Development of follicular dendritic cells in lymph nodes depends on retinoic acid-mediated signaling

Jasper J. Koning, Anusha Rajaraman, Rogier M. Reijmers, Tanja Konijn, Junliang Pan, Carl F. Ware, Eugene C. Butcher and Reina E. Mebius
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Original submission

First decision letter

MS ID#: DEVELOP/2021/199713

MS TITLE: Development of follicular dendritic cells in lymph nodes depends on retinoic acid mediated signaling

AUTHORS: Jasper J Koning, Anusha Rajaraman, Rogier M Reijmers, Tanja Konijn, Junliang Pan, Carl F Ware, Eugene C Butcher, and Reina E Mebius

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

It has been previously shown that retinoic acid (RA) is essential for induction of early expression of CXCL13 which attracts lymphoid tissue-inducer (LTi) cells and is essential for lymph node development, in stromal organizer cells during ontogeny (van de Pavert et al., Nat.Immunol.2009). LTi cells produce lymphotoxins which act on stromal organizer cells to differentiate into lymph node mesenchymal stromal cells, including CCL19-expressing fibroblastic reticular cells (FRCs) in T cell zone, CXCL13-expressing follicular dendritic cells (FDCs) in germinal centers (GC), and marginal reticular cells (MRCs) in B cell zone located outside the GC. However, roles of RA in lymph node development after birth remain unclear.
In this paper, the authors used in vitro cultures of mouse embryonic mesenchymal cells and showed that RA induced differentiation of embryonic mesenchymal cells into the CXCL13-expressing FDCs and inhibited their lymphotoxin-mediated differentiation into CCL19-expressing FRCs. In addition, they showed that CXCL13 expression was reduced at the age of 2 weeks when RA receptor (RAR) signaling was blocked in Nestin-expressing cells early after birth using Nestin-CreERT2-RAR-dominant negative (DN) mice.
Furthermore, the authors showed that treatment of animals with RAR inhibitor BMS493 from day 4 after birth caused reduction in expression of CXCL13 and RARbeta, ratio of B cell follicle volume over total lymph node volume, and generation of FDCs, but did not alter total lymph node volume and expression of CCL19 in LNs. This is an interesting paper, showing the role of RA in FDC development after birth.

Comments for the author

1. The authors should show which cells express Cre in lymph nodes in 2 week-old tamoxifen-treated Nestin-CreERT2-RAR-DN mice or Nestin-CreERT2 mice crossed to reporter mice by histological analysis.
2. The authors should show expression levels of Stra8 (a target of RA), CXCL13, CXCL12, GP38, IL-7, CCL19 and CCL21 in sorted lymph node stromal cells, such as CD45-Ter119-CD31-gp38+ cells in control and BMS493-treated animals.
3. The role of CXCL13 in lymph node development is different between mesenteric and cervical lymph nodes and other lymph nodes. How about the development of FDCs in mesenteric and cervical lymph node in CXCL13 deficient mice?

Minor points
1. Line 230, Please spell out “DN”.
2. I could not see any asterix in Figure 2B.
3. The authors should describe which lymph nodes (MLN?) are shown in Figure 5B-D.
4. Please describe the age of mice shown in Figure 5C in the Figure legend.

Reviewer 2

Advance summary and potential significance to field

Our understanding of the origins of lymphoid stromal cells are still in their infancy. Some key signalling pathways have been identified, but the ontogeny and specific signals for each stromal subtype are not fully understood. This manuscripts describes the specific requirement of retinoid acid signalling for the development of FDCs, and defines the timescale during which these signals are required during lymph node development.

Comments for the author

As presented, this manuscript is intriguing and important, but is not fully convincing in some areas. Additional experiment will make these important findings more robust.
Suggested revisions:
1. The pretreatment with retinoic acid showed a strong block in the induction of FRC specific chemokine and cytokines. but the reasons for this are not fully explored. Can the authors determine which RA-stimulated signalling causes the inhibition of the response to LT stimulation? This question seems to be key to the findings of the paper.
2. The data in figure 2 suggests that at birth stromal cells are mixed, and are edited or further differentiate into FRC and FDCs in the postnatal period. Previous studies have shown that FRC neighbouring B cell follicles in adult lymph nodes can express FDC markers and B cell growth factors such as BAFF. Can the authors extend their analysis in figure 2 to show whether individual stromal cells at this early stage are expressing both FDC and FRC markers, or whether these cells types are already distinct from one another but are situated in the same niche at this time?
3. The data show that RA can be produced in a broad range of cell types, and that expression changes between developing LNs and adult LNs. However these data do not resolve which cell type is the key source prior to birth, or what timing is important for the specification. Can the authors block RA production in DCs or stromal cells (BECs) to determine which is important? If not I would suggest moving this data to a supplementary figure.
4. Genetic experiments to delete RA receptor are lacking controls showing the efficiency of the ablation.
Please show what proportion of cells are targeted. If this approach is very inefficient changing to dosage, the number of doses or the route of administration should improve the deletion efficiency. Currently, since no change in mRNA for Cxcl13 is observed, it is unclear whether this experiment has been technically successful.
5. Data using pharmacological inhibition of RA receptor are very striking. These data could be extended by treating mice at a range of time points both before and after birth, and also comparing LNs after a short term treatment, to see if FDC numbers recover.

First revision

Author response to reviewers' comments

Point by point reply to the reviewers.
We would like to thank the reviewers for their time and feedback on the paper. We will provide our response to the questions and issues raised in blue:

Reviewer 1 Comments for the Author:
1. The authors should show which cells express Cre in lymph nodes in 2 week-old tamoxifen-treated Nestin- CreERT2-RAR-DN mice or Nestin-CreERT2 mice crossed to reporter mice by histological analysis.
We do agree that insight in which cells are being targeted would be useful. Unfortunately, we are currently unable to perform additional experiments to show which cells in lymph nodes are being targeted in tamoxifen treated Nestin-CreERT2 mice when induced from day 5 onwards for 5 consecutive days. Previously, we have shown that induction of Cre expression in Nestin-Cre ERT2 mice at 2 weeks after birth, mainly labeled endothelial cells in lymph nodes when crossed to reporter mice, when analyzed 4 weeks later (Koning et al, Journal of Immunology 2016). Therefore, induction of Cre expression at 2 weeks after birth is too late for targeting FRCs. In the same paper, we also showed that in nestin-GFP mice, at day of birth, the vast majority of stromal cells, including non- endothelial stromal cells, express GFP, indicating that they still express nestin at that time. For this reason, we decided here to induce nestin specific deletion shortly after birth from day 5 onwards for 5 consecutive days.
Others have shown efficient targeting of nestin^{pos} cells in Nes-Cre-ERT2 mice shortly after birth using similar induction protocols (Isern et al, eLife 2014, Ding et al, Gastroenterology, 2020). In our

study, analysis was done when animals were 14 days, or 21 days old and therefore we don't expect to see Cre-ERT2 expression in induced cells anymore, excluding the staining for Cre protein as an approach to address this question using the existing remaining frozen tissues.

2. The authors should show expression levels of Stra8 (a target of RA), CXCL13, CXCL12, GP38, IL-7, CCL19, and CCL21 in sorted lymph node stromal cells, such as CD45-Ter119-CD31-gp38+ cells in control and BMS493- treated animals.
As far as we can find, Stra8 expression is absent in LN FRC (i.e. in datasets of scRNAseq analysis of lymph node FRCs from Perez- Shibayama et al. Science Immunology, 202 and the database from the (https://www.immgen.org/), and its expression is restricted to germ cells. We therefore feel that determining the expression levels of Stra8 will not generate any meaningful data. However, CXCL13 is also a direct target of RA (van de Pavert et al. Nat. Immunol. 2009) and restricted in its expression to follicular dendritic cells and marginal reticular cells, which have been described as precursors for follicular dendritic cells by the Bajénoff lab (Jarjour et al. J.Exp. Med 2014). The other markers, are mainly expressed by T cell area stromal cells and to some extent by (lymphatic) endothelial cells (Cxcl12, Ccl19, podoplanin) or dendritic cells (Ccl19). The effect of BMS493 treatment results in a significant reduction in CXCL13 expression, while the T cell stromal cell/(lymphatic)endothelial associated markers are unaffected. We feel that the effect of BMS493 on CXCL13 expression that we report here would not change if we would repeat this analysis on sorted lymph node stromal cells.

3. The role of CXCL13 in lymph node development is different between mesenteric and cervical lymph nodes and other lymph nodes. How about the development of FDCs in mesenteric and cervical lymph node in CXCL13 deficient mice?
We believe that the reviewer is stating that if CXCL13 is not necessary for all lymph nodes to form, perhaps CXCL13 is not necessary for all FDCs to develop. CXCL13 deficient mice were first described in 2000 by the Cyster group (Ansel et.al. Nature 2000). In this manuscript the authors show that FDCs are absent in the remaining LNs in CXCL13 deficient mice, due to the lack of LTα1ß2 on the cell surface of B cells. LTα1ß2 expression in turn, is induced on B cells via CXCL13. Thus, in the absence of CXCL13, FDCs cannot form, since it is a prerequisite for FDC development.

Minor points
1. Line 230, Please spell out "DN".
We have changed this accordingly

2. I could not see any asterix in Figure 2B.
We have replaced the asterix by arrows to hopefully better indicate the cells of interest.

3. The authors should describe which lymph nodes (MLN?) are shown in Figure 5B-D.
In fig 5B & C we have mentioned that these are peripheral lymph nodes (meaning brachial, axillary and inguinal lymph nodes as specified in the Method section) in the main text as well as in the figure itself. To further clarify which type of lymph nodes are used, we now have mentioned this in the figure legend of Fig 5D as well.

4. Please describe the age of mice shown in Figure 5C in the Figure legend.
We have added this info to the figure legend.

Reviewer 2 Comments for the Author:
As presented, this manuscript is intriguing and important, but is not fully convincing in some areas. Additional experiment will make these important findings more robust.

Suggested revisions:

1. The pretreatment with retinoic acid showed a strong block in the induction of FRC specific chemokine and cytokines. but the reasons for this are not fully explored. Can the authors determine which RA-stimulated signalling causes the inhibition of the response to LT stimulation? This question seems to be key to the findings of the paper.
We agree with the reviewer that identifying the molecular mechanism of RA specific repression of chemokine and cytokine signaling in precursor stromal cells would be extremely interesting. And

this particular point has been the focus for quite a while. However, as RA mediated regulation of gene transcription is quite complex, it turned out to be a question that could not easily be answered.
What we do show is that activation of the alternative NfKb pathway seemed unaffected although nuclear translocation of p52 and RelB was lower (not significant) in cells that were pre-treated with retinoic acid. It might very well be that RA mediated signaling specifically prevents RelB access to particular promotor regions, thereby regulating transcription of FRC specific genes. Although we made several attempts to elaborate this, we were not successful. Because of the complexity of both the Nfkb signaling pathway, as well as the retinoic acid signaling pathway, this is material for additional studies and we leave this question to be answered by others.

2. The data in figure 2 suggests that at birth stromal cells are mixed, and are edited or further differentiate into FRC and FDCs in the postnatal period. Previous studies have shown that FRC neighbouring B cell follicles in adult lymph nodes can express FDC markers and B cell growth factors such as BAFF. Can the authors extend their analysis in figure 2 to show whether individual stromal cells at this early stage are expressing both FDC and FRC markers, or whether these cells types are already distinct from one another but are situated in the same niche at this time?
Indeed, in the dataset from Perez-Shibayama et al (Science Immunol. 2020) T/B zone reticular cells (TBRCs) in **adult naïve lymph nodes**, which are neighboring the B cell follicles, are expressing mRNA for BAFF and Mfge8 while of these TBRCs only one subset (TBRC1) additionally shows CCL19 expression. All TBRCs however lack CXCL13 expression, which is restricted to FDCs, IFRCs and MRCs and all these cells in turn lack BAFF expression. At this adult stage there are no stromal cell subsets that express both CXCL13 and CCL19 and therefore mRNA for CXCL13 and CCL19 form good markers to distinguish T- or T/B reticular stromal cells from FDCs, This is particular relevant, since in the first week after birth there are no good cell surface markers to identify FDCs or their precursors in lymph nodes. Markers that are generally used to identify FRCs, such as podoplanin, are also expressed by FDCs, although at lower level. Markers that are expressed by FDCs, such as Cr2 (8C12), are only expressed by adult, fully mature FDCs and cannot be detected at week 3 of age or earlier. And indeed, we observed that at day of birth, CXCL13 and CCL19 expressing stromal cells are mixed. This is in line with already published data, showing that after birth, segregation of B and T cell areas occurs, which is mediated by chemokines produced by stromal cells (Cupedo et al. J. Immunol. 173:4889-96).

Therefore, in figure 2, we focused on the expression of these specific chemokines which can be used to discriminate between FRCs (CCL19 pos), FDCs (CXCL13pos) and MRCs (Tnfsf11pos). We do show in figure 2 that at day of birth (fig. 2A) there are cells that express both Cxcl13 and Ccl19. Also, 1 week after birth we can find a substantial amount of cells within the presumptive B cell follicle that express both Ccl19 and Cxcl13 (fig. 2B). These cells are now indicated with an arrow to better visualize them. In B cell follicles at later stages, there is a clear distinction in expression of Cxcl13 and Ccl19 with Cxcl13 being expressed by stromal cells in B cell follicles and Ccl19 being expressed by stromal cells outside these areas, in line with observations by others (Perez-Shibayama et al (Science Immunol. 2020).

3. The data show that RA can be produced in a broad range of cell types, and that expression changes between developing LNs and adult LNs. However these data do not resolve which cell type is the key source prior to birth, or what timing is important for the specification. Can the authors block RA production in DCs or stromal cells (BECs) to determine which is important? If not I would suggest moving this data to a supplementary figure.
We have blocked RA **signaling** in both DCs and BECs by intercrossing the RAR-dn with CD11c-Cre-ERT2 as well as Cadh5-Cre-ERT2 mice, which didn't allow us to solve the question raised (data not shown). Of note: overexpression of RAR-dn in a particular cell subset is described to block cell-autonomous RA mediated signaling, but not their RA production.
We have not blocked RA **production** in DCs or BECs as suggested by the reviewer and have explained in the main text the reason for this using the following text; *"The multicellular distribution of retinoic acid producing enzymes imposes an impossibility to specifically assign the importance of these cellular sources for postnatal FDC development since the functional consequences of cell specific Aldh1 deletion in one cell subset, could be rescued by the expression of another Aldh1 enzyme in the same cell type, or by the expression of the same and other enzymes in another subset."*
We therefore feel that all cellular subsets that are shown in figure 3 to express the enzymes that

can convert retinal into retinoic acid can contribute to the generation of retinoic acid in the early week after birth. Therefore we feel that this data is important and should be part of the main text. On this point we are disagreeing with the reviewer that we should move these data to a supplementary figure, since we identify now also endothelial cells as potential source of RA production within lymph nodes, which has not been reported before.

4. Genetic experiments to delete RA receptor are lacking controls showing the efficiency of the ablation. Please show what proportion of cells are targeted. If this approach is very inefficient changing to dosage, the number of doses or the route of administration should improve the deletion efficiency. Currently, since no change in mRNA for Cxcl13 is observed, it is unclear whether this experiment has been technically successful.
We do agree that additional data on the efficiency of cell specific targeting would be useful. Although we are currently unable to perform additional experiments to show which cells in lymph nodes are being targeted, others have shown efficient targeting of nestin^{pos} cells in Nes-Cre-ERT2 mice shortly after birth using similar induction protocols (Isern et al, eLife 2014, Ding et al, Gastroenterology, 2020). In our study, analysis was done when animals were 14 days, or 21 days old and therefore we don't expect to see Cre-ERT2 expression in induced cells anymore. In 2 week old lymph nodes we do see reduced CXCL13 expression, which is significant (although not indicated but now added to figure 4A), suggesting that targeted deletion was successful.

5. Data using pharmacological inhibition of RA receptor are very striking. These data could be extended by treating mice at a range of time points both before and after birth, and also comparing LNs after a short term treatment, to see if FDC numbers recover.
We have previously shown that treating pregnant mice with BMS eventually resulted in reduced lymph node sizes in adult mice (vd Pavert, Nature 2014). In this manuscript we that also LTi cell differentiation is hampered when BMS is administered that early, which contributes to the reduction in lymph node size. In Figure 5, we do show that treating mice for 7 days, and leaving them untreated for another 21 days, does result in development of FDCs, although the area occupied by B cell follicles in lymph nodes is reduced compared to controls, indicating a reduced FDC development after birth upon 7 days of pharmacological inhibition. We also show that suboptimal doses do affect development of B cell follicles in a similar manner. We know from vitamin deficient mice, in which mice are raised on a vitamin A deficient diet and reach their vitamin A deficient state around 10 weeks after birth, that FDCs could still be found. This would suggest that vitamin A is not necessary for FDCs to stay around once they are formed Therefore, we believe that only shortly after birth, vitamin A signaling pathway is crucial for development of FDCs.

Second decision letter

MS ID#: DEVELOP/2021/199713

MS TITLE: Development of follicular dendritic cells in lymph nodes depends on retinoic acid mediated signaling

AUTHORS: Jasper J Koning, Anusha Rajaraman, Rogier M Reijmers, Tanja Konijn, Junliang Pan, Carl F Ware, Eugene C Butcher, and Reina E Mebius

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referee 2s' comments can be satisfactorily addressed. Please attend to her/his comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Reviewer 1

Advance summary and potential significance to field

This paper have found the important role of RA in FDC development in lymph nodes after birth.

Comments for the author

The authors have given a satisfactory response to my concerns.

Reviewer 2

Advance summary and potential significance to field

This manuscript shows an important role for retinoid acid signalling in the development of FDCs. While the interaction between RA and LT signalling pathways remains mechanistically unresolved, the current manuscript does highlight the importance of these pathways for different stromal subtypes. The paper raises some important next questions for the stromal immunology field.

Comments for the author

The additional data in figure 4A is sufficient to show that the Cre model has significantly reduced CXC13 expression in vivo. I do not require any further experiments to be added at this point.
I only suggest that the authors ensure that some of the explanations laid out in their rebuttal be incorporated into the manuscript where appropriate, to make clear to the reader where the limitations are of this study, and where the next questions for the field arise.

Second revision

Author response to reviewers' comments

Point by point reply to the reviewers.
We would like to thank the reviewers for their time and feedback on the paper. We will provide our response to the questions and issues raised in blue:

Reviewer 1 Advance Summary and Potential Significance to Field:
This paper have found the important role of RA in FDC development in lymph nodes after birth.

Reviewer 1 Comments for the Author:
The authors have given a satisfactory response to my concerns.

We thank the reviewer for this positive feedback.

Reviewer 2 Advance Summary and Potential Significance to Field:
This manuscript shows an important role for retinoid acid signalling in the development of FDCs. While the interaction between RA and LT signalling pathways remains mechanistically unresolved, the current manuscript does highlight the importance of these pathways for different stromal

subtypes. The paper raises some important next questions for the stromal immunology field.

Reviewer 2 Comments for the Author:
The additional data in figure 4A is sufficient to show that the Cre model has significantly reduced CXC13 expression in vivo. I do not require any further experiments to be added at this point.
I only suggest that the authors ensure that some of the explanations laid out in their rebuttal be incorporated into the manuscript where appropriate, to make clear to the reader where the limitations are of this study, and where the next questions for the field arise.

We thank the reviewer for this positive feedback. We have incorporated the following changes and explanations (highlighted in yellow) into the manuscript

- line 159; some -> a substantial number
- line 344: Identifying the molecular mechanism of RA specific repression of chemokine and cytokine signaling in precursor stromal cells would be extremely interesting. It might very well be that RA mediated signaling specifically prevents RelB access to particular promotor regions, thereby regulating transcription of FRC specific genes although this requires further study.
- line 382: It remains to be determined which of the ALDH expressing cell types is the key source for retinoic acid production that mediates FDC development after birth. This would require deletion of multiple retinoic acid producing enzymes in specific cell types shortly after birth.
- line 481: To delete RA receptor signaling postnatally in nestin expressing cells, tamoxifen was dissolved in corn oil (Sigma, C8267) at a final concentration of 10 mg/ml and mothers were treated via oral gavage with 1mg tamoxifen (Sigma, T5648) per day for 5 consecutive days, starting at day 5 after delivery.

Third decision letter

MS ID#: DEVELOP/2021/199713

MS TITLE: Development of follicular dendritic cells in lymph nodes depends on retinoic acid mediated signaling

AUTHORS: Jasper J Koning, Anusha Rajaraman, Rogier M Reijmers, Tanja Konijn, Junliang Pan, Carl F Ware, Eugene C Butcher, and Reina E Mebius
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.