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Astrocyte-Microglia Crosstalk and Microglial Exhaustion in Alzheimer's Disease—An In Vitro Study

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Abstract. Astrocytes and microglia interact through direct contact, reciprocal signaling, and factors released into the extracellular environment. These interactions may influence how the brain responds to amyloid-beta (Aβ) in Alzheimer's disease (AD). Habib et al. identified a disease-associated astrocyte (DAA) state in 5xFAD mice with changes in genes related to inflammation, complement, endocytosis, lysosomal activity, and amyloid biology. Astrocytic PD-L1 and microglial PD-1 may suppress inflammatory signaling and support microglial Aβ uptake. Based on these findings, this proposal uses a two-part experimental design. Experiment 1 first tests whether DAA-conditioned medium or recombinant PD-L1 changes the resulting microglial phenotype; experiment 2 compares four culture conditions and the effects of Aβ protein on microglial phenotype, as well as the effects of different culture conditions on astrocytes. The predicted pattern is that DAA-conditioned medium and PD-L1 will improve several microglial readouts after prior Aβ exposure, while direct co-culture will preserve more function during ongoing Aβ stress than medium transfer alone. This prediction provides new insights to future therapeutic approaches, since it establishes the importance of PD-L1 in the regulation of microglial inflammation, and the effects of the interactions between microglia and astrocytes.

Keywords: Alzheimer's disease, Amyloid-beta, Astrocytes, Microglia, Direct co-culture

1. Introduction

1.1. Astrocyte-microglia interactions in Alzheimer's disease

Alzheimer's disease is associated with extracellular Aβ protein accumulation, neuronal dysfunction, and changing responses from several non-neuronal cell types. Microglia are resident immune cells that can detect extracellular material, release inflammatory signals, and take up Aβ. Astrocytes support extracellular homeostasis and neuronal metabolism, but they can also influence immune activity through surface molecules and released factors. The effect of either cell type may change with the disease environment and cannot be classified as completely protective or completely harmful from a single marker [1, 2].

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Habib et al. used single-nucleus RNA sequencing to identify several astrocyte states in 5xFAD mice [3]. Disease-associated astrocytes (DAAs), increased with age and pathology, is a phenotype of astrocytes exclusively present in AD patients. DAAs expressed genes that encoded molecules including Gfap, Vim, Serpina3n, Ctsb, Apoe, Clu, and other molecules involved in complement-related pathways. This mixed program suggests that plaque-associated astrocytes may simultaneously show adaptive, inflammatory, and potentially harmful features. The study established an in vivo transcriptional state, but it did not directly test what those astrocytes do to microglia.

To examine this unresolved functional relationship, the present study uses an in vitro culture model in which astrocyte medium generated after treatment with Aβ, TNF-α, and IFN-γ is defined as DAA-conditioned medium. This is an operational treatment definition. Expression of several DAA-related markers after this treatment would not prove that the cultured astrocytes are identical to the broad in vivo DAA state described by Habib et al. [3]. Throughout this proposal, any conclusion about a true DAA identity will require broader validation [4, 5].

1.2. PD-L1/PD-1 signaling and microglial Aβ uptake

Kummer et al. reported PD-L1-positive astrocytes and PD-1-positive microglia near Aβ plaques and found that loss of PD-1 increased inflammatory signaling while reducing CD36 expression and Aβ uptake [6]. Independent studies have shown that astrocyte-derived complement signaling can alter microglial phagocytosis and amyloid pathology [7] and that CD36 upregulation enhances microglial Aβ phagocytosis [8]. These results support the possibility that astrocytic PD-L1 helps regulate microglial inflammatory state and Aβ uptake.

Kummer et al. did not show that PD-L1 explains all astrocyte effects [6]. Direct co-culture also permits physical contact, continuous release of factors, and reciprocal signaling that cannot be reproduced by a single transfer of conditioned medium. Previous AD-related co-culture models have used multi-cell systems to examine interactions that are absent from single-cell cultures [9]. This leads to the central logic of the two experiments: first, to test whether DAA-conditioned medium or recombinant PD-L1 improves an Aβ-associated microglial phenotype after washout; and second, to test whether direct astrocyte-microglia co-culture differs from separate culture and astrocyte-medium transfer during a shared Aβ environment.

1.3. Research question and hypotheses

The primary question of the research is how do DAA-conditioned medium, recombinant PD-L1, direct astrocyte-microglia co-culture, and separate-culture astrocyte-medium transfer affect microglial inflammation and Aβ phagocytosis in this Aβ-exposed culture model.

The research posits four main hypotheses: first, after 2-3 days of synthetic Aβ42 exposure, microglia will show an exhaustion-like pattern: higher TNF-α, IL-1β, and PD-1; lower Aβ phagocytosis; and lower CD36 and P2RY12. This pattern will be described as an operational Aβ-associated phenotype rather than definitive chronic microglial exhaustion. Second, following washout, DAA-conditioned medium and recombinant PD-L1 will each shift the Aβ-exposed microglia toward the untreated control pattern, with lower inflammation and PD-1 together with higher Aβ phagocytosis, CD36, and P2RY12. Third, during the second experiment, direct co-culture of astrocytes and microglia in the Aβ condition will partially preserve microglial function compared with the separate-culture and astrocyte-medium-transfer condition. The no-Aβ groups are expected to retain the healthiest microglial pattern. Lastly, Aβ exposure and the presence or absence of direct

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microglial interaction will alter DAA-related astrocyte-marker expression and the proposed astrocyte Aβ-production readout. Higher values are predicted in the Aβ direct co-culture group than in the Aβ separate-culture group.

2. Materials and methods

The materials used in the experiment are primary microglia from neonatal wildtype mice, primary astrocytes from same mice, synthetic Aβ-42 oligomers, recombinant PD-L1 protein, and 24-well plates with culture medium.

The experiments can be divided into 2 sections. The first section determines the effects of astrocytic PD-L1 to restore function in microglia co-cultured with amyloid plaques. In the first section, microglia from neonatal wildtype mice are obtained and placed in 24-well plates with culture medium. The healthy microglia are then divided into 2 groups: One control group with no additional treatment, and one experimental group that receives synthetic Aβ42 oligomers into their cultures. The microglia are then cultured for 2-3 days, while they respond to the changes in their environment.

Next, several methods that assesses the condition of the microglia take place. The following tests are done to both control and experiment groups: First, ELISA method is used to measure the amount of TNF-α and IL-1γ, marker proteins for inflammation. Then. Aβ Phagocytosis Assay is used to test the efficiency of Aβ phagocytosis of the microglial cells. Finally, Flow cytometry is used to test for the amount of microglia that have the CD36 and P2RY12 proteins expressed, where the former indicates proteins involved in Aβ phagocytosis and the latter indicates how far the microglial cells deviates from their normal state into an exhausted state. The test results from the experimental group will then be compared to that of the control group, to confirm that the microglia treated with Aβ protein had entered the exhausted state.

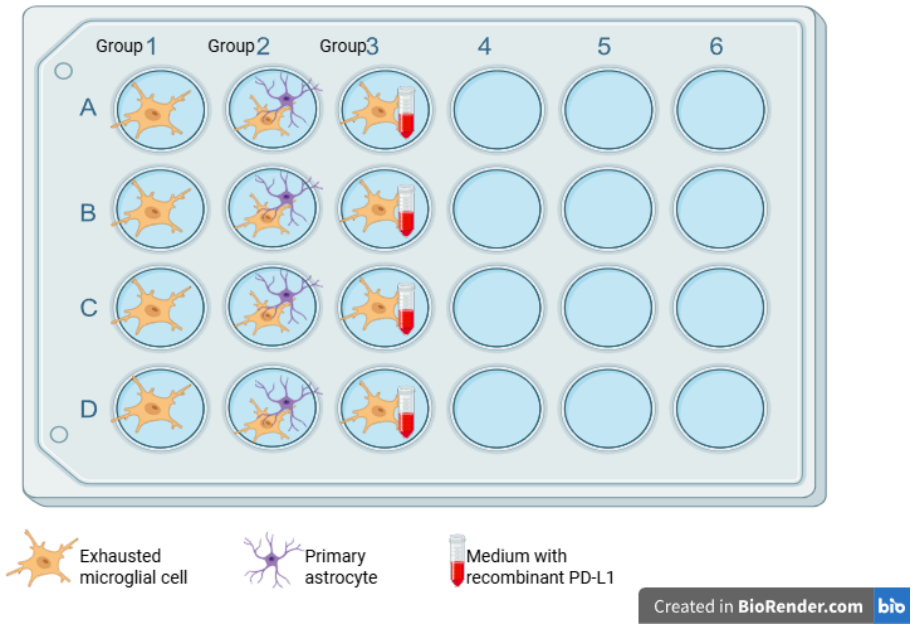


Figure 1. Experimental setting of the 3 groups of exhausted microglial cells under different conditions in the first section of the experiment; Created in https://BioRender.com

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After the tests, the experimental microglia are washed thoroughly to make sure the Aβ oligomers in them are completely removed. The washed experimental cells are again divided into 3 groups as shown in Figure 1: One control with no further treatment, one astrocyte group that receives disease associated astrocyte medium into their cultures, and one PD-L1 group that receives only PD-L1 in their cultures. The cells are then placed for 1-2 days, while the effects of the astrocytes take place. Then, the same tests that were run before the astrocytes were added to the microglia are going to be repeated on the 3 groups of microglia, and results will be cross-compared.

For the second section of the experiments, there will be 4 groups of cells with the arrangement shown in Figure 2: Group 1 with microglia and astrocytes from the same mice grown together in a culture without Aβ treatment, Group 2 with microglia and astrocytes grown separately in different cultures without Aβ treatment, group 3 with microglia and astrocytes grown together, with Aβ treatment, and group 4 with microglia and astrocytes grown separately in cultures treated with Aβ and transferring the astrocyte medium with the products of the astrocyte to the culture with microglia. The cells are then cultured for 2-3 days, waiting for the effects to take place.

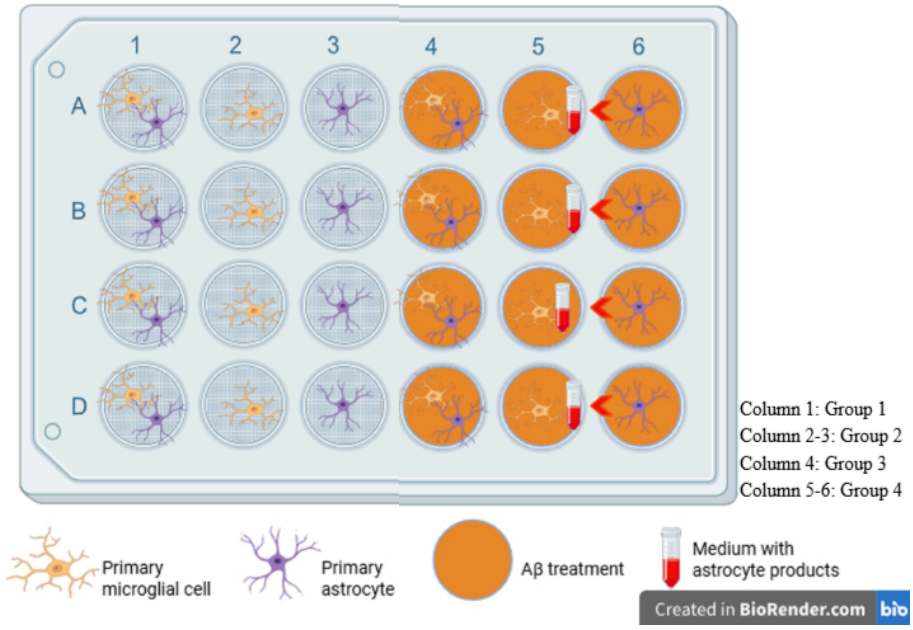


Figure 2. Experimental setting of the 4 groups of microglial cells and astrocytes under different conditions in the second section of the experiment; Created in https://BioRender.com

Following the 2-3 day incubation period, the microglial outcome measures used in the first section, except PD-L1 expression, will be repeated across all experimental groups. Astrocyte responses will also be evaluated by measuring DAA-related marker expression and the proposed astrocyte Aβ-production endpoint, where the former is used to check if interactions between microglia and astrocytes will affect the astrocytes' phenotype, and the latter will test our hypothesis about the astrocytes that they will indirectly lead to the production of more Aβ proteins, through a cascade of signal transduction caused by prolonged inflammation. Results from all four groups will then be compared across conditions. The statistical analysis methods that will be used in the experiment include: one way ANOVA, which compares the means for the three groups; Shapiro–Wilk test to test for normality; and Grubbs' test to test if there are any outliers.

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3. Results

3.1. Results of the first section of the experiment

For the first section of the experiment, the expected results, displayed in Table 1, are related with the effectiveness of astrocytes restoring results in exhausted microglia in the experimental group compared to that of the control group.

Table 1. Expected results: astrocytic PD-L1 restore function in microglia co-cultured with amyloid plaques

Group	Inflammation	PD-1 expression	Phagocytosis of Aβ by microglia	Expression of CD36	Expression of P2RY12
1	High	High	Low	Low	Low
2	Low	Low	High	High	High
3	Low	Low	High	High	High

In the exhausted microglia that were not treated with astrocytes, the level of marker proteins for inflammation and the expression is expected to be high, while the phagocytosis of Aβ by the microglia, and the expression of CD36 and P2RY12 are expected to be low. This is the manifestation of microglial cells in exhausted state, where cells continuously engage in immune responses and become inefficient in engulfing Aβ, and deviate from their normal and healthy state.

For the exhausted microglia that got the treatment from either astrocyte directly and the medium containing products of astrocytes, the expected outcome would be very different than the group without astrocyte intervention. This shows that astrocytes, along with their products, such as PD-L1, are capable of regulating the immune mechanisms of microglia in certain ways.

3.2. Results of the second section of the experiment

For the second section of the experiments, test results are analyzed in 2 parts, classified by the type of cells involved.

3.2.1. For the microglial cells:

Table 2. Expected results: the interactions between DAA and microglia in plaque environment: microglial outcomes

Groups	Inflammation	Phagocytosis of Aβ by microglia	Expression of CD36	Expression of P2RY12
1	Low	High	High	High
2	Low	High	High	High
3	Moderate-high	Moderate	Moderate	Moderate
4	High	Low	Low	Low

The test results shown in Table 2 indicate that direct astrocyte co-culture and the astrocyte medium transfer had different effects on regulating the immune mechanisms of microglia.

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3.2.2. For the astrocytes:

Table 3. Expected results: the interactions between DAA and microglia in plaque environment: astrocyte outcomes

Group	DAA Marker Expression (Serpina3n, VIM, C3)	Astrocyte Aβ Production
1	Low	Low
2	Low	Low
3	High	High
4	Moderate	Moderate

These test results shown in Table 3 predict the changes of the astrocytes' states after the experiment, where there is a difference between the outcomes of the group 3 astrocytes cultured together with microglia and the group 4 astrocytes cultured alone, which indicates that there are certain ways in which microglia can affect astrocytes.

4. Discussion

Astrocytes' role in Alzheimer's disease affecting microglia is then revealed: it plays a double role which leads to both the production of beneficial factors that protects the microglia, and harmful factors that could lead to neuroinflammation and more production of Aβ proteins [4, 7].

The results of the first section of the experiment show that astrocytes, and more specifically PD-L1, are the key protective factors involved in restoring function in exhausted microglia [6]. Because after the addition of astrocytes or PD-L1 into the cultures of exhausted microglia, the inflammation and expression of PD-1, the receptor of PD-L1 decreased, signifying the decrease in exhaustion of the microglia, CD36 expression and the phagocytosis of Aβ increased, signifying the efficiency of the microglia engulfing Aβ proteins increased [6, 8], and the increase of P2RY12 expression may indicate a shift toward a less activated state, although P2RY12 should not be considered solely a marker of resting microglia because P2RY12-positive microglia in AD brains can also express activation markers [10].

The results of the section of the experiment show the dual role of astrocytes: on one hand, the astrocytes restored the microglia into their ground state, just like that in the first section. On the other hand, the astrocytes cultured with Aβ manifested the harmful side, releasing more DAA markers such as serpina3n and complement genes, causing neuroinflammation and more production of Aβ proteins. This is where the position of astrocytes in regulating the immune response of the microglia gets tricky. Both the positive and negative impact are significant, and this evidence reinforces that the use of astrocytes alone without intervention in the therapy of Alzheimer's disease is not sufficient [3, 4, 7].

The disparities in outcomes arise from the difference between direct support via co-culture and indirect support via medium transfer only. The recovery of the microglia needs continuous and steady supply of PD-L1 from the astrocytes; and the medium contains only limited resources with limited supply, therefore the recovery rate and effectiveness of the microglia that received the medium must be less than that of the microglia that were co-cultured with astrocytes. This conclusion provides insight for the different situations in pathological individuals. It provides

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evidence for the interactions between astrocytes and microglia and the corresponding effects, for instance, some microglia are located by proximity to astrocytes while others are not, this difference arises the need of different treatment and that is where the results of different groups in the second part of the experiment can contribute [9].

The reason for the disparity of the outcomes for the astrocytes cultured together with the microglia and the astrocytes cultured alone is that there is a mutual influence between the 2 types of cells. While the astrocyte produces factors that changes the microglia, the microglia may be also influencing the astrocytes by releasing more pro-inflammatory factors, causing the astrocytes to switch into DAA phenotype and express more enzymes that will lead to the production of more Aβ proteins [4, 5, 11].

There are limitations to this study, though. PD-L1 is a ligand that will trigger responses in the microglia, which might conversely cause even more exhaustion to the cells. Because there are factors that readily worsen the condition of the microglial cells, such as the presence of Aβ proteins, the experiment cannot ensure whether PD-L1 also deteriorates the condition of the microglia by consistently triggering the PD-1 receptors on the microglial cells, causing the microglial cells to be more exhausted in terms of their immunological functions. This is a potential disadvantage of the usage of PD-L1 in AD therapy because excessive inflammation leads to neuronal damage and loss of immune homeostais, and may contribute to the progression of the disease in patients.

This research performed a test on the limited effects from limited factors of interaction between microglia and astrocytes in vitro. In the future, more tests that involve in vivo experiments could be done to discover more specific mechanisms of this communication, since in vivo experiments would better approximate the pathological microenvironment, as they preserve the spatial and cellular heterogeneity that is lost in simplified in vitro systems. The significance of this research on future therapeutic approaches is that it suggests that the therapists could focus on balancing the benefits of the astrocytes in their regulation on microglial immune responses and the downturns of DAAs releasing factors that enhances the production of Aβ proteins [9].

5. Conclusion

This research provided new insights to future research in treating Alzheimer's disease by separating PD-L1 effects from other astrocyte signals, and comparing direct contact with soluble-factor signaling; as well as how different states of microglia and astrocytes can affect AD progression. Furthermore, by providing the evidence about the merits and demerits of astrocytes in AD, potential future directions for AD therapeutics are set.

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