European Journal of Immunology

Lymph node biopsy analysis reveals an altered immunoregulatory balance already during the at-risk phase of autoantibody positive rheumatoid arthritis.

Tamara H. Ramwadhdoebe, Janine Hähnlein, Karen I. Maijer, Leonard J. van Boven, Danielle M. Gerlag, Paul P. Tak, MD and Lisa G.M. van Baarsen

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Handling Executive Committee member: Prof. Iain McInnes

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 - 05-Apr-2016

Dear Mrs. Ramwadhdoebe, Dr. Van Baarsen,

Manuscript ID eji.201646393 entitled "Lymph node biopsy analysis reveals an altered immunoregulatory balance already during the at-risk phase of autoantibody positive rheumatoid arthritis." 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.

You will see that Referee 1 questions the validity of the data in Figures 3 and 4, but has suggested that should you improve the data presentation and the data still hold up, the story is interesting. Referee 2,

however, feels that the study was set up in a way in which the data generated are difficult to really interpret into a strong message:

"I'm unsure what the key finding is here. The LN biopsy system is novel, but the authors provide neither a strong hypothesis to test nor a broad immune profiling that would justify the approach. As such this work would probably appeal to RA specialists rather than the broader immunology community."

The Executive Editor agrees with this analysis but also sees that biopsies are difficult to obtain. Having said that, we strongly encourage you to address the referees' comments as thoroughly as possible with experimental data, as without significant major alterations the submission will not be accepted. 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 referee(s) 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,
Karen Chu

European Journal of Immunology

On behalf of Prof. Iain McInnes

Dr. Karen Chu
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Reviewer: 1

Comments to the Author
This is a well written and generally clearly presented paper. The work is novel in that -to my best knowledge- this is the first study of T cell phenotype and cytokine profile on T cells from human lymph nodes of patients at risk of RA or with early RA. This is a highly innovative and unique approach that will be of interest to many readers in the field.

There are some areas however that require improvements or further clarification:

For all flow analysis, because the LN samples and PB samples were collected over time, how did the authors control for variation in the machine or laser settings? Did they use CS&T beads and application settings to control for this? If not, how did they control for this?

Page 6, lines 134-136: the authors state they could not investigate expression of chemokine receptors in PB samples as liquid nitrogen storage alters the expression of these molecules. Can the authors elaborate on this finding and provide appropriate references or show the data as supplementary figure?

Page 6, lines 137-140, and figure 2: the authors refer to CXCR3+CCR6-CCR4- cells as Th1, CXCR3-CCR6-CCR4+ cells as Th2, and to CXCR3-CCR6+CCR4- cells as Th17. I highly recommend that the authors refer to cells based on their selected phenotype rather than their 'immunological name'

especially in the absence of staining for specific cytokine profiles. It cannot be assumed that all CXCR3-CCR6+CCR4- cells are IL-17+, and vice versa not all IL-17+ are CCR6+, so this needs to be amended throughout in the manuscript.

Page 7/figure 3: It is crucial to include representative flow plots here as was done for figures 1 and 2, so that readers can judge the flow staining patterns and the quality/quantity of the data.

Page 8: co-expression of IL-17 and IL-10 or IFNg and IL-10; the authors state that the data need to be interpreted with caution as the frequencies are very low. It is particularly crucial to include representative flow plots here as was done for figures 1 and 2, so that readers can judge the quality of the data.

On page 11, lines 244-246, of the discussion the authors discuss that the reduced production of pro inflammatory cytokines in LN T cells from at risk and early RA patients could be due to exhaustion. If this is a possible explanation for the findings presented, then it needs to be speculated/explained how these exhausted cells still cause arthritis? Later on in lines 260-261 the authors speculate however that the Th cells in at risk patients may differentiate towards a pro inflammatory phenotype. How does that fit with the hypothesis of exhausted cells?

Minor:
Page 10, Lines 232-233: sentence needs checking
Page 11, line 263: during of…. remove 'of'

Reviewer: 2

Comments to the Author
The hypothesis that autoimmunity develops in the LN is widely accepted, yet no direct evidence exists to demonstrate this. The authors have analysed T cell populations in the LN and compared this with PBMC in groups of healthy controls, those at risk of RA (seropositives) and those with RA. They find changes in T cell phenotypes associated with RA and RA risk groups, some appear unique to LN, others to PBMC and

some present in both. The study groups are selected out of practicality rather than addressing the specific hypothesis proposed by the authors. Being seropositive, the RA risk group are by definition known to have a systemic immune disorder, therefore is examining the LN any more relevant than studying PBMC? Consequently, the authors fail to produce a strong conclusion from the data regarding the relative merits of analysing LN versus PBMC responses in RA.

Specific Points

Results – the phrase 'trend' is meaningless in the context used.

Methods – the comparisons between LN and PBMC results would have been much stronger if paired samples were analysed. Can this be done?

Figure 2 – can the authors justify the selection of these markers for Th cell phenotyping in LN? As well as being indicative of T cell subsets in PBMC, chemokine receptors are also important in controlling cell localisation in tissue. Therefore applying the same criteria to PBMC and LN cells is simplistic. The following data in Figure 3, supports this conclusion (not that they are 'resting memory cells' p10 ln220). It would have been more illuminating to analyse the Tcm/Tem phenotypes of the cells in these samples.

Figure 3 – I found the discrepancy between the MFI and % data confusing. For example in LN samples % IL-10 positive cells is decreased in RA/RA risk, whereas the MFI data suggests an increase. How were these figures calculated and could the authors show representative FACS plots?

First Revision – authors' response
19-Jun-2016

Reviewer: 1

Comments to the Author
This is a well written and generally clearly presented paper. The work is novel in that -to my best knowledge- this is the first study of T cell phenotype and cytokine profile on T cells from human lymph nodes of patients at risk of RA or with early RA. This is a highly innovative and unique approach that will be of interest to many readers in the field.

There are some areas however that require improvements or further clarification:

For all flow analysis, because the LN samples and PB samples were collected over time, how did the authors control for variation in the machine or laser settings? Did they use CS&T beads and application settings to control for this? If not, how did they control for this?

Indeed, since samples were collected over time it was highly important to control for day to day measurement variation. Therefore, CS&T beads were run daily and the same machine with dedicated cytometer configuration was used for the measurements of the samples throughout the study with regular control runs to check compensation controls. We added this information to the methods section.

Page 6, lines 134-136: the authors state they could not investigate expression of chemokine receptors in PB samples as liquid nitrogen storage alters the expression of these molecules. Can the authors elaborate on this finding and provide appropriate references or show the data as supplementary figure?

We have added the appropriate reference to the text which describes alterations in chemokine expression after cryopreservation on human lymphocytes. Because of these possible effects of cryopreservation on chemokine receptor expression we only investigated chemokine receptor expression on freshly isolated lymph node tissue samples. (Costantini et al. Journal of immunological Methods Vol 278 issue 1,2 July 2003 p145-153)

Page 6, lines 137-140, and figure 2: the authors refer to CXCR3+CCR6-CCR4- cells as Th1, CXCR3-CCR6-CCR4+ cells as Th2, and to CXCR3-CCR6+CCR4- cells as Th17. I highly recommend that the authors refer to cells based on their selected phenotype rather than their 'immunological name' especially in the absence of staining for specific cytokine profiles. It cannot be assumed that all CXCR3-CCR6+CCR4- cells are IL-17+, and vice versa not all IL-17+ are CCR6+, so this needs to be amended throughout in the manuscript.

We agree with the reviewer and changed the wording accordingly.

Page 7/figure 3: It is crucial to include representative flow plots here as was done for figures 1 and 2, so that readers can judge the flow staining patterns and the quality/quantity of the data.

We have included representative dot plots.

Page 8: co-expression of IL-17 and IL-10 or IFNg and IL-10; the authors state that the data need to be interpreted with caution as the frequencies are very low. It is particularly crucial to include representative flow plots here as was done for figures 1 and 2, so that readers can judge the quality of the data.

We have included representative dot plots

On page 11, lines 244-246, of the discussion the authors discuss that the reduced production of pro inflammatory cytokines in LN T cells from at risk and early RA patients could be due to exhaustion. If this is a possible explanation for the findings presented, then it needs to be speculated/explained how these exhausted cells still cause arthritis? Later on in lines 260-261 the authors speculate however that the

Th cells in at risk patients may differentiate towards a pro inflammatory phenotype. How does that fit with the hypothesis of exhausted cells?

It may seem contradictory to find exhausted cells despite the presence or development of arthritis, however studies in chronic viral infections have demonstrated that exhausted $CD4^+$ T cells, while impaired in producing pro-inflammatory cytokines like IFN-γ, may still maintain the capacity to produce IL-21. If IL-21 production in lymph node is sustained this may drive B cell differentiation and proliferation and therefore, induction of autoimmune responses and inflammation may still occur. Furthermore, we only observe this exhaustion in lymph node T cells and not in peripheral blood T cells, suggesting that the lymph node environment influenced their response in vitro. Apparently, during the earliest phases of rheumatoid arthritis lymph node T cells are less capable of producing cytokines upon in vitro stimulation compared with healthy controls. On the other hand, the frequency of pro-inflammatory lymph node $CXCR3^+CCR6^-CCR4^-$ (Th1 profile) cells measured directly ex vivo is increased in RA patients compared with healthy controls, while the frequency of regulatory T cells is lower in lymph node biopsies of RA-risk individuals. So, although we observe ex vivo higher frequencies of pro-inflammatory T cells (Th1 but also $CD8^+CD69^+$ T cells and $CD8^+$ memory T cells see Ramwadhdoebe et al., CTI 2016), upon in vitro stimulation these cells produce less cytokines suggesting an exhaustive phenotype. This is an intriguing phenomenon which requires further detailed and longitudinal analyses. More research is needed to identify the exact phenotype of the $CD4^+$ T cells in terms of cytokine production (IL-21) and transcription factor expression. Since this requires time-consuming sampling of new fresh lymph node biopsies we cannot perform these analyses in the cohort used in this manuscript, but we will address this in future studies.

We have revised these sections of the discussion to explain the interpretation of our results in a better context.

Minor:
Page 10, Lines 232-233: sentence needs checking Page 11, line 263: during of…. remove 'of'

We have revised these sentences.

Reviewer: 2

Comments to the Author
The hypothesis that autoimmunity develops in the LN is widely accepted, yet no direct evidence exists to demonstrate this. The authors have analysed T cell populations in the LN and compared this with PBMC in groups of healthy controls, those at risk of RA (seropositives) and those with RA. They find changes in T cell phenotypes associated with RA and RA risk groups, some appear unique to LN, others to PBMC and some present in both. The study groups are selected out of practicality rather than addressing the specific hypothesis proposed by the authors. Being seropositive, the RA risk group are by definition known to have a systemic immune disorder, therefore is examining the LN any more relevant than studying PBMC? Consequently, the authors fail to produce a strong conclusion from the data regarding the relative merits of analysing LN versus PBMC responses in RA.

Many studies in RA patients have compared immune activation of peripheral blood samples with immune activation at the site of pathology, the synovium, and found that immune activation is especially increased in target tissue of RA. Previous studies from our department revealed that changes in gene

expression and TCR repertoire in the synovium are present in very early stages of arthritis. Synovial tissue harbors an increased inflammatory gene expression profile while this is not clearly detectable in peripheral blood (van Baarsen et al. A&R 2010 June vol 62 no 6; 1602-1607). In addition, in early RA the synovial tissue harbors expanded clones of T cells, B cells and plasma cells while these are not present in peripheral blood (Doorenspleet et al. ARD 2014 April 73 (4) 756-762: Klarenbeek et al. ARD 2012 June 71 (6) 1088-1093). Thus early changes in immunoregulatory profile may not be detectable in peripheral blood but can be found in peripheral tissue. Since we also found that the synovium of RA-risk individuals does not display overt infiltration of immune cells (van de Sande et al. Ann. ARD 2011; 70: 772-777: de Hair et al A&R 201 March vol 66 no 3; 513-522) and the production of autoantibodies takes place in lymphoid tissue we reasoned that the earliest steps in immune cell activation need to be studied in lymph node tissue samples, rather than the synovium or peripheral blood. Indeed, the exhaustion phenotype was only observed in lymph node T cells and not in peripheral blood T cells, indicating the importance of lymph node tissue analyses and suggesting that the lymph node environment influenced their response in vitro. We changes some of our sentences in the introduction and discussion section to better clarify this point.

Specific Points

Results – the phrase 'trend' is meaningless in the context used.

We have rephrased the description of the data that were not significant.

Methods – the comparisons between LN and PBMC results would have been much stronger if paired samples were analysed. Can this be done?

Of course we agree with the reviewer on this point. Unfortunately, we have no PBMCs available from the analyzed lymph node biopsies.

Figure 2 – can the authors justify the selection of these markers for Th cell phenotyping in LN? As well as being indicative of T cell subsets in PBMC, chemokine receptors are also important in controlling cell localisation in tissue. Therefore applying the same criteria to PBMC and LN cells is simplistic. The following data in Figure 3, supports this conclusion (not that they are 'resting memory cells' p10 ln220). It would have been more illuminating to analyse the Tcm/Tem phenotypes of the cells in these samples.

We agree with the reviewer that a combination of markers like CCR7, CD69 and CD45RO (or CD27 with CD45RA) would have enabled us to identify more clear the different Tcm/Tem subsets. However, due to the limitations of the size of the lymph node biopsy obtained we were forced to make a relatively small and careful selection of markers to use in our phenotypic analysis. We would like to examine more detailed effector versus memory subtypes in future studies, which will require new inclusions and lymph node biopsy sampling.
We have chosen to analyse a set of chemokine receptors to be able to distinguish T helper 1, T helper 2 and T helper 17 cells without the need of ex vivo stimulation which is required for cytokine analyses. The profiling based on chemokine receptors has been earlier described in literature to work in both peripheral blood and lymph node samples. We have rephrased the definition of the different cell types in the text based on the corresponding chemokine receptor expression detected. Although we cannot exclude that chemokine receptor expression in lymph node tissue may be different in peripheral blood, we believe that the differences that we have detected within the lymph node analyses are specific for the investigated subtype, namely the $CXCR3^{+}CCR6^{-}CCR4^{-}$ T helper 1 profile. However, based on our

findings shown in figure 3, we cannot exclude the possibility that these cells may be impaired in their IFN-γ production. We have included this statement in the text.

Figure 3 – I found the discrepancy between the MFI and % data confusing. For example in LN samples % IL-10 positive cells is decreased in RA/RA risk, whereas the MFI data suggests an increase. How were these figures calculated and could the authors show representative FACS plots?

We have added representative FACS dot plots of the cytokine measurements to show the expression patterns for the different cytokines. The % of cytokine producing cells was calculated within the total $CD4^+$ T cell population by gating on the cytokine positive $CD4^+$ T cells. The gMFI data was calculated by dividing the geometric mean fluorescent intensity of the cytokine positive population by the geometric mean fluorescent intensity of the cytokine negative population in order to adjust for experimental variation

Second Editorial Decision

18-Jul-2016

Dear Dr. van Baarsen,

Thank you for submitting your revised manuscript ID eji.201646393.R1 entitled "Lymph node biopsy analysis reveals an altered immunoregulatory balance already during the at-risk phase of autoantibody positive rheumatoid arthritis." to the European Journal of Immunology. Your manuscript has been re-reviewed and the comments of the referee(s) are included at the bottom of this letter.

Although the referees have recommended publication, some revisions to your manuscript have been requested. Therefore, I invite you to respond to the comments of the referee(s) and revise your manuscript accordingly.

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.**

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. We look forward to receiving your revision.

Yours sincerely,
Nadja Bakocevic

on behalf of
Prof. Iain McInnes

Dr. Nadja Bakocevic
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Reviewer: 1

Comments to the Author
My comments have been satisfactorily addressed. I would only suggest that the authors indicate the percentage values in the quadrants of all representative face data plots as these are currently omitted. And there is a small typo on page 11, line 256: 'Is has > 'It has

Reviewer: 2

Comments to the Author
The authors have made changes to the text of the MS to address some of the points raised. In response to these:

the production of autoantibodies takes place in lymphoid tissue we reasoned that the earliest steps in immune cell activation need to be studied in lymph node tissue samples• . Can the authors provide a reference to animal models or clinical studies that demonstrate this point (e.g. compared with bone marrow)? [p4 line88]

Results the phrase non significant increase is as meaningless as trend.

Figure 2 The profiling based on chemokine receptors has been earlier described in literature to work in both peripheral blood and lymph node samples• Can the authors provide references in justification (particularly given the statement?
This statement seems to contradict the following point Although we cannot
exclude that chemokine receptor expression in lymph node tissue may be different in peripheral blood. This point really requires clarification and justification with reference to previous studies or assay standardization.

Figure 3 Im no clearer on the justification for the normalized gMFI used in this study or what this parameter tells us in addition to % positive. My previous point stands; For example in LN samples % IL-10 positive cells is decreased in RA/RA risk, whereas the MFI data suggests an increase• . This requires reference to an authoritative study demonstrating use of this parameter. Subsequently, the authors cannot justify selection of one parameter over the other when performing data interpretation.

Second Revision – authors' response
22-Aug-2016

We would like to thank the reviewers for their positive comments on our revised manuscript and the Editorial Board for giving the opportunity for submitting a revision of our paper. All suggestions of the reviewers have been addressed in the manuscript including references to support our statements.

In addition, we would like to mention that flow cytometry data based on percentage of cytokine producing cells (%) or amount of cytokine production per cell (based on gMFI) are independent of each other, result in different outcome measurements and are commonly used in immunological studies.

European Journal of Immunology

Third Editorial Decision

25-Aug-2016

Dear Dr. van Baarsen,

It is a pleasure to provisionally accept your manuscript entitled "Lymph node biopsy analysis reveals an altered immunoregulatory balance already during the at-risk phase of autoantibody positive rheumatoid arthritis." 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). 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. Iain McInnes

Dr. Nadja Bakocevic
Editorial Office
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