**Lenalidomide beneficially alters IL-16 methylation status and IL-16 levels under rotenone insult in N9 cells**

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**ABSTRACT:** The role of immune cells in the central nervous system and their interaction with peripheral monocytes are extensively investigated in the recent decade. Both animal models of parkinsonism and post-mortem studies demonstrated that peripheral immune cell recruitment occurs due to neuroinflammation seen in the Parkinson's disease pathology. Understanding these interaction mechanisms and possible modulators that have played a role in these processes is important for treatment options. As an immune anchor of the central nervous system, microglia are suggested to be one of the possible modulators of this interaction. IL-16 was recently described as a novel regulator for T cell recruitment and probably had a role in sporadic Parkinson's disease seen in the Chinese population. With the knowledge of epigenetic alterations in Parkinson's disease is seen in the immune cells, in the present study, we investigated possible changes in IL-16 levels with or without lenalidomide treatment in the rotenone-induced N9 microglial cells by enzyme-like immunosorbent assay (ELISA). Additionally, we also evaluated the methylation status of the IL-16 gene promoter after drug treatments by methylation-specific-polymerase chain reaction (ms-PCR) analysis. Our results indicate that rotenone causes an increase in the IL-16 release, and lenalidomide attenuates that release in microglial cells. Additionally, rotenone changes the methylated status of IL-16 to unmethylated status, which explains increased IL-16 levels. However, lenalidomide treatment inhibited an increase in rotenone-induced IL-16 levels. Therefore, the present study suggests that lenalidomide improves the methylation status of IL-16 and prevented the increase in the IL-16 levels due to the rotenone insult.

**KEYWORDS:** Rothenone; lenalidomide; IL-16; methylation; N9 cells; Parkinson's disease; microglia.

1. **INTRODUCTION**

Microglia are considered as primary cellular defense cells in the central nervous system [1]. Especially, their responsive nature to the local changes and modulatory role, they are essential for homeostasis. Microglial activation, which is named microgliosis, is occurred after disease states such as brain injury, oxidative stress, infection, tumors, and neurodegeneration [2]. After revealing that peripheral immune cells can communicate with the central nervous system, microglial activation and its interaction with peripheral lymphocytes are extensively investigated [3]. Because central-mediated antigen presentation is essential for microgliosis and neuroinflammation, possible mechanisms or mediators are considered important future drug targets.

Interleukin-16 (IL-16) is one of the proinflammatory cytokines which also known as lymphocyte attractant factor (LCF) [4]. Several studies demonstrated that IL-16 acts as an immunomodulatory cytokine that contributes to peripheral CD4+ cell recruitment and activation at the sites of inflammation or autoimmune diseases [5,6]. Additionally, IL-16 was also demonstrated to be a migratory signal modulator and regulate the secretion of other proinflammatory cytokines [7]. Especially in the human brain, the attraction of peripheral CD4+ to the central nervous system under neuroinflammatory circumstances is reported to be modulated by IL-16 [8]. Therefore, IL-16 could be an essential target for neuroinflammation and neurodegenerative diseases as a bridge between peripheral and central nervous system communication. Recent studies demonstrated that microglia could release IL-16 under inflammatory and malignant conditions [9]. Therefore, it is rational to think that IL-16 could regulate microglial-mediated neuroinflammation seen in neurodegenerative diseases. In Parkinson's disease, oxidative stress and inflammation causes impairment in the blood-brain barrier.
integrity and migration of peripheral lymphocytes to the central nervous system and exacerbates neuron loss [10]. Microgliosis is especially considered responsible for this central and peripheral communication due to the innate migratory and immune regulator roles. Thus, compounds that inhibit microgliosis or cytokines which have a role in communication are essential candidates for future therapy options.

Rotenone is a potent mitochondrial complex I inhibitor which is widely used to mimic Parkinson’s disease [11]. Additionally, rotenone is also a useful tool to understand the inflammatory-immune response in microglial cells. Therefore, in this study, to understand possible IL-16 response in microglial cells rotenone was selectively used. In the last decades, epigenetic changes have emerged as vital genetic alterations for the start and progression of neurodegenerative disorders or neuroinflammation [12]. Mainly, methylation is a significant change that directly regulates cell communication and behavior. As a result of methylation, gene transcription is repressed. That balance is tightly regulated in the microglial cells to maintain homeostasis of the central nervous system’s environment. Recent studies suggested that epigenetic alterations in microglial cells could have a role in microgliosis and its interaction with peripheral cells [13]. The methylation status of numerous mediators in the microglia is investigated.

Lenalidomide, a novel analog of thalidomide, is an immunomodulatory drug currently used in multiple myeloma treatment. The effect of lenalidomide on the central nervous system, particularly in the neuroinflammation context, is described in previous studies [14]. Additionally, neuroprotective effects of lenalidomide on Parkinson’s disease is showed in cell lines and animal models [15]. However, the possible role of lenalidomide on IL-16 expression is suggested in studies conducted on multiple myeloma, the effect of lenalidomide treatment on IL-16 levels in microglia is still missing. Significantly, the protective effects of lenalidomide on rotenone-induced inflammation are well described [15,16]. So, we used rotenone to induced microglial cells to the inflammatory state in the present study. Additionally, based on the immunomodulatory effects of lenalidomide on microglia and its previously described effects on methylation status on different cell types, we sought to investigate the effect of lenalidomide treatment IL-16 levels and its methylation status in rotenone-induced microglial cells.

2. RESULTS

2.1. Lenalidomide rescues rotenone-induced loss in cell viability

Cell viability was assessed by MTT test (Figure 1). Due to the rotenone at the 30 (57.7±2.21), 100 (31.6±6.65), 300 (21.2±7.31), 1000 (18±3.33) nM concentrations caused more than 70% loss in cellular viability, 10 nM (76.3±3.78) concentration was selected for further experiments (Figure 1A). Lenalidomide was co-treated with rotenone at the 1, 3, 10, and 30 µM concentrations (Figure 1B). Our results demonstrated that lenalidomide at the 10 (85.7±2.37) and 30 (89.3±4.51) µM concentrations significantly inhibited rotenone-induced loss in N9 cells (p=0.018 and p<0.001, respectively, Figure 1B). Therefore, 10 nM rotenone and 10 µM lenalidomide concentrations were selected for further experiments.

2.2. Rotenone-induced increase in IL-16 levels attenuated by lenalidomide treatment

IL-16 levels were determined by ELISA (Figure 2). Our results demonstrated that rotenone (311±27.2 pg/mL) caused a significant increase in the IL-16 levels compared to the control (32.1±9.17 pg/mL, p<0.001, Figure 2). Although single lenalidomide (32.5±13.5 pg/mL) treatment did not cause any change in cell media, co-treatment with rotenone prevented the rotenone-induced increase in IL-16 levels (154±25.3 pg/mL, p<0.001, Figure 2).

2.3. Lenalidomide rescues rotenone-induced methylation status of IL-16 promoter

Methylation-specific PCR results demonstrated that the IL-16 promoter was 67.08±6.14% methylated and 32.91±14% unmethylated in the control group (Figure 3). IL-16 methylation status was found 30.83±7.26% methylated and 69.16±7.26% unmethylated in the rotenone group (Figure 3). Although methylation status of IL-16 was found to be 73.3±18.05% methylated and 26.6±18.05% unmethylated in single lenalidomide treatment, it was found 66.6±23.57% methylated and 33.3±23.56% after co-treatment of rotenone and lenalidomide (Figure 3).
Figure 1. Cell viability results after rotenone (A) and lenalidomide (B) co-treatment. Rotenone dose-dependently decreased cellular viability in investigated doses (A). Lenalidomide at the dose of 10 and 30 µM improves the cellular viability of rotenone-induced microglial cells (B). Statistical differences were evaluated by one-way ANOVA test, and all data are represented as mean±SD. ***p<0.001, *p<0.05 versus control.

Figure 2. IL-16 expression in the N9 microglial cell media after drug treatments. Rotenone remarkably increased IL-16 levels. In contrast, lenalidomide prevented IL-16 increase. Differences between groups were evaluated by Kruskal-Wallis, and all data are represented as mean±SD. ***p<0.001 versus control and ###p<0.001 versus rotenone.
3. DISCUSSION

In the recent decade, the knowledge that the brain is an immune-privileged organ, has changed after identifying different interactions between peripheral immune cells and the central nervous system [17]. Accumulating evidence suggests that innate and adaptive responses in the microglia causes alterations in the central nervous system and attract peripheral T cells to ongoing neuroinflammation. Gaining a better knowledge in between these two compartments is now considered fundamental for understanding neuroinflammation, neurodegeneration, and in our case, Parkinson’s disease. This interplay needs signaling elements, pathways, or mechanism that is added to the literature day by day [18]. In physiological conditions, neurons are protected by the peripherally insults or immune cells by the blood-brain barrier, the blood-cerebrospinal fluid barrier, and meninges. Homeostatic conditions maintain the permeability of these structures to protect neurons from unintended harmful stimulus. Nevertheless, these changes in these intact structures' permeability are shown to be observed under disease conditions, such as neuroinflammation, neurodegeneration, or infection [19]. However, astrocytes in the end foot of the blood-brain barrier are considered as a significant regulator during disease states, microglia also showed to be another essential cell type due to their migratory and secretory phenotypes [20,21]. Furthermore, released cytokines or other mediators from the microglia can also change permeability during Parkinson’s disease [22]. Therefore, identifying primary cytokines responsible for these interactions is crucial for future therapeutic interventions. In this study IL-16, which is one of these candidates expressed by monocytic cells, was investigated. IL-16 is a ligand for CD4 T cell cells and is shown to contribute to these cells’ activation in the inflammation sites and recruit other T cells [23].

In physiological conditions, IL-16 in human microglia is minimally expressed, but its secretion and attraction of peripheral CD4 T cells across the blood-brain barrier are shown in disease states [9]. Due to their innate ability to trigger inositol triphosphate generation, translocation of protein kinase C and secretion of proinflammatory cytokines control various aspects of T cells in the central nervous system. Guo et al. demonstrated the expression of IL-16 by microglial cells in several different insults [24]. Their study suggested that IL-16 could describe microglial cell activation as a valuable marker for ongoing microgliosis during
disease states. Additionally, increased IL-16 during inflammatory states also has a role in antigen presentation mediated by MHCII expression [25]. Therefore, activation of IL-16 in the microglial cells is essential for more than one aspect of the central nervous system's immune regulation. However, reports about IL-16 expression in the microglia are limited. This study investigated IL-16 expression specifically on the microglial cell to delineate its expression under rotenone insult. Our results align with previous studies that IL-16 expression increases during neuroinflammation and that increase alleviated by lenalidomide treatment.

Epigenetic alterations in Parkinson's disease are considered vital for disease progression and epidemiology. Several studies investigated the role of epigenetic modifications, especially methylation and acetylation, and suggested that these modifications could change the response of microglia's inflammatory conditions [12]. Due to the methylation has been shown to affect gene expression robustly, delineating possible status of IL-16 methylation in microglia provides valuable knowledge about the inflammatory role of microglia. Therefore, in this study methylation status of IL-16 expression in microglial cells was investigated. Our results align with IL-16 levels in the cell media, and rotenone treatment altered methylated IL-16 to an unmethylated state. Additionally, our results also support the hypothesis of increased antigen presentation and immune activation after rotenone treatment, reported in previous studies on Parkinson's disease. In contrast, the co-treatment of lenalidomide with rotenone caused a change in methylation status to the methylated state, which was parallel with IL-16 levels. Neuroprotective effects of lenalidomide are showed in Parkinson's disease models [15]. However, none of these models have investigated antigen presentation or IL-16 expression, but our results seem to make sense when considered together with lenalidomide's immunomodulatory effects. However, our results also in line with attenuated interleukin-16 expression after lenalidomide treatment on multiple myeloma cells.

4. CONCLUSIONS

In conclusion, lenalidomide protects against rotenone-induced cell loss in microglial cells. As far as we have known this is the first study that investigates the effect of rotenone and lenalidomide treatment on IL-16 expression on microglial cells. Our results suggest that inhibitory effect of lenalidomide on rotenone-induced IL-16 increase might be modulated with increasing methylation status of IL-16 promoter. Nevertheless, further studies must be investigated under microglial stimulants such as TNF-α, interferon-γ, LPS, or alpha-synuclein, an intercellular protein related to Parkinson's disease pathology, to show possible differences.

5. MATERIALS AND METHODS

5.1. Chemicals

Rotenone, lenalidomide, dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich. Dulbecco's modified eagle medium (DMEM, 11965084), fetal bovine serum (FBS, 11550356), penicillin/streptomycin (10003927), Dulbecco's phosphate-buffered saline (dPBS, 14190144), trypsin-EDTA (25200072) used in the cell culture were obtained from Gibco (Fisher Scientific, USA). Rotenone and lenalidomide dissolved in the DMSO to prepare x50 stock solution, and aliquots were stored at -80°C. On the day of the experiments, rotenone and lenalidomide were freshly diluted in cell mediums.

5.2. Drug treatments and cell viability

N9 murine microglial cells were kindly provided by Dr. Paola Ricciardi-Castagnoli (Cellular Pharmacology Center, Milan, Italy). Cells were seeded to the 75 cm² flasks in 10% FBS and 1% penicillin/streptomycin containing DMEM and incubated at 37°C on 5% CO₂. After reaching 80% confluency, cells were washed with dPBS and detached by 0.25% trypsin-EDTA for seeding needed plates for further experiments. Cells were seeded to 96-well plates for the MTT test. After 24-hour drug treatment, cells were washed with dPBS to avoid chemical interaction. Medium-diluted MTT (0.5 mg/mL) was administered to cells for 4 hours in a dark area. Formazan crystals were dissolved with DMSO, and optic density was determined spectrophotometrically at 570 nm (TECAN, Austria). Concentrations of rotenone (1, 3, 10, 30, 100, 300 and 1000 nM) and lenalidomide (1, 3, 10, 30 µM) treatments were selected according to the viability results under constant cell culture conditions (pH 7.4, 37°C).
5.3. Determination of IL-16 levels

Cells were seeded to 12-well plates for drug treatments. Following drug treatments, mediums were isolated. Centrifuged medium supernatants were used for the determination of IL-16 levels by ELISA. IL-16 ELISA kit (Sun-red Co., Wuhan, China) was used for analysis with strictly following the manufacturer's instructions.

5.4. DNA isolation and bisulfite modification

Cells were seeded in 6-well plates for DNA isolation and bisulfite modification. Cells were briefly washed with dPBS and detached by 0.25% trypsin-EDTA solution. Following the elimination of trypsin-EDTA with centrifugation, cells were diluted in 1 mL dPBS. DNA was isolated with DNA Mini Kit® (Geneid, Taiwan), strictly following the manufacturer's instructions. DNA concentration was determined by a NanoDrop spectrophotometer (Jenway Genova Nano, England). To sensitively determine methylation status to distinguish methylated sequences bisulfite modification was employed [26]. According to the manufacturer protocol, following DNA isolation (500 ng/20 μL), bisulfite modification was performed by EpiJET® Bisulfite Conversion Kit (Thermo Fisher Scientific, Lithuania).

5.5. Methylation-specific polymerase chain reaction (PCR) analysis

IL-16 promoter methylation status was analyzed with methylation (M) and unmethylation (U) – specific primers. Methylation-specific PCR primers were selected from a freely available Urogene network. Polymerization was performed by Taq DNA Polymerase (Thermo Fisher Scientific, Lithuania) under the following cycling conditions; initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95°C for 40 s; 57°C for 40 s and 72°C for 70 s. The final extension was carried out at 72 °C for 7 min and then maintained at 4°C. Then, PCR products were separated on a 2% agarose gel. DNA ladder (Thermo Fisher Scientific, Lithuania) was used as a size marker for PCR products. Methylation status was evaluated by the detection of M and U PCR products after amplification.

5.6. Statistical analysis

All experimental data were analyzed by SPSS (v21.0, Illinois, USA). All data were expressed as mean±SD. Following the determination of data distributions, Kruskal-Wallis and One-Way (ANOVA) tests were performed. Multiple comparisons were employed by Tukey’s and Mann-Whitney U post hoc analysis.

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