의학석사 학위논문

인터루킨-22와 인터루킨-22수용체가 뇌에서의 염증반응 조절에 미치는 영향에 관한 연구

The role of interleukin-22 and its receptor on the regulation of inflammatory responses in the brain

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The role of interleukin-22 and its receptor on the regulation of inflammatory responses in the brain

by

Dahae Lee

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Master of Science in Medicine (Anatomy) at Seoul National University College of Medicine

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ABSTRACT

The role of interleukin-22 and its receptor on the regulation of inflammatory responses in the brain

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Interleukin (IL)-22 is a member of IL-10 family, is a potent mediator of inflammatory responses. It is produced by activated $CD4^+$ T cells and natural killer (NK) cells and takes effect on non-hematopoietic cells mainly stromal and epithelial cells. And, IL-22 receptor consists of IL-22Rα and IL-10Rβ. It is known that IL-22Rα expression is restricted to non-hematopoietic cells in the skin, pancreas, intestine, liver, lung and kidney. Even though IL-22 is mainly involved in the development of inflammatory responses, but there is a no report regarding their roles on inflammatory responses in the brain. In the present study, I investigated the role of IL-22 and its receptor on inflammatory responses in the brain using mouse microglia cell line, BV2 and mouse hippocampal neuronal cell line, HT22.

When BV2 and HT22 were treated with rIL-22 (20 ng/ml), the expression of cyclooxygenase (COX)-2 was increased and its expression is followed by the increase of PGE2 production. Next, I examined the production of pro-inflammatory cytokines, such as IL-6 and TNF-α and pro-inflammatory chemokines by IL-22 treatment. As a result, I confirmed that IL-6, TNF-α, and MIG/CXCL9 are remarkably increased. Taken together, IL-22Rα is spontaneously expressed on cells in the brain, especially microglia and neuron, and it is closely involved in the development of inflammatory responses after interaction with IL-22.

Keywords: IL-22, IL-22Rα, Inflammation, BV2, HT22

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LIST OF ABBREVIATIONS

IHC: immunohistochemistry

PFA: paraformaldehyde

ELISA: enzyme-linked immunosorbent assay

HRP: horse radish peroxidase

IL: interleukin

PBS: phosphate buffered saline

PBST: PBS containing 0.1% Tween 20

rIL-22: recombinant interleukin-22

RT-PCR: reverse transcription-polymerase chain reaction

Th2: type 2 helper T cell

INTRODUCTION

Interleukin (IL)-22, a member of the IL-10 cytokine family, is produced by several subsets of lymphocytes such as $CD4^+$ T helper 17 (Th17) cells and Th22 cells, natural killer (NK) cells, $CD8^+$ cytotoxic T cells, γδ T cells and lymphoid tissue inducer (LTi)-like cells (Dumoutier *et al.* 2001, Kim *et al.* 2017, Kotenko *et al.* 2001, Ouyang and O'Garra 2019). In addition, it is recently reported that IL-22 is the production of increased in activated macrophage (Akil *et al.* 2015). Moreover, IL-22 is closely related with autoimmune diseases such as rheumatoid arthritis (RA) (Roeleveld and Koenders 2015), skin inflammatory diseases (Ma *et al.* 2008) and Crohn's disease (Torres *et al.* 2017) by promoting inflammatory responses. According to report by (Boniface *et al.* 2005), IL-22 up-regulates the production of acute-phase proteins in hepatoma cells. It suggests that IL-22 is involved in the inflammatory responses. However, there are several reports regarding anti-inflammatory role of IL-22 in inflammation bowel diseases (He *et al.* 2018, Mizoguchi *et al.* 2018, Seiderer *et al.* 2007).

The biological role of IL-22 was originally described in hepatoma, keratinocytes and pancreatic acinar cells (Boniface *et al.* 2005, Brunner *et*

al. 2019, Fujita 2013), thereafter reported to be involved in the pathogenesis of numerous inflammatory diseases, notably in skin inflammation such as psoriasis (Ito *et al.* 2019, Ma *et al.* 2008). IL-22R is a heterodimeric form of IL-22Rα and IL-10Rβ (Jang *et al.* 2016, Nograles *et al.* 2009, Ouyang and O'Garra 2019). It is generally known that IL-10Rβ is expressed in all cells, including immune cells. And IL-22Rα is not expressed on immune cells, but selectively found its expression epithelial cells in the studied lung (Hebert *et al.* 2018), kidney (Weidenbusch 2018) and colon cells (Akil *et al.* 2015, Weidenbusch 2018). For this reason, the role of IL-22Rα have been studied on non-hematopoietic organs. Still, there are no reports regarding the expression of IL-22Rα and its role in brain. In this study, I examined whether IL-22α is expressed in the brain. And so, then its role on the development of inflammatory responses in the brain also investigated.

It is widely known that microglia are important cell during the development a progression of inflammatory responses in the brain (Dzamba *et al.* 2016, Perry *et al.* 2010). Microglia, the resident macrophages of the central nervous system (CNS), are exquisitely sensitive to brain diseases and injury, altering their morphology and phenotype to adopt activated state in response to pathophysiological brain

insults (Forloni *et al.* 2016). The origin of microglia is generally considered to be bone marrow-derived monocytes and it is a non-neuronal cell scattered in the central nervous system (CNS), and immune cells that protect the living body by reacting to brain infection and damage (Baek and Kim 2016, Conley and Diaz-Arrastia 2017). When the brain is injured by brain diseases, microglia morphologically transform into "activated microglia" which show retracted processes and enlarged cell bodies and become proliferative at the injured site (Al-Dahhak *et al.* 2018, Cho *et al.* 2011, Espinosa-Fernandez *et al.* 2019, Henn *et al.* 2009). These activated microglia appear to be implicated in many pathological states, in particular, their cytotoxic and inflammatory roles through the production of IL-6, IL-1β and TNF-α (Dzamba *et al.* 2016, Lee *et al.* 2006). The activated microglia release various bioactive molecules including nitric oxide (NO) and reactive oxygen species (ROS). And Cyclooxygenase (COX), also known as an enzyme for prostaglandin (PG) H (Bitto *et al.* 2017, Shi *et al.* 2012, Woodling *et al.* 2014). COX-1 and COX-2 are present in several regions of human brain, even so COX-2 is the major inducer of inflammatory processes in the hippocampus (Javed *et al.* 2012, Sil and Ghosh 2016, Woodling *et al.* 2014).

The hippocampus is critical for learning, memory and cognition (Amani

et al. 2019, Busche 2018, Liu *et al.* 2013). In particular, hippocampus plays an important role in spatial memory, enabling information integration and memory from short-term memory to long-term memory (Arrieta-Cruz and Gutierrez-Juarez 2016). However, there is no report IL-22Rα expression in hippocampus and its role on inflammatory responses that is closely related Alzheimer's disease. Therefore, I have studied the role of IL-22 and its receptor on inflammatory responses in the brain using murine microglia cell line, BV2 and murine hippocampal neuronal cell line, HT22.

MATERIALS AND METHODS

Cell culture The murine microglia cell line, BV2, murine hippocampal neuronal cell line, HT22 and murine hepatoma cell line, Hepa1c1c7 were maintained in DMEM (HyClone, Queensland, Australia) supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and antibiotics (100 U/ml of penicillin and 100 μg/ml streptomycin; Welgene, Namcheon-myeon, South Korea) at 37°C in a humidified atmosphere containing 5% CO_2. HT22 was kindly provided by professor Mook, Inhee (Seoul National University College of Medicine).

Animals C57BL/6 mice were maintained in specific pathogen free condition at the animal facility in the Seoul National University College of Medicine. The animal protocol for experiments was reviewed and approved by Ethics Committee of the Seoul National University.

Immunohistochemistry (IHC) Mice were sacrificed with perfusion with 4% paraformaldehyde (PFA). Brains were removed and post-fixed in 4% PFA overnight at 4°C. After transferred to 15% sucrose and placed at room temperature for overnight. And then stored in 30% sucrose at 4°C. Fixed

tissues were embedded in paraffin and sectioned with 4 μm thickness. After deparaffinization and hydration, epitope of antigen was retrieved by heating with 0.1 M citrate buffer (pH 6.0) with microwave. After blocking endogenous peroxidase with H_2O_2 and inhibiting nonspecific signals with 5% goat serum contained blocking solution (Vector Laboratories, Burlingame, CA, USA) for 1 hr at room temperature, tissue sections were incubated with primary antibody against IL-22 receptor alpha (IL-22Rα) antibody (1:150; Abcam, Cambridge, UK) at 4°C for overnight in a humidified chamber. And then incubated with biotinylated goat anti-rat immunoglobulin as a secondary antibody (1:250; Vector Laboratories) for 1 hr at room temperature. ABC solution (Vector Laboratories) was loaded on sections for 30 mins and DAB kit (Vector Laboratories) was used for chromogenic detection. After counter staining with hematoxylin, dehydration and clearing, tissue sections were mounted (Life technologies, Frederick, MD. USA). For image analysis, visualizing an Olympus AX-70 microscope equipped with a motorized stage (Olympus, Melville, NY, USA) and the MCID 6.0 Elite Imaging Software (GE healthcare, Piscataway, NJ, USA).

Flow cytometry analysis Cells were resuspended in ice cold fluorescence

activated cell sorting (FACS) buffer containing 0.5% BSA and blocked at 4°C for 10 mins with FcR blocking reagent (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). For analysis, cells were stained with rabbit developed polyclonal anti-IL-22Rα Ab in 2.5 μg/10^6 cells (Abcam) at 4°C for 30 mins. After washed cells three times with FACS buffer at 1500 rpm (3 mins/each), and then stained with mouse developed anti-rabbit IgG-FITC 1:400 as a secondary antibody (Santa cruz Biotechnology, Santa Cruz, CA, USA) on ice for 30 mins. And washed twice with FACS buffer (3 mins/each). The cells were analyzed by Attune NxT Flow Cytometry (Thermo scientific, Wilmington, DE, USA). FlowJo software (Tree Star, Ashland, OR. USA) was used for data analysis.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) To examine the expression of IL-22Rα, IL-10Rβ and COX-2 in BV2 and HT22 were performed. Briefly, cells were cultured for 24 hrs and 48 hrs after the treatment of rIL-22 (20 ng/ml). Total cellular RNA was extracted from 1 x 10^6 cells using TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using 1 μg of total RNA in a first-strand complementary DNA synthesis reaction with AMV Reverse Transcriptase (Promega, Madison, WI, USA). The primer used for the RT-PCR was as

follows: 5′- CTG CAA CCT GAC TAT GGA GA-3′ (forward) and 5′-TTC ACT CGG CAC ACG TAG GG-3’ (reverse) for IL-22Rα (425 bp) / 5′- AAG CAG AGT CCT GAA GAC AA-3′ (forward) and 5′-AGA TCA CTG TGA TCC TCC TG-3’ (reverse) for IL-10Rβ (310 bp) / 5′- ACA CAC TCT ATC ACT GGC ACC -3′ (forward) and 5′- TTC AGG GAG AAG CGT TTG C -3’ (reverse) for COX-2 (274 bp) / 5′- GAG AGT GGT GCC AGT CTA GT-3′ (forward) and 5′-GCC ACA CTC CTC CAC AAT CA-3’ (reverse) for β-actin (207 bp). The PCR amplification process consisted of 35 cycles of 94°C for 15 s; 57°C for 45 s; and 72°C for 1 min for IL-22Rα / 30 cycles of 94°C for 15 s; 58°C for 45 s; and 72°C for 1 min for IL-10Rβ / 35 cycles of 94°C for 15 s; 55°C for 45 s; and 72°C for 1 min for COX-2 / 35 cycles of 94°C for 15 s; 58.1°C for 45 s; and 72°C for 1 min for β-actin. PCR products were separated by electrophoresis on 1.5% agarose gel and visualized by staining with Red Safe (Intron biotechnology, Seongnam, Korea). And PCR products were electrophoresed and the density of each band was analyzed by Image J software (NIH, Bethesda, MD, USA).

Cell counting kit-8 (CCK-8) assay Cells were cultured in the presence or absence with 5, 10, 20 ng/mL of rIL-22 (R&D systems, Minneapolis, MN, USA) for 72 hrs. CCK-8 solution was added to each well of the plate and

then absorbance was measured at 450 nm using the SpectraMax iD3 and normalized with Softmax Pro software (Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis Cells (1 x 10^6) were lysed and proteins were extracted using lysis buffer containing 50 mM Tris-HCL (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktails. The protein concentration was measured with BCA assay. An equal amount of protein (30 μg/sample) was dissolved in a 10% polyacrylamide-SDS gel with 100 V for 24 hrs and transferred onto a nitrocellulose membrane. Blocking was performed for 1 hr at room temperature with 5% non-fat milk in PBS containing 0.1% Tween 20 (PBST). The blocked membrane was incubated with anti-IL-22Rα Ab (1:5000; Abcam), anti-IL-10Rβ Ab (1:5000; Sigma, St. Louis, MO, USA), anti-β-actin Ab (1:5000; Sigma) at 4°C for overnight. After washing 3 times (5 mins/each) with 0.1% PBST, membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:10,000; Cell signaling Technology, Boston, MA, USA) for IL-22Rα / HRP-conjugated anti-mouse IgG Secondary Ab (1:10,000; Cell signaling Technology) for IL-10Rβ and β-actin as a secondary Ab at RT for 1 hr. The membrane was

washed 3 times (5 mins/each) and the immune reactive proteins were visualized with the electrochemical luminescence (ECL) detection system (Thermo scientific). The bands were analyzed for their density using the Image J software (NIH). Results were expressed as relative intensity and each band was adjusted to that of β-actin.

Enzyme-Linked Immunosorbent Assay (ELISA) Cells were seeded in 6-well at 2 x 10^5 cells/well with or without recombinant IL-22 (R&D systems), and allowed to grow to confluence for 24 hrs and 48 hrs. After the culture supernatants of BV2 and HT22 were collected, the concentration of IL-6 and TNF-α (BioLegend, San Diego, California, USA) and PGE2 (R&D systems) in the culture supernatants were measured by ELISA. ELISA was performed according to the manufacturer's instruction and the relative absorbance was measured at 450 nm and concentrations were calculated using the SpectraMax iD3 (Molecular Devices).

Mouse pro-inflammatory chemokine panel assay BV2 cells were seeded in 6-well at 2 x 10^5 cells with or without recombinant IL-22 (R&D systems) and allowed to grow to confluence for 48 hrs. The production of 13 kinds of chemokines were measured with LEGENDplex™ analysis kit

(BioLegend). Beads for chemokines were prepared by vortexing for 30 secs and added to culture supernatants in 96 well plate. The plate was covered with aluminum foil to protect the plate from light with gentle agitation at 800 rpm on a plate shaker for 2 hrs at room temperature. After centrifuge the plate at 1050 rpm for 5 mins, supernatants were discarded immediately. Plate was washed 4 times (5 mins/each) with washing buffer. Detection antibodies were added to each well, and then shake at 800 rpm at RT for 1 hr. And add streptavidin-phycoerythrin (SA-PE) to each well directly and shake the plate on a plate shaker at 800 rpm at RT for 30 mins. After 30 mins, resuspended the beads by pipetting. Since the beads are differentiated by size and internal fluorescence intensity on a flow cytometer, analyte-specific populations can be segregated and PE fluorescent signal quantified. So resuspended cells were analyzed by FACS Attune NxT Flow Cytometry (Thermo scientific), FlowJo software (Tree Star) and LEGENDplex^{tm} analysis software were used for data analysis.

Statistical analysis Data were presented as means ± SDs. Unpaired *t* test was used to compare two groups. Statistical analysis was carried out using Graph Pad Software Prism version 6.01 (Graph Pad Software, Le Jolla, CA, USA)

Results

IL-22Rα is constitutively expressed on murine microglia cell line, BV2 and hippocampal neuronal cell line, HT22

It is known that IL-22Rα is expressed on non-hematopoietic cells, such as pancreatic epithelial cells, hepatocytes and keratinocytes. However, it is still unknown that whether it is expressed on the cells in the brain. Therefore, IL-22Rα expression in murine microglia cell line, BV2 and murine hippocampal neuronal cell line, HT22 at transcriptional and translational levels was examined by RT-PCR, western blotting and flow cytometry analysis. As shown in Fig. 1, I confirmed that IL-22Rα mRNA is expressed in both of cell lines. I also confirmed that it is expressed on the surface of BV2 and HT22 by western blotting and flow cytometry analysis (Fig. 2 and 3).

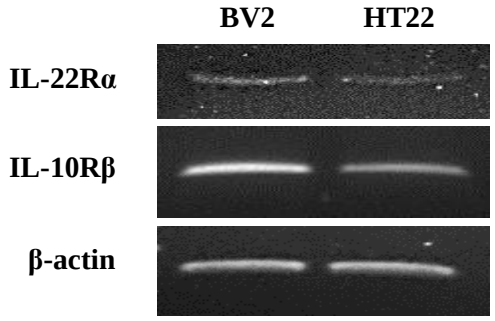


Fig. 1 The expression of IL-22Rα in BV2 and HT22 at transcriptional level

Cells (1 x 10^6) were collected and total RNA was extracted by Trizol. And then cDNA was made by reverse transcription. RT-PCR was performed by using the specific primer for IL-22Rα and IL-10Rβ as described in *Materials and Methods*. Result is representative of three independent experiments.

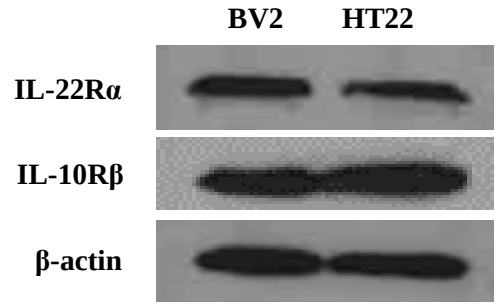


Fig. 2 The expression of IL-22Rα in BV2 and HT22 at translational level: Western blot analysis

Cells (1 x 10^6) were lysed and protein was extracted for western blot analysis as described in *Materials and Methods*. βeta-actin was used as a loading control. Result is representative of three independent experiments.

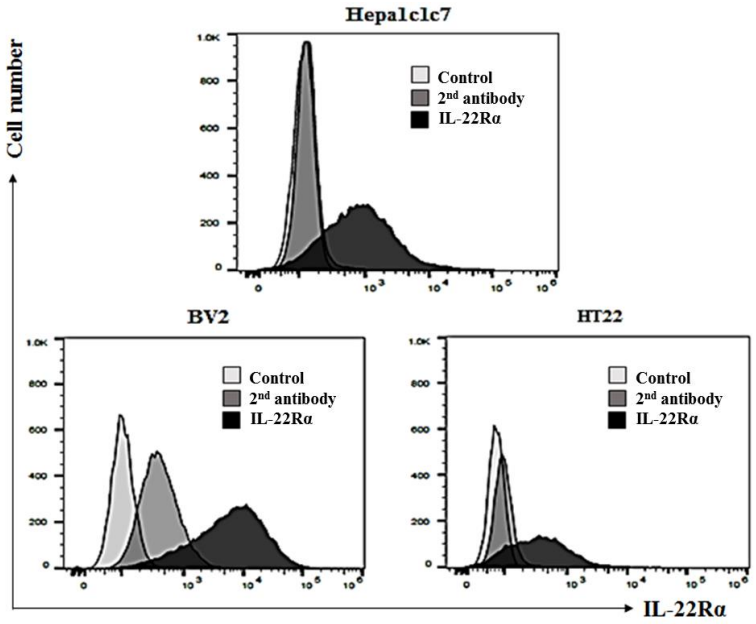


Fig. 3 The expression of IL-22Rα in BV2 and HT22 at translational level: Flow cytometry analysis

Cells (1 x 10^5) were collected and resuspended in FACS buffer containing 0.5% BSA. After cells were treated with Fc blocking reagent at 4°C for 10 mins, and then stained with rabbit developed anti-mouse IL-22Rα Ab (2.5 μg/10^6) on ice for 30 mins. FITC-conjugated anti-rabbit Ab was used as secondary Ab (1:40). IL-22Rα expression was determined by flow cytometry analysis. Result is representative of three independent experiments.

IL-22Rα is constitutively expressed in mouse brain tissue

Since I have already confirmed that IL-22Rα is expressed in murine microglia cell line, BV2 and murine hippocampal neuronal cell line, HT22. IL-22Rα expression in the brain tissues were examined by immunohistochemistry. It is shown that IL-22Rα is constitutively expressed in mouse brain tissues, especially in hippocampus and cerebellum (Fig. 4).

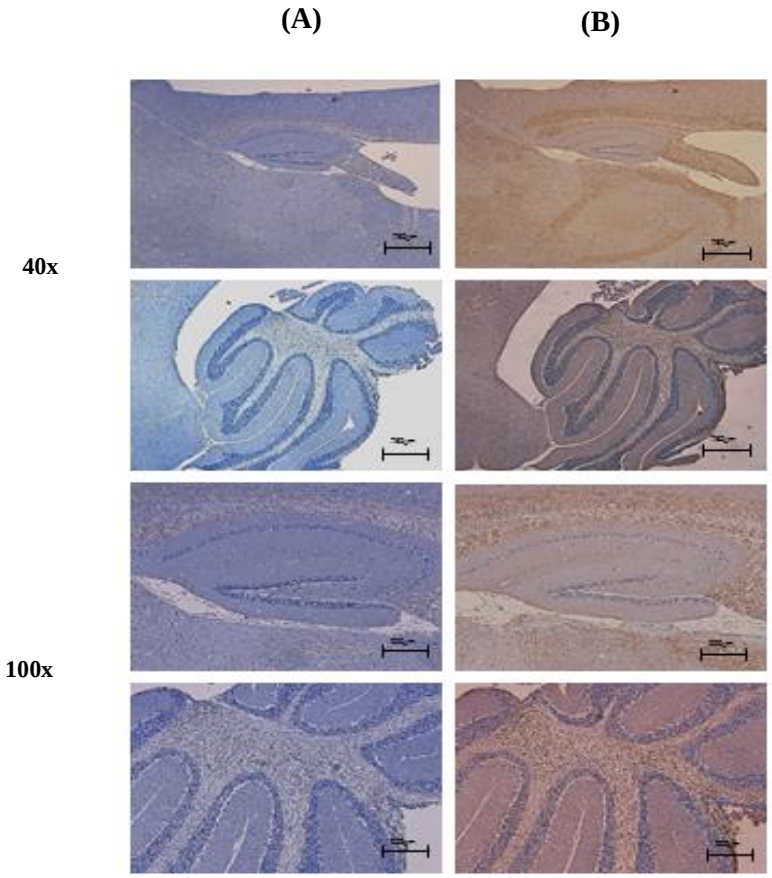


Fig. 4 IL-22Rα expression in mouse brain tissue

Brain tissues were freshly isolated and fixed in 4% PFA at 4°C. Paraffin-embedded tissues were sectioned with 4 μm thickness. Sections were incubated with primary antibodies against IL-22Rα (1:100) at 4°C for overnight in a humidified chamber. Then, sections were incubated biotinylated anti-rabbit antibody (1:150) for 1 hr at RT. ABC solution was loaded on sections for 30 mins and DAB kit. (A) Control (B) IL-22Rα

IL-22 increases the proliferation of BV2 and HT22 via the interaction of IL-22Rα

The role of IL-22Rα on the surface of BV2 and HT22 was investigated after treatment of 0, 5, 10, and 20 ng/ml of recombinant IL-22 for 72 hrs by CCK-8 assay. When cells were exposed to IL-22, I found that the proliferation of BV2 and HT22 was increased, even though there was no dose-dependent fashion (Fig. 5).

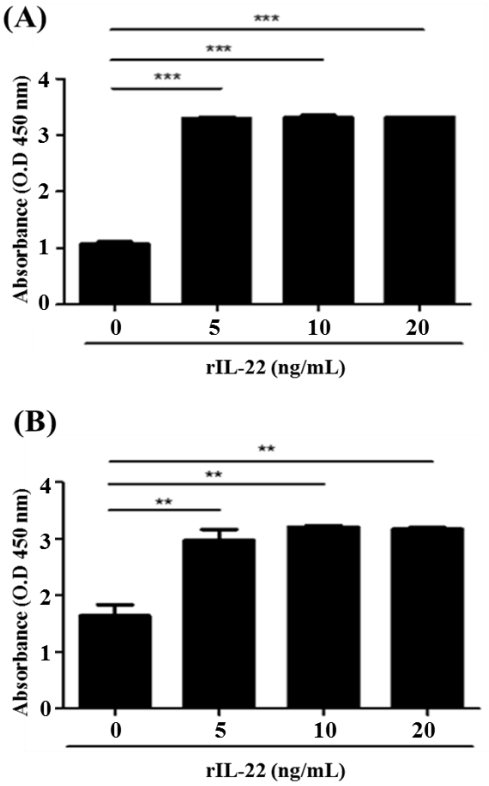


Fig. 5 The proliferation of BV2 and HT22 by the treatment of IL-22

Cells (1.25 x 10^3) were cultured in 96 wells in the presence of 0, 5, 10 and 20 ng/ml of recombinant IL-22 for 72 hrs. The proliferation of BV2(A) and HT22(B) was estimated using the CCK-8 assay following the manufacturer's protocol. The absorbance at 450 nm was detected using a microplate reader. **p<0.01, ***p<0.001.

IL-22 increases COX-2 mRNA expression in BV2 and HT22 via the interaction of IL-22Rα

IL-22 is one of the important pro-inflammatory cytokines. It is known that COX-2 is rarely expressed in steady state, but it is rapidly up-regulated under inflammatory condition. For this reason, I examined whether COX-2 mRNA expression is increased in BV2 and HT22 after treatment of IL-22 for 1, 3, 6, and 12 hrs by RT-PCR. When BV2 exposed to 20 ng/ml of IL-22, COX-2 mRNA expression was remarkably increased at 1 hr after IL-22 treatment (Fig. 6). In case of HT22, COX-2 mRNA expression was also remarkably increased at 6 hrs after IL-22 treatment (Fig. 7).

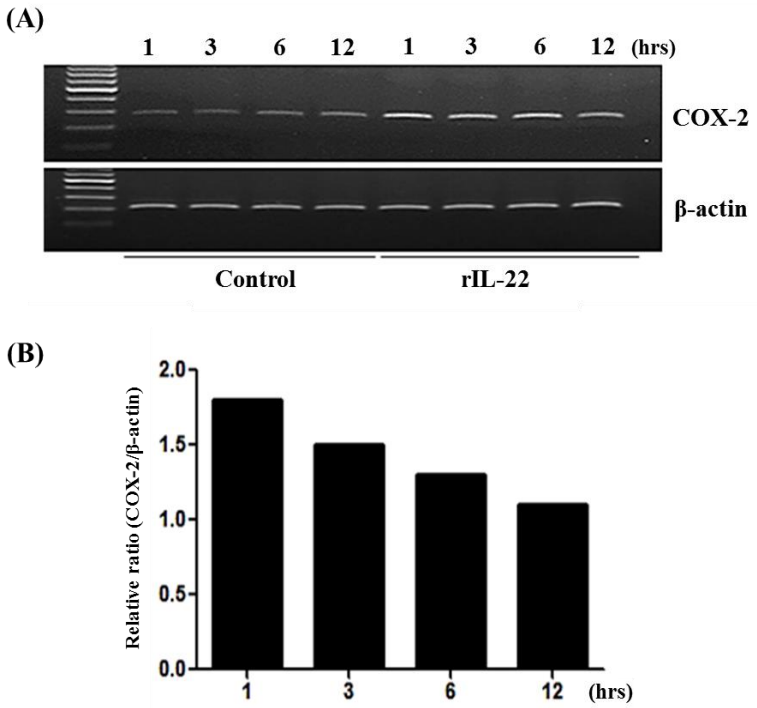


Fig. 6 The increase of COX-2 mRNA expression in BV2 by the treatment of IL-22

BV2 (1 x 10^5) was treated with 20 ng/mL of IL-22 for 1, 3, 6, and 12 hrs and total RNA was extracted and cDNA was made. RT-PCR was performed by using the specific primer for IL-22Rα as described in *Materials and Methods.* (A) The expression of IL-22Rα after electrophoresis PCR product on 1.5% agarose gel and visualization by staining with Red safe. (B) Densitometry analysis for the relative expression of each band against that of β-actin. Results are representative of three independent experiments.

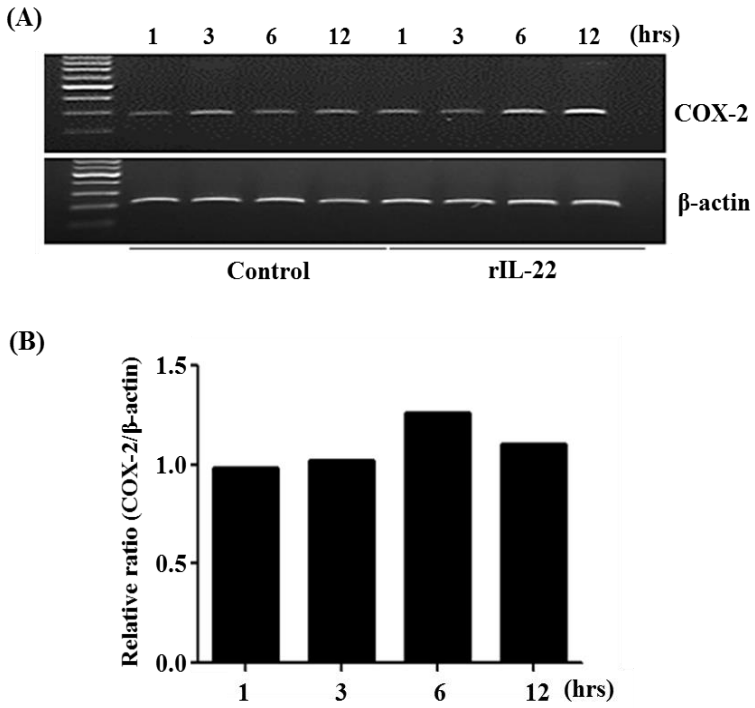


Fig. 7 The increase of COX-2 mRNA expression in HT22 by the treatment of IL-22

HT22 (1 x 10^5) was treated with 20 ng/mL of IL-22 for 1, 3, 6, and 12 hrs and total RNA was extracted and cDNA was made. RT-PCR was performed by using the specific primer for IL-22Rα as described in *Materials and Methods.* (A) The expression of IL-22Rα after electrophoresis PCR product on 1.5% agarose gel and visualization by staining with Red safe. (B) Densitometry analysis for the relative expression of each band against that of β-actin. Results are representative of three independent experiments.

IL-22 increases prostaglandin E2 (PGE2) production in BV2 and HT22 via the interaction of IL-22Rα

Based on the increase of COX-2 by IL-22 treatment shown in Fig. 6 and 7, PGE2 production from BV2 and HT22 after exposure to 20 ng/ml of IL-22 for 24 hrs and 48 hrs was examined by ELISA. As I expected, the production of PGE2 in IL-22 from BV2 and HT22 was remarkably increased at 24 hrs after the treatment with IL-22 (20 ng/ml) (Fig. 8). However, there was no significant difference between 24 hrs and 48 hrs.

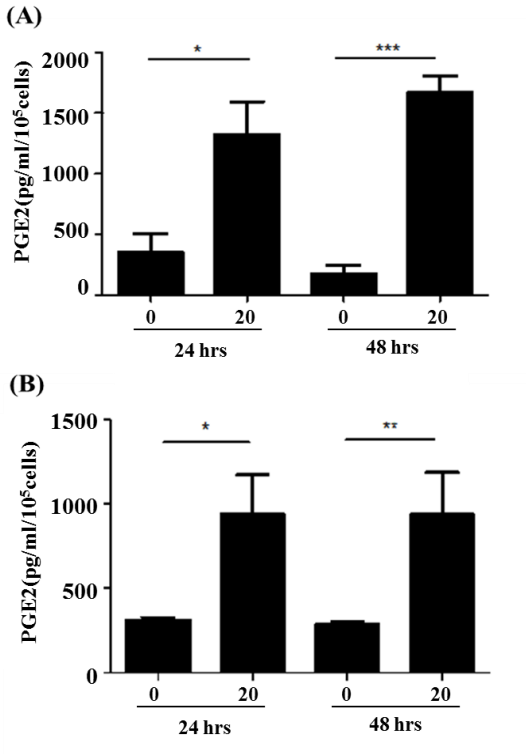


Fig. 8 The increase of PGE2 mRNA expression in BV2 and HT22 by the treatment of IL-22

One hundred thousand of BV2(A) and HT22(B) were treated with 20 ng/ml of IL-22 for 24 hrs and 48 hrs. And then, culture supernatants were collected and the changes in the production of PGE2 were measured by ELISA as described in the *Materials and Methods*. Each sample is in triplicates and results are representative of three independent experiments. Data are presented as the means ± SD. *$p<0.1$, **$p<0.01$, ***$p<0.001$

IL-22 increases IL-6 and TNF-α production in BV2 and HT22 via the interaction of IL-22Rα

It is widely known that COX-2 and PGE2 play an important role in the inflammatory response, but IL-6 and TNF-α are also known to play an important role. Therefore, I investigated whether IL-22 also increases IL-6 and TNF-α production in BV2 and HT22 via the interaction with IL-22Rα. As shown in Fig. 9, IL-6 production from both of cell lines were remarkably increased by IL-22 treatment and it is especially more increased from hippocampal neuronal cell line, HT22 than microglia cell line, BV2. In case of and TNF-α production, its production was remarkably increased from both of cell lines upon IL-22 treatment and it was also higher in HT22 than BV2 (Fig. 10).

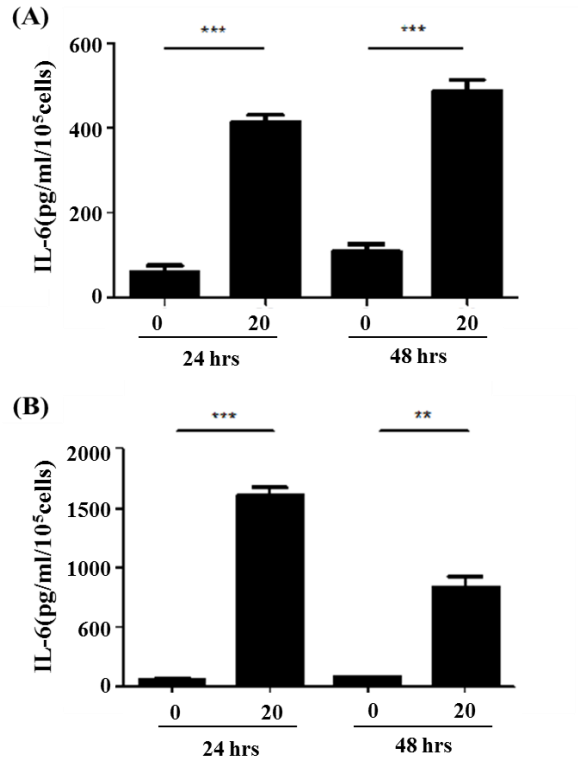


Fig. 9 The increase of IL-6 production from BV2 and HT22 by the treatment of IL-22

One hundred thousand of BV2(A) and HT22(B) were treated with 20 ng/ml of IL-22 for 24 hrs and 48 hrs. And then, culture supernatants were collected and the changes in the production of IL-6 were measured by ELISA as described in the *Materials and Methods*. Each sample is in triplicates and results are representative of three independent experiments. Data are presented as the means ± SD. **p<0.01, ***p<0.001

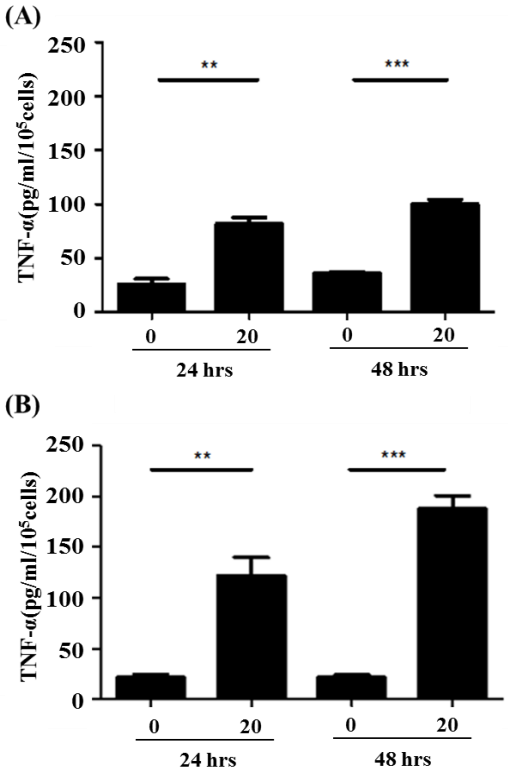
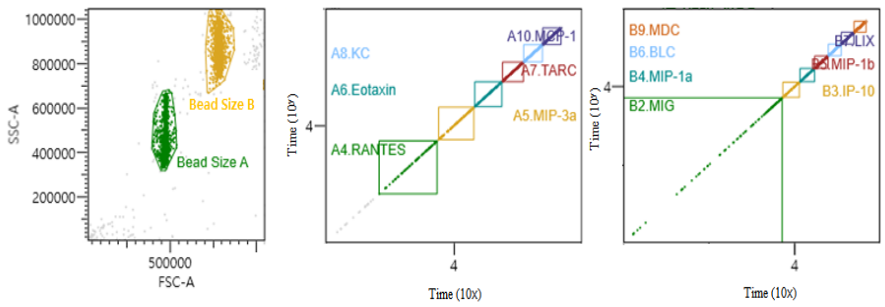


Fig. 10 The increase of TNF-α production from BV2 and HT22 by the treatment of IL-22

One hundred thousand of BV2(A) and HT22(B) were treated with 20 ng/ml of IL-22 for 24 hrs and 48 hrs. And then, culture supernatants were collected and the changes in the production of IL-6 were measured by ELISA as described in the *Materials and Methods*. Each sample is in triplicates and results are representative of three independent experiments. Data are presented as the means ± SD. **$p<0.01$, ***$p<0.001$

IL-22 increases MIG/CXCL9 production from microglia cell line, BV2

The most important thing to alleviate the inflammatory condition is to control the infiltration of inflammatory cells into the inflammatory site. Therefore, the production of inflammatory chemokines from BV2 upon IL-22 treatment by chemokine beads array and ELISA. Among 13 types of chemokines which were analyzed, MIG/CXCL9 was definitely increased from microglia cell line, BV2 upon IL-22 treatment (Fig. 11). To confirm the result of chemokine beads array, the production of MIG/CXCL9 from BV2 upon IL-22 treatment was also measured by ELISA. Consistent with the result of chemokine beads array shown in Table 1, IL-22 remarkably increased the production of MIG/CXCL9 from BV2 (Fig. 11)



	Control	rIL-22
RANTES	ND	ND
MIP-3α	ND	ND
Eotaxin	ND	ND
TARC	ND	ND
KC	ND	ND
MCP-1	ND	ND
MIG	11.62	58.04
IP-10	ND	ND
MIP-1α	ND	ND
MIP-1β	ND	ND
BLC	ND	ND
LIX	ND	ND
MDC	ND	ND

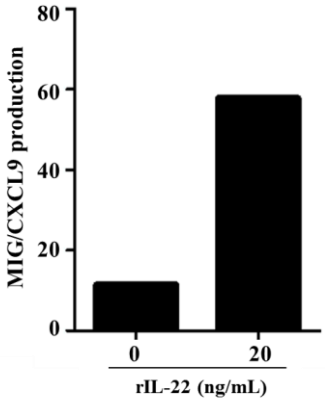


Fig. 11 The increase of MIG/CXCL9 production from murine microglia cell line, BV2 by the treatment of IL-22

BV2 (1 x 10^5) was treated with 20 ng/ml of IL-22 for 48 hrs as described in *Materials and Methods*. And then the production of inflammatory chemokines was analyzed by the mouse pro-inflammatory chemokine panel, a multiplex beads-based assay panel, using fluorescence-encoded beads suitable for use on various flow cytometers. This panel allows simultaneous quantification of 13 mouse chemokines, including MCP-1 (CCL2), MIP-1β (CCL4), MIG (CXCL9), MIP-1α (CCL3), MIP-3α (CCL20), LIX (CXCL5), KC (CXCL1),

BLC (CXCL13), MDC (CCL22), RANTES (CCL5), IP-10 (CXCL10), EOTAXIN (CCL11), TARC (CCL17). ND: not detected

Discussion

IL-22 is a cytokine belonging to the IL-10 family, and it is released from activated T cells and NK cells (Dumoutier *et al.* 2001, Kotenko *et al.* 2001, Lejeune *et al.* 2002, Ouyang and O'Garra 2019), but recently it has been reported to increase in activated macrophage (Akil *et al.* 2015). It is known to play a pro-inflammatory as wells as anti-inflammatory role. Most of them are pro-inflammatory, and related papers include previous studies showing correlations between skin (Fujita 2013, Kim *et al.* 2017, Ma *et al.* 2008), uveitis (Ke *et al.* 2011, Kim *et al.* 2016), AD patients (Brunner *et al.* 2019, Nograles *et al.* 2009), liver injury (Gao and hepatology 2012, Mo *et al.* 2018), and IL-22. It is known that IL-22 has an anti-inflammatory effect on inflammation bowel diseases (Lalli *et al.* 2012, Mizoguchi *et al.* 2018, Seiderer *et al.* 2007). The cell surface-standing IL-22 receptor complex consists of the receptor chains IL-22Rα and IL-10Rβ (Dumoutier *et al.* 2001, Lejeune *et al.* 2002). IL-10Rβ is expressed in all cells, including immune cells (Dean *et al.* 2018, Ip *et al.* 2017), IL-22Rα is known to be expressed in skin, kidney, intestine, liver and pancreas among

non-hematopoietic cells (Nagalakshmi *et al.* 2004, Trifari and Spits 2010). Therefore, studies on the relationship with IL-22R in relation to other organs were conducted, but since there is no report related to IL-22Rα in the brain. In this experiment, the microglia cell line BV2 was used because it is known to protect the living body by showing an initial response to infection (Henn *et al.* 2009, Kang *et al.* 2004, Lee *et al.* 2006, Ning *et al.* 2018). As it remains clear, experiments were conducted on the role of inflammatory responses between IL-22 and IL-22Rα in the brain using murine microglia cell line, BV2 and murine hippocampal neuronal cell line, HT22.

As shown in Figure 1, IL-22Rα expression in BV2 and HT22 was identified at the transcriptional level. At the same time, expression of IL-10Rβ was also confirmed. As such, IL-22Rα was expressed at the protein level (Figure 2). It is known that IL-22Rα is released from the liver, so use the hepatoma cell line, hepa1c1c7 as a positive control (Mo *et al.* 2018, Wolk *et al.* 2007). The levels of IL-22Rα were similar to those of the hepatoma cell line in BV2 and HT22 (Figure 3). Previous data were identified at the cellular level. So far, we have seen expression in tissues related to non-hematopoietic cells such as skin and pancreas (Ito *et al.* 2019,

Tan *et al.* 1997). Since there is no report of expression in the cells that make up the brain tissue, this experiment (Figure 4) was conducted to confirm whether IL-22Rα is expressed in the brain tissue. IL-22Rα was identified by immunohistochemistry method after brain extraction from normal C57BL/6 mouse by brain perfusion. So in the Figure 4, we can see that IL-22Rα is expressed in the hippocampus and cerebellum. Of the known brain regions, hippocampus plays an important role in spatial memory enabling information integration and memory from short-term memory to long-term memory (Ahmadian *et al.* 2018, Apostolova *et al.* 2012, Ardekani *et al.* 2019). And cerebellum is involved in playing the largest role in motor control (Adeghate *et al.* 2013, Lopez Gonzalez *et al.* 2016). Hippocampus can be thought of as Alzheimer's disease and cerebellum as Parkinson's disease (Figure 4). The study focused on hypotheses related to the role of inflammatory responses in the brain with IL-22R. Therefore, our results are very meaningful in that they suggest the possibility of expression in brain tissue. In Figure 5, proliferation was confirmed by CCK-8 assay to determine what happens in BV2 and HT22 by treating recombinant IL-22 by concentration. The effect of recombinant IL-22 was confirmed by treatment with 5, 10, 20 ng/mL concentration. As a result, proliferation

occurred at 5, 10 and 20 ng/mL in both BV2 and HT22 using without rIL-22 as a control. So in this Figure 5, we can see the proliferation of the cells while processing IL-22. Another important factor related to inflammation, inflammatory prostaglandin signaling plays and important role in pre-clinical development of neurodegenerative diseases (Belkhelfa *et al.* 2014, Heppner *et al.* 2015, Woodling *et al.* 2014). And Cyclooxygenase (COX)-2, the inducible form of the COX enzyme for prostaglandin synthesis, rarely expressed in steady state and up-regulated and induced rapidly by stimulation of mitogen, cytokine and lipopolysaccharide (Bitto *et al.* 2017, Maesaka *et al.* 2013, Sil and Ghosh 2016). So we did the stimulation in this study via recombinant IL-22 (20 ng/ml). The increase in COX-2 was also confirmed through experiments and there is a time difference between BV2 and HT22. (Figure 6. BV2 and Figure 7. HT22) Overexpression of COX-2 eventually leads to increased prostaglandin formation, so the PGE2 ELISA was conducted (Maesaka *et al.* 2013, Shi *et al.* 2012, Sil and Ghosh 2016). The experiment was confirmed that the increase in 24 hrs and 48 hrs (Figure 8). It is known that IL-22 is involved in the induction of inflammatory response (Hazen *et al.* 2019, Zheng *et al.* 2007). Therefore, we investigated the role of IL-22 on the production of inflammatory

cytokines TNF-α and IL-6 from recombinant IL-22 (20 ng/mL) treated in BV2 and HT22 (Figure 9. IL-6 / Figure 10. TNF-α). In this experiment, the microglia have a function of protecting the living body (Anastasio 2015, Li and Barres 2018), but if activated too much, IL-6, IL-1β, TNF-α and other inflammatory cytokines, such as neuronal death and chronic inflammation is known to be a major cause of degenerative brain disease (Acx *et al.* 2017, Aguilar *et al.* 2014, Apostolova *et al.* 2012). The study of chemokines and their receptors in the central nervous system (CNS) is not only relevant for the understanding of brain physiology and pathophysiology, also lead to the development of targeted treatments for neurodegenerative diseases (Acx *et al.* 2017, Ali *et al.* 2019, Reale *et al.* 2012). Therefore, we confirmed what chemokine is expressed through beads array experiments. So MIG/CXCL9 was discovered through experiments. The results show that BV2 increases in (Figure 11). Further research on these results will be needed. In our case, IL-22Rα expressed in BV2 and HT22 increased the inflammation-related factors. However, inflammation in the brain is very important in relation to Alzheimer's or Parkinson's, a major degenerative brain disease (Arshavsky 2014, Forloni *et al.* 2016). So far, pathological studies of Alzheimer's and Parkinson's

studies have shown that increased COX-2, PGE2, IL-6, and TNF-α are very important in our results. It was not clearly explained, but based on our results, it is highly likely that this is due to the action of IL-22 and IL-22Rα. For this reason, further experiments involving IL-22 and IL-22Rα in Alzheimer's or Parkinson's animal models would be very meaningful. It is then thought that it is very important to regulate IL-22Rα, but it is also important to study the case where the IL-22Rα is increased. As a result of experiments, it was confirmed that IL-23 increased IL-22Rα in BV2 and HT22 (Data was not shown). Thus, regulation of IL-22Rα expression through the regulation of IL-23 may be a starting point for regulating inflammation in the brain and consequent degenerative brain diseases such as Alzheimer's and Parkinson's (Murphy *et al.* 2003, Neurath and reviews 2019, Yen *et al.* 2006). However, if IL-22Rα is already expressed, there may be a method of blocking it using a neutralizing antibody, soluble IL-22 binding protein.

Taken together, this study is based on cell lines and animals, but it may be more meaningful to identify human brain tissue donated to the brain tissue bank. When this process occurs in the brain as various inflammatory reactions occur in the living body, the cells that are important in this

process are macrophages present in the brain and non-neuronal cells differentiated from the monocyte and scattered in the central nervous system (Berridge 2013, Li and Barres 2018, Prinz *et al.* 2017). The problem is that they are difficult to pass blood-brain barrier because they are large in size, using nanotechnology. In conclusions, IL-22Rα on the surface of BV2 and HT22 might be an important role on their proliferation and the production of inflammatory mediators (IL-6, TNF-α and MIG/CXCL9) after interaction with IL-22.

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국 문 초 록

인터루킨-22와 인터루킨-22 수용체가 뇌에서의 염증반응 조절에 미치는 영향에 관한 연구

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해부학 전공 이 다 해

인터루킨-22는 인터루킨-10 패밀리에 속하는 염증 반응에 있어서의 매개인자로 작용하는 것으로 알려져 있다. 주로 활성화된 $CD4^+$ T세포와 자연살해세포에서 분비되고 비조혈세포인 간질 및 상피세포에 주로 영향을 미친다. 인터루킨 22에 대한 수용체인 인터루킨-22 수용체 α는 피부, 췌장, 소장, 간, 폐와 신장 등의 비조혈모세포에서 발현된다고 알려져 있으나, 뇌에서의 발현과 관련된 연구는 아직 보고된 바가 없다. 따라서, 본 연구에서는 인터루킨-22 수용체 α가 발현되는지의 여부를 확인하기 위해 생쥐 미세아교세포주인 BV2와 해마신경세포주인 HT22를 사용하여 연구를 진행하였다. 흥미롭게도, BV2와 HT22에서 인터루킨-22 수용체 α가 발현한다는 것을 유전자와 단백질 수준에서

유전자중합효소연쇄반응법, 면역블로팅 및 유세포 분석을 통하여 확인할 수 있었다. 생체 내의 뇌 조직 측면에서 인터루킨-22 수용체 α의 발현을 확인하기 위해 쥐의 뇌를 추출하여 면역조직화학방법으로 염색하여 인터루킨-22 수용체 α가 발현이 되는지 확인한 결과, 해마와 소뇌에서 인터루킨-22 수용체 α가 발현되어 있는 것을 확인할 수 있었다. 다음으로, 재조합 인터루킨-22가 처리된 BV2와 HT22에서 염증성 사이토카인인 종양괴사인자-α와 인터루킨-6의 분비가 현저하게 증가되는 것을 확인하였다. 이와 동시에 염증반응 유발과 진행에 있어서 중요한 역할을 하는 것으로 알려져 있는 사이클로옥시게네이즈-2와 프로스타글란딘 E2의 발현과 생성도 증가됨을 관찰하였다. 또한 염증성 케모카인인의 생성도 증가됨을 확인하였다. 결론적으로, 인터루킨-22 수용체 α는 뇌조직을 구성하는 미세아교세포와 해마신경세포에서 발현되고, 인터루킨-22의 자극에 의해 종양괴사인자-α, 인터루킨-6, 사이클로옥 시게네이즈-2 그리고 프로스타글란딘 E2의 생성 증가를 통해 뇌에서의 염증 반응 유도와 진행에 관여함을 확인할 수 있었다.

주 요 어: 염증, 증식, BV2, HT22, IL-22, IL-22Rα
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