Author's response to reviews

Title: Cord blood neutrophils display a galectin-3 responsive phenotype accentuated by vaginal delivery

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Author's response to reviews: see over
Dear Editor,

We thank you and the reviewers’ for your helpful remarks on our manuscript (9658055069551562) entitled “Cord blood neutrophils display a galectin-3 responsive phenotype accentuated by vaginal delivery”. We take this opportunity to reply to the remarks and to submit a revised manuscript to be considered for publication in BMC Pediatrics.

On the subsequent pages of this letter, we provide a response (in plain text) to each of the reviewer’s remarks (in italics).

We hope that the revised manuscript, with changes made according to the reviewer’s remarks, now is acceptable for publication in BMC Pediatrics.

Sincerely yours,

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Referee #1

Major comments and questions:

1. It is not clear how the different cells have been used. Overall, there were 18 CS and 20 vaginal delivery neutrophils, and 33 controls. Since the variation in galectin-3, L-selectin and IL-8 is rather large, it would be valuable to know if there is a correlation between galectin-3 in plasma/blood, ROS production, and any of the other parameters. It is understandable that it was not possible to perform all the analysis on the cord blood cells in parallel, but some correlative analysis could be done.

Reply: The reviewer has a very good point in that it would be interesting to look for correlations between galectin-3 and ROS production or other activation parameters. However, the galectin-3 assay required plasma samples, which were not collected for the primary cohort in which the cell parameters were measured. Thus, we have no data of all parameters in the same donor, as the samples were collected at different time points. We have added this information to the Materials and Methods section (pg. 6).

2. Why was only CS cord, and not vaginal delivery blood analyzed for galectin-3?

Reply: Cord blood plasma obtained both after CS and after vaginal deliveries were in fact analyzed for galectin-3. Galectin-3 levels in plasma obtained from CS is shown in Fig. 1 and Table 1, while levels in plasma from cord blood after vaginal delivery is shown in Table 1.

3. When discussing the mechanisms for the priming status and role of galectin-3, one could discuss their earlier data showing that alkaline phosphatase increased in CS, reflecting some degranulation (gelatinase?). Is there some evidence for degranulation in the neonatal neutrophils, or does the "primed" state reflect a maturation process?

Reply: Our previous studies show that there is an increase in ALP in cord blood neutrophils from both groups, CS and vaginal delivery, although published in two different papers; Khalfan L et al, Pediatr Allergy Immunol, 1992, DOI: 10.1111/j.1399-3038.1992.tb00032.x, and Khalfan L et al, Pediatr Allergy Immunol, 1995, PMID: 8750312. The data also indicated that after vaginal delivery, the neutrophils are primed in response to fMLF, and the conclusion drawn was that increased ALP was correlated to increased fMLF response (priming). However, in CS neutrophils, that also were high in ALP, there was no increase in fMLF response, showing that the ALP activity is uncoupled from the priming associated with vaginal delivery. We have in fact measured ALP in the present material, and found the levels to be equally high between the CS and the vaginal delivery cohorts, thus strengthening this conclusion. For sake of clarity regarding the presented data, we have chosen not to show those data in the current manuscript. With regard to the reviewers question on evidence for degranulation in neonatal neutrophils, we have no proof of a degranulation process taking place in the cord blood cells. However, the fact that they show and increased response to galectin-3 strongly indicates prior degranulation, as a prerequisite for a galectin-3 response in adults is that the cells have mobilized granules, described by us in Karlsson A et al, Blood, 1998, PMID: 9558402.

We have added a sentence stating the possibility of degranulation associated with pre-priming in cord blood neutrophils to the Discussion (pg. 12).

4. The authors say have put forward the idea that icROS can suppress inflammation (ref 24). Is the increased icROS in neonatal cells in fact a pro-inflammatory or anti-inflammatory response?

Reply: The field of intracellular ROS production and its importance in inflammation is expanding right now, and there are indications that icROS can affect innate immune processes in both a pro-inflammatory and an anti-inflammatory manner. The idea that icROS can suppress inflammation is primarily based on two reports where patients presenting a specific decrease/lack of icROS, but not ecROS, suffer from hyper-inflammation. The first report (Matute JD et al., Blood, 2009, PMID: 19692703) describes a selective deficiency in icROS in a patient with a novel form of chronic granulomatous disease (CGD), where the NADPH-oxidase lacked a functional p40phox subunit. This specific lack of icROS production correlated with a hyper-inflammatory phenotype (granulomatous
colitis) in this patient. The second report (Ferguson PI et al, Arthritis Rheum, 2008, PMID: 18821685) describes the inflammatory symptoms in a patient suffering from SAPHO (Synovitis Acne Pustulosis Hyperostosis Osteitis), a syndrome associated with strong pro-inflammatory features, and that was shown to produce decreased amounts of, specifically, icROS. These studies can be interpreted as if icROS under normal circumstances (in healthy individuals) suppress inflammation.

However, we have recent data on neutrophils isolated from patients suffering from the autoinflammatory periodic fever syndrome PFAPA (Periodic Fever Aphthous stomatitis Pharyngitis cervical Adenitis), where an increased icROS production (with unaltered ecROS production) is associated with the hyper-inflammatory state of these patients during febrile flares (Sundqvist M et al, Ann Paeditr Rheum, 2012, DOI: 10.5455/apr.112620120441 (abstract), this study is currently a manuscript under revision for publication in Arthritis Rheum, and can be sent to the reviewer if requested). These data thus give another conclusion than above; that icROS is a pro-inflammatory signal.

For the neonatal neutrophils after vaginal delivery, the increased icROS production is associated with an increase in L-selectin cleavage, suggesting that the cells are primed, which per se is a pro-inflammatory feature as it leads to increased responsiveness and thereby risk for tissue damage. We thus lean toward that the increased icROS production is of pro-inflammatory nature in the neonatal cells, but this remains to be further studied.

We have increased our discussion on icROS as a regulator of inflammation, in the Discussion (pg. 13).

5. **Minor point. Define “GPCR”**.

**Reply:** The abbreviation GPCR has been defined on pg. 4 of the manuscript.
Referee #2

Major comments and questions:

1. Authors state that the neutrophils in the adults are in the “resting state” and furthermore state that the cord blood neutrophils obtained from the CS delivery are also in the resting state with obviously some difference in response to galectin-3. Can neutrophils in the context of pregnancy (an immune tolerance model) be considered in a resting state (Page 9, para 3)?

Reply: The reviewer is of course right in that neutrophils in the context of pregnancy cannot be considered to be in a resting state, or resting environment perhaps. The text that is referred to is “As previously shown, resting adult neutrophils were unresponsive to galectin-3, while their LPS-primed phenotype responded readily [5] (Fig. 2A). Interestingly, for the CSCB neutrophils the picture was different. The resting cells responded (Fig. 2A) at levels slightly above but significantly increased as compared to adult neutrophils (Fig. 2B)”. We understand that our wording has made the text somewhat confusing. By resting CSCB cells we meant resting in the context of absence of in vitro priming.

We have changed the word “resting” to “non-stimulated” in the Result section (pg. 9) in order to clarify our reasoning.

2. Galectin-3 levels in the plasma: In figure 1 and Table 1, the investigators reported galectin-3 levels in cord blood derived from 8 samples from CS and VD, while they obtained 18 samples from caesarean delivery and 20 samples from VD. Provide complete data on all samples to be able to make meaningful conclusions.

Reply: Please see the response to reviewer #1, question 1. The samples for cell analysis and galectin-3 levels in plasma were collected at different time points, and we did not collect plasma from the first cohort, making it impossible to provide data on galectin-3 levels in those samples. We have added this information to the Materials and Methods section (pg. 6).

3. Galectin and ROS production: Plasma samples from VD had lower galectin-3 levels compared to CS (Table 1), however the ROS production measured in-vitro was significantly higher compared to CS samples (Figure 5). Is this response found exclusively in an in-vitro model? What is the relevance in an in-vivo setting?

Reply: There is no significant difference in the levels of plasma galectin-3 between VD and CS samples (Table 1; ns = no statistically significant difference), while there is a difference in ROS production in response to exogenous galectin-3. This indicates that the galectin-3 levels in plasma are not altered by the same mechanisms that drive neutrophil priming, but is a delivery-independent feature in the circulation of the fetus at a time point close to delivery.

We have added this conclusion to the Discussion (pg. 13).

Our ex-vivo system is, we believe, the model closest to an in-vivo setting as can possibly be tested. The in-vivo situation can never be fully appreciated by experimentation, but the relevance of the ex-vivo findings can nevertheless be extrapolated to a theoretical understanding of reality. Thus, we believe that the data on neutrophil priming and galectin-3 levels in plasma are relevant in an in-vivo setting, and have referred in the text to a number of others studies that support the presence of such features in other settings of inflammation (e.g., Yektaei-Karin E et al, Pediatr Allergy Immunol, 2007, PMID: 18078418, Frolova L et al, Inflamm Res, 2009, PMID: 19271150, Malamitsi-Puchner A et al, Early Hum Dev, 2005, PMID: 15814224, Khalfan L et al, Pediatr Allergy Immunol, 1992, DOI: 10.1111/j.1399-3038.1992.tb00032.x, Khalfan L et al, Pediatr Allergy Immunol, 1995, PMID: 8750312, Demmert M et al, Clin Exp Immunol, 2012, PMID: 22236000).

4. The most important question to address is the biological significance of the findings in this study? If blood samples were to be obtained after birth (CS and VD), would the investigators have found similar results? Are the findings merely a reflection of the fetal milieu? No additional data was
provided with regard to maternal inflammatory response (MIRS) or fetal inflammatory response syndrome. This is particularly important as CBVD neutrophil phenotype was different compared to CBCS.

Reply: We believe that the biological significance of our findings is that the differences in neutrophil physiology between CS and VD may be of importance in the neonate’s first interaction with the outer world and may thus have implications for development of immunity. Also, the fact that cord blood neutrophils after CS are different from neutrophils in adults may be a basis for an increased understanding of neonatal innate immunity and immune defence.

We have changed the text in the conclusion in order to more clearly describe our reasoning regarding these matters (pg. 15).

The question of whether a neonate shows the same results after birth can only be speculated upon, since it is not possible to test this due to the unavailability of blood samples of needed size from healthy babies. The transfer into an environment outside the womb will affect the innate immune defence in a number of ways, in part due to the exposure to and establishment of commensal flora. Possibly, babies delivered vaginally may have a different initial preparedness for this exposure (indicated by our result that these cells are more primed).

We have included this last part in our conclusion (pg. 15).

With regard to MIRS and fetal inflammatory response syndrome (FIRS), all samples in this study are collected from healthy babies with healthy mothers. The MIRS and FIRS are very rare conditions and fall outside the scope of this study in its entirety.

We have included a sentence to the Materials and Methods section stating that all mothers included in the study were previously healthy and without signs of infection at time of delivery (pg. 6).

5. To suggest that the changes in the neutrophil phenotype are in anticipation of “infectious challenges” is incomprehensible

Reply: We have rephrased the sentence in the conclusion to more carefully describe our hypothesis derived from the achieved data (pg. 15).