NEUROPROTECTIVE PROPERTIES OF FINGOLIMOD IN AN EXPERIMENTAL MODEL OF MULTIPLE SCLEROSIS-INDUCED OPTIC NEURITIS

BY

AMRITHA ANAND CANDADAI

(Under the direction of Priya Narayanan)

ABSTRACT

Multiple Sclerosis (MS) is a highly disabling neurological disease characterized by demyelination, inflammatory responses, and neurodegeneration. Visual dysfunction resulting from Optic Neuritis (ON) is one of the most common clinical manifestations of MS. Current medications available for MS specifically target the inflammatory phase and have limited effects on long-term disability. There is a gap in knowledge in identifying an agent that focuses on the neurodegenerative phase of the disease. Fingolimod is an immunomodulatory agent approved for MS therapy. The objective of the current study was to evaluate the potential neuroprotective properties of Fingolimod. An *in vitro* model of ON was established utilizing the R28 retinal neuronal cell line. Neuronal damage was induced by treating with Tumor Necrosis Factor α (TNFα). Cell viability studies showed that Fingolimod treatment significantly reduced TNFα-induced neuronal death. Studies on signaling pathways demonstrated that Fingolimod attenuated the TNFα-induced changes in cell survival and cell stress signaling molecules. Furthermore, immunofluorescence studies performed using various neuronal markers indicated that Fingolimod treatment protected the R28 cells against the TNFα-induced neurodegenerative changes. In conclusion, our study suggests that Fingolimod exhibited neuroprotective properties in an experimental model of optic neuritis.

Index Words: Multiple Sclerosis; Neuroprotection; Fingolimod; Optic Neuritis

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DEDICATION

This thesis is wholeheartedly dedicated to my loved ones – thank you for being my strength and backbone during this adventure.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

1. Introduction

1.1. Multiple Sclerosis: A Clinical Perspective

Multiple Sclerosis (MS) is a chronic, autoimmune, inflammatory, neurodegenerative disease characterized by the demyelination of neurons. [1,2] This CNS disease affects approximately 400,000 people in the United States and 2.1 million people worldwide [3], showing a higher rate of incidence in women as compared to men. [4,5] Presently, the exact etiology causing the initiation of the disease has not been identified. However, research points to a combination of genetic predisposition coupled with a non-genetic or environmental trigger factor. [6,7]

Being a disease involving neurological deficit and related implications, MS notably affects the quality of life of patients and causes socioeconomic burden for the individual and for society. [3,8] This makes the demand for effective and "patient-friendly" therapy grow larger, thereby justifying the vital need to understand the disease in depth.

A patient may experience their first neurological attack in the form of a "Clinically Isolated Syndrome" or CIS. Recurring episodes are addressed by using the McDonald Criteria, which provides established guidelines to successfully diagnose the disease. Diagnosis is achieved through the history and neurological examination in addition to, a) Neurological damage depicted by the presence of T2-weighted MRI lesions, that are specifically identified as either disseminated in space (DIS) or disseminated in time (DIT); b) Cerebrospinal fluid levels, particularly the presence of oligoclonal bands or high immunoglobulin (IgG) index. Detailed parameters are employed to

further sub-classify the diagnosis into one of four types of MS: i. Relapsing-Remitting (RRMS), ii. Secondary Progressive (SPMS), iii. Primary-Progressive (PPMS), iv. Progressive Relapsing (PRMS). [9]

Clinical manifestations of the disease are highly variable and unpredictable, depending on the area in the CNS that is damaged. Signs and symptoms include fatigue, numbness/tingling, spasticity, vision disability, gait difficulty, pain, bladder dysfunction, sexual dysfunction, emotional or cognitive changes. Less common symptoms include speech difficulty, tremors, and seizures. [10] Hence, once the symptoms have been well-established, a comprehensive care system is vital for the patient.

1.2. Pathogenesis of Multiple Sclerosis

The understanding of the pathophysiology of MS and potential molecular mechanisms involved in disease progression has evolved over decades of research. Overlapping processes and cross-linking responses contribute towards ultimate outcome, which can be categorized into demyelination, inflammation, axonal dysfunction, and neurodegeneration.

I) Inflammation

An initiating factor or "trigger" causes the primary activation of autoreactive $CD4^+$ T cells in the periphery. [11] Once activated, the T cells infiltrate into the CNS by penetrating the blood-brain barrier (BBB). This migration across the BBB is mediated by the interaction of very late antigen-4 (VLA-4) present on T cells and vascular cell adhesion molecule-1 (VCAM-1) present on the capillary endothelial cells on the basement membrane. [13,14] Inside the CNS, antigen-presenting

cells (macrophages, B cells, and dendritic cells) expressing MHC Class II complex interact with T cells, causing the activation of a pro-inflammatory cascade. [15] The subsequent inflammatory response is driven by the release of cytokines and chemokines, further activation of B cells, monocytes, resident microglia, and macrophages. [16,17] The resident glial cells maintain the degree of constant inflammation even in the absence of infiltrated lymphocytes. [18] This cumulatively results in the disruption of myelin, oligodendrocytes, and axonal bundles.

II) Demyelination

Myelin is a protein- and lipid-rich substance produced by oligodendrocytes that is uniformly present around axons of nerve cells in the form of a 'myelin sheath.' [53] Myelinated axons are majorly populated in white matter (the name derived from whitish appearance due to myelin) and are found in limited amounts in gray matter. Multiple myelin sheath layers cover the length of axons and are separated from each other by short gaps called nodes of Ranvier. This sheath serves its purpose by insulating axons and increasing the rate of impulse conduction through the nerve. Damage to the myelin disrupts nerve impulse conduction and is a factor contributing to neurological disturbances seen in demyelinating diseases such as MS. [54]

A study published in 1999 showed that both healthy individuals and MS patients presented a similar amount of myelin-reactive T-lymphocytes in circulation. [12] Since demyelination in MS is a characteristic event, this implies that there exist other factors distinctive of MS or other demyelinating diseases that cause the specific antigenicity towards the myelin sheath. The autoimmune response and specific antigenicity towards myelin components can be explained by a combination of three mechanisms: epitope spreading [21,22], bystander activation [21,22], and molecular mimicry [23]. In MS and other autoimmune diseases, $CD4^+$ T cells are activated by a

"primary epitope" (in this case, an antigen peptide specific to myelin protein components), causing their infiltration into the CNS and migration into the target tissue. [19,20] This leads to the inflammatory cascade of events characterized by the release of cytokines and chemokines, coupled with further recruitment of inflammatory cells from the periphery, thereby ultimately causing the activation of secondary myelin-specific epitopes *(epitope spreading)*, damage to nearby cells or tissues mediated by phagocytic mechanisms and release of TNF-α, reactive radical species, proteolytic enzymes *(bystander activation)*, and further cross-activation of autoreactive T and B lymphocytes *(molecular mimicry)* due to tissue/cellular debris presented on resident and peripheral antigen-presenting cells. [21]

III) Neurodegeneration & Axonal Disruption

The concepts of inflammation and neurodegeneration are not mutually exclusive in MS. Although we understand the different mechanisms contributing to both, the exact flow of events remains unclear and is up for debate. Several possibilities can explain the interplay between the two events and include: a) inflammation causes neurodegeneration at a later stage, b) primary neurodegenerative mechanisms induce a secondary inflammatory cascade, c) other factors cause simultaneous stimulation of both events. CNS diseases such as MS, Parkinson's Disease, Alzheimer's, although triggered by different and varying etiologies, have some similar mechanistic pathways that instigate neuronal injury. A therapeutic agent with neuroprotective action that can significantly control the progressive stages of MS would be appreciably beneficial in managing the harsh and diverse complications faced by a patient. In order to get there, understanding the potential cellular mechanisms contributing to neuronal injury is crucial.

Damage to the axons in an MS brain is one of the hallmark events that point towards progressive neuronal degeneration. Typical axon loss is constant throughout the MS pathogenesis from initiation of myelin-specific auto-immune attack to inflammation and degenerative mechanisms, making it appear as both the cause and consequence of neurodegeneration. Neurons have a high energy demand but low energy reserves. [45] Due to this, neurons are highly prone to energy deficits and require a constant level of energy to function. Axons are crucial to mitochondrial energy transport and signal conduction. Axonal transport is dependent on structural and chemical components including microtubules, [46,47] calcium sensor proteins [48] and anchoring proteins[49]. A faulty transport system is implicated in neurodegenerative mechanisms. Along with a defective transport system, the cumulative effect of local pro-inflammatory mediators, demyelinating epitopes, ROS producers and consequent apoptotic cascades as previously described all result in disrupted axons and eventually neurodegeneration.

1.3. Mechanisms of Dysfunction

MS pathogenesis involves multiple processes, signaling pathways, and biochemical changes that advance at the molecular, cellular, and ultimately the systemic level that cumulatively results in a dysfunctional environment. The different known mechanisms of dysfunction contributing to disease progression are detailed below.

i) Mitochondrial Injury

Neurons and in particular, axons depend on oxidative energy metabolism to carry out their functions with respect to signal conduction mediated by mitochondrial ATP production. [24] Not only are mitochondria essential for cellular energy production, they also play a significant role in other cellular events such as fatty acid oxidation, amino acid biosynthesis, steroid metabolism,

calcium handling, and production of reactive species. [25,26,27] The mitochondrial respiratory chain present in the inner mitochondrial membrane holds an integrated system of protein-coenzyme complexes, most of which are coded by mitochondrial DNA and are responsible for catalyzing the conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). [38,43] Disruption in the structure and function of this electron respiratory chain pathway results in mitochondrial damage. [26] Interruption in ATP production causes a dysfunctional Na^+/K^+ ATPase pump in the mitochondrial matrix, which alters the charge flow and causes an increase in intra-axonal Ca^{2+}. This suboptimal ion channel regulation promotes the release of proteases, phospholipases, and other degradative enzymes that initiate axonal degeneration. [28] Moreover, generation of toxic reactive oxygen species (ROS) in the respiration chain complexes I and III has been implicated in cellular stress-induced apoptosis. [29] ROS causes the subsequent activation of pro-apoptotic mediators such as cytochrome-c which stimulates caspase activation and cell death. [29] Mitochondrial DNA mutations or deletions found in MS neurons have also been investigated as they are associated with accelerated aging of the cells. [30] Damaged mitochondrial DNA can augment oxidative stress-induced apoptosis by decreasing the expression of critical proteins necessary for electron transport. [44] This leads to a vicious cycle of ROS-mediated neurodegeneration. Finally, a study demonstrated that both anterograde and retrograde mitochondrial transport were reduced in a chronic experimental model of MS. [50] A dysfunctional transport within cells causes the accumulation of proteins and organelles and results in axonal swelling. This can be confirmed by assessing the accumulation of amyloid precursor protein (APP), one of the markers for axon damage. Studies have shown accumulation of APP in MS brains at both early and post-mortem stages of disease as a distinctive feature of mitochondrial dysfunction-induced neurodegeneration. [51,52]

ii) Oxidative Stress

Oxidation is one of the vital cellular processes that preserves cellular energy and function. Within a cell (in this case, neuron), there exists an oxidative gradient maintained by "pro-oxidant" and "anti-oxidative" molecules that regulate their levels in order to facilitate different processes and maintain homeostasis. [31]. An imbalanced environment induces stress, mediated by the generation of unstable, reactive agents such as reactive oxygen and nitrogen species (ROS/RNS) – superoxides (O_2^-), hydroxyl radicals (OH^-), peroxynitrites ($ONOO^-$) and hydrogen peroxide (H_2O_2). Once the primary demyelination is initiated, infiltrated lymphocytes and macrophages, along with locally present pro-inflammatory astrocytes and microglia orchestrate the progressive neurological damage. All these inflammation-inducing processes involve ROS-induced cell/tissue injury. Microglial activation is well-recognized in ROS-mediated pathways. [32,33,34] The expression of major ROS mediators, NADPH oxidase and myeloperoxidase have been shown in the microglia of active MS lesions. [35] Studies have also shown the presence of oxidized lipids in myelin membranes, dying oligodendrocytes, axons and their association with neurodegeneration and lesion formation. [36,37] As reviewed previously, the mitochondrial ROS are prominent in the event of oxidative stress. Excessive ROS affects the mitochondrial respiratory chain assembly by deactivating enzymes NADH oxidase, cytochrome c oxidase and ATP synthase, all of which are integral in the production of mitochondrial energy. [38,39] Furthermore, ROS alters the mitochondrial membrane permeability and structure due to lipid peroxidation of mitochondrial phospholipids [40], oxidation of thiol groups on oxidative translocators [41] and modifying the structure and biochemical functions of fundamental mitochondrial respiratory enzymes, which

increases the proton permeability of the inner mitochondrial membrane, disrupts structure and impairs mitochondrial function. [42]

1.4. Optic Neuritis in MS

As described earlier, the complications associated with MS are wide-ranged due to the diverse and disseminated nature of the disease. One of the frequent clinical manifestations is visual dysfunction. Studies have shown that approximately 20% of patients present with inflammation of the optic nerve aka optic neuritis as their first symptom of MS. [59,60] Another study conducted by the North American Research Committee on Multiple Sclerosis (NARCOMS) showed that of the 9107 patients participating in the study, 60% reported signs of vision impairment, and 14% of these depicted moderate/severe/very severe impairment of vision. [61] Based on the available research, visual function is significantly correlated to MS disease progression.

Tests such as Low-contrast letter acuity (LCLA) help get an idea on vision-specific quality of life aspects that are of clinical significance to patients. LCLA coupled with optical coherence tomography (OCT) for retinal structural integrity and electroretinography for electrophysiological events together provide necessary information on vision-associated complications in MS patients and successfully demonstrated their relevance in progressive stages of the disease. [57,58] Optic neuritis, is characterized by thinning of the nerve fiber layer [62], degeneration of retinal ganglion cells (RGCs) [63], and loss of inner retinal function secondary to optic nerve degeneration. [64,65] A recent study from our lab validated MS-induced retinal neurodegeneration in an experimental murine model and revealed a neuroprotective effect by the targeted deletion of Arginase-2 gene.[66]

1.5. Current Treatment: Disease-Modifying Therapies

As of now, no cure exists to treat patients diagnosed with MS. Therefore, management of the disease is accomplished by one of these three strategies: controlling exacerbations, alleviating disease progression with disease-modifying therapies (DMTs), and symptomatic therapy. Use of therapeutic agents that help slow down the progression of disease is a beneficial approach. Since these therapeutics function by altering the rate of progression of disease, they are given the term "disease-modifying therapies" or DMTs. Disease modification is achieved by breaking the chain of pathophysiological activity, and thereby reducing impact on the patient's long-term disability. [67] Treatments are pharmacologically and biologically diverse and choice of agent depends on various factors such as severity/stage of disease, cost, adverse effect profiles, and patient preference. [69]

Table 1. Disease Modifying Therapies in MS: Provided by Multiple Sclerosis Coalition consensus paper titled "The Use of Disease-Modifying Therapies in Multiple Sclerosis: Principles and Current Evidence; SUMMARY". According to the MS Coalition, there are fifteen DMTs approved by the US Food & Drug Administration (updated September 2019). (Abbreviations: s.c.:subcutaneous, i.m.: intramuscular)

SELF-INJECTIBLES	ORAL	INTRAVENOUS
Daclizumab (Zinbryta®)	Dimethyl fumarate (Tecfidera®)	Ocrelizumab (Ocrevus™)
Glatiramer acetate (Copaxone® and Glatopa®)	Fingolimod (Gilenya®)	Alemtuzumab (Lemtrada®)
Interferon beta 1-a, s.c. (Rebif®)	Teriflunomide (Aubagio®)	Mitoxantrone (Novantrone®)
Interferon beta 1-a, i.m. (Avonex®)		Natalizumab (Tysabri®)
Interferon beta 1-b (Betaseron® and Extavia®)		
Pegylated Interferon beta 1-a (Plegridy®)		

As discussed earlier, MS pathogenesis is majorly dominated by the binary action of inflammation and neurodegeneration and is characterized by demyelination. All approved DMTs, although effective in reducing frequency and severity of relapses, function by suppression of the immunological mechanisms in MS pathology, and none are known to target the neurodegeneration of MS. [68] This makes them partially effective as the more progressive mechanisms driven by neurodegeneration remain untargeted. Therefore, an agent with additional neuroprotective action may offer advantages over existing therapies in reducing ultimate MS disability.

For this purpose, the drug of interest in my study is Fingolimod (Gilenya®), an approved DMT for relapsing forms of MS. Its success as an immunomodulatory agent combined with neuroprotective potential has previously been demonstrated in various disease models other than MS (see Section 1.7). This prospect coupled with its prior approval in MS therapy makes Fingolimod a strong candidate for a safe and efficacious neuroprotective strategy in MS as well as other neurodegenerative diseases. The next section explores characteristic features of fingolimod and its potential action as a neuroprotective agent.

1.6. Immunomodulator in MS Therapy: Fingolimod

Discovery and Development: The concept of isolating immunosuppressants from naturally derived fungal extracts began in 1971 with the discovery of cyclosporin A. [72] This motivated researchers to apply a similar strategy to identify therapeutics for autoimmune conditions such as MS, rheumatoid arthritis, and systemic lupus erythematosus, which eventually lead to the synthesis of Fingolimod (code named: FTY720). Fingolimod is a derivative of ISP-1 (aka myriocin), a fungal metabolite of the Chinese herb I. sinclarii as well as a structural analog of sphingosine. [70,73] 0.5

mg fingolimod (FTY720/Gilenya; Novartis) was the first oral DMT approved in September 2010 by the U.S. Food & Drug Administration for first-line treatment of relapsing-remitting MS. [73] Mechanistically, Fingolimod functions by binding to the sphingosine-1-phosphate (S1P) receptors and subsequently inhibiting the infiltration of lymphocytes from peripheral blood into the CNS. Importantly, its highly lipophilicity facilitates the drug to easily penetrate through the blood-brain barrier and reach the CNS tissues in appreciable concentrations. [76]

Receptor Biology: S1PRs are widely expressed in immune, neural, endothelial, and smooth muscle cells. [73] The receptors exist in the form of five subtypes ($S1P_{1\text{-}5}$); in the CNS, they are abundantly expressed by microglia ($S1P_{1,2,3,5}$), astrocytes ($S1P_{1,2,3}$), oligodendrocytes ($S1P_{1,3,5}$), neurons ($S1P_{1,3}$), and neural progenitor cells ($S1P_{1,2,3,5}$). [74] In physiology, the receptor family has been implicated in the regulation of different processes such as cellular trafficking, modulation of barrier integrity, and maintenance of vascular tone. [75]

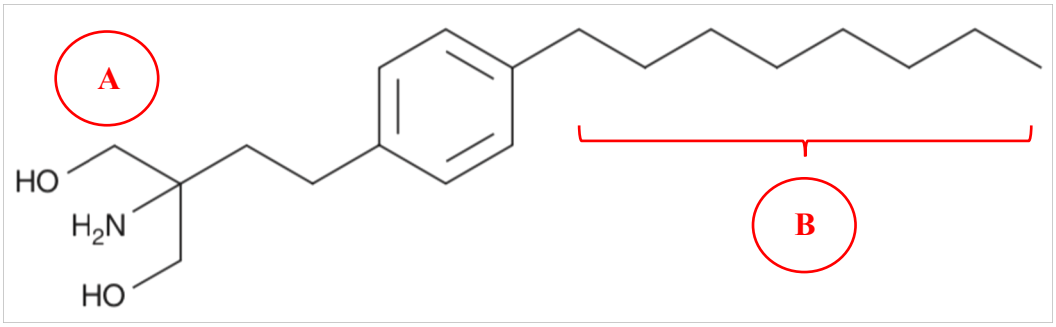

Figure 1. Structure of Fingolimod. IUPAC name: {2-amino-2-[2-(4 octylphenyl)ethyl]- 1,3-propanediol} Consists of a polar head (A) and lipophilic tail (B) demonstrating high lipophilicity, thereby rapid penetration across the blood-brain barrier. In the CNS, this precursor gets phosphorylated in the presence to S1K to fingolimod phosphate. Phosphorylated fingolimod acts as a ligand on S1P receptors to mediate downstream signaling pathways. Abbreviations: S1K: Sphingosine-1-kinase, S1P: Sphingosine-1-phosphate.

Mechanism of action: Fingolimod's endogenous structural analogue, sphingosine, is a metabolite component of a cell membrane protein called sphingomyelin. Sphingosine and fingolimod alike are substrates for sphingosine kinases that phosphorylate them into their active forms: sphingosine-1-phosphate and fingolimod phosphate, respectively. Once activated, fingolimod phosphate serves as a direct agonist of S1P receptor subtypes $S1P_1$, $S1P_3$, $S1P_4$, $S1P_5$. [74] This ligand-receptor interaction causes the modulation of cellular T-cell trafficking and prevents the lymphocytes from being infiltrated into the brain from the periphery due to MS-initiating trigger(s). This is of crucial significance because it inhibits the consequent activation of various infiltrated T-cell driven immunological mechanisms that result in a pro-inflammatory and neurodegenerative state in the CNS, as demonstrated in progressive MS pathology. Therefore, fingolimod functions as an immunomodulatory or immunosuppressive agent and is suitably beneficial in decelerating disease advancement and ultimate disability.

1.7. Evidence of Neuroprotection with Fingolimod

In the last decade or so, there has been a surge of new data with respect to the possibility of neuroprotective effects of fingolimod, in addition to its established immunomodulation. Fingolimod has been shown to prevent neurodegenerative mechanisms targeting an inflammatory CNS state in *in vitro, in vivo,* and clinical settings, some examples of which have been highlighted below.

Studies on Parkinson Disease models have shown a positive impact with fingolimod. One study demonstrated a reduction in motor deficit functions and loss of dopaminergic neurons in the

substantia nigral region, along with reversal of inflammation in the presence of fingolimod. [77] Similar results were replicated in a different study *in vitro* using SN4741 dopaminergic cell line which showed fingolimod attenuating mitochondrial damage-induced neuronal loss. [78] Mechanistically, it was found that the protective effects of fingolimod in PD were correlated with the activation of survival pathway mediated by AKT/ERK1/2 and increased expression of a neuron-specific brain-derived neurotrophic factor (BDNF). [79] Also, another study showed that long-term treatment with oral fingolimod reduced the formation of α-synuclein aggregates (a toxic form of the normally occurring protein in neural tissues) and increased levels of BDNF in transgenic mice overexpressing mutant human α-synuclein. [80] In a model of Alzheimer's, fingolimod was able to reverse the effect of damage by modulating the levels of different markers such as GFAP (astrogliosis marker), taurine (anti-inflammatory marker) and neuronal markers N-Acetyl aspartate and glutamate. [81] A meta-analysis was conducted by Liu et al (2013) which included 9 studies that focused on quantification of infarct volume and neurological deficit scoring in a model of transient middle cerebral artery occlusion (MCAO) of ischemic stroke challenged with fingolimod. The study concluded that fingolimod could be a possible candidate for stroke due to its protective effects on neurological deficit and infarct volume in eight of the nine included studies. [82] When germinal matrix hemorrhage was induced in neonatal rat pups, long-term cerebral inflammation and behavioral deficits were significantly improved with fingolimod-stimulated activation of S1PR/AKT/Rac1 signaling pathway. Maintenance of blood-brain barrier integrity and brain edema formation were also associated with fingolimod protection. [83] To assess the effect of fingolimod on short and long-term axonal dysfunction, the cuprizone-induced demyelination model was employed. This study found that although myelin recovery after acute cuprizone exposure was not affected, fingolimod could significantly enhance chronic

remyelination. Accumulation of amyloid precursor protein-positive (APP+) spheroids (an indication of neurodegenerative pathology) was reduced in the presence of fingolimod treatment, demonstrating a favorable impact on axonal degeneration. The authors of this study believe that fingolimod can help maintain CNS integrity by directly communicating with resident cells in the CNS and think that further studies are required to better define these interactions. [84] Fingolimod promoted neurogenesis and oligodendrogenesis by demonstrating specific differentiation and growth of NSCs in culture. Additionally, it enhanced the production of neuroblasts and OGDs post-induction of excitotoxic brain injury. [86] From the perspective of visual impairment, fingolimod-mediated protection of retinal ganglion cell loss and reduction in structural and functional deficits of the inner retina were portrayed in experimental glaucoma. [85] The RAGE (receptor for advanced glycation end products) axis is well-studied and linked with inflammatory and neurodegenerative diseases. A clinical study involving 17 patients of relapsing-remitting type of MS assessed their serum expression of isoforms of RAGE and its ligands. The study found increased serum levels of sRAGE (soluble RAGE) and esRAGE (endogenous secretory RAGE) in patients after 12 months of treatment with fingolimod. It also showed that there was a reduced expression of high mobility group box 1(HMGB1) and pentosidine, both of which are non-AGE ligands of the receptor. These findings suggested to the authors that fingolimod can mitigate neurodegeneration and inflammation via the RAGE axis, whose dysfunction is pronounced not only in MS, but also other neuroinflammatory conditions. [87]

Promising outcomes with fingolimod represent the need for further investigation in order to confirm the theories on its action as a neuroprotective agent. In the present study, we will

investigate the neuroprotective properties of Fingolimod and the associated molecular mechanisms.

2. Objectives and Central Hypothesis

Vision impairment due to optic neuritis is one of the most common presenting symptoms of multiple sclerosis (MS), a neuroinflammatory CNS disorder characterized by demyelination. Current medications available for MS solely target the inflammatory phase but not the neurodegenerative phase. Our **rationale** behind the study is the need for identifying agents capable of targeting both the neurodegenerative and inflammatory stages of the disease in reducing ultimate MS disability. Fingolimod, a Sphingosine-1-Phosphate receptor agonist is currently approved for MS therapy as an immunomodulating agent. It functions by regulating lymphocyte infiltration into the CNS and thus suppressing the inflammatory cascade. Studies conducted in experimental models of Alzheimer's, Parkinsons Disease, and stroke demonstrated neuroprotective actions of fingolimod. The **objective** of this study is to assess the potential neuroprotective properties of fingolimod and to explore its underlying mechanisms in an in vitro model of MS-induced optic neuritis. Our **central hypothesis** (Fig 2) is that fingolimod treatment reduces MS-induced neuronal damage in the experimental model of optic neuritis.

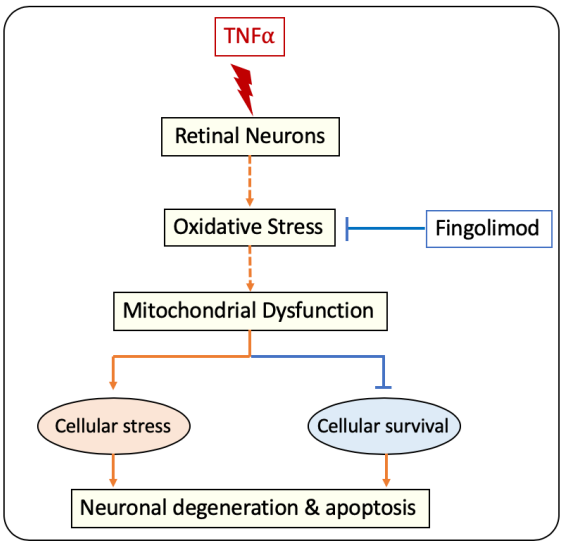

Fig 2. Hypothesis: Fingolimod inhibits TNFα-induced neuronal damage by the regulation of cellular stress and survival signaling.

3. Study Design

An *in vitro* model was standardized in our laboratory to address the goals of our study. The "R28" rat neuroretinal cell line (Kerafast) maintained in low-glucose DMEM medium and differentiated to neuronal phenotype with 25 µg/mL laminin (Sigma 11243217001) and 250 mM modified cyclic adenosine monophosphate (pCPT-cAMP) (Sigma C3912) treatments. TNF-α was used as the insult to induce cell injury and degeneration and our study design (Figure 3) resulted in four groups as shown below:

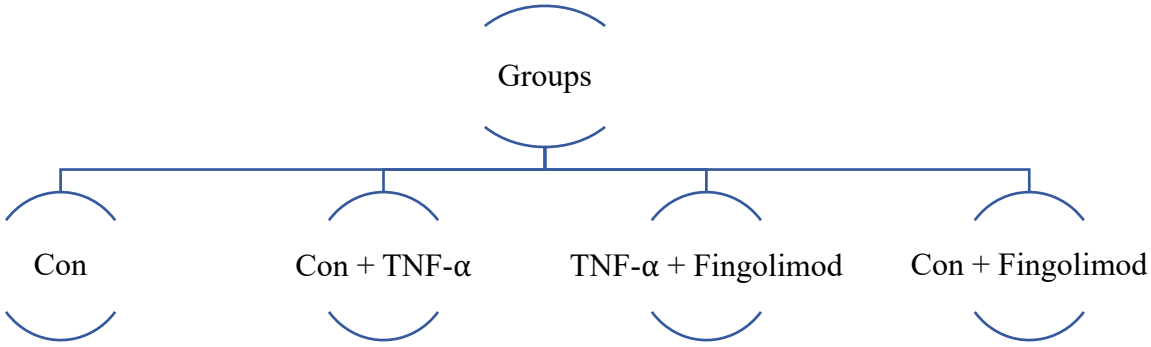

Figure 3. Study design showing control and experimental groups

Compostion of culture medium (500 mL)

- 420 mL DMEM low-glucode medium (Hyclone SH30021.01)
- 15 mL Sodium bicarbonate (7.5% stock solution, w/v) (Sigma S8761)
- 50 mL Fetal calf serum (Hyclone SH30073.02)
- 5 mL MEM non-essential amino acids (GIBCO 11140-050)
- 5 mL MEM vitamins (GIBCO 11120-052)
- 5 mL L-glutamine (200 mM stock) (GIBCO 25030-081)
- 0.625 mL Gentamicin (80 mg/mL stock) (GIBCO 15750-060)

4. Specific Aims

Aim 1: To determine the neuroprotective effect of Fingolimod in retinal neurons *in vitro*.

Utilizing the *in vitro* experimental model, the impact of Fingolimod on changes in neuronal damage, cell survival, and stress signaling molecules were investigated. Immunofluorescence studies were performed to assess neurodegenerative and/or cell survival changes, and Western blot analysis were employed to study signaling pathways involved.

Aim 2: To investigate the molecular mechanisms of Fingolimod-mediated neuroprotection.

Studies were performed to investigate the molecular mechanisms regulating Fingolimod-mediated neuroprotection. Effect of Fingolimod on oxidative stress and mitochondrial superoxide formation, in response to the injury were assessed.

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CHAPTER 2

NEUROPROTECTIVE PROPERTIES OF FINGOLIMOD IN AN EXPERIMENTAL MODEL OF MULTIPLE SCLEROSIS-INDUCED OPTIC NEURITIS[1]

[1] ***Candadai, Amritha A. et al.,*** **Manuscript to be submitted to the *Journal of Pharmacology*.**

Abstract

Purpose: Vision impairment associated with optic neuritis is prevalent in approximately 20% of patients with multiple sclerosis (MS). All approved disease modifying therapies (DMTs) available for MS function by suppressing inflammation and have unknown impact on the long-term neurodegenerative phase. Any agent with additional neuroprotective properties may offer advantages over existing therapies in reducing ultimate MS disability. The current study was undertaken to determine the neuroprotective potential of fingolimod (FTY), a Sphingosine-1-Phosphate receptor agonist currently approved as an immunomodulator in MS therapy. ***Methods:*** Utilizing the rat retinal neuronal cell (R28) line, an *in vitro* model to assess MS-mediated neurodegeneration was established. Neuronal damage was induced by treating with TNFα at 10 ng/mL. Cell viability was quantified using Trypan blue method. Changes in signaling pathways were elucidated using Western blot and differences in neuronal morphology were assessed by immunofluorescence. ***Results and Conclusion****:* Treatment of R28 cells with TNFα caused significant cell death, while FTY treatment increased the cell survival. The upregulation observed in phospho-p38 MAPK in response to TNFα treatment was reduced in the presence of Fingolimod. Further, the levels of cell survival markers (phospho-Akt and Bcl-xL) were decreased, while the expression of cleaved caspase-3 (a cell death marker) was increased in TNFα-treated R28 cells. These changes were reversed in response to Fingolimod treatment. Immunofluorescence studies demonstrated that Fingolimod treatment protected the retinal neurons against the TNFα-induced neurodegenerative changes. ***Translational Impact:*** Fingolimod's prior FDA approval for immunomodulation in MS is an advantage over other the therapeutic strategies for

neuroprotection. Establishing its additional neuroprotective properties is promising for outcomes in MS as well as other neurodegenerative diseases.

INTRODUCTION

Multiple sclerosis is a demyelinating, neurodegenerative, autoimmune disease of the central nervous system (CNS), prevalent in about 400,000 people in the US and 2.1 million people worldwide. [1,2,3] At present, the main cause for the CNS disease has not been detected, however, MS research indicates a combination of genetic factors simultaneous with environmental triggers. [4,5] MS patients experience a wide range of symptoms that have a detrimental impact on their sensory and motor functions. Approximately 20% of MS patients present with symptoms of vision deficits associated with optic neuritis. [6,7] Parameters of visual function are utilized as important outcome measures in MS studies.[8] Although the current MS therapeutics are successful in suppressing the inflammatory pathology, they have a limited effect on long-term neurodegenerative phases of disease. There exists a gap in our knowledge in identifying an agent that effectively targets both aspects of the disease.

An immunomodulator approved for relapsing-remitting MS therapy in 2010, Fingolimod (FTY) is a sphingosine analogue that functions by suppressing lymphocyte infiltration into the CNS. [9-11] Once phosphorylated into its active form by sphingosine-1-kinase, it acts as an agonist on sphingosine-1-phosphate receptors.[12-15] Besides its success as an immunomodulator, FTY exhibits neuroprotective properties as previously evidenced in models of Alzheimer's, stroke and Parkinsons disease.[16-21] The aim of our study is to assess the neuroprotective potential of FTY in an *in vitro* model of MS-induced optic neuritis.

The R28 rat neuroretinal cell line treated with TNFα was standardized to mimic MS-mediated neuronal injury *in vitro*. Studies by Seigel et al demonstrate the activity of neuronal markers in

mRNA, protein, and functional levels in the cell line.[23,24] The expression of neuron-specific markers (MAP2, Syntaxin, NSE, Nestin) as well as neurotransmission receptors (dopamine, serotonin, acetylcholine, glycine receptors) justify the use of these cells to study CNS function.[22] Utilizing the *in vitro* experimental model of optic neuritis established in our laboratory, the current study investigated the neuroprotective properties of fingolimod. Immunofluorescence studies were performed to validate changes in neuronal morphology and Western blot studies assessed the changes in cell survival and stress signaling pathways involved, and investigate the impact of FTY in reducing TNFα-induced neuronal injury.

MATERIALS AND METHODS

Cell culture

Immortalized R28 retinal precursor cells (heterogenous population of cells derived from the parent cell line E1A-NR.3), purchased from Kerafast® (Kerafast, Inc., Bostan, MA), were used. The cells were maintained in low-glucose DMEM medium (Hyclone SH30021.01) supplemented with 10% fetal calf serum (Hyclone SH30073.02), 0.225% Sodium bicarbonate (Sigma S8761), 1X MEM non-essential amino acids (GIBCO 11140-050), 1X MEM vitamins (GIBCO 11120-052), 0.5 mM l-glutamine (GIBCO 25030-081), 50 µg gentamicin (GIBCO 15750-060). The cells were differentiated to neuronal phenotype with the help of 25 µg/mL laminin (Sigma 11243217001) and 250 mM modified cyclic adenosine monophosphate (pCPT-cAMP) (Sigma C3912) treatments.

Experimental model of optic neuritis

Dose-response experiments were conducted to standardize the *in vitro* treatment of TNFα (recombinant rat Tumor Necrosis Factor α) (R&D Systems 510-RT) to induce neuronal injury in

R28 cells. On day 0, cells were grown on 6-well cuture plates until about 60-70% confluency (24 hours). Treatments with TNFα at doses of 5, 10, 25, 50 ng/mL were initiated on day 1, followed by a 24 hour incubation period. Next, cell viability with different doses of TNFα was compared against a control group with no treatment that depicted normal growth and differentiation.

Treatment with Fingolimod

Once the effective dose of TNFα was established, experiments were set up to identify an appropriate treatment concentration of Fingolimod (Cayman Chemicals 11975) in R28 cells. Upon reaching the desired confluency of 60-70%, cells were pre-treated with fingolimod at concentrations of 2.5, 5, 10, 25, 50, and 100 nM for a 1 hour incubation period prior to treatment with TNFα at its selected dose as described previously. Cell viability differences were compared among a control group (having no treatment), a TNFα-treated group, and group co-incubated with TNFα and varying FTY concentrations. FTY treatment alone at higher doses of 100 nM, 200 nM, and 500 nM were implemented to test its cytotoxicity.

Cell viability

The degree of viability of R28 cells post-treatment with TNFα and FTY was determined by using the Trypan blue method. On day 2 of the experimental setup, cells were trypsinized and collected in labelled tubes respective to their grouping. Equal volumes of sample of cell suspension and trypan blue dye were thoroughly mixed using a micropipette, from which 10 μL was injected into a cell counting chamber (Fischer Scientific 02-671-55A) for manual counting. Trypan blue dye stains dead cells blue and the number of viable cells in all four 16-squared tiles of the chamber were counted. This was repeated in triplicates for each cell suspension sample and cell viability

was plotted as percentage with respect to 100% control. All graphs are represented as Mean ± SEM.

Western blot analysis

Cells were homogenized and the lysate was collected in RIPA buffer (EMD Millipore 20-188) containing protease (ThermoFisher Scientific 78430) and phosphatase inhibitors (ThermoFisher Scientific 78428). Protein estimation was performed using Biorad protein assay kit. Samples with equal amount of protein were prepared by using 4X Laemmli buffer (BioRad 161-0747) containing β-mercaptoethanol (Fisher Scientific O3446I-100). Samples were separated on SDS-PAGE and transferred to nitrocellulose membranes (BioRad 1706404). Membranes were blocked in 5% milk (BioRad 1706404) in tris-buffered saline with tween-20 (TBS-T) and incubated with respective primary antibodies (Table 1) overnight at 4ºC. Membranes were washed with 1X PBS and incubated in appropriate secondary antibodies (Table 1). Signals were detected using enhanced chemiluminescence (ECL) solution (ThermoFisher 32106). Image J software was utilized to conduct densitometric analysis and the intensity measurements were normalized to loading control. The expression levels of proteins normalized with loading control were plotted with respect to 100% control.

Chamber slide preparation

R28 cells were grown on 8-well glass slides with removable chamber (Thermofischer 154941) at a density of 15,000 to 20,000 cells per well. Post-24 hour incubation, cells were treated using the same study design described previously, resulting in four groups based on treatments: Control (no treatment), TNFα group, TNFα + FTY group, Control + FTY group. This was followed by another

24 hour incubation period, after which the culture media was removed and cells were washed with 1X PBS. Then, cells were fixed with 2% paraformaldehyde for 10 mins, followed by wash with PBS and stored in humidified containers at 4ºC.

Immunoflourescence staining

Chamber slides were brought to room temperature and washed with PBS before initiating the staining protocol. Permeabilization was achieved using 0.1% Triton X-100 in PBS for 5 mins, followed by a PBS wash and blocking with 10% donkey serum at room temperature for 1 hour. After, wells were washed and incubated with respective primary antibodies (Table 2) overnight. The following morning, the wells were incubated with appropriate secondary antibodies (Table 2) for 2 hours. Wells were washed, dried, and the chambers were separated from the glass slide using a removal apparatus provided in the kit. Cells were covered with cover slip using mounting medium containing DAPI stain and stored in 4ºC.

RESULTS

I. Neuronal injury induced by TNFα

Percentage of cell viability using the Trypan blue method was assessed for dose-response studies of TNFα at doses of 0, 5, 10, 25, and 50 ng/mL. We found that TNFα showed a significant reduction in cell viability at doses 10 ng/mL (44.6 ± 24.8 %), 25 ng/mL (39.8 ± 8.7 %), and 50 ng/mL (60.2 ± 13.0 %) ($p<0.05$ vs. Control) (Fig 4). Our findings suggested that TNFα at 10 ng/mL desirably reduced the percentage of viable cells by nearly half that of the control group.

II. Fingolimod reduces TNFα-induced neuronal injury

Cells were pre-treated with FTY at concentrations 0, 2.5, 5, 10, 25, 50, and 100 nM for 1 hour prior to TNFα induction (10 ng/mL). Our results showed that FTY concentrations at 25 nM (79.7 ± 17.7%) and 50 nM (71.0 ± 32.3 %) significantly reversed TNFα-induced injury to near control levels ($p<0.001$) (Fig 5A). Experiments with high dose FTY treatment alone suggested its cytotoxicity at doses above 100 nM. Doses of 100 nM, 200 nM, and 500 nM showed a decreasing viable cell count of 14.5×10^4, 3.5×10^4, and 2×10^4, respectively compared to an average viable cell count of 47.5×10^4 in control cells without FTY treatment. Based on our findings, 25 nM was chosen as the dose of FTY to be used in further studies. (Fig 5B)

III. Fingolimod attenuates cellular stress and survival signaling.

Changes in phosphorylated p38 MAP kinase expression were assessed to characterize cellular stress by Western blotting. Fig 6A shows that TNFα augmented the expression of p-p38 MAPK and this increased expression was remarkedly controlled in the presence of FTY. Moreover, we found that FTY treatment alone had no effect on levels of p-p38 MAPK. Our quantification demonstrated the increased levels of p-p38/total p38 with TNFα at $p<0.05$ versus control group (Fig 6B). In the presence of FTY, levels of p-p38/total p38 were significantly reduced at $p<0.05$ versus the TNFα group (Fig 5B). Also, FTY treatment alone showed no significant changes in p-p38 MAPK expression when compared to control.

Changes in phosphorylated AKT levels were tested in order to assess cell survival using Western analysis. Fig 6C shows a decreased expression of p-AKT with TNFα induction. Consistently, our quantification data showed a significant decrease in levels of p-AKT/t-AKT in the presence of

TNFα at $p<0.05$ versus control (Fig 6D). However, we did not see a significant change in p-AKT/t-AKT levels upon TNFα and FTY co-treatment.

IV. Effect of Fingolimod on TNFα-induced neurodegeneration

Immunofluorescence studies were conducted to study changes observed in neuronal morphology of R28 cells among the different treatment groups. Immunoflurorescence staining with neuronal nuclei marker (NeuN) (red) overlayed with Dapi staining (blue) revealed neuronal loss induced by TNFα, which was attenuated with FTY treatment (Fig 7A – 7D). Secondly, we characterized the expression of neurofilament marker Tuj1 in R28 cells. Fig 7E – 7H show Tuj1 staining (green) overlayed with Dapi nuclear staining (blue). Tuj1 expression was downregulated due to axonal injury in TNFα-treated cells, however, we observed that FTY was able to protect the cells against neurofilament damage. Consistently, neuron-specific enolase (NSE) marker (green) was found to be less expressed in the presence of TNFα, and the damage to neurons was prevented by FTY treatment (Fig 7I – 7L).

V. Effect of Fingolimod on neuronal cell death

In order to evaluate apoptotic changes, Western analyses using apoptotic marker cleaved caspase-3 (CC-3) and anti-apoptotic marker Bcl-xL were conducted. We observed an upregulated expression on CC-3 along with a corresponding downregulated expression of Bcl-xL in the presence of TNFα alone. In the co-treatment group with TNFα and FTY, we observed a reversal in changes with respect to CC-3 and Bcl-xL (Fig 8A). Our quantification data showed an increase in the levels of CC-3/B-Actin significantly at $p<0.005$ versus control, and FTY significantly reversed this effect at $p<0.05$ versus TNFα group (Fig 8B). With respect to Bcl-xL protein levels,

TNFα caused a significant decrease in levels of Bcl-xL/B-Actin at $p<0.05$ versus control. However, we did not find a significant difference in the TNFα+FTY co-treatment group compared to the TNFα group (Fig 8B).

Immunofluorescence staining with CC-3 was consistent with our findings with protein levels, demonstrating an increased CC-3 expression (red) in the TNFα group compared to control, followed by its suppressed expression in the presence of TNFα and FTY (Fig 8D – 8G).

DISCUSSION

The present study was conducted to assess the potential neuroprotective action of fingolimod in an experimental model of optic neuritis. Utilizing the R28 neuroretinal cell line, an *in vitro* experimental model of neurodegeneration was standardized to assess the impact of fingolimod on TNFα-induced neuronal damage. Utilizing cellular and molecular approaches, our study demonstrates neuroprotective properties of fingolimod in MS-induced optic neuritis.

Lack of effective treatment strategies to reduce neurodegeneration continue to be a major problem in the field of MS research. It is vital to understand the underlying mechanisms of MS-induced neuronal damage and dysfunction. Even though research on the pathophysiology of MS and associated molecular mechanisms have evolved over decades of research, the field lacks an *in vitro* model to study the neurodegeneration. Synthetic molecules such as trimethyltin[25], oxaliplatin[26], and cuprizone[27], although successful in creating a neurodegenerative environment, do not accurately represent the neuroinflammatory changes observed in an MS brain. In MS, inflammatory leukocytes are believed to infiltrate the CNS to mediate demyelination and neuronal degeneration via cytokines upon activation of T lymphocytes and antigen-presenting cells

(APCs).[28] Tumor necrosis factor α (TNFα) is one of the primary cytokines that is present in elevated levels in active MS lesions, serum and cerebrospinal fluid of MS patients.[29] Studies conducted on BV-2 microglial cell line [30] and primary mixed neuronal and glial cultures [31] show the effect of TNFα-induced damage and apoptosis. Our study demonstrated the effect of TNFα-induced neuronal apoptosis in R28 neuroretinal cells. The R28 cell line is an immortalized, rat retinal origin, heterogenous, precursor cell line with differentiation potential. R28 cells were differentiated to neuronal phenotype with addition of modified form of cAMP and laminin and grown using DMEM. Studies by Seigel et al demonstrate the expression of neuron-specific markers such as MAP2, Syntaxin, NSE, and Nestin along with neurotransmission receptors such as dopamine and serotonin, to name a few.[46,47] Our study further characterized the expression of neurofilament marker Tuj1, along with NSE and NeuN in these cells.

Axonal damage is one of the hallmark events that drives progressive neuronal degeneration in MS. Typical axon loss is constant throughout the MS pathogenesis from initiation of myelin-specific auto-immune attack to inflammation and degenerative mechanisms, making it crucial to the consequences of neurodegeneration. In MS, one of the major clinical presentations observed in patients is optic neuritis. Studies have shown that approximately 20% of patients present with inflammation of the optic nerve as their first symptom of MS. [32,33] Another study conducted by the North American Research Committee on Multiple Sclerosis (NARCOMS) showed that of the 9107 patients participating in the study, 60% reported signs of vision impairment, and 14% of these depicted moderate/severe/very severe impairment of vision. [34] Based on the available research, visual function is significantly correlated to MS disease progression.

Current MS therapies function by suppressing the inflammatory pathways of disease and have little impact on long-term neuronal damage, causing a major gap in knowledge and emphasizes the need for a neuroprotective therapeutic agent. Therefore, our study focused on assessing the neuroprotective effect of fingolimod in an *in vitro* model of MS-induced optic neuritis. Fingolimod, a sphingosine-1-phosphate (S1P) receptor modulator, has previously shown to prevent neurodegenerative mechanisms targeting an inflammatory CNS state in *in vitro, in vivo,* and clinical setting, as detailed below. Studies on Parkinson Disease models have shown a positive impact with fingolimod. [35-37] One study demonstrated a reduction in motor deficit functions and loss of dopaminergic neurons in the substantia nigral region, along with reversal of inflammation in the presence of fingolimod. [35] Similar results were replicated in a different study *in vitro* using SN4741 dopaminergic cell line which showed fingolimod attenuating mitochondrial damage-induced neuronal loss. [36] Mechanistically, it was found that the protective effects of fingolimod in PD were correlated with the activation of survival pathway mediated by AKT/ERK1/2 and increased expression of a neuron-specific brain-derived neurotrophic factor (BDNF). [37] Also, another study showed that long-term treatment with oral fingolimod reduced the formation of α-synuclein aggregates (a toxic form of the normally occurring protein in neural tissues) and increased levels of BDNF in transgenic mice overexpressing mutant human α-synuclein. [38] In a model of Alzheimer's, fingolimod was able to reverse the effect of damage by modulating the levels of different markers such as GFAP (astrogliosis marker), taurine (anti-inflammatory marker) and neuronal markers N-Acetyl aspartate and glutamate. [39] A meta-analysis was conducted by Liu et al (2013) which included 9 studies that focused on quantification of infarct volume and neurological deficit scoring in a model of transient middle cerebral artery occlusion (MCAO) of ischemic stroke challenged with fingolimod. The study concluded that fingolimod could be a

possible candidate for stroke due to its protective effects on neurological deficit and infarct volume in eight of the nine included studies. [40] Promising outcomes with fingolimod represent the need for further investigation in order to confirm the theories on its action as a neuroprotective agent.

Mitochondrial dysfunction and associated oxidative stress-induced neurodegeneration play a vital role in MS pathogenesis. The neuroinflammation-driven MS pathology causes a disruption in neuroaxonal homeostasis, marked by elevated oxidative stress, increase in reactive oxygen species, and consequent damage to mitochondria. [41,42] This leads to a metabolic stress environment mediated by mitochondrial DNA damage (abnormal mitochondrial gene expression), impaired energy production and homeostasis, increase in free radicals, and damage to cellular structure. [43] Studies have also shown the presence of oxidized lipids in myelin membranes, dying oligodendrocytes, axons and their association with neurodegeneration and lesion formation. [44,45]

In response to fingolimod treatment, our studies found a reduction in the phosphorylation of p38 MAP kinase (a cellular stress signaling pathway) in TNFα-treated R28 cells. Further, fingolimod upregulated the levels of phosphorylated Akt, indicating its impact on the survival signaling. TNFα-induced cell death was confirmed by the upregulation of cleaved caspase-3 (a cell death marker) expression along with reduced levels of Bcl-xL (an anti-apoptotic protein). These changes were observed to be reversed in response to fingolimod treatment, supporting its neuroprotective function. Fingolimod treatment also protected the retinal neurons against the TNFα-induced neurodegeneration studied by the expression of neuronal markers and changes in their morphology. One limitation of our study is that it did not elucidate the role of cell survival/stress signaling pathways that are directly associated with TNFα and fingolimod action. The

concentration of Fingolimod used in our study (25 nM) corresponds to 7.6875 ng/mL. However, the concentration of active fingolimod phosphate in adult MS patients is 1.35 ng/mL.[48] This difference in human systemic concentration versus *in vitro* concentration in our models is an interesting aspect of the study and suggest that repurporsing may be needed for the neuroprotective action of Finglimod in MS patients. However, further studies are required to confirm the results.

Overall, our study investigated the potential neuroprotective effects of fingolimod in an *in vitro* setting of neurodegeneration. The R28 neuroretinal cell line was characterized as a successful platform for evaluating neuronal properties, neuronal damage in the presence of TNFα and its suppression with fingolimod. Based on the cellular and molecular analysis, fingolimod demonstrated a potential to be investigated as a novel neuroprotective strategy in conditions like MS. Our future studies will elucidate mechanistic pathways driven by oxidative stress and mitochondrial dysfunction.

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Conflict of Interest

Authors declare that there are no financial or any other conflicts of interest.

Figure Legends

Fig 4. Neuronal injury induced by TNFα. Dose-response effect of TNFα treatment on R28 cells assessed by Trypan blue staining method. Neuronal damage was induced by treating R28 cells with TNFα for 24h at doses 0, 5, 10, 25, and 50 ng/mL. Quantification showed that TNFα at 10 ng/mL showed a marked reduction in cell survival and was chosen as the effective dose. [N=3; *$p<0.05$ vs. dose 0] Data presented as Mean ± SEM.

Fig 5. Fingolimod reduces TNFα-induced neuronal injury. Dose-response effect of Fingolimod by Trypan blue staining method. Cells were pre-treated with Fingolimod, followed by TNFα (10 ng/mL) and cell viability was assessed at 24h. **A)** Cell viability in response to fingolimod at concentrations 25, 50, and 100 nM. [$^{\#}p<0.005$ vs. Con; *$p<0.05$ vs. TNFα]. **B)** Fingolimod at 25 nM was chosen as the optimum dose [N=3; *$p<0.001$ vs. Con; $^{\#}p<0.01$ vs TNFα]. Data presented as Mean ± SEM. **C)** Cytotoxicity with fingolimod. Fingolimod at higher concentrations of 100 nM, 200 nM, and 500 nM demonstrated decreased percentage cell count of 30.5%, 7.4%, and 4.2% respectively when compared with 100% cell count. (N=1)

Fig 6. Effect of fingolimod on cellular stress and survival signaling. Western blot analysis showing effect of fingolimod on TNFα-induced cellular stress and survival signaling. **A)** The

upregulation observed in phosphor-p38 MAPK level in response to TNFα treatment was attenuated in the presence of findolimod. **B)** Quantification of Western analysis showed increased level of phospho-p38 in the presence of TNFα and a significant reduction in response to fingolimod treatment [N=6; *p<0.05 vs Con, #p<0.05 vs. TNFα]. **C)** Reduced phospho-Akt expression in response to TNFα treatment. **D)** Western quantification demonstrated a significant decrease in phospho-Akt levels with TNFα treatment [N=4; *p<0.05 vs. Con]. Data presented as Mean ± SEM. Representative images are presented.

Fig 7. Fingolimod treatment reduced TNFα-induced neurodegeneration. Immunofluorescence staining of R28 cells following treatment with TNFα and fingolimod. Immunofluorescence staining using neuron-specific nuclei marker NeuN (A-D), neuron-specific beta-tubulin class III staining with Tuj1 (E-H), and neuron-specific enolase staining with NSE (I-L). Fingolimod depicts protection against neurodegeneration. [DAPI: 4',6-diamidino-2-phenylindole]. N=3 per group and representative images are presented.

Fig 8. Effect of fingolimod on TNFα-induced neuronal death. A) Expression of cleaved caspase-3 was increased, and corresponding Bcl-xL expression downregulated in response to TNFα treatment. **B)** Quantification of the change in cleaved caspase-3 expression with TNFα treatment and the effect of fingolimod. [N=3; *p<0.005 vs. Con; #p<0.05 vs. TNFα]. **C)** Quantification of Bcl-xL levels showed a significant downregulation in levels in the presence of TNFα [N=3; *p<0.05 vs. Con]. **D-G)** R28 cells immunostained using cleaved caspase-3 following treatment with TNFα and fingolimod. The increased presence of cleaved caspase-3 positive cells (arrows) in the TNFα group is reduced with fingolimod treatment.N=3 per group and

representative images are presented. [CC-3: Cleaved caspase-3; DAPI: 4',6-diamidino-2-phenylindole]

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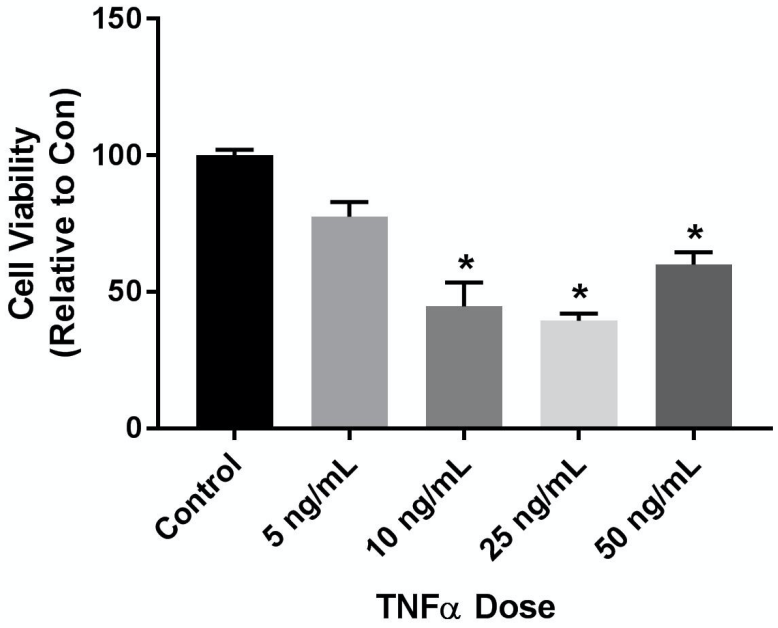
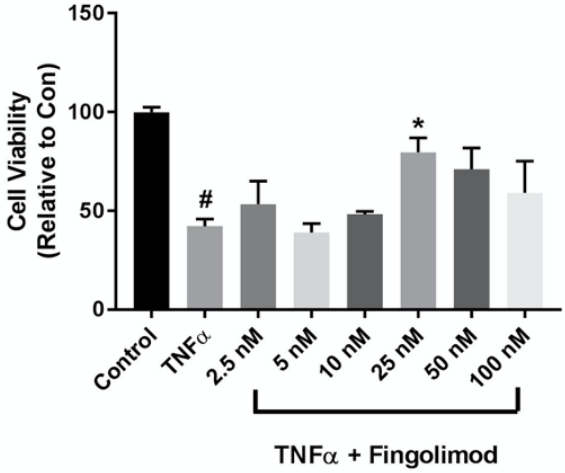
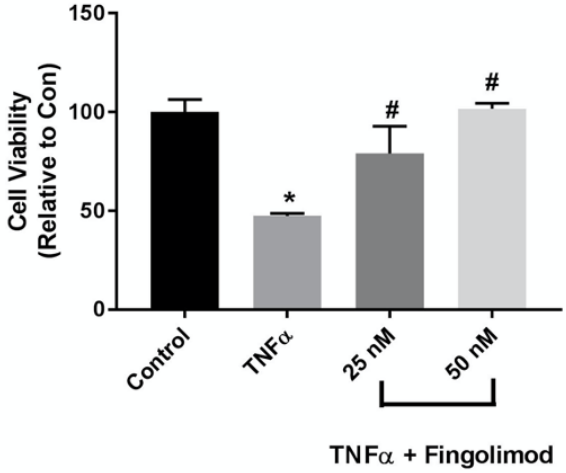


Figure 4. Neuronal injury induced by TNFα





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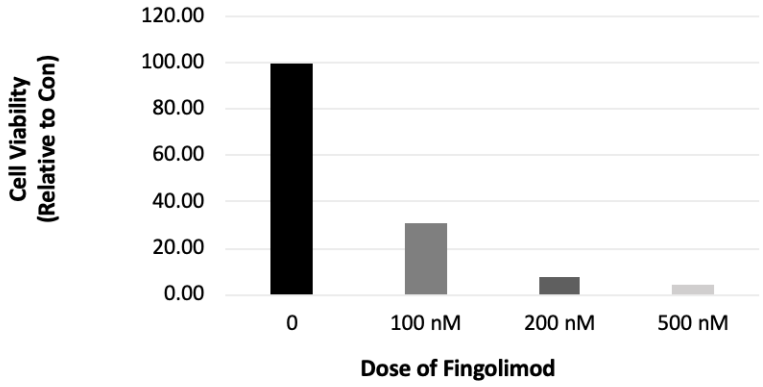

Dose	Cell Count	% Cell Count
0	475000	100
100 nM	145000	30.5
200 nM	35000	7.4
500 nM	20000	4.2

Figure 5. Fingolimod reduced TNFα-induced neuronal injury

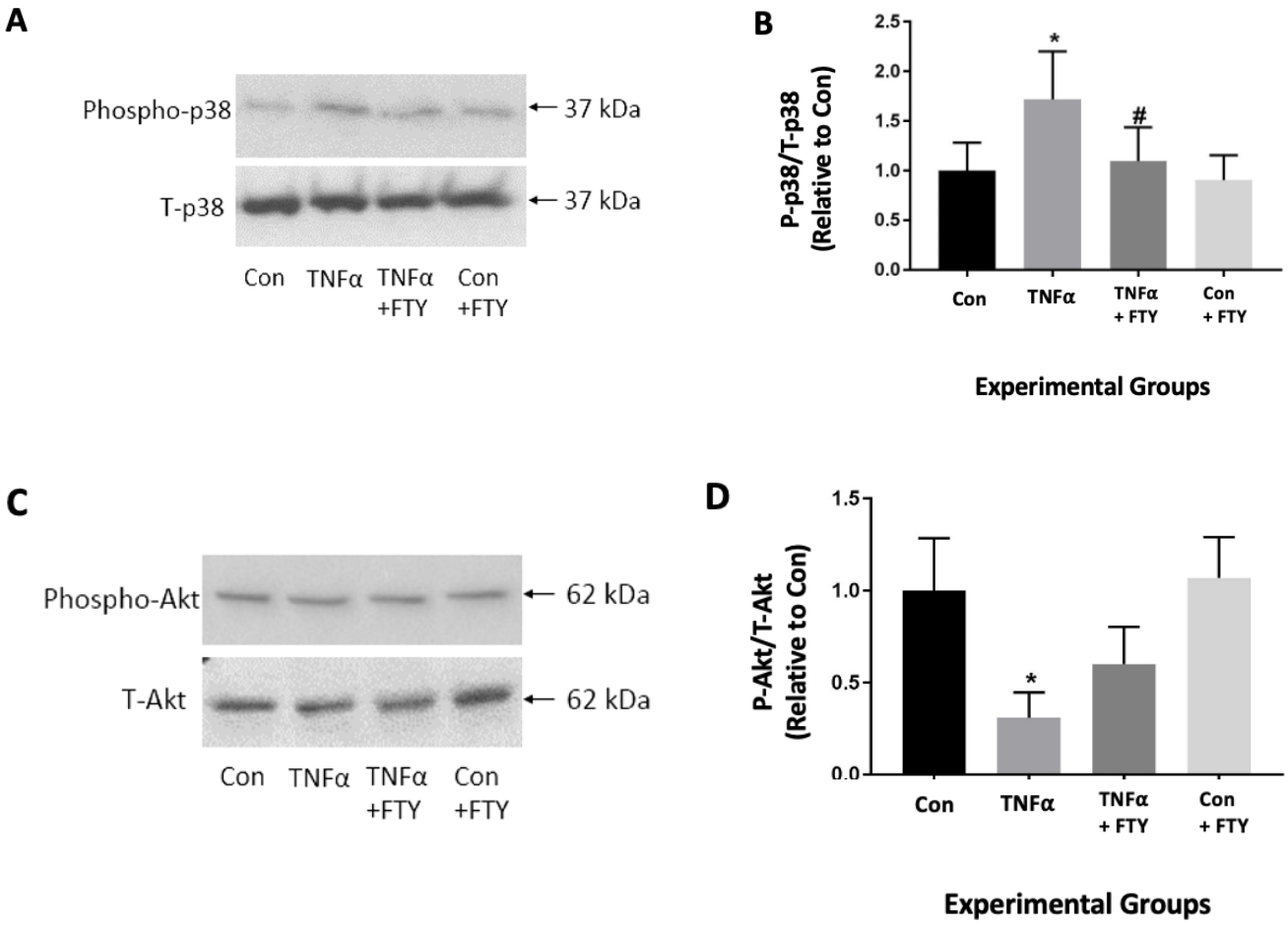

Figure 6. Effect of Fingolimod on cellular stress and survival signaling

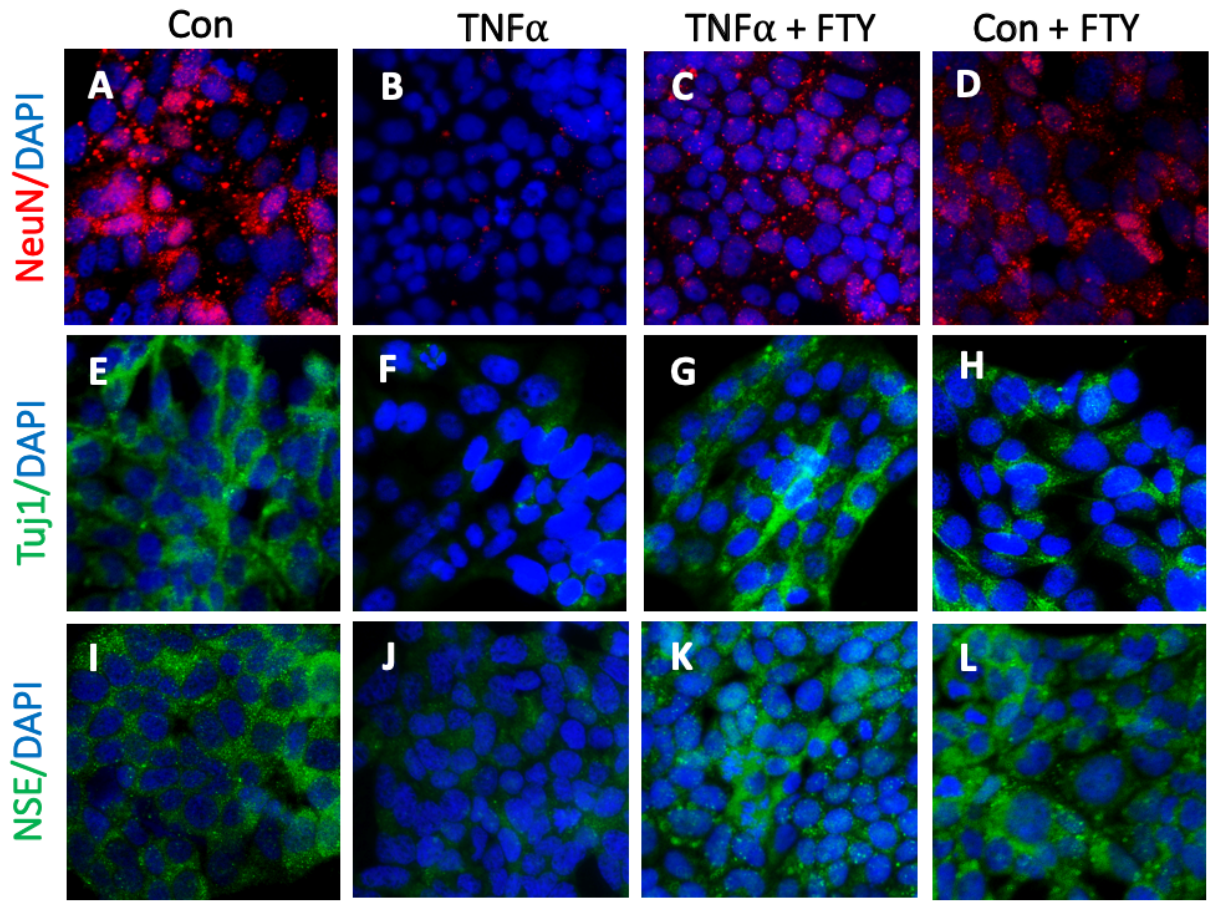

Figure 7. Fingolimod treatment reduced TNFα-induced neurodegeneration

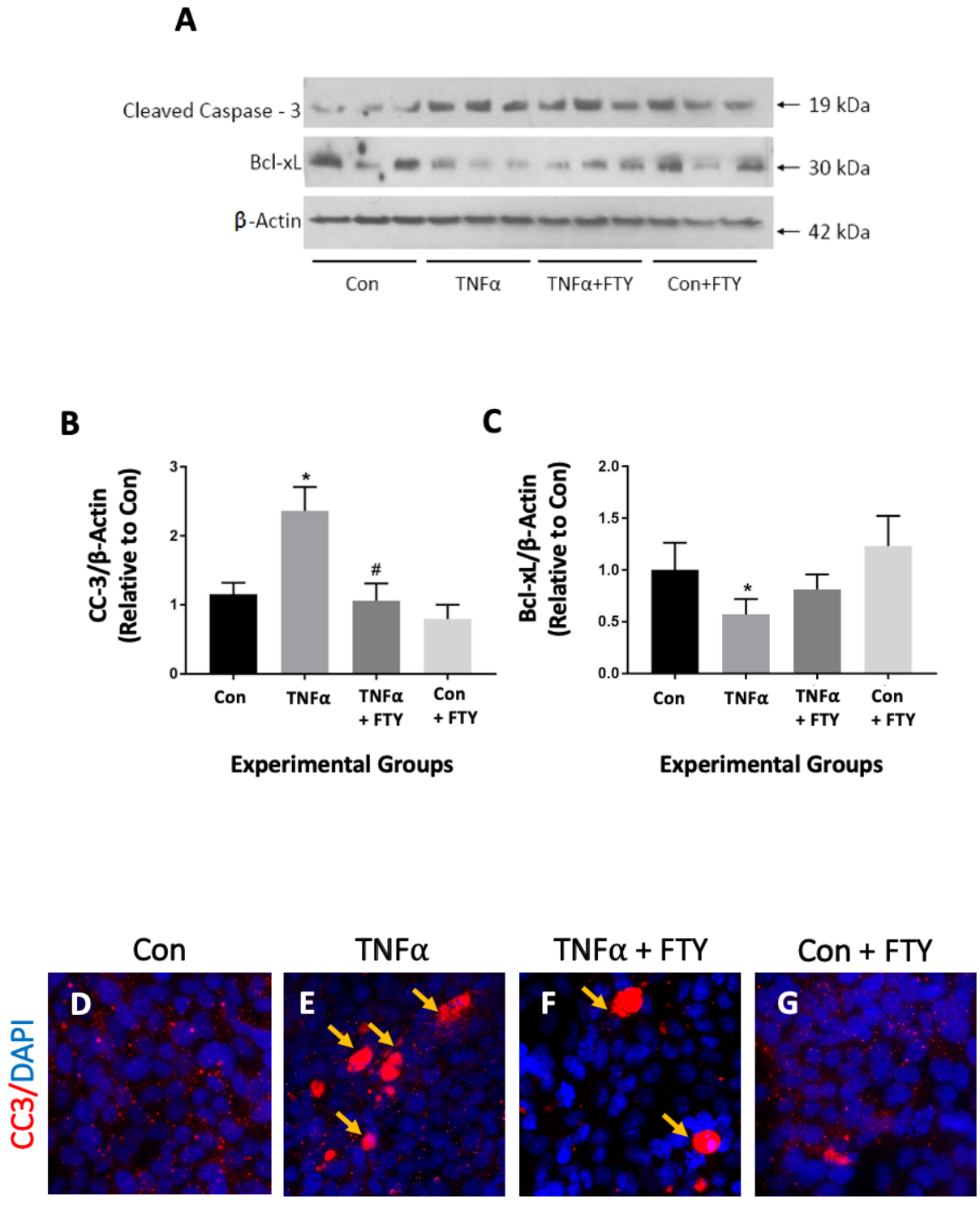

Figure 8. Effect of Fingolimod on TNFα-induced neuronal death

Table 2. Antibodies used

Antibody used	**Catalogue No.**	**Company**	**Dilution**
Phospho-P38 MAP Kinase	4511S	Cell Signaling	1:1000
Total P38 MAP Kinase	9212S	Cell Signaling	1:1000
Phospho-Akt	4060S	Cell Signaling	1:1000
Total Akt	9272S	Cell Signaling	1:1000
Bcl-xL	2764S	Cell Signaling	1:1000
Cleaved caspase - 3	9664S	Cell Signaling	1:1000
β-Actin	A1978-200UL	Sigma	1:10000
NeuN	10782-604	VWR	1:500
Tuj1	MAB1195	R&D Systems	1:500
NSE	NSE	Aves Lab	1:500
Secondary antibody used			
Goat anti-Rabbit IgG (H+L) HRP Conjugate	170-6515	Bio-Rad	1:2000
Goat anti-mouse IgG (H+L) HRP Conjugate	172-1011	Bio-Rad	1:2000