Intestinal mucosal adherence and translocation of commensal bacteria at the early onset of type 2 diabetes: molecular mechanisms and probiotic treatment


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

1st Editorial Decision 17 March 2011

Thank you for the submission of your manuscript "Increased intestinal adherence and translocation of commensal bacteria at the early onset of type 2 diabetes: molecular mechanisms and probiotic treatment". We have now heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise some concerns on the study, which should be addressed in a revision of the manuscript.

In particular, reviewer #2 highlights that the effect of treatment with another bacterial strain should be investigated while reviewer #3 points to an alternative hypothesis for the involvement of NOD1 in metabolic improvement.

On a more editorial note, we agree with reviewer #3 that the manuscript would benefit from improved clarity and would thus encourage you to edit the text and title accordingly. Please also include an ethics statement for animal experiments as described in our Instructions to Authors (http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1757-4684/homepage/ForAuthors.html)

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the space and time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged differently with the editor.
I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

REFEREE REPORTS:

Referee #1:

Amar and colleagues clearly demonstrate that the early onset of HFD-induced hyperglycemia is characterized by an increased bacterial translocation from intestine towards tissues, fueling a continuous metabolic bacteremia. The bacterial translocation is ruled through CD14 and Nod1, while leptin is suggested to participate although it remains to be defined if leptin works directly or indirectly.

The manuscript is very well written and easy to follow; overall the conclusions are very well supported by the data.

Still the reviewer has some minor suggestions:
1) the discussion section should be improved by discussing potential mechanisms connecting LPS/CD14/Nod1 to obesity. In particular:

1a. Please cite the possible role of Adam17 as transducer of LPS effects in adipose tissue. The following manuscripts should be discussed and cited:
- Tissue inhibitor of metalloproteinase 3 deficiency causes hepatic steatosis and adipose tissue inflammation in mice.


1b. Please cite the inflammasome activation as a potential mechanism connecting bacterial DNA to obesity. The following manuscripts should be discussed and cited:
- The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance.
- The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and insulin sensitivity.

Referee #2:

Amar et al evaluated the presence of live commensal bacteria in the gut of different animal models in association with high fat diet. The hypothesis is that the presence of these bacteria is pathogenic at the metabolic level, leading to development of type 2 diabetes. The administration of a probiotic decreased the inflammatory status leading to metabolic improvement.

The hypothesis is plausible and the results support it.
General Comments

The effects of probiotic treatment were, at least, controversial. A 1-month daily treatment of HFD-induced diabetic mice with B420 reduced the number of GFP-E. coli in different segments of small intestine mucosa. However, the quantity of Bifidobacterium species was not significantly increased. The authors support the effects of probiotics on previous publications (Collado et al., 2007) and on the effects of pro-inflammatory cytokines. However, glucose intolerance was moderately blunted (I do not grasp the full meaning of this sentence) and fasting glycemia remained unaffected. A control treatment with another bacteria strain would have been desirable. This part weakens authors' findings.

While the effects of probiotics on proinflammatory cytokines are well studied, no mention is made about the effects of live bacteria (E.coli) on inflammatory status. Is there any relationship between DNA content in intestinal mucosa and in circulation with parameters of inflammation?

The conclusions on leptin are entirely based on the studied effects in ob/ob mice. The authors would need to deliver leptin and demonstrate that leptin reduces bacterial translocation to conclude that leptin is involved.

Although the authors write about adipose tissue inflammation in the introduction, this has been scarcely reported.

Other comments

Page 6, line 5: Please expand "sensitive to HFD". In terms of weight, metabolic dysregulation??

Referee #3 - Comments on Novelty/Model system:

The use of GFP labeled bacterium to determine transit of bacteria from the gut to the blood and adipose tissue was novel. The use of probiotics is of interest. Also CD14 and NOD mutants are of interest.

Referee #3 Other Comments:

This manuscript examines transit of bacteria from the gut into blood and adipose tissue with a high fat diet and in ob/ob mice. In general the observations are of interest but there are some issues

1) There appears to be inappropriate attention to certain details. For example on 6, the authors state"Concentration of total bacteria DNA in ob/ob mice when compared with WT mice (Fig 1A, B,F and G. Figure1A and B refers to high fat diet so this sentence does not make sense. "In the double mutant mice translation of GFP-Ecoli to MAT was blunted when compared with ob/ob " figure 2E describes a GTT -no data on GFP bacteria.

3) was MLN defined?

4) It appears that some of the figures in supplemental figures appear in the actual figures which is confusing-Is this correct? if so why is this done

This attention to detail detracts from the manuscript, making it difficult to understand the manuscript and causes concern for the reviewer.

5) If NOD and CD14 both have significant impact how do the authors reconcile the two receptors being involved? A hypothesis/model would be useful.

6) At 4 weeks of HFD diet there is no difference in bacterial DNA in MAT of NOD1 ko mice, how does this affect the hypothesis about its role in bacteria translocation. Doesn't this suggest that an
intracellular response in MAT to the bacterial ligand as an explanation for metabolic improvement. Or at least raise the question even when blood DNA is reduced.

1st Revision - Authors’ Response 25 May 2011

Thanks for giving us the opportunity to revise and improve our manuscript. We have performed all experiments required by the reviewers and added some more that we thought were pertinent. Therefore we do consider that the manuscript is much improved and I take the opportunity to thank the reviewers for their excellent job.

Comments from the Editor

- In particular, reviewer #2 highlights that the effect of treatment with another bacterial strain should be investigated while reviewer #3 points to an alternative hypothesis for the involvement of NOD1 in metabolic improvement.

⇒ We addressed these important points.

- On a more editorial note, we agree with reviewer #3 that the manuscript would benefit from improved clarity and would thus encourage you to edit the text and title accordingly. Please also include an ethics statement for animal experiments as described in our Instructions to Authors (http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1757-4684/homepage/ForAuthors.html)

⇒ The text has been edited by a native from the UK. We have filed the form corresponding to the ethics statement for animal experiments.

Referee #1:

Amar and colleagues clearly demonstrate that the early onset of HFD-induced hyperglycemia is characterized by an increased bacterial translocation from intestine towards tissues, fueling a continuous metabolic bacteremia. The bacterial translocation is ruled through CD14 and Nod1, while leptin is suggested to participate although it remains to be defined if leptin works directly or indirectly.

⇒ This is an important question. We have performed some experiments using a probiotic treatment where the Lactococcus lactis that we used produces leptin locally into the intestine (Bermudez et al Applied and Environmental Microbiology 2007). This process does not allow significant amount of leptin to be detected into the blood. In the present experiment we treated daily C57 HFD-fed mice with the leptin-producing probiotic and its corresponding non-producing control for 8 weeks. The data show that body weight gain, oral glucose tolerance, fed plasma insulin were lower in the leptin group than in the controls. In addition, intestinal bacterial adherence (ileum), and mesenteric white adipose tissue translocation of E.coli-GFP were reduced. Importantly the accumulation of alive bacteria (E. coli-GFP) was reduced in the mesenteric adipose tissue whereas the total bacterial DNA concentration was normal which suggests that leptin has restored the activity of the immune system, allowing the destruction of the translocated bacteria into the adipose fat pad, preventing hence an exacerbated inflammatory reaction since cytokine mRNA concentrations were reduced in the leptin group. Furthermore, we performed similar experiments in ob/ob mice. The data show that body weight and fat mass gain, fed plasma insulin, were reduced whereas no major changes were observed on glucose tolerance. In addition, intestinal bacterial adherence was reduced in both ileum and caecum as well as adipose tissue bacterial translocation. Similarly to what observed in the HFD mice the total amount of bacterial DNA remained the same but alive bacteria were reduced suggesting, as well, an increased bactericidal activity of the immune system in response to leptin. Hence, the overall inflammatory cytokine mRNA concentration was reduced accordingly by leptin-producing probiotic. These data have been added to the text (Fig 6, 7 described page 10) and discussed (page 16).
The manuscript is very well written and easy to follow; overall the conclusions are very well supported by the data.

Still the reviewer has some minor suggestions:
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   - Tissue inhibitor of metalloproteinase 3 deficiency causes hepatic steatosis and adipose tissue inflammation in mice.
   - Mice heterozygous for tumor necrosis factor-alpha converting enzyme are protected from obesity-induced insulin resistance and diabetes.

1b. Please cite the inflammasome activation as a potential mechanism connecting bacterial DNA to obesity. The following manuscripts should be discussed and cited:
   - The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance.
   - The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and insulin sensitivity.

We addressed these points into the discussion section (page 12). This much improves the quality of the text. We greatly thank this reviewer for pertinent comments.

Referee #2:

Amar et al evaluated the presence of live commensal bacteria in the gut of different animal models in association with high fat diet. The hypothesis is that the presence of these bacteria is pathogenic at the metabolic level, leading to development of type 2 diabetes. The administration of a probiotic decreased the inflammatory status leading to metabolic improvement.

Comments

- The effects of probiotic treatment were, at least, controversial.
  A 1-month daily treatment of HFD-induced diabetic mice with B420 reduced the number of GFP-E. coli in different segments of small intestine mucosa. However, the quantity of Bifidobacterium species was not significantly increased.

We do agree that the lack of Bifidobacterium species increase (beside the tendency observed, but non significant) in the mucosa during the probiotic treatment of HFD-fed mice is controversial. In other sets of experiments (performed during other projects) in normal chow fed-mice the bacterial adherence of Bifidobacterium species was increased, however this was not frankly observed here in HFD condition. This could be explained by the fact that the HFD is deleterious for the survival of Bifidobacteria. Indeed we previously demonstrated (Cani et al Diabetes 2007) that the number of Bifidobacterium species is reduced by several Log in response to a fat-enriched diet. Therefore, our data suggest that the “therapeutic efficacy” of the probiotic treatment might not be directly related to an increased survival of the probiotic in the mucosa. Since this reviewer’s point is important we
added a note in the discussion section (page 17) as follows “. However, we did not observe a dramatic increase in the concentration of Bifidobacterium spp. in the mucosa of HFD-fed treated mice when compared with non treated mice. This was expected since we previously described that fat-diet strongly reduces the amount of the Bifidobacterium genus (Cani et al, 2008). Therefore, the mechanisms through which the probiotic regulates glucose metabolism do not seem to be related to an increased mucosal concentration of the probiotic and hence remain yet to be discovered”.

The authors support the effects of probiotics on previous publications (Collado et al., 2007) and on the effects on pro-inflammatory cytokines. However, glucose intolerance was moderately blunted (I do not grasp the full meaning of this sentence) and fasting glycemia remained unaffected.

⇒ We do agree that the effect of the probiotic treatment on the control of glucose tolerance is moderate. This is due most likely due to the short term treatment that we applied. The aim of this short term treatment was to uncover any improvement of insulin action which precedes the secondary improvement of the glucose tolerance. This is a general physiological concept developed in the field of diabetes i.e. insulin resistance which precedes the occurrence of diabetes. This is most likely true as well for remission from diabetes. We previously described that in human a reduction of insulin resistance leads to a normal glucose tolerance (Burcelin et al Diabetes Care 1993) and conversely a slight increase in insulin resistance was associated with relapses from remission in patients. Therefore, we here show that in diabetic mice the reversal of insulin resistance precedes the improvement of glucose tolerance and later on the diabetic state. Since we do agree with this reviewer that it is an important argument we commented this point in the discussion section (page 17) as follows “This impacted the overall glycemic control since glucose intolerance reduced, although moderately, in the probiotic-treated HFD-fed mice when compared with non-treated HFD-fed mice. Our data suggest that the reversal of diabetes by probiotic treatment required first a normalization of mucosal dysbiosis, which was followed by reduced bacterial translocation, tissue inflammation, insulin resistance, and secondarily glycemia.”

To this reviewer, we did perform some dose response curves of Bifidobacterium treatments. The data on this figure demonstrate a dose dependent effect of a one month treatment of diabetic mice on glucose tolerance.

![IPGTT after one month of treatment with Bifido 420 group 1](image)

Intraperitoneal glucose tolerance in mice in which diabetes was induced by a high-fat diet and then treated with increasing doses of probiotics.

This set of data is provided to the reviewer only, since we don’t have all assays related to the other doses of probiotic.

- A control treatment with another bacteria strain would have been desirable. This part weakens authors' findings.
We studied similarly the effect of NCFM (*Lactobacillus* strain) on glucose metabolism but could not detect any anti-inflammatory activity nor antidiabetic effect of this probiotic. Therefore, we did not add the data to this manuscript. We however added a line in the discussion section (page 17) as follows “In a different set of mice treated with a strain of *Lactobacillus* (NCFM) we could not observed any effect on inflammation (Not shown).”

- While the effects of probiotics on proinflammatory cytokines are well studied, no mention is made about the effects of live bacteria (*E. coli*) on inflammatory status.

⇒ 10⁹ live *E. coli*/mouse were used over a single dose and 2 hours later the mice were sacrificed and studied. To our knowledge acute treatment does not induce a state of inflammation. However, since we cannot rule out this important suggestion we added a comment at the end of the discussion section (page 17), as follows “We cannot rule out that an acute administration of probiotic could have a direct impact on inflammation, therefore a similar reasoning could be made with regards to the gavage of *E. coli*. However, we do not have any evidence for this assumption.”

- Is there any relationship between DNA content in intestinal mucosa and in circulation with parameters of inflammation?

⇒ We have quantified in following mouse models: 1. Normal chow control, 2. High-fat diet one week, and 3. High-fat diet 4 weeks, the 16S rRNA DNA in mucosa of ileum, blood and mesenteric adipose tissue. For each mouse we quantified the mRNA coding for different cytokines in the mesenteric adipose depot and analyzed correlations with the bacterial DNA content in the lumen and the mucosa of ileum.

The data show that the mRNA concentrations coding for the cytokines were not increased after only one week of HFD but there was a clear tendency after 4 weeks of HFD. The major actor of inflammation, TNF-α, was however significantly increased. Therefore, the TNF-α mRNA concentration correlated positively with the bacterial DNA content in the lumen and the mucosa of ileum.

The conclusions on leptin are entirely based on the studied effects in ob/ob mice. The authors would need to deliver leptin and demonstrate that leptin reduces bacterial translocation to conclude that leptin is involved.

⇒ This is certainly an important comment about the role of leptin. We have performed some experiments using a probiotic treatment where the *Lactococcus lactis* that we used produces leptin locally into the intestine (Bermudez et al Applied and Environmental Microbiology 2007). This process does not allow significant amount of circulating leptin to be detected. In the present experiment we treated daily C57 HFD-fed mice with the leptin-producing probiotic and its corresponding non-producing control for 8 weeks. The data show that body weight gain, oral glucose tolerance, fed plasma insulin were lower in the leptin group than in controls. In addition, intestinal bacterial adherence (ileum), mesenteric white adipose tissue translocation of *E. coli*-GFP was reduced. Importantly the accumulation of alive bacteria (*E. coli*-GFP) was reduced in the mesenteric adipose tissue whereas the total bacterial DNA concentration was normal which suggests that leptin has restored the activity of the immune system, allowing the destruction of the translocated bacteria into the adipose fat pad, preventing hence an exacerbated inflammatory reaction since cytokine mRNA concentrations were reduced in the leptin group.

Furthermore, we performed similar experiments in ob/ob mice. The data show that body weight and fat mass gain, fed plasma insulin, were reduced whereas no major changes were observed on glucose tolerance. In addition, intestinal bacterial adherence was reduced in both ileum and caecum as well as adipose tissue bacterial translocation. Similarly to what observed in the HFD mice the total amount of bacterial DNA remained the same but alive bacteria were reduced suggesting, as well, an increased bactericidal activity of the immune system in response to leptin. Hence, the overall inflammatory cytokine mRNA concentration was reduced accordingly by leptin-producing probiotic. These data have been added to the text (Fig 6 and 7 described page 10) and discussed (page 16).
- Although the authors write about adipose tissue inflammation in the introduction, this has been scarcely reported.

⇒ We previously published that one month high-fat diet induced some degree of adipose tissue inflammation in a similar model (Cani et al Diabetes 2007). We here performed similar analyses and show as well that the cytokine mRNA concentrations in the mesenteric adipose tissue (MAT) of mice fed a high-fat diet for four weeks were moderately, but significantly increased for TNF-α and IFN-γ. We further analyzed mice after only 1 week of HFD and could not detect any significant increase in the expression of markers of inflammation (Fig 5B). This suggests that the inflammatory process progressively developed. We do agree that it is an important notion that need to be discussed. This is the reason why we added these results to the manuscript (Fig 5B described page 9).

Other comments

- Page 6, line 5: Please expand "sensitive to HFD". In terms of weight, metabolic dysregulation??
⇒ Corrected (page 7).

Referee #3:

1) There appears to be inappropriate attention to certain details. For example on 6, the authors state "Concentration of total bacteria DNA in ob/ob mice when compared with WT mice (Fig 1A, B,F and G. Figure 1A and B refers to high fat diet so this sentence does not make sense.
⇒ The text has been clarified (page 8).

2) "In the double mutant mice translocation of GFP-E. coli to MAT was blunted when compared with ob/ob " figure 2E describes a GTT - no data on GFP bacteria
⇒ The text has been corrected (page 8).

3) Was MLN defined?
⇒ Mesenteric lymph nodes, now described (pages 6, 13).

4) It appears that some of the figures in supplemental figures appear in the actual figures which is confusing-Is this correct? if so why is this done
This attention to detail detracts from the manuscript, making it difficult to understand the manuscript and causes concern for the reviewer.
⇒ We are sorry for this confusion. The difference between figure in the text and the supplementary figures are related to a different time course. These are the same experiments but the one in the text is shown 2 hours after gavage with GFP-E. coli, while in supporting information the data were obtained 5 hours after gavage. We added on the figure the information related to the time course used.

5) If Nod and CD14 both have significant impact how do the authors reconcile the two receptors being involved? A hypothesis/model would be useful.
⇒ This is an important remark. It has been described that bacterial recognition involves numerous TLRs and NLRs specialized in the recognition of different bacterial antigens, on the cell surface (TLRs) and in the cytosolic compartment (NLRs). As the PRRs CD14/TLR4 and Nod1 recognize different PAMPs delivered by the same gram-negative bacteria, lipid A portion of LPS and meso-diaminopimelic acid (meso-DAP) moiety of peptidoglycan (PGN), respectively, it is probable that they are non-redundant systems (Kufer & Sansonetti Current Opinion in Microbiology 2007).
Consistently, we here described that CD14, and Nod1 are two receptors separately involved, but both necessary to induce the full inflammatory response from immune cells against translocating gram-negative bacteria. These findings are in line with our previous results demonstrating that diet-induced changes in intestinal microbiota, such as a rise in the gram-negative to gram-positive ratio, may be an important factor in triggering adiposity and insulin resistance (Cani et al Diabetes 2007) (Amar et al American Journal of Clinical Nutrition 2008). Moreover, the increase of gram-negative bacteria translocation that we found here in blood and MAT of HFD-fed mice before the onset of diabetes, reinforce the implication of gram-negative bacteria signaling, through not only their LPS but also their PGN, in the onset of HFD-induced adiposity and insulin resistance.

In addition, a cross-talk between TLR and NLR have been described. Priming of cells with PGN and products that are sensed by cytosolic-localized members of the NLR family has a synergistic effect on TLR signaling and vice versa (Kufer & Sansonetti Current Opinion in Microbiology 2007). For example, Nod1 and Nod2 mRNA expression is induced by TLR4 activation and TNF-α (Takahashi Y et al Journal of Veterinary Medical Science 2006). In addition, the Nod2 agonist, muramyl dipeptide (MDP) enhances Myd88 expression, a key adaptor protein for TLR4 signaling (Takada H et al Journal of Endotoxin Research 2002). As Myd88 is a common adaptor protein in all TLR pathways (except TLR3) we thought to compare glucose metabolism in wild-type mice and Myd88 knockout mice. The data show that the mutant mice were diabetic and glucose intolerant (Fig 3A), hyperinsulinemic in the fasting state (Fig 3D), and insulin resistant (Fig 3E). This set of data is surprising but suggests that over the course of the maturation of the immune system bacterial fragment-induced inflammation is a mechanism which could prevent the dysregulation of glucose homeostasis as if it was allowing a proper maturation of the immune system as observed in type 1 diabetic animal models (Wen et al Nature 2008).

Furthermore, we also quantified bacterial translocation in Myd88 mice. The data show that the total bacterial content was increased in the mutant mice and alive bacteria, as quantified by using E. coli-GFP, was also increased suggesting that the immune system transports and does not destroy the bacteria. We added these data (Fig 3 described pages 7 and 8) and the corresponding comments in the discussion section (page 15).

We modified “Furthermore, the fact that both CD14 and Nod1 were necessary for the induction of diabetes in response to the fat-enriched diet, suggests that a crosstalk between the two non-redundant pathways might be involved, to synergize for a full inflammatory response to the translocated gram-negative bacteria. Myd88 is a good candidate; however, surprisingly the intolerance to glucose as well as the fasted glycemia were higher in the knock-out model than in wild-type mice.”

6) At 4 weeks of HFD diet there is no difference in bacterial DNA in MAT of NOD1 ko mice, how does this affect the hypothesis about its role in bacteria translocation. Doesn't this suggest that an intracellular response in MAT to the bacterial ligand as an explanation for metabolic improvement. Or at least raise the question even when blood DNA is reduced?

⇒ This is indeed a very important remark from this reviewer. The accumulation of bacterial DNA is increased in wild-type mice in response to HFD (Fig 1B). To discover the genes involved in this process we challenged this phenotype when studying mutant mice. Our data show indeed, as noticed by this reviewer, that the bacterial DNA content remains the same between HFD and NC mice in the Nod1 ko mice (Fig 1E). Therefore, the mutation prevents the increased bacterial DNA accumulation induced by the HFD, suggesting that the corresponding protein is indeed involved in the process of bacterial translocation. We precised these comparisons in the text (page 8).
Editor
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REFEREE REPORTS:

Referee #2 (Comments on Novelty/Model System):
This is a remarkable manuscript.

Referee #2 (Other Remarks):
The comments were adequately addressed.
I have no further comments.

Referee #3 (Comments on Novelty/Model System):
Clever model for examining effects of bacteria in type 2 diabetes

Referee #3 (Other Remarks):
The authors have answered response as requested. The paper uses thoughtful techniques to address a process that has potential significance to the development of type 2 diabetes.