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Investigating the Molecular Response of Microglia to Lipopolysaccharide-induced Neurotropic Activation: Implications for Blood-Brain Barrier Breakdown

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ABSTRACT

We investigated the molecular response of microglia, a key player in brain defense, to neurotropic activation by lipopolysaccharide. We found increased expression of pro-inflammatory and regulatory signaling markers, as well as blood brain barrier (BBB) breakdown. Specifically, we observed significant increases in TNF-α (>4-fold) and IL-1β (>8-fold), which indicate inflammation, and in IL-6 and MCP-1, which signal leukocyte recruitment and infiltration. We also saw an increase in MMP-2 expression and a decrease in Occludin expression, indicating BBB breakdown. However, we found no changes in MMP-9 or its inhibitor TIMP-1. Our study is the first to uncover the molecular mechanisms of early immune response and its effect on barrier proteins of the microglial cell, and will be useful for understanding the brain's immune response to infectious diseases.

Keywords: Microglia, tight junction protein, cytokine, blood-brain barrier

1. INTRODUCTION

Microglia, the resident immune cells of the central nervous system (CNS), play a crucial role in maintaining the homeostasis of the brain and responding to various pathological conditions. Microglia, the resident immune cells of the CNS, play a crucial role in defending against infection and inflammation in the immune-privileged environment of the CNS. As members of the macrophageal lineage, microglia are uniquely equipped to respond to and combat pathogens and other immune challenges within the CNS [1]. Microglial cells possess the capability to migrate in response to even the smallest insult or injury in the CNS, specifically the brain [2]. To gain an understanding of the microglial cells' immune response, many models, such as three-dimensional models based on collagen, have been studied using both animal and cell-based methods [2,3]. After encountering foreign materials such as

Lipopolysaccharide (LPS), microglial cells become activated and release a range of cytokines and chemokines necessary for restoring immune balance. These stimuli can be either short-lived or long-lasting injuries, resulting in increased neuroinflammation. Microglial cells also play a significant role in the neuronal inflammasome, controlling processes such as apoptosis and cell death [4].

The blood-brain barrier, or BBB, is a complex structure that acts as a barrier between the bloodstream and the brain. It is made up of specialized cells called endothelial cells, which line the blood vessels in the brain, and the tight junctions between these cells. These structures work together to create a barrier that is both physical and immunological in nature. The BBB is highly selective, allowing certain molecules to cross the barrier while preventing others from passing through. This is accomplished through a combination of passive diffusion and active transport. Passive diffusion is the natural movement of molecules from an area of higher concentration to an area of lower concentration. However, the BBB prevents this process for many substances, including gases and ions, that might be harmful to the brain. On the other hand, the BBB allows certain essential nutrients to cross the barrier through active transport. This is a process by which molecules are moved across the barrier with the help of specific transporters, which recognize and bind to specific molecules. This enables the brain to receive the essential nutrients it needs to function properly while keeping harmful substances out. Overall, The blood brain barrier plays a crucial role in maintaining the delicate environment of the central nervous system, by regulating the transfer of molecules between the blood and the brain [5]. In addition to microglial cells, the blood-brain barrier is composed of various other elements such as astrocytes, podocytes, pericytes, pinocytic vesicles, and tight junction proteins [6]. The glial cells are the ones that react to any small changes in the microenvironment of the central nervous system. It has been observed that changes in the levels of cytokines can affect the permeability of the blood-brain barrier by regulating the tight junctions in the endothelial cells present in the brain's blood vessels [7].

BV-2 cells are a type of mouse microglia that have been genetically modified to be immortalized, which means they can continue to grow and divide in laboratory conditions [8,9]. These cells are widely used in scientific research for studying various pharmacological and immunological processes [10]. They are known to produce a variety of proteins and chemicals, such as chemokines, that are involved in maintaining the immune system and the blood-brain barrier (BBB). Some of the key cytokines produced by BV-2 cells include TNF-α and IL-1β, which have pro-inflammatory roles, while others like IL-6 are involved in the recruitment of immune cells to sites of injury [11]. BV-2 cells are also known to secrete enzymes called metalloproteinases (MMPs)[12] and a protein called Occludin [13], which are thought to play important roles in the breakdown of the BBB.

In the present study, we aimed to investigate the early immune response of microglia under laboratory defined conditions, using the BV2 cell line as a model. We standardized the stimulation of these cells and measured their response in order to establish a well-defined model of microglial activation. Our results demonstrate that this model can be useful for studying various CNS inflammation and infectious conditions, as it allows for the controlled elicitation of immune response in microglia. We propose that this model can provide valuable insights into the mechanisms of microglial activation and aid in the development of new therapeutic strategies for CNS disorders.

2. METHOD AND MATERIALS

2.1. Cell characterization and maintenance for immune response studies

The Murine microglial cell line (BV2) was chosen as the substrate for the study because it is a well-established model for studying the immune response of microglia. These cells were grown in a specific type of medium, Dulbecco's Modified Eagle Medium (DMEM, Himedia), which contains certain chemicals and supplements that help to support the growth of the cells. Additionally, the medium was supplemented with 7.5% sodium bicarbonate (Gibco) and 10% Fetal Bovine Serum (FBS, Gibco) which helps to provide the necessary nutrients and growth factors for the cells. The cells were grown in an incubator at 37°C with 5% CO2 to mimic the conditions of the body. Once the cells reached confluency, they were sub-cultured or propagated by using mechanical disruption procedures or scraping and subsequently centrifuged at 1500rpm for 10 minutes. The cells were then split in the ratio of 1:2 to 1:4 as per the requirement for the experiment.

2.2. Effects of lipopolysaccharide stimulation on the BBB using BV2 cells

BV-2 cells are a type of cell culture that are commonly used in research to study the blood-brain barrier. In this study, the researchers seeded BV-2 cells in a 25 cm2 flask and allowed them to grow overnight in a culture medium called DMEM, which is enriched with 2% fetal bovine serum (FBS). The next day, the cells were treated with a stimulus called Lipopolysaccharide (LPS), which is a type of molecule that can induce inflammation. The LPS was added to the culture medium at a concentration of 100 ng/ml and the cells were incubated with this stimulus for 4 hours. To ensure a fair comparison, an identical set of control flasks were also set up and treated with culture medium alone, without the addition of LPS. This will allow the researchers to observe the effects of LPS specifically, without the influence of other variables [3].

2.3. RNA isolation and cDNA synthesis for gene expression analysis in BV2 cells

In order to analyze gene expression, the researchers first extracted total RNA from the cells using the RNAeasy Mini kit (Qiagen, India). The kit is designed for the isolation of high-quality RNA from a wide range of samples, including cells. The process began by lysing 106-108 cells and then extracting the RNA as per the manufacturer's protocol. After the extraction, the RNA was converted to cDNA (Takara Bio, India) using a reverse transcription process. The cDNA was then stored at -80°C until it was ready to be used for gene expression analysis. This step is important because it enables researchers to analyze the genes that are being transcribed in the cells, which can provide valuable insights into cellular processes and responses.

2.4. qPCR analysis of BBB breakdown and immune response in BV2 cells treated with lipopolysaccharides

In the present study, gene expression was divided into three distinct categories in order to better understand the specific changes occurring in the cells being studied. The first category, labeled as "Inflammatory," includes a group of genes that are known to play a role in initiating and exacerbating inflammation within the body. These genes include Interleukin-1 beta (IL-1β), Tumor Necrosis Factor alpha (TNF-α), Interleukin-10 (IL-10), Inducible nitric oxide synthase (iNOS), Interferon gamma (IFN-γ), and Interleukin-18 (IL-18). The second category, labeled as "Regulatory," includes genes that play a role in regulating the immune response, such as Monocyte chemoattractant protein-1 (MCP-1) and Interleukin-6 (IL-6). The final category, labeled as "BBB

breakdown," includes genes that have been linked to the breakdown of the blood-brain barrier, such as Occludin, Matrix metalloproteinases (MMP2, MMP9) and Tissue Inhibitor of Metalloproteinase (TIMP1). To analyze the expression of these genes, the researchers used a technique called real-time PCR, which allows for the quantification of specific RNA molecules in a sample. They used a kit from Takara Bio (India) and followed the primers and cycling conditions that were described in previous studies [14.15]. A housekeeping gene, GAPDH, was also used as a control to ensure that the changes in gene expression were specific to the genes of interest. To obtain accurate results, three separate BV2 flasks were used for each gene and the ΔCt value, which represents the difference in cycle threshold between the target gene and the housekeeping gene, was calculated. Fold change was then determined using the ΔΔCt method and a change was considered significant if the ΔΔCt value was greater than 2 [16].

3. RESULT AND DISCUSSION

3.1. Morphological changes in BV2 cells following lipopolysaccharide stimulation

Non-stimulated or unstimulated BV2 cells, a commonly used microglial cell line, appeared diverse in terms of shape and structure. Without any external stimulus, the cells displayed a range of morphologies, from spherical to ameboid, and some cells even had protrusions. However, upon exposure to LPS (100ng/mL), a well-established pro-inflammatory stimulus, the cells underwent a significant change in their appearance. The protrusions on the cells were greatly reduced and the overall shape of the cells became more spherical or flattened. Additionally, if the cells had "spikes" present, they were observed to be thickened and shortened as shown in Figure 1. This change in cell morphology after LPS exposure is likely a result of the inflammatory response induced in the cells.

3.2. Evaluation of GAPDH as a housekeeping gene in Lipopolysaccharide-exposed BV2 cell

In order to assess the impact of LPS (lipopolysaccharide) exposure on cellular gene expression, we performed a series of experiments on BV2 cells, a commonly used model for studying microglial activation. One of the initial steps in these experiments was to determine an appropriate reference

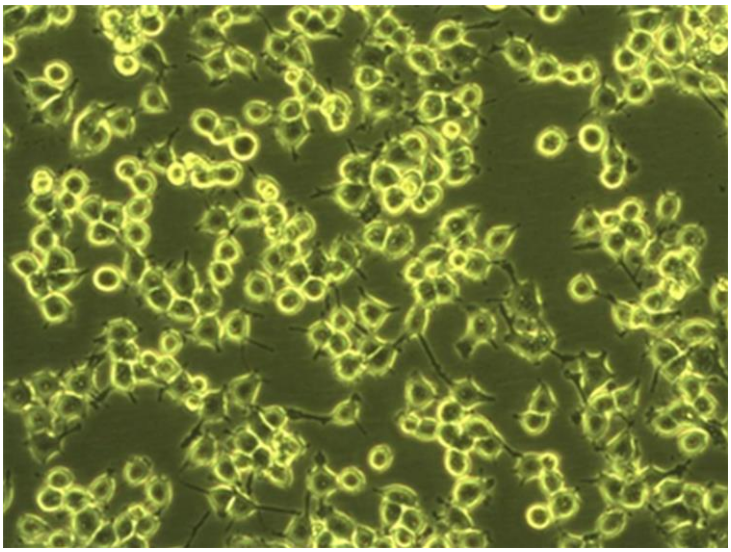

Figure 1. BV2 (Murine microglial) cells grown in DMEM with FBS, cells show heterogeneous morphology with presence of prominent 'spikes' or protrusions.

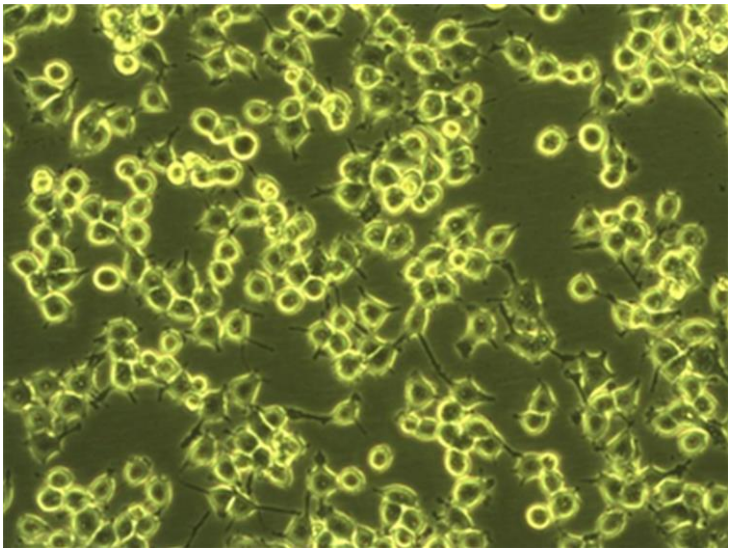

Figure 2. BV2 cells 4 hours after exposure to LPS (100ng/mL), cells show distinct flattening and increased appearance of spherical cells, with reduction in the number of spikes

gene, or "housekeeping" gene, to normalize our data. To do this, we compared the expression of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in untreated and LPS-exposed cells after a 4 hour exposure period. By analyzing the Ct value, a measure of the amount of starting template in the PCR reaction, we observed a fold change of 1.03 between the two groups. This relatively small difference in GAPDH expression between the two groups indicates that it would be a suitable reference gene for normalizing our data. As a result, all further gene expression measurements were normalized to the respective GAPDH levels in order to accurately compare changes in expression between the untreated and LPS-exposed cells.

3.3. Analysis of pro-inflammatory, regulatory, and BBB breakdown markers

In the present study, we exposed BV2 cells to LPS (100ng/mL) for a period of 4 hours in order to induce an inflammatory response. Our observations showed a shift in favor of pro-inflammatory markers, with the primary marker being TNF-α as shown in Table 1. However, it's worthy to note that this early response did not elicit any signs of oxidative stress related apoptosis, as seen by an insignificant rise in the mRNA levels of iNOS. This led us to investigate downstream markers in order to better understand the specific changes that were occurring in the cells. Upon further examination, we observed a similar shift in the regulatory markers under investigation towards inflammation and chemotaxis dependent recruitment of neutrophils and other mononucleocytes as shown in Table 2. In particular, the increment in the expression of IL-6, which is a marker indicated for increased vascular permeability, from microglial cells led us to assess the levels of gelatinases and occludin associated with the breakdown of the blood-brain barrier (BBB). In agreement with our hypothesis, we indeed observed the markers to be regulated in favor of this compromise. Though we had expected both gelatinases under evaluation (MMP-2 and MMP-9) to increase, we did not observe any significant rise in the MMP-9 mRNA levels as shown in Table 3. We presume that our analysis period of 4 hours was insufficient to detect this increase, since its inhibitor TIMP-1 had already started being down-regulated due to LPS induction. This suggests that a longer period of LPS exposure may be necessary in

Table 1. Gene expression of cytokines involved in inducing inflammation after LPS exposure for 4 hours.

Target gene	ΔCt Base line (Mean ± SD)	ΔCt LPS (Mean ± SD)	Fold Change
IL-1β	10.69±0.89	9.34±1.66	2.57
TNF-α	5.93±1.29	3.88±1.64	4.16
IL-10	10.23±0.22	11.92±0.43	-3.24
IFN-γ	8.33±0.80	11.91±0.87	-12.00
*i*NOS	7.84±0.71	7.74±0.96	1.07
IL-18	12.60±0.59	12.26±0.41	1.25

Table 2. Gene expression of chemokines involved in regulating inflammation after LPS induction for 4 hours.

Target gene	ΔCt Base line (Mean ± SD)	ΔCt LPS (Mean ± SD)	Fold Change
IL-6	11.35±1.43	10.19±0.97	2.23
MCP-1	5.40±0.55	4.70±0.45	1.62

Table 3. Gene expression of proteins involved in BBB compromise after LPS induction for 4 hours

Target gene	ΔCt Base line (Mean ± SD)	ΔCt LPS (Mean ± SD)	Fold Change
Occludin	8.28±0.64	11.53±0.45	-9.55
MMP-2	15.42±0.54	12.39±0.80	8.13
MMP-9	12.58±0.76	13.42±1.32	-1.79
TIMP-1	11.81±0.21	15.45±0.71	-12.49

order to fully observe the effects of MMP-9 on BBB breakdown.

Lipopolysaccharide (LPS) is a substance known to have pro-inflammatory effects on the body. Researchers have used in-vitro and ex-vivo models to study the immune response generated by LPS [1]. Microglia, a type of immune cell in the brain, play a crucial role in this response by secreting various cytokines to other cells such as endothelial cells, astrocytes, and podocytes. This helps to contribute to the maintenance of the blood-brain barrier (BBB). Recently, a three-dimensional collagen construct of microglial cells from mice has been created to study the activation of these cells due to LPS. In the present study, the researchers aimed to understand and identify the immune mechanisms involved in three categories: inflammation, regulation, and BBB breakdown. To do this, they used BV-2 cells, which are microglial cells of murine origin [3]. Cytokines have been found to play a role in various forms of neurodegeneration, and are induced in response to brain injury, having diverse actions during cellular injury and repair [17].

The present study aimed to investigate an early microglial response to stimuli in relation to inflammation, immune regulation, and blood-brain barrier (BBB) breakdown. To do this, the researchers activated microglial cells for 4 hours using Lipopolysaccharide (LPS) [17]. At the end of this induction period, they observed a pro-inflammatory response, indicated by an increase in the cytokine TNF-α and a decrease in the cytokine IL-10 [20]. These two cytokines have opposite effects on the body, with TNF-α

being neurotoxic and IL-10 being protective. The researchers also observed that the anti-microbial cytokine IFN-γ was down-regulated more than ten-fold [19]. They suggest that this may be due to the short induction period or the presence of another cytokine, IL-15, which has been known to down-regulate the expression of IFN-γ [21]. Additionally, there was no evidence of oxidative stress to the cells after LPS insult within the exposure period, as observed by an insignificant upregulation in the expression levels of iNOS. This differs from previous studies that have reported a significant upregulation in iNOS levels after longer exposure to LPS or higher concentrations of the polysaccharide [18]. The researchers also investigated the gene expression of IL-1β, a potent signaling molecule that is normally expressed at low levels but is rapidly induced in response to local insults [10]. This makes it an optimal marker for the determination of inflammation in the cells in the present study. The researchers found that inflammation by LPS was confirmed by a significant increase in the mRNA levels of IL-1β. Though IL-1β alone might not contribute to apoptosis of the neuronal cells, it might act synergistically with other Th1 cytokines [10], and more importantly, it is implicated in activation of microglia and self-perpetuating neurotoxicity [23].

Our findings of increased TNF-α and IL-1β gene expressions led us to investigate whether there was a corresponding increase in the expression levels of IL-18, which is known to enhance the expression of these two cytokines [24]. Although we did observe a relative increase in the mRNA levels of IL-18, it was not statistically significant. We hypothesize that the peak IL-18 gene expression may have occurred earlier than the end-time of the LPS exposure. Additionally, we also investigated the downstream effects of this inflammation chain by studying the induction of IL-10 gene expression by TNF-α. After 4 hours, we observed that the negative feedback mechanism had not yet started, as indicated by a relative decrease in the mRNA levels of IL-10.

The blood–brain barrier (BBB) plays a crucial role in preventing the influx of most compounds from blood to brain. However, mechanisms that can cause the breakdown of the BBB include increased vessel permeability, leukocyte infiltration of the central nervous system (CNS), and loss of tight junction proteins. The proper function of BBB is dependent on the function of various cell types in the CNS, such as Pericytes, podocytes, astrocytes, and microglial [25].

In our study, we aimed to understand the regulatory mechanism in microglia that could lead to the breakdown of the BBB during brain inflammation. To do this, we investigated the gene expression of MCP1, a member of the C-C chemokine family that is secreted by microglial cells as an anti-viral defense mechanism. We observed a one and half fold increase in the levels of MCP1, which although not statistically significant, may be an early indicator of BBB breakdown. MCP-1 is known to regulate the infiltration of monocytes, T lymphocytes, and natural killer cells in the neuronal locales through chemo-attraction [26]. Additionally, we also found that vessel permeability is governed by IL-6, which is also known to play a role in amplification of leukocyte recruitment, contributing to local inflammatory responses. In our study model, we observed a two-fold increase in the IL-6 [27] gene expression levels after LPS induction, when compared to the baseline, indicating a shift towards the breakdown of the BBB in the microglial environment [28].

In our study, we placed equal importance on the function of tight junction proteins (TJ proteins) and matrix metalloproteinases, as they play crucial roles in

regulating the integrity of the BBB. TJ proteins are specialized multi-protein complexes located at the apical contact point between cells in epithelial and endothelial tissues, and act to seal the intercellular space, creating a permeability barrier required for transport processes [29]. Among these proteins, Occludin, a tetraspan membrane protein, plays a key role in regulating what enters and exits the neurovascular unit of the brain, and its expression is directly associated with the integrity of the BBB [30]. In the endothelium, proteolytic cleavage of occludin to inactive fragments, which is mediated by matrix metalloproteinases (MMPs), particularly gelatinases (MMP-2 and MMP-9), leads to barrier disruption. In our analysis, we observed a significant decrease in the genomic expression of Occludin, which would be a precursor to low protein levels and a compromised BBB. As a downstream investigation, we decided to study the expression of the gelatinases in a BV2 cell culture model.

Previous studies have shown that MMP-2 plays a role in the degradation of occludin and the alteration of BBB integrity during the early stages of ischemia, while MMP-9 and other factors contribute to further degradation and long-term changes [31]. Our study on LPS-induced inflammation evaluated the early responses at 4 hours of exposure. We found a significant increase in MMP-2 levels, while MMP-9 levels remained similar to the baseline. This is consistent with previous research that suggests MMP-2 plays a role earlier than MMP-9 [31]. As there was no significant change in MMP-9 mRNA levels, we also looked at the expression of its corresponding inhibitor TIMP-1 [32], which was significantly down-regulated, indicating that the low levels of MMP-9 may increase with longer exposure to an inflammation stimulant like LPS.

4. CONCLUSION

In conclusion, our study has shown that BV2 cells are a suitable model for investigating the effects of LPS-induced inflammation on the blood-brain barrier. By using these cells, researchers can study the microglia's role in the breakdown of the blood-brain barrier, as well as other conditions such as neurotoxicity, viral and bacterial brain infections, and immune responses in a controlled and consistent manner, as opposed to using animal models which can vary batch to batch.

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6. CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

7. SOURCE/S OF FUNDING

The current research project was approved by Institutional Bio-Safety Committee (IBSC) vide Ref. HITRT/IBSC/2017/C-2.

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