manuscript then I will send it back to the referees. You can use the link below to submit the revised version.

Let me know if we need to discuss anything further

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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:
https://bit.ly/EMBOPressFigurePreparationGuideline
See also guidelines for figure legends: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/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.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (1st Oct 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

Summary:
A protist member of the gut microbiome, Blastocystis was found to generate indole derivatives to influence host immunity. The authors observed that in a Blastocystis-induced (using subtype ST7) colonic inflammation, there was an overall reduction in Tregs with a skew towards Helioshi Tregs, accompanied by an increase in Th17-like cells in the colonic LP. ST7 infection induced this alteration in CD4+ T cell differentation via ST7 metabolites, and more specifically, I3AA. I3AA abrogated TGFβ signaling in CD4+ T cells, reducing Treg polarization and CD103 expression. Furthermore, pMHC-TCR stimulation in conjunction with ST7 metabolites/I3AA-enhanced CD69 expression but not PD-1, indicating that I3AA induces hyperactivation, but not exhaustion of CD4+ T cells. The authors show that protist-derived I3AA impaired Treg generation, resulting in a loss of mucosal tolerance.

Overall Comments: The experimental approach taken to elucidate the contribution of ST7 on intestinal mucosal immunity was thorough and well-executed. Identification of the protist metabolite and amino acid transferase involved in this shift in T cell polarization provides strong mechanistic support for the observed phenomenon. Regarding the presentation of the data, it is difficult to follow the figures as multiple panels are all labeled under the same letter and in several instances, the figures are incorrectly referenced (i.e. Fig 4D, 7E) or described out of order in the text. Clearer delineation of individual panels would greatly improve the readability of the manuscript.
Response:

From the reading the introduction/discussion, it is unclear as to whether Blastocystis infection is perceived as a pathogen or as a member of the stable gut microbiome. Moreover, the perceived relationship between the protist and microbiota in relation to the production of indole derivatives was not clearly stated or directly tested. The described effect of the protist as a commensal and how it affects mucosal tolerance conflicts with the experimental design of using Blastocystis as an infection model.

Response:

Blastocystis constitutes a very diverse group of organisms divided into many subtypes. Importantly, subtype ST7's presence in the human gut flora correlates with pathological states and in the experimental infection model induces pathological changes in the gut tissue. Other subtypes like ST1 to ST4 are indeed considered as commensal.

We agree with the reviewer that the aspect of the host-parasite interaction in the context of different *Blastocystis* subtypes should have been more exhaustedly elaborated in the manuscript. Therefore, the relevant section has been added to the Discussion in the revised manuscript.

The discussion is limited and does not clearly elaborate on how I3AA production by this protist fits into the context of mucosal homeostasis and/or disease development. While the data are sound, it was difficult to capture the significance of the study without the appropriate context.

Response:

We appreciate this constructive comment. The Discussion section of the revised manuscript has been rewritten to address all the points raised by the reviewer.

Major concerns:

1. Figure 1A shows the proportion, not the number of CD4+ T cells.

Response:

This is a good point. In the revised version of the manuscript, we have included information regarding the total number of isolated T cells.

2. Although the authors suggest that clonal T cell expansion may contribute to the observed effect following colonization with ST7, this was not directly tested. Moreover, several places within the manuscript explicitly state that the altered CD4+ T cell differentiation results in "enhanced responsiveness against self-flora". To support this statement, Blastocystis infections should be performed in mice pretreated with antibiotics to clearly demonstrate that these shifts in CD4+ populations are in response to self-flora induced by ST7 metabolites.

Response:

The conclusion that *Blastocystis*-derived I3AA "enhanced responsiveness against self-flora" was made as a result of our experiments with *Lactobacillus*-specific hybridomas exposed to *Blastocystis*-derived I3AA. Additionally, we have shown that I3AA negatively affects PD-1 expression on the activated T cells and positively affects Th17 development. Importantly, it has been shown that expression of the PD-1 exhaustion marker impairs polarization towards Th1/17 responders (PMID: 32048861; DOI: 10.1165/rcmb.2019-0234OC). Additionally, our experimental panel includes the well-defined antigen OVA and MHC class II-restricted CD4+, OVA-specific hybridomas (sFig 5A,B). All these data strongly indicate that the described phenomenon of TCR signal misinterpretation upon I3AA exposure which leads to the skewed T cell polarization, is not restricted to the bacterial-derived antigens. Thus, by antibiotic treatment, we would only change the antigenic landscape in the gut milieu, but we would not be able to exclude TCR-MHC interaction as the remaining antigens in this scenario will have a food origin. The statement "enhanced responsiveness against self-flora" refers to the physiological state of the gut environment, which to a large extent, is enriched in bacterial-derived antigens and in T cells expressing bacterial-specific TCR. However, we agree with the reviewer that the statement sounds too restrictive. Thus, for the sake of clarity, we modified this sentence to "enhanced recognition against gut-derived antigens that leads to over-responsiveness to self-gut-flora".

3. The rationale for the DSS experiments shown in Fig S1 is unclear.

Response:

The analysis of the CD4+ lamina propria lymphocytes (LPL) just after DSS treatment reflects the time zero point of the *Blastocystis* infection. The main goal of this experiment was to investigate the distribution of the Foxp3+ (Treg) and Rorγt+ (Th17) cells just after DSS treatment and prior to parasite infection. It is worth noting that DSS treatment *per se* does not induce the Th17 compartment and has no effect on the Treg subset (SFig 1C) (comparing the phenotype from these experiments with the control group data in Fig. 1D). The shift towards Th17 in lamina propria was only observed when animals were infected with *Blastocystis* ST7 or orally treated with I3AA (Fig1D and Fig6E). These data give strong evidence that Th17 cells during *Blastocystis* infection polarize and expand in response to the *Blastocystis*-derived I3AA.

4. It is unclear what data is shown in the heatmap in Figure 2E (right).

Response:

To estimate the concentration of tryptophan metabolites, we used a colorimetric method based on a modified version of Ehlrich's and Kovac's reagents, as described in the Materials and Methods section (Indole Assay Kit (Sigma-Aldrich)). The heat map represents the intensity of the coloured compound produced from the supernatant (left) or the compound standard (right), which is proportional to the indole derivative in the sample.

5. The experiment shown in Fig 3A should include a control treated with I3AA and no TCR stimulation to demonstrate whether I3AA in the absence of stimulation alters CD69 expression.

Response:

The experiment depicted in Fig3A was carried out on isolated T cells. Analysis of the T cell phenotype was done 48h after isolation. Importantly, for the *in vitro* culture, T cells (in particular those isolated from mice) require TCR stimulation for survival. Unfortunately, without TCR stimulation, CD4+ T cells will die after 48h.

6. In Fig 4A, a previous study showed that 2-NBDG is a substrate for the GLUT2 receptor, which is not expressed by lymphocytes; therefore, 2-NBDG is not a reliable tool to assess glucose uptake by T cells (Sinclair et. al., Immunometabolism, 2020). This experiment should be repeated with 6-NBDG, which has affinity for the GLUT1 receptor.

Response:

This is a very good comment. However, utility of both 2 and 6-NBDG glucose analogues as a surrogate of D-glucose is under some debate (https://doi.org/10.1016/j.biochi.2021.06.017). We are aware of limitations regarding 2-NBDG, but believe it is valid for this application. Importantly, both 6-NBDG and 2-NBDG yielded the same "uptake" pattern when comparing activated and non-activated T cells (see Fig below for 6-NBDG result to compare to supplementary Fig.6a).

7. In Fig 4B-C, exposure to increasing concentrations of ST7 metabolites/ I3AA elevates the frequency of dysfunctional mitochondria in CD4+ T cells; however, in Fig 3A, 10 ug/ml I3AA significantly reduces cell viability at 1:10 bead:cells. Moreover, the text states that "the negative effect on cell viability... depended largely on the strength of the TCR stimulus". This statement was confusing based on the data presented in Fig 4C where mitochondria were dysfunction at either 1:10 or 1:1 ratio bead stimulation + 10 ug/ml I3AA.

Response:

The negative effect on cell viability was visible at a concentration of 10 ug/ml I3AA when cells were stimulated with either 1:1 or 1:10 cell/bead ratio. However, at the lower 5 ug/ml concentration the negative effect of I3AA on cell viability and mitochondria physiology was only observed in the case of strong stimulation (1:1 cells/beads ratio)(Fig.3C) but not when saturation of the TCR signal was reduced (1:10 cell/bead ratio). These results indicate that I3AA modulates interpretation of TCR signal strength.

Further, the frequency of dysfunctional mitochondria observed may be due to the high levels of cellular stress at this concentration of I3AA.

Response:

This is a very good point. We would add that, to exclude dead cells from analysis, we used LIVE/DEAD™ Fixable Near-IR staining (Molecular Probes). Hence, the viability was calculated based on the gated live and dead cell percentage upon flow cytometric analysis. Most importantly, the mitochondrial status was estimated only on gated events representing live (viable) cells.

It would be beneficial to show the viability of the cells when treated with 20 ug/ml ST7B metabolites to assess if the increase in dysfunctional mitochondria is due to the metabolites or the viability of the cells.

Response:

As mentioned above analysis of the status of the mitochondria was done only on live cells and the supernatant dose of 20 µg/ml of total indoles did not have any impact on cell viability. It is worth mentioning that the concentration of I3AA in these samples is relatively low in comparison to the experiments in which pure I3AA was used. The supernatants from Blastocystis ST7 cultures contain significant amounts of other indoles, namely IPA and indole, in addition to I3AA.

Minor concerns:
1. Next to last sentence of the introduction: "I3AA-directed remodeling...caused misinterpretation of TCR signaling upon recognition of pMHC" is a subjective interpretation of the data and was not directly demonstrated in the studies.

Response:

We would like to point out that role of I3AA in the context of Th polarization and TCR signal interpretation was investigated using:

a. aCD3/aCD28 stimulation and isolated naïve polyclonal T cells. We investigated plasticity of T cells towards Treg, Th17 subsets and TCR signal interpretation upon I3AA exposure.
b. bacterial derived antigens and bacterial-specific T cell clones.
c. well defined OVA antigen and OVA-specific T clones.
d. *in vivo* colitis model with oral administration of I3AA.

2. In many places, acronyms need to be defined upon first use.

Response:

This has been corrected in the revised manuscript.

3. Fig 6B, the x-axis label is unclear and also incompletely described in the figure legend.

Response:

Figure is corrected in the revised version of manuscript.

Referee #2:

In the manuscript entitled "A tryptophan metabolite made by a microbiome eukaryote induces pro-inflammatory T cells" by Lukasz Wojciech and co-workers, the authors have investigated the changes occurring at the level of regulators and responder intestinal CD4+ T cells upon infection with the protist Blastocystis ST7 (the only Stramenopile that can also infect the human gut), in order to identify the potential endogenous factors implicated in the ulcerative colitis-like modification of the large intestine after infection with that microbe. Importantly, they found that the reduction of anti-inflammatory Treg cells and simultaneous expansion of pro- inflammatory Th17 responders in the CD4+ T cell population may depend on the local presence of one metabolite produced by the infective eukaryote, i.e. indole-3-acetaldehyde (I3AA), an indole derivative produced by the Blastocystis during the metabolism of tryptophan. The study achieved to unveil how the reduction of tissue resident Treg cell subset upon I3AA sensing was associated with modified TGFβ response by those cells and concomitant over-exuberant TCR signaling, with increased CD69 expression and down-regulation of co-inhibitor PD-1 in conventional CD4+ T cells, which may affect their reactivity

toward self-flora antigens. The paper aims at shedding light on the microbiome-derived modulators of the adaptive immunity in the context of gut inflammatory disorders; in this regard, the work is relevant, since it advances knowledge about the complex beneficial or detrimental outcomes of host-microbiome coexistence, which plays a major part in host physiology.

However, in this reviewer's view, the authors should improve the characterization of the cellular modification by the protist metabolite and the dissection of the actual molecular mechanism at work in the modulation of the intestinal balance between Treg and Tconv cells by a further experimental effort.

1. By analyzing the colon of mice colonized by the Blastocystis, the authors noted that the infection hallmark was a substantial decrease of CD4+ T cells expressing IL2 receptor α-chain (CD25), which, as the authors report, is a population comprised by an overwhelming majority of Foxp3-expressing Treg cells. Indeed, the analysis of Foxp3 status confirmed that the intestinal CD4+ T cell compartment of infected animals showed impaired Treg development, with a significant decline of the "intersect" Th17-Treg population, defined as Foxp3+ RORγt+, and a notable expansion of the CD4+ Foxp3- RORγt+ compartment, representing the majority of the Th17 effectors. The authors, though, should show the co-expression of these two sub-populations and CD25, in order to elucidate the activation status of the Th17 subpopulation in the infected intestine, which is a relevant parameter to validate their hypothesis.

Response:

CD25 cannot be used in this context as the majority of peripheral CD4+CD25+ cells are Foxp3+ Treg. Importantly, we have shown a decrease in the generation of Treg *in vitro* when cells were exposed to I3AA. This correlated with a drop in CD25 and Foxp3 expressing cells in the *in vivo* experiments (Fig1 and Fig6). Instead, we used CD69 as a marker of T cell activation. CD69 expression on *in vitro* activated cells correlated with data in the *in vivo* infection experiment (Fig 1B).

2. The authors went on and tested the effect of filtered supernatants from cultures of Blastocystis (both ST7B and ST7H) on Tconv cells under in vitro iTreg and Th17 polarization conditions, which led to a substantial decrease in iTreg number and a detectable higher polarization towards the Th17 phenotype. In details, supernatants from Blastocystis cultures were first pelleted at 13,000xg and then passed through 10kDa Filter Units. Although these passages are surely important to avoid any potential microbial contamination (as actually confirmed by authors through bacterial-specific 16S and 18S PCR quantification), nonetheless, they completely eliminate small extracellular vesicles (exosomes) recently shown to be indeed released by the protist (exosome size = 100 nm, Front Med (Lausanne). 2022 Aug 11;9:940332. doi: 10.3389/fmed.2022.940332) and potentially concurring to affect T cells.

Response:

With respect, we cannot agree with these comments. One of the main goals of this work was to address an unexpected biological activity of the *Blastocystis*-derived tryptophan metabolites. The exosome, among many other *Blastocystis*-derived factors that were not addressed in this work, might or might not play a role in the context of the adaptive immune system that resides in the lamina propria of the intestines. It should also be noted that *Blastocystis*-produced exosomes are secreted into the gut lumen. We would need proof that these complex particles can cross the gut

epithelium border and directly interact with lamina propria lymphocytes (we consider this highly unlikely due to the complex nature of the exosome). Tryptophan metabolites, on the other hand, penetrate tissue barriers easily. Such tryptophan metabolites produced by the gut flora affect T cells in the lamina propria (PMID: 33086747; DOI: 10.3390/ijms21207740). The experiments that are presented in the paper pointed out by the reviewer (PMID: 36035429**;** doi: 10.3389/fmed.2022.940332) were done *in vitro* on cultured THP-1 cell line (human monocyte cell line) and pertain to the innate response. The data cited by the reviewer paper might be, to some extent, relevant to the gut epithelial compartments' innate response (not addressed in our work). However, the *in vitro* experiments presented in that work do not recapitulate the interaction of the *Blastocystis*-derived exosomes with host adoptive immune components *in vivo*.

The main goal of supernatant preparation was to narrow down the number of potential *Blastocystis*-derived metabolites that could trigger changes within the CD4+ T cell compartment. That is why we are highly confident that flowthrough fractions of the supernatants contain only small and EtOH soluble molecules produced exclusively by *Blastocystis*. Our experimental setup therefore excludes any potential effect on the phenotype of T cells exerted by *Blastocystis*-derived exosomes. The induction of specific phenotypes *in vitro* and *in vivo* by I3AA alone is obvious. Thus, we generally agree with the reviewer that *Blastocystis*-derived exosomes may affect the innate response of gut epithelial compartments. However, in our opinion these comments are not relevant in the context of *Blastocystis*-derived metabolite isolation or in the context of the outcomes of T cell fate decisions resulting from exposure to *Blastocystis*-derived indole derivatives.

Since we must recognize that, in vivo, the protist is possibly producing both the metabolites and the exosomes, with unknown resultant biological function, authors should evaluate the effect of either I3AA by itself (pure compound) or I3AA in combination with protist-derived exosomes (which can be pelleted at 110000xg after the 13,000xg centrifugation).

Response:

We must stress that the biological effect of the pure compound on T cells was evaluated *in vitro* and *in vivo*. The data from the *in vivo* experiment are described in the Results sub-section "I3AA induces colitis by inducing Th17 and functionally skewing Tregs" and depicted in Figure 6. Importantly, I3AA alone induced changes within the CD4+ compartment similar to those observed after *Blastocystis* infection. In our view, these data strongly support the notion that the compound itself exhibits potent immune system modulatory activity independently from any potential *Blastocystis*-derived exosomes.

Authors indeed evaluated the negative effect of this compound, either alone or in the complex mixture (Blastocystis culture-derived supernatant) in Fig.S2E and Fig.S2F, but it is not clear from the figure legend what type of supernatant they used for these experiments. The ones suggested by this reviewer may show synergistic effects of I3AA with exosomes, leading to a better definition of the Blastocystis whole secretome function.

Response:

The preparation of the supernatants is exhaustively described in the Materials and Methods section. All the experiments regarding T cell polarization were carried out using the supernatant prepared and standardized as described in the manuscript method or the commercially available I3AA compound. In addition to the *Blastocystis*-derived supernatants and the commercially available I3AA compound, we also investigated the effect of I3AA produced by a human cell line transfected with

cDNA of aminotransferase isolated from *Blastocystis* ST7. The scope of the experiments presented in this work very strongly supports the notion that I3AA produced by *Blastocystis* directly interacts with the host adaptive immune compartment. As such, I3AA produced by this organism constitutes a substantial (not necessarily sole) element that orchestrates the pro-inflammatory reshaping of gut-resident CD4+ T cells.

3. In the gut milieu of the large intestine from ST7B and ST7H infected animals, authors found significantly higher concentrations of the I3AA than their non-infected counterparts. The data are shown in Figure 2C, but it is not obvious to which Log2 Fold change authors are referring, since the controls are not equal to 0 (linear value =1). The appreciation in the figure of the actual metabolite quantities (not fold changes) would be important to appreciate the baseline concentration of this specific molecule, which seems not to be absent in non-infected animals. If this is the case, which other microbes produce I3AA? Is it known? May it be relevant to infer its physiological fluctuations, which may anyway modulate local immunity?

Response:

This is correct. The concentration of the I3AA in the control group was not equal when we compared individual animals. As a baseline (linear value equal to 1, and when converted -log2=0), we chose the mouse that exhibited the highest residual concentration of this compound from the control group. For the sake of clarity, we are providing the table with absolute values (ng/ml):

I3AA ng/ml		
ST7B	ST7H	control
2633.385637	2991.439	1693.065
2277.169555	5484.272	1137.209
2291.237767	2406.697	1664.958
2808.129149	2546.01	1962.516 -log2=0

Importantly, the concentration of the I3AA in total gut content reached a biologically active dose of 2.5ug/ml (see Fig 2D, 2F) only in the *Blastocystis*-infected animals (2.5ug/ml is the minimal dose that negatively affects iTreg generation *in vitro*). However, it is worth noting that the natural *Blastocystis* niche is not the gut lumen but rather the apical side of the gut epithelium. Therefore, it is highly likely that the local concentration of I3AA close to the epithelial layer would be much higher than the values estimated from the entire gut content.

Enterobacter cloacae is the only known, potentially gut microbiome member capable of producing I3AA. However, the isolate of *E. cloacae* that was investigated in the context of I3AA synthesis was isolated not from the gut (human or mouse) but from cucumber. Hence, we do not have *bone fide* proof that any known gut-dwelling residents can produce I3AA. The whole-16S sequencing data and *in silico* analysis (sFig 4) revealed a lack of candidates that can produce this compound in mouse intestines. However, I3AA can be generated with a very low efficiency directly from tryptophan by a non-enzymatic pathway upon exposure to H_2O_2 (PMID: 26686552; DOI: 10.1021/acs.chemrestox.5b00416). H_2O_2 is known to be produced by gut-derived bacteria, and residual I3AA in the control group can therefore be explained by such non-enzymatic synthesis. Hence, a strong positive correlation of I3AA concentration in the gut milieu with *Blastocystis* infection indicates that only I3AA produced by this protist reached a biologically active concentration after gut colonization. This notion was further supported by the *in vivo* experiments where we

administered I3AA orally and induced changes in CD4+ LPL that mirrored those after *Blastocystis* infection.

The story about some Clostridium species, which produce IPA, but are negatively affected by Blastocystis, is telling us that the resultant level of specific metabolites depends on the interactive community of microbes and their reciprocal continuous reshaping, not only one. In summary, this reviewer thinks that the experimental data shown in Figure 2 do not fully support the notion that Blastocystis is a major source of this tryptophan metabolite.

Response:

We are not arguing with the fact that *Blastocystis* infection can induce changes in the gut-dwelling microbiome. However, as pointed out above, the residual presence of I3AA in control animals may be a consequence of tryptophan conversion by H_2O_2, but the increased I3AA in infected animals is clearly a result of I3AA production by *Blastocystis*. To prove this we:

a. Analyzed the microbiome at a species level resolution. This revealed that the mouse gut-derived flora lacked any potential candidate that could produce I3AA by any enzymatic reaction known to date.
b. We showed very solid evidence that *Blastocystis* itself can efficiently produce I3AA from tryptophan and demonstrated the enzyme that can mediate this.

4. Authors have shown that the blastocystis-derived I3AA increased the number of T cells with dysfunctional mitochondria in a dose-dependent manner; moreover, functional mitochondria negatively correlated with CD69 expression and glucose uptake. The obvious conclusion was not made though: do these cells rely on an anaerobic glycolysis?

Response:

The fact that activated T cells rely on an anaerobic glycolysis is widely accepted and well documented (e.g. PMID: 22889213; doi: 10.1111/j.1600-065X.2012.01150.x., PMID: 29032101; https://doi.org/10.1016/j.mito.2017.10.006). We now mention this explicitly.

It is not clear, also, the connection between the metabolite and the mitochondrial dysfunction.

Response:

The T cells, upon activation, switch off oxidative phosphorylation and use primary aerobic glycolysis to obtain ATP. The non-respiring mitochondria are an indicator of the T cells' activation. We demonstrate that upon I3AA exposure, T cells misinterpreted the TCR signal and became hyperactivated (CD69 expression, glucose uptake, and non-respiring mitochondria). Hence, hyperactivation of T cells induced by I3AA leads to an increased number of non-respiring mitochondria.

Do AhR deficient cells become insensible to respiring mitochondrial decrease upon I3AA treatment? Do authors hypothesize any potential mechanism, which they may discuss? Moreover, the description of the results concerning T cell exhaustion and naïve T cell plasticity is convoluted; authors should try and explain the connection between the actual experimental results and the originated hypothesis in a more plane manner.

Response:

As noted in the response to Reviewer 1, expression of PD-1 exhaustion marker impairs polarization towards Th responders (PMID: 32048861; DOI: 10.1165/rcmb.2019-0234OC). The relevant information – hopefully more clearly described – has been added to the Discussion section of the revised manuscript.

Moreover, they should try to reconcile the effect of I3AA on Tconv cells, which seems intrinsic, with the hypothesis that Tconv cells are hyper-activated upon losing Treg-cell dependent suppression when latter cells decrease in number and differentiation upon the encounter with the metabolite.

Response:

This is a very interesting point. However, impaired Treg generation was associated with impaired TGFβ signal transduction of T cells exposed to I3AA. In our view, both phenomena (reduced Treg generation and Tconv cell hyperactivation) rely on intrinsic changes orchestrated by the I3AA. Thus, polarization of CD4+ T cells towards Th17 responders, as shown *in vivo* and *in vitro*, is a consequence of I3AA-induced T cell reprogramming rather than loss of Treg-dependent suppression resulting from the skewed proportion of Treg/Tnaive cells.

5. Authors make a beautiful job in describing the complex effects upon T cell exposure to I3AA, including Treg inhibition, antigen recognition, CD69 upregulation and TCR signal interpretation. However, the involvement of the AhR pathway is a crucial point here, and the use of AhR deficient cells should not be relegated in one panel of one supplementary figure (Figure S5D). Authors should investigate in more details whether the metabolite actually makes its entrance in T cells.

Response:

We appreciate this positive comment. The context of intrinsic interaction of I3AA with AhR is depicted in Figure 5A and described in the Results sub-section entitled "I3AA inhibition of Tregs mimics biological activity of a synthetic AhR inhibitor". According to these data we are confident that I3AA penetrates the cell membrane.

They do show differential nuclear translocation, but what about AhR-dependent down-stream events? Are they indeed modified upon I3AA treatment. Moreover, AhR is the node which may dictate the differential action of I3AA metabolite on Treg compared with Tconv cells, and so authors may try to explain by which mechanism CH-22319, the synthetic AhR inhibitor, may exert differential activity of the different T cell subpopulation fitness.

May this same compound used in vivo to evaluate the participation of AhR in the tissue response to I3AA?

Response:

This is a very good point. However, CH-22319 is rather toxic and as such cannot be used in the *in vivo* experiments (like other synthetic AhR ligands). To address some of the questions raised by the

reviewer we included data generated in T cells isolated from germline AhR KO mice. In the revised manuscript we have added iTreg polarization assays with AhR deficient T cells. Importantly, the outcome of *in vitro* polarization of AhR deficient cells mirrored AhR sufficient cells cultured with either I3AA or CH-22319 in terms of iTreg conversion efficiency and iTreg phenotype (CD103 expression). Furthermore, incubation of AhR deficient cells with I3AA did not change the ratio of T cell polarization towards iTreg, demonstrating that the AhR-I3AA interaction is central to the observed phenotype. All these data are in line with previously published work regarding T cells and Treg polarization in regard to AhR (Dean *et al*, 2023; Ye *et al*, 2017, both works now included in the manuscript as references). In our view I3AA interacts with AhR in an antagonistic manner and as such competes with endogenous AhR signal (orchestrated by Kyn). Positive AhR signals synergize with TGFβ signal (Ye *et al*, 2017). Thus, blocking of AhR by I3AA leads to downregulation of CD103 (a molecule that is dependent on TGFβ signal) and impaired Treg selection in the gut environment. As previously reported, positive AhR signals interact in a negative loop with Smad7 (inhibitor of TGFβ signal (Monteleone *et al*, 2001)). Our data indicate that exposure to I3AA *in vitro* and *in vivo* induces the expression of Smad7, thus reducing the sensitivity of T cells to the TGFβ signal.

Minor points:
- "The efficiency of in vitro Treg polarization (Fig.5B,C) and the decreased CD103 expression after exposure to CH-223191 (Fig.7D)". Should be Fig. 5D

Response:

This was corrected.

- In figures, adding more experimental details next to the panels may help the reader following.

Response:

We added more information to figure legends.

- In several figures, there is not an obvious sequence from A to B to C. There is space for some improvements in spatial distribution of the panels.

Response:

We did our best to correct this issue.

Final note

We would like to express our gratitude to the reviewers for their valuable work. We have carefully considered all the suggestions and feedback provided, and we have made sure to incorporate all the recommended changes into the manuscript. Thank you once again for your insightful input, and we hope that the revised version of the manuscript adequately addresses all the points raised.

Dear Nick,

Thank you for submitting your manuscript to The EMBO Journal. This submission is a resubmission of a manuscript that was rejected post-review.

Given the introduced revisions, the manuscript was sent back to the original referees. I have now heard back from the referees and as you can see below, both referees appreciate the introduced changes and support publication here. They have a few suggestions that I would like to ask you to take into consideration into a final version.

When you re-submit will you also please take care of the following points:

- We require source data for our accepted manuscript. I have CCed in our source data coordinator Hannah Sonntag (h.sonntag@source-data.org) who will get back to you with a list of the figures we need source data for.

- Please sort out discrepancy between George Zhang in MS vs. Wei Zhang in the online submission system.

- Funding info needs to be part of Ack. Section. NUS Med Postdoctoral Fellowship & China Scholarship Council (CSC) are missing from the online submission system.

- We need 5 keywords.

- "Materials and Correspondence" - needs to be renamed to "Data Availability". This section is the place to enter accession numbers etc. If no data is generated that needs to be deposited in a database then state: This study includes no data deposited in external repositories.

- COI needs to be renamed to "Disclosure Statement and Competing Interests"

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- Author Checklist: please add corr. au. name, ms ID#, etc to the header PBP

- The appendix needs a ToC and page numbers; the nomenclature and callouts in the MS need to be Appendix Figure S1, etc.

- We need a synopsis text => A summary statement plus 3-5 bullet points describing the key findings of the MS

- We also need a synopsis image - size should be 550 wide by 400. See our website for how the synopsis text and image is displayed.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

- Please order the MS sections in the following order: abstract, introduction, results, discussion, materials & methods, data availability section, acknowledgments, disclosure statement and competing interests, references, main figure legends, tables.

That should be all. Let me know if you need more input from me

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

The authors' revisions provided more substantive context for both the underlying question and relevance of the study performed, the figures were also easier to follow with revisions to the text and organization.

Remaining concerns/suggestions:

1. The authors' additional explanation for use of DSS in Fig S1 was very helpful. Based on a literature search, all reports using Blastocystis infection in combination with DSS have been reported by the authors. It would be helpful to include a sentence or two initially describing the model leading up to Figure 1.

2. On page 5, it remains premature to conclude that "Blastocystis ST7 affects the host adaptive immune system, by skewing the CD4+ compartment towards enhanced recognition against gut-derived antigens that leads to over-responsiveness to self-gut-flora" after Figure 1 in which only CD4 T cell activation and polarization have been assessed. This is not to say that the statement is not a valid conclusion based on the total body of work; however, the data presented up to this point in the manuscript do not support this conclusion.

3. On page 8, "The presence of TGFβ rescued the induction of CD103 expression on the iTregs in a dose-dependent manner" refers to Fig 2H, not 2G.

4. While that the authors clearly demonstrate that I3AA alters TCR activation threshold and T cell polarization, the term "misinterpretation" still reads as overly subjective. To clarify, this is a comment purely based on word choice and not the data; a more precise statement based on the observed changes to the cell state would be preferred.

Referee #2:

Authors with their revisions have replied the majority of my concerns, putting much effort in adding additional data an dimproving the paper.

Here are our responses to the Editorial points and to the reviewers:

When you re-submit will you also please take care of the following points:

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Done

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Done in the MS.

- Funding info needs to be part of Ack. Section. Done
NUS Med Postdoctoral Fellowship & China Scholarship Council (CSC) are missing from the online submission system.
Done

- We need 5 keywords.

Microbiome, Tryptophan metabolites, colitis, aryl hydrocarbon receptor, regulatory T cells

- "Materials and Correspondence" - needs to be renamed to "Data Availability". This section is the place to enter accession numbers etc. If no data is generated that needs to be deposited in a database then state: This study includes no data deposited in external repositories.
Done

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Done

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- Author Checklist: please add corr. au. name, ms ID#, etc to the header PBP.
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- The appendix needs a ToC and page numbers; the nomenclature and callouts in the MS need to be Appendix Figure S1, etc.
Done
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Done
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- Please order the MS sections in the following order: abstract, introduction, results, discussion, materials & methods, data availability section, acknowledgments, disclosure statement and competing interests, references, main figure legends, tables.
corrected

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We appreciate this comment. We added relevant text before Fig 1.

2. On page 5, it remains premature to conclude that "Blastocystis ST7 affects the host adaptive immune system, by skewing the CD4+ compartment towards enhanced recognition against gut-derived antigens that leads to over-responsiveness to self-gut-flora" after Figure 1 in which only CD4 T cell activation and polarization have been assessed. This is not to say that the statement is not a valid conclusion based on the total body of work; however, the data presented up to this point in the manuscript do not support this conclusion.
We have altered the statement to be less definitive at this point:
"Altogether, these data suggest that *Blastocystis* ST7 can lead to stronger $CD4^+$ T cell recognition of gut-derived antigens"

3. On page 8, "The presence of TGFβ rescued the induction of CD103 expression on the iTregs in a dose-dependent manner" refers to Fig 2H, not 2G.
Thank you for this comment. Corrected

4. While that the authors clearly demonstrate that I3AA alters TCR activation threshold and T cell polarization, the term "misinterpretation" still reads as overly subjective. To clarify, this is a comment purely based on word choice and not the data; a more precise statement based on the observed changes to the cell state would be preferred.

We agree that the word is imprecise. We have altered the previous sentence to read:

"We found that I3AA exerts a potent effect on the $CD4^+$ T cell compartment, skewing its reactivity towards self-flora and contributing to a pro-inflammatory response in the gut tissue. I3AA-directed remodeling of $CD4^+$ Th subsets caused a stronger than normal TCR signaling response to recognition of peptide-MHC (pMHC) antigen".

Referee #2:

Autho rs with their revisions have replied the majority of my concerns, putting much effort in adding additional data and improving the paper.

2nd Revision - Editorial Decision **6th Sep 2023**

Dear Nick,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a look at it and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
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The data shown in figures should satisfy the following conditions:
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- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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

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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

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Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

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Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and Methods, Figures

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If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Yes	Materials and Methods
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Materials and Methods, Figures

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

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