Understanding the Effect of Cytokines on bEND.3 Cells in the Blood Brain Barrier in Neuropsychiatric Systemic Lupus Erythematosus

by
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A thesis submitted to the Department of Biomedical Engineering,
Cullen College of Engineering
in partial fulfillment of the requirements for the degree of

Bachelor of Science

in Biomedical Engineering

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University of Houston
May 2023

ACKNOWLEDGEMENTS

I would like to express my immense gratitude to my advisor and Principal Investigator, Dr. Chandra Mohan, for the opportunity, training, tools, and resources I needed to conduct research and projects, and I would like to thank Dr. Yaxi Li for giving me the opportunity to be a part of the project and for letting me work with her O-Link Proteomics project results in this thesis (Chapter 4, Figures 4.1-4.5) to build my research upon. I would also like to extend my gratitude to the other members of Dr. Mohan's, Dr. Shereen Majd's and Dr. Mohammad Reza Abidian's laboratory at the University of Houston, as this project would not have been possible without their guidance, resources, and support. Thank you to the Office of Undergraduate Research, especially Dr. Rikki Bettinger, for being incredibly supportive of my endeavors.

Finally, I would like to thank Dr. Anto Sam Crosslee very especially for his generous help as my primary mentor. He gave me the guidance and advice I needed to be a part of the lab, despite my never-ending questions. His willingness to tirelessly support me, teach me, and answer all my questions despite setbacks is what motivated me to continue in research and push through to the end.

The results in this project are a product of the collective efforts of all the researchers in this laboratory at the University of Houston and would not have been possible without everyone's collaboration.

ABSTRACT

Neuropsychiatric systemic lupus erythematosus (NPSLE) is an autoimmune condition that can develop as a result of systemic lupus erythematosus (SLE). NPSLE can occur in between 12-90% of all people with SLE and can manifest as symptoms as insignificant as headaches or as detrimental as cognitive impairment and cognitive decline. The development of NPSLE has been attributed to the degradation of the blood brain barrier (BBB), a vascular network around the brain that mediates the movement of molecules into and out of the brain. In NPSLE the permeability of the BBB increases, allowing pro-inflammatory cytokines and other immune cells to cross into the brain, causing damage to neurons and sections of the brain. In this study, we attempted to investigate the mechanisms with which BBB breach can happen by screening serum from NPSLE patients for elevated protein biomarkers, and then using those to determine their effects on bEND.3 cells in the BBB through cytotoxicity assays. Cytokines IL-2, IL-17 and TGF-α were found to be elevated and therefore, MTT assays were used to determine the amount of cell death experienced by bEND.3 when exposed to these cytokines for different periods of time. The results showed vague trends and patterns but generally remained inconclusive. On the whole, shorter exposure times and lower concentrations of cytokines induced greater cell death. In some cases, results exhibited cell proliferation instead of cell death, by having cell viability percentages larger than that of the untreated control. Flow cytometry revealed the bEND.3 cells had been contaminated by RAW 264.7 macrophages, shedding light on the nature of the strange results. While the results on the effect of cytokines on bEND.3 remains inconclusive, the data presents some possibilities about the role of cytokines in cell proliferation of immune cells.

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CHAPTER 1: INTRODUCTION

The blood brain barrier (BBB) is a collection of capillaries that enables a tight control of the movement of molecules, cells and ions between the blood and the brain [1]. The vascularization of the brain allows for selective permeability and precise control allows for healthy neuronal function and the maintenance of homeostasis of the central nervous system [1], [2]. The two-way system enables not only the entry of required molecules to the brain but the excretion of carbon dioxide and acts as a barrier to prevent the entry of toxins and pathogens. This musculature is formed by three different cells: endothelial cells (ECs), astrocyte end-feet and pericytes. These cells coordinate a series of metabolic, physical and transport properties that regulate interactions with vascular, immune, and neural cells, enabling healthy movement of molecules between the brain and the blood brain barrier [1].

The effects of systemic lupus erythematosus (SLE) on the brain, spinal cord or the nerves is called neuropsychiatric systemic lupus erythematosus (NPSLE). Cognitive impairments and other neuropsychiatric symptoms NPSLE occur in a wide range, from 12%-90% of all people with SLE [3]-[15]. The wide range of manifestation occurrences are due to the different studies conducted with a systematic method involving inconsistent application criteria and sample populations [3]-[16]. The mechanisms that cause NPSLE are unclear but are likely to be caused by a combination of different factors such as blood brain barrier breach, pathogenic autoantibodies that target parts of the nervous system including endothelial cells, or neuronal damage. So far, research has focused largely on autoantibody associations and their effects on the immune system in patients with NPSLE [3]. There are certain types of cytokines that contribute to the

disruption to the blood brain barrier, such as interleukin 2 (IL2) and interleukin 17 (IL17), which is so far the most researched. Molecules such as neurturin (NRTN) and artemin (ARTN) are neurotrophic factors involved neurogenesis, as part of the superfamily of Transforming Growth Factor- β (TGF- β) [17]. TGF-α, which is not very well researched within the context of BBBB, has neurotrophic properties that contribute to the repairing and proliferation of neurons and cells in adult brain injuries [18].

However, there remains a gap in knowledge where little to no research has been done on these biomolecules in the context of NPSLE. Our research attempted to start filling in the gap and identify clear markers of BBB breach in NPSLE as well as identify the serum proteins and autoantibodies that are predictive of BBB breach in SLE.

Currently, there are several models that help determine NPSLE diagnoses but they strongly depend on opinion-based subjective diagnoses by experts who observe the symptoms [19]. There remains a need to diagnose NPSLE objectively as well as subjectively, taking a "multidisciplinary approach" to devise different methods of diagnoses to be used in conjunction for a more reliable decision [19]. This research attempts to find gold-standard markers that can be detected in blood during blood tests for CSF leakage in the CNS. These markers include cytokines such as IL2, IL17 and other various biomolecules. IL-17 is a proinflammatory cytokine and therefore contributes to the pathogenesis of inflammatory diseases. IL-2, despite showing promising results as a type of cancer immunotherapy, simultaneously causes neuropsychiatric changes that result in the development of brain edema. Autoimmune cells are not meant to cross the BBB and cause neuroinflammation if they do.

Previous research confirms the concept that IL-2 plays a significant role in several chronic neurological autoimmune diseases, including NPSLE. The mechanism continues

to remain unknown, however. Wylezinki and Hawinger conducted their experiments in cell cultures of primarily bovine and murine cells along with human bone marrow microvascular endothelial cells (BMEC) and human fibroblast growth factors (FGF) and investigated how IL-2 disrupted the blood brain barrier and altered vasocirculation in the brain. [20]

This thesis aims to more closely investigate and understand the mechanisms and workings of cytokines that are involved in NPSLE specifically relevant to the biomarkers and proteins found in blood and serum. The experiments will attempt to recreate mechanisms found in the BBB and investigate the effect of the cytokines on an *in vitro* representation through the use of bEND.3 cells, mouse brain endothelial cells found making up the vascular structures of the BBB.

CHAPTER 2: REVIEW OF RELEVANT LITERATURE

In "Blood-brain barrier disruption and neuroinflammation as pathophysiological mechanisms of the diffuse manifestations of neuropsychiatric systemic lupus erythematosus," the authors discuss the role of the blood-brain barrier (BBB) in neuropsychiatric systemic lupus erythematosus (NPSLE) [21].

One potential mechanism of BBB disruption in NPSLE is activation of the complement system. The complement system is a group of proteins involved in immune defense, and its activation can lead to the recruitment of immune cells and the release of inflammatory mediators, both of which can contribute to BBB disruption. Studies have found evidence of complement activation in the brains of patients with NPSLE, as well as elevated levels of complement proteins in the cerebrospinal fluid of these patients. The authors suggest that inhibition of complement activation may be a potential therapeutic target for preventing or treating BBB disruption in NPSLE [21].

The authors also discuss other potential mechanisms of BBB disruption in NPSLE. For example, proinflammatory cytokines, such as tumor necrosis factor-alpha and interleukin-1 beta, can activate endothelial cells and lead to increased permeability of the BBB. Antibodies against the BBB, such as anti-N-methyl-D-aspartate receptor antibodies, have also been implicated in BBB dysfunction in NPSLE. In addition, oxidative stress and mitochondrial dysfunction may play a role in BBB disruption by impairing the function of endothelial cells [21].

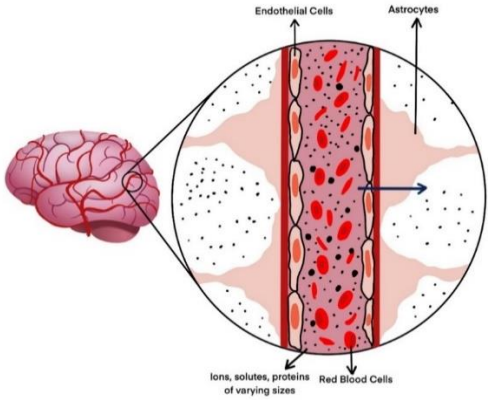


Figure 2.1. Visual representation of the components and movement in the BBB. The arrow in the center indicated the movement of pro-inflammatory biomolecules into the matter of the brain.

"The immunologic etiology of psychiatric manifestations in systemic lupus erythematosus: A narrative review on the role of the blood brain barrier, antibodies, cytokines and chemokines" by Deijns et al. is a review article that explores the link between systemic lupus erythematosus (SLE) and psychiatric manifestations [22]. The article starts by discussing the clinical presentation of neuropsychiatric symptoms in SLE, which can vary widely and include cognitive dysfunction, mood disorders, seizures, and psychosis. The authors then delve into the pathophysiology of SLE, which involves the production of autoantibodies that can target various organs, including the CNS [22]. The BBB is an important barrier that normally prevents the entry of immune cells and molecules into the brain but can become disrupted in SLE due to the presence of autoantibodies, cytokines, and chemokines. [22]

The authors review several studies that have investigated the role of autoantibodies in neuropsychiatric SLE, including anti-NMDA receptor antibodies, anti-ribosomal P antibodies, and anti-endothelial cell antibodies. These autoantibodies can

cross the BBB and cause damage to neurons and glial cells in the brain, leading to cognitive dysfunction and mood disorders [22].

The article also discusses the role of cytokines and chemokines in SLE-associated neuropsychiatric symptoms. Inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) have been implicated in the pathogenesis of depression and anxiety in SLE patients. Chemokines such as CCL2 and CXCL10 can attract immune cells to the brain and contribute to neuroinflammation. Despite that, only a few number of cytokines and chemokines have been found to have a correlation with NPSLE, and the authors note that there is no significant association between many cytokines and the pathogenesis of the disease [22].

The authors propose that a better understanding of the immunological mechanisms underlying neuropsychiatric symptoms in SLE is crucial for the development of more effective treatments. They suggest that targeting the BBB, autoantibodies, cytokines, and chemokines may be potential strategies for treating these patients. The review article provides a comprehensive overview of the current state of knowledge on the immunologic etiology of psychiatric manifestations in SLE and highlights the need for further research in this field, including researching the role of antibodies in NPSLE in more detail [22].

"The influence of cytokines on the integrity of the blood-brain barrier *in vitro*" is a research article that investigates the effects of cytokines on the blood-brain barrier (BBB) using an *in vitro* model. The study focuses on three pro-inflammatory cytokines - IL-6, IL-1β, and TNF-α - and their effect on the BBB, which is essential for protecting the central nervous system from harmful substances [23].

The results of the study showed that exposure to the cytokines led to the loss of integrity of bEnd.3 endothelial cells, which are used as an *in vitro* model of the BBB. This was measured by a decrease in trans-endothelial electrical resistance (TEER), which is a measure of the tightness of the cell layer. The study also found that the cytokines led to changes in the expression of tight junction proteins, which are essential for the proper functioning of the BBB [23].

The authors concluded that the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α are capable of disrupting the integrity of the BBB *in vitro* by altering the expression of tight junction proteins. This may have implications for the role of cytokines in the pathogenesis of neurological disorders that involve BBB dysfunction [23].

Another paper by Roche et al. aimed to investigate the effects of a combination of cytokines on the integrity of the *in vitro* blood-brain barrier (BBB) model using bEnd.3 endothelial cells. The researchers used interleukin-6 (IL-6), interleukin-17 (IL-17), and tumor necrosis factor alpha (TNF-α) to create an inflammatory stress environment [24].

The researchers conducted a series of experiments to examine the effect of the cytokine combination on the BBB model. In one experiment, they used a trans-endothelial electrical resistance (TEER) measurement to determine the integrity of the BBB. The TEER values are inversely proportional to the permeability of the endothelial cell layer, so lower TEER values indicate a more permeable BBB. The results showed a significant decrease in TEER values in the cytokine-treated group compared to the control group [24]. Cytotoxicity results showed some changes in the cell number through methyl-thiazolyl-tetrazolium (MTT) tests. IL-6 and IL-17 resulted in an average 70% cell viability.

In another experiment, the researchers measured the permeability of the BBB by adding a fluorescent dye to the cell culture media and measuring its penetration into the endothelial cell layer. The results showed that the cytokine-treated group had a higher level of fluorescent dye penetration compared to the control group, indicating that the BBB had become more permeable. In addition to the experiments described earlier, the researchers also investigated the effects of the cytokine combination on the expression and localization of tight junction proteins in the bEnd.3 endothelial cells. Tight junction proteins play a crucial role in maintaining the integrity of the BBB by forming a barrier between adjacent endothelial cells [24].

The researchers used immunofluorescence staining to visualize the expression and localization of the tight junction proteins occludin and claudin-5. They found that the cytokine combination decreased the expression and disrupted the localization of occludin and claudin-5. In the control group, occludin and claudin-5 were located at the cell-cell junctions, indicating intact tight junctions. However, in the cytokine-treated group, occludin and claudin-5 were no longer localized at the cell-cell junctions, indicating disrupted tight junctions [24].

The researchers also conducted experiments to investigate the underlying mechanisms of the BBB disruption. They found that the cytokine combination caused an increase in the production of reactive oxygen species (ROS) and activation of the nuclear factor kappa B (NF-κB) signaling pathway, both of which are associated with oxidative stress and inflammation [24].

An aptamer is a short single-stranded DNA or RNA molecule that can bind to a specific target molecule, such as a protein or a small molecule. In an aptamer-based screen done by Mohan Lab, 1,129 proteins were screened and 40 were found to be

elevated in the cerebrospinal fluid (CSF) of patients with NPSLE. Validation through enzyme-linked immunosorbent assay (ELISA) confirmed that nine proteins, including IgM and macrophage colony-stimulating factor (M-CSF), were significantly elevated in a predominantly White NPSLE cohort compared to patients with other neurological diseases. CSF IgM and M-CSF were found to be the most discriminatory proteins for NPSLE. A second NPSLE cohort in Hong Kong showed that CSF IgM and lipocalin 2 were the most "discriminatory" proteins. Elevated CSF complement C3 was associated with an acute confusional state. Additionally, eleven molecules elevated in NPSLE CSF were also elevated in the choroid plexus, suggesting a common cause or root. [25]

Another study by Dr. Chandra Mohan's team in Mike et al. investigated whether lipocalin-2 (LCN2) plays a role in the pathogenesis of NPSLE. The researchers used a lupus mouse model and found that LCN2 deficiency attenuated depression-like behavior and impaired spatial and recognition memory. Whole-brain flow cytometry showed that LCN2 deficiency did not reduce brain infiltrating leukocytes in the mice. RNA sequencing revealed that LCN2 regulated genes that are key mediators of the neuroinflammatory cascade. The study also found that LCN2 is upregulated in the cerebrospinal fluid of NPSLE patients across different ethnicities, identifying CSF LCN2 as a novel biomarker for NPSLE. [26]

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Proximity Extension Assay (O-Link Proteomics)

To investigate the serum from 16 patients with SLE and extensive BBB damage, 49 patients with only SLE and 9 healthy patients to identify trends and patterns within the proteins present, a Proximity Extension Assay (PEA) was used to that translates protein information by linking protein-specific antibodies to DNA-encoded tags. O-Link (Olink, Uppsala, Sweden) proteomics is a targeted protein screening that allows the detection of disease-specific proteins using matched pairs of antibodies that are linked to oligonucleotides binding to a matching detection protein.

3.2.1. Incubation

An Incubation mix was prepared according to the ratios given in the table below, and vortexed and spun down. 47 uL of Incubation mix was transferred to each well of an 8-well strip using reverse pipetting, and 3 uL was transferred to each well of a 96-well plate. This was the Incubation Plate. 1 uL of each sample was added to the bottom of each well, with three wells (pink), two wells (orange) and three wells (green) were used to add 1 uL Negative Control, 1 uL pooled plasma sample as Sample Control and 1 uL Interplate Control respectively (as shown below). The plate was sealed with an adhesive plastic film, spun at 400 x g for 1 minute at room temperature, then incubated overnight at +4^{o}C.

Table 3.1. Volumes of reagents required for creating the Incubation mix for O-Link

Incubation mix	per 96-well plate (µL)
Incubation Solution	280.0
Incubation Stabilizer	40.0
A-probes	40.0
B-probes	40.0
Total	400.0

3.2.2. Extension

Table 3.2. Volumes of reagents required for creating the Extension mix for O-Link

Extension mix	per 96-well plate (µL)
High Purity Water	9385
PEA Solution	1100
PEA Enzyme	55
PCR Polymerase	22
Total	10 562

An extension mix was prepared according to the volumes shown in Table 3.2. The Incubation plate was brought to room temperature and centrifuged for 1 minute at 400 g, then Vortexed and Extension mix was transferred to the wells. The plate was sealed, Vortexed, then spun down and incubated in a thermal cycler with the PEA program.

3.3.3. Detection

A 96.96 Dynamic Array™ Integrated Fluidic Circuit (IFC) was prepared and primed according to the manufacturer's instructions. The appropriate steps were taken to transfer the Detection mix to a Detection plate, transfer the contents of the Incubation plate to the Detection plate, load the chip in the Fluidigm IFC Controller HX and run Run the Olink Protein Expression 96×96 Program (50°C 120 s, 70°C 1800 s, 25°C 600 s, 95°C 300 s (95°C 15 s, 60°C 60 s) x40) in the Fluidigm Biomark™ Reader according to the manufacturer's instructions.

3.2 Cell Culture and Growth

The experiments were conducted on endothelial cells (bEnd.3, mouse endothelial cell), (ATCC, Manassas, VA, USA). Sterile techniques were used throughout the entire cell culture process to attempt to prevent contamination and ensure the integrity of the cells. The laminar flow hood was cleaned with 70% ethanol and the UV light was turned on for 15-30 minutes to prepare a sterile workspace. The appropriate number of bEND.3 cells, often $2x10^6$ cells, were transferred to a new culture dish or flask using sterile technique and following the manufacturer's instructions for cell seeding density. The dish or flask was filled with cell culture media that had been prepared by adding 10% FBS to the Dulbecco's Modified Eagle Medium (DMEM) (Grand Island, NY, USA) according to the manufacturer's instructions, and 5% 10,000 units/mL Penicillin, 10,000 μg/mL Streptomycin solution. The DMEM was stored at 4°C until needed. The dish or flask with the cells was then placed in the incubator.

The cells were monitored daily for signs of growth and subcultured when they reached 80-90% confluency, often 2-3 times a week. To subculture the cells, the cell culture media was aspirated, and the cells were rinsed with sterile Phosphate Buffer Solution (PBS). 0.25% Trypsin-EDTA solution, which was diluted to 0.05% in later passages, was added to the cells and incubated for 5-7 minutes. The cells were then observed under a light microscope to ensure detachment from the surface of the dish or flask. Fresh cell culture media was added to neutralize the trypsin-EDTA solution and the cells were transferred to a new culture dish or flask at the appropriate seeding density. The new dish or flask was incubated at 37^oC and the cells were monitored daily for signs of growth.

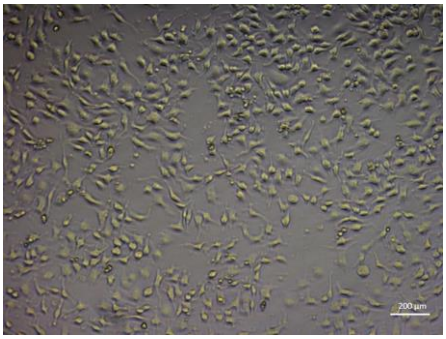
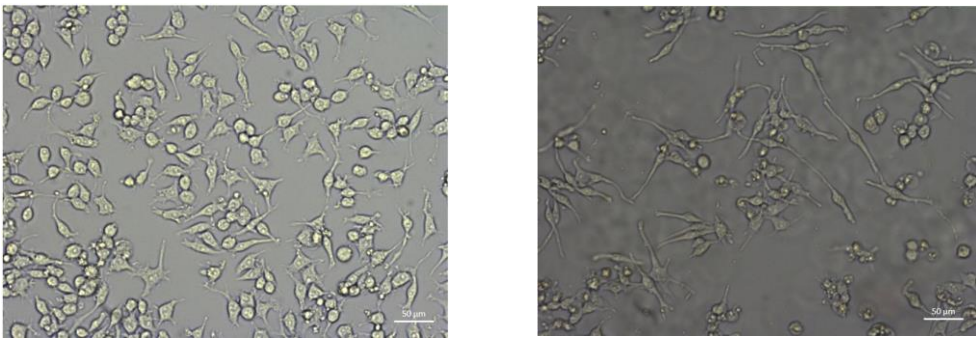


*Figure 3.1. Microscope image of bEND.3 cells at 50x magnification (*scale bars are estimates)*

In order to attempt to improve adherence and proliferation of the cells, 6-well plates were coated with a mixture of 50% poly-D-lysine and 50% autoclaved water on glass inserts/cover slips and bEND.3 cells were seeded and cultured in 37^{o}C and 5% CO_2 over the course of a week. Media was changed every 2 days and the cover slips were imaged.



(a) (b)

*Figure 3.2. Images of the bEND.3 cells (a) without poly-D-lysine coating (b) on the poly-D-lysine coating glass cover slips at 200x magnification (*scale bars are estimates)*

3.3 Transendothelial Electrical Resistance (TEER) Measurements

It is critical that the model used to simulate the BBB is able to simulate human cells as accurately as possible. In order to accomplish this Corning™ Transwells™ (Corning, NY, USA) were used, a versatile tool that was built with human microvascular endothelial cells (HMVEC) on the top layer to create a monoculture. Cell proliferation on the membranes allow the investigation of the permeability and disruption of the BBB *in vitro.* After the seeding of the cells, it takes 14 days for the BBB to develop. The results fluctuated heavily over the days, remaining the same on average.

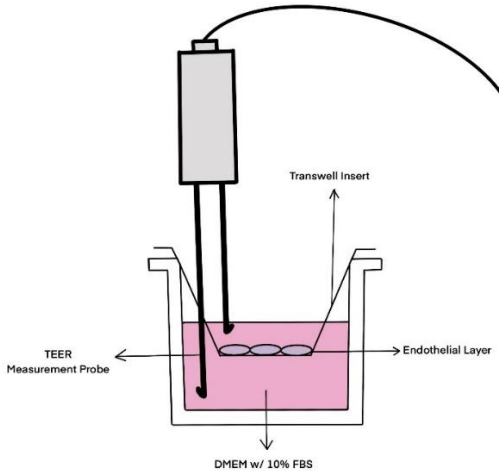


Figure 3.2. The use of the TEER probe to measure difference in resistance between the membrane and the media.

12 mm Corning™ Transwells™ with pore sizes of 0.4 µm were developed by coating with attachment factors (Cell Systems, Kirkland, WA, USA). The cultures were always incubated at a temperature of 37 °C. They were produced as contact co-cultures and contained cells from two different species (human astrocytes and bovine microvascular endothelial cells (BMVEC)), and as well as monocultures with only

bEND.3. Cytokines were added to the Transwells™ from the top, and the media was changed every 3 days. The experiment required regular monitoring of cell growth and conditions. Permeability of blood brain barrier was measured using a transepithelial electrical resistance (TEER) machine. For measurements, the cells were FBS-starved for at least 24 hours.

3.4. Cytotoxicity Assay

An MTT kit was obtained to carry out a cytotoxicity assay at passage 4 of the bEND.3 cells. The MTT assay is a colorimetric assay that measures the activity of mitochondrial enzymes, which is an indicator of cell viability. In this assay, the cells were treated with different concentrations of the cytokines at different time points, and then incubated with MTT for one hour, which is converted to formazan by the mitochondrial enzymes. The cells and crystals were dissolved using an isopropyl solution with 0.04% HCl. The amount of formazan produced is proportional to the number of viable cells, which can be measured by spectrophotometry.

In the MTT assay, two absorbances were measured: the first one was the absorbance of the MTT formazan product, which is generated by the reduction of MTT by viable cells, and the second one is the absorbance of the cloudiness of the reagents, which was used to normalize the results based on the corrected OD of the untreated controls. The first absorbance is typically measured at a wavelength of 570 nm, while the second absorbance is measured at a wavelength of 630 nm or higher, depending on the solvent used for solubilization. The difference between the two absorbance values can be used to calculate the cell viability or proliferation rate.

3.5. Flow Cytometry

The endothelial cells were phenotyped using flow cytometry. First, the cells were harvested with 0.05% trypsin. Then, they were incubated with 3% Bovine Serum Albumin (BSA) for 30 minutes. The antibodies used were chosen to prevent more than one antibody per color channel. CD140b (blue), PE CD31 (brown), and PE F4/80 (red) were used to detect mesangial cells, endothelial cells, and macrophages respectively. The cells were analyzed using a flow cytometer. The gating strategy used involved manually selecting the population of interest and drawing a gate to exclude any debris or unwanted cells from the analysis. It identified cell populations based on forward and side scatter, followed by analysis of fluorescence intensity to determine the proportion of cells positive for the given markers.

CHAPTER 4: RESULTS

4.1 Proximity Extension Results (O-Link Proteomics)

Results were obtained in O-Link's Normalized Protein eXpression (NPX). A high NPX value pertains to a high protein concentration. Similarly, fold change (FC) is a measure describing the extent of change between an original and a final value detected. The higher the fold change, the higher the difference of protein concentrations detected in serum of healthy patients versus those of patients with BBBB or cognitive deficit, respectively. The volcano plot shows data points with low p-values associated with highly significant proteins appearing towards the top of the plot.

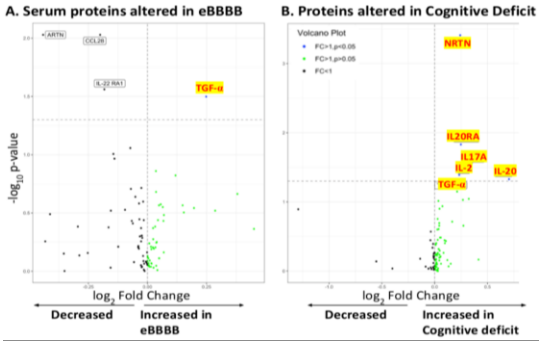
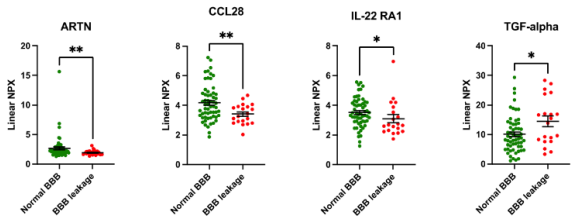


Figure 4.1. Volcano plots for the proteins detected from OLink showing (A) proteins altered in patients with eBBBB (B) proteins altered in samples from patients with severe cognitive deficit.

In Figure 4.1 (A) the proteins appearing high on the volcano plot are those that appear in higher concentrations in people with eBBBB than those who are healthy, and some appear in lower concentrations. The proteins include ARTN, IL-22 RA1, CCL28 appearing in lower concentrations, while TGF-α appears as a +0.25 log2 fold change.

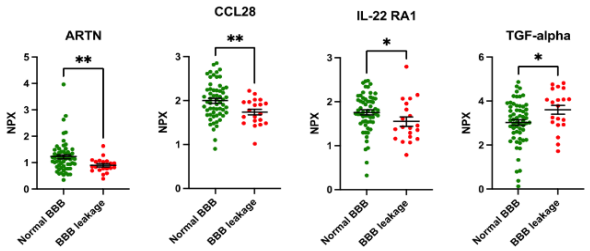
In cognitive deficit, the proteins that showed a quantity increase were much more in number, including NRTN, IL-20 RA, IL-17A, IL-2, IL20 and TGF-α. With the

exception of TGF-α, the cytokines each had a FC of greater than 1 and a p-value of less than 0.05, associating with both a higher concentration changes from the serum samples without cognitive deficit, but also low p-values show a higher significance of the cytokines in the sample.



(a) (b) (c) (d)

Figure 4.2. Results of Mann-Whitney tests for NPX linear data from serum samples obtained from BBBB patients for (a) ARTN (b) CCL28 (c) IL-22 and (d) TGF-α.



(a) (b) (c) (d)

Figure 4.3. Results of Mann-Whitney test results from serum samples with BBBB leakage versus no BBB breach for (a) ARTN (b) CCL28 (c) IL-22 and (d) TGF-α.

The serum cytokines identified were further tested to research individual concentrations in serum samples from patients with and without BBB breach. The median concentrations of the cytokines obtained from patients with a breach is higher with some but lower than healthy concentrations in others. TGF- α shows the largest difference in NPX and linear NPX values compared to non-BBB breach samples, going as high as 4 on the NPX scale whilst its concentrations remain at 3 on the NPX scale in figure 4.3.

Artemin concentrations decreased in samples with blood brain barrier leakage and had the smallest concentration change compared to the other cytokines. NPX data numbers remained almost exactly the same in people with BBB breach and no breach, being 1 and 1.2 respectively.

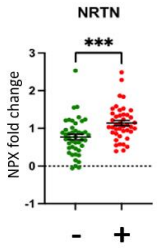
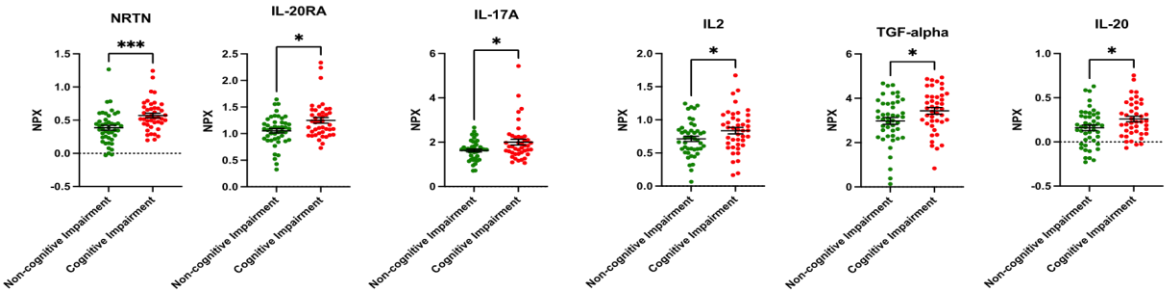


Figure 4.4. Changes in serum neurturin (NRTN) with samples from patients with cognitive impairment versus patients with little to no cognitive impairment. (-) indicates no cognitive impairment (+) indicates cognitive impairment.

ARTN's counterpart from the glial cell line-derived neurotrophic factor (GDNF) family, NRTN was also investigated specifically from samples obtained from people with degrees of cognitive impairment rather than outright evidence of a BBB breach. The NRTN concentration increases in samples with cognitive impairment.



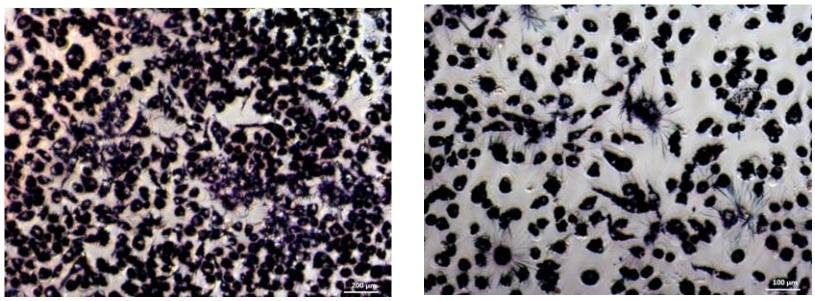
(a) (b) (c) (d) (e) (f)

Figure 4.5. Changes in cytokine concentration of serum with cognitive impairment versus without cognitive impairment in (a) NRTN (b) IL-20RA (c) IL-17A (d) IL-2 (e) TGF-α and (f) IL-20.

4.2. Transendothelial Electrical Resistance (TEER) Results

To measure membrane permeability, a TEER probe was used to measure the difference in resistance between media and the membrane with seeded cells. The longer end of the probe was placed in the media and the shorter end was positioned within the Transwell™ insert. The TEER results were inconclusive. There was little evidence of a forming blood brain barrier, as the resistance measurements did not change on a day-to-day basis. The readings fluctuated between 18 to 24 units of ohms per square centimeter ($\Omega \bullet cm^2$), with the average being 22 $\Omega \bullet cm^2$ over the course of 3 days. It takes 14 days for a blood brain barrier to fully form, as the endothelial cells continue to proliferate and form junctions, yet despite that, there was no increase in resistance. Microscopy revealed the presence of clumped cells.

4.3. Cytotoxicity Results



(a) (b)

*Figure 4.6. Microscopic image of the MTT crystals forming in the endothelial cells after 24 hours at (a) 50x magnification and (b) 100x magnification (*scale bars are estimates)*

Cytotoxicity was used to measure the cell death of the bEND.3 cells under the stress induced by the inflammatory cytokines. The weaker the color developed, the higher the spectrophotometer intensity, the higher the cell death.

4.3.1. Relative cytotoxicity of cytokines at 24, 48 and 72 hours

24-hour

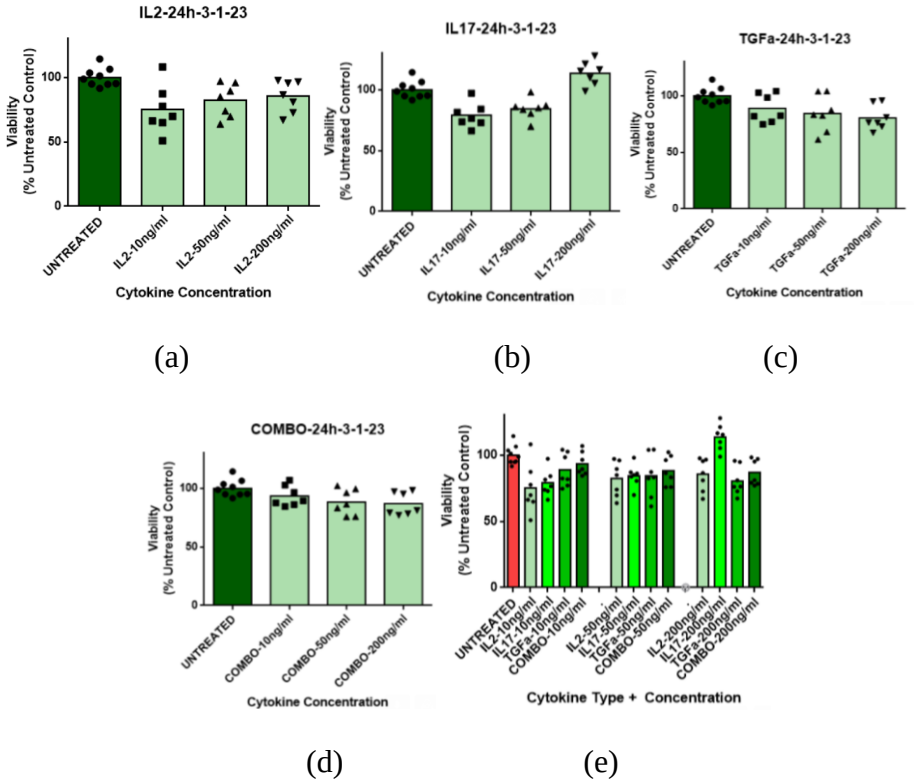


Figure 4.7. MTT results for the cytotoxicity of bEND.3 after 24 hours of exposure to 10, 50 and 100 ng/ml of (a) IL-2 (b) IL-17 (c) TGF-α at (d) a combination of 1L-2, IL-17 and TGF-α and (e) a comparison of the results.

Cytotoxicity measurements were done by testing for the rate of cell death by exposing the seeded cells to the cytokines IL-2, IL-17, and TGF-α at concentrations of 10, 50 and 200 ng/ml each. The results demonstrate that each of the cytokines show significantly different effects on the bEND.3 cells dependent on their concentrations. IL-2 shows a significant increase in cell viability as cytokine concentration increases, despite the fact that cell viability decreased overall for all concentrations. 10 ng/ml shows a large standard deviation and range of results, with one reading going up to 110% of the viability (as a percentage of the untreated control). Both IL-2 and IL-17 show an upward

trend, with viability of the seeded cells increasing as the dosage of the cytokines become more concentrated. IL-17 at 200 ng/ml also seems to show higher viability than the control itself, being at 110% of the untreated control.

TGF-α and the combination cytokines in Figure 4.7c and 4.7d show trends opposite of the IL-2 and IL-17 results. The percentage viability decreases, with both having a concentration of 10 ng/ml be the one that has the highest viability and 200 ng/ml giving the lowest viability. When compared in unison, IL-2 at a low concentration of 10 ng/ml has the lowest concentration of all the cytokines and concentrations investigated in these set of results.

48-hour

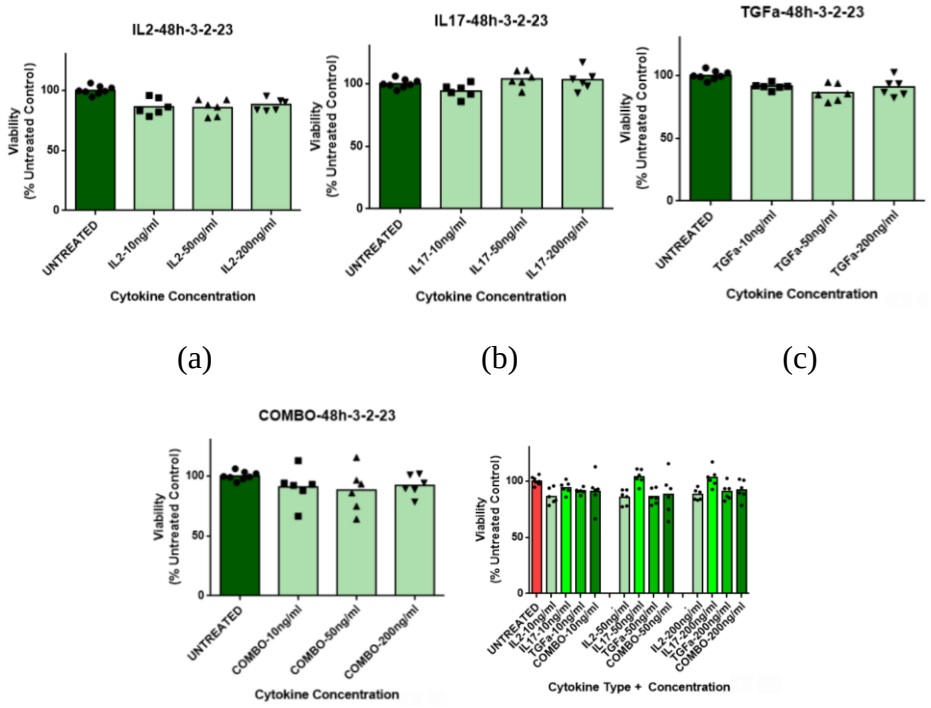


Figure 4.8. MTT results for the cytotoxicity of bEND.3 after 48 hours of exposure to 10, 50 and 100 ng/ml of (a) IL-2 (b) IL-17 (c) TGF-α at (d) a combination of 1L-2, IL-17 and TGF-α and (e) a comparison of the results.

At 48 hours, the results of the cytotoxicity tests show varying trends in cell death and cell proliferation. At this time-point, Il-2 seems to reduce cell viability by the same amount for each concentration, being close to 80-90% alive as a percentage of the untreated control. Il-17 has a lesser effect on the seeded cells, causing almost no cell death, and in fact showing cell proliferation at 50 ng/ml and 200 ng/ml. Results of the TGF-α and the combination of cytokines show an average downward trend, with cytotoxicity increasing and viability decreasing as the concentration of the cytokines increases, however the difference in cytotoxicity of the variable concentrations is not significant. IL-2 at 10 ng/ml again represents the cytokine and concentration that presents the highest level of cell death at 48 hours.

72-hour

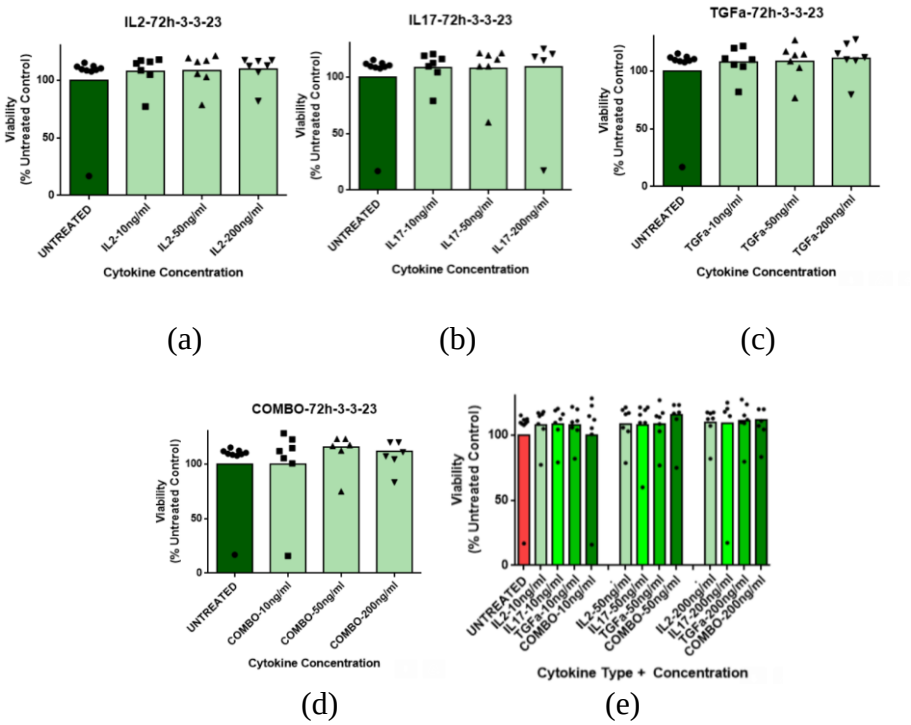


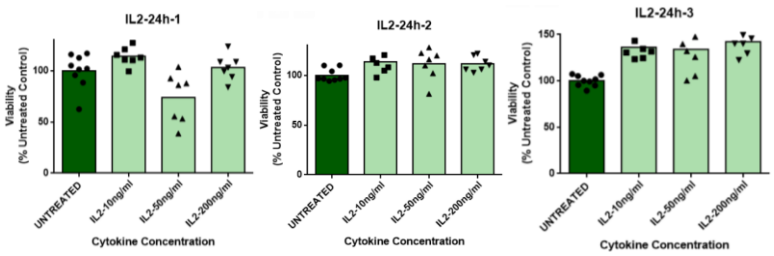
Figure 4.9. MTT results for the cytotoxicity of bEND.3 after 72 hours of exposure to 10, 50 and 100 ng/ml of (a) IL-2 (b) IL-17 (c) TGF-α at (d) a combination of 1L-2, IL-17 and TGF-α and (e) a comparison of the results.

At 72 hours, there remains little evidence of cytotoxicity in all three cytokines or the experiments with the combination of the three cytokines tested together. Except at a combination concentration of 10 ng/ml, every single iteration of cytokine and concentration shows an increase in cell proliferation rather than evidence of cell death. While 10 ng/ml of the combination cytokines has a viability practically the same as that of the control, the rest of the iterations show cell viability of 110% of the untreated control. This set of results also has a high number of anomalies, with each category having a largely anomalous reading that shows cell viability to be as low as 10-20% of the untreated control. Despite that single anomaly, the rest of the readings in each concentration of each cytokine remains largely the same.

4.3.2. Relative cytotoxicity follow-up assays at 24 hours

Follow-up cytotoxicity assays were conducted at 24 hours three times. The passage number, cell seeding density and cytokine concentrations remained the same as the previous experiments.

IL-2



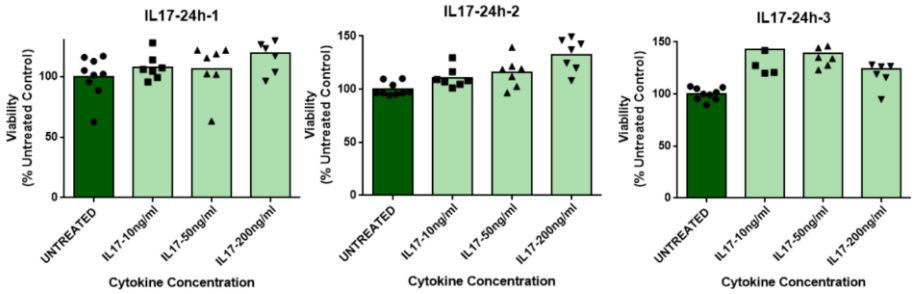
(a) (b) (c)

Figure 4.10. MTT replicates for the cytotoxicity of 10, 50 and 200 ng/ml concentrations of IL-2 on bEND.3 after 24 hours of exposure

These results fail to exhibit a trend. Figure 4.10a, b, and c represent replicates of the same experiment. Despite that, each result manifests very differently. In all 3 repeats,

IL-2 at 50 ng/ml has the highest cell death, with its viability being 70% of that of the control, but in (b) and (c) they still have viabilities of that larger than the control. Along with that, the last two figures also show very little actual difference between the results of IL-2 at 50 ng/ml, as compared to IL-2 at the other concentrations. In the last replicate (c), each of the three concentrations show a significantly higher rate of cell proliferation than its counterparts, reaching viability percentages of up to 130% of the untreated control. The individual readings also have a large range and exhibit a high standard deviation. The concentration with the highest standard deviation is also IL-2 at 50 ng/ml for all three repeats.

IL-17

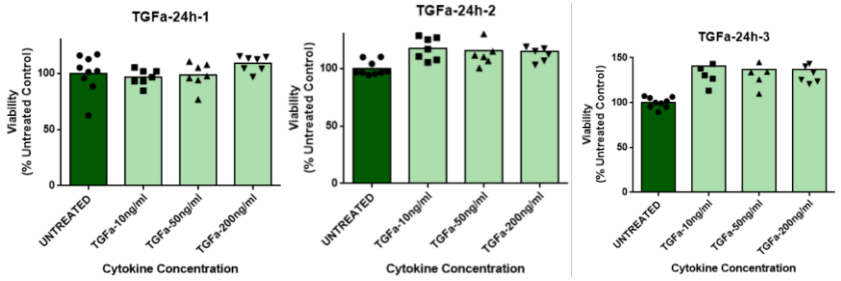


(a) (b) (c)

Figure 4.12. MTT replicate results for the cytotoxicity of 10, 50 and 200 ng/ml concentrations of IL-17 on bEND.3 after 24 hours of exposure.

IL-17 exhibits a general trend in only two of its replicates. The first two show a general increase in viability while figure 4.12c shows a decrease. It is interesting to note that the range of readings and standard deviations remain similar for all of them, except IL-17 at 50 ng/ml in figure 4.12a. The general viabilities of these readings also surpass those of the control, with figure xc showing a cell viability percentage being almost 150% of the untreated control.

TGF-α

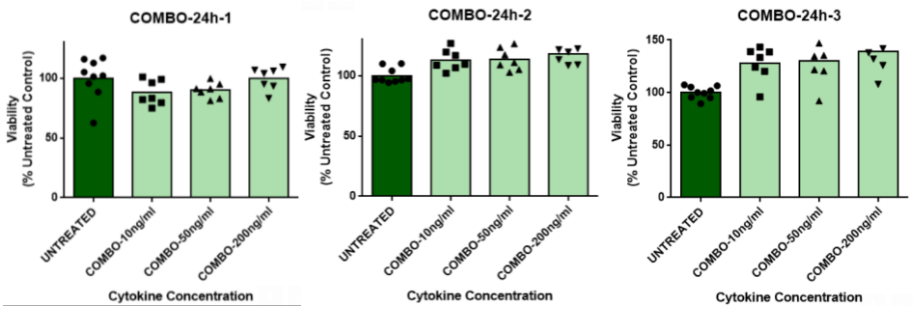


(a) (b) (c)

Figure 4.13. MTT replicate results for the cytotoxicity of 10, 50 and 200 ng/ml concentrations of TGF-α on bEND.3 after 24 hours of exposure.

TGF-α exhibits a wide range of results. Figure 4.13a shows a general increase in the viability of the seeded cells and figures 4.13b and 4.13c show the opposite, with viability decreasing as concentration of the cytokines increases. The individual reading values remain within a range, and there are no anomalous results in these readings, however the trends do not correspond with each other. In the third replicate experiment (figure xc) the cell viability becomes almost as high as 150% of the untreated control. The concentration with the highest cell death was TGF-α at 10 ng/ml, but only exhibits this in one replicate (figure 4.13a).

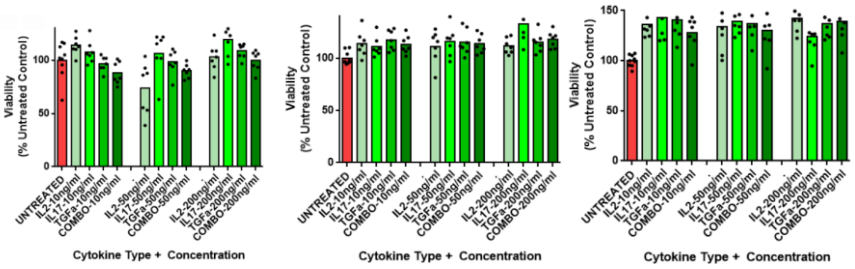
Combination



(a) (b) (c)

Figure 4.14. MTT replicate results for the cytotoxicity of 10, 50 and 200 ng/ml concentrations of IL-2, IL-17 and TGF-α on bEND.3 after 24 hours of exposure.

Only one replicate of this experiment (figure 4.14a) exhibits actual cell death at each of the three concentrations. The rest of the replicates exhibit cell viabilities as higher than that of the untreated control, going as high as 125-130%. Despite that, all three of these replicates demonstrate an increase in cell proliferation as the concentration of the cytokines increases.



(a) (b) (c)

Figure 4.15. Combined comparison of MTT replicates for the cytotoxicity of 10, 50 and 200 ng/ml concentrations of a combination of IL-2, IL-17 and TGF-α on bEND.3 after 24 hours of exposure.

These comparison graphs demonstrate that the cell viabilities at different concentrations vary significantly. The combination cytokine tests show a fair amount of cell death, being significant in figure 4.15a and 4.15c, while figure 4.15a also shows the large amount of cell death by IL-2 at 50 ng/ml. These concentrations do not follow a single trend or pattern but vary greatly instead depending on the concentrations administered to the seeded cells in the wells.

4.3. Flow Cytometry Results

Due to the inconsistent nature of the cytotoxicity results, flow cytometry was conducted to allow a closer analysis of the cells. Forward scatter (FSC) detects the cell size and granularity, while side scatter (SSC) reflects the internal complexity and granularity of the cells. There is different fluorescent intensity in different channels.

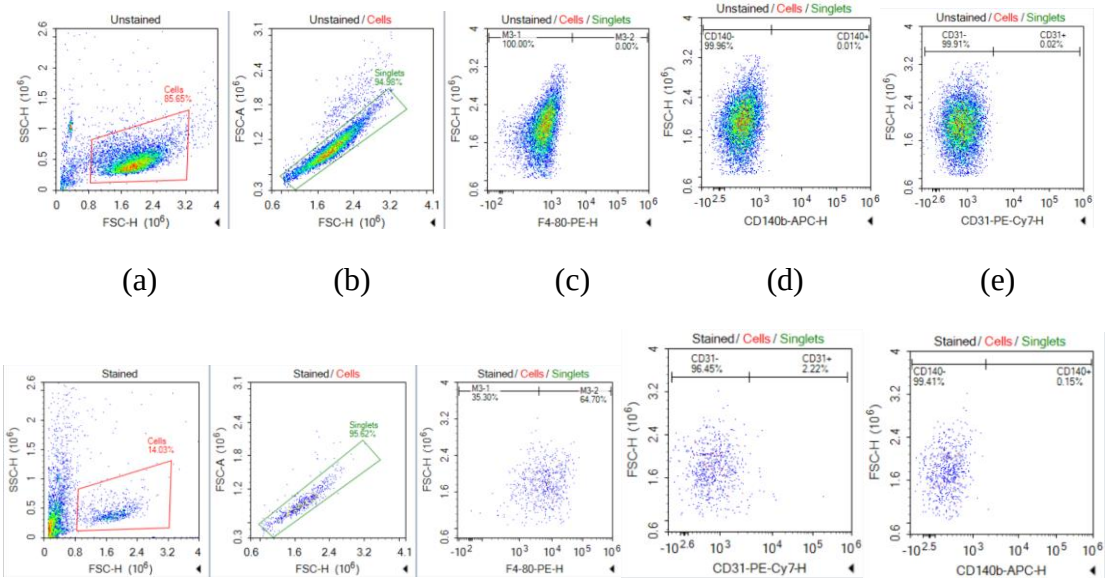


Figure 4.16. Flow cytometry of (a)-(e) unstained cells and (f)-(j) stained cells for binding of different markers for different cell types, including endothelial, macrophage and mesangial cells.

In the scatter plot, the unstained cells are located on the bottom left corner of the graph, while the stained cells are shifted to the right. This shift occurs because the fluorescent signal emitted by the stained cells is detected and measured by the fluorescence detector in figure 4.16 (h) and is plotted on the x-axis of the scatter plot. The intensity of the fluorescence signal is proportional to the number of antibodies bound to the cell, which correlates with the abundance of the labeled macrophages.

The results show a shift in the scatter plot for F4/80 markers, indicating that the flow cytometry had phenotyped macrophages. The results revealed that the cells used for the experiments contained 95-99% RAW 264.7 macrophages and only 1-5% bEND.3 cells.

At this point, no further assays were performed on the cells.

CHAPTER 5: DISCUSSION

O-link proteomics is a tool for discovering new biomarkers and exploring the underlying biological mechanisms of diseases. By measuring a large number of proteins in a single assay, it allows for the identification of protein patterns or signatures that may be associated with disease states.

The O-Link Proteomics assays carried out by the lab signify the top cytokines present in serum of NPSLE patients. The NPX data and the p-values signify that there is a high correlation between the presence of IL-2, IL-17 and TGF-α and symptoms of cognitive impairment/cognitive decline. In order to determine the origin of these cytokines, and the mechanism of their production and role in cognitive impairment and cognitive decline, these results acted as the basis for the follow-up assays, starting with permeability.

TEER was attempted to be used to investigate the changes in the integrity of the BBB using physical permeability tests. The TEER probes would measure the resistance of the bEND.3 membranes in relation to the DMEM surrounding the Transwell™ inserts.

The technique proved difficult to produce results from. The greater the degradation of the membrane, the larger the pores and the greater the permeability of the membrane. Permeability would be quantified using electrical resistance; greater permeability means a lower relative resistance between the membrane inserts and the surrounding DMEM media. The results fluctuated heavily over the days, remaining the same on average. This gave poor indication of a developing blood brain barrier, and therefore it would be difficult to investigate the degradation of a barrier that there was no evidence of being developed. Voirin et al. suggested that TEER results could be less

reliable than other methods of analysis due to limitations of the technique, such as difficulty collecting measurements, the effects of temperature change on the readings, the technique of the experimenter and its difficult reproducibility. Therefore, TEER was not considered a technique that was essential to collecting useful data on the effects of the cytokines on the integrity of the bEND.3 barrier [24].

The cytotoxicity study evaluated the cytotoxicity of bEND.3 cells by exposing them to three different cytokines, namely IL-2, IL-17, and TGF-α, at different concentrations. The results indicate that each cytokine showed a distinct effect on the viability of the cells. The results achieved in these sets of experiments vary greatly in comparison to literature published. There has been no identifiable trend or pattern in these results.

The 24-hour measurements in the preliminary assays conducted offer the most promising results by demonstrating a clear trend between the cytokines and their concentrations. There is a significant loss in cell number and the viability of the cells, with cell viability going as low as 75% in both IL-2 and IL-17 at 10 ng/ml each. In both cases, they seem to induce the same effect on the cells, with a gradually increasing rate of cell proliferation as their concentrations increase. Unlike TGF-α and the combination experiments, the higher the concentrations the higher the cell growth. In the latter two, TGF-α and combination cytokines seem to induce a greater rate of cell proliferation in lower concentrations and lower rate of growth in concentrations as high as 200 ng/ml.

Despite such large increases in concentrations, the changes in cell viability are often less significant in comparison to such large concentration increases. It seemed as if lower concentrations tended to perform better on inducing cell apoptosis than higher concentrations. This pattern was also followed by weaker effects of the cytokines as

exposure times increased, despite the fact that it was hypothesized that if the bEND.3 were exposed to the cytokines for longer, cell death would increase. At 48 hours, the results followed the trend found in the 24-hour results, but less closely. IL-2 and IL-17 showed more cell death at lower concentrations and TGF-α and the combination of cytokines showed less cell death at lower concentrations. At 72 hours, the concentrations of cytokines seemed to influence the cell apoptosis and cell viability very little. In this case, all produced graphs show a cell viability that is higher than the control itself. This may be due to the fact that in 72 hours the cells were dividing, especially as cells susceptible to cell apoptosis by the pathways induced by the cytokines would die and cells resistant to the stress induced by the cytokines would be able to divide more freely, passing along a possible resistance gene and/or mutation, and producing an overall sample resistant to the inflammatory agents.

These results suggest that the effects of cytokines on cell viability are complex and multifaceted. The observed effects of IL-2 and IL-17 on cell viability may be attributed to their pro-survival and anti-apoptotic properties. On the other hand, the observed effects of TGF-α and the combination cytokines may be attributed to their pro-apoptotic and cytotoxic properties. The results also suggest that the optimal concentration of cytokines for inducing cytotoxicity in bEND.3 cells may vary depending on the specific cytokine and the concentration used.

With the relative success of the 24-hour timepoints in the preliminary MTT assays, follow-up assays were conducted under identical conditions as before to test for reproducibility. In this case, the results were far different than those produced in the preliminary tests. These assays can be compared to the initial 24-hour timepoint assays done. Figure 4.5a shows IL-2 at 50 ng/ml having a cell viability of 75%, a value that

occurs again for IL-2 at 50 ng/ml in figure 4, however larger concentrations consistently encourage cell proliferation rather than cytotoxicity, the opposite of what is expected. Often, IL-2 and IL-17 come across as the cytokines that have the largest amount of cell death, but usually at lower concentrations, while TGF-α and the combination cytokines have the opposite trend. This may be due to the fact that TGF-α is a more protective cytokine and is said to encourage growth of cells rather than induce cytotoxicity, while IL-2 and IL-17 more openly are regarded as pro-inflammatory cytokines and invoke inflammatory responses in cells and tissue. The combination cytokines might display their strange results due to the polarizing nature of the reagent created by mixing pro-inflammatory cytokines with pro-endothelialization growth factors, hence the large range in cell viability results. Along with that, the results clearly indicate that it was the presence of the cytokines that induced cell proliferation, as the control groups remained in relative constancy with the cell viability values of the other readings, and where the cell viability clearly and significantly increased in the presence of the cytokines.

In multiple instances in the results, lower concentrations of cytokine exposure seemed to induce a higher level of cell apoptosis, even at varying exposure periods. One possible reason why lower concentrations of cytokines may cause more cytotoxicity in bEND.3 cells than higher concentrations is that these cells may have a specific threshold for cytokine activation. At low concentrations, the cytokines may not be activating the necessary pathways for cell survival, but at higher concentrations, the activation of these pathways may be sufficient to promote cell survival and even proliferation. However, at very high concentrations, the cytokines may activate pathways that lead to cell death.

Another possibility is that bEND.3 cells may have a unique cytokine receptor expression pattern or sensitivity to certain cytokines at lower concentrations. For

example, some cytokines may activate specific receptors that are more highly expressed on bEND.3 cells at low concentrations but may activate other receptors at higher concentrations, leading to different cellular responses. It is possible that the combination of different cytokines at lower concentrations may have synergistic or additive effects on bEND.3 cells, leading to increased cytotoxicity compared to a single cytokine at a higher concentration. This could be due to interactions between the different cytokines or the activation of different pathways by the combination of cytokines.

The flow cytometry was conducted as a follow-up to analyze the cells more closely after such inconclusive results. The results show that more than 95% of the cells were RAW 264.7 macrophages. This poses the possibility that at the time of the follow-up cytotoxicity assays, the results were not reproducible from the preliminary ones because the number of macrophages in relation to the number of bEND.3 cells increased significantly, producing irreplicable results. It provides an explanation for the phenomena that had been occurring where the cell viability of all the cytokine concentrations were increasing rather than decreasing, producing effects that lead to the production of immune cells by the cytokines, as well as the production of more cytokines [27]-[28]. While, to our knowledge, there seems to be no evidence of IL-17, IL-2 and TGF-α being involved in the direct proliferation of macrophages, it is possible that they trigger a mechanism or pathway that causes either prevents the hindering of growth or stimulate growth themselves.

This does, however, make much of the cytotoxicity results significantly unreliable, as they could have been contaminated with an unknown number of macrophages for an indiscernible amount of time.

CHAPTER 6: CONCLUSIONS

25% of all patients with SLE have extensive BBB breach, which causes harsh neurological consequences. Despite the condition being so common, there is very little understanding of the molecular basis of a disease as prevalent as SLE and therefore NPSLE. The research outlined in this thesis attempted to first find a molecular signature of BBB breaches and CSF leakage in the CNS, and then by investigating cytokines like IL-2 and IL-17, it attempted to identify how the cytotoxic nature of these cytokines affect endothelial cells as a representation of a BBB. This also attempts to define cognitive impairment and help us gain a better understanding of how one would measure cognitive decline.

The research also attempted to investigate more closely the mechanisms of cytotoxicity induced on bEND.3 cells using cytokines such as IL-2, IL-7 and TGF-α. After an initial screening, that found these proteins as cytokines that highly correlated to serum of people with symptoms of cognitive decline and cognitive impairment, these cytokines underwent an attempt to be validated. While there are many different ways to investigate the mechanisms of how cytokines influence mouse bEND.3 as a representation of a BBB, these experiments specifically worked with cell death and cell proliferation, and attempting to find a pattern or trend when the cells were exposed for different periods of time. The results were inherently inconclusive, varying from inducing a high amount of cell apoptosis to instead stimulating cell proliferation.

Upon revelation of the flow cytometry results, the MTT assays as a method of understanding the mechanisms of cell death in bEND.3 under the influence of cytokines

became unreliable in this case. However, the significant trends and patterns outlined in this thesis about the changes in cell proliferation and the stimulation of cell growth under exposure with different concentrations of cytokines reveals important information about the interactions between cytokines and immune cells such as macrophages, and the mechanisms of cell proliferation and possibly even cell activation.

Despite this unexpected setback, previous research and literature provide results that can be used to plan follow-up experiments that more directly relate to studying the integrity of the blood brain barrier. These include more mechanical and physiological methods of measuring the degree of degradation of the BBB by exposure to cytokines. Assays can include fluorescence permeability by measuring how much dextran-FITC, a fluorescent probe, passes through, with the hypothesis that the more probes pass through, the greater the degradation of the BBB membrane. Another method includes the measurement of the quantity of proteins produced when tight junctions form, including occludin, claudin-5, and ZO-1, using gene expression and rt-qPCR techniques.

Additionally, we can investigate whether the addition of neurotrophic growth factors changes or repairs the Transwell™-simulated blood brain barrier, including investigating the mechanisms of TGF-α. Protective factors like NRTN and ARTN can be investigated in conjunction with inflammatory cytokines to determine the mechanisms of pathogenesis of NPSLE on bEND.3 cells as well as astrocytes in a co-culture.

Further research will attempt to identify whether and how neurotrophic factors like ARTN and NRTN can be introduced to simulated BBB breaches, or in times of cytokine flares in serum, to act as anti-cytokine therapy, control flares of the established linked cytokines, and encourage reparations of any damage done to the BBB. The study on the whole aims to shed light on the mechanism of NPSLE, investigate how cytokines

and neurotrophic factors interact with each other within a simulated BBB, and investigate methods of repairing disrupted barriers.

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