Maternal B cell signaling orchestrates fetal development in mice

Mandy Busse, Stefanie Langwisch, Kerry Tedford, Klaus Dieter Fischer and Ana Claudia Zenclussen

DOI: 10.1242/dev.199783

Editor: Florent Ginhoux

Review timeline

Original submission:	6 May 2021
Editorial decision:	7 July 2021
First revision received:	23 July 2021
Accepted:	25 August 2021

Original submission

First decision letter

MS ID#: DEVELOP/2021/199783

MS TITLE: Maternal B cell signaling orchestrates fetal development in mice

AUTHORS: Mandy Busse, Stefanie Langwisch, Kerry Tedford, Klaus Dieter Fischer, and Ana Claudia Zenclussen

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The question addressed in the manuscript was to understand whether maternal B cell absence or B cell impaired signaling (TLR or IL-10 secretion) had effects on several gestational outcomes such as number of implantation, placental thickness, diameter and area, uterine artery resistance and fetal growth.
They show that:
1) B cell specific MyD88 and IL-10 signaling is necessary for an appropriate development in utero
2) IL-10 production by B cells is needed for uterine blood flow regulation during gestation.
3) alterations in B cell-specific expression of CD19, MyD88 and IL-10 enhance the susceptibility towards LPS-induced preterm delivery.
This paper contribute to the understanding of the immunology during pregnancy, highliting cellular populations and signaling pathways essential for a successful pregnancy.

Comments for the author

1) Figure 1 is really hard to understand. We don't know how many dams were maited per groups, how many pregnant dams you obtained or what was the percentage of successful pairing in each group. For example : figure B) is about the number of dams with dead implantation. Why do you only present the results for WT and BIL-10-/- groups and not the other groups ? Why do you have only 8 pregnancies in the WT group and 3 in the BIL-10-/- when you show 34 WT dams in figure D) and 9 BIL-10-/- in figure C). In Figure D) why do you only show the results for WT and MyD88-/- groups and not the others ?
To summarize, you should better make a big general table mentioning for each group all the gestational outcomes : 1) the number of maited dams, 2) the number of successful pairing, the time for vaginal plug, the number of dead implantation, the number of moribund dams, the number of implantation…..
Also in the text you say "the mean number of implantation in WT dams was 8,733".
3 numbers after the comma is not relevant. You should better write 8±stand dev.

2) Figure 2 : legend : the number of n per group is not clear. What "n=4-6 dams and n=3-11 implantation/dams per day" means ?

3) Figure 5 : Not having all the groups at all the concentrations make the reading of the figure very complicated.

4) There is a huge imbalance between the quality of the introduction and the discussion. Please, enrich a bit the introduction.

Minor comments :
- please rewrite the first sentence of the introduction.
- the figures are in the wrong order : 1-5-2-3-4
- define "gd" for gestational day in the abstract.

Reviewer 2

Advance summary and potential significance to field

Thank you for the opportunity to review the interesting work by Busse and colleagues. This study investigates the effect of various B cell related deletions on successful implantation and susceptibility to preterm birth. By highlighting the necessity of the individual pathways to correct implantation the study offers an important contribution to our understanding of uterine immunity in reproductive health. We think that some important information on the pregnancy outcomes should be added in order to draw conclusions on how the different pathways affect fetal development.

Comments for the author

Figure 1B: The table only gives information on BIL-10-/- mice. Does this mean that the number of pregnancies with resorptions was unchanged in CD19-/-, BMyD88-/-, MyD88-/-? Please include the

information on all mice strains. Consider combining this information in a single table for a more clear overview.

Page 5 Lines 32 – 34: Is this a commonly used technique? If so, please include references. Do you see in your data that fetal size (during gestation and at the end of the experiment) is indeed reduced when the implantation sites where smaller? Did you weigh the pups on gd17?

Line 23-25: According to the text, the authors could discriminate between fetus, placenta and decidua basalis at gd10. Thus, this fits with the analysis of the implantation sites (Fig2.) Consider adding the information of Figure 4 to Figure 2 or otherwise switching Figure 3 and 4. The part on uterine artery resistance now breaks the train of thought on morphology of the implantation sites.

Figure 3: What's the sample size shown in this figure? Please show dot plots rather than bars.

Figure 4 is now restricted to assessment of the placenta. An addition of parameters on fetal morphology would be valuable.

Figure 5 and the respective text: It might be clearer to start with the lowest concentration of LPS which does only induce preterm delivery in the two most susceptible strains.

Page 8, line 12: Relates to previous comment regarding Figure 4. Consider adding fetus size rather than the complete implantation site when referring to inappropriate fetal growth.

Page 10, line 10 – 15: See previous comments, where are the data on fetal size shown?

Page 10, line 17-24: According to Figure 2E, it seems like BMyD88 deficiency affects the size of the implantation site to a lesser extent than BIL10-/-.

Are the BMyD88-/- mice still able to produce IL-10 (through a different way of activation)?

Related to this, are there any controls to show that the knockouts lack indeed the desired pathways?

Page 11, line 25-28: Did you check the phenotype of the individual KO mices'

B cells or their capacity to produce IL-10 after in vitro stimulation?

Page 13, line 4-5: How was blinding achieved? Does this software allow for unbiased stereology?

Discussion: It is remarkable that BIL-10 -/- mice showed no difference in number of implantation sites but are severely affected when assessing implantation areas and uterine artery resistance. How do the authors explain this discrepancy?

Other points

Page 5 line 13: Consider showing only 1 decimal (instead of 8.733, might be confused with thousand delimitator)

Figure 5: In the previous figures, the x axis was labeled. These figures were easier to read. To be consistent, consider adding the labels (in the same sequence as the other figures) below the axis in Figure 5, too.

Page 5 line 12: Does this include dead pups?

Page 5, line 31; page 8, line 34; page 11 line 10: This should be "have/was shown".

Page 8, line 33-34: Add "in mice".

Page 9, line 15: Please include references.

Page 10, line 1-3, line 27 These sentence are difficult to read, please revise.

What was the motivation to sacrifice at gd17 rather than allowing the mice to deliver naturally?
Title: Rather than fetal development, consider referring to placentation/development of the implantation site as there is currently limited focus on the pups. This might change if more details on fetal outcomes are added.

First revision

Author response to reviewers' comments

Maternal B cell signaling orchestrates fetal development in mice by Mandy Busse, Stefanie Langwisch, Kerry Tedford, Klaus Dieter Fischer, and Ana Claudia Zenclussen

Response to Reviewers´queries

Reviewer 1:

Advance Summary and Potential Significance to Field:

The question addressed in the manuscript was to understand whether maternal B cell absence or B cell impaired signaling (TLR or IL-10 secretion) had effects on several gestational outcomes such as number of implantation, placental thickness, diameter and area, uterine artery resistance and fetal growth.
They show that: 1) B cell specific MyD88 and IL-10 signaling is necessary for an appropriate development in utero 2) IL-10 production by B cells is needed for uterine blood flow regulation during gestation. 3) alterations in B cell-specific expression of CD19, MyD88 and IL-10 enhance the susceptibility towards LPS-induced preterm delivery. This paper contribute to the understanding of the immunology during pregnancy, highliting cellular populations and signaling pathways essential for a successful pregnancy.

Reviewer 1 Comments for the Author:

1) Figure 1 is really hard to understand. We don't know how many dams were maited per groups, how many pregnant dams you obtained or what was the percentage of successful pairing in each group. For example : figure B) is about the number of dams with dead implantation. Why do you only present the results for WT and BIL-10-/- groups and not the other groups ? Why do you have only 8 pregnancies in the WT group and 3 in the BIL-10-/- when you show 34 WT dams in figure D) and 9 BIL-10-/- in figure C). In Figure D) why do you only show the results for WT and MyD88-/- groups and not the others ? To summarize, you should better make a big general table mentioning for each group all the gestational outcomes : 1) the number of maited dams, 2) the number of successful pairing, the time for vaginal plug, the number of dead implantation, the number of moribund dams, the number of implantation…..

Also in the text you say "the mean number of implantation in WT dams was 8,733". 3 numbers after the comma is not relevant. You should better write 8±stand dev.

Answer:

First, we would like to thank the Reviewer for the time and the dedication to review our manuscript and for the helpful comments.

We thank the Reviewer for the suggestion to make the data more comprehensible by including the requested information. Following this suggestions, we changed Figure 1 to Table 1 and included there all relevant pregnancy characteristics and all investigated mouse strains (Table 1A).

We added the headline “ultrasound measurements” to Table 1B) to clarify that these results were obtained by Vevo measurements.

Following the suggestions, we also included more WT mice to ensure that we have always comparable Vevo measurements with the other strains at the same gestational days. As for the question, the number of pregnancies between B) and C) varies because C) is a summary of all matings within the indicated strains at gd16/17 while in B) only matings that were followed by ultrasound were included. As not all animals were followed up by ultrasound because of animal regulations and because we harvested samples at different gestational days, the number of measured animals is always lower than the total number of animals included in the experiment.

The previous Figure 1A was put in the supplement (Supplementary Figure 1A). Also as suggested, we now only one digit after the point and added the SD.

2) Figure 2: legend : the number of n per group is not clear. What “n=4-6 dams and n=3-11 implantation/dams per day” means ?

Answer:

For the experiments, 4-6 dams were included. On gd5, usually only 3-5 implantations were detectable via ultrasound due to their small size. On gd8 and gd10, the size of the implantations increased and therefore, the chance to detect and measure them. This is why, depending from the investigated strain, 6-11 implantations were detectable.

3) Figure 5 : Not having all the groups at all the concentrations make the reading of the figure very complicated.

Answer:

We apologize for this. After discussing this internally, we have now updated the Figure and used different colors to better visualize the different strains. We hope that this is now satisfactory.

4) There is a huge imbalance between the quality of the introduction and the discussion. Please, enrich a bit the introduction.

Answer:

Thanks for this suggestion. We have now enriched the introduction with knowledge about ultrasound and intrauterine growth restriction.

Minor comments :

- please rewrite the first sentence of the introduction.

- the figures are in the wrong order : 1-5-2-3-4

- define “gd” for gestational day in the abstract.

Answer:

Thanks for these suggestions that helped improving the quality of the paper. We rewrote the first sentence of the introduction. We apologize for the wrong order of the figures. Please note that they have now changed as for your suggestions. We defined “gd” for gestational day in the abstract.

Reviewer 2

Advance Summary and Potential Significance to Field:

Thank you for the opportunity to review the interesting work by Busse and colleagues. This study investigates the effect of various B cell related deletions on successful implantation and susceptibility to preterm birth. By highlighting the necessity of the individual pathways to correct implantation, the study offers an important contribution to our understanding of uterine immunity in reproductive health. We think that some important information on the pregnancy outcomes should be added in order to draw conclusions on how the different pathways affect fetal development.

Reviewer 2 Comments for the Author:

1. Figure 1B: The table only gives information on BIL-10-/- mice. Does this mean that the number of pregnancies with resorptions was unchanged in CD19-/-, BMyD88-/-, MyD88-/-? Please include the information on all mice strains. Consider combining this information in a single table for a more clear overview.

Answer:

We would like to thank this Reviewer for the time and the dedication to review our manuscript. Also thanks for addressing this point. We changed Figure 1 to Table 1, which now includes all relevant general pregnancy characteristics of all investigated mouse strains (Table 1A). Results which were obtained by Vevo measurements are shown in Table 1B for a better clarification.

2. Page 5 Lines 32 – 34: Is this a commonly used technique? If so, please include references. Do you see in your data that fetal size (during gestation and at the end of the experiment) is indeed reduced when the implantation sites where smaller? Did you weigh the pups on gd17?

Answer:

Thanks for the question, Yes, this is commonly used. We have now included references. Indeed, the pups from WT dams are bigger than the ones from the other strains. We now include the weights from the pups on gd17 and added the information as Supplementary Figure 2.

3. Line 23-25: According to the text, the authors could discriminate between fetus, placenta and decidua basalis at gd10. Thus, this fits with the analysis of the implantation sites (Fig2.) Consider adding the information of Figure 4 to Figure 2 or otherwise switching Figure 3 and 4. The part on uterine artery resistance now breaks the train of thought on morphology of the implantation sites.

Answer:

We thank the reviewer for this suggestion. We switched the Figures as suggested and it reall reads better.

4. Figure 3: What's the sample size shown in this figure? Please show dot plots rather than bars.

Answer:

We apologize for this omission. We added the number of dams in the Figure legend (previously Figure 3). We decided to show bars instead of dot plots due to the huge number of strains and gestational days which might result in confusion. As the data arise from multiple measurements for each animal and several animals per group, the use of bars is correct.

5. Figure 4 is now restricted to assessment of the placenta. An addition of parameters on fetal morphology would be valuable.

Answer:

We thank the Reviewer for the suggestion to add parameters of fetal morphology. We measured the size of the amniotic cavity (length and width) which contains the fetus and changes in size as the

fetus grows. A direct measurement of the fetus was not performed since the small -and sometimes not optimally measurable- fetal size in BMyD88$^{-/-}$, MyD88$^{-/-}$ and BIL-10$^{-/-}$ mice.

6. Figure 5 and the respective text: It might be clearer to start with the lowest concentration of LPS which does only induce preterm delivery in the two most susceptible strains.

Answer: When discussing this issue within our team, we considered that it would be relevant to start with the highest LPS concentration to show that basically it is possible to induce preterm delivery in WT mice if the concentration of applied LPS is high enough. By decreasing the LPS concentration we showed that no fetal death is taking place in the WT while CD19-/- and B<MyD88-/- still have suffer from fetal death and so we could reveal the importance of the B cells and B cell-specific molecules involved in LPS-induced preterm delivery.

7. Page 8, line 12: Relates to previous comment regarding Figure 4. Consider adding fetus size rather than the complete implantation site when referring to inappropriate fetal growth. Page 10, line 10 – 15: See previous comments, where are the data on fetal size shown?

Answer:

As for gd 5, the complete implantation is detectable already at this stage. It would be however technically not very accurate to measure fetus (at this stage, still embryo). Measuring whole implantation sites allows to compare the growth of the fetus and placental structures, which are absolutely necessary for fetal survival, on gd5 and gd8. Due to the decreased implantation and fetus size in BMyD88$^{-/-}$, MyD88$^{-/-}$ and BIL-10$^{-/-}$ mice, we would be able to measure the size of the amniotic cavity with accuracy only from gd10 on and would thereby lose the important parameters from earlier pregnancy days.

8. Page 10, line 17-24: According to Figure 2E, it seems like BMyD88 deficiency affects the size of the implantation site to a lesser extent than BIL10-/-. Are the BMyD88-/- mice still able to produce IL-10 (through a different way of activation)?

Answer:

This is a really great question. We have preliminary data from ongoing studies that suggest that cells from BMyD88-/- mice are able to produce IL-10, but significantly less than WT dams. Please see this confidential information here. This, however, does not change the conclusions made in this paper.

We have removed unpublished data provided for the referees in confidence.

9. Related to this, are there any controls to show that the knockouts lack indeed the desired pathways?

Answer:

Yes, we checked the genotype of the individual KO mice (CD19 and MyD88 or CD19 and IL-10) before including them in our study. Moreover, among several other parameters, expression of CD19 and absence of IL-10 production by peritoneal and splenic B cells following 5h stimulation with PMA+ionomycin+Brefeldin A were measured by flow cytometry.

10. Page 13, line 4-5: How was blinding achieved? Does this software allow for unbiased stereology?

Answer:

When measuring the mouse, only a number (mouse identification number), which does not allow to draw any conclusions about the strain, and the gestational day was added to the software. The software allows unbiased stereology. Besides, only one operator (Dr. Busse) performed the measurements to avoid operator variances.

11. Discussion: It is remarkable that BIL-10 -/- mice showed no difference in number of implantation sites but are severely affected when assessing implantation areas and uterine artery resistance. How do the authors explain this discrepancy?

Answer:

Thanks for this question. Indeed, it is not a discrepancy, they are rather different parameters that give information in either number of implantations regardless their size or implantation areas regardless how many implantations a given female has.

The number of implantations in BIL-10 -/- mice is decreased, just not statistically significant. On the other site, it might be reasonable to speculate that WT mice having more implantations might have also smaller fetuses since there is only limited space for them. But indeed, it is the other way, they have more and bigger implantations, indicating that the lack of IL-10 signaling in B cells affect implantation size. Alterations in uterine artery resistance are found also in human pregnancies complicated by PE or IUGR. It remains to be investigated how IL-10-producing B cells contribute to these pathologies.

Page 11, line 25-28: Did you check the phenotype of the individual KO mices' B cells or their capacity to produce IL-10 after in vitro stimulation?

Answer:

We used MyD88-/- mice to show that the BMyD88-/- mice lack indeed MyD88. Deficiency of IL-10 expression by B cells was confirmed by flow cytometry of B cells, with T cells used as controls following 5h stimulation with PMA+ionomycin+Brefeldin A. IL-10 was measured by flow cytometry. We further checked the genotype of the individual KO mice (CD19 and MyD88 or CD19 and IL-10) before including them in our study.

Other points:

a) Page 5 line 13: Consider showing only 1 decimal (instead of 8.733, might be confused with thousand delimitator)

Answer: Thanks for this suggestion. We now show only one decimal.

b) Figure 5: In the previous figures, the x axis was labeled. These figures were easier to read. To be consistent, consider adding the labels (in the same sequence as the other figures) below the axis in Figure 5, too.

Answer: We labeled the X axis and added colors to improve understanding.

c) Page 5 line 12: Does this include dead pups?

Answer: The dead pups were excluded, we added this information to the Figure Legend.

d) Page 5, line 31; page 8, line 34; page 11 line 10: This should be "have/was shown".

Answer: We apologize for these grammatical errors and corrected them.

e) Page 8, line 33-34: Add "in mice".

Answer: We added "in mice".

f) Page 9, line 15: Please include references

Answer: We included references on page 9, line 15.

g) Page 10, line 1-3, line 27 These sentence are difficult to read, please revise.

Answer: g) We re-wrote the sentences on page 10, lines 1-3 and line 27.

h) What was the motivation to sacrifice at gd17 rather than allowing the mice to deliver naturally?

Answer: When designing the experiments, we aimed to obtain results that allow us to understand the participation of B cells in immune balance during pregnancy and concretely the pathways involved in B cell mediated immune balance. Letting the mice give birth, we would not be able to obtain material (placenta, fetuses) as they are not available after natural birth. Besides, the situation in terms of cell activation, cytokine secretion would not be comparable among the groups as some will give birth earlier.

We have recently shown in human maternal and cord blood, that IL-10 producing B cells are decreased in preterm birth. Therefore, we addressed the question whether the deficiency of IL-10 producing B cells or the deficiency of B cell-specific MyD88 expression (MyD88 is an important molecule in the signal transduction following LPS stimulation and this is often used to generate murine B10 cells) alters the susceptibility towards LPS-induced preterm delivery.

Title: Rather than fetal development, consider referring to placentation/development of the implantation site as there is currently limited focus on the pups. This might change if more details on fetal outcomes are added.

Answer: We hope now that we have provided enough parameters on fetal size/ weight of the pups to prove that we determined alterations in fetal development and would like therefore to keep the original title.

We would like to thank both Reviewers and the Editor for the helpful comments that helped improving the quality of our paper and also making it more amenable to read for the broad audience of “Development”.

Second decision letter

MS ID#: DEVELOP/2021/199783

MS TITLE: Maternal B cell signaling orchestrates fetal development in mice

AUTHORS: Mandy Busse, Stefanie Langwisch, Kerry Tedford, Klaus Dieter Fischer, and Ana Claudia Zenclussen
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The question addressed in the manuscript was to understand whether maternal B cell absence or B cell impaired signaling (TLR or IL-10 secretion) had effects on several gestational outcomes such as number of implantation, placental thickness, diameter and area, uterine artery resistance and fetal growth.
They show that: 1) B cell specific MyD88 and IL-10 signaling is necessary for an appropriate development in utero 2) IL-10 production by B cells is needed for uterine blood flow regulation during gestation. 3) alterations in B cell-specific expression of CD19, MyD88 and IL-10 enhance the susceptibility towards LPS-induced preterm delivery. This paper contribute to the understanding of

the immunology during pregnancy, highliting cellular populations and signaling pathways essential for a successful pregnancy.

Comments for the author

Thanks to the authors for taking my suggestions into account. The manuscript has evolved a lot, greatly facilitating reading and understanding.
I do not have any other further concerns.

Reviewer 2

Advance summary and potential significance to field

We thank the authors for addressing all our queries well. The article presents valuable results and an important contribution to the field. Congratulations on this manuscript.

Comments for the author

Minor remarks:
- The authors confirm that various flow-cytometry based control experiments have been performed. This is noteworthy and only strengthens the presented work. Please include this information in the Methods and Supplementary Material.
- p.4 l.29 should be “a” instead of “an”, Discussion p.10, l.7 “deficiencies”,
p.10, l.10: suggestion to add “on pregnancy outcome” after “the impact of B cell subpopulations”.
- p.10, l.16: “… such as CD28” overexpression/reduction? Please clarify.
- p.11, l.1 “shown” instead of “showed”
- p.11, l.1-3: Suggestion to change the sequence of this sentence: “… IL-10-producing B cells with regulatory capacity expanded in the uterine B cell population, which was shown by…”
- p.13, l.30: “Even though” instead of “despite”