

Chronic schistosomiasis during pregnancy epigenetically reprograms T-cell differentiation in offspring of infected mothers

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Handling Executive Committee member: Prof. Maria Yazdanbakhsh

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision - 21-Dec-2016

Dear Dr. Prazeres da Costa,

Manuscript ID ej.201646836 entitled "CHRONIC HELMINTH INFECTION DURING PREGNANCY EPIGENETICALLY REPROGRAMMS T CELL DIFFERENTIATION IN OFFSPRING" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referees concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this

will result in delays in the re-review process.**

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Nadja Bakocevic

On behalf of Prof. Maria Yazdanbakhsh

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Reviewer: 1

Comments to the Author

This is an interesting paper and nicely relates work in the mouse model to studies in humans.

In line with this, it would be helpful in the abstract to mention that this work was conducted in the mouse model; also in the introduction to be clear as to which work was conducted in human populations and which in mouse models.

As you point out, DNA methylation is understood to have an important role in T cell differentiation - was it not possible for you to look at this, alongside the histone modifications?

I defer to those with expertise in mouse models to provide more comments on technical aspects of the experiments.

Reviewer: 2

Comments to the Author

The paper by Klar et al focusses on epigenetic imprinting of the neonatal immune system by maternal schistosoma infections in mice. This is a follow-up study on a previous observation that different phases of maternal schistosoma infections (pre-patent, acute or chronic infection) lead to a differential susceptibility or protection against allergic airway inflammation. Here the authors present experiments in which they investigate the role of in utero epigenetic imprinting of the immune system in offspring. Naive T cells were isolated and stimulated under Th1 or Th2 differentiating conditions and subsequent analysis showed less Th2 differentiation in offspring from chronically infected mothers. This corresponded to less histone acetylation at the promoter of Th2 cytokines.

The data presented here provide novel data and as such are interesting, though there are a few unclarities:

- it is unclear at what moment the naive T cells from the offspring were analysed, i.e. at what age? Is the epigenetic imprinting stable over time in the offspring?
- the stimulation protocol to reach differentiated Th1 or Th2 cells is unclear. In what way are the sorted naive T cells stimulated (the polarizing conditions are clear, but not the activation, cell numbers, length of stimulation)?
- What are the raw cytokines levels (pg/ml) in the stimulated cultures in Fig 2? Now only ratios are presented.
- In Fig 2 in some displays sufficient data points are provided (e.g Th2 development), but for others only very few data points (i.e. mice) are displayed and showing a considerable spread, e.g. IFN γ , T-bet and GATA-3. How many separate litters do these data points represent? I assume that offspring from at least 3 different litters should be analysed to provide representative data.
- Chronic helminth infections are linked to an increased immune regulation by e.g. Treg and Breg cells. It would be interesting to see in what way histone modification linked to immune regulation e.g. the cytokine IL-10, TGF β or FoxP3 are influenced in the offspring?

First Revision – authors' response - 03-Feb-2017

Point-by-point letter:

We thank the reviewers for taking the time to thoroughly read our manuscript and are pleased that they acknowledge the novelty of our work. We have addressed all the raised issues and our answers are detailed in the sections below.

Reviewer #1:

This is an interesting paper and nicely relates work in the mouse model to studies in humans.

1. In line with this, it would be helpful in the abstract to mention that this work was conducted in the mouse model; also in the introduction to be clear as to which work was conducted in human populations and which in mouse models.

We thank the reviewer for this comment regarding the clarity of the conducted experiments and modified the abstract (page 2, lines 44 and 46) as well as the introduction (pages 3 and 4, lines 65, 71, 73, 77, 85) accordingly.

2. As you point out, DNA methylation is understood to have an important role in T cell differentiation - was it not possible for you to look at this, alongside the histone modifications?

We agree that this is a very interesting point. The number of naïve T-cells (CD4⁺CD62L⁺) of individual mice obtained from spleens via MoFlo or MACS sorting for the epigenetic experiments were not high enough to perform both chromatin immunoprecipitation (ChIP) and DNA methylation via pyrosequencing simultaneously. For ChIP, we needed about 1x10⁵ cells for *each antibody* used. To cover all histone acetylation changes possible, 3 different antibodies per histone were used. Since we investigated histone acetylation at both histones H3 and H4 (Pan acetylation) and used a mock control antibody (IgG) and two input controls (IC) to ensure the reliability and repeatability of our data for each sample, we needed almost 1x10⁶ CD4⁺CD62L⁺ T cells per mouse. Thus, it would not have been feasible for us to spare enough CD4⁺CD62L⁺ T cells to run DNA methylation studies simultaneously to our epigenetic and functional *in vitro* studies. Thus, we are currently setting up the ATAC method (assay for Transposase Accessible Chromatin Buenrostro et al. Nat Methods 2013) which allows high throughput sequencing of smaller cell samples (1x10⁴).

Reviewer #2:

The paper by Klar et al focusses on epigenetic imprinting of the neonatal immune system by maternal schistosoma infections in mice. This is a follow-up study on a previous observation that different phases of maternal schistosoma infections (pre-patent, acute or chronic infection) lead to a differential susceptibility or protection against allergic airway inflammation. Here the authors present experiments in which they investigate the role of *in utero* epigenetic imprinting of the immune system in offspring. Naïve T cells were isolated and stimulated under Th1 or Th2 differentiating conditions and subsequent analysis showed less Th2 differentiation in offspring from chronically infected mothers. This corresponded to less histone acetylation at the promoter of Th2 cytokines.

The data presented here provide novel data and as such are interesting, though there are a few unclarities:

1. It is unclear at what moment the naïve T cells from the offspring were analysed, i.e. at what age? Is the epigenetic imprinting stable over time in the offspring?

This is a valid point and important question. In our experiments, offspring were analyzed at the age of 8 weeks. This is now stated in the material and methods part. Due to the fact that mice in that age already developed a mature immune system we have good evidence that this effect is stable over time. This is also enforced by the results of our previous paper, showing effects on the

development of allergic airway inflammation in 6-8 weeks old, adult offspring from schistosome infected female mice (Straubinger et al., JACI, 2014). Thus we argue, that our observations are not only a transient effect, but rather impact the immunity of these offsprings throughout life and might impact the responses against a variety of immunological challenges.

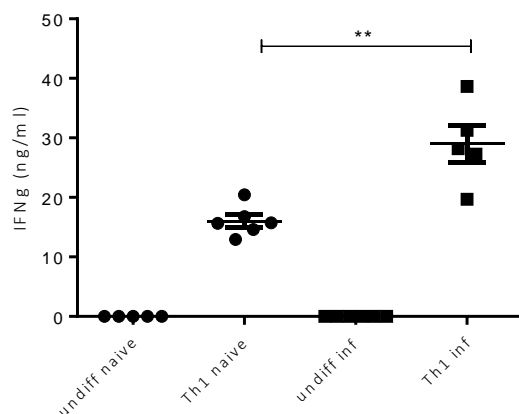
2. The stimulation protocol to reach differentiated Th1 or Th2 cells is unclear. In what way are the sorted naïve T cells stimulated (the polarizing conditions are clear, but not the activation, cell numbers, length of stimulation)?

We greatly appreciate the detailed and constructive comment of the reviewer regarding the T cell differentiation protocol. We modified the part of the T cell differentiation assays and added cell numbers and length of stimulation (page 8, line 189 – 194).

For T_H1 conditions, 2×10^6 splenocytes (FACS and RT-PCR/ELISA analysis) were incubated with $10 \mu\text{g/ml}$ $\alpha\text{-IL-4}$, 20 ng/ml IL-12 and 20 ng/ml IFN- γ for 3 days. For the differentiation control a T_H0 /undiff condition was performed with $10 \mu\text{g/ml}$ $\alpha\text{-IFN-}\gamma$ and $10 \mu\text{g/ml}$ $\alpha\text{-IL-4}$ for 3 days, respectively. For T_H2 conditions, $10 \mu\text{g/ml}$ $\alpha\text{-IFN-}\gamma$, $10 \mu\text{g/ml}$ $\alpha\text{-IL-12}$ and 25 ng/ml IL-4 were added to 3×10^6 splenocytes (FACS-analysis) or 1.5×10^6 (RT-PCR/ ELISA) for 6 days and again for the differentiation control a T_H0 /undiff condition was performed with $10 \mu\text{g/ml}$ $\alpha\text{-IFN-}\gamma$ and $10 \mu\text{g/ml}$ $\alpha\text{-IL-4}$ for 6 days, respectively.

3. What are the raw cytokines levels (pg/ml) in the stimulated cultures in Fig 2? Now only ratios are presented.

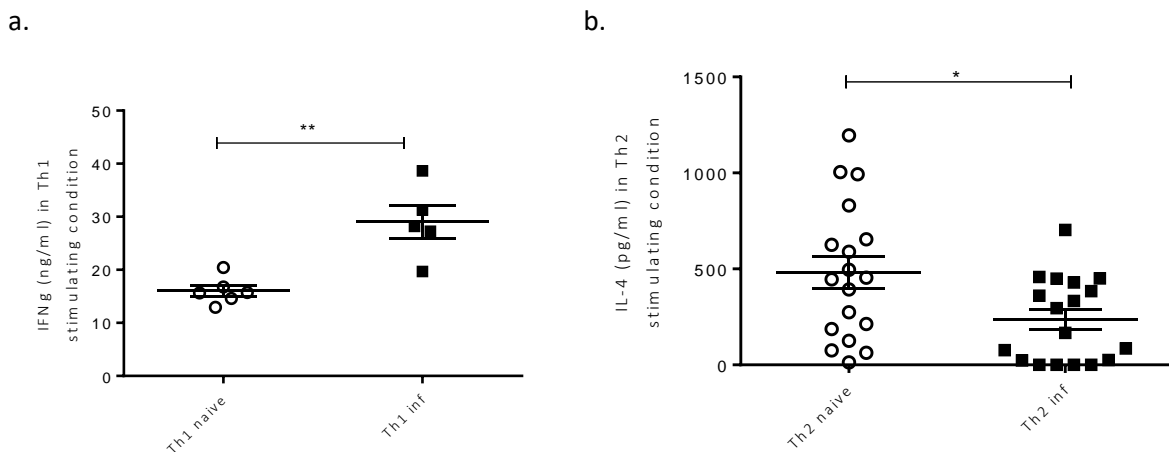
We greatly appreciate this question by the reviewer. Indeed we spotted a mistake in our manuscript with regards to the IFN- γ ELISA data. By mistake we depicted the graph with the raw cytokine data (ng/ml) and labeled the axis wrong. However, our conclusion of an increased Th1 differentiation capacity would not have been different as you can see from the raw data below (in fact, when building the fold change (undiff/Th1) the values would have been even greater):



Maternal *S. mansoni* infection leads to stronger T_H1 cell differentiation of $CD4^+CD62L^+$ T cells. Cytokine levels of IFN- γ under Th0 (undiff.) and Th1 conditions in offspring from naïve and infected mothers are detected by ELISA. Asterisks show statistical differences indicated by brackets. (** $p < 0.01$).

Data shown as mean \pm SEM depicting one of two representative experiments. (Offspring: $n \geq 5$, mothers: $n \geq 3$ per group).

For the Th1 condition, IFN- γ levels ranged (under Th1 differentiating condition) from 15pg/ml to 38.63 ng/ml. For the Th2 condition, IL-4 levels ranged (under Th2 differentiating conditions) from 15pg/ml (according to sensitivity and detection limit) to 495.00 pg/ml. After revising this issue we decided to show this results as raw cytokine data for Th1 and Th2 differentiated conditions after background subtraction of undifferentiated T cells, respectively. We agree with the reviewer that this way the reader does not have to question the range of the values and can clearly see the effect in a less complicated graph. Thus, and as the reviewer suggested we exchanged the graphs in the manuscript to show cytokine levels in the stimulated cultures. Please see new Figures 3 b and e (and graphs below) and corresponding adjustments in the figure legend (pages 14+15, Fig.2).



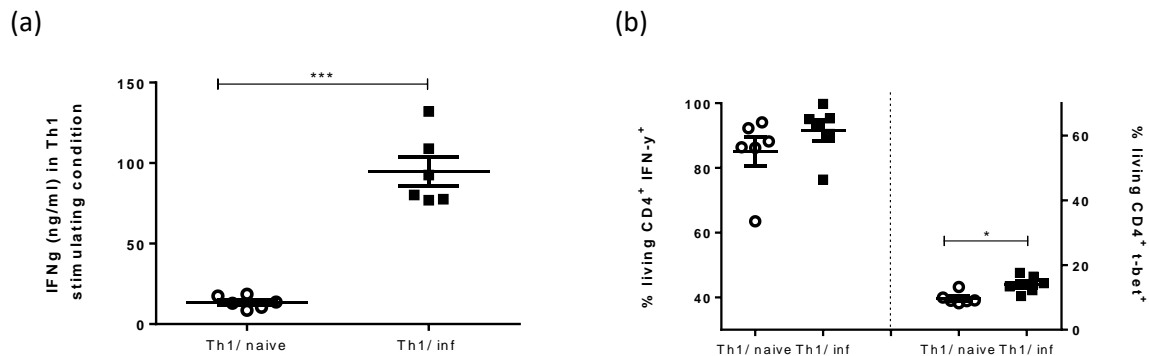
Maternal *S. mansoni* infection leads to stronger T_H1 and impaired T_H2 cell differentiation of $CD4^+CD62L^+$ T cells. Cytokine levels of IFN- γ (a) and IL-4 (b) under Th1 and Th2 differentiating conditions are detected by ELISA and shown after subtraction of corresponding cytokine levels of undifferentiated T cells. Asterisks show statistical differences indicated by brackets. (* $p < 0.05$, ** $p < 0.01$). Data shown as mean \pm SEM depicting one of two representative experiments (IFN- γ) or pooled data from two experiments with similar outcomes (IL-4). Each experiment was conducted with offspring: $n \geq 5$, mothers: $n \geq 3$ per group.

4. In Fig 2 in some displays sufficient data points are provided (e.g Th2 development), but for others only very few data points (i.e. mice) are displayed and showing a considerable spread, e.g. IFN- γ , T-bet and GATA-3. How many separate litters do these data points represent? I assume that offspring from at least 3 different litters should be analysed to provide representative data.

We thank the reviewer for spotting these differences. Our conclusions on the results of Fig. 2 b, c, e, f (showing individual symbols) were drawn on the basis of two independent experiments with similar outcomes (meaning two experiments for Th1 and two experiments for Th2 conditions). The readouts based on FACS analyses for the Th2 condition were comparable between the two independent experiments, which allowed us to pool the data. For the Th1 condition, the percentage of living $CD4^+$ IFN- γ^+ or t-bet $^+$ cells varied between the two experiments; however, the significant difference in the transcription factor t-bet was present in both experiments. By pooling the data points of the two Th1 condition experiments we will falsely miss the fact of significant differences between the groups due to a high variation. This concept is also true for the readouts in Fig. 2e

showing cytokine levels, which varied between the two Th1 experiments, and thus, prevented us from pooling the data to not oversee the true effect of significant differences. Conclusively, Th1 differentiation *in vitro* is more likely to be influenced by ambient parameters such as cell density or pre-activation during splenocyte preparation, which cannot be fully controlled in lab practice, although we used highly standardized protocols. Importantly, each experiment was conducted with offspring: $n \geq 5$ and mothers: $n \geq 3$ per group.

However, the reviewer is right that this was not stated correctly in the manuscript and we have now changed this in the figure legend on pages 14+15.



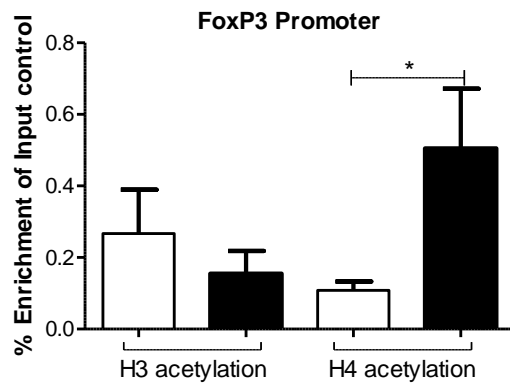
Second representative experiment:

Maternal *S. mansoni* infection leads to a more pronounced Th1 cell differentiation of CD4 $^{+}$ CD62L $^{+}$ T cells. Cytokine levels of IFN- γ under Th1 condition are detected by ELISA (a). Frequencies of CD4 $^{+}$ effector cells were analyzed by ICS for IFN- γ , t-bet (Th1) (b). Asterisks show statistical differences indicated by brackets. (* $p < 0.05$, *** $p < 0.001$). Data shown as mean \pm SEM.

5. Chronic helminth infections are linked to an increased immune regulation by e.g. Treg and Breg cells. It would be interesting to see in what way histone modification linked to immune regulation e.g. the cytokine IL-10, TGF β or FoxP3 are influenced in the offspring?

We strongly agree with the reviewer regarding the aspect of possible changes in immune regulation within these offspring, since this is a hallmark in chronic helminth infection and might be transferred to the offspring. We are currently working on this hypothesis and performed a ChIP based histone acetylation analysis of FoxP3 promoter region of sorted splenic CD4 $^{+}$ CD62L $^{+}$ T cells before differentiation (Fig. 1). Our preliminary data reveals that offspring from chronically infected mothers show increased H4 acetylation Foxp3 promotor region compared to those from naïve offspring. This could translate to active transcription resulting in elevated protein levels and eventually stronger capacity of immune regulation. As we follow the line of argumentation we agree that the concept of altered immune regulation in these offspring is of great interest. As the reviewer mentioned, immune regulation is facilitated not only by Treg cells, but also other immune cell types such as Breg cells. To get a full picture on this aspect we are currently performing experiments addressing these developmental aspects in different cell populations of the offspring of schistosome-infected mothers. These experiments are quite challenging and beyond the scope of this short communication, which mainly focuses on T cells their altered differentiation capacity as a novel

concept and finding. However, we agree that this paper is the basis for further interesting research questions that the reviewer raises.



CD4⁺CD62L⁺ MACS or MoFlow sorted splenocytes from offspring with maternal infection and naive mothers were analyzed by ChIP assay. H3 and H4 acetylation and RT-PCR was performed to assess the percent enrichment at the FoxP3 promoter region. Results were pooled from three independent experiments and depicted as mean \pm SEM. Statistical significances between the indicated groups (offspring: $n = 16$ vs. 16 , mothers: $n = 9$ vs. 6) are obtained after using Mann-Whitney test (FoxP3). (* $p < 0.05$).

References

Straubinger, K., S. Paul, O. Prazeres da Costa, M. Ritter, T. Buch, D. H. Busch, L. E. Layland, and C. U. Prazeres da Costa. 2014. Maternal immune response to helminth infection during pregnancy determines offspring susceptibility to allergic airway inflammation. *Journal of Allergy and Clinical Immunology* 134: 1271-1279.e1210.

Second Editorial Decision - 10-Feb-2017

Dear Dr. Prazeres da Costa,

It is a pleasure to provisionally accept your manuscript entitled "CHRONIC HELMINTH INFECTION DURING PREGNANCY EPIGENETICALLY REPROGRAMS T CELL DIFFERENTIATION IN OFFSPRING" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1521-4141/accepted](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted)). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Nadja Bakocevic

on behalf of
Prof. Maria Yazdanbakhsh

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