OPEN PEER REVIEW REPORT 1

Name of journal: Neural Regeneration Research
Manuscript NO: NRR-D-20-00218
Title: Dynamics of systemic immune responses after murine spinal cord injury
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Reviewer's country: UK
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COMMENTS TO AUTHORS

The value of the manuscript is not clear as it does not seem to add to the existing body of work on the effects of CNS injury on systemic immunity. Unless, the MS undergoes major revisions suggested, it might be required to reject the MS.

Summary of aims and main findings

In this study, the authors explore the effects of spinal cord injury on systemic immunity in the acute and post-acute phase of injury. By examining changes physiological parameters, the immune composition of the blood, the functional responsiveness of splenic T cells and circulating cytokines they characterise the immune response following spinal cord injury. Their work adds weight to the growing body of evidence that demonstrates systemic immune dysregulation following CNS injury.

Strengths

The study adds weight to mounting evidence that highlights the role of CNS injury in driving systemic immune alterations which could potentially impact infection susceptibility.

Limitations

Whilst the study recapitulates observations from other groups, there is limited novelty in the results presented. Based on the existing literature it is unclear what value this study adds. It is purely observational and there is no attempt to dissect potential mechanisms. Major revisions with significant reorganisation of the manuscript are required to be even properly reviewed.

Detailed report on study

Additional comments that warrant further responses are detailed below

Figure-wise analysis

Figure 1

(A, B) Immunohistochemistry for microglia using P2RY12 along with Iba1 for mononuclear phagocytes and their quantification will be useful to assess the extent of inflammation. At what time point were these images taken?

In (A) there are no inflammatory foci- I am unclear what the authors mean when they refer to the H&E image showing evidence of immune cells infiltrating the injured site.

In (B) what are the two colours representing? There is no key/legend.

It is unclear why (C) is presented as it is unrelated to the message of the figure. This could be removed.

Pick a style of formatting and stick to it. Decide whether you would like to abbreviate "day" to "d" or not.

In the temperature curve the authors jump from d-1 to d1 but in the CRP graph they measure concentrations on d0. d0 is a critical point when the surgery was performed and if this data is presented then the value on the day of surgery will guide how to interpret the graph.

Details of statistics used must be provided in the legend and not just level of significance.

Figure 2

Please shorten the legend, no need to keep using the phrase dynamic changes.

Why is n = 4/group? Why are 2 animals excluded compared to the previous graph of n = 6 per

group?

What about the cell numbers of lymphocytes, neutrophils and monocytes? Which of these is the dominant factor in decreasing the cell numbers or are all cells equally affected? Details of statistical tests are missing here and present only comparisons to baseline as this is what is also discussed in the text.

Figure 3

The figure is of very low quality and reading the FACS plots is impossible.

The comments here are based on what is possible to read from the existing manuscript but higher quality images will be required. Please see:
https://www.ncbi.nlm.nih.gov/pubmed/21278737 on how to format FACS data for a paper.

The nomenclature of T cells based on the markers used is not entirely correct. Th17 cells are also capable of producing IFN. Please see https://www.ncbi.nlm.nih.gov/pubmed/21278737 and https://www.ncbi.nlm.nih.gov/pubmed/29802020. Unless the authors are able to provide FACS data with transcription factor staining for t-bet and ROR, with the limited markers used they cannot designate cells they have identified as Th1 or Th17. The terminology in the paper must be changed to IL-17+ CD4 T helper cells or IFN+ CD4 T helper cells. No comment on lineage/polarisation can be made with the existing data. No details of statistics again. Comparing every group to every other group and showing these comparisons on the graph is unnecessary and very confusing for the reader. Given the narrative is mainly a description of changes with respect to baseline please make only these comparisons.

Figure 4

Please do not include graphs of cytokines that are not altered. They are not discussed and are not essential to the narrative. They can be moved to a supplementary figure if required. No details of statistics again. Comparing every group to every other group and showing these comparisons on the graph is unnecessary and very confusing for the reader. Given the narrative is mainly a description of changes with respect to baseline please make only these comparisons.

General comments on manuscript

The line numbering is significantly off set from the text which makes commenting on text in certain lines difficult. This needs to be corrected.

The quality of figures is very poor in some instances and higher resolution images will be required for further review (specifically Fig. 3 as lack of clarity impedes the examination of the data).

Remove p values from the text, add details of statistics to figure legends and use clear and standard nomenclature eg, when describing cells the “+” needs to be a superscript eg CD4+ and not CD4+. The reference list is sufficient overall covers relevant literature in an unbiased manner. There are a few more citations that the manuscript would benefit from and these are outlined below.

Methods are sufficiently detailed for replication but need to be shortened. See below for detailed comments.

Abstract

Overall, the abstract will benefit from shortening.

It is unclear why in the methods the model is described as “high level spinal cord”. This is confusing as high level could imply higher thoracic or cervical vertebrae. Delete this phrase.

The methods section is overly detailed. Such detailed information regarding flow cyto metry is not required.

The results section is also very confusing and needs to be reorganised. Every single change is described. Some of the conclusions are unclear as the language is not sufficient. Others are unsubstantiated by the results. The observations cannot be described as immunosuppression as the data are nuanced. They can be described as immune dysregulation or alterations.

Designating the response as immune suppression based on lymphopenia is incorrect unless increased infection susceptibility can be demonstrated. No evidence of intra spinal inflammation has been investigated so no comment on systemic immunity and its relationship with intra spinal immunity can

be made.

Introduction

Like other sections, the introduction needs major revision and copy editing.

The progression of narrative is also not clear: the 1st paragraph introduces spinal cord injury, the 2nd discusses immune alterations in the spinal cord post-injury and the 3rd is on interactions between CNS immunity and systemic immunity in repair.

The authors do not examine neurological recovery post SCI and do not asses: inflammation in the spinal cord, behaviour or neuronal repair in anyway. Paragraph 3 is more tuned towards the discussion section- it is not directly related to the narrative and would fit better after results have been presented.

There is no description of the spectrum of immune alterations after SCI, this is directly related to what the authors go on to examine and there should be a discussion of the existing knowledge on the effect of spinal cord injury on systemic immunity in general. There should also be a focus on the effect of SCI on T cell function. These can also be used For example please see:

o https://www.ncbi.nlm.nih.gov/pubmed/21734283
o https://www.ncbi.nlm.nih.gov/pubmed/29282251
o https://www.ncbi.nlm.nih.gov/pubmed/19636355
o https://www.ncbi.nlm.nih.gov/pubmed/28316792
o https://www.ncbi.nlm.nih.gov/pubmed/27810921
o https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5029282/
o https://www.ncbi.nlm.nih.gov/pubmed/28920935

This sentence should be deleted: “Immediately after SCI, the blood spinal cord barrier (BSCB) is broke down, the immune cells including granulocytes, lymphocytes, and blood monocytes infiltrates through the defective BSCB into the injured sites of spinal cord, where they synergistically induce pro- and anti-inflammatory responses”. What do the authors mean by pro- and anti-inflammatory responses and it is unclear how they can be synergistic or whether the cells are being referred to here.

Methods

Could the authors clarify if they used power calculations to determine sample sizes? If not, can the authors please explain why as ARRIVE guidelines dictate use of power calculations. https://www.nc3rs.org.uk/sites/default/files/documents/Guidelines/NC3Rs%20ARRIVE%20Guidelines%202013.pdf

CRP measurement: it is stated a rat ELISA kit for CRP was used. This is incorrect as a mouse ELISA kit was used based on the supplied catalogue number. The catalogue number has a typo: it is SEKM-0059
https://solarbio.en.alibaba.com/product/60788265053-810230692/Mouse_CRP_Elisa_Kit.html?spm=a2700.icbuShop.41413.13.478d18a2v3CgOO.

Flow cytometric analysis:

o This section needs to be re-written. The step-wise progression in staining cells is incomprehensible. Here is an example of a paper on how to style the flow cytometry section: https://www.ncbi.nlm.nih.gov/pubmed/28423340
o Was a cell viability dye used and if not, why? The lack of a cell viability dye can confound results and so it is important clarify.

Statistics: The authors state that they used a Student’s t test to perform statistical analyses on their data and that they did this by pair-wise comparisons of their groups.

o This is incorrect and ALL data in figures 1-4 need to be re-analysed.
o The data from the study must be tested for normality first and if the data are not normally distributed, a non-parametric test must be used.
o Further, if the groups have unequal standard deviation, a test which does not assume equal standard deviations between groups must be used.
o Appropriate post hoc tests must be used for pair-wise comparisons as this corrects for multiple comparisons. Eg Tukey’s test.

Results

Section: Pathological changes completely transected SCI

o The authors talk of immune cell infiltration. No evidence of inflammation can be seen in the H&E image. This comment is not valid unless the authors can provide data on IHC for immune cell markers CD45, CD11b, P2RY12, Iba1 or CD3, etc.

In fact, it would be good to see CD4 with IFN-g and IL-17 staining in the spinal cord sections.

o The description of temperature changes is unrelated to the narrative of the paper and should be removed. If required, the data can be presented as a supplementary figure as validation of the success of the SCI surgery with the outcome of disrupted thermoregulation.

o Remove mean values of CRP from text. This has already been provided in the figure. Describe that overall trend individual values at every time point don't need to mentioned!

Section: The Change of subpopulation blood cells changes in peripheral blood after SCI.

o The authors make the following comment: "it keeps elusive whether there is a dynamic change of WBC in peripheral blood". This is untrue, please remove this comment. There is a lot of data on peripheral blood cells in the literature. As an example please see:

https://www.ncbi.nlm.nih.gov/pubmed/27810921 and

https://www.ncbi.nlm.nih.gov/pubmed/28920935. o Please remove the mean+/- SEM information in the text as this information is available in the main figure.

o Again, this section is verbose for the information that it provides. This section can be shortened as such length is not necessary to describe the alterations in the frequencies of 3-4 cell types.

Section: The dynamic change of Th1 andTh17 subsets of T cells (CD4 + IFN-γ + and CD4 + IL17A +) and The increase of Tregs subset (CD4 + CD25 + FOXP3 +) cells after SCI

o Merge the two sections, their separation does not make sense.

o This section needs a complete overhaul as the text is very confusing to follow and some of the comments are factually incorrect.

o The section needs to be shortened: don't describe every single change it is confusing, have a clear narrative, remove mean % values as these are already in the figure, say significant and remove the p values, stop clarifying IFN- +cells every single times as Th1 subset.

o Overall it comes across that the authors are unaware of T cell biology based on the way flow cytometry was performed and the way T cells are described:The authors make the following comment, "In mice, T cells are characterized with the positive expression of CD4 surface marker.

Effective T-cell responses to pathogen and injury require the secretion of cytokines (Bethea and Dietrich, 2002; Bareyre and Schwab, 2003; Gonzalez et al., 2003; Donnelly and Popovich, 2008)."

This needs to be deleted as it is just plain wrong. T cells are characterised by the TCR complex CD3 and they are further distinguished based on their expression of either TCR or TCR. CD4+and CD8+T cells and based on their transcription factors they can be classified into a number of different polarisation states. Please see:

https://www.ncbi.nlm.nih.gov/pubmed/22341735. Please comments on figure 3. The distinction between Th1 and Th17 cells is not as clear as the authors make it out to be as lineage tracking experiments have shown IL-17+cells are capable of producing IFN. Can the authors present the data as follows: gate on CD4+cells. In the next gate plot IFN-g on the X axis and IL-17 on the Y axis. Please quantify the % of IL-17+, IFN+and IFN+IL-17+cells over time.

When discussing Tregs. Please refer to and cite papers by Ethan M. Shevach, John O'Shea and Alexander Rudensky and reorganise this section too.

Section: The Expression of pro-and anti-inflammatory Cytokines through T cells immune response mediate in the peripheral circulation system

o "After SCI, the inflammatory reactions contributes substantially to the secondary injury and its intervention enhances functional recovery in SCI models (Bethea and Dietrich,2002; Gonzalez et al., 2003; Brambilla et al., 2005). The pro- and anti-inflammatory cytokines coordinately take part in inflammations after SCI."

This contradicts both what the authors say early but also many other groups that have highlighted how inflammation plays a role in injury as well as repair. Please remove or rephrase this to reflect that.
o “whereas, IL-10 is a potent anti-inflammatory cytokine suppressing the activities of immune cells” This is not true as IL-10 is an immunoregulatory cytokine and work by Werner Muller has shed light on the functions of IL-10. o “Our results showed SCI caused a paradox of pro- and anti-inflammatory cytokine expressions”- The authors refer to a paradox, I am not clear what this paradox is as overall they go on to show a decrease in the functional responsiveness of splenocytes in terms of their ability to produce cytokines in culture. This fits with the previous data where they demonstrate fewer leukocytes post SCI.
o The authors say “In our investigation, SCI did not markedly affect the expression of IL-10 in T cells derived spleen after stimulation at indicated time points”. This comment is wrong as the production of IL-10 cannot be ascribed to Tregs in a mixed splenocyte culture.

Discussion

The authors go back and forth between different results sections in no logical order. This needs to be corrected.

The authors repeatedly refer to a paradox of pro and anti-inflammatory cytokines which. The spectrum of cytokines measured seems fine but what the authors refer to is unclear.

The authors discuss autonomic dysfunction in the setting of systemic immune suppression but do not talk about this in the introduction. Discussing these mechanisms in the introduction is critical as it sets the scene for the study.
https://www.ncbi.nlm.nih.gov/pubmed/28920935

A discussion of how SCI is part of a wider phenomenon of how systemic immune alterations are driven by CNS injury in general including TBI and stroke would be beneficial.

The authors mention how studies have neglected systemic immunity and that this is the key paper on the effect of SCI on systemic immunity. Based on the existing literature I am not convinced this is true.

Conclusions

Needs to be clarified- this is not the study on systemic immune alterations post-stroke. There are others and that this study adds to the body of work. But even at this stage it is not clear what this study adds to the existing body of literature.

References

Satisfactorily referenced.

Other references that would be beneficial to include have been suggested in the appropriate sections.