Peer Review File

Article information: https://dx.doi.org/10.21037/atm-23-1960

Reviewer A

The review by Brint et al, discusses the structure of inflammasomes and signaling pathways that may play a potential role in the etiology of MS. In addition, the authors analyze treatment options of IC100 and DMF for MS. The review is out of date and does not include recent information on inflammasome structure and signaling, and, importantly, does not include several reports on the mechanism of action of IC 100 and the role of inflammasome proteins as biomarkers of MS. The authors are encouraged to revise the manuscript to include this information.

Major Points:

Comment 1. The literature is full of similar reviews describing the structure of the inflammasomes and their role in processing inflammatory cytokines as described in the manuscript. Therefore, it is recommended that the authors include new and exciting information on inflammasome structure and signaling pathways. Please see: Cell 186, 2288-2312, 2023.

Reply 1: We agree with and thank the reviewer for the suggestion. We added information regarding NLRP3 components being used as disease biomarkers, as well as more recent structural information from cyro-em experiments. We also added info about disk shape, ubiquitination and nek7. To bring this review up to date, critical new references were introduced to the manuscript, including five published in 2023.

Comment 2. The authors should include information described on the mechanism of action of IC 100 (Transl Res. 2023 Jan;251:27-40. doi: 10.1016/j.trsl.2022.06.016. Epub 2022 Jul 3). For example, how IC 100 enters cells, the biodistribution and the action of IC 100 on ASC specks. In addition, it would be prudent to include information of the role of inflammasome proteins as biomarkers in MS patients as described: Front Neurol. 2018 Mar 19;9:135. doi: 10.3389/fneur.2018.00135. eCollection 2018.

Reply 2: We are grateful for the critical suggestion. We have incorporated the references and the related knowledge in the manuscript. More specifically, the mechanism of action for IC100 was expanded upon, including CNS and cell entry and the ability to avoid proteasomal degradation.

Comment 3. Line 403 states: “We were able to visually identify the specific residue locations of succination by DMF and compare them with the residue locations where Caspase 1 cleaves GsdmD. The validity of this mechanism rested on whether DMF could block access to Caspase 1. These residues should be reported and depicted in a diagram.

Reply 3: We thank the reviewer for the suggestion. In Figure 5, we labeled and

incorporated the primary residue Cysteine 192, which is spatially close to the cleavage site of caspase 1.

Comment 4. Line 412 states: “Using UniProt’s alignment tool, we found that the NLRP3 and ASC pyrin domains contained 21 matching residues (22.581%), and subsequently marked them within our PyMol structure. This comparison led us to conclude that the ASC pyrin domain contains a significant area of uniquely organized residues, to which IC100 can specifically target.” The authors should include a diagram depicting this alignment and what specific residues in the Pyrin domain they discuss.

Reply 4: We included the alignment as follows and decided to remove the sentence in the manuscript. The information was not used again in other places in the manuscript, and we felt it did not contribute to any conclusions and was only distracting.

```
CLUSTAL O(1.2.4) multiple sequence alignment


sp|Q96P20|NLRP3_HUMAN|1-93       MKMASTRCKLARYLEDLEDVDLKKFKMHLEDYPPQKGCIPLPRGQTEKADHVDLATLMID    60
sp|Q9ULZ3|ASC_HUMAN|1-91         --MGRARDAILDALENLTAEELKKFKLKLLSVPLREGYGRIPRGALLSMDALDLTDKLVS    58
                                   *. :*  :   **:*   :*****::* . * ::*   :***   . * :**:  ::.

sp|Q96P20|NLRP3_HUMAN|1-93       FNGEEKAWAMAVWIFAAINRRDLYEKAKRDEPK       93
sp|Q9ULZ3|ASC_HUMAN|1-91         FYLETYGAELTANVLRDMGLQEMAGQLQAATHQ       91
                                 *  *  .  ::. ::  :. :::  : :    :
```

Comment 5. A discussion of MCC950 does not seem warranted since this drug has toxic side effects and has dropped by drug companies for further development. There are new and improved variants of MCC950 that show promise and have been taken forward for clinical trials. These new drugs should be discussed.

Reply 5: We removed MCC950, and instead, we included melecules in favor of more relevant treatment options such as Ocrelizumab and Fingolamide.

Comment 6. The diagrams are very simplistic. They should be revised to incorporate more detailed signaling molecules and inflammasome inducers.

Reply 6: In response to the reviewer’s suggestion, all figures have been updated, and the more complex mechanism was depicted in Figure 1.

Reviewer B

The article entitled “Multiple Sclerosis: The NLRP3 Inflammasome and Therapeutics” is a review article that discusses the involvement of the NLRP3 inflammasome in multiple sclerosis and treatments that can reduce the expression and/or activation of NLRP3.

It is an interesting article, but I believe that important modifications should be made so that the quality of the presentation improves:

Comment 1. In the abstract the NLRP3 inflammasome is not mentioned at any time and, however, due to the title of the article, it deals precisely with this inflammasome.

Reply 1: We agree with the reviewer and would like to thank the reviewer for this critical suggestion. NLRP3 has been incorporated into the abstract: "NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) are sensors to control the cell's inflammatory response. Activation of NLRP3 leads to the formation of the NLRP3 inflammasome complex, which leads to Caspase 1 and Gasdermin D (GsdmD) activation".

Comment 2. In text, there are several quotes that appear without numerical format:
- Page 3; Lines 27-28
- Page 12; Lines 263-264
- Page 13; Lines 269

Reply 2: We apologize for these issues. Corrections regarding citations have been made.

3. Since the regulation of the inflammasome is a step that occurs prior to its activation, perhaps it would be better to put this section before the section in which the activation is explained.

Reply 3: We thank the reviewer for the valuable suggestion. The section describing regulation of the inflammasome has been moved before the section describing activation.

4. Perhaps in the introduction section the information on the relationship between the NLRP3 inflammasome and multiple sclerosis should be expanded, for example, with the places where NLRP3 is usually expressed in this disease.

Reply 4: Following the reviewer's suggestion, information describing NLRP3 expression in monocytes has been added.

Reviewer C

The review on the topic of the role of NLRP3 inflammasome in the pathogenesis of MS is timely and important as multiple studies have reported on the role of NLRP3 inflammasome activation in the migration of inflammatory cells to the CNS.

Comment 1: The review is very long and not focused, it would be better to focus on NLRP3 inflammasome only as stated in the title. The focus is apparently on Gasdermin D, which mediates pyroptotic cell death. However, majority of inflammatory cells, predominantly myeloid peripheral blood cells and microglia do not undergo cell death, but NLRP3 inflammasome activation primarily mediates cytokine secretion (IL1b) and cell migration.

Reply 1: We thank the reviewer for the critical review and suggestions. In response, the title has been updated to reflect the discussion around NLRP3's role

in pyroptosis. We updated the manuscript with more recent publications. Additionally, we significantly revised the manuscript and we hope it is more focused and readable. We agree with the reviewer that under physiological conditions, “majority of inflammatory cells, predominantly myeloid peripheral blood cells and microglia do not undergo cell death, but NLRP3 inflammasome activation primarily mediates cytokine secretion (IL1b) and cell migration”. However, in the case of MS, NLRP3 can drive GsdmD cleavage and pyroptosis in microglia (McKenzie et al., *J Neuroinflammation* 17, 253 (2020)).

Comment 2: Multiple statements in the introduction are not accurate from clinical standpoint: “MS is neurodegenerative disease accompanied by inflammation”. “MS particularly affects the nerves of the CNS”; “MS can be detected by LP to look at elevated oligoclonal levels and using MRI”. The disease is caused by inflammation, it primarily causes myelin loss and can be diagnosed only in the presence of demyelinating brain and spinal cord lesions detected by MRI.

Reply 2: We apologize for the inaccurate description of MS diagnosis. Information regarding disease identification in clinically correct terms has been added to the introduction: “Multiple sclerosis (MS) is characterized by both inflammation and immune-mediated neurodegeneration within the central nervous system (CNS). Inflammation is not merely a consequence but a critical driving force in the disease's pathogenesis(1). Magnetic Resonance Imaging (MRI) reveals characteristic lesions or plaques in the central nervous system that indicate demyelination and inflammation, supporting the diagnosis of MS(2). Plaques are regions of nerves where the myelin has been removed, preventing them from conducting the electrical signals necessary for proper function and are associated with inflammation and the accumulation of inflammation-related cells(3).”

Comment 3: The review is very long and not well organized. Many references are listed without explanation or clear link to MS (EBV reactivation is associated with NLRP3 inflammasome, Burton, 2019).

Reply 3: We thank the reviewer for the critical suggestions. In response, we significantly revised the manuscript, hoping it would be more focused and in better shape. Additionally, this section in the introduction was expanded upon to link MS, NLRP3, and MS.

Comment 4: The section describing priming and activation steps of NLRP3 inflammasome activation are not clearly written, NFKB is a priming signaling pathway leading to the expression of inflammasome components, activation step is related to the activation of inflammasome and pro-caspase mediated cleavage of caspase 1, IL1b and GSDMD.

Reply 4: This section has been rewritten for clarity: “The cause of MS-related neurodegeneration is in question. Still, there is evidence that supports genetic and environmental causes, such as low vitamin D serum levels, smoking, obesity, and

Epstein-Barr Virus infection (EBV)(4). Evidence that EBV, a herpesvirus, may be responsible for triggering the autoimmune response and lead to demyelination(5). The study utilized a large sample size (over 10 million) over the course of 20 years and found that the risk of MS increased 32-fold after infection with EBV. Additionally, connections have been made between EBV reactivation and the NLRP3 inflammasome, a possible initiator of the MS-associated inflammatory response(6)."

Comment 5: Most importantly, it is not discussed that GSDMD-mediated cell death is not required for IL1b secretion from inflammatory myeloid cells, which are expanded in MS.

Reply 5: We thank the reviewer for the suggestion. We added, "It is important to note that GsdmD-mediated cell death is not a requirement for elevated IL-1β secretion from inflammatory myeloid cells of patients with MS.", to clarify the nonessentiality of pyroptosis in MS.

Comment 6: Many references are old, and some new references are not listed, even though they address specifically the NLRP3 inflammasome activation in monocytes in RRMS (Seyedsadr M. et al, PNAS 2023) line 90-91 in the manuscript.

Reply 6: We agree with and thank the reviewer for the suggestion. We added information regarding NLRP3 components being used as disease biomarkers, as well as more recent structural information from cyro-em experiments. We also added info about disk shape, ubiquitination and nek7. To bring this review up to date, critical new references were introduced to the manuscript, including five published in 2023.

Comment 7: It would be most important to discuss other, more prevalent pathways of IL-1 induction, which does not require inflammatory cell death.

Reply 7: We thank the reviewer for the suggestion. We added an important reference to let interested readers find these information. Axel Weber et al. ,Interleukin-1 (IL-1) Pathway. *Sci. Signal.*3,cm1-cm1(2010).

Comment 8: The role of NLRP3 inflammasome in the neuronal cell death does not cite multiple relevant references (Wu et al, Neurosci Lett 2023, Vontell et al, Brain Pthol, 2023; Li et al, Behav Brain Res, 2020). It would be important to report papers which identified NLRP3 inflammasome components expression in neurons.

Reply 8: We thank the reviewer for the critical references and these papers have now been cited in addition to others.

Comment 9: Regarding MS treatment, the key references and focus of discussion should be on S1P inhibitors, which are reported to inhibit NLRP3 inflammasome activation (Weichand et al, JEM, 2017, DI Menna, Pharmacol Res, 2013). Also, Bruton Tyrosine kinase inhibitors, new treatment for MS with documented inhibition of NLRP3 inflammasome activation (Liu et al, J Allergy Clin Imm, 2017,

Ito, Nat Comm 2015, Ghosh et al, J Neuroinflamm, 2021) is not included.

Reply 9: S1P inhibitor have been added to the Treatment section and all references mentioned in this comment have been added.

Comment 10: The manuscript should be shortened, focused on the key mechanisms related to NLRP3 inflammasome activation role in MS, multiple grammar and English edits are required. Since NLRP3 inflammasome activation is best characterized in myeloid cells, it would be preferable to focus on these cells in the peripheral circulation and within the CNS (microglia).

Reply 10: Based on the reviewer's suggestion, we had significantly truncated, modified and updated the manuscript. We hope it is now up to the journal's standard.