EMBO reports

Manuscript EMBOR-2011-35482

Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma

Shannon L. Russell, Matthew J. Gold, Martin Hartmann, Benjamin P. Willing, Lisa Thorson, Marta Wlodarska, Navkiran Gill, Marie-Renee Blanchet, William W. Mohn, Kelly M. McNagny and B. Brett Finlay

Corresponding author: B. Brett Finlay, University of British Columbia

Review timeline:

Submission date:	13 October 2011
Editorial Decision:	11 November 2011
Revision received:	26 January 2012
Editorial Decision:	10 February 2012
Revision received:	19 February 2012
Accepted:	21 February 2012

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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11 November 2011

Thank you for your patience while your study has been under peer-review at EMBO reports. We have now received reports from the three referees that were asked to assess it, which you will find at the end of this email. As you will see, all the referees find the topic of interest and are supportive of publication of the work here. However, there are a few issues that need to be addressed in a revision before we can consider your study for publication in EMBO reports.

After reading the reports, I think that including a Vancomycin only control group in figure 1, analyzing Treg numbers in the lungs (I wouldn't remove the data in the colon, just add the lung data to figure 3) and characterizing the microbiota of mice treated with antibiotics only during adult life, would be important for publication in EMBO reports. In addition, please add a more thorough discussion and contextualization of the current state of the field and possible mechanisms, as referee 1 suggests. I also feel it would be important to specifically discuss in the Results section that antibiotics were given to the breeding pairs and after birth, that is, the mice were also exposed in utero.

Undoubtedly, our short length clearly hindered the discussion of your results in the context of previous literature. As you have only 3 figures and a small table, I think we can increase our length

limit to 35,000 characters. In this regard, we are currently changing our reference format to one that will save a considerable amount of space, so that more text can be accommodated. Please format the references as indicated at the end of this email, which will also allow a more thorough discussion.

If the referee concerns can be adequately addressed, we will be happy to accept your manuscript for publication. Please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I am happy to be the bearer of positive news! I look forward to seeing a revised manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

This is a very interesting study and builds upon the research published by a number of investigators that changes in the gut microbiota can modulate murine allergic responses in the lungs. The data is technically sound, but is missing one control group (described below). The major concern for this manuscript is the quality of the academic discussion in the introduction and discussion. This is not the first study of its kind in the field and little information is provided to the reader on potential mechanismS.

MAJOR CONCERNS (A-G)
A. The authors omit almost all of these other studies in their introduction and discussion, including the original studies by Noverr et al. demonstrating that antibiotics can promote the development of lung allergic responses and the studies by Hunt, Forsythe and Arnold that introduction of oral bacteria can ameliorate OVA-allergic responses in the lungs. These citations need to be added and discussed in both the introduction and discussion to put this current study in context.

1. Noverr, M. C., N. R. Falkowski, R. A. McDonald, A. N. McKenzie, and G. B. Huffnagle. 2005. Development of allergic airway disease in mice following antibiotic therapy and fungal microbiota increase: role of host genetics, antigen, and interleukin-13. Infect Immun 73:30-38.
2. Noverr, M. C., R. M. Noggle, G. B. Toews, and G. B. Huffnagle. 2004. Role of antibiotics and fungal microbiota in driving pulmonary allergic responses. Infect Immun 72:4996-5003.
3. Hunt, J. R., R. Martinelli, V. C. Adams, G. A. Rook, and L. R. Brunet. 2005. Intragastric administration of Mycobacterium vaccae inhibits severe pulmonary allergic inflammation in a mouse model. Clin Exp Allergy 4. Forsythe, P., M. D. Inman, and J. Bienenstock. 2007. Oral treatment with live Lactobacillus reuteri inhibits the allergic airway response in mice. Am J Respir Crit Care Med 175:561-569.
5. Arnold, I. C., N. Dehzad, S. Reuter, H. Martin, B. Becher, C. Taube, and A. Muller. 2011. Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells. J Clin Invest 121:3088-3093.
6. Karimi et al. (already cited in the manuscript)

Other points to address:
B. Figure 1: Vancomycin only is needed to demonstrate that the increase in allergic parameters are not due to the antibiotic alone (without allergen challenge)

C. The Treg changes in the colon (Figure 3) are not relevant to the other data in this manuscript and

this figure should be dropped. There is no data linking Treg numbers in the colon to the increase in lung inflammation. Inclusion of this data is an over-interpretation of their data.

D. Do the authors have any information about Treg numbers in the lungs?

E. Mice do not develop asthma. Asthma is a human disease. The text should be modified to refer to this as allergic airway disease or experimental murine allergic asthma.
***Clin Exp Allergy. 2008 May;38(5):829-38. Ovalbumin-sensitized mice are good models for airway hyperresponsiveness but not acute physiological responses to allergen inhalation. Zosky GR, Larcombe AN, White OJ, Burchell JT, Janosi TZ, Hantos Z, Holt PG, Sly PD, Turner DJ.

F. The pyrosequencing data presented is only analyzed down to the family level, not the genus level; thus, references to lactobacilli and other genera (from the pyrosequencing data) in the discussion should be changed to use family-level designations.

G. The discussion is very light on substance. More context and integration is needed. More discussion is needed about what has already been published that could link vancomycin-induced microbiota changes to immunologic changes in the lungs. Between Huffnagle, Forsythe, Lambrecht, Holt, Rook and Artis, there is quite a bit written about mechanisms of allergic response development in the lungs and how other changes at other sites in the body may augment or protect against allergy.

Referee #2:

The gut microbiota composition has been considered to be readily changeable by antibiotics ingestion, and the change could influence the host immune status. However there is no report to formally address this issue. In the present study, the authors examined mice treated with vancomycin or streptomycin starting from the neonatal periods and clearly showed that vancomycin treatment induced dramatic change in the composition of the gut microbiota, accompanied by a significantly higher susceptibility to allergic airway disease. Interestingly, in vancomycin-treated mice, the intestinal microbiota lacks Clostridium and Bacteroides but has an increase in Lactobacillus and Proteus. The authors further showed that these microbiota situation resulted in a reduction of colonic Treg cells, which was consistent with the previous reports (Mazmanian, Nature, Science, PNAS and Honda, Science) and might be responsible for the high susceptibility of vancomycin-treated mice to allergic airway disease. This paper is very interesting and provides a significant amount of information. As vancomycin is one of the most frequently used antibiotics, this study has many clinical implications.
I have only two comments:
1. The authors used the term 'early life antibiotics' or 'neonatal vancomycin treatment'. However, looking at the method section, the authors kept the mice on vancomycin from birth through adult. Therefore, the term 'neonatal treatment' is misleading and gives us an impression that the authors treated mice only during neonatal period. The authors should make clear this point.
2. If possible, it would be very informative to show the microbiota composition in mice treated with vancomycin only during adult life by pyrosequencing of 16S rRNA genes, and discuss why adult vancomycin treatment did not influence the susceptibility to allergic airway disease.

Referee #3:

The manuscript by Russell et al. represents an important advance in our understanding of the pathogenesis of allergic asthma. The hypothesis is clear and the experiments performed clearly show the effects of early life antibiotics on the microbiota that affect asthma susceptibility.

Several points that should be considered to improve the paper are as follow:

Abstract/Intro/Discussion- The work from this study are most consistent with the "disappearing microbiota" hypothesis advanced by Blaser and Falkow, not the earlier "hygiene hypothesis." In fact, the data provide strong experimental support for the phenomena hypothesized (Nature Rev

Microbiol 2009).

Line 64- The early life mice also were exposed in utero. This should be pointed out in the Methods or Results section.
Line 101- Do the Permanova analyses refer to the ileal, fecal, or both combined? Unifrac analyses of the pairwise differences would provide another measure of the changes in community composition.
Line 116- The taxonomic levels in Table 1 that were identified should be clarified.
Line 199- The definition of vancomycin as narrow spectrum and streptomycin as broad spectrum are anachronistic. As the current study and others cited show, these definitions are not accurate.
Line 393- Data on antibiotic effects in the older mice also should be shown and discussed.

1st Revision - authors' response 26 January 2012

Responses to reviewers' comments:

We would like to thank the reviewers for their very positive and constructive comments. As a result, this revised manuscript is now much improved. We agree with the reviewers that more contextualization was required in the introduction and discussion sections of the report to reflect the current state of the field. We have included antibiotic only control groups where we thought they provided useful information to the reader and included Treg numbers in the lungs to complement colon data. Characterizing the microbiota of mice treated with antibiotics only during adult life was also included, and we think this data has actually added strength to the ideas originally presented. With the addition of this new data, we were able to provide more mechanistic insights that we believe make our story more compelling and more relevant to the field.

Specific Reviewer Comments

Referee #1:

"This is a very interesting study and builds upon the research published by a number of investigators that changes in the gut microbiota can modulate murine allergic responses in the lungs. The data is technically sound, but is missing one control group (described below). The major concern for this manuscript is the quality of the academic discussion in the introduction and discussion. This is not the first study of its kind in the field and little information is provided to the reader on potential mechanisms."

1. Reviewer comment: "The authors omit almost all of these other studies in their introduction and discussion, including the original studies by Noverr et al. demonstrating that antibiotics can promote the development of lung allergic responses and the studies by Hunt, Forsythe and Arnold that introduction of oral bacteria can ameliorate OVA-allergic responses in the lungs. These citations need to be added and discussed in both the introduction and discussion to put this current study in context."

   1. Noverr, M. C., N. R. Falkowski, R. A. McDonald, A. N. McKenzie, and G. B. Huffnagle. 2005. Development of allergic airway disease in mice following antibiotic therapy and fungal microbiota increase: role of host genetics, antigen, and interleukin-13. Infect Immun 73:30-38.
   2. Noverr, M. C., R. M. Noggle, G. B. Toews, and G. B. Huffnagle. 2004. Role of antibiotics and fungal microbiota in driving pulmonary allergic responses. Infect Immun 72:4996-5003.
   3. Hunt, J. R., R. Martinelli, V. C. Adams, G. A. Rook, and L. R. Brunet. 2005. Intragastric administration of Mycobacterium vaccae inhibits severe pulmonary allergic inflammation in a mouse model. Clin Exp Allergy 4. Forsythe, P., M. D. Inman, and J. Bienenstock. 2007. Oral treatment with live Lactobacillus reuteri inhibits the allergic airway response in mice. Am J Respir Crit Care Med 175:561-569.
   5. Arnold, I. C., N. Dehzad, S. Reuter, H. Martin, B. Becher, C. Taube, and A. Muller. 2011. Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells. J Clin Invest 121:3088-3093.
   6. Karimi et al. (already cited in the manuscript)

   Answer: Because our word limit was significantly increased we were able to expand the

introduction section to address the reviewer's concerns. We added a significant amount of detail to the introduction and discussion sections, specifically highlighting some of the key studies involved in shaping the current state of the field. All of the articles mentioned above have been cited either in the introduction or in the discussion of this manuscript. The suggestions made have added considerable strength to the findings in this study.

2. Reviewer comment: "Figure 1: Vancomycin only is needed to demonstrate that the increase in allergic parameters are not due to the antibiotic alone (without allergen challenge)"

   Answer: This was a control that we included in our original experiments, yet decided not to initially include in the manuscript. Total cell counts from bronchoalveolar lavage (BAL) of PBS (vehicle) + Vancomycin and PBS + Streptomycin treated animals have been included in Figure 1A. Because we could not detect any differences between these controls and the untreated control in any of the parameters tested (i.e. BAL counts, eosinophils, serum IgE, histology) the remaining data was put into a supplementary figure (Fig S2). Because eosinophil numbers and OVA-specific IgE levels were below detection for all three controls, these graphs were not included, and only pathology scores and the corresponding representative histological sections were included in Fig S2.

3. Reviewer comment: "The Treg changes in the colon (Figure 3) are not relevant to the other data in this manuscript and this figure should be dropped. There is no data linking Treg numbers in the colon to the increase in lung inflammation. Inclusion of this data is an over-interpretation of their data."

   Answer: There have been a number of recent reports that show how changes in specific members of the microbiota result in a reduction of colonic Treg cells (Mazmanian, Nature, Science, PNAS and Honda, Science). Here we want to show that our findings are consistent with these previous reports, and add that these observations are associated with enhanced allergic airways disease. We also thought that comparing Treg numbers in streptomycin- and vancomycin-treated animals would provide some new insights to what has already been published. We would like to suggest that our findings support evidence for a gut-lung axis, a model proposed by several experts in the field (there are several review articles on the subject, including one referenced in our manuscript by Forsythe, 2011 CHEST). This gut-lung axis may help us explain why Treg numbers in the gut or spleen (as a result of probiotic administration, for example) may influence the outcome of an inflammatory reaction occurring in the lung. We have acknowledged the reviewer's concern by expanding on our explanation as to why the colonic Treg data may be relevant to the study, and have performed several additional animal experiments to obtain Treg numbers in the lung which have been added to this figure (Fig 3B). This addition allows us to more thoroughly discuss the significance of our findings.

4. Reviewer comment: "Do the authors have any information about Treg numbers in the lungs?"

   Answer: Yes, this data has been added to Figure 3 (see comment #3 above), and subsequently discussed in the body of the manuscript. A more thorough discussion has been included to describe possible mechanisms that may be influencing the results presented in this study.

5. Reviewer comment: "Mice do not develop asthma. Asthma is a human disease. The text should be modified to refer to this as allergic airway disease or experimental murine allergic asthma."

   ***Clin Exp Allergy. 2008 May;38(5):829-38. Ovalbumin-sensitized mice are good models for airway hyperresponsiveness but not acute physiological responses to allergen inhalation. Zosky GR, Larcombe AN, White OJ, Burchell JT, Janosi TZ, Hantos Z, Holt PG, Sly PD, Turner DJ.

   Answer: We agree with the reviewer. All references in the text referring to the murine model of allergic asthma used in this study have been changed to suitably reflect this important point.

6. Reviewer comment: "The pyrosequencing data presented is only analyzed down to the family level, not the genus level; thus, references to lactobacilli and other genera (from the

pyrosequencing data) in the discussion should be changed to use family-level designations."

Answer: The pyrosequencing data was analyzed down to the genus-level, however for simplicity with respect to Fig. 2C, the data was grouped according to family. We realize that this was not described effectively in the text, so it has been changed to the following so that it can be more easily interpreted:
Page 7, Lines 137-142
"The 16S rRNA sequences were taxonomically identified according to the Greengenes database [21] to the genus-level using a naïve Bayesian classifier. The 16S rRNA genes and their frequencies classified at the family-level (for simplicity) in feces from control, streptomycin- or vancomycin-treated animals further emphasizes how significantly the bacterial community in the gut changed after antibiotic treatment, particularly in the animals that received vancomycin (Fig 2C)."

7. Reviewer comment: "The discussion is very light on substance. More context and integration is needed. More discussion is needed about what has already been published that could link vancomycin-induced microbiota changes to immunologic changes in the lungs. Between Huffnagle, Forsythe, Lambrecht, Holt, Rook and Artis, there is quite a bit written about mechanisms of allergic response development in the lungs and how other changes at other sites in the body may augment or protect against allergy."

   Answer: We completely agree with the reviewer. Because we were granted more space, we were able to significantly expand the discussion (*pages 9-12*). We included a lot more background, describing what is known about microbial influences on the immune system, both inside and outside the intestinal compartment. We also included relevant literature linking intestinal bacteria to changes in allergy, and in models of asthma. We used these studies to draw more in-depth conclusions about our data, and used it to provide more detailed mechanistic insights.

Referee #2:

"The gut microbiota composition has been considered to be readily changeable by antibiotics ingestion, and the change could influence the host immune status. However there is no report to formally address this issue. In the present study, the authors examined mice treated with vancomycin or streptomycin starting from the neonatal periods and clearly showed that vancomycin treatment induced dramatic change in the composition of the gut microbiota, accompanied by a significantly higher susceptibility to allergic airway disease. Interestingly, in vancomycin-treated mice, the intestinal microbiota lacks Clostridium and Bacteroides but has an increase in Lactobacillus and Proteus. The authors further showed that this change in microbiota resulted in a reduction of colonic Treg cells, which was consistent with the previous reports (Mazmanian, Nature, Science, PNAS and Honda, Science) and might be responsible for the high susceptibility of vancomycin-treated mice to allergic airway disease. This paper is very interesting and provides a significant amount of information. As vancomycin is one of the most frequently used antibiotics, this study has many clinical implications."

8. Reviewer comment: "The authors used the term 'early life antibiotics' or 'neonatal vancomycin treatment'. However, looking at the method section, the authors kept the mice on vancomycin from birth through adult. Therefore, the term 'neonatal treatment' is misleading and gives us an impression that the authors treated mice only during neonatal period. The authors should make clear this point."

   Answer: We agree with the reviewer. We decided to keep our terminology (i.e. 'neonatal') the same because we thought it was the most easily interpreted description we could think of. However to clarify this important experimental detail, we included additional descriptions of the antibiotic administration protocol in the results section,
   Page 7, lines 87-89:

   "To determine whether antibiotic perturbations of intestinal microbiota affect asthma, experimental allergic asthma was induced in mice exposed to vancomycin or streptomycin as neonates (including exposure *in utero*) or only as adults."

And in the methods, *page 13, lines 284-287*:
"C57BL/6J breeding pairs were given vancomycin or streptomycin (Sigma-Aldrich, St. Louis, MI) at 200 mg/liter in drinking water. Pups born from respective breeding pairs were reared on antibiotic-treated water with their littermates for the duration of the experiment. Hence, our term "neonatal exposure" refers to antibiotic exposure both *in utero* and after birth. Alternatively, 7-week-old C57BL/6J female mice were given vancomycin or streptomycin (Sigma) at 200 mg/liter in drinking water two days prior to sensitization with OVA, hence "adult exposure".

We hope this describes the antibiotic administration regime more clearly.

9. Reviewer comment: "If possible, it would be very informative to show the microbiota composition in mice treated with vancomycin only during adult life by pyrosequencing of 16S rRNA genes, and discuss why adult vancomycin treatment did not influence the susceptibility to allergic airway disease."

   Answer: As suggested by the reviewers, we added this important set of controls to our existing pyrosequencing dataset. We had to sequence an entirely new set of data, which included generating 31, 327 new pyrotags, and then redo all of the statistics presented in the previous manuscript with the new data included. The adult data has been added to Figure 2C for comparison with the neonatal antibiotic data. Also, we have included a PCO plot in Figure S3 that compares these two datasets using relevant statistics. We were able to draw more conclusions between the neonatal vancomycin disease phenotype and the adult vancomycin disease phenotype with these sequencing results. We think that by adding this data to our study, we have actually made a more compelling argument for our conclusions and the related mechanistic insights.

Referee #3:

"The manuscript by Russell et al. represents an important advance in our understanding of the pathogenesis of allergic asthma. The hypothesis is clear and the experiments performed clearly show the effects of early life antibiotics on the microbiota that affect asthma susceptibility."

10. Reviewer comment: "Abstract/Intro/Discussion- The work from this study are most consistent with the "disappearing microbiota" hypothesis advanced by Blaser and Falkow, not the earlier "hygiene hypothesis." In fact, the data provide strong experimental support for the phenomena hypothesized (Nature Rev Microbiol 2009)."

    Answer: We absolutely agree with the reviewer. To reflect the significance of this review written by Blaser and Falkow to our present study, we have included several references to their "disappearing microbiota" hypothesis in our manuscript. A significantly more detailed introduction was written to provide more support for our findings. We have referenced the review on several occasions in the text, including *page 2, line 40-47*: "Blaser and Falkow have recently revisited the hygiene hypothesis, suggesting that it may not be a decline in childhood infections that is important, rather, it is how modern societal practices are causing the disappearance of ancestral species of our indigenous microbiota who may confer benefits beyond our current understanding [3]. The loss of these ancestral species could be causing a rapid reorganization of the microbial hierarchy in our guts faster than our immune systems can adapt, promoting the emergence of dysregulated immunological disorders like allergic asthma. "

11. Reviewer comment: "Line 64- The early life mice also were exposed in utero. This should be pointed out in the Methods or Results section."

    Answer: Refer to comment #8 above.

12. Reviewer comment: "Line 101- Do the Permanova analyses refer to the ileal, fecal, or both combined?

Answer: The PERMANOVA analysis was run on fecal and ileal data combined, to reflect the data presented in Figure 2B. This has been clarified in the methods section, so that it is more easily understood. *Pages 15-16, lines 340-344:* "Global community structure comparisons from fecal and ileal samples were made using PCO [43], PERMANOVA [44] and Simpson's diversity index [45] implemented in PRIMER6+[46]. Taxa-treatment association analysis [47] in conjunction with additional statistical analyses (see suppl methods) were performed to determine antibiotic-related indicator species (fecal samples only)."

As an aside, the indicator analysis was run on fecal samples, *and* fecal + ileal samples, however no differences in the bacterial indicators were detected, so we only put in the statistics from the fecal-only data in Table 1.

13. Reviewer comment: "Unifrac analyses of the pairwise differences would provide another measure of the changes in community composition."

    Unifrac measures are statistical tests based on multiple sequence alignments (MSA's). Because of our space limitations, we were limited in the number of statistical tests we could describe in the text. If space permitted, we could have included these types of tests. We chose a variety of MSA-independent approaches in our study (such as pairwise comparisions using PERMANOVA), and decided that these tests sufficiently answered the questions we wanted to ask.

14. Reviewer comment: "Line 116- The taxonomic levels in Table 1 that were identified should be clarified."

    Answer: We agree with the reviewer. To clarify the taxonomic levels we describe in Table 1, we added a column to the table called "Rank", which describes at what taxonomic level the corresponding OTU could be confidently classified (e.g. f, family; o, order, etc.).

15. Reviewer comment: "Line 199- The definition of vancomycin as narrow spectrum and streptomycin as broad spectrum are anachronistic. As the current study and others cited show, these definitions are not accurate."

    Answer: We changed the quoted text to reflect the reviewer's wishes. Rather than describing the antibiotics as "narrow" and "broad" spectrum, we have described them as such on *page 13, line 289*:
    "Vancomycin, an antibiotic that directly targets Gram-positive bacteria, and streptomycin, an antibiotic that directly targets both Gram-positive and Gram-negative bacteria, were chosen…"

16. Reviewer comment: "Line 393- Data on antibiotic effects in the older mice also should be shown and discussed."

    Answer: We agree with the reviewer. The other reviewers have also suggested this. Refer to comment #9.

2nd Editorial Decision 10 February 2012

Thank you for your patience while we have peer-reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports, although referee 1 requests a couple of minor modifications, which can be easily addressed. I wouldn't change the mention to asthma from title and abstract, but would qualify this to reflect the reality in mouse in the manuscript text.

Regarding your question about short pyrosequencing reads, we do request that they be deposited in a public database. I have discussed this issue with my colleagues of Molecular Systems Biology, who also encounter this issue repeatedly after the closure of the SRA, and they have pointed me to the

following editorial

http://genomebiology.com/2011/12/3/402

This lists places were you can deposit your data, such as the EBI. Our policy is that you should make your reads available if at all possible, so please submit them to one of these databases and provide the accession code with your final version.

Thank you for your contribution to EMBO reports. I'm happy to be the bearer of good news!

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

There are two important concerns about precision from the previous review that the authors have said they answered, but they have not.

5. Reviewer comment: ""Mice do not develop asthma. Asthma is a human disease. The text should be modified to refer to this as allergic airway disease or experimental murine allergic asthma.""
***Clin Exp Allergy. 2008 May;38(5):829-38. Ovalbumin-sensitized mice are good models for airway hyperresponsiveness but not acute physiological responses to allergen inhalation. Zosky GR, Larcombe AN, White OJ, Burchell JT, Janosi TZ, Hantos Z, Holt PG, Sly PD, Turner DJ.
Answer: We agree with the reviewer. All references in the text referring to the murine model of allergic asthma used in this study have been changed to suitably reflect this important point.
-----> NONE OF REFERENCES ABOUT MOUSE ASTHMA HAVE BEEN REMOVED, INCLUDING IN THE TITLE. THIS IS NOT ACCURATE AND NEEDS TO BE CHANGED.

4. Reviewer comment: ""Do the authors have any information about Treg numbers in the lungs?""
Answer: Yes, this data has been added to Figure 3 (see comment #3 above), and subsequently discussed in the body of the manuscript. A more thorough discussion has been included to describe possible mechanisms that may be influencing the results presented in this study.
-----> THE DATA HAS BEEN ADDED TO FIGURE 3 (3B), HOWEVER, THERE IS NO TEXT IN THE RESULTS DESCRIBING THAT THIS ANALYSIS HAS BEEN DONE OR WHAT THE RESULTS WERE. IT NEEDS TO BE POINTED OUT IN THE RESULTS THAT THE CD4+CD25+FOXP3+ DID NOT CHANGE IN THE LUNG CELL POPULATIONS THEY ANALYZED.

Finally, there is another point of precision regarding the immunology:
-----> the authors need to correct the terminology: CD4+CD25+ FoxP3+ T cells are just that - this is a phenotype analysis. Treg are defined by function, not surface phenotype. Yes, most Tregs are CD4+CD25+FoxP3+ T cells; however, not all CD4+CD25+FoxP3+ T cells are Tregs. Immunology journals are very particular about this designation. Flow cytometry does not identify Tregs. This doesn't change the results of the study or its implication. The text should be corrected to read something like: "there was a decrease in the number of CD4+CD25+FoxP3+ T cells, which is a cell-surface phenotype expressed by many Treg cells"

Referee #2:

The revised manuscript contains a set of extremely well controlled, important and interesting findings. In my view, the findings are sufficiently novel and of broad interest to be published in EMBO reports.

Referee #3:

Authors have well-responded to my comments and those of the other reviewers. I recommend acceptance of this important report.

2nd Revision - authors' response 19 February 2012

We would like to sincerely thank you and the reviewers for all of the valuable insights and helpful comments provided to us in the reviews and comments.

Based on to your recommendations, we have made a few changes to the revised manuscript in hopes that we have now adequately addressed all of the reviewers' comments. Specifically, we have better highlighted the experimental nature of the murine asthma model wherever it was mentioned in the text, as well as touched on the phenotypic nature of the tissue-specific Treg experiments. For your convenience, we have attached a version of the manuscript with tracked changes, so that it is clear to you where we have made these changes. We have also deposited our pyrosequencing data in the Sequence Read Archive (SRA) on the NCBI website, a public database suitable for the deposition of short sequence reads. The accession number for this deposition has been referenced in the results section of the manuscript. We hope that you will now find the manuscript acceptable for publication in EMBO reports.

In addition, we have prepared a cover suggestion that we think highlights the novelty of the work and its contribution to the exciting field of microbiome research. One of our graphic artists is currently putting the finishing touches on the artwork, so we will send it to you within the week for consideration in the next available issue.

We would like to thank you again for considering our work suitable for publication in EMBO Reports. We look forward to hearing from you soon.

3rd Editorial Decision 21 February 2012

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

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

Yours sincerely,

Editor
EMBO Reports

