Commensal microbiota influence systemic autoimmune responses

Jens Van Praet, Erin Donovan, Inge Vanassche, Michael Drennan, Fien Windels, Amélie Dendooven, Liesbeth Allais, Claude Cuvelier, Fons van de Loo, Paula Norris, Andrey Kruglov, Sergei Nedospasov, Sylvie Rabot, Raul Tito, Jeroen Raes, Valerie Gaboriau-Routhiau, Nadine Cerf-Bensussan, Tom Van de Wiele, Gerard Eberl, Carl Ware and Dirk Elewaut

Corresponding author: Dirk Elewaut, University Hospital Ghent

Review timeline:
- Submission date: 24 February 2014
- Editorial Decision: 19 March 2014
- resubmission: 03 September 2014
- Editorial Decision: 02 October 2014
- Revision received: 12 November 2014
- Accepted: 01 December 2014

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

1st Editorial Decision 19 March 2014

Thank you for submitting your manuscript to The EMBO journal. Your manuscript has now been seen by the three referees and I am afraid that their recommendations are not very positive at this stage.

The referees clearly find the analysis interesting. However they also raise similar issues with the paper and find that the link between commensal microbiota and anti-nuclear antibody responses is not sufficiently substantiated by the data presented. Given the raised concerns and as it is not trivial to address them, I unfortunately see no other option but to reject the present submission.

I am very sorry that I can’t be more positive at this stage.

REFEREE REPORTS

Referee #1:

Van Praet et al report that the systemic immune responses specifically anti-nuclear antibody (ANA) responses are influenced by commensal microbiota. They showed that about ~25% of the lymphotoxin (LT) receptor β deficient mice spontaneously develop ANAs. Similarly blocking LT-signaling at late stages of development also resulted in spontaneous ANA production. Thymic
transplantation experiments showed that spontaneous ANA production doesn't depend on T cells. The antibiotic treatment of Ltbr-/- mice has slightly reduced prevalence of ANAs. ANA positive Ltbr-/- mice showed expanded segmented filamentous bacteria (SFB) levels in their intestine. The experiments presented in the manuscript suggest that ANAs are produced in a subset of Ltbr-/- mice. However, the main conclusion of the paper, whether commensal microbiota is involved in this process is not unequivocally shown.

Major concerns

1. There is no data that directly support the involvement of commensal microbiota is shown. Figure 3a shows that GF mice treated LTβR-Fc also still developed ANA (23%) similar to all other genetic mutants which are only slight lower than wild type mice treated with LTβR-Fc. This argues against their hypothesis about the role of commensal microbiota in the generation of ANA. Could this be a stochastic event given the fact that only 1/4 th of Ltbr-/- show ANAs? Are there any differences in the single ANA or multiple ANA antibodies between GF and wild type mice?

2. It is known that the microbial alterations happen in mutant mice that show defects in the lymphoid architecture development. This is likely due to impaired anti-microbial immune responses. The reduction in the IgA levels in ANA+ mice in fact shows that the microbiota is not contained by the immune system leading to altered microbiota composition. It is worth to note that IgA deficient mice show expanded SFB load in the intestine (Suzuki et al 2004 PNAS). This could perhaps lead to systemic invasion of microbiota or microbial products that stimulate autoimmune B cells. The experiments showing the altered microbiota (e.g. SFB) doesn't provide evidence for their involvement in the ANA production. Could authors directly show if the altered microbiota is involved in inducing ANA by transplanting Ltbr-/- or SFB and wt mice microbiota to GF mice and measure ANA production after LTβR-Fc injection?

3. The authors claim that systemic autoimmune responses are affected but surprisingly no antibody mediated pathologies are shown. The possibility that the polyreactive intestinal B cell derived antibodies are present in the serum of Ltbr-/- mice due to impaired architecture rather than active regulation of autoantibody responses should also considered.

Minor concerns

1. The ANA titers or the values and details about how many mice showed which ANA antibodies should be shown
2. The histopathology of LTβR-Fc treated mice which shows the loss of PP, CP and ILF should be shown as a figure
3. Figures and tables could be separated throughout the manuscript

Referee #2:

The manuscript by Elewaut and colleagues examines Ltbr ko mice that they found have antinuclear antibodies. A small percentage of mice have this phenotype (20-25%) They show this effect can be mimicked by antisera to Ltbr-FC and that the effect is does not require any defects produced by the thymus. They then show that the phenotype is influenced by the microbiota based on germ free and antibiotic studies. They then correlate reduced serum IgA levels with ANA levels in humans and mice. The finding of ANA in this model is potentially interesting. There are many issues that should be addressed before publication.

Major issues

1. Since the microbiota is eventually evoked as a mechanism, cage and maternal effects must be addressed. The numbers are small for most experiments, as cage effects could be in play here. This issue must be addressed.

2. It is not clear if littermate controls were used in these experiments and if so what are the effects on the phenotype. Again, this must be addressed experimentally as the microbiota appears to play a role in the phenotype. Littermate effects must be tested in these models.
3. The numbers of mice is quite small and negative findings could simply be due to type 1 error (ie B-Ltb/- mice).

4. What are the titers of the anti-nuclear antibodies? The bands in Fig C are not that robust in most cases. It is not clear how robust the titers are in this experiments and comparing experiment to experiment. Does the Plus/Minus system lose important statistical detail?

5. The argument explaining the chimera data is not clear given that the Rorgt cells are key in Fig 1E.

6. The effects of broad spectrum antibiotics are modest. No analysis (other than enlarged cecums) is offered to show the effects of the antibiotics on the microbiota and that the effects were consistent from experiment to experiment and cage to cage. This issue must be addressed experimentally.

7. The germ free LTbr-RC treated has the same percentage of ANA positive mice as in Fig. 1. This would suggest there is no effect of the microbiota. Why do the authors interpret this finding as that there is an effect of the microbiota. The interesting group may be the conventionalized mice Why does this group have higher numbers of ANA positive mice.

8. The microbiota analysis is weak. Sequencing by 16S would be more complete and informative than DGGE.

9. The IgA effects in Fig 4 are not well connected to the manuscript. It does not add much.

Referee #3:
The manuscript by Van Praet et al., describes an association of commensal microflora with systemic autoimmune responses. They observed development of antinuclear antibodies in Ltbr deficient mice which lack secondary lymphoid organs. They report non-participation of central tolerance but involvement of commensal microflora in these mice. Finally they also draw parallel between mouse and human conditions with respect to IgA levels. The study is interesting but the strength of results reported are little less convincing. They tried different elegant in vivo approaches which helped understand the issue only partially.

Major points:
1. Time of measurement of immune responses need to be more homogenized if possible in between experiments.

2. Fig 1e and f indicates development of ANA does not depend on T or B cells but on both hematopoietic and non hematopoietic compartments. Is it possible to look into or comment on innate immune cells (possibly RORyt+?). Since there is a connection with commensal microbiota later this might be an important direction to investigate.

3. For assessing central tolerance, the authors might look into absolute cell nos. of T and B cells and also APCs.

4. Fig 3 shows moderate (around two times) of increased frequency of ANA development. However the pathological reflections in Ltbr/- mice are not obvious (Figs S3 and S4). This non association of pathology along with mild variation (in absence of statistical quantification) contributed by microbiota decreases the strength of the presented case. Would colonizing germ free mice with SFB and treating with LTbR-Fc result in more discerning results?

Minor points
The authors might want to discuss cellular parameters resulting in this breach of tolerance.
Referee #1:

Major concerns 1. There is no data that directly support the involvement of commensal microbiota is shown. Figure 3a shows that GF mice treated LTbR-Fc also still developed ANA (23%) similar to all other genetic mutants which are only slight lower than wild type mice treated with LTbR -Fc. This argues against their hypothesis about the role of commensal microbiota in the generation of ANA. Could this be a stochastic event given the fact that only 1/4 th of LTbR -/- show ANAs? Are there any differences in the single ANA or multiple ANA antibodies between GF and wild type mice?

Answer: Several lines of evidence support the involvement of commensal microbiota in the observed phenotype, including new data as requested by the referees. First, we show that antibiotic treated LTbR -- mice have about 50% reduced frequency of ANA compared to wild-type mice (Figure 3a upper panel). Secondly, we corroborated these results in independent experiments by treating both germ-free and wild-type mice with LTbR -Fc. (Figure 3a lower panel). The data from these independent experiments clearly show that commensal microbiota are critical for the observed phenotype. Additionally, new experiments were conducted in germfree mice that were monoclonized with SFB versus a non-adherent non-pathogenic E. Coli strain in the presence of LTbR-Fc. They showed that SFB unlike E. Coli has the ability to potently induce ANA in LTbR-Fc treated mice. Altogether the data strongly support the role of microbiota and particularly the role of SFB.

While in theory we agree with the referee that the generation of ANA in LT deficient mice could be a stochastic event similar to what has been described in other mice models for generalized autoimmunity such as MRL/lpr mice, the analysis of microbiota and link to ANA induction strongly argue against this. Mice with multiple ANA reactivities have a profoundly distinct intestinal microbiome, enriched with SFB, compared to ANA negative mice. These observations together with functional data including the monocolonization experiments argue against a stochastic event. Regarding the difference multiple ANA between GF en wild -type mice, we found that only 1 out of 13 germfree mice (7,6%) developed multiple ANA versus 3 out of 15 controls (20%). Overall our data are consistent with a critical role of commensal microbiota in ANA induction.

2. It is known that the microbial alterations happen in mutant mice that show defects in the lymphoid architecture development. This is likely due to impaired anti-microbial immune responses. The reduction in the IgA levels in ANA+ mice in fact shows that the microbiota is not contained by the immune system leading to altered microbiota composition. It is worth to note that IgA deficient mice show expanded SFB load in the intestine (Suzuki et al 2004 PNAS). This could perhaps lead to systemic invasion of microbiota or microbial products that stimulate autoimmune B cells. The experiments showing the altered microbiota (e.g. SFB) doesn't provide evidence for their involvement in the ANA production. Could authors directly show if the altered microbiota is involved in inducing ANA by transplanting LTbR +/- or SFB and wt mice microbiota to GF mice and measure ANA production after LTbR -Fc injection?

Answer: We thank the referee for raising this interesting point. To address this issue, we performed additional experiments wherein mono-colonized mice were treated with LTbR-Fc. ANA formation in mice colonized with SFB was compared with a non-adherent non-pathogenic E. coli. We treated mice during pregnancy and their pups in the neonatal period to prevent formation of PP and ILF. We found that the percentage of mice in the group with at least one ANA was higher in the SFB group versus the E. coli group (Fig 3d). These data were added to the manuscript.

3. The authors claim that systemic autoimmune responses are affected but surprisingly no antibody mediated pathologies are shown. The possibility that the polyreactive intestinal B cell derived antibodies are present in the serum of LTbR +/- mice due to impaired architecture rather than active regulation of autoantibody responses should also considered.
Answer: This is indeed an aspect that was not discussed in the paper. We agree this is important point and therefore adapted the text.

Addition: It was reported that intestinal IgA and IgG production plasma cells, while mostly antigen specific, include a relatively high frequency of cells secreting autoreactive and polyspecific antibodies (Benckert et al, 2011; Scheid et al., 2011). This is in contrast with the bone marrow IgG plasma cells where autoreactivity is relatively rare. Because we were unable to find immune-mediated pathology despite the presence of multiple ANA reactivity, this could point to production of polyreactive intestinal B cells due to impaired architecture rather than active regulation of autoantibody responses. In such a scenario, SFB could alter the threshold for induction of ANA given its marked ability to generate potent Th17 responses.

Minor concerns

1. The ANA titers or the values and details about how many mice showed which ANA antibodies should be shown

Answer: Regarding the LTbR -/- mice, these data were included in figure 1b: this figure shows the percentage of 6 months-old mice that develops a specific ANA. However, we adapted the figure so the individual titers are visible. We determined the titers semi-quantitatively and added this in the method section. The maximum titers of the mice for the various reactivities were: SmB (1:12800), RNP-A (1:3200), RNP-70k (1:6400), Ro52/SSA (1:12800), CenpB (1:12800), Topo-I/Scl70 (1:6400) and Jo-1 (1:12800). In LTbR-Fc treated 3-months-old wild-type mice, these titers were largely comparable: RNP-A (1:12800), RNP-70k (1:6400), RNP-C (1:12800), CenpB (1:12800) and Topo-I/Scl70 (1:6400). A notice on the comparability on the spectrum of the reactivities and titers in different presented experiments is now included in the manuscript.

2. The histopathology of LTbR -Fc treated mice which shows the loss of PP, CP and ILF should be shown as a figure

Answer: The figure is included below. As such data have been previously reported by Gérard Eberl and most recently by the team of Valerie Gaboriau-Routhiau and Nadine Cerf-Benussussan we would therefore not include this in the present manuscript.
Legend:
(A) Hematoxylin and eosin staining of intestinal rolls obtained from mice monocolonized by SFB and treated or not with LTβR-Ig to inhibit the development of PP (PP−), of PP and cryptopatch-derived ILF (PI−), or of all gut lymphoid tissue (including tertiary lymphoid tissue) (PI−Lt). Black squares indicate magnified areas of interest. Red circles indicate visible lymphoid follicles, including tertiary lymphoid structures in PI− SFB-mice.
(B) Whole small intestines from mice colonized for 20 days, treated or not before birth with LTβR-Ig. Arrows indicate PP (only visible in untreated mice).

3. Figures and tables could be separated throughout the manuscript
Answer: we feel this is an editorial issue. If the editor wants us to separate these, we will adapt the text as such.

Referee #2:

Major issues

1. Since the microbiota is eventually evoked as a mechanism, cage and maternal effects must be addressed. The numbers are small for most experiments, as cage effects could be in play here. This issue must be addressed.

Answer: This is an important remark. To address this issue, we analyzed in detail the ANA data of 369 consecutively born LTβR−/− mice in our animalarium. The overall prevalence of at least one ANA at the age of 3 months was 23% (85/369). There was some variation between litters from different breeding cages (one male breeder and two female breeders). However, looking at the ANA frequency of pups born from the same breeding cage, we observed no clear differences. We thus found a maternal effect, but no cage effect. Therefore, in further subsequent experiments we choose a minimum of 2 litters to be analyzed. In addition, we performed a number of complementary approaches to solidify the role of microbiota including experiments in germfree mice as well as the opposite approach in monocolonization experiments, which rule out these effects. This will be added to the manuscript.

Addition: Ltbr−/− mice receiving antibiotics had a reduced prevalence of ANA compared to a large series of 369 untreated consecutively born three-months-old animals (Fig. 3a, upper panel). In this series, we some variation in ANA positivity between litters, but no significant differences in prevalence between cages weaned from the same breeding cage. This points to a maternal but not a cage effect on the phenotype. Therefore, we opted to analyze offspring of at least 2 litters in further experiments.

2. It is not clear if littermate controls were used in these experiments and if so what are the effects on the phenotype. Again, this must be addressed experimentally as the microbiota appears to play a role in the phenotype. Littermate effects must be tested in these models.

Answer: We indeed tested littermate controls and apologize this was not clarified in the original manuscript. In the cell specific knock-out mice using the various cre lines we used littermate controls (cre negative) in our analysis. In contrast to the RORγt-cre+ mice, ANA were not observed in cre negative littermate controls (see figure 1e). We adapted the text.

Addition: To this end, we generated mice deficient in LTβ in T cells (T-Ltb−/−), B cells (B-Ltb−/−) or RORγt+ cells (Roryt-Ltb−) and littermate controls.

3. The numbers of mice is quite small and negative findings could simply be due to type 1 error (ie B- Ltb−/− mice).

Answer: Unfortunately, we had no additional mice from the cell specific LTb deficient mice in our colony for analysis readily available as with the exception of the LTbfl/fl RORγt-cre, the other lines has been discontinued. However, we were able to test T cell-specific and ROR-gt LTa deficient animals of 3 months of age we obtained from our collaborators Nedospasov and Krukov in Berlin. Contrary to the RORγt-cre LTa mice (2/14 mice, anti-RNPA and C, titers 1:12800), none of the T cell specific LTa ko mice (0/7) in their colony developed ANA. None of the littermate controls provided (0/15) developed ANA. The prevalence overall in this colony was somehow lower than in mice tested in other colonies (Gent and Jouy-en-Josas, Paris), which was usually at least 25%. We anticipate this reflects primarily differences in the microbial composition. As we were not able to obtain serum samples from all controls we had in our colony (particularly the MB1-cre) we would rather not present these data in the presented manuscript and provide this primarily for the referee’s attention.
4. What are the titers of the anti-nuclear antibodies? The bands in Fig C are not that robust in most cases. It is not clear how robust the titers are in this experiments and comparing experiment to experiment. Does the Plus/Minus system lose important statistical detail?

Answer: We semi-quantitatively determined the titers using a dilution of the strong anti-RNPA reference sample and added this information to the method section. The maximum titers of the mice for the various reactivities were SmB (1:12800), RNP-A (1:3200), RNP-70k (1:6400), Ro52/SSA (1:12800), CenpB (1:12800), Topo-I/ScI70 (1:6400) and Jo-1 (1:12800). In LtBr-Fc treated 3-months-old wild-type mice, these titers were largely comparable: RNP-A (1:12800), RNP-70k (1:6400), RNP-C (1:12800), CenpB (1:12800) and Topo-I/ScI70 (1:6400). We added a figure to the revised manuscript as illustration. However, as the titers do not have a Gaussian distribution, the statistical analysis will require the use of a non-parametrical test with 4 categories: negative, 1:12800, 1:6400 and 1/3200. Given the few mice with a value of 1:6400 or 1:3200, such a chi-square test will have the same outcome as the Fisher’s exact tests with 2 categories (positive and negative) performed in the paper. Moreover, in human disease and in mice models these titers are not correlated with disease activity (von Muhlen & Tan, 1995).

5. The argument explaining the chimera data is not clear given that the RORgt cells are key in Fig 1E.

Answer: For clarity, the cell-specific experiments using various cre lines were designed to delineate the cellular source of LTb. The chimera data were focused on determining impact of haematopoetic versus stromal LTB expression. We show that in addition to a role on stromal cells, there is also a role of LTBr expression on hematopoetic cells, such as dendritic cells. Previously, Summers-deLuca L et al. have reported as role of LT BR for optimal function of DC. Thus, expression of LTBr on both stromal and haematopoetic cells are therefore important.

6. The effects of broad spectrum antibiotics are modest. No analysis (other than enlarged cecums) is offered to show the effects of the antibiotics on the microbiota and that the effects were consistent from experiment to experiment and cage to cage. This issue must be addressed experimentally.

Answer: To address the effects of broad spectrum antibiotics, we performed two independent experiments wherein we treated 4 different litters. The overall frequency of ANA positivity was 13% (4/32), which is half of the frequency observed in 369 consecutively born LtBr-/- mice in our facility (23%). The frequencies of the different litters were 0/8, 0/8, 2/8 and 2/8. In the two litters that had positive mice, the frequency in the cages was 0/4, 2/4, 1/4 and 1/4. We added this information the manuscript.

Addition: Ltbr/- mice receiving antibiotics had a reduced prevalence of ANA compared to a large series of 369 untreated consecutively born three-months-old animals (Fig. 3a, upper panel).

7. The germ free LtBr-Fc treated has the same percentage of ANA positive mice as in Fig. 1. This would suggest there is no effect of the microbiota. Why do the authors interpret this finding as that there is an effect of the microbiota. The interesting group may be the conventionalized mice. Why does this group have higher numbers of ANA positive mice.

Answer: We thank the referee for raising this point. It is important to highlight that the germ free experiments were conducted in another facility (INRA, Jouy-en-Josas) where mice were conventionalized with microbiota from mice kept in their facility. Therefore, some caution needs to be taken when directly comparing the absolute values of ANA frequencies between different experiments as they were done in different institutions. Rather, the results should be compared to their respective controls. We have now indicated this in the text. In all cases, LtBr-Fc administration resulted in induction of ANA, when germfree mice received the same regimen, frequency was reduced by more than 50%. In addition, we also noticed higher frequencies in mice monoclonized with SFB versus non pathogenic E.Coli in germ-free mice treated with LtBr-Fc. Thus, clearly microbiota have a strong impact on the observed induction of ANA in the absence of LT.

8. The microbiota analysis is weak. Sequencing by 16S would be more complete and informative than DGGE.
Answer: We initially used DGGE as a screening assay to detect difference between wild-type, ANA positive LTbR−/− and ANA negative LTbR−/−. As requested by this referee, we now conducted 16S rRNA sequencing. We added these data to the revised version. The results showed that Candidatus Arthromitus abundance in luminal, mucosal and fecal samples of multiple ANA positive and ANA negative LTbR−/− mice. The data were added the manuscript.

9. The IgA effects in Fig 4 are not well connected to the manuscript. It does not add much.

Answer: We agree with the referee that the precise role of IgA is not entirely clear. It could be anticipated that reduction in the IgA levels in ANA positive mice in fact shows that the microbiota is not contained by the immune system leading to altered microbiota composition. As also highlighted by referee 1, IgA deficient mice show expanded SFB load in the intestine (Suzuki et al., 2004 PNAS). We anticipate that this could lead to direct systemic invasion of microbiota or microbial products that stimulate autoimmune B cells. However, we have no direct evidence that IgA would contain microbiota, thereby leading to bacterial translocation. Therefore as suggested by the referee, we would rather not put emphasis on these data, particularly as mice lacking all GALT and therefore IgA still developed ANA when monocolonized with SFB. This argues against a critical role for IgA. The discussion has been adapted accordingly.

Addition: ILF and PP contain the stromal micro-environment for IgA production, a critical antibody that helps maintain gut homeostasis (Eberl, 2007). Furthermore it was shown that SFB can also induce IgA responses via de novo induction of tertiary lymphoid tissue in contrast to E. Coli in mice lacking PP and cryptopatch-derived ILF (Lécuyer, 2014). Because an aberrant expansion of SFB was reported in IgA-deficient mice, it could be anticipated that IgA was protective against ANA induction. Yet ANA are present at comparable levels in mice with gut tertiary follicles which have substantial concentrations of IgA in feces as in mice lacking all gut lymphoid tissues which have hardly any IgA detectable in the feces. This argues against a critical role for IgA in LT dependent ANA induction.

Referee #3:

Major points:

1. Time of measurement of immune responses need to be more homogenized if possible in between experiments.

Answer: We performed ANA tests in all full knock-out models (LTbR−/−, LTα−/−, LTβ−/− and cell-specific LTb−/−) at the age of 3 months. For LTbR-Fc treated mice, this was also 3 months. This was stated in the text as 12 weeks. We agree that this is confusing and have therefore adapted the text.

2. Fig 1e and f indicates development of ANA does not depend on T or B cells but on both hematopoietic and non hematopoietic compartments. Is it possible to look into or comment on innate immune cells (possibly RORgt+?). Since there is a connection with commensal microbiota later this might be an important direction to investigate.

Answer: We indeed looked for the role of LT expression on innate lymphoid cells in the model. To this end, we created mice deficient in LTb on RORgt expressing cells. We found a similar frequency and distribution of ANA in the mice compared to LTbR−/− mice. These data are shown in figure 1e.

3. For assessing central tolerance, the authors might look into absolute cell nos. of T and B cells and also APCs.

Answer: The gold standard to assess a defect in central tolerance are thymic transplantation experiments. We therefore transplanted thymic grafts from LTbR−/− and wild-type animals in nude mice. After transplantation, recipient mice develop T lymphocytes because the presence of a functional thymus. We did not observe ANA in nude mice grafted with a LTbR−/− thymus. We ascertained T cell development in recipient mice and could not find any differences in the absolute numbers of T (liver and spleen) and B cells (spleen) (see below), nor in the number and the distribution of thymocytes (as shown in figure 2a and 2b). Unfortunately, we did not specifically look in these experiments in to APCs.
**Figure:** Absolute number of T cells (liver, spleen) and B cells (spleen) of recipient nude mice transplanted with thymi of wildtype or LTbR mice.

A. Spleen

B. Liver

4. Fig 3 shows moderate (around two times) of increased frequency of ANA development. However the pathological reflections in LTbR -/- mice are not obvious (Figs S3 and S4). This non association of pathology along with mild variation (in absence of statistical quantification) contributed by microbiota decreases the strength of the presented case. Would colonizing germ free mice with SFB and treating with LTbR -Fc result in more discerning results?
Answer: We thank the referee for raising these interesting points. We have extensively searched for organ damage associated with the appearance of ANA but as outlined in the paper there was no real evidence for this. There are potentially many reasons for this including background dependency to induce organ damage (eg complement activation, …). Alternatively, as suggested by referee 1, the absence of immune-mediated pathology could point to production of polyreactive intestinal B cells due to impaired architecture rather than active regulation of autoantibody responses. We have added this to the revised version.

To more directly address the role of SFB we performed monoclonization experiments with SFB as suggested by the referee. Mono-colonized mice were treated with LTR-Fc ANA formation in mice colonized with SFB was compared with *E. coli* MG1655 (Fig. 3). We treated mice during pregnancy and their pups in the neonatal period to prevent formation of PP and ILF. We found that the percentage of mice in the group with at least one ANA was markedly higher in the SFB group versus *E. coli* group. These data were added to the manuscript.

**Minor points**

**The authors might want to discuss cellular parameters resulting in this breach of tolerance.**

*Answer:* We have added a few sentences to the discussion.

*Addition:* It was reported that intestinal IgA and IgG production plasma cells, while mostly antigen specific, include a relatively high frequency of cells secreting autoreactive and polyspecific antibodies (Benckert et al, 2011; Scheid et al., 2011). This is in contrast with the bone marrow IgG plasma cells where autoreactivity is relatively rare. Because we were unable to find immune-mediated pathology despite the presence of multiple ANA reactivity, this could point to production of polyreactive intestinal B cells due to impaired architecture rather than active regulation of autoantibody responses. In such a scenario, SFB could alter the threshold for induction of ANA given its marked ability to generate potent Th17 responses.

---

2nd Editorial Decision 02 October 2014

Thank you for submitting your manuscript to the EMBO Journal. This submission is revised version of MS 88320. The manuscript has now been re-reviewed by referees #1 and 2. Referee #3 was not available to re-review the present submission.

I have now heard back from the two referees and as you can see below, both referees appreciate the introduced changes. However, referee #2 still has some concerns regarding the role of microbiota in the observed phenotype. Should you be able to address the raised issues then I would like to invite you to submit a suitably revised manuscript for our consideration.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

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

**REFEREE REPORTS**

Referee #1:

Authors have now carefully revised the manuscript with additional experiments. All my original comments were satisfied in the revision. The manuscript now stands a good chance for publication.

Referee #2:
I still have a few concerns regarding the claims made that the microbiota significantly influences this particular phenotype (ANA titers) with LTbeta inhibition.

1. There is a maternal effect that is mentioned in the rebuttal letter, but this is not delineated in any clear manner within the paper. This unexplained variability makes interpretation of the treatment with antibiotics (Fig 3A) difficult, especially since this is relatively a much smaller sample size and this could still be driven by undefined maternal effects. This point really needs to be addressed as it may be the key to linking microbes to this phenotype. Also the antibiotics used (and method) was not written anywhere that I could find.

2. The small sample size for many of the LTbeta deficient mice was not adequately addressed. This is potentially an artifact of maternal effects as noted above.

3. The fact that germ free ko mice have the same penetrance as conventionally raised mice is a significant point against a primarily microbially driven phenotype. There is some evidence that the phenotype can be modestly modulated by microbes is suggested. However, the relative evidence of the antibiotic experiment in conventionally raised mice and the conventionalization of germ free mice is less impressive than LTbeta inhibition itself. Thus, the LTbeta defect appears to be the primary driver (without it there is no defect). Thus any connection to the microbiota here appear to be overstated.

4. The sequencing results are very confusing. For example, methylbacterium is an environmental microbe and may just be a contaminant (not typically in mice). Carthormitus is found in termites. This is not SFB (Candidatus Savagella).

1st Revision - authors' response 12 November 2014

I still have a few concerns regarding the claims made that the microbiota significantly influences this particular phenotype (ANA titers) with LTbeta inhibition.

1. There is a maternal effect that is mentioned in the rebuttal letter, but this is not delineated in any clear manner within the paper. This unexplained variability makes interpretation of the treatment with antibiotics (Fig 3A) difficult, especially since this is relatively a much smaller sample size and this could still be driven by undefined maternal effects. This point really needs to be addressed as it may be the key to linking microbes to this phenotype. Also the antibiotics used (and method) was not written anywhere that I could find.

Answer: To provide the reviewer a better insight into the variability between litters, we again used the data of the 369 consecutively born LTβR−/− mice in our facility in Gent. As stated in the manuscript, the overall frequency was 23%. This cohort included 31 litters of at least 6 mice. The mean frequency of ANA prevalence of these litters was 26% (95% CI 18-35%). Thus, the overall frequency of the four litters of the antibiotic treated mice (12%) lies outside this 95% confidence interval (p=0.002, one-sample t-test).

Mice were treated with ampicillin, streptomycin and colistin (Sigma-Aldrich) in the drinking water at a concentration of 1 g/l, 5 g/l and 1 g/l, respectively. This was already stated in the methods section under ‘Antibodies, fusion protein and antibiotics’.

2. The small sample size for many of the LTbeta deficient mice was not adequately addressed. This is potentially an artifact of maternal effects as noted above.

Answer: We agree that the sample size of the T, B and RORgt cell specific LTβ deficient mice seems small. However, both knockout (cre positive) and controls (cre negative) were weaned from the same breeding pairs, thus any maternal effects on both groups are equal. Furthermore, we made any effort to find more samples of T and B cell specific LT deficient mice. Unfortunately, we lost the cell specific LTβ deficient mice in our animal facility and none of our collaborators is breeding these lines. Nevertheless, we were able to test samples of T cell and B cell specific LTα knockout mice. As shown in Fig. 1a, LTα deficient mice have the same autoimmune phenotype. In total, none of T cell deficient LTα knock-out mice developed ANA at the age of 3 months (data of 7 T cell deficient LTα knock-out mice were already shown in the first point to point reply). Moreover, a
thymic transplantation experiment argued against a role for a defect in central T cell tolerance in the phenotype. Regarding B cell specific LTα knockout mice, none of 6 mice developed ANA at the age of 3 months. We think these additional data augment the power of our observations and lower the chance of a type II statistical error. As mentioned in the first reply, RORgt cell specific LTα knockout mice do develop ANA.

**Addition to the main text:** As we were able to test only small sample sizes of cell specific Ltb−/− mice, we also collected sera from cell specific Lta−/− mice. Alike cell specific Ltb−/− mice, only Rorγt-Lta−/− but neither T-Lta−/− nor B-Lta−/− developed ANA at the age of three months (data not shown).

3. The fact that germ free ko mice have the same penetrance as conventionally raised mice is a significant point against a primarily microbially driven phenotype. There is some evidence that the phenotype can be modestly modulated by microbes is suggested. However, the relative evidence of the antibiotic experiment in conventionally raised mice and the conventionalization of germ free mice is less impressive than LTbeta inhibition itself. Thus, the LTbeta defect appears to be the primary driver (without it there is no defect). Thus any connection to the microbiota here appear to be overstated.

**Answer:** We agree with the reviewer that the deficiency in LT is the primary driver of the model. However, we confirmed the hypothesis that microbiota influence the development of ANA in the model by three different independent experiments. The experiments with the antibiotics treated mice (Fig. 3a, upper panel), germfree mice (Fig. 3a, lower panel) and monocolonized mice (Fig. 3d) all point to the same biologic hypothesis. Thus, these data support our conclusion that LT dependent systemic autoimmune responses are associated with increased SFB colonization; thus enforcing a combined interplay of LT and microbiota.

4a. The sequencing results are very confusing. For example, methylobacterium is an environmental microbe and may just be a contaminant (not typically in mice).

**Answer:** With regard to *Methylobacterium*, as the reviewer points out, there are indeed some species associated to contamination (Barton 2006 and Salter 2014). For this reason, we revisited our data to assess the likelihood that these patterns were driven by contamination. We should note that, from a theoretical perspective, a contaminant has very little chance to have a great effect in samples with high amounts of biomass (like faecal material), as they should be overpowered by the endogenous DNA and have minimal representation in the result. After careful evaluation, we conclude that the patterns seen here are not driven by contamination, based on 3 lines of evidence: (i) given that samples were handled as one pool, we would expect that, if *Methylobacterium* were a contaminant we should see similar abundance across all samples, not the very specific signal we see in Fig. 3b; (ii) Kaakoush (2012) reports *Methylobacterium adhaesivum*, which has 100% of identity with one of our *Methylobacterium* sequences (Suppl. Fig. S6, OTU_2794), present in the gut of children affected with Crohn’s disease. This species appears to be part of the dysbiotic flora of vertebrates exposed to inflammation, suggesting it is possible that it was part of the flora of mice used in this experiment; (iii) A phylogenetic analysis of *Methylobacterium* sequences reported as contaminants (Barton 2006) and our sequences identified as *Methylobacterium*, show two separate clusters: *Methylobacterium* OTUs from our mice data and contaminants (Fig. S5).

**Addition to the main text:** The presence of the *Methylobacterium* genus has been associated with contamination in samples with low biomass (Barton et al, 2006; Salter et al, 2014). However, we feel that this is not the case here – the faecal material used in this study has large biomass that should dilute the contaminant rather than allowing for its dominance in the profile. Furthermore, a Neighbor-Joining tree shows that the observed Methylobacterium forms a separate cluster than the one produced by the reported contaminants (Fig. S5).
Fig S5. Neighbor-Joining tree generated from Methylobacterium 16S rRNA sequences of the mice data and sequences reported as contaminants (Barton et al, 2006). We can see two clusters: pink squares are Methylobacterium OTUs (operational taxonomic unit, gene rated from the illumina MiSeq data processed with LotuS pipeline) from our mice data and black circles are the sequences from contaminants (Barton et al, 2006).

4b. C arthromitus is found in termites. This is not SFB (Candidatus Savagella).

Answer: The naming of this bacterial group is currently in transition – Thompson (2012) has indeed recently suggested to rename Candidatus arthromitus isolated in vertebrate intestines to Candidatus savagella. At the same time, the closest relative to the sequence isolated in our study comes from the genome of SFB-Mouse-NL, a commensal murine bacterium associated to maturation of gut immune function published recently (Bolotin 2014), which, in this study is reported as Candidatus arthromitus – this is the reason why we originally stuck to that naming. Also, the large majority of hits in the greengenes database come from sequences annotated as C. arthromitus, but coming from mouse samples. In conclusion, we think the identified bacterium is indeed SFB and thus that the main results are not affected, but have expanded the text to clarify the naming issues.

Addition in the methods section: From the greengenes database (v. 2013), we identify Candidatus arthromitus (ID: 376862) that, according to Thompson (2012), should be Candidatus savagella. Greengenes reference sequence for Candidatus arthromitus (ID 376862) is 100 % identical to EU791150.1 (isolated from mouse distal small intestine and reported as uncultured Bacteria) and 99% to several Candidatus arthromitus sp. SFB-mouse sequences (NR_074545.1, NR_074460.1, AP012209.1, AP012202.1, D86305.1, LK932435.1, CP008713.1, EU791182.1). All these sequence entries are annotated as Candidatus arthromitus in the NCBI record. Also, the closest sequence coming from full genomes is that from a SFB-Mouse-NL, a commensal bacterium associated to maturation of gut immune function published recently (Bolotin 2014) and reported as Candidatus arthromitus.

Addition in the legend of Fig. 3: Please note that Candidatus arthromitus, according to Thompson (2012), should be renamed Candidatus savagella.

References:
Thank you for submitting your manuscript to The EMBO Journal. Your revision was re-reviewed by referee #2. I have now heard back from the referee who supports publication here. I am therefore very pleased to accept the paper for publication.

REFEREE REPORT

Referee #2

It is disappointing that with such a large cohort of mice that a careful examination of maternal effect on the phenotype was not reported (or perhaps the data was not collected).

The goal of my comments about possible contamination in the sequencing results were that hopefully the authors have gone back and carefully re-examined their raw data and analysis. My hope is that this is correct and will stand up over time.

2nd authors’ response 12 December 2014

1. It is disappointing that with such a large cohort of mice that a careful examination of maternal effect on the phenotype was not reported (or perhaps the data was not collected).

Given the space limitations of the article we opted to refer to these observations rather in the text. In any case, we carefully examined the impact of maternal and cage dependent effects in this cohort of mice, as referred to in the manuscript.

2. The goal of my comments about possible contamination in the sequencing results were that hopefully the authors have gone back and carefully re-examined their raw data and analysis. My hope is that this is correct and will stand up over time.

As outlined in the paper the link of SFB and ANA has been approached by 3 independent approaches: 1. DGGE, cloning of sequencing of differentially expressed bands; 2. Direct 16S rRNA sequencing and 3. confirmation by SFB qPCR on independent samples on 3 different components (luminal, mucosal, faecal). Therefore, the suggestion of this referee of potential contamination is surprising and can be ruled out, especially as further functional experiments using SFB monoclonized mice confirmed the results by yet another approach.