# ENTANGLEMENT OF EPSTEIN-BARR VIRUS WITH ALZHEIMER'S DISEASE

**M.SC. THESIS**

By

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**DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING**

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# ENTANGLEMENT OF EPSTEIN-BARR VIRUS WITH ALZHEIMER'S DISEASE

**A THESIS**

*Submitted in partial fulfilment of the requirements for the award of the degree*
***of***
**Master of Science**

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# INDIAN INSTITUTE OF TECHNOLOGY INDORE

## CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **ENTANGLEMENT OF EPSTEIN-BARR VIRUS WITH ALZHEIMER'S DISEASE** in the partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from September 2021 to May 2023 under the supervision of **Dr. Hem Chandra Jha**, Associate Professor, Department of Biosciences and Biomedical Engineering.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Priyanka Patra
16/05/23

**Signature of the student with date**
**(PRIYANKA PATRA)**

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This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

16/5/2023

Signature of the Supervisor

**Dr. Hem Chandra Jha**

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**PRIYANKA PATRA** has successfully given his/her M.Sc. Oral Examination held on **9th May 2023**

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# PUBLICATIONS

Manuscripts from thesis

**P. Patra**[#], A. Rani[#], N. Sharma, C. Mukharjee, H.C. Jha, "Unravelling the connection of Epstein-Barr virus and its glycoprotein $M_{146-157}$ peptide with neurological ailments," *ACS Chemical Neuroscience* ([#]Equal contribution).

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Manuscripts apart from thesis

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# WORKSHOPS ATTENDED

**Volunteered** at SERB sponsored **KARYASHALA** entitled "Isolation and characterization of viruses and bacteria from various samples", IIT Indore (12-17 July).

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# ACRONYMS

**ACA** Acute Cerebellar Ataxia

**AD** Alzheimer's Disease

**ADEM** Acute Disseminated Encephalomyelitis

**AFM** Atomic Force Microscopy

**ALS** amyotrophic lateral sclerosis

**Apo** Apolipoprotein

**APP** Amyloid precursor protein

**Aβ** Amyloid-β

**BACE-1** Beta site APP cleaving enzyme-1

**BCRF-1** BamHI C fragment rightward open reading frame 1

**BHRF-1** BamHI H fragment rightward open reading frame 1

**BL** Burkitt's lymphoma

**BRLF-1** BamHI R fragment in leftward open reading frame-1

**BZLF-1** BamHI Z fragment leftward open reading frame-1

**CNS** Central nervous system

**CSF** Cerebrospinal fluid

**CTD** C-terminal domain

**DMSO** Dimethyl sulfoxide

**EBNAs** EBV nuclear antigens

**EBV** Epstein-Barr virus

**GBS** Guillain-Barre syndrome

**gM** glycoprotein-M

**gp350** glycoprotein-350

**HHVs** Human Herpesviruses

**HSV** Herpes simplex virus

**IL** Interleukin

**IM** Infectious mononucleosis

**JNK** Jun N-terminal kinase dependent

**LCV** Lymphocryptovirus

**LMPs** latent membrane proteins

**LRP1** Low Density Lipoprotein Receptor-Related Protein 1

**MAPK** Mitogen-activated protein kinase

**MMP** Mitochondrial membrane potential

**MS** Multiple sclerosis

**NF-kB** Nuclear factor kappa-light-chain-enhancer of activated B cells

**PD** Parkinson's Disease

**PFA** Paraformaldehyde

**ROS** Reactive oxygen species

**RS** Raman spectroscopy

**Th S** Thioflavin-S

# ENTANGLEMENT OF EPSTEIN-BARR VIRUS WITH ALZHEIMER'S DISEASE

# ABSTRACT

Epstein-Barr virus (EBV) is a member of herpesviridae and is the first known oncogenic virus. The virus undergoes both latent and lytic cycles. Infrequent reactivation of the virus from the latent cycle causes a serious health condition called infectious mononucleosis (IM) or glandular fever. Patients suffering from IM are found to be at a high risk for Alzheimer's disease (AD) but the mechanism is still elusive. The present study deals with understanding the unrevealed association of EBV with AD. A 12-amino-acid containing peptide derived from EBV-glycoprotein M (gM), $_{146}$SYKHVFLSAFVY$_{157}$ accelerated the aggregation of A$\beta_{42}$. Exposing the neuronal cells to EBV and gM$_{146-157}$ was capable of initiating inflammatory response in vitro. Additionally, the exposure significantly altered the mitochondrial biology by decreasing the mitochondrial membrane potential (MMP). Besides, exposure to EBV and gM$_{146-157}$ manifested upregulation in the synthesis of amyloid precursor peptide (APP) and Apolipoprotein E (ApoE4) in the *in-vitro* system. Subsequently, intranasal administration of EBV and gM$_{146-157}$ exposed IMR-32 have significantly increased the anxiety like behaviour in female Swiss albino mice. Taken together, the study underlined the mysterious association of EBV with neuroinflammation, mitochondrial dysfunction and ultimately AD.

**Key words:**

Alzheimer's disease, Amyloid beta, Amyloid precursor protein, Apolipoprotein E, Epstein-Barr virus, Glycoprotein M

# CHAPTER 1

## Introduction

Epstein-Barr virus (EBV) also known as human herpes virus 4 (HHV4) is a ds DNA virus, member of the genus lymphocryptovirus (LCV) of the γ-herpesvirus subfamily [1]. It has been reported as a class I carcinogen since its discovery [2]. Along with its role in cancers, it is also notoriously known to be associated with several neurological anomalies like multiple sclerosis (MS), Alzheimer's disease (AD), Parkinson's disease (PD), Acute Disseminated Encephalomyelitis (ADEM), Acute Cerebellar Ataxia (ACA) to name a few [3]. The opportunistic pathogen infects the host during adolescence and generally remains asymptomatic. Being ubiquitous it is present in about 90-95% of the population [4]. Infrequent activation of this virus leads to serious health conditions like infectious mononucleosis (IM) [5]. A significant association of IM has been reported with an increased risk of AD [6]. Jha et. al. have demonstrated the infection of EBV into neuronal cells namely, Ntra2, SH-SY5Y and primary human foetal neurons [7]. Further, EBV also infects glial cells and leads to the activation of an inflammatory cascade [8]. Additionally, the elevated presence of anti-amyloid beta antibodies is found to be secreted by EBV-transformed B-cell lines isolated from AD patients [9]. An increased number of Anti-EBV IgG levels have been reported in Korean elderly suffering from amnestic mild cognitive impairment (aMCI) in comparison to the healthy individuals suggesting some role of the virus in the conversion of cognitively normal state to the condition of aMCI [10].

An *in-silico* study conducted in our lab led to the discovery of a peptide fragment with 12-amino acids ($_{146}$SYKHVFLSAFVY$_{157}$) derived from the glycoprotein M (gM) of EBV, which showed a high tendency to form amyloid-like aggregates [11]. These aggregates are composed of $A\beta_{42}$ peptides which are the cleavage products of amyloid precursor protein (APP) [12]. More than the production of Aβ peptides their clearance is a matter of serious concern. The mechanism of clearance for fibrillar Aβ

and soluble Aβ are different but are majorly mediated by Apolipoprotein E (ApoE) [13]. The brain second last producer of ApoE after the liver and ApoE is the most abundant apolipoprotein in the brain [14], [15]. The molten globule form of ApoE4 makes it the least capable for clearance among the others (AopE2 and ApoE3) [16]. Additionally, the C-terminus of ApoE3 is demonstrated to have interactions with viral proteins like EBV nuclear antigen 1 (EBNA1) and BamHI Z fragment leftward open reading frame-1 (BZLF1), these interactions are postulated to interfere with the clearance of amyloid beta, while the deeper mechanistic details are still elusive [17].

Along with the accumulation of Aβ peptides and p-tau tangles, neuroinflammation is the third core pathophysiology involved in AD pathogenesis [18]. Activation of inflammatory molecules further leads to impairment in mitochondrial biology [19]. Molecules like il-6 and tgf-β are reported to be responsible for the dysfunction of mitochondria in AD patients [20]. Increase in amyloid deposits also negatively influences mitochondrial function [21]. Apart from the above-mentioned cellular responses, AD significantly impairs the mental health of the subjects. AD patients have higher anxiety levels and treatment with anti-anxiety drugs deals the development of the disease [22], [23].

The present study focuses on defining mechanisms involved in the association of EBV with AD and plotting them together. We have thus evaluated the potential of the $gM_{146-157}$ in seeding the aggregation of $A\beta_{42}$ and their characterization through atomic force microscopy (AFM). Alterations in the mRNA expression of inflammatory molecules have been assessed post-exposure of EBV and of $gM_{146-157}$. We have then studied the alteration in the mitochondrial membrane potential (MMP) as this deeply colocalises with neurodegeneration. APP was evaluated at the protein level through western blot and immunofluorescence. Along with this the protein level of the AD risk factor ApoE4 has been evaluated. Further to validate the effect *in-vivo* female Swiss albino mice were intranasally administered with IMR-32

(neuronal) cells exposed to EBV and $gM_{146-157}$. Mice which were subjected to the exposure of EBV and $gM_{146-157}$ showed significantly increased anxiety like behaviour.

# CHAPTER 2

## Review of literature

Epstein-Barr virus (EBV) is a double stranded DNA (184 kb) virus of the family Herpesviridae (Figure 1) [24]. It was first discovered in Burkett lymphoma and was then reported as a class I carcinogen [2], [25]. Electron microscopic studies of the tumour samples by Epstein and Barr lead to the discovery of EBV [26]. EBV infects the host and stays in the latent state in 95% of the population [4]. The virus spread through intimate oral contact among adolescents or by exchange of other body fluids like, blood and genital secretions but how the pre adolescents acquire the virus is still elusive [27]. The virus is known to infect B-cells and nasopharyngeal epithelial cells [28]. The virus maintains a perfect balance the lytic and latent cycles to gain a life-long persistence inside the infected host body. Nevertheless, factors like co-infection mediated immunological stress, immunosuppression and other stimulations muddle this balance leading to the activation of virus [29]. Infrequent activation of the virus causes a severe health condition called infectious mononucleosis (IM) [30]. Reactivation of virus is also linked with lymphatic and epithelial malignancies [2], [31]. Apart from IM in long run it is known to be cause of several neurodegenerative disorders. EBV is known to be a causative agent of various neurodegenerative disorders like AD, PD, MS, EBV encephalitis, acute cerebellar ataxia and Guillain-Barre syndrome (GBS) to name a few [3].

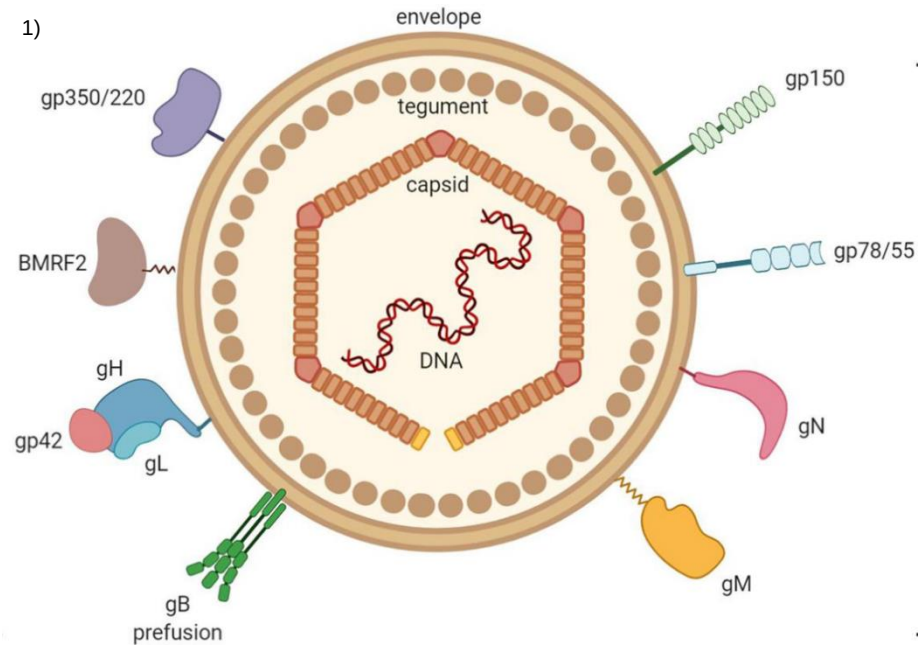



**Figure 1: Structure of Epstein-Barr virus**

**Source of the image:** Main Targets of Interest for the Development of a Prophylactic or Therapeutic Epstein-Barr Virus Vaccine [32].

Conversion of latent to lytic cycle can occur in the B-cells mediated by BZLF1 and BamHI R fragment in leftward orientation (BRLF1), which upregulate the expression of viral genes like BamHI fragment C rightward open reading frame 1 (BCRF1) and BamHI fragment H rightward open reading frame 1 (BHRF1) and decrease the expression of host factors which are crucial in inflammation [33]. These conditions lead to cell death and egress of virion particles. Viral proteins like gp350/220, BZLF1, BRLF1 promote lytic replication in infected lymphocytes circulating it to the central nervous system (CNS) [34]. The above-mentioned processes induce pathogenesis which colocalizes with neurocognitive deterioration, neuroinflammation and damage [3]. The virus employs its envelope glycoproteins gp 350/220, gp42, gHgL and gB in order to interact with host cell receptors like cluster differentiation 21 (CD21), human leukocyte antigen (HLA)-DR, and integrins to name a few [35]. Jha et. al. demonstrated the infection of EBV into SH-SY5Y, Ntra2, primary human foetal neurons and primary neurons [7]. Infection of EBV into glial cells illustrated activation of inflammation cascade[8].

Alzheimer's disease is named after its discoverer Dr Alois Alzheimer, who first characterized the disease based on the study of a patient named Auguste Deter. He described the illness as progressive and irreparable damage inflicted on the neurons, leading to cognitive impairment [36]. The pathological markers of the disease are, presence of amyloid beta (Aβ) plaques outside the neurons and presence of sticky phosphorylated tau tangles outside the neurons [37]. The Aβ plaques are composed of Aβ peptides of 37 to 49 residues with $A\beta_{42}$ being the most prevalent [38]. Sequential cleavage of the APP by α-/β- secretase and γ-secretase leads to the production of these peptide fragments. Cleavage by α- and γ-secretase is called non-amyloidogenic cleavage and by β- and γ-secretase is termed amyloidogenic cleavage (Figure 2) [12]. These peptides then undergo aggregation to form dimers, oligomers and fibrils [39]. These fibrils form sticky plaques which get deposited between neurons, deteriorate inter-neuronal communication and eventually lead to neuronal death [40].

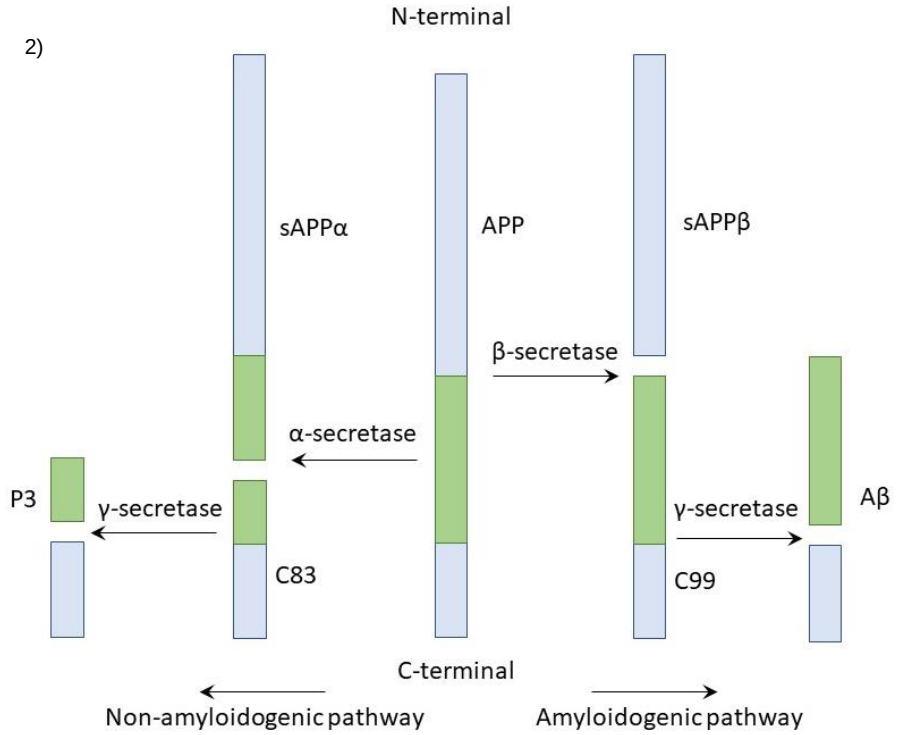



**Figure 2: Cleavage of amyloid precursor protein**

Cleavage of APP through non-amyloidogenic and amyloidogenic pathway by α-/γ-secretase and β-/γ-secretase respectively.

AD just like various other neurological disorders can be familial and sporadic. The familial causes of AD can be like trisomy of the 21$^{st}$ chromosome (Down's Syndrome), mutations in APP, components of β-secretase/ γ-secretase [41], [42]. Presence of one or more alleles of ApoE4 is also a major genetic risk factor for AD. The clearance of Aβ is a matter of more serious concern than its production. The clearance of fibrillar Aβ occur through receptor mediated phagocytosis and that of soluble Aβ through fluid phase micropinocytosis. Macrophages are majorly responsible for the clearance of Aβ [13]. The receptor mediated clearance of Aβ is mediated by a polymorphic and multifunctional protein apolipoprotein E (ApoE) [43]. The brain is next after liver in synthesis of ApoE4. It plays an indispensable role in lipid transport in CNS. There are 3 alleles of ApoE majorly found in humans E2, E3 and E4. The C-terminal of ApoE4 has high affinity towards hydrophobic moieties and the N-terminal is the receptor-binding domain. These alleles differ just at two locations in their amino acid sequence i.e., 112 and 158. The more prominent molten globule form of ApoE4 makes it a poor mediator for Aβ clearance [16]. ApoE4 is also reported to stimulate the amyloidogenic cleavage of APP in an Low Density Lipoprotein Receptor-Related Protein 1 (LRP1) dependent pathway [44]. Thus, E4 is the most potent in increasing the deposits of Aβ in the interneuron space [45].

Numerous studies have elaborated the pathological events involved in familial form of the disease but the mechanistic details of the sporadic form are still elusive. Factors like surrounding environment, lifestyle, pathogenic infections, stress, epigenetics and traumatic brin injury can all lead to AD [42]. Pathogenic invaders like Herpes Simplex Virus 1 (HSV1), Human Herpes Virus 6 (HHV6), *Chlamydia pneumoniae*, *Helicobacter pylori* and *Spirochetes* to name a few [46]. Interestingly, a significant association of EBV mediated IM has been reported with an

increased risk of AD [6]. At the same time, high amount of anti-EBV antibodies were found in patients suffering from cognitive impairment [10]. A 12 amino acid peptide fragment derived from the EBV gM ($_{146}$SYKHVFLSAFVY$_{157}$) was discovered to have self- aggregative properties similar to the golden standard of aggregation and was cytotoxic to neuronal cells with an $IC_{50}$ value of 37.03 μM [11]. The cellular pathways involved in the pathogenesis of EBV are elusive but there have been studies conducted to understand the mechanistic details involved in infection and pathogenesis of HSV1, which is also a member of Herpesviridae. Infection of HSV1 into neuronal and glial cells leads to increased synthesis of Aβ peptides and upregulation in activity of β- and γ-secretase [47]. Its infection also leads to synaptic dysfunction in cultured cortical neurons [48]. This increase was restored upon treating the cells with antiviral, acyclovir [49]. Intriguingly, infection load of HSV1 in ApoE4 is transgenic mice is greater than in ApoE3 [50].

The pathological consequence of AD in synaptic damage and neurodegenerations. Preceding these conditions occurs neuroinflammation. In-spite of having different cellular mechanisms neuroinflammation appears to be at intersection of almost all of the neurodegenerative disorders [51]. Inflammation of neurons has been postulated to have an indispensable role in the developing sporadic form of AD [18]. Particularly, the pro-inflammatory molecules colocalize with the pathology in AD mice models and human subjects [52]. Inflammatory molecules were found to be associated with neuritic plaques and drugs against inflammation have been found to decelerate the development of AD [18], [53]. Inflammatory mediators like acute phase proteins were reported to colocalize with Aβ plaques which included- α1-antichymotrypsin (ACT), α2-macroglobulin (A2M), intracellular adhesion molecule (ICAM-1), apolipoprotein B, clusterin, serum amyloid β component and heparan sulphate proteoglycan [54]. Earlier studies have suggested that IL-6 results in accumulation of the acute phase molecules in dementia of the Alzheimer's type (DAT) patients [55]. It has been stated that cytokine promotes the production of

Aβ peptides possibly initiate a positive feedback loop where these peptide deposits further promote cytokine synthesis by activation of microglia [56]. Introducing recombinant IL-1 promoted the expression of APP in HUVEC cells, in a protein kinase C-mediated pathway [57]. Additionally, the increased expression of IL-1β, was observed to hinder microglial Aβ clearance [58]. IL-1β further can induce the synthesis of IL-6, astrocyte proliferation and synthesis of nerve growth factors [59]. Both IL-1β and TNF-α were reported to promote γ-secretase-mediated cleavage of APP through a Jun N-terminal kinase (JNK)-dependent Mitogen-activated protein kinase (MAPK) pathway [60]. Expression of murine IL-4 (mIL-4) in the hippocampus leads to aggravation of amyloid burden *in-vivo*. This is postulated to as a result of reduced clearance of Aβ by microglial cells [61], [62].

Along with inflammation there are other major cellular nuances mitochondrial dysfunction was been depicted to be a preceding marker in case of various neurological disorders like Huntington's disease (HD), Parkinson's disease (PD), AD, Friedreich's ataxia and amyotrophic lateral sclerosis (ALS) [63]. Increase in Aβ levels reduce the membrane potential of the mitochondrial membrane [64]. The Aβ peptides are found to colocalize within the mitochondrial membrane disrupting the potential. This leads to the production of reactive oxygen species (ROS) in the neuronal cells [65]. The above-mentioned events suffocate the neurons to death, damaging a greater part of brain. These conditions in long run severely hamper the mental health of the patient. Reduced cognizance and increased anxiety [22]. Further, anti-anxiety drugs were reported to be potent in impairing the development of AD [23]. Further, earlier reports have suggested a significant association between attachment anxiety and titre of EBV viral capsid antigen (VCA) in human subjects [66].

So, its high time to take up the challenge of understanding the association of EBV with neuropathies like AD.

# CHAPTER 3

## Aim and Objectives

**Aim**

- To unravel the mysterious connection of Epstein-Barr virus with Alzheimer's disease.

**Objectives**

- Examination of EBV-$gM_{146-157}$ mediated acceleration in the aggregation of $A\beta_{42}$, in solution.
- Assessment of inflammatory cascade initiated by exposure of EBV and $gM_{146-157.}$
- Evaluation of amyloid precursor protein and apolipoprotein E4 level upon exposure to EBV and $gM_{146-157}$.
- Understanding the association of EBV with AD via *in-vivo* model system.

# CHAPTER 4

# Materials and Methods

## 4.1 Materials

### 4.1.1 Equipment used

4.5µm Nitrocellulose membrane: (Cat. no.#1620115, BIORAD laboratories Inc. India)
6-well plate: (Corning, Merck)
Cell culture discs: (Corning, Merck)
Centrifuge tubes (15/50 ml) and stands: (Tarsons, Genaxy)
Coverslips: (Blue Star)
Glass slides: (Blue Star)
Microcentrifuge tubes and stands: (Tarsons, Genaxy)
Pipettes and tips: (Eppendorf, Sartorius, Pfact, Cole-Parmer)
Serological pipettes: (Cole-Parmer)
Western blot protein separation unit: (BIORAD laboratories Inc. India)
Western blot transfer unit: (BIORAD laboratories Inc. India)

### 4.1.2. Instruments used

AFM: (Solver-Pro, NT-MDT, Russia)
Centrifuge and ultracentrifuge: (Eppendorf, Remi, Thermo-Fisher and Bechman coulter)
Confocal microscope: (OLYMPUS IX83, Shinjuku, Tokyo, Japan)
Fluorescence microscope: (OLYMPUS IX83, Shinjuku, Tokyo, Japan)
Gel documentation system: (BIORAD laboratories Inc. India)
Heat block; (NEOLAB)
Microplate reader spectrophotometer: (Agilent BioTek synergy H4 hybrid microplate reader)
Nanodrop Spectrophotopmeter: (Thermo Fisher Scientific)
PCR thermocycler; (Thermo Fisher Veriti 96-well thermo cycler)
pH meter: (P200, Cole-Parmer)
Real-time PCR system: (Agilent Technologies)

Refrigerators: (-80°C, -20°C,0°C and 4°C

### 4.1.3. Reagents used

1M Tris-HCL (pH-6.8); (Tris- HCL, Cat. no.#TC073, Himedia Laboratories Pvt. Limited, India)
20 % SDS; (Cat. no.#0210291890, MPBIO)
40% Acrylamide: (Cat. no.#1610144, BIORAD laboratories Inc. India)
Antibiotics cocktail: (Cat. no.#A001A, Himedia Laboratories Pvt. Limited, India)
Anti-fade: (Cat. no#P36930, Gold antifade mountant, Thermo Fisher Mountant)

Beta-mercaptoethanol: (Cat. no.#MB005, TCI)
Bovine Serum Albumin (BSA): (Cat. no#MB083, Himedia Laboratories Pvt. Limited, India)
Bromophenol blue: (Cat. no.#MB123, Himedia Laboratories Pvt. Limited, India)
Chloroform: (Cat. no. # 102431, Sigma-Aldrich)
Dulbecco's Modified Eagle Medium (DMEM): (Cat. no.#AL006, Himedia Laboratories Pvt. Limited, India)
Foetal Bovine Serum (FBS): (Himedia Laboratories Pvt. Limited, India)
Glycerol: (Cat. no.#29054500, Himedia Laboratories Pvt. Limited, India)
Isopropanol: (CAS no.#67-63-0, Sigma-Aldrich)
Methanol: (Cat. no.#106007, Sigma-Aldrich)
Molecular grade ethanol: (Cat. no.#32221, Honeywell International Inc., USA)
PCR cDNA 1st strand synthesis kit: (Cat. no.#6110A, Takara Bio Inc., USA)
Phenylmethylsulfonyl fluoride (PMSF): (Cat. no.#329-98-6, Sigma-Aldrich)
Phosphate Buffer Saline (PBS): (Himedia Laboratories Pvt. Limited, India)

Ponceau S-stain: (Cat. no.#ML045, Himedia Laboratories Pvt. Limited, India)

Protease and Phosphatase Inhibitors: (Cat. no.#P5726 and PIC0002, Sigma-Aldrich)

RIPA (Radio Immuno Precipitation Assay) Buffer: (Cat. no#N653; Thermo Fisher Scientific)

Sodium dodecyl sulfate (SDS): (Cat. no.#MB010, Himedia Laboratories Pvt. Limited, India)

SYBR green: (Cat. no.#A25742; Applied biosystem Powerup, Thermo Fisher Scientific)

Target gene-specific primers: (Integrated DNA Technologies, Inc.)

Thioflavin S (Th S): (Cat. no.#T1892, Sigma-Aldrich)

Tris-HCL (pH 6.8 and 8.8): (Cat. no.#MB030, Himedia Laboratories Pvt. Limited, India)

Triton X100: (Cat. no.#MB031, Himedia Laboratories Pvt. Limited, India)

TRIzol reagent: (Cat. no.#MB601, Himedia Laboratories Pvt. Limited, India)

Trypsin 0.05%: (Cat. no.#TCL033, Himedia Laboratories Pvt. Limited, India)

## 4.2. Methodology

### 4.2.1. Preparation of peptide aggregates

Peptides of EBV-gM$_{146-157}$ and A$\beta_{42}$ were purchased in lyophilized form, from S BioChme Group Labs, Athani, Thrissur, India and Sigma (PP69, Sigma-Aldrich) respectively. Aggregates of EBV-gM$_{146-157}$, (125 μM) were prepared as mentioned previously [11]. 221 μM of A$\beta_{42}$ was prepared in 50 mM tris HCl (pH≥9.0).

### 4.2.2. Thioflavin S assay

A$\beta_{42}$ was mixed with aggregates of EBV-gM$_{146-157}$ in two sets such that the final concentration of EBV-gM$_{146-157}$ was kept constant (20 μM) and that of A$\beta_{42}$ was 2 μM and 4 μM. Pure solutions of EBV-gM$_{146-157}$ (20

μM), $A\beta_{42}$ (2 μM) and $A\beta_{42}$ (4 μM) were also prepared as control. These solutions were then allowed to incubate at 37°C for 24 h. On getting tangled with mature aggregates, Th S (dissolved in 50% methanol) emits fluorescence in the range of 440–450 nm upon getting excited at 391 nm. The solutions were subjected to 0.05% of ThS (from 0.1% stock) and were excited at 391 nm. The prepared samples were loaded in quartz cuvettes of 400 μl. The slit width of the fluorometer was set at 1nm for both excitation and emission. The fluorescence emission was recorded within the range of 400 and 600 nm. The emission was recorded similarly as described earlier [11]. The aforementioned experimentation was done in two replicates.

#### 4.2.3. Atomic force microscopy

Customized silicon wafers were prepared with dimensions 1cm X 1cm X 2 mm to coat the samples. Aggregates of EBV-$gM_{146-157}$ (62.5 μM) were mixed with peptides of $A\beta_{42}$ (10 μM) and incubated at 37°C for 24 h and pure EBV-$gM_{146-157}$ aggregates and pure $A\beta_{42}$ peptides were taken as control. 10 μl of sample was coated on the wafer and was air-dried for 24 hrs in a dust-free environment. Atomic force microscopy (AFM) was then done to capture the images. Experiment was performed in two biological replicates.

#### 4.2.4. Cell culture

The human neuroblastoma cell line (IMR-32) was procured from the National Centre for Cell Science, Pune, India. HEK 293T cells stably transfected with the bacterial artificial chromosome (BAC) green fluorescent protein (GFP)-EBV was used for purification of the virus. Dulbecco's modified Eagle's medium (DMEM; Himedia Laboratories Pvt. Limited, India) supplemented with 10% fetal bovine serum (FBS; Himedia Laboratories Pvt. Limited, India), 50 U/ml, 100 μg/ml and 2 mM of penicillin, streptomycin and L-Glutamine respectively was used to culture all the above-mentioned cells. The environment for growing the cells was humified with 5% $CO_2$ at 37°C. Experimental setup for *in-vitro* (Figure 3).

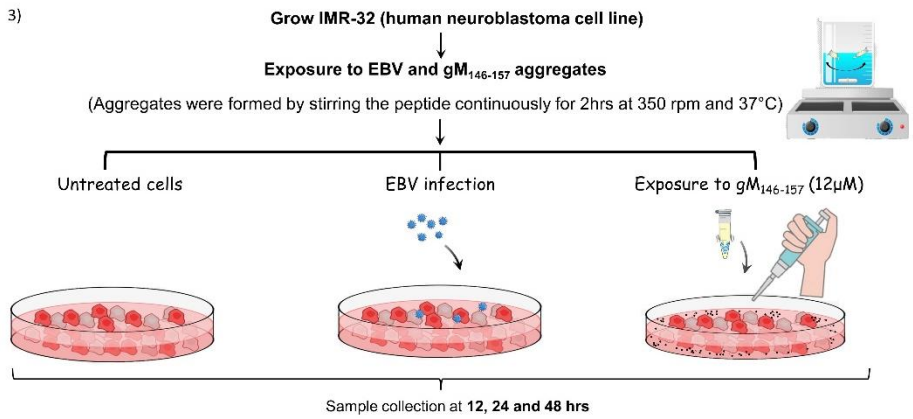



**Figure 3: Experimental setup for *in-vitro* demonstration of the association between exposure of EBV and $gM_{146-157}$ and AD pathology on IMR-32 cells**

### 4.2.5. Quantitative real time-polymerase chain reaction

RNA isolation

qRT-PCR was performed on three biological and two technical replicates. IMR-32 cells were seeded on 6-well plate and were exposed to EBV and $gM_{146-157}$ separately for 12, 24 and 48 h. Cells were scraped post exposure, washed with PBS and cell pellets were made. The cell palettes were then subjected to total RNA isolation through TRIzol reagent. 300 µl of TRIzol reagent was used according to 3 x $10^5$ cells. Cells were incubated in it for 5-10 min at room temperature. 100 µl (according to the volume of TRIzol added) of chloroform was added to the cell lysate and allowed to incubate at room temperature for 10 min. The cell lysate was then centrifuged at 12,000 g for 15 min at 4°C and the aqueous phase was transferred to a new set of MCTs. This was followed by the addition of isopropanol and incubation for 10 min at room temperature. The MCTs were then centrifuged at 12,000 g and 4°C for 15 min. The supernatant was discarded and the pellet was washed with 75% ethanol and centrifuged at 8000g for 7.5 min. The supernatant was discarded and the pellet was air-dried. 20 µl of distilled water was added to each pellet and allowed to dissolve on the heating block at 42°C for 5-10 min. The quantity and purity of RNA were measured by nanodrop.

1st strand cDNA synthesis

First-strand cDNA synthesis was performed using the reverse transcription kit (PrimeScriptTM RT Master Mix; Takara, India). A total of 4 µg of RNA (template) was used for cDNA synthesis. Random hexamers (1 µM) and dNTPs mixture (0.5 mM) were added to the RNA samples and allowed to incubate on the heating block at 65°C for 5 min. This was allowed by the addition of 5 X reverse transcriptase buffer, reverse transcriptase enzyme (200 units) and RNase A inhibitor (20 units). The final volume of the mixture was 20 µl.

The program set on the PCR thermocycler was such that it contained the following stages

Stage I – 30 °C - 10 minutes (Annealing)

Stage II – 42 °C - 60 minutes (Polymerisation)

Stage III – 70 °C – 15 minutes (Enzyme deactivation)

Storage at 4 °C - ∞

Quantitative real-time polymerase chain reaction

The cDNA was diluted with distilled water (40 µl per 1 µg of DNA). The SYBR green was first mixed with an equal volume of water. This was followed by the addition of gene-specific primers both forward and backward with final concentration 500 nM each (separately). The primer and SYBR mix were equally divided into the number of sample tubes (7) and 2 µl of the samples were added to respective sample tubes. This mixture was loaded onto a PCR plate in duplicate such that the final volume is 10 µl.

The real-time PCR system was programmed at 10 min at 95°C followed by (15 s at 95°C, 20 s at 55°C, 20 s at 72°C) × 40 cycles. Relative expression of the gene was studied by the $2^{-\Delta\Delta Ct}$ method. ΔCt the difference between Ct value of the target gene and GAPDH. Further, ΔΔCt is the difference between ΔCt of the sample and that of

the control [67]. The values thus obtained were used to obtain the fold change. The above-mentioned experiment was performed in two biological and two technical replicates.

### 4.2.6. Western blot

Western blot was performed in three biological replicates. IMR-32 cells were seeded in 60 mm plates and allowed to grow. The cells were then exposed to EBV and $gM_{146-157}$ followed by the scrapping of the cells at 12, 24 and 48 h. The cells were washed with PBS and subjected to lysis by Radioimmunoprecipitation assay (RIPA) buffer [10 mM Tris (pH 7.5), 150- mM sodium chloride, 2 mM EDTA, 1% NP-40], supplemented with (PMSF) and halt (protease and phosphatase inhibitor). The cell lysate was then centrifuged at 12,000 rpm and 4°C for 25 minutes. The supernatant was collected and normalized (after protein estimation using Bradford) according to the standard using 4 x loading dye. This was followed by heating the samples at 95°C. Protein samples were loaded onto 10% acrylamide gel and the western blot was performed followed by the transfer of proteins on 0.45 μM nitrocellulose membrane. 5X ponceau S staining was performed to evaluate to locate and identify the proteins transferred onto locate and identify the proteins on the blot. The blot was then blocked with 4.5% BSA for 1 h, to prevent the non-specific binding of antibodies. This was followed by incubation with primary antibodies (1:1000) against the target proteins and with their respective secondary antibodies conjugated with horseradish peroxidase for 1 h (1:3000). Antibodies against APP (CT695, Invitrogen) rabbit, ApoE4 (4E4, Cell Signalling Technology, Danvers, MA, USA) mouse, NF-kB (D14E12 from Cell Signalling Technology) rabbit, Danvers, MA, USA), and GAPDH (14C10, Cell Signalling Technology, Danvers, MA, USA) were used. Secondary anti-mouse (7074, Cell Signalling Technology, Danvers, MA, USA) and anti-rabbit (7076, Cell Signalling Technology, Danvers, MA, USA) antibodies have been used. Chemiluminescent detection was done employing Pierce ECL western blotting substrate (1705061; Bio-Rad Laboratories, Inc., USA). Analysis and quantification of the blots were done using

Image J software (National Institute of Health, Bethesda). This experiment was performed in three biological replicates.

### 4.2.7. Immunofluorescence

IF has been carried out to demonstrate the localization of APP level inside the cell post-exposure to EBV and $gM_{146-157}$. 2.5 $x10^4$ cells were seeded on coverslips in a 6-well plate and were subjected to the exposure of EBV and $gM_{146-157}$ for 12, 24 and 48 h. The coverslips were then harvested and fixed with 4% paraformaldehyde solution for 20 min. The cells were then subjected to 0.2% freshly prepared triton x100 in order to permeabilize the cell membrane. This was followed by blocking through 1% BSA for 1 h followed by washing with 1XPBS. The cells were then incubated with primary antibodies at room temperature for 2 h and were washed thrice. This was followed by incubation with secondary antibodies conjugated with different fluorophores and was supplemented with (4',6-diamidino-2-phenylindole) DAPI (4083, Cell Signalling Technology). The coverslips were then mounted on glass slides with the help of a drop of antifade mounting media. The cells were then observed under CLSM (FluoView 1000, Olympus America Inc., USA). Analysis and quantification of the images were carried out using Image J software (National Institutes of Health, Bethesda, MA, USA). The fluorescence intensity was measured and plotted in comparison to the unexposed control.

### 4.2.8. Mito-tracker assay

The cells were subjected to mito-tracker red and green staining in order to study the mitochondrial potential and mass respectively. 0.05 x 106 cells were seeded onto a 24-well plate and were exposed to EBV and $gM_{146-157}$ for 12, 24 and 48 h. The cells were then washed with PBS. Mito-tracker red and green were prepared in DMEM at 500 nM and 300 nM concentrations along with DAPI. The cells were then incubated with this solution for 60 min and were washed with PBS. This was followed by visualization of the cells under the fluorescence microscope (OLYMPUS IX83). Analysis and quantification of the images were

done using Image J software and the fluorescence intensity was measured and plotted in comparison to the unexposed control.

### 4.2.9 Animal subjects

Adult female Swiss Albino mice weighing 20-30 g, were housed in polypropylene cages and acclimated for a week before experimentation in a 14 h light: 10 h dark in an environment with a temperature of 23 ± 2 °C and humidity under control, with free access to laboratory feed and drinking water. Animals were maintained as per the guidelines of the Institutional Animal Ethics Committee (IAEC), the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), and the Ministry of Environment and Forests, Government of India, New Delhi. The experimental protocol was approved by the IAEC of the School of Life Sciences, Devi Ahilya University, Indore. (Registration no. 779)

Precisely, a total of five groups were made, four groups (immunosuppressive control, only IMR-32 exposed, EBV infected and $gM_{146-157}$ subjected mice) having 6 mice along with one neat control group (negative control) (Figure 4). For immunosuppression, mice were subjected to peritoneal cyclosporin (working concentration; 10 mg/kg) on an alternate day for 10 days and then xenograft of IMR-32 prior-exposed with EBV or $gM_{146-157}$ for 24 hrs was administered intranasally. Three booster doses were given for the above-mentioned cells or cells exposed to EBV/$gM_{146-157}$ every five days. Besides, to avoid the fungal infection ketoconazole (working concentration; 5-10 mg/kg) was administered from the $10^{th}$ day of immunosuppression till 15 days. Further mice were maintained and the behavioural assay was recorded for 5 weeks before sacrifice.

G1 – Intranasally administered with PBS.

G2 – Intranasally administered with $5 \times 10^6$ IMR-32 cells.

G3 – Intranasally administered with $5 \times 10^6$ IMR-32 cells which were infected with EBV for 24 h.

G4 – Intranasally administered with 5 x $10^6$ IMR-32 cells which were exposed to $gM_{146-157}$ for 24 h.

### 4.2.10. Elevated plus maze

The mice were placed on the centre of the elevated maze facing towards one of the open arms and allowed 1 minute of free exploration. Parameters like first arm entry, number of entries, time taken in travel to ends of arm and time spent at the ends were noted. The assay was performed after training the mice for two weeks. Then, the assay was performed twice before the immunosuppression, twice after the immunosuppression, 5 times during the exposure and 3 times after the exposure.

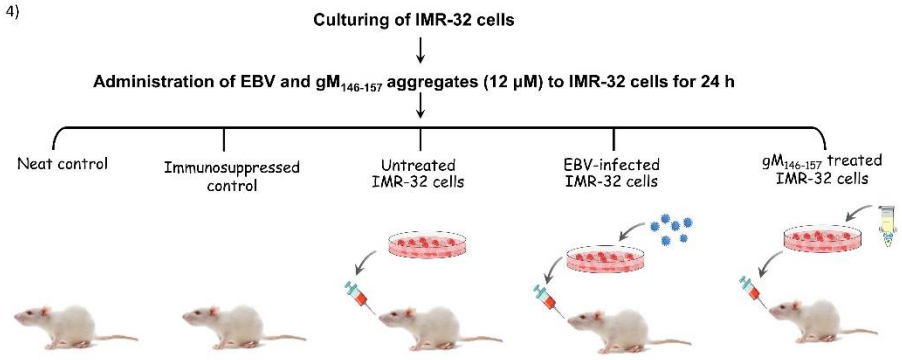



**Figure 4: Experimental setup for *in-vivo* demonstration of the association between exposure of EBV and $gM_{146-157}$ with AD in female Swiss albino mice**

### 4.2.11. Statistical analysis

All the *in-vitro* experiments were carried out in three biological replicates. Data were presented as means ± standard error mean (SEM) of three independent experiments. One-way ANOVA followed by post hoc analysis was performed to compare the differences in the mean values of IMR-32 cells with IMR-32 cells exposed to EBV/$gM_{146–157}$. The statistical significance of p-values <0.05, <0.01 and <0.0001 were considered to be */#, **/## and ***/### for upregulation/downregulation respectively.

# CHAPTER 5

# Results

## 5.1. Epstein-Barr virus glycoprotein M derived fragment ($_{146}$SYKHVFLSAFVY$_{157}$) accelerates the aggregation of Amyloid $\beta_{42}$

Thioflavin S assay was performed in order to evaluate the increase in aggregates as a result of incubation of $A\beta_{42}$ (2 µM and 4 µM) with 20 µM of $gM_{146-157}$ (125 µM) aggregates. Th S binds to mature aggregates emits fluorescence in the range of 450-550 nm upon getting excited at 391 nm [68]. Increase in fluorescence emission and a blue shift of 21 nm and 13 nm was observed in $gM_{146-157}$ + $A\beta_{42}$ 2 µM and 4 µM respectively in comparison to pure $A\beta_{42}$ samples (both 2 µM and 4 µM). (Figure 5) Further, to characterize the morphology of the aggregates the samples were subjected to AFM. We found these aggregates to be having spherical structures as it was reported in the case of $gM_{146-157}$ [11]. Oligomeric forms of these spherical structures were found in $A\beta_{42}$ sample and these oligomeric structures increased in number as a result of incubation of $A\beta_{42}$ with $gM_{146-157}$. (Figure 6)

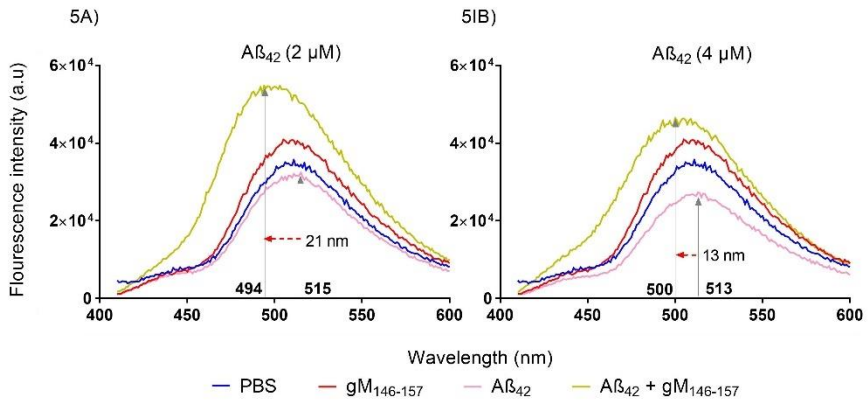



**Figure 5 : Thioflavin S Fluorescence emission of after the incubation of 24 h**

5A) Emission spectrum of $A\beta_{42}$ (2 µM) and 5B) emission spectrum of $A\beta_{42}$ (4 µM). Emissions of pure gM, $A\beta_{42}$ (2 µM) and $A\beta_{42}$ (4 µM) were

at around 515 nm. Incubation of $A\beta_{42}$ (2 µM) and $A\beta_{42}$ (4 µM) with $gM_{146-157}$ lead to an increase in fluorescence emission and blue shift of 21 nm and 13 nm respectively.

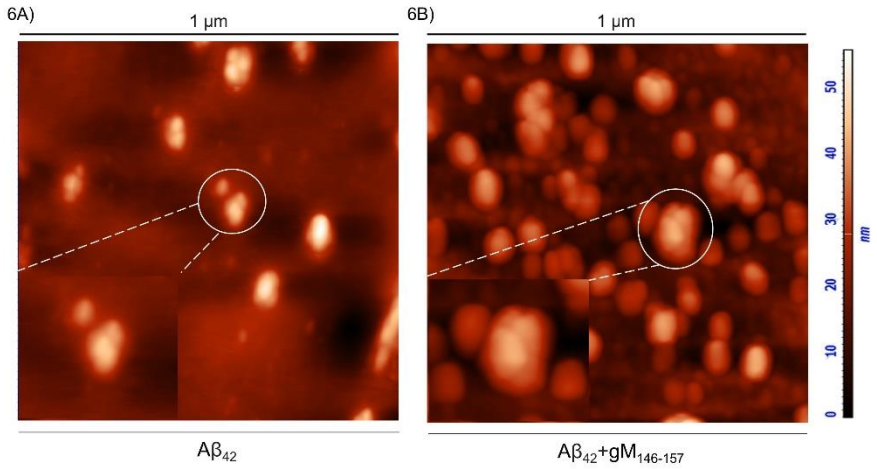


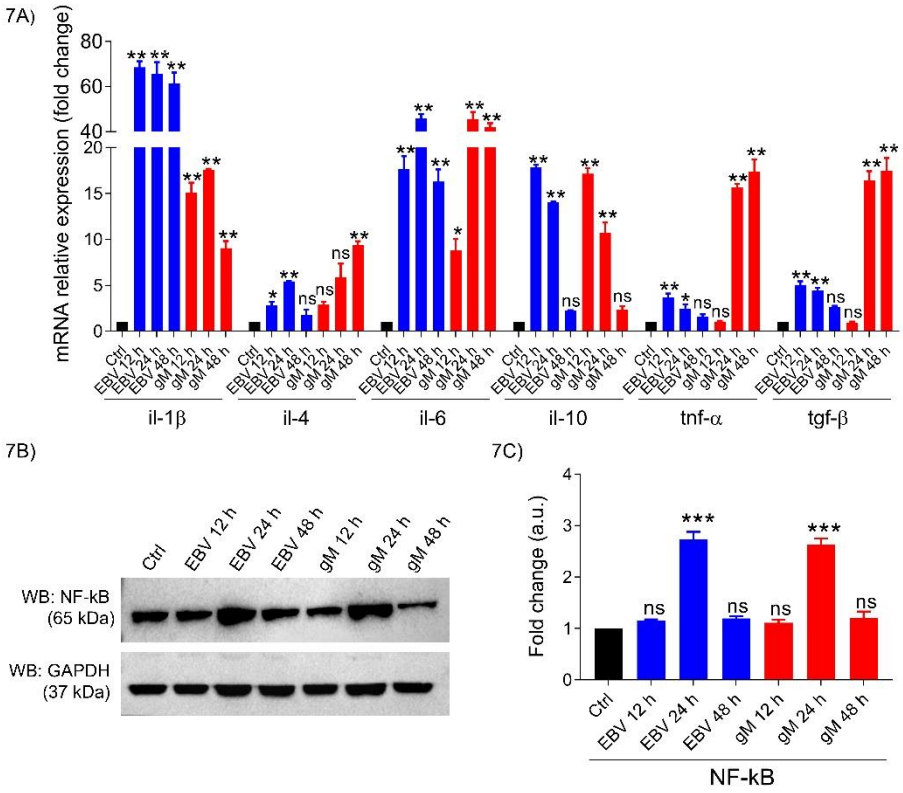

**Figure 6: Images obtained from atomic force microscopy**

AFM of aggregates formed in A) $A\beta_{42}$ and B) $A\beta_{42}$+$gM_{146-157}$. An increase in spherical oligomeric structures was suggested as a result of incubation of $A\beta_{42}$ with $gM_{146-157}$.

### 5.2. Exposure of Epstein-Barr virus virion and its peptide $gM_{146-157}$ were found to instigate immune response in neuronal cells (IMR-32)

The IMR-32 cells were exposed to EBV and $gM_{146-157}$ for 12, 24 and 48 h. The $IC_{50}$ of the $gM_{146-157}$ was found to be 37.03 µM, and a subtoxic dose of 12 µM was used for further experiments where >90 % of the cells were alive [11]. Neuroinflammation is found to be one of the preceding factors for various kinds of neurological disorders [51]. In order to assess the alterations in the inflammatory molecules we have performed qRT-PCR for genes namely, *il-1β, il-4, il-6, il-10, tnf-α* and *tgf-β* (Figure 7A). A significant upregulation was observed in pro-inflammatory molecules i.e., *il-1β* and *il-6* as a result of exposure to EBV and $_{gM146-157}$ at all the considered time points ($p<0.01$). An increase in transcript level of *tnf-α* and *tgf-β* was observed at transcript level as

result of exposure of $gM_{146-157}$ at 24 and 48 h of timepoint ($p<0.01$). At the same time there was an increase in the expression of il-10 at 12 and 24 h post exposure ($p<0.01$). Increase in the mRNA level of il-4 was not much significant (p=ns) (Figure 7A). Further, we have also evaluated NF-kB levels after the exposure of EBV and $gM_{146-157}$. A significant increase in NF-kB level was observed at 24 h post exposure of EBV and $gM_{146-157}$ ($p<0.0001$). (Figure 7B and 7C)



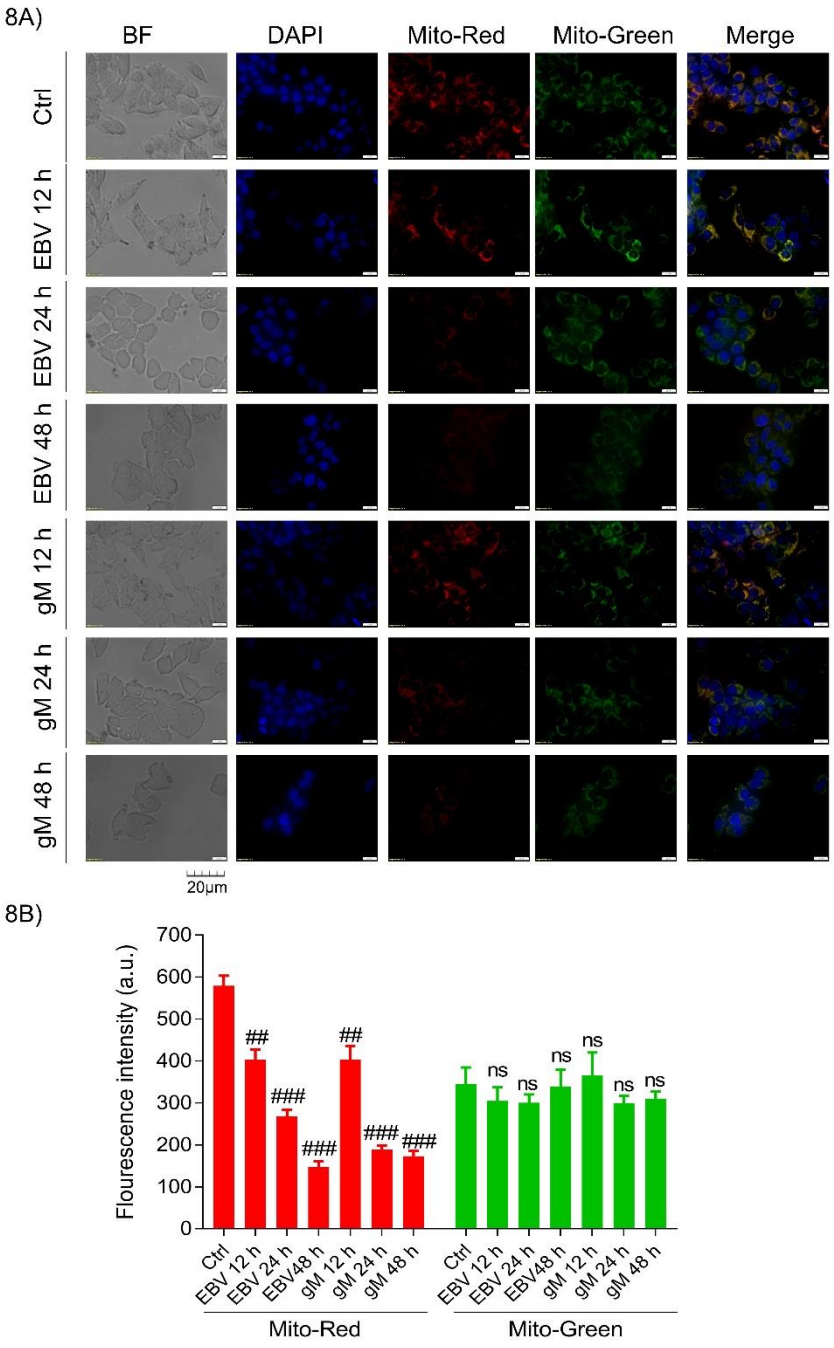

**<u>Figure 7</u>: Assessment of nuances in inflammatory cascade after exposure of EBV and $gM_{146-157}$ separately on IMR-32 cells for 12, 24 and 48 h**

7A) Infection of EBV increased the expression of pro-inflammatory cytokines (il-1β and il-6) ($p<0.01$). Similar results were found upon exposure of $gM_{146-157}$ ($p<0.01$) Exposure of $gM_{146-157}$ has also significantly enhanced the expression of tnf-α and tgf-β at 24 and 48 h ($p<0.01$). 7B) Western blot panels representing NF-kB and its 7C) quantification. NF-kB increased was found to be increased at 24 h post exposure of EBV and $gM_{146-157}$ ($p<0.0001$). The statistical significance

of p-values <0.05, <0.01 and <0.0001 were considered to be */#, **/## and ***/### for upregulation/downregulation respectively. The experiment was performed in 3 biological and 2 technical replicates.

### 5.3. Subjecting neuronal cells to the exposure of EBV and $gM_{146-157}$ leads to deterioration in its mitochondrial biology

Mitochondrial dysfunction and disruption have been deeply colocalized with various kinds of neurological disorders [63]. Particularly increase in amyloid beta peptides results in reduction of mitochondrial membrane potential (MMP) [64]. In order to evaluate the changes in mitochondrial potential and mass we have performed mito-tracker red and green staining respectively [69]. The mito-tracker red is a mitochondrial potential dependent dye with absorption and emission intensities 588 and 564 nm [70]. Mito-tracker green stain binds to mitochondrial proteins covalently, interacting with the free thiol groups and thus accumulates selectively inside the mitochondrial matrix [69]. We have observed a significant decrease in the fluorescence emission mediated by mito-tracker red after the exposure of EBV and $gM_{146-157}$ at 12 ($p<0.01$), 24 ($p<0.0001$) and 48 h ($p<0.0001$) (Figure 8A and 8B) which further indicates a cutback in MMP. While there was no significant change observed in the fluorescence emission of mito-tracker green (Figure 8A and 8B).




**<u>Figure 8</u>: Evaluation of changes in mitochondrial potential and mass through mito-tracker red and mito-tracker green stains**

8A) Fluorescence emission mediated by mito-tracker red and mito-tracker green on IMR-32 cells exposed to EBV and $gM_{146–157}$ separately for 12, 24 and 48 h along with its 8B) quantification. A significant decrease in mito-tracker red fluorescence emission was observed at 12 ($p<0.01$), 24 ($p<0.0001$) and 48 h ($p<0.0001$) for both EBV and

$gM_{146–157}$ exposed cells. There was no significant change observed in mitochondrial mass. The statistical significance of p-values <0.05, <0.01 and <0.0001 were considered to be */#, **/## and ***/### for upregulation/downregulation respectively. The experiment was performed in 3 biological replicates.

### 5.4. Epstein-Barr virus and $gM_{146-157}$ found to increase the protein level of amyloid precursor protein (APP) and apolipoprotein E4 (ApoE4)

Alterations in the level of ApoE4 were evaluated by performing western blot and found appreciable increase at its protein level at 12 ($p<0.01$), 24 ($p<0.0001$) and 48 h ($p<0.0001$) of EBV infection. A similar rise was observed at all the time points post exposure of $gM_{146-157}$ ($p<0.0001$). (Figure 9AI, 9AII and 9AIII) In order to evaluate the changes in production of Aβ peptides we have performed western blotting against the amyloid precursor protein (APP) and further studied the localization of APP through immunofluorescence. We have found a significant enhancement in the protein level of APP at 24 ($p<0.01$) and 48 h ($p<0.0001$) of EBV infection (Figure 9AI, 9AII and 9AIII). Likewise, a remarkable increase in APP levels was observed at 12 ($p<0.0001$) and 24 h ($p<0.0001$) post exposure of $gM_{146-157}$. Similar increase was observed in the immunofluorescence assay against APP at 12, 24 and 48 h of EBV and $gM_{146-157}$ exposure ($p<0.01$) (Figure 9BI and 9BII).

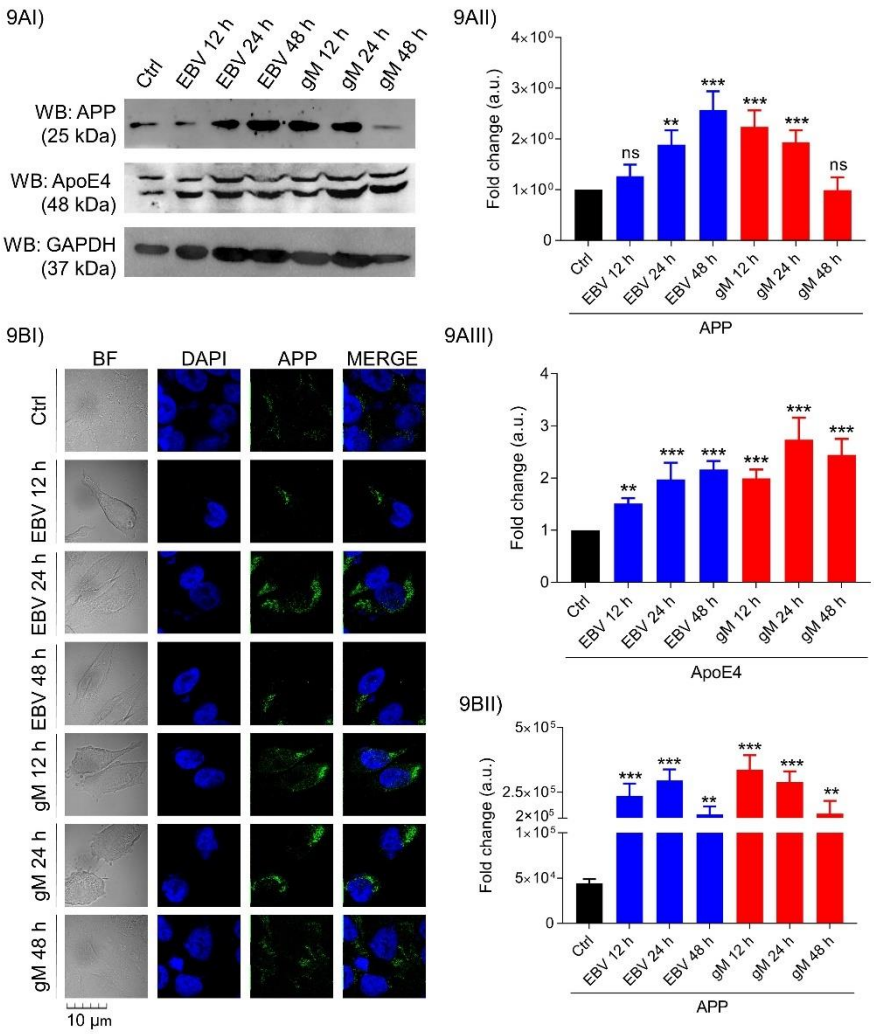



**Figure 9: Alterations in the level of pathogenic markers of Alzheimer's disease through western blot**

AI) Changes in the protein level of APP (25 kDa) and ApoE4 (48 kDa) and a housekeeping protein (GAPDH) through western blotting along with the quantification AII) ApoE4 and AIII) APP. A significant rise in the protein level of APP was observed as a result of the exposure. BI) Immunofluorescence of APP after infection with EBV and $gM_{146–157}$ at 12, 24 and 48 h along with BII) quantification It showed a significant increase at 12 ($p<0.0001$) and 24 ($p<0.0001$) and 48 h ($p<0.01$). Given plots; *x*-axis, time-dependent EBV infection; *y*-axis, fold change with respect to EBV infected samples. The statistical significance of p-values <0.05, <0.01 and <0.0001 were considered to be */#, **/## and ***/### for upregulation/downregulation respectively. The experiment was

performed in 3 biological replicates.

### 5.5. Intra-nasal administration of EBV and gM$_{146-157}$ exposed IMR-32 results in increased anxiety related behaviour in Swiss albino females

Elevated plus maze assay was employed to study the anxiety and depression like behaviour in mice after exposure. Increase in percentage of open arm entry was manifested in G3 and G4 during after the exposure ($p<0.0001$) (Figure 10A and 10B) We observed a significant decrease in the number of entries for G3 and G4 during ($p<0.01$) and post exposure ($p<0.05$) (Figure 10C). Similarly, the time consumed in travelling to the ends and time spent at the ends were also observed to be increased for these groups during and after the exposure ($p<0.05$) (Figure 10D and 10E).

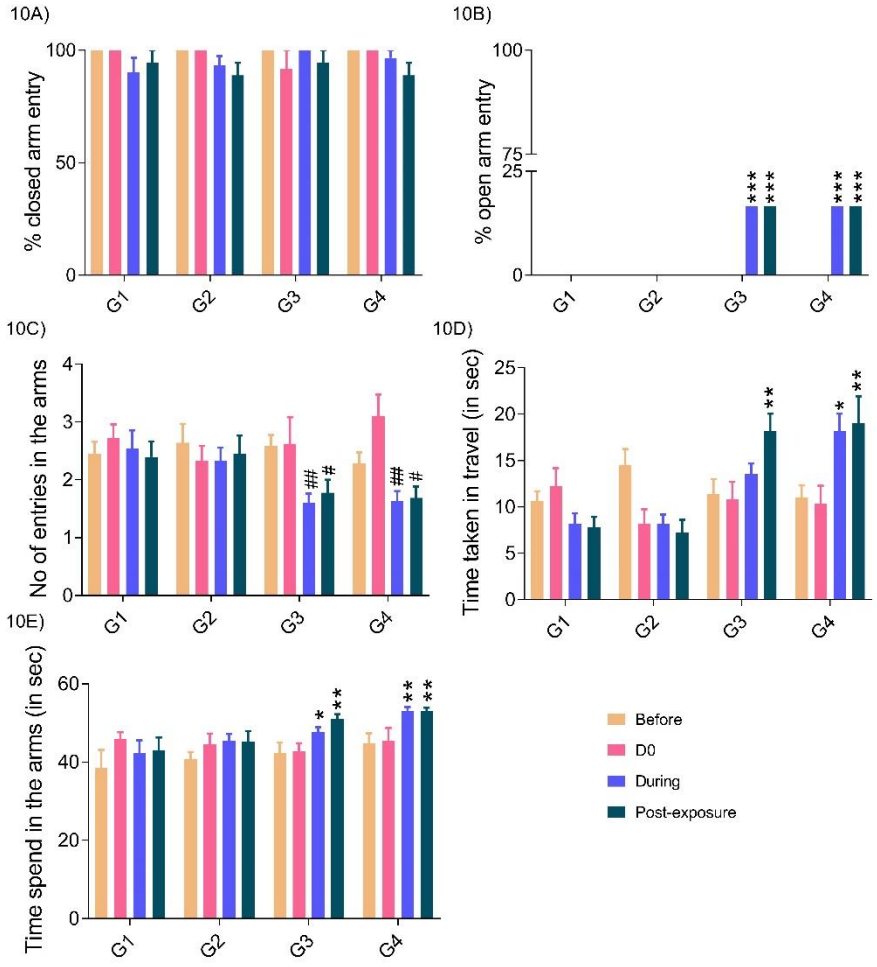


**Figure 10: Evaluation of anxiety like behaviour through elevated plus maze assay**

G1 – Intranasally administered with PBS, G2 – Intranasally administered with 5 x $10^6$ IMR-32 cells, G3 – Intranasally administered with 5 x $10^6$ IMR-32 cells which were infected with EBV for 24 h, G4 – Intranasally administered with 5 x $10^6$ IMR-32 cells which were exposed to $gM_{146-157}$ for 24 h.

Intranasal administration of EBV and $gM_{146-157}$ exposed IMR-32 cells increased anxiety like behaviour in mice during and post- exposure. A) Percentage of closed arm entry B) Percentage of open arm entry C) Total number of entries D) Time taken to travel to the end E) Time spent at the arm end. The statistical significance of p-values <0.05, <0.01 and <0.0001 were considered to be */#, **/## and ***/### for upregulation/downregulation respectively.

# CHAPTER 6

## Discussion

A plethora of studies have demonstrated the association of various pathogens with neurological disorders. After demonstration EBV infects brain cells, the entanglement of EBV with several neurological ailments has achieved an ample amount of attention [7]. A peptide fragment derived from the EBV glycoprotein M ($_{146}$SYKHVFLSAFVY$_{157}$) was discovered to have self-aggregative properties comparable to A$\beta_{42}$ [11]. In the present study A$\beta_{42}$ aggregate seeding property of g$M_{146\text{-}157}$ was evaluated by performing the thioflavin S assay. Increase in Th S-mediated fluorescence was observed as a result of incubating A$\beta_{42}$ with g$M_{146\text{-}157}$ in comparison to pure A$\beta_{42}$. AFM was performed for these samples in order to characterize the morphology of aggregates. These aggregates were found to be spherical structures as reported for 125 μM of g$M_{146\text{-}157}$ and oligomers of these aggregates were observed in pure A$\beta_{42}$ samples on subjecting to aggregation[11]. Interestingly, increase in these oligomers was found as a result of incubating g$M_{146\text{-}147}$ with A$\beta_{42}$. Fragments derived from gB and gK i.e., $_{22}$GVGADVRGSVMVGKGVALVIG$_{42}$ and $_{208}$LYHRPAIGVIVGCELMLRFVAVGLIVGT$_{235}$ respectively, of HSV1 were also found to be having self-aggregative properties and the former was also reported to increase thioflavin positive aggregates on incubation with A$\beta_{42}$ in comparison to pure A$\beta_{42}$ [71], [72].

In spite of the conviction that the brain is an immune-privileged site, immune responses occur routinely in the brain and play an indispensable role in protection against infectious agents. Regardless of the differences in pathophysiology of various neurodegenerative disorders, all of them share neuroinflammation as a common feature [51]. A significant rise in the mRNA expression of pro-inflammatory molecules namely, il-1β and il-6 has been observed as a result of exposure to EBV and g$M_{146\text{-}157}$. Adding to this exposure of g$M_{146\text{-}157}$ has also resulted in a remarkable

upregulation in the expression of tnf-α and tgf-β. IL-1 was earlier reported to upregulate the expression of APP through a protein kinase C dependent pathway which utilizes the AP-1 binding site of APP [57]. IL-1β further can increase the production of IL-6 and also promote the proliferation of astrocytes [59]. Along with IL-1β, IL-6 is also known to enhance the synthesis of APP [73]. TNF-α significantly increases the production of Aβ peptides by upregulating beta-site amyloid precursor protein cleaving enzyme 1 (BACE1), a component of β-secretase [74]. Interestingly, both TNF-α and IL-1β were found potent in stimulating γ-secretase mediated cleavage of APP [60]. On the other hand, IL-6 was also described to play a significant role in the development, differentiation, regeneration and degeneration of neurons in the peripheral nervous system (PNS) and CNS [75]. A rise in the transcript level of il-4 was also observed post-exposure to EBV and $gM_{146-157}$. Earlier studies have shown that IL-4 escalates the burden of Aβ by decreasing scavenging by glial cells [62]. A prominent increase in the level of NF-kB was also observed after the exposure of EBV and $gM_{146-157}$. NF-kB plays a major role in pathogenesis of AD. Its activation induces the transcription of pro-inflammatory cytokines following the canonical pathway [76]. Studies have shown the p65 unit of NF-kB to bind with promotor of BACE1 and promote its expression [77].

Apart from inflammation, mitochondrial anomalies are also a common feature of degenerating neurons in the case of various neurodegenerative disorders including AD and PD [19]. In case of AD, mitochondrial dysfunction mediated rise in oxidative stress produces lipid peroxidation product 4-hydroxynonenal, which enhances the activity of secretase by covalently modifying the γ-secretase complex [63]. In the present experimental set-up mito-tracker assay revealed a significant reduction MMP post exposure of EBV and $gM^{146-157}$ at all the considered time points. Interestingly, similar reduction in MMP has been reported in AD cybrids in comparison to the control. Additionally, subjection to high levels of Aβ dissipation of MMP [21].

Further, western blot results have revealed a significant increase in protein levels of APP (25 kDa C-terminal) and ApoE4 as a result of exposure to EBV and $gM_{146-157}$ at all the considered time points. Immunofluorescence assay against APP on IMR-32 cells exposed to EBV and $gM_{146-157}$ also showed similar results. An earlier study by Wozniak et.al. demonstrated increased expression of amyloid beta peptides and upregulation of secretases as a result of HSV-1 infection in cultured neuronal and glial cells [47]. At the same time, HSV1 replication inhibitors like, acyclovir and helicase primase inhibitor ($BAY_{57–1293}$) significantly decreases the level of Aβ in cell culture [49], [78]. Similarly, exposure to $gB_{22-42}$ results in increased production of APP and its breakdown products [71].

There can be various ways to interpret the results but the recent evidences suggest that Aβ is an anti-microbial peptide (AMP), a form of innate immune response which undergoes fibrillation for the protection from infectious and foreign agents. There have been reports which stated, oligomers of Aβ peptides have antimicrobial properties and increase in Aβ is directly properties is directly proportional to resistance against infectious agents [79]. Increased Aβ was found to negatively impact the replication of HSV1 by inhibiting it [80], [81]. Eimer et. al. have also demonstrated that fibrillation of Aβ occurs when its oligomers entrap HSV1 as a protective measure [82].

Alzheimer's pathology is characterized by reduced cognizance, and increased anxiety and depression like behaviour in the subjects. Anxiety like symptoms have been reported in 40% of AD patients and can be potent factor in conversion of cognitive impairment to Alzheimer's [22]. The mice were subjected elevated plus maze assay in order to study the behavioural changes. The number of entries into the arms taken by the EBV and $gM_{146-157}$ exposed groups were found to be significantly reduced. Mice in these groups also took longer time to cover the distance from the centre of the maze to any of the arm ends. Time spent at the arm ends was also found to be increased for these groups. Thus,

subjecting the mice to elevated maze manifested a significant increase in anxiety and depression like behaviour during and after the exposure to EBV and $gM_{146-157}$.

# CHAPTER 7

# Conclusion

The Epstein-Barr virus has been potent in gaining continuous attention for its mysterious involvement in various neurological diseases. There have been shreds of evidences suggesting the association of the virus with the AD yet the deeper mechanistic details were not very well defined. The present study deals with understanding the role of EBV and its derived $gM_{146-157}$ in the pathogenesis or progression of AD. Taking together the results have shown that incubating aggregates of $gM_{146-157}$ with $A\beta_{42}$ peptides leads to increased aggregation of $A\beta_{42}$. These aggregates are spherical structures and are found to be oligomeric in $A\beta_{42}$ samples. Incubation of $gM_{146-157}$ leads to an increase in these aggregates. Exposure of EBV and $gM_{146-157}$ can lead to the activation of an inflammatory cascade in neuronal cells. Reduced mitochondrial membrane potential was observed after the exposure of EBV and $gM_{146-157}$ at all the time points. It has also been observed that exposing the neuronal cells to EBV and $gM_{146-157}$ results in a significant rise in the protein level of APP and ApoE4. Further, intranasal administration of IMR-32 cells exposed to EBV and $gM1_{46-157}$ led to a prominent increase in the anxiety like behaviour in female Swiss albino mice. Conclusively, for the first time this study highlights the role of EBV and $gM_{146-157}$ in increasing the risk of the AD.

11)

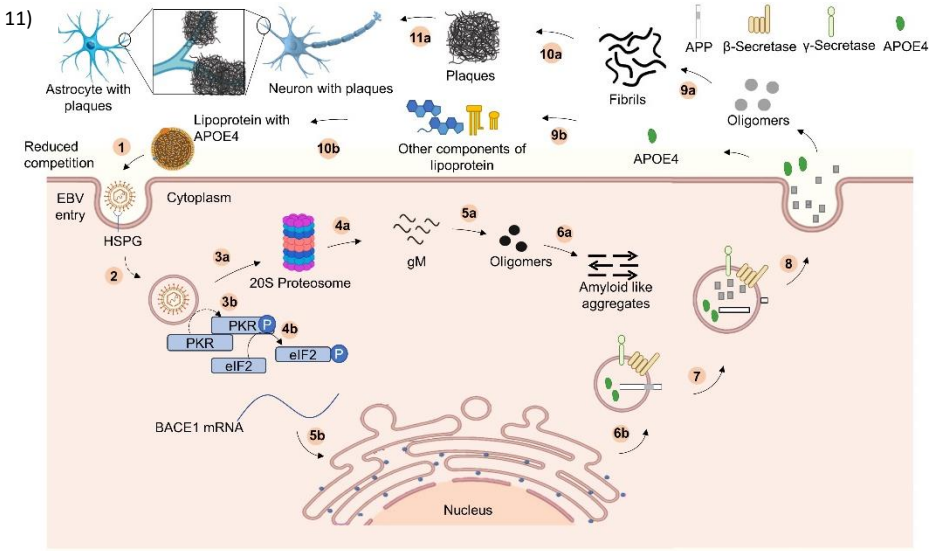



**Figure 11: Illustration of possible cascade followed by EBV after infection into neurons**

EBV possibly activates PKR which then undergoes autophosphorylation and activates eIF2 by phosphorylation. eIF2 positively influence translation of BACE1 mRNA leading to increase in amyloid fragment production. EBV infection increases production of ApoE4 which being a bad competitor might further increase virus entry into cell [83].

# CHAPTER 8

## Scope for future work

The study has tried to unravel the role of EBV in AD and the mechanism involved in the pathogenesis. There can be more studies done to understand these association *in-vivo*. Immunohistochemistry of the collected mice brain tissue against APP. There can be work done to understand the relation between EBV titre and stage specific progression of AD in clinical subjects. There can be experiments done to understand to role of ApoE4 in infection of EBV into neuronal cells. This can be done by creating knockdown for ApoE4 in neuronal cells, infecting them with EBV and quantifying the expression of viral genes. This can also be studied *in-vivo* by creating mice transgenic to ApoE4 which can be then compared with normal and ApoE3 transgenic mice. One can evaluate the change in expression of β- and γ- secretase as a result of exposure to EBV and $gM_{146-157}$. Evaluation of the antimicrobial property of $A\beta_{42}$ peptides against EBV and the restoration of APP levels as result of treatment with anti-viral drugs on EBV infected neuronal cells.

# ANNEXURES

| Sr. no | Genes | Primer Sequence |
|---|---|---|
| 1 | Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) | F: TGCACCACCAACTGCTTAG |
| | | R: GATGCAGGGATGATGTTC |
| 2 | Interleukin 1 beta (IL1β) | F: GGGCCTCAAGGAAAAGAATC |
| | | R: TTCTGCTTGAGAGGTGCTGA |
| 3 | Interleukin 6 (IL6) | F: TACCCCCAGGAGAAGATTCC |
| | | R: TTTTCTGCCAGTGCCTCTTT |
| 4 | Interleukin 10 (IL10) | F: TGCCTTCAGCAGAGTGAAGA |
| | | R: GGTCTTGGTTCTCAGCTTGG |
| 5 | Interleukin 4 (IL 4) | F: GCCACCATGAGAAGGACACT |
| | | R: ACTCTGGTTGGCTTCCTTCA |
| 6 | Tumor necrosis factor alpha (TNFα) | F: CAGAGGGCCTGTACCTCATC |
| | | R: GGAAGACCCCTCCCAGATAG |
| 7 | Transforming growth factor beta (TGFβ) | F: GGGACTATCCACCTGCAAGA |
| | | R: CCTCCTTGGCGTAGTAGTCG |

**Table 1: List of primers used in qRT-PCR**

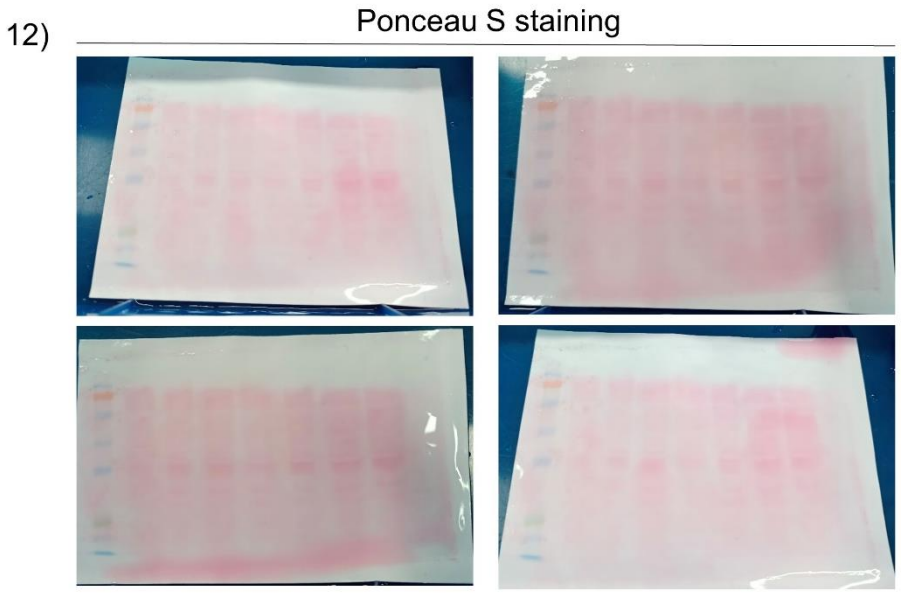



**Figure 12: Images of the blots after ponceau S staining**

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