THE ROLE OF COMPLEMENT SYSTEM COMPONENTS C5a AND C5aR IN AN ALZHEIMER CELL MODEL DEVELOPED FROM PROGENITOR NEURAL CELLS

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ABSTRACT

THE ROLE OF COMPLEMENT SYSTEM COMPONENTS C5a AND C5aR IN AN ALZHEIMER CELL MODEL DEVELOPED FROM PROGENITOR NEURAL CELLS

Alzheimer's disease (AD) is a neurodegenerative disease characterized by plaques and neurofibrillary tangles (NFT) resulting from the accumulation of amyloid beta peptides. According to data from the World Health Organization (WHO) (2020), there are currently more than 55 million people worldwide suffering from AD. The complement system, consisting of more than 25 proteins, is one of the important components of inflammatory responses. C5a, a complement system anaphylatoxin, is one of the inflammatory mediators of the innate immune system. C5aR1 (CD88), the classical receptor of C5a, is also known to play a role in neurodegenerative diseases. Demonstration of complement components and activation in the brain of Alzheimer's patients has led to various speculations regarding the role of this inflammatory pathway in neurodegenerative mechanisms. It is not clear how the role of the complement system affects the pathogenesis of the disease. The aim of this study was to determine how C5a caused a change in AD characteristics in an Alzheimer's cell model developed from human progenitor neural cells. There was no previous study on the complement system relationship with an Alzheimer's cell model developed from human progenitor neural cells. The effect on the presence and inhibition of C5a by exposing cells to C5a and then suppressing it with a C5a antagonist, PMX53, was examined in an Alzheimer's cell model developed in immunology lab of Yeditepe University. It was also analyzed whether the antagonist, PMX53, had an effect alone. Alzheimer's cell groups had much higher C5aR1 expression than the control group. When C5a was added to the cells, no significant difference was observed in the C5aR1 level compared to the Alzheimer group. When PMX53 was added, C5aR1 level decreased, but when C5a and PMX53 were added together, there was an increase in C5aR level. While some of the results were compatible with the literature, some were contradictory, but they proved that the complement system has a significant effect on Alzheimer's disease.

ÖZET

PROJENİTOR NÖRAL HÜCRELERDEN GELİŞTİRİLMİŞ BİR ALZHEIMER HÜCRE MODELİNDE KOMPLEMAN SİSTEM BİLEŞENLERİ C5a VE C5aR'NİN ROLÜ

Alzheimer hastalığı (AD) amiloid beta peptidlerinin birikmesi sonucu oluşan plaklar ve nörofibriler yumaklar (NFT) ile karakterize edilen nörodejeneratif bir hastalıktır. Dünya Sağlık Örgütü'nün (WHO) (2020) verilerine göre, şu anda dünya çapında AD'den muzdarip 55 milyondan fazla insan olduğu biliniyor. Kompleman sistem 25'ten fazla proteinden oluşan, inflamatuar yanıtların önemli bileşenlerinden biridir. Bir kompleman sistem anafilatoksini olan C5a'da, doğuştan gelen bağışıklık sisteminin inflamatuar aracılarından biridir. C5a'nın klasik reseptörü olan C5aR1 (CD88)'in de nörodejeneratif hastalıklarda rol oynadığı bilinmektedir. Alzheimer hastalarının beyninde kompleman bileşenlerinin ve aktivasyonunun gösterilmesi, bu inflamatuar yolağın nörodejeneratif mekanizmalarda rolüne ilişkin çeşitli spekülasyonlara neden olmuştur. Kompleman sistemin rolünün hastalık patogenezine etkisinin ne yönde olduğu net değildir. Bu çalışmanın amacı, insan progenitör nöral hücrelerden geliştirilen Alzheimer hücre modelinde C5a'nın AD karakteristik özellikleri üzerinde nasıl bir değişikliğe yol açtığını saptamaktı. Daha önce insan progenitör nöral hücrelerinden geliştirilen bir Alzheimer hücre modeliyle kompleman sistem ilişkisine dair bir yapılan bir çalışma bulunmuyordu. Alzheimer hücre modelinde hücreleri C5a'ya maruz bırakıp daha sonra bir C5a antagonisti olan PMX53 ile baskılayarak C5a varlığında ve inhibisyonunda nasıl bir etki oluşturduğuna bakıldı. Aynı zamanda antagonist olan PMX53'ün tek başına bir etkisi olup olmadığı da analiz edildi. Alzheimer hücre grupları kontrol grubuna göre çok daha fazla C5aR1 ifadesine sahipti. Hücrelere C5a eklendiğinde C5aR1 seviyesinde Alzheimer grubuna göre anlamlı bir fark gözlenmedi. PMX53 eklendiğinde ise C5aR1 seviyesinde düşüş görüldü fakat C5a ve PMX53 beraber eklendiğinde C5aR seviyesinde artış vardı. Sonuçların bazıları literatürle uyumlu iken bazıları çelişkili bulundu, fakat kompleman sistemin Alzheimer hastalığında önemli bir etkisi olduğunu kanıtlar nitelikteydi.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv

ABSTRACT v

ÖZET vi

TABLE OF CONTENTS vii

LIST OF FIGURES ix

LIST OF TABLES xi

LIST OF SYMBOLS/ABBREVIATION xii

1. Introduction 1

1.1. ALZHEIMER'S DISEASE 1

1.1.1. Overview 1

1.1.2. Alzheimer's Disease Hypotheses 2

1.1.3. Alzheimer's Causes and Risk Factors 4

1.2. COMPLEMENT SYSTEM 7

1.2.1. Overview 7

1.2.2. Complement System Pathways 8

1.2.3. Complement System Regulation 11

1.2.4. Complement Anaphylatoxins 13

2. LITERATURE REVIEW 16

2.1. RECENT RESEARCH ON THE ROLE OF THE COMPLEMENT SYSTEM IN ALZHEIMER'S DISEASE 16

3. AIM OF THE STUDY 18

4. MATERIAL AND METHOD 19

4.1. MATERIAL AND CHEMICALS 19

4.1.1. Instruments 19

4.1.2. Equipments 19

4.1.3. Solutions and Kits 20

4.2. METHODS 20

4.2.1. Culture of Rencell VM cells 21
4.2.2. Immunophenotyping with Flow Cytometry 23
4.2.3. Western Blot Testing 26
4.2.4. Confocal Microscopy 28
5. RESULTS 30
5.1. Culture Of Rencell Vm Cells 30
5.2. Detection Of Differentiation In Rencell Vm Cells 30
5.3. Analysis of the Effect of C5a and PMX53 on Cell Culture Model of Alzheimer's Disease 32
5.3.1. Effect of C5a and Its Inhibitor PMX53 on Viability in Cell Culture Model of Alzheimer's Disease 33
5.3.2. Effect of Complement Component C5a and its Inhibitor PMX53 on C5aR Expression in a Cell Culture Model of Alzheimer's Disease 33
5.3.3. Effect of Complement Component C5a and its Inhibitor PMX53 on Amyloid Precursor Protein, Amyloid Beta 40 and Amyloid Beta 42 Protein Levels 35
5.3.4. Effects of C5a and PMX53 application on p-tau isoforms in Alzheimer's Disease cell culture model created from Rencell VM cell line 37
5.3.5. Visualizing the effects of C5a and PMX53 in a Cell Culture Model of Alzheimer's Disease 37
6. LIMITATIONS 39
7. DISCUSSION 40
8. CONCLUSION 44
REFERENCES 45

LIST OF FIGURES

Figure 1.1. Alzheimer's disease risk factors…..……5

Figure 1.2. Alzheimer Disease Genetic Factors. Mutations in the APP, PSEN1 and PSEN2 genes alter APP processing and are important genes in the development of Alzheimer's disease. Even though mutations in the APOE, TREM2, and CD33 genes are more common than in other genes, the chance of developing AD in those who have these mutations is lower……..…..……..……..……..……..……..…….……7

Figure 1.3. Complement activation pathways…….....9

Figure 1.4. C1 complex. The C1 complex consists of the C1q molecule and the C1r and C1s zymogens. ….....….....9

Figure 1.5. Membrane attack complex (MAC)….....10

Figure 4.1. Methods used in this thesis project.....20

Figure 4.2. (a) Antibody titration results for C5aR. The best results were obtained with 0.25 µl of C5aR antibody.....24

Figure 4.3. Antibody titration results for (a) APP, (b) Aβ40 and (c) Aβ42. The best result were seen in all groups at a ratio of 1:1000.....24

Figure 4.4. Example western blot images. 28

Figure 5.1. Morphological monitoring of the differentiation process induced by the withdrawal of additives from the medium in the AD cell model. All photos were taken with JuLi imaging device (NanoEntek, South Korea) at 4X zoom. Cells gain neuronal morphology by the second day, and neuronal morphology becomes evident on the 5th and 7th days.....30

Figure 5.2. Morphological monitoring of the differentiation process induced by the withdrawal of additives from the medium in the AD cell model with a fluorescent filter. All photos were taken with JuLi imaging device (NanoEntek, South Korea) at 4X zoom.....31

Figure 5.3. Analysis of the effect of adding C5a peptide or PMX53 to the medium on viability of neurons. C5a peptide at all applied doses or PMX53 applied at IC50 dose did not significantly affect cellular viability.....32

Figure 5.4. Analysis of C5aR expression in the cellular model. **a.** Sample flow cytometry dials for analysis of C5aR levels in the control, Alzheimer's, C5a, PMX53, and C5a+PMX53 groups. **b.** Intergroup comparison of C5aR levels in control, Alzheimer's, C5a, PMX53 and C5a+PMX53 groups. Under normal conditions, cells do not express C5aR, whereas receptor expression is increased in Alzheimer's cell models. *p<0,05; ****p<0,0001; #p<0,05; ##p<0,01; ###p<0,001; ####p<0,0001.....33

Figure 5.5. Analysis of amyloid precursor protein (a), amyloid beta 40 (b) and amyloid beta 42 (c) protein levels in Control, AD, C5a, PMX53 and C5a+PMX53 groups.....34

Figure 5.6. Analysis of the levels of APP, aβ40 and aβ42 proteins between groups. *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001; #p<0,05; ##p<0,01; ###p<0,001; ####p<0,0001.....35

Figure 5.7. Effects of C5a and its antagonist PMX53 on p-tau isoforms in Alzheimer's disease cell culture model created from RenCell VM cell line. **a.** It caused an increase in Ser202/Thr205 p-tau isoform in both AD and C5a+PMX53 groups. **b.** No significant effect of C5a or PMX53 was detected in the Ser199 isoform. **c.** There was a significant increase in Thr181 isoform in all groups except PMX53. **d.** Example Western Blot images. . *p<0,05; **p<0,01; ***p<0,001; #p<0,05.....35

Figure 5.8. Analysis of aβ42 protein level and C5aR level by confocal microscopy. Cells were labeled with C5aR1 or aβ42 antibodies. The nucleus is distinguished by DAPI. Images were taken at 40X zoom.....37

LIST OF TABLES

Table 1.1. Complement anaphylatoxin functions.....15

Table 4.1. Cell viability measurement by spectrophotometer after addition of MTS to the cells.....22

Table 4.2. Experimental group.....22

Table 4.3. Median values for C5aR.....24

Table 4.4. Median values for APP, Aβ40 and Aβ42.....25

Table 4.5. Bovine serum albumin dilution chart provided by Thermo Fisher Scientific, Pierce™ BCA Protein Assay Kit.....27

LIST OF SYMBOLS/ABBREVIATION

Ach	Acetylcholine
AchE	Acetylcholinesterase
AD	Alzheimer Disease
APOE	Apolipoprotein E
APP	Amyloid Precursor Protein
APS	Ammonium persulfate
Aβ	Amyloid beta
BACE	β-site APP cleavage enzyme
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
C1-INH	C1 inhibitor
C4bp	C4-binding protein
C5a	Complement component 5
C5aR1	C5a receptor 1
C5aR2	C5a receptor 2
C5L2	C5a receptor-like 2
CD	Cluster of differentiation
CD33	Transmembrane receptor
CD35	Complement receptor 1
CD46	Complement regulatory protein
CD55	Complement decay-accelerating factor
CD59	Protectin
CD88	Anti-Complement receptor C5aR
CFH	Complement Factor H
CFI	Complement Factor 1
ChaT	Choline acetyltransferase
CNS	Central nervous system
CR1	Complement Receptor 1
CrrY	Complement Receptor 1-releated Gene / Protein Y
CVD	Cardiovascular disease
Cy3	Cyanine-3

DAF	Decay Accelerating Factor
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimetil sülfoksit
DNA	Deoksiribonükleik asit
DPBS	Dulbecco's phosphate-buffered saline
EGF	Epidermal Growth Factor
EOAD	Early-onset Alzheimer Disease
FITC	Fluorescein isothiocyanate
FS	Forward scatter
GFP	Green fluorescent protein
GPI	Glycosyl-phosphatidyl-inositol
GPR77	G protein-coupled receptor 77
H2O	Hydrogen dioxide
HBSS	Hanks' Balanced Salt Solution
HRP	Horseradish peroxidase
IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LOAD	Late-onset alzheimer disease
MAC	Membrane attack complex
MACIF	Membrane attack complex inhibitory factor
MASP	Mannan-binding lectin-associated serine protease
MBL	Mannose binding lectin
MCP	Membrane Cofactor Protein
MTS	Cell proliferation assay reagent
NaCI	Sodium chloride
NFT	Neurofibrillary tangles
NK	Natural killer

PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PHF	Paired helical filaments
PMX205	Complement C5a receptor antagonist
PMX53	Complement C5a receptor antagonist
PSEN	Presenilin
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulfate
SS	Side scatter
Tau	Tubulin associated unit
TBS-T	Tris-buffered saline-tween 20
TEMED	Tetramethylethylenediamine
TREM	Triggering receptor expressed on myeloid cells
VAchT	Vesicular acetylcholine transporter
WHO	World Health Organization

1. INTRODUCTION

Although the central nervous system has been thought to be a privileged organ in terms of immunity for many years, it actually contains many immune system components. Recent research has shown that both glia and nerve cells in the brain can produce immunoglobulins and complement system components. This suggests that it is not necessary to cross the blood-brain barrier for the development of inflammatory molecular mediators [1]. At the same time, the complement system, which is seen as an important component of inflammatory responses, is thought to have an important role in Alzheimer's disease. It has been shown that the C5a component formed after the activation of the complement system and its interaction with its receptor, C5aR (CD88), are associated with some neurodegenerative diseases, including Alzheimer's [2,3].

1.1. ALZHEIMER'S DISEASE

1.1.1. Overview

Dementia, conversationally called "dotage", is that the general name of disease that have an effect on daily life activities and cause amnesia [4]. The most common dementia subtype is Alzheimer's (60 percent of all dementias) [5]. Alzheimer's and other dementias are the seventh leading cause of death. According to the World Health Organization (WHO) (2020), there are currently more than 55 million people worldwide who suffer from AD [6]. This number is expected to increase as life expectancy increases worldwide [7]. Alzheimer's disease (AD), named after the German psychiatrist Alois Alzheimer, is a slowly progressive neurodegenerative disease that affects large areas of the cerebral cortex and hippocampus, characterized by neurotic plaques and neurofibrillary tangles as a result of amyloid-beta peptides [8,9].

The situation gets worse over time as Alzheimer's is a progressive disease. First of all, unnoticed changes begin to occur in the brain. These changes progress over time, resulting in some noticeable symptoms. The duration between the start of these brain alteration and the onset of symptoms is thought to be 20 years or more. Memory loss and language impairments are common symptoms that appear after years of these changes. Damage to

neurons in the areas of the brain responsible for cognitive function causes these symptoms [10].

1.1.2. Alzheimer's Disease Hypotheses

There are different hypotheses about the development of Alzheimer's Disease, which will be explained in detail.

1.1.2.1. Amyloid Hypothesis

For decades, it was established that abnormal β-sheet deposition in the central nervous system has a high association with dementia, giving rise to the amyloid hypothesis [9]. For more than 25 years, the amyloid hypothesis, also known as the amyloid cascade hypothesis or the Aβ hypothesis, has been regarded as the most recognized mechanism for the pathogenesis of AD [11].

Studies of proteolytic processing have revealed that Aβ is a normal product of APP (amyloid precursor protein) metabolism and is produced at high levels in neurons as well as other cell types throughout a person's lifespan. The amyloid precursor protein gene (APP) on chromosome 21 is known to be causally associated with early-onset autosomal dominant familial AD [12]. However, the neuronal function of APP is still unclear, although it is thought to have a role in synaptic plasticity [8].

APP is a 695 amino acid membrane protein that is cleaved in the amyloidogenic pathway by the β-site APP cleavage enzyme (BACE) and γ-secretase enzymes. BACE operates on the N-terminal domain of the Aβ sequence to process APP, whereas γ-secretase, a multiprotease complex that contains PSEN1/2, acts on the transmembrane domain of APP via endopeptidase/carboxypeptidase cleavages. As a result of γ-secretase-mediated cleavages in variable regions of APP, two different-length peptides Aβ-40 and Aβ-42 are formed, the most common forms in the brain [13].

According to the amyloid hypothesis, age or pathological conditions reduce the degradation of Aβ, which is produced from APP by β- and γ-secretase, resulting in the accumulation of Aβ peptides (Aβ40 and Aβ42) [9]. An increase in the Aβ42/Aβ40 ratio causes the formation

of Aβ amyloid fibrils, which causes neurotoxicity and tau pathology, eventually leading to neuronal cell death and neurodegeneration [9,11].

1.1.2.2. Tau-related Hypothesis

The Tau hypothesis is based on Tau (tubulin-associated unit), the main component of neurofibrillary tangles (NFT), one of the neuropathological features of AD [7,14]. The paired helicoidal filaments (PHF), which are fundamentally made of hyper-phosphorylated Tau, are the major components of NFTs [14].

Tau is a microtubule-associated protein found essentially in axon-rich neurons, where it acts as a scaffolding protein [7,15] and regulates tubulin group stability [11]. Tau is inappropriately phosphorylated in pathological situations, damaging the axons of neurons and resulting in neurodegeneration because tau is crucial for the maintenance of neuronal integrity and homeostasis [7,14,15].

Several lines of data support the idea that disruption of Tau homeostasis is a fundamental event in AD. Tau anomalies are present in various neurodegenerative conditions besides Alzheimer's, including frontotemporal dementia, cortico-basal degeneration, multiple system atrophy, and motor neuron disease. As a result, these disorders are known as tauopathies. Neuropathological investigations have shown that the evolution of NFT distribution in the brain coincides with the clinical course of cognitive deficits in Alzheimer's disease. Furthermore, intra-neuronal hyper-phosphorylated Tau has been observed in the brains of people with very mild dementia who do not have Aβ pathology. As a result, Tau hyper-phosphorylation may be the first step in the physiopathology of Alzheimer's disease; other pathological processes, such as abnormal APP metabolism leading to excessive Aβ synthesis, may be secondary to the disruption of neuronal homeostasis. Nonetheless, the larger body of evidence linking amyloid pathology in AD, as well as the lack of genetic mutations in the Tau gene associated with Early or Late-Onset AD, cast doubt on the concept that Tau pathology is the first event in AD [14].

1.1.2.3. Cholinergic Hypothesis

Acetylcholine is a neurotransmitter that is active throughout the cortex, basal ganglia, and basal forebrain [16,17). According to the "Cholinergic Hypothesis," disruption in cholinergic function is crucial in Alzheimer's disease, particularly in brain areas involved in learning,

memory, behavior, and emotional reactions, such as the neocortex and the hippocampus [18].

In the 1970s, neocortical and presynaptic cholinergic deficiencies were notified to be related the enzyme choline acetyltransferase (ChAT), which is responsible for acetylcholine synthesis (ACh) [14]. The most obvious clinical finding in Alzheimer's disease is brain atrophy, which occurs when the levels of acetylcholine (ACh), a neurotransmitter responsible for the conduction of electrical impulses from one nerve cell to another, are reduced due to rapid hydrolysis by the acetylcholinesterase (AChE) enzyme [18]. The ChAT enzyme synthesizes ACh in the cytoplasm of cholinergic neurons from choline and acetyl-coenzyme A and transports it to synaptic vesicles via the vesicular acetylcholine transporter (VAChT). ACh is involved in various physiological processes in the brain, including memory, attention, sensory information, learning, and other essential activities. Degeneration of cholinergic neurons has been discovered to occur in Alzheimer's disease, causing changes in cognitive function and memory loss [9].

1.1.3. Alzheimer’s Causes and Risk Factors

Alzheimer's disease has been identified as a complex disease with multiple risk factors including growing age, gender, genetic factors, head injuries, vascular diseases, infections, and environmental factors (10).

1.1.3.1.Age

Many factors contribute to neurodegeneration, but the most important risk factor is aging. Understanding the fundamental mechanisms of aging is critical for the initiation and progression of neurodegenerative disorders. Many neurodegenerative disorders, including the most common neurodegenerative diseases, Alzheimer's and Parkinson's, are more common in elderly [19].

The majority of those suffering from Alzheimer's disease are 65 or older. This condition affects only a small percentage of young people. Age is a significant risk factor for the establishment and progression of Alzheimer's disease [20].

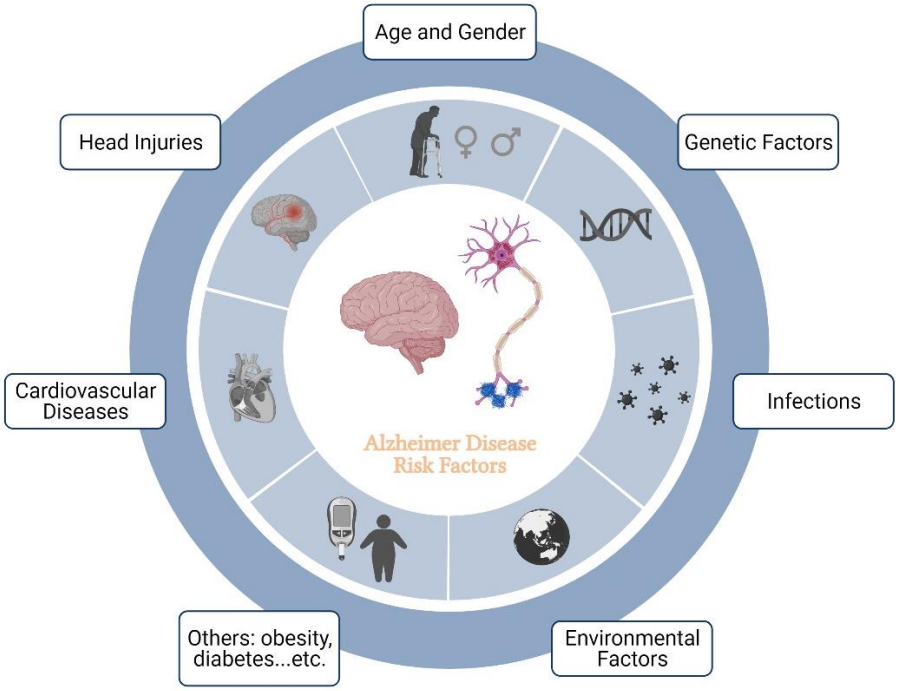

Figure 1.1. Alzheimer's disease risk factors [9]. Created with BioRender.com.

1.1.3.2.Environmental Factors

Environmental risk factors such as air pollution, nutrition, infections, metals, and many more can cause oxidative stress and inflammation, increasing the likelihood of developing Alzheimer's disease [21,22]. There is a relationship between oxidative stress, neuroinflammation, and neurodegeneration in persons exposed to air pollution, as well as the existence of hyper-phosphorylated tau and Aβ diffuse plaques in the frontal cortex. Air pollution can lead to an increase in Aβ42 production and accumulation, as well as reduced cognitive performance [23].

Increasing studies in recent years support that nutrition and diet are important risk factors in AD. A variety of dietary components, including antioxidants, vitamins, polyphenols, and fish, have been shown to reduce the incidence of Alzheimer's disease, while saturated fatty acids, high-calorie intake, and excessive alcohol use have been identified as risk factors [24]. Also, by participating in the construction of cellular proteins such as APP and Aβ, metal ions such as zinc and copper can aggravate Alzheimer's disease. Additionally, metal ions may interact with many additional AD-related processes, including as those involved in

neurofibrillary tangle formation, APP secretase cleavage, and Aβ proteolytic degradation [25]. In addition, chronic infections of the central nervous system (CNS) can lead to the formation of Aβ plaques and NFT, making them risk factors for Alzheimer's disease [26].

1.1.3.3. Genetics

The presence of Alzheimer's disease in a first-degree relative significantly increases a person's risk of developing AD. For this reason, it has been determined that genetic variables have an important effect on the development of AD. Many genes are known to increase the likelihood of developing dementia. These identified genes provide a better understanding of the biological processes of the disease. There are two types of AD genetics. The first is Early-Onset AD (EOAD), which occurs before the age of 65, and the second is Late-Onset AD (LOAD), which is more prevalent [27]. Late-Onset AD does not exhibit a consistent inheritance pattern [28]. Amyloid precursor protein (APP), Presenilin-1 (PSEN-1) and Presenilin-2 (PSEN-2) are genes linked to Early Onset AD, but Apolipoprotein E (APOE) is linked to both Early Onset AD and Late-Onset AD [29].

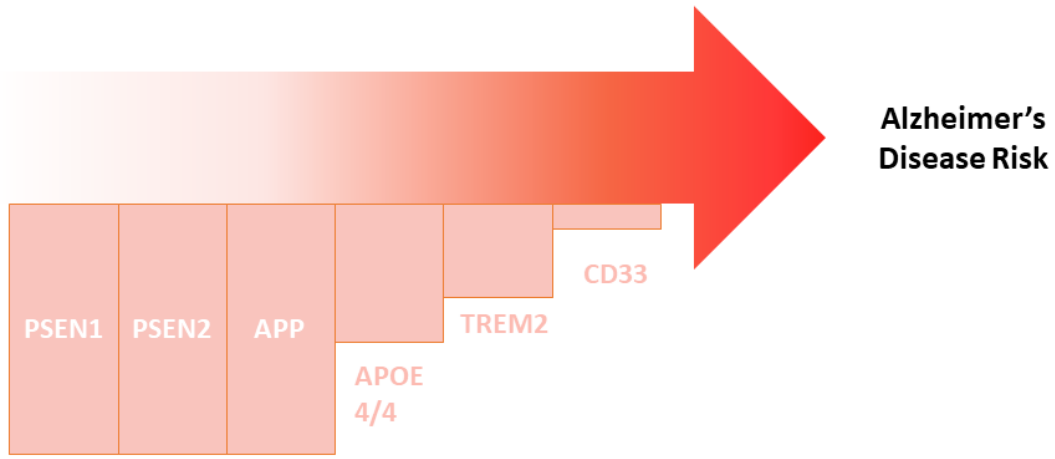

Figure 1.2. Alzheimer Disease Genetic Factors. Mutations in the APP, PSEN1 and PSEN2 genes alter APP processing and are important genes in the development of Alzheimer's disease. Even though mutations in the APOE, TREM2, and CD33 genes are more common than in other genes, the chance of developing AD in those who have these mutations is lower [30].

1.1.3.4. Other Medical Conditions

To add to this list, older people with Alzheimer's disease (AD) frequently have medical disorders such as cardiovascular disease (CVD), obesity, diabetes, and others. All of these medical disorders enhance the likelihood of acquiring Alzheimer's [9].

1.2. COMPLEMENT SYSTEM

1.2.1. Overview

Complement is a component of the innate immune system that reacts with each other to opsonize pathogens in the body and serves as the body's initial line of defense. Plasma proteins produced mostly by the liver or membrane proteins expressed on cell surfaces make up the complement system [31]. In plasma and on cell surfaces, the complement system contains over 30 proteins [32].

The complement system has three physiological activities in terms of immunological homeostasis. To begin with, this system is an important component of innate immunity, serving as the initial line of defense against infections and non-microbial stressors. Secondly, it serves as a link between the innate and adaptive immune systems and third, immune complexes and inflammatory damage products are excreted, promoting immune surveillance [32,33].

The complement system can be activated in three ways: classical, lectin, or alternative pathway. Although it begins in three different ways, they all lead to the same terminal channel (Figure 3) [31].

The classical pathway was the first complement pathway discovered and is activated by binding of the antibody to the cell surface and ends with the lysis of the same cell. The components of the classical pathway are denoted by the letter “C” followed by a number. Components are called from C1 to C9, although this is based on the order in which they were discovered rather than the reaction sequence. In the alternative pathway, which is the second pathway discovered, proteins are called factors and different capital letters are used instead of numbers (e.g. Factor B, Factor D). While the system is activated in both the classical

pathway and the alternative pathway, the complement proteins are cleaved and lowercase "a" and lowercase "b" letters are added to the cleavage products. The large piece is denoted by the letter b and the small piece by the letter a (e.g. C3a/C3b or Bb/Ba). This condition is shown exactly the opposite in C2 as an uncommon situation. C2a is the large piece and C2b is the small piece. Finally, in the lectin pathway, the first enzymes to be activated are known as MASP-1 (mannan-binding lectin-associated serine protease-1) and MASP-2 (mannan-binding lectin-associated serine protease-2). After these first enzymes, the same continues in the classical way [32].

1.2.2. Complement System Pathways

1.2.2.1. Classical Pathway

The classical pathway is involved in both innate and adaptive immunity. The classical pathway is initiated by a single IgM or multiple IgGs that complex to an antigen or pathogen-associated molecular model (PAMP). IgG1, IgG2, IgG3, and IgM are the IgGs that activate the classical pathway. IgG4, IgA, IgD, and IgE are unable to bind or activate complement.

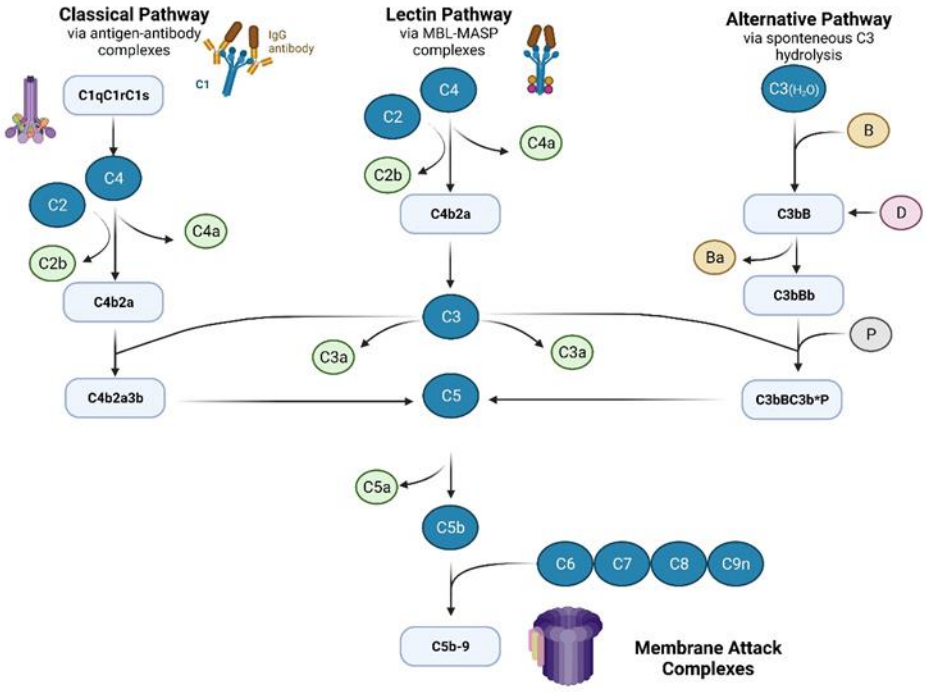

Figure 1.3. Complement activation pathways [40]. Created with BioRender.com

While more than one IgG is required for complement activation, a single IgM is sufficient for this activation [34].

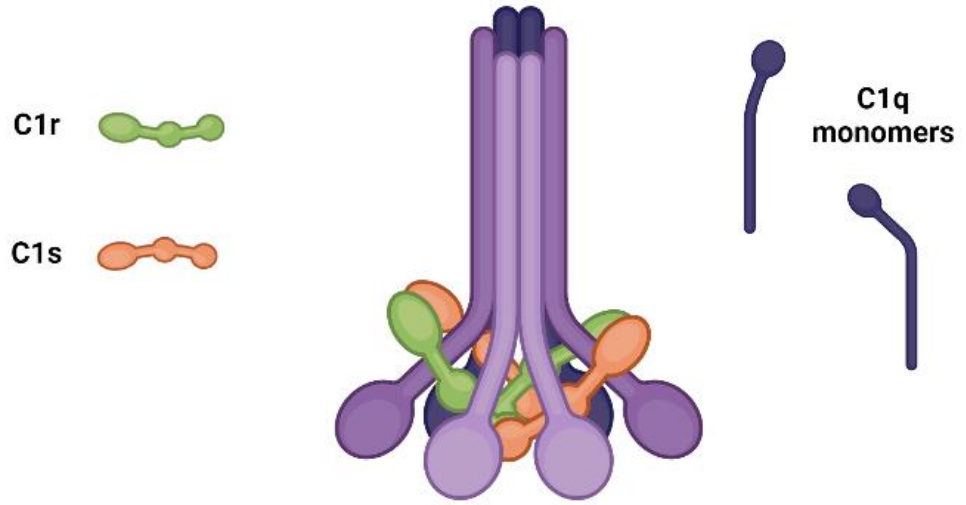

Figure 1.4. C1 complex. The C1 complex consists of the C1q molecule and the C1r and C1s zymogens. Created with BioRender.com

C1q, the first component of this pathway, binds to antibodies that form complexes with antigens, linking the adaptive humoral immune response to the complement system. C1q is part of the C1 complex, which consists of a single C1q molecule attached to two molecules of the C1r and C1s zymogens. However, C1q consists of 6 tail and head parts. The heads can bind directly to the constant Fc regions of immunoglobulin molecules or to the surface of certain pathogens, thereby triggering complement activation in the absence of antibodies [35]. This changes the conformation of C1r, which then cleaves and activates the C1s zymogen. When active, the C1s enzyme acts on the classical pathway's following two components, C4 and C2. To put it another way, the C1 enzyme degrades C4 and C2. As a result of this fragmentation, C3 convertase is formed by two big fragments, C4b and C2a. The cleavage products combine to form the C4b2a complex. This complex remains on the surface of the pathogen as C3 convertase of the classical pathway. The most important activity of this complex is to cleave large numbers of C3 molecules to produce C3b molecules covering the pathogen surface. C3b then joins the C4b2a complex. The other cleavage product, C3a, is an anaphylatoxin and initiates a local inflammatory response. When C3b joins C4b2a, the C4b2a3b complex is created. This complex is also responsible for the formation of C5 convertase. In other words, the enzyme that breaks down C5 into C5a and C5b is formed. As with the previous model, the newly formed C5 convertase cleaves

C5 into C5a and C5b. As with C3, C5a is an anaphylatoxin. C5b is the initiator of the membrane attack complex called MAC. (figure.) Then C6 is quickly added to the accumulated C5b. Following that, C7, C8 are added, and finally C9 is attached, creating an asymmetrical pore that leads to targeted fragmentation of the pathogen [33,34].

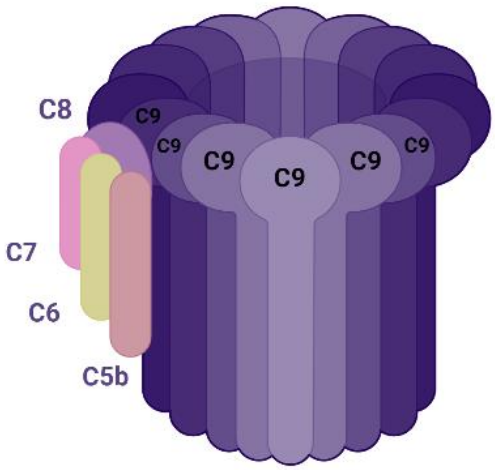

Figure. Membrane attack complex (MAC). Created with BioRender.com.

1.2.2.2. Alternative Pathway

The reason why this pathway is called the alternative pathway is that it was discovered as an 'alternative' pathway for complement activation after the classical pathway was defined. Antigen recognition is not required for activation of this pathway. C1, C2 and C4 components are not active in this pathway. This pathway is initiated by the spontaneous hydrolysis of C3. It is automatically activated through the hydrolysis of a disulfide bond on a complete C3 protein to form C3 (H_2O). Factor B binds to the hydrolyzed C3 formed. Binding of factor B then allows a plasma protease called factor D. The factor then splits B into Ba and Bb, and Bb remains associated with C3 (H_2O) to form the C3Bb complex. This complex is a liquid phase C3 convertase. Although in small amounts, it can degrade many C3 molecules into C3a and C3b. This alternative pathway results in the formation of C3 convertase, C3bBb. This complex is stabilized by another serum protein, properdin. In the alternative pathway, C5 convertase continues to cleave another C3 molecule forming C3bBbC3b. The alternative pathway C5 convertase also cleaves C5 into C5a and C5b. Because the alternative pathway is antigen-independent, it often functions as an amplification loop for the classical and lectin pathway. Despite changes in convertase

molecular assembly, all three pathways lead to the same terminal pathway after C5 cleavage to produce MAC [37,38].

1.2.2.3. Mannose-Binding Lectin (MBL) Pathway

A protein very similar to C1q called the mannose-binding lectin (this protein may also be ficolin) binds to their surface and initiates complement activation. MBL or ficolin associates with a number of monosaccharides, similar to C1q-IgM recognition. When MBL binds target carbohydrates, the lectin pathway proteases MASP-1 and MASP-2 are activated sequentially by MASP-1 autoactivation followed by MASP-2 activation. When this MBL complex binds to a pathogen surface, MASP-1 and MASP-2 are activated to cleave C4 and C2. Thus, the MB-lectin pathway initiates complement activation in the same way as the classical pathway. Parts C4 and C2 are divided into C2b/C2a and C4b/C4a as in the classical pathway. C3 convertase is formed when C2a and C4b join [37,39]. From here, it takes place in the same way as the classical pathway.

1.2.3. Complement System Regulation

Complement system activation is controlled by several regulatory proteins. Complement system regulators are divided into membrane-bound complement regulators and fluid phase complement regulators [41].

1.2.3.1. Membrane-bound Complement Regulators

CD35 (Complement Receptor 1 [CR1])

CD35 (complement receptor 1 [CR1]) is a 190-kDa transmembrane glycoprotein found in both classical and alternative pathways [37,42]. Human CR1 is expressed in leukocytes and podocytes in the glomeruli of the kidney, B cells, erythrocyte surface, follicular dendritic cells, polymorphonuclear cells and phagocytic macrophages. CD35 accelerates the degradation of the C3/C5 converter and acts as a cofactor for factor 1 in C3b and C4b degradation [37,38,42]. CD35 also functions as an immunological adhesion receptor and is thought to influence the development of autoimmunity [37,43].

CD46 (Membrane Cofactor Protein [MCP])

Membrane cofactor protein, commonly known as complement inhibitor, is a transmembrane glycoprotein with a molecular weight of 51-68 kDa [44–46]. Except for erythrocytes, CD46

is expressed in all nucleated cell types. It also acts as a cofactor for factor 1-mediated cleavage of C3b and C4b [38,44,46]. This regulator is more widely expressed than CD35. This has an important role in protecting the host cell from excessive complement activation. [45].

It is implicated in the down-modulation of adaptive T helper type 1 immune responses by modulating the production of interferon (IFN)- and interleukin (IL)-10 in T helper cells. CD46 deficiency is a risk factor for a variety of diseases caused by complement-mediated 'self-attack.' [37].

CD55 (Decay Accelerating Factor [DAF])

DAF is a cell membrane anchored glycosyl-phosphatidyl-inositol (GPI)-associated 70 kDa glycoprotein that is widely expressed except for natural killer cells (NK) and a specific subset of T cells [37,38,44]. The main role of CD55 is to prevent the formation of C3 and C5 converters and accelerate the degradation of pre-existing C3 and C5 converters. Thus, activation of classical and alternative C3 and C5 converters is prevented [38,47].

CD59 (Protectin)

CD59, also known as membrane attack complex inhibitory factor (MACIF), is an 18-25 kDa GPI-linked glycoprotein. Protectin is expressed in nearly all human cells and tissues (circulating blood cell, endothelial cell, most epithelial cells) [37,44,48]. Protectin/CD59 is also found in neural tissues apart from these cells. In a previous study, its expression has been demonstrated to be enhanced in regions of deteriorated brain tissue in Alzheimer's disease [44,49].

CrrY (Complement Receptor 1-releated Gene / Protein Y)

CrrY is a transmembrane protein widely expressed in rodents to compensate for deficient expression of CD55 and CD46. CrrY mimics the actions of DAF and MCP in human cells, which control C3 accumulation [37].

1.2.3.2. Fluid-phase complement regulators

C1 Inhibitor (C1-INH)

C1-INH is a 105 kDa serine protease inhibitor. C1-INH is secreted from hepatocytes, macrophages, fibroblasts, platelets, magakaryocytes, and endothelial cells. It acts on the classical pathway by preventing the C1 complex from interacting with each other. For this

reason, although it is normally seen as a regulator of the classical pathway, some studies show that it also acts on the mannose-bound lectin pathway by binding to MASP-1 and MASP-2 [50].

Complement Factor I (CFI)

CFI, a serine protease, is a regulator of 88 kDa found in all three complement pathways [51]. CFI is secreted by hepatocytes, macrophages, lymphocytes, endothelial cells, fibroblasts, and skeletal myoblasts [52]. It inhibits the synthesis of C3 and C5 convertase enzymes [50].

Complement Factor H (CFH)

CHF is a major regulator of the alternative complement pathway. They bind to sialic acid, glycosaminoglycans, and heparin-containing surfaces. They can discriminate between host and foreign surfaces by acting on both soluble and membrane-bound proteins in this way. Since it contains several C3b binding sites, it prevents factor B from binding and forming C3 convertase [50].

C4-binding protein (C4bp)

C4bp is a regulator of both the classical and lectin pathway. It binds to C4b and causes the C3 convertase to degrade faster [37,53]. It is synthesized mostly in the liver and less frequently by activated monocytes [54].

1.2.4. Complement Anaphylatoxins

Anaphylatoxins, also known as C3a, C5a, and C4a molecules, are polypeptides generated when the complement system is activated. Anaphylatoxins play a significant part in the host defense system since they have a wide variety of biological actions. They are also described as immunomodulators, as they have the ability to modulate the humoral and cellular immune response. These immunomodulators are also potent inflammatory mediators that induce multiple inflammatory responses [55,56].

The anaphylatoxins are released when their parent compounds (C3, C4 and C5) are activated. The C3 convertases of the classical or alternative pathways generate C3a directly, but considerable C5 cleavage and the release of C5a require the participation of the surface-fixed activation fragment of C3, C3b. As a result, C5a formation is only conceivable after C3 activation and does not always imply that C3a has also been generated. C4a is exclusively

produced by active C1 during classical pathway activation. C4a is extremely similar to C3a, functioning on the same receptors, and is most likely of modest significance due to its lack of biological action [57]. Since this research study is focused on C5a and its receptor C5aR1, we will focus on them rather than all of the anaphylatoxins.

1.2.4.1. C5a Anaphylatoxins

As is known, the C5 protein is one of the key components in the complement system. The C5 protein is cleaved to C5a and C5b by C5 convertase [58]. C5a is a 74 amino acid protein and an inflammatory anaphylatoxin produced by activated neutrophils, macrophages, platelets and coagulation cascade proteins [59]. The complement factor C5a is a prominent effector of all complement activation pathways, recruiting and activating inflammatory cells and causing significant immunoinflammatory consequences. C5a is also generated locally in the CNS, and the C5aR (CD88) is found on neurons and glia. This anaphylatoxin has been implicated in the pathophysiology of neurodegenerative illnesses in vivo, which is consistent with its presence in the CNS [60,61]. C5a has two different receptors, C5aR1 (CD88) and C5L2 (GPR77) [62].

Based on this background information literature was searched as explained in next section.

C5a Receptors

C5a works by activating high-affinity C5a receptors (C5aR and C5L2). C5aR is a G-protein-coupled receptor with seven transmembrane segments that belongs to the rhodopsin family; C5L2 is similar but not G-protein-coupled. C5a binds to C5L2 with strong affinity, however there have been little biological reactions to this interaction. C5L2 is found on neutrophils as well as immature dendritic cells. C5aR expression was first discovered in myeloid cells such as neutrophils, eosinophils, basophils, and monocytes. C5aR has recently been discovered on a range of nonmyeloid cells in several organs, including the lung and liver. C5aR expression is widely upregulated after the start of sepsis, implying the relevance of C5a/C5aR signaling in the pathogenesis of sepsis-induced inflammation and the development of multiple organ failure (MOF) [61].

Table 1.1. Complement anaphylatoxin functions [63].

Function	**C3a**	**C4a**	**C5a**
Defined receptor	+	-	+
Chemotaxis	+	?	+
Smooth-muscle contraction	+	?	
Vascular permeability	+	?	+
Myeloid cell activation	+	-	+
Platelet activation	+	-	+
CD4/CD8 and γδ T cell modulation	+	-	+
Induction of acute phase response (cytokine production)	+	-	+
Developmental homing	+	-	+
Regeneration	+	-	+
Antimicrobial	+	+	-
Cross-desensitization		?	

2. LITERATURE REVIEW

2.1. RECENT RESEARCH ON THE ROLE OF THE COMPLEMENT SYSTEM IN ALZHEIMER'S DISEASE

The complement system is thought to play a role in neurological diseases such as Alzheimer's. However, the complement system's function in these disorders appears to be rather complicated. According to research, the complement system has both harmful and beneficial impacts on neurological illnesses [63]. Studies have shown that anaphylatoxins are produced during most pathological events in the CNS. Animal models are a valuable tool for studying the role of the complement system in disease pathogenesis and processes. C5a, the complement activation product, has been the subject of numerous recent CNS research since it was discovered that C5a receptors are present in the brain and that CNS cells respond to C5a [63].

C5/C5a loss in mice rendered animals resistant to cerebral malaria and significantly decreased neutrophil recruitment and CNS damage in a traumatic brain injury model, according to one of these investigations. This supports the idea that C5a may increase inflammation in the CNS through mononuclear phagocytes or infiltrating neutrophils. Another research found that therapy with medications that suppress complement system activation, such as heparin and enoxaparin, reduced Aβ accumulation and astrocyte activation in the AD animal model. This result shows that inhibition of the complement system may cause a decrease in AD pathology [64].

The complement system may contribute to the etiology of Alzheimer's disease by activating glia. Another study aimed to provide further evidence of complement involvement by switching an AD mouse model to a C1q knockout to generate an AD mouse incapable of activating the classical complement pathway. These C1q-deficient mice had an increase in neuronal integrity and a significant decrease in glial activation, unlike the other control mice. Because C5a is chemotactic for microglia and astrocytes, it is hypothesized to be a key signal for glial recruitment surrounding Aβ plaques in AD. The increased area of inflammation around the plaque may then cause neurotoxicity and subsequent symptomatic cognitive loss of AD [65]. In addition to all these, local inflammation is thought to play a role in the

destruction of brain neurons in Alzheimer's disease. It has been shown that the classical pathway of the complement system can be activated by β-amyloid deposits and neurofibrillary tangles, especially in the AD brain. At the same time, it has been determined that all components in the classical pathway are found in the neurons of the AD brain. In addition, it has been stated that the alternative complement pathway is activated. Membrane attack complex (MAC), the end product of the system, was also discovered in the neuronal membranes of AD brains. After C5 separates into two by C5 convertase and the production of C5a and C5b, MAC formation begins via C5b. Therefore, the finding of MAC in AD brain supports the idea that C5a is also released in the brain [66].

C5aR (C5a receptors) have been detected in human and rodent hippocampal pyramidal and granular cells, cortical pyramidal neurons, oligodendrocytes, astrocytes, and microglia, as well as human neuroblastoma cells [66]. The C5a receptor antagonist PMX-205, a different derivative of PMX-53, has been shown to reduce disease activity in various animal models of CNS disease [55,67]. PMX-205 was demonstrated to significantly reduce fibrillar Ab and inflammatory glia in the cortex and hippocampus of mice. These pathological changes suggest that cognitive performance in the disease has a positive effect. All these observations provide important evidence for the role of C5aR/CD88 in AD. These results show that inhibition of complement receptors in complement-mediated inflammation can reduce neuroinflammation and can be used as a therapeutic tool in other neurodegenerative diseases mediated by complement-mediated inflammation [55].

Based on the limited information from previous studies, we have decided to explore the role of C5a-C5aR1 interaction in cellular model of AD developed by Başak Aru, Immunology Department, Faculty of Medicine, Yeditepe University.

3. AIM OF THE STUDY

In the studies conducted so far, changes in the complement system components in Alzheimer's disease have been detected, and these changes have been explored and observed mostly on animal models. However, it is not clear whether the effects of these changes are related to pathogenesis of the disease. The results of the studies conducted contradict each other on this issue. However, none of the studies evaluating the role of complement system components in Alzheimer's have been performed on cultured human progenitor neural cells. Here, we aim to reveal if there is a change in amyloid plaques in the presence and absence of C5a in the Alzheimer's cell model developed from human progenitor neural cells (Model developed from ReNCell VM cell line in Immunology Laboratories of Faculty of Medicine, Yeditepe University. This project was supported within the scope of TUBITAK ARDEB 1001 program. [Project Number: 217S663]).

4. MATERIAL AND METHOD

4.1. MATERIAL AND CHEMICALS

4.1.1. Instruments

Centrifuge (HITACHI, CT6EL), Indonesia

Mini Centrifuge (Isolab 7000rpm), Germany

+4°C refrigerator (Arcelik), Turkey

+4°C/-20°C refrigerator (Arcelik), Turkey

-80°C freezer (ESCO Lexicon ULT Freezer), USA

Mini shaker (IKA MS2),

Spectrophotometer (BioTek Epoch), USA

Inverted Microscope (Olympus CKX41), USA

Class II Type A2 Laminar Air Flow (ESCO, LA2-4A1),

Galaxy 170 S incubator (New Brunswick, 272007456),

DxFLEX Flow Cytometry System (Beckman Coulter), USA

JuLI Br Imaging System (NanoEnTek, JULI FL), Korea

Western Blotting system (Bio-Rad),

Confocal Microscopy System (Zeiss LSM780)

4.1.2. Equipments

12x75 mm flow tubes (Beckman Coulter),

Micro pipettes 1000 µl (Thermo Scientific), 200 µl (Thermo Scientific), 20 µl (Gilson), 10 µl (Gilson),

Centrifuge Tubes, 15mL (BluCAPP), 50 mL (BluCAPP, Falcon),

Serelogical pipettes 5 mL (CAPP Harmony), 10 mL (CAPP Harmony), 25 mL (CAPP Harmony),

Tissue Culture Flasks 25 - 300 cm^2 (TPP), Cell culture dish 60 mm (TPP, SPL)

4.1.3. Solutions and Kits

Cell culture reagents (Dulbecco's Phosphate Buffered Saline Solution – DPBS),

DMEM/F12 without HEPES (Thermo Fisher Scientific 11320074), Laminin (Sigma L2020-1MG), Complement Component C5aR Antibody (Novus NB100-66539),

Recombinant C5a protein (Sino Biological, 10604-HNAE), RenCell maintenance medium (Merck SCM005), PMX53 (Sigma 5336830001),

Goat anti-mouse seconder antibody-Cy3 (Thermo Fisher Scientific A10521).

4.2. METHODS

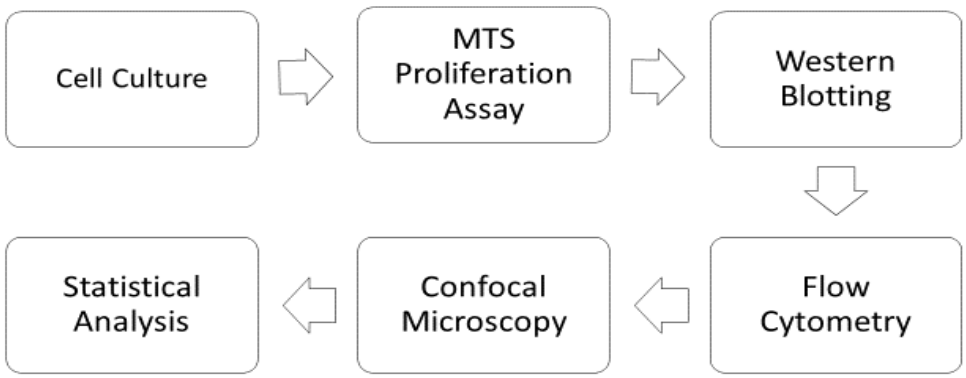

Figure 4.1. Methods used in this thesis project.

4.2.1. Culture of Rencell VM cells

These cells were produced and characterized for the project numbered 217S663, which was previously supported under the TÜBİTAK ARDEB 1001 program. All cell culture procedures were conducted according to the manufacturer's recommendations.

In order to reproduce the cells, flasks coated with laminin (Sigma #L2020-1MG) and a 96-well plate were used, and the cells were cultured with RenCell Maintenance Media (Merck #SCM005) as the medium. The seeding density of the cells is 300,000 cells per well in a 96 plate and 600,000 cells in a T25 flask. Viability is checked with trypan blue before seeding. The medium was supplemented with bFGF and EGF growth factors at a final concentration of 20 ng/mL in order for the cells to maintain their progenitor properties and proliferate. Neuronal differentiation was induced by removal of bFGF and EGF additives from the medium. All media, including the medium of control cells, were changed every other day during cell growth and during the 2-week differentiation period.

4.2.1.1.C5a and PMX53 dose determination in RenCell VM cells

The ideal dose at the end of the neuronal differentiation period was determined as 0.1 ng/mL, 1 ng/mL, 10 ng/mL, 100 ng/mL as 4 doses for C5a (Sino Biological, #10604-HNAE) and 20 nM for PMX53 (Sigma #5336830001) as a result of the literature search. PMX53 is a potent C5a receptor antagonist (IC_{50} = 20 nM). Viability was measured the MTS method, which is a colorimetric method, by applying the media prepared with these determined dose intervals to the cells. Cells treated with medium containing solvent (DMSO) for PMX53, and medium containing solvent (PBS) for C5a at equal concentrations with substance doses were used as control. After 2 hours, the viability was measured in the spectrophotometer at a wavelength of 490nm. At the end of the 24th and 48th hours, the medium in the wells was emptied and the medium containing 10 percent MTS was added. As a result of the viability measurement, 100 ng/mL was chosen for C5a for use in further studies, and it was decided to continue with the 20 nM recommended by the manufacturer for PMX53.

Table 4.1. Cell viability measurement by spectrophotometer after addition of MTS to the cells.

24 hours					48 hours				
0.1	**1**	**10**	**100**	**PMX53**	**0.1**	**1**	**10**	**100**	**PMX53**
109,59	91,92	87,22	93,98	100,19	113,75	105,09	105,09	106,82	115,48
98,31	88,16	89,66	99,62	100,19	121,62	117,05	103,36	109,82	111,55
94,55	96,99	93,42	96,24	99,62	118,94	122,09	110,44	109,82	112,96

4.2.1.2.Analysis of the effect of identified substances on RenCell VM cells

Geltrex ECM substrate was used to reflect the extracellular matrix in the differentiation process of RenCell VM cells. In the study, as recommended by the manufacturer, Geltrex was diluted 1/100 in DMEM/F12 medium and applied to 15 culture petri dishes (60 mm). At the end of the overnight incubation, they were removed from the petri dishes and the cells were seeded into petri dishes at 500,000 cells per petri dish in RenCell growth medium containing EGF and bFGF. After the cells were adhered, they were replaced with medium without EGF and bFGF.

4.2.1.3.Addition of C5a and PMX53 to test groups

At the end of the 1-week differentiation period, chemicals were added to each group;

For the C5a group, 3 mL of medium containing C5a was added to 3 plates. (max. concentration 100 ng/mL),

For the PMX53 group, 3 mL of medium containing PMX53 was added to 3 dishes. (20 nM concentration),

For the C5a+PMX53 group, 3 mL of medium containing C5a+PMX53 was added to 3 plates.

The culture media were freshly prepared and changed to contain the same proportions of substance. The medium of the control group and AD control group were also changed every two days with RenCell VM medium.

Table 4.2. Experimental group

Control group	RenCell VM cells without chemicals
Alzheimer's group	Neurons of Alzheimer's model developed from RenCell VM cells without chemicals
C5a group	Addition of C5a at a concentration of 100 ng/mL to the AD model neurons
PMX53 group	Addition of PMX53 at a concentration of 20 nM to the AD model neurons
C5a+PMX53 group	Addition of C5a and PMX53 together to the AD model neurons

4.2.2. Immunophenotyping with Flow Cytometry

Flow cytometry is a technique for swiftly analyzing single cells or particles that are suspended in a buffered salt-based solution and flow through single or multiple lasers. Each particle is examined for visible light scatter as well as one or more fluorescence characteristics. Visible light scatter is assessed in two directions: forward (Forward Scatter or FSC) to show the relative size of the cell, and at 90° (Side Scatter or SSC) to reveal the internal complexity or granularity of the cell. Light scattering is unaffected by fluorescence. Transfection and production of fluorescent proteins (for example, Green Fluorescent Protein, GFP), staining with fluorescent dyes (e.g., Propidium Iodide, DNA), or staining with fluorescently attached antibodies are used to prepare samples for fluorescence measurement (e.g., CD3 FITC). Flow cytometry is a strong instrument with applications in immunology, virology, molecular biology, cancer biology, and infectious disease surveillance (69).

4.2.2.1.Antibody Titration Test

The ideal antibody usage dose was determined by performing the antibody titration test. First, the cells were removed and centrifuged, and the remaining pellet was suspended with isoflow. The suspended cell solution was placed in the tubes. 3 different doses (1µl, 0.5µl, 0.25µl) of anti-C5aR (Novus #NB100-66539, clone P12/1) or anti-APP (Cell Signaling Technology, #2450S) together with anti-aβ40 (Cell Signaling Technology, #12990S) and anti-aβ42 (Cell Signaling Technology, #149748S) (1:500, 1:1000 and 1:2000) were added to the tubes and incubated for 1 hour. Anti-mouse secondary antibody - Cy3 (Thermo Fisher Scientific, #A10521) was added as a secondary antibody for C5aR. After PE Anti-rabbit

(Abcam, #ab7007) and Alexa Fluor 647 anti-mouse (Cell Signaling Technology, #4410) secondary antibodies were used to label APP and aβ40 or aβ42 antibodies after 1 hour of incubation period, analysis was performed in a flow cytometry system (Figure 4.2.). The rate of C5aR at 0.5 µl dose was not significant. The best results were seen at 0.25 µl in all groups (Table 4.3.). For APP, aβ40 and aβ42, the best results were seen in all groups at a ratio of 1:1000 (Figure 4.3. and Table 4.4.)

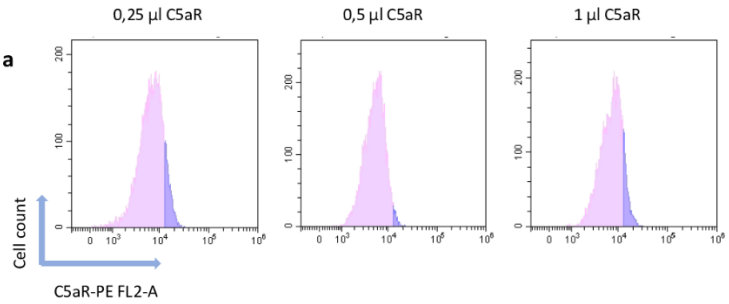

Figure 4.2. (a) Antibody titration results for C5aR antibody. Optimum concentration was with 0.25 µl for C5aR antibody.

Table 4.3. Median values for C5aR.

	Median Value		
	0,25 µl	**0,5 µl**	**1 µl**
C5aR	14867.1	14152.7	14439.7

4.2.2.2.Analysis of C5aR level by flow cytometry

The media of cells in 60 mm dishes were collected and the dishes were washed with PBS. Cells were incubated with 1 ml of accutase for 1 min and removed. The collected cells were centrifuged and the supernatant was poured, and the cells at the bottom were suspended with DPBS and transferred to flow tubes. 0.25 µl of C5aR antibody was added to the tubes and incubated for 1 hour. After 1 hour of incubation, anti-mouse secondary antibody - Cy3 was added to each tube as a secondary antibody and allowed to incubate for 1 hour again. The tube of each group was divided into 3 tubes and read with the Beckman Coulter DxFlex flow cytometer system. The study was performed in triplicate and CytExpert for DxFlex software was used in the analysis.

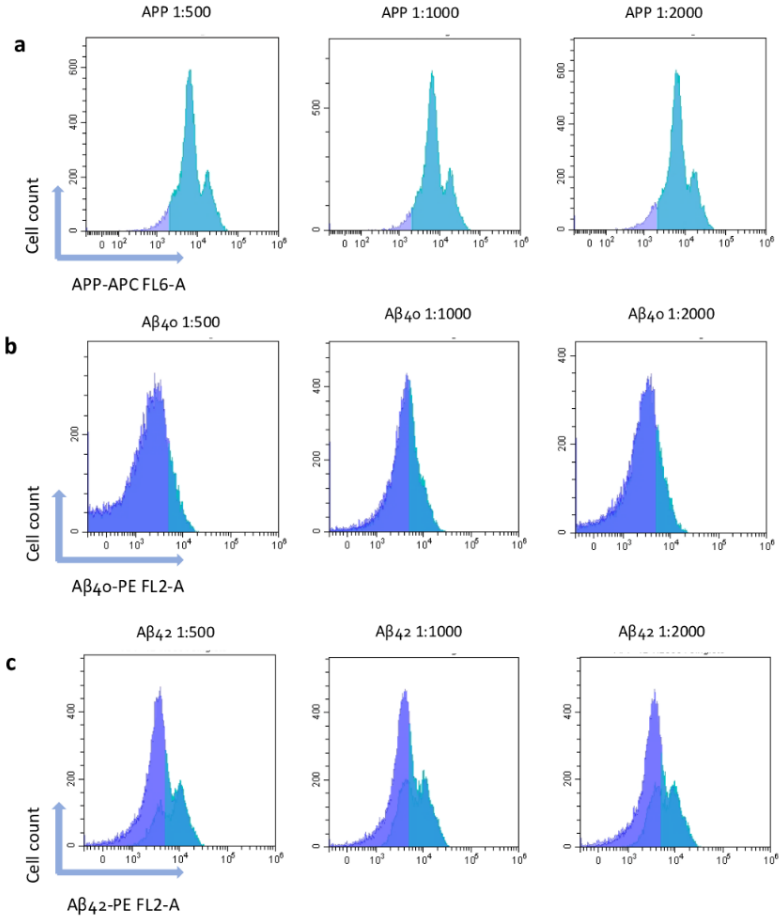

Figure 4.3. Antibody titration results for (a) APP, (b) Aβ40 and (c) Aβ42. The best result were seen in all groups at a ratio of 1:1000.

Table 4.4. Median values for APP, Aβ40 and Aβ42.

	Median Value		
	1:500	**1:1000**	**1:2000**
APP	6829.9	6992.4	6674.4
Aβ40	6855.2	7206.9	6971.7
Aβ42	9151.0	9423.0	8985.0

4.2.2.3.Analysis of amyloid precursor protein, amyloid beta 40 and amyloid beta 42 protein levels

In order to determine the intracellular protein level by flow cytometry, the cells were lifted from petri dishes with Accutase, suspended in a 4°C chilled paraformaldehyde (PFA) solution, and incubated at 4°C for 30 minutes and fixed. The fixative was removed and cells were permeabilized with DPBS containing 0.1 percent Triton X-100 by incubation for 1 hour at 4°C. For amyloid plaque deposition analysis, cells were labeled with APP together with aβ40 or aβ42 antibodies by incubation for 1 hour at 4°C. Primary antibody solutions were removed by centrifugation and cells were labeled with secondary antibody by incubation for 30 minutes in the dark at room temperature. Goat anti-rabbit AF647 (Thermo Fisher Scientific #A21096) antibody was used as secondary antibody. Then, measurements were made with flow cytometry.

4.2.3. Western Blot Testing

In research, Western blot is frequently used to separate and identify proteins. Through gel electrophoresis, a mixture of proteins is sorted based on molecular weight, and hence on type, in this procedure. These findings are then transported to a membrane, where each protein forms a band. The membrane is then treated with antibodies that recognize the protein of interest. The unattached antibody is washed away, leaving just the antibody that is bound to the protein of interest. The developed film then detects the attached antibodies. Exclusively one band should be observed since the antibodies only bind to the protein of interest. Because the thickness of the band correlates to the amount of protein present, performing a standard can reveal how much protein is present [69].

4.2.3.1.Analysis of Tau and phospho-Tau protein levels

Western blot was used for the analysis of tau isoforms, three different phosphorylated tau isoforms (p Thr181, Novus Biologicals, Cat. No: NB100-82245; p Ser202-Thr205, Thermo Fisher Scientific, Cat. No: MN1020, p Ser199, Cell Signaling Technology, Cat. No: 29957,) were analyzed. Protein extraction buffer (2X concentrate); 1 mL Triton X-100, 5 mL 10 percent NP-40, 0.5 mg SDS, 4.38 g NaCl, 5 mL 1 M Tris-base (pH: 7.4), 11.5 mL 87 percent glycerol on top of 50 mL distilled water prepared by adding The buffer was passed through a 0.4 micron filter and stored at 4°C. Prior to protein extraction, an equal volume of

extraction buffer was mixed with Halt™ Protease and Phosphatase Inhibitor Cocktail at 100X concentration (Thermo Fisher Scientific, Cat. No. 78446). The medium was removed from the petri dishes containing the cells, HBSS was added on the cells, and after five minutes of incubation, the cells were scraped with a scraper and the cells were removed. HBSS was removed by centrifugation and lysis was performed by adding 150 µL of protein extraction buffer to the pellet, on ice. The study was carried out in triplicate, the samples were pooled and protein isolation was done. Protein lysates were transferred to chilled sterile 2 mL Eppendorf tubes and centrifuged for 25 minutes at 14000 g at 4 °C. Supernatants were collected, transferred to new sterile Eppendorf tubes and stored at -80 °C. Protein concentrations were measured with the Pierce BCA assay Kit (Thermo Fisher Scientific). For this purpose, bovine serum albumin supplied with the kit was diluted with distilled water as specified in the kit and added to the wells of the 96-well plate in duplicate at a volume of 25µl/well (Table 1.1). The samples were diluted 10 times with distilled water and added to the wells in duplicate, at a volume of 25µl/well. Kit contents Reagent A and Reagent B were mixed at a ratio of 50:1 and added to the wells at 200 µL/well. The plate was incubated for 35 minutes in a 37°C cell culture incubator, then allowed to come to room temperature in the dark and the absorbance was measured at 562 nm wavelength in the spectrophotometer. Protein concentrations were calculated with the slope of the standard curve.

Table 4.5. Bovine serum albumin dilution chart provided by Thermo Fisher Scientific, Pierce™ BCA Protein Assay Kit.

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20–2,000 µg/mL)			
Vial	**Volume of Diluent (µL)**	**Volume and Source of BSA (µL)**	**Final BSA Concentration (µg/mL)**
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

For Western Blot, a 12 percent bis-acrylamide gel was poured by mixing 30 percent Acrylamide/Bis Solution, 37.5:1 (Bio-Rad), Tris, distilled water, Sodium dodecyl sulfate (SDS), tetramethylethylenediamine (TEMED) and 10 percent ammonium persulfate (APS). 50 µg of denatured protein sample was loaded into each well and the gel was run for 3.5 hours in an 80 V electric field. A protein standard (PageRuler Prestained Protein Ladder, 10 - 180 kDa protein ladder) was used in each gel. Proteins were transferred to 0.45 µm PVDF

membranes (Thermo Fisher Scientific) for 1 hour at 300 mA electric field by wet transfer method. Membranes were blocked with TBS-T (Tris buffered saline-Tween 20) containing 3 percent Blocker BSA (Thermo Fisher Scientific, Catalog No: 37520) on a shaker at room temperature and overnight with primary antibodies prepared in TBS-T containing 3 percent Blocker BSA. incubated at 4 °C on a shaker. Membranes washed with TBS-T were labeled with HRP (horseradish peroxidase) conjugated secondary antibodies by incubation for 1 hour at room temperature on a shaker. Vilber Fusion Pulse imaging system was used for imaging. The chemiluminescent signal was obtained with the Thermo Fisher Scientific SuperSignal™ West Femto Maximum Sensitivity Substrate. Band intensities were obtained with the ImageJ analysis program.

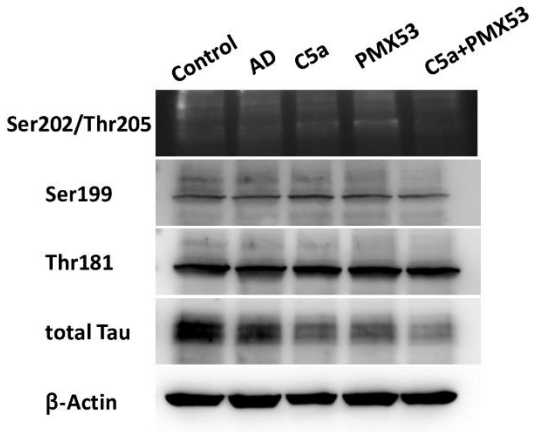

Figure 4.4. Example Western Blot images.

4.2.4. Confocal Microscopy

Aβ42 proteins were visualized by confocal microscopy to determine cellular localization. For this purpose, cells were seeded on Geltrex coated fluorescent imaging slides (Millicell EZ SLIDE 8-well glass) at 2×10^3/well and differentiation was induced. Fixation was carried out by incubation at +4°C for 30 min using 3 percent glutaraldehyde solution. In order to prevent autofluorescent radiation, unbound aldehyde groups were removed by applying 0.1 M Glycine solution for 30 minutes. The permeability of the cell membranes was ensured by incubation with 0.1 percent Triton x-100 solution for 45 minutes at +4 °C. After permeabilization, cells were incubated with TBS-T containing 1 percent bovine serum albumin for one hour and then incubated with primary antibody at 4°C overnight. Following

removal of the primary antibody solution, cells were labeled with secondary antibody by incubation for 1 hour in the dark at room temperature. DAPI at a concentration of 300 nM was used for nuclei marking, slides were covered with Sigma Aldrich FluoroShield mounting medium and viewed with a Zeiss LSM770 confocal microscope.

4.3. STATISTICAL ANALYSIS

Student's T-Test and One-Way Analysis of Variance (ANOVA) test was utilized for statistical analysis and all statistical analysis was performed by GraphPad Prism 8.4.2 software. The P value less than 0.05 was accepted as statistically significant.

5. RESULTS

5.1. Culture Of Rencell Vm Cells

T25 culture flask and 96-well plate were covered with laminin dissolved in DMEM/F12 medium (concentration: 20 ng/mL) by incubating overnight in a 37°C cell culture incubator. bFGF and EGF were added to the medium (RenCell Maintenance Media) at a final concentration of 20 ng/mL. This prepared medium was added to the laminin-coated flask and to each of the 96-well plate wells. The cells were cleared from the freezing solution by centrifugation, suspended in the medium and transferred to flask and 96-well plate. Pre-seeding viability and cell count were checked with trypan blue on a JULI imaging device. The seeding density of the cells was 300,000 cells per well and 600,000 cells per flask in a 96-well plate. Cells began to adhere within the first hours after seeding. The medium was changed every other day and the cells were expected to be confluent.

In order to initiate neuronal differentiation, the exchange continued with the medium without growth factors bFGF and EGF.

5.2. Detection Of Differentiation In Rencell Vm Cells

Cell bodies were elongated after 24 hours of incubation with differentiation medium. Images of the 7-day differentiation process of cells in the AD cell culture model can be observed in Figure 5.1, and fluorescent images can be observed in Figure 5.2. At the end of the 7-day period, cell morphologies were visualized and elongation and neurite-like structure development were detected in the cell body.

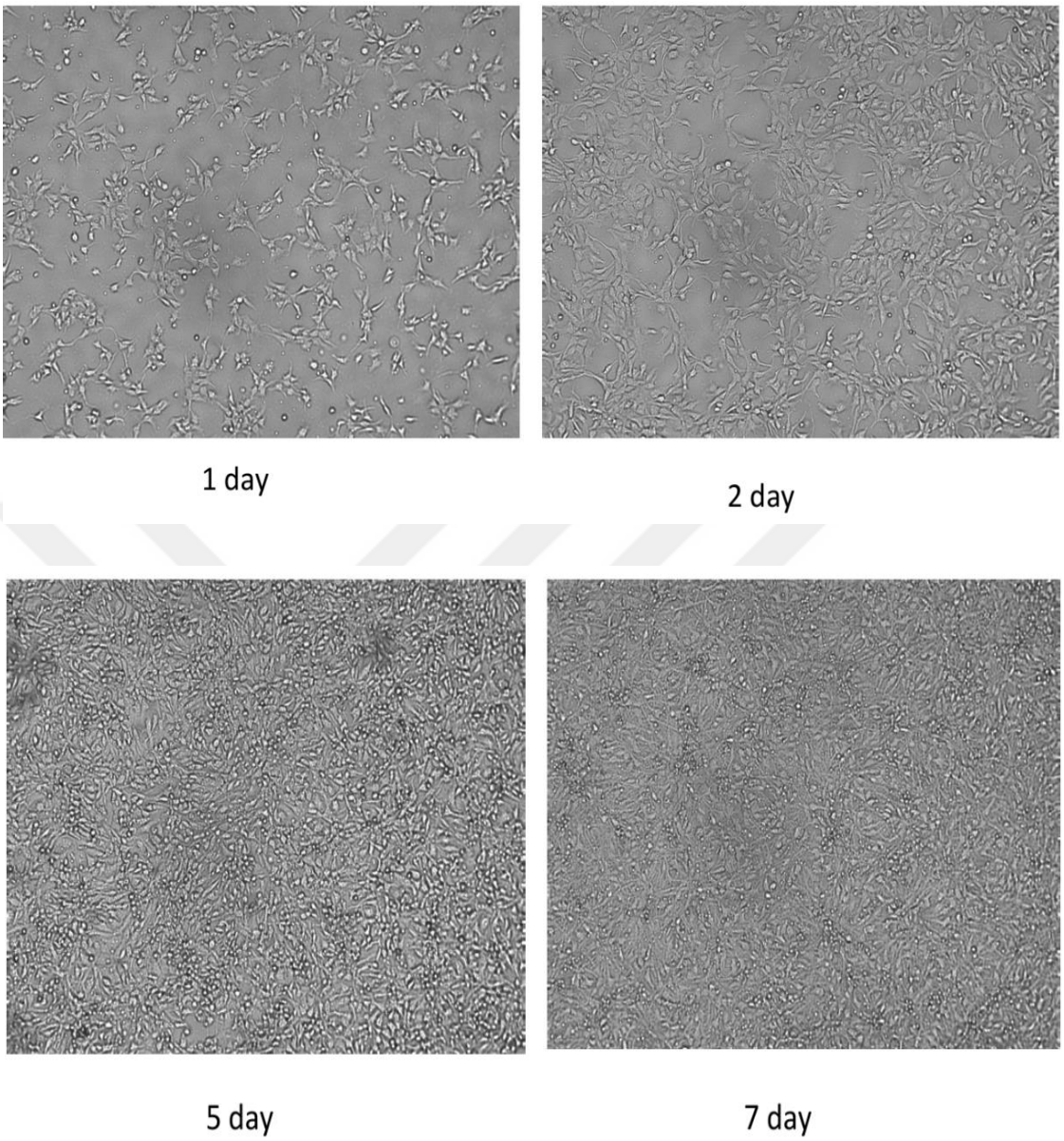

Figure 5.1. Morphological monitoring of the differentiation process induced by the withdrawal of additives from the medium in the AD cell model. All photos were taken with JuLi imaging device (NanoEntek, South Korea) at 4X zoom. Cells gain neuronal morphology by the second day, and neuronal morphology becomes evident on the 5th and 7th days.

5.3.Analysis of the Effect of C5a and PMX53 on Cell Culture Model of Alzheimer's Disease

MTS Cell Proliferation Colorimetric Assay, flow cytometry and Western blotting methods were used to determine the effects of C5a and PMX53 on cells. Viability was measured by the MTS Cell Proliferation Colorimetric Assay, levels of 40-amino-acid and 42-amino-acid amyloid-β isoforms (Aβ40 and Aβ42) and APP (amyloid precursor protein) were measured by flow cytometry, and tau concentration was measured by Western blotting.

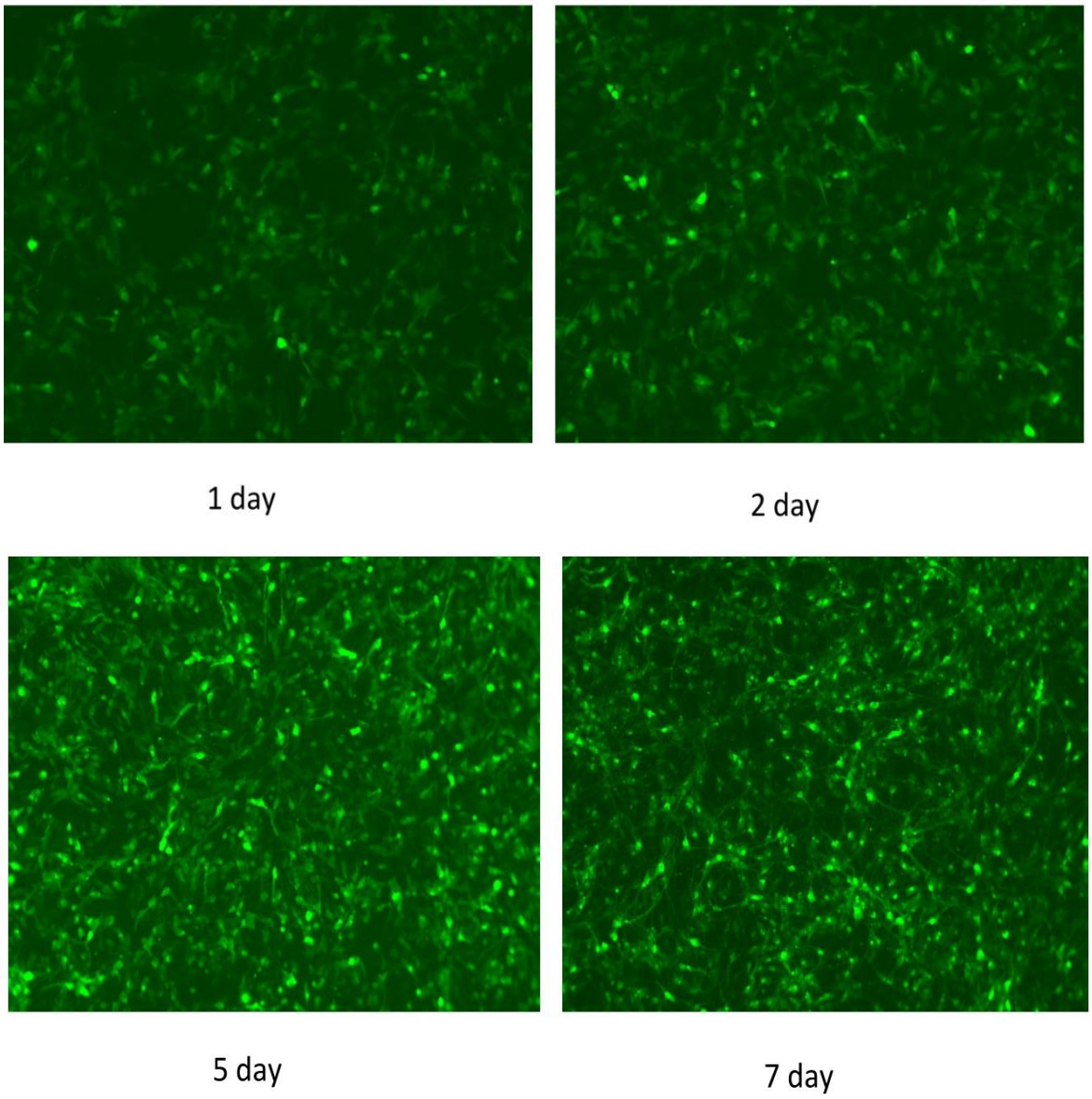

Figure 5.2. Morphological monitoring of the differentiation process induced by the withdrawal of additives from the medium in the AD cell model with a fluorescent filter. All photos were taken with JuLi imaging device (NanoEntek, South Korea) at 4X zoom.

5.3.1. Effect of C5a and Its Inhibitor PMX53 on Viability in Cell Culture Model of Alzheimer's Disease

In the RenCell VM AD model created, the effect of C5a peptide at different doses (0.1, 1, 10 and 100 ng/mL) and the IC50 concentration specified by the manufacturer, of PMX53, a non-competitive C5aR inhibitor, on viability were analyzed. There was no significant difference between the doses in the 24 and 48 hour incubation periods (p>0,05) (Figure 5.3.)

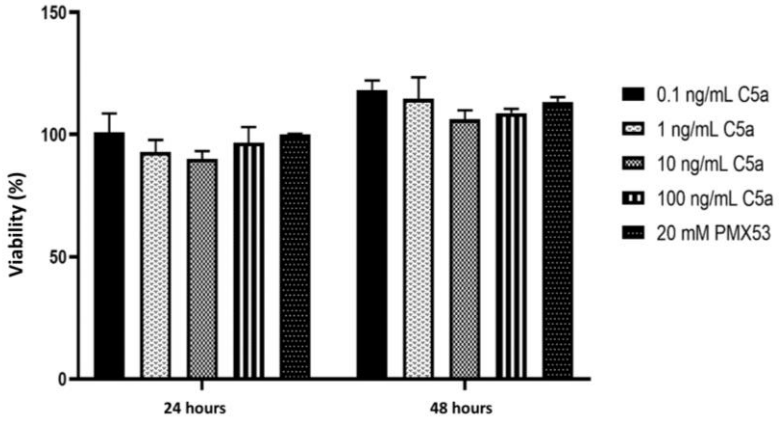

Figure 5.3. Analysis of the effect of adding C5a peptide or PMX53 to the medium on viability of neurons. C5a peptide at all applied doses or PMX53 applied at IC50 dose did not significantly affect cellular viability.

5.3.2. Effect of Complement Component C5a and its Inhibitor PMX53 on C5aR Expression in a Cell Culture Model of Alzheimer's Disease

As part of the data obtained from the MTS analysis, 100 ng/mL of C5a differentiated cells were added to the medium and incubated for 1 week, changing the medium every 48 hours.

Addition of C5a appears to cause increased expression of C5aR in cells.

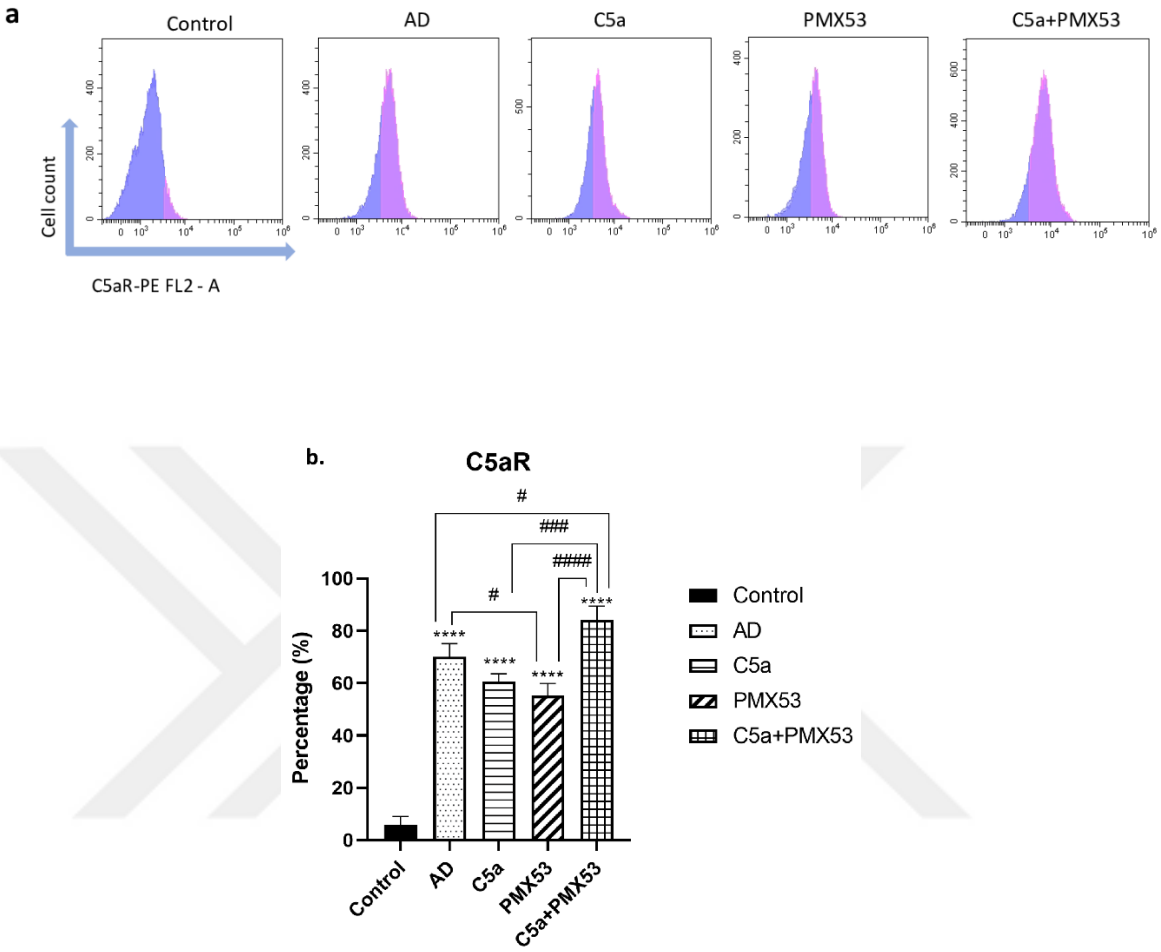

Figure 5.4. Analysis of C5aR expression in the cellular model. **a.** Sample flow cytometry dials for analysis of C5aR levels in the control, Alzheimer's, C5a, PMX53, and C5a+PMX53 groups. **b.** Intergroup comparison of C5aR levels in control, Alzheimer's, C5a, PMX53 and C5a+PMX53 groups. Under normal conditions, cells do not express C5aR, whereas receptor expression is increased in Alzheimer's cell models. *p<0,05; ****p<0,0001; #p<0,05; ##p<0,01; ###p<0,001; ####p<0,0001

It was found that C5aR level was higher in AD group than PMX53 group (p=0.0132) and lower than C5a+PMX53 group (p=0.02). There was no significant difference between AD and C5a groups (p>0.05). There was a significant increase between the C5a and C5a+PMX53 groups (p=0.0005) and between the PMX53 and C5a+PMX53 groups (p<0.0001). As a result, C5aR level was found to be higher in the C5a+PMX53 group

compared to the other groups. There is a difference between C5a and PMX53 groups, but this difference is not significant (p=0.58).

5.3.3. Effect of Complement Component C5a and its Inhibitor PMX53 on Amyloid Precursor Protein, Amyloid Beta 40 and Amyloid Beta 42 Protein Levels

In protein expression analyzes performed in all groups, increases in aβ40 and aβ42 protein levels were found to be significantly higher than in the control group ($p<0.0001$). There was a significant decrease in APP protein level only in the PMX53 group compared to the control ($p<0.0001$). It was not determined that the other groups made a significant difference in terms of APP protein level compared to the control ($p>0.05$). In the analyzes performed between groups, there was no significant difference between the aβ40 protein levels between the C5a and AD groups (p=0.0004), between the C5a and C5a+PMX53 groups (p=0.0021), and between the C5a and PMX53 groups (p=0.0054) found to make a difference. When the results are examined, it is seen that there is a significant decrease in the C5a group compared to the other groups.

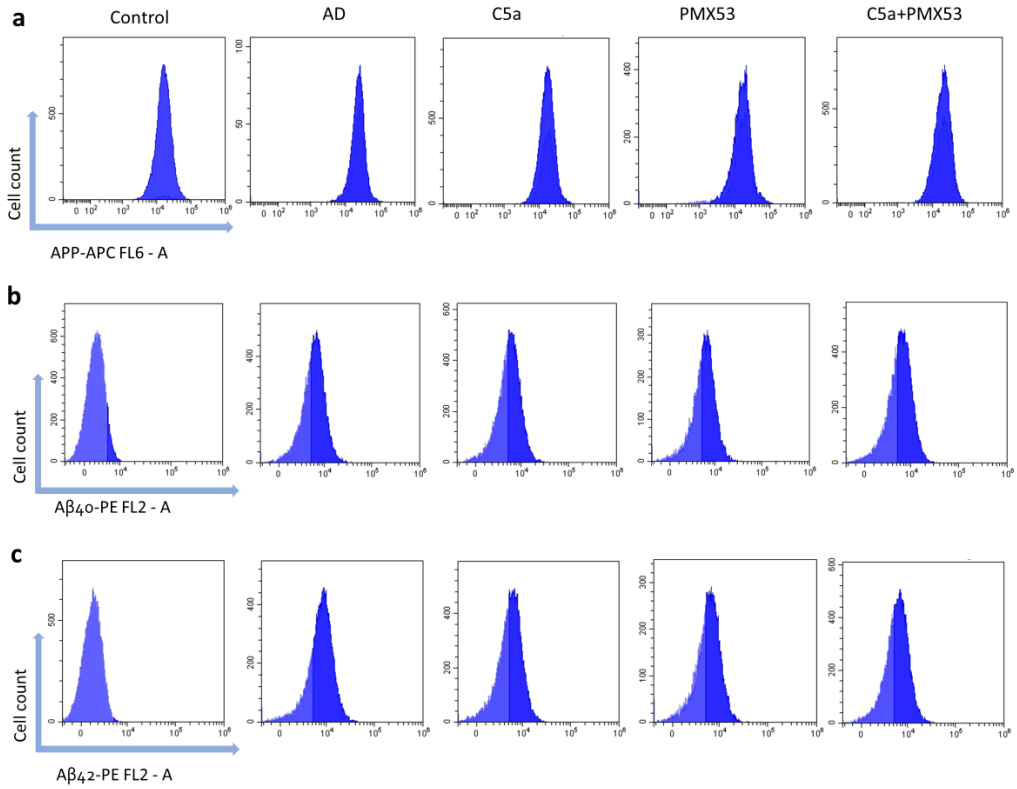

Figure 5.5. Analysis of amyloid precursor protein (a), amyloid beta 40 (b) and amyloid beta 42 (c) protein levels in Control, AD, C5a, PMX53 and C5a+PMX53 groups.

It was found that Aβ42 protein level was significantly lower in the C5a group compared to the AD group ($p<0.0001$), while it created a significant difference in the C5a+PMX53 group ($p=0.0035$) and PMX53 group ($p=0.0214$). In addition, aβ42 level was decreased in C5a+PMX53 ($p=0.003$) and PMX53 ($p=0.0006$) groups compared to AD group. There was no significant difference between C5a+PMX53 and PMX53 groups ($p>0.5$).

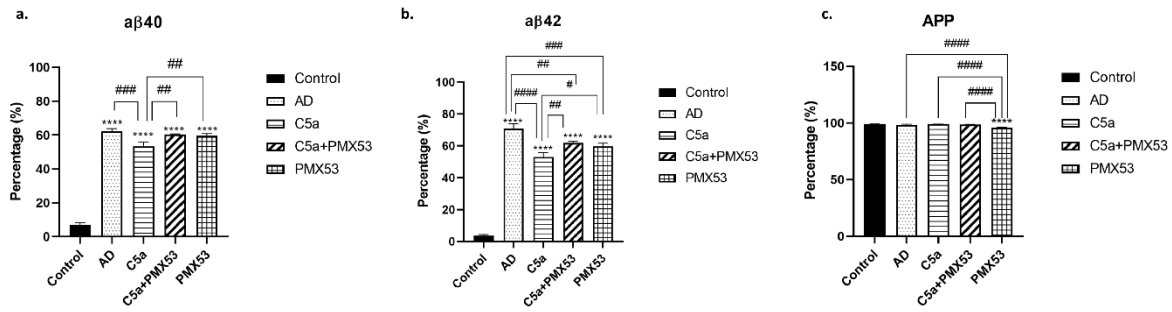

Figure 5.6. Analysis of the levels of APP, aβ40 and aβ42 proteins between groups. *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001; #p<0,05; ##p<0,01; ###p<0,001; ####p<0,0001

5.3.4. Effects of C5a and PMX53 application on p-tau isoforms in Alzheimer's Disease cell culture model created from Rencell VM cell line

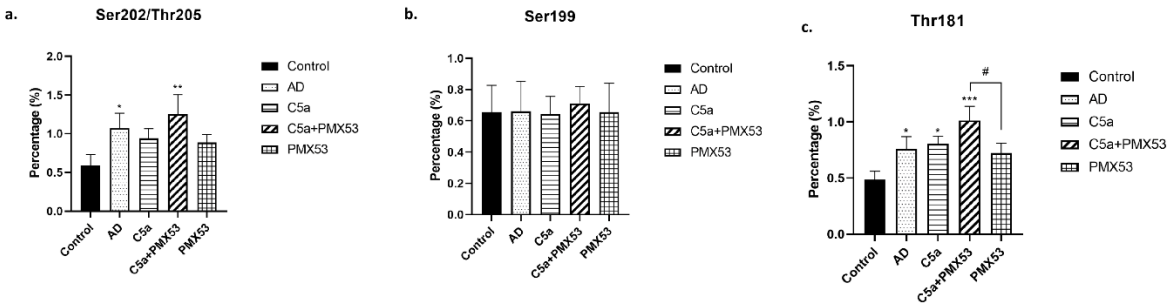

Figure 5.7. Effects of C5a and its antagonist PMX53 on p-tau isoforms in Alzheimer's disease cell culture model created from RenCell VM cell line. **a.** It caused an increase in Ser202/Thr205 p-tau isoform in both AD and C5a+PMX53 groups. **b.** No significant effect of C5a or PMX53 was detected in the Ser199 isoform. **c.** There was a significant increase in Thr181 isoform in all groups except PMX53. *p<0,05; **p<0,01; ***p<0,001; #p<0,05

When the effects of C5a and PMX53 treatments on p-tau isoforms were analyzed, there was a significant increase in Ser202/Thr205 isoform only in the AD group (p=0.0422) and C5a+PMX53 group (p=0.0061). No significant difference was observed in the other groups. No significant effect of C5a or PMX53 was detected in the Ser199 isoform (p>0.05). There was a significant increase in the Thr181 isoform between the control group and the AD group (p=0.0346), between the C5a group (p=0.0142) and between the C5a+PMX53 group (p=0.0004). No significant difference was observed in the PMX53 group (p>0.05).

5.3.5. Visualizing the effects of C5a and PMX53 in a Cell Culture Model of Alzheimer's Disease

Homogeneous staining of C5aR1 indicates the presence of the receptor on the membrane. When C5a peptide is given to cells, it is seen that the signal is increased and there is a decrease in the level of aβ42. This result are in agreement with the flow cytometry results.

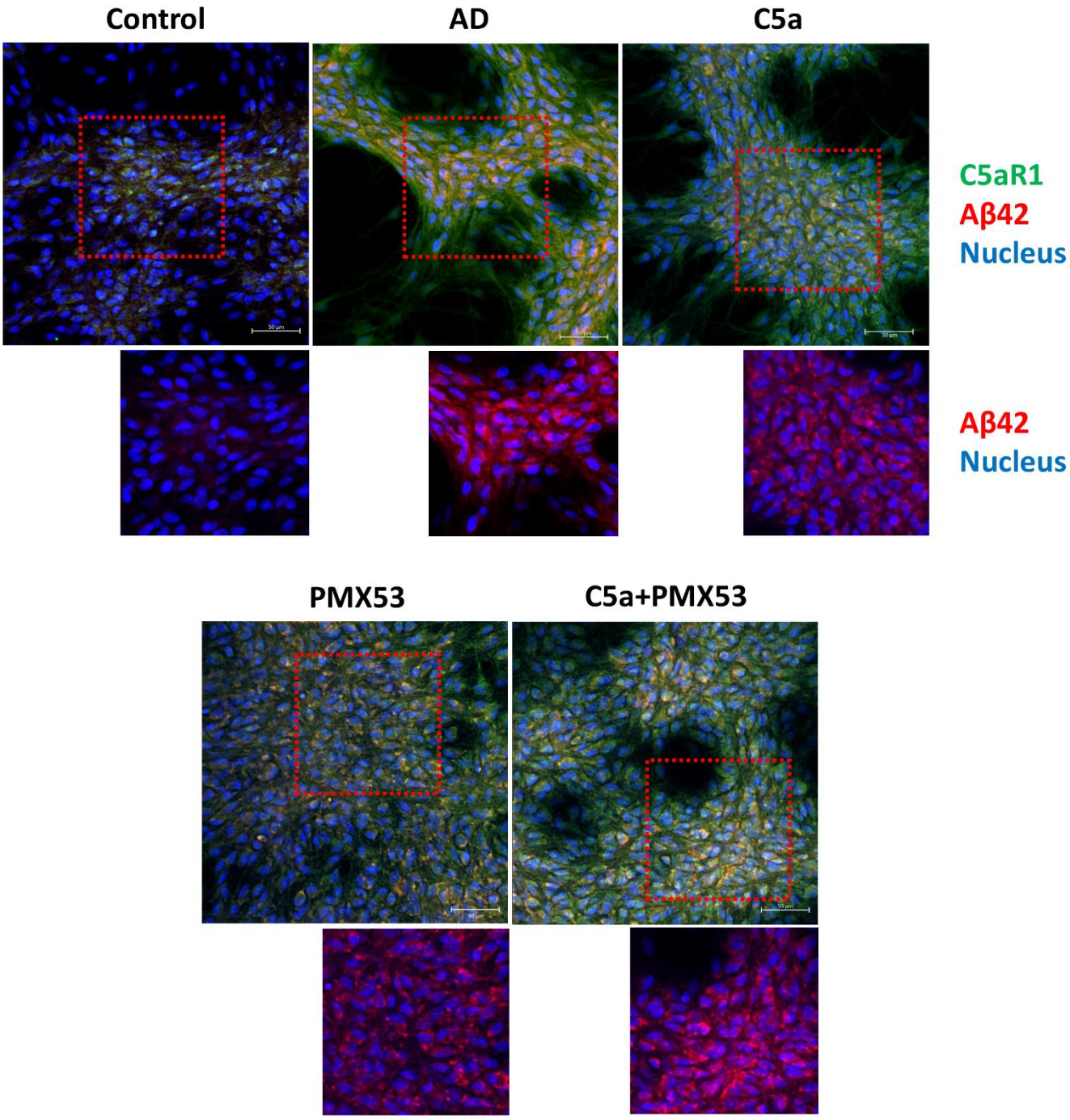

Figure 5.8. Analysis of aβ42 protein level and C5aR level by confocal microscopy. Cells were labeled with C5aR1 or aβ42 antibodies. The nucleus is distinguished by DAPI. Images were taken at 40X zoom.

6. LIMITATIONS

- This project was funded through the remaining budget of the TÜBİTAK 1001 project "'Evaluation of Cellular Stress and Inflammasome Activation in Alzheimer's Model Developed from Human Neuronal Precursor Cells' Project No: 217S663." awarded to Prof. Dr. Gülderen Yanıkkaya Demirel, Immunology Department, Faculty of Medicine, Yeditepe University which was rather limited.
- A more comprehensive reagent portfolio covering all of the complement proteins and their regulatory proteins would have provided more information about the role of the complement system in Alzheimer's Disease.
- This study could include the molecular methods for examining the signaling pathways inside the neurons in culture. Again due to limited budget, we were not able to make these evaluations.

7. DISCUSSION

AD is a complex, multifactorial disease, and available data indicate that the disease is unique to humans. Age of onset, rate of progression, and pathology of the disease vary among patients. It is an irreversible neurodegenerative disease associated with aβ accumulation and hyperphosphorylated tau [70]. It is known that the number of people aged 65 and over living with Alzheimer's dementia in 2022 is approximately 6.5 million in the USA exclusively [71]. Pathological accumulations of aβ and phosphorylated tau that lead to AD occur in a sequential process. First, monomers accumulate within neurons to form oligomers, and then these oligomers combine to form amyloid plaques and NFTs [70]. Aβ, which is first degraded by the β-secretase-1 and then γ-secretase enzymes and released as monomeric aβ, is the processing product of APP. As a result of γ-secretase-mediated cleavages in variable regions of APP, two different-length peptides aβ-40 and aβ-42 are formed, the most common forms in the brain. Defects such as decreased destruction of aβ or increased aβ production play a role in AD pathology. Such defects cause accumulation of aβ40 and aβ42. An increase in the Aβ42/Aβ40 ratio causes the formation of Aβ amyloid fibrils that cause neurotoxicity and tau pathology, eventually leading to neuronal cell death and neurodegeneration [9,11].

There is currently a significant need to develop new treatment modalities for AD, as there is not yet a therapeutic approach to treat the disease. The existence of complement activation products has been proven, suggesting that the complement system-mediated synapse pruning as well as the activation of the complement cascade may be the cause of many other neurological disorders in the brain. Therefore, modulation of certain complement components specific for different stages of the disease leads to the hypothesis that it can be considered as a new and effective therapeutic approach to the disease [72].

Although these 3 proteins are known as anaphylatoxins, there is little evidence for a role of C4a in this activation [73].

Studies with experimental animals have shown that C5a has an important role in host defense in studies using C5a deficient animals or mice with deletion of C5aR expression. C5a activity occurs when it interacts with two receptors identified as C5aR and C5L2 [73]. C5aR and C5L2 are seven transmembrane segment proteins with similar cDNA homology [74].

In our study, it was observed that there was an increase in C5aR expression in all groups compared to the control group. While the cells did higher express low level of C5aR under normal conditions, it was observed that the cells expressed C5aR in the Alzheimer's model. However, contrary to expectations, there was no significant difference between the AD and C5a groups. Cells given the C5a peptide were expected to have increased C5aR expression. Lack of this increase may be due to presence of another C5a receptor, C5L2. Studies using C5L2 show that C5L2 has the ability to bind C5a with an affinity similar to that of C5aR. In mouse neutrophils, C5L2 has been shown to affect the signaling activity of the C5a receptor [75]. That is to say, C5L2 may be playing a role in optimizing C5a signals. In our study, we were not able to measure C5L2 receptor.

GPCR (G-protein-coupled receptor) heterodimerization, such as that seen between $GABA_B$ receptor subunits, may enhance receptor expression on the cell surface. Although the interactions between C5L2 and classical anaphylatoxin receptors have not been fully determined, the possibility of heterodimerization exists. Chen et al. shows that C5L2 can work cooperatively with C5aR-mediated signals [76].

In addition, the human C5aR N-terminal is the recognition site for C5a, and antibodies recognizing the C5aR N-terminal domain may interfere with C5a binding. Studies show that the extracellular C5a/C5aR interaction is considerable in determining high binding affinity, but it is not sufficient for the receptor to be activated [77].

In addition to all these, neurons obtained from progenitor neuron cells were used in the study. This may have an effect on the responses of the cells. Different results may be obtained by using mature neuron cells. Also, C5aR1 may not interact with C5a, subsequently added to the cell environment. Since C5aR1 is an endogenously expressed receptor [78], it can be thought that it may only interact with C5a inside the cell.

In recent years, research has been carried out to understand C5a receptor antagonists. However, only a few C5a receptor antagonists have been described [77]. The cyclic hexapeptides PMX53 and PMX205 are inhibitors of C5aR1. These inhibitors are widely used to study the role of C5aR1 [79]. PMX53 is the most extensively studied antagonist with low nanomolar potency and a wide range of efficacy. These features make the PMX53 an ideal tool for research [80].

In our study, PMX53 antagonist was used. When PMX53 was added to the cell medium, it was observed that there was a decrease in C5aR1 level compared to the Alzheimer group. Considering that PMX53 is a C5aR1 antagonist, this was an expected result. In this case, it can be said that PMX53 inhibits C5aR1. However, when C5a and PMX53 were added to the cell environment together, it was observed that the level of C5aR1 increased in compare to the other groups. The reason for this may be that the co-existence of C5a and PMX53 in the cell environment increases the binding strength of the C5a and C5aR1 interaction.

In the protein expression analyzes performed between all groups, as expected, aβ40 and aβ42 protein levels were significantly higher in all other groups compared to the control group. Significantly elevated levels of these proteins indicate the occurrence of an amyloid pathology. However, there was no significant difference in APP protein level between the groups except for PMX53. This finding may be attributed to the effect of PMX53 on orphan receptor Mas-related gene 2 (MrgX2), a G protein-coupled receptor (GPCR) that is expressed on nociceptive neurons and mast cells [82,83]. Previously, role of PMX53 was investigated on human mast cells that are known to express C5aR where authors revealed that mast cell degranulation is mediated by MrgX2 activation upon PMX53 treatment [82]. In another study, it is also shown that even if PMX53 reaches serum levels high enough to block C5aR, it does not reduce synovial inflammation, which indicates opposite actions of PMX53 on C5aR and MrgX2 simultaneously [84]. There was a significant decrease in aβ40 and aβ42 levels in the C5a cell group compared to all other groups. When the other groups were compared among themselves, no difference was observed in the aβ40 level, while there was a significant decrease in the aβ42 level of PMX53 and C5a+PMX53 compared to the AD group. This result is consistent with studies indicating that administration of a complement receptor suppressor, PMX53, into the cell environment reduces neuroinflammation and fibrillar aβ [55]. However, the absence of this difference in aβ40 may be owing to structural differences between aβ40 and aβ42. Aβ40 is the most common amiloid beta isoform in the brain, while aβ42 is increased in AD. The aggregation mechanisms of Aβ40 and aβ42 are different from each other. Aβ40 can exist as a monomer, dimer, or tetramer, but aβ42 exists as stable pentamers or hexamers and can readily form high molecular weight oligomers and protofibrils. Since aβ40 tetramers are not prone to interact with additional monomers and dimers, fibril formation is slower than aβ42 [81]. The difference in this fibril formation rate may also have made a difference in the mechanism of

action of PMX53. The fact that the levels of aβ40 and aβ42 in the cell groups with C5a peptide added were significantly lower than in all groups except the control, is inconsistent with literature. But it is consistent with another study that advocated a neuroprotective role for C5a. In that study, it is stated that C5a may provide a protective effect against glutamate-induced neurotoxicity in AD in-vitro and in-vivo. It is argued that the mechanism that creates this effect in C5a-mediated neuroprotection is caused by the activation of MAP-kinase [3]. The fact that protein levels in both aβ40 and aβ42 were lower in C5a added cell groups than in all groups seems to be consistent with this publication. To clear the contradictory status of the these findings, more exploration on this issues are necessary.

When the effects of C5a and PMX53 application on p-tau levels, another AD marker, were examined, it was observed that our AD model has significantly increased phospo-Ser202/Thr205 isoform in the AD group while combinational C5a+PMX53 treatment further increased its' expression, though the increase was not significant. On the other hand, both C5a and PMX53 treatments decreased phospo-Ser202/Thr205 levels, indicating both have the ability to supress tau phosphorylation. A significant increase in the phospho-Thr181 levels was also observed in the AD group and C5a+PMX53 treatment further enhanced this isoform while PMX53 treatment was able to lower phospho-Thr181 isoform similar to the the control level. These data may also suggest possible roles of MrgX2 in the elimination of tau tangles, either by supressing tau phosphorylation, or facilitating the removal of these structures. Our findings may also suggest a link between pain perception and AD pathogenesis when considering the role of MrgX2 is involved in nociception [83]. However, currently there are no studies that have investigated the involvement of MrgX2 and its' downstream targets in AD, thus, further studies are needed to confirm these hypotheses.

Our results indicate that there is a possible role of complement system in development of Alzheimer's Disease. Due to limited budget, we were only able to explore the role of C5a and it's receptor (C5aR1). In compliance with previous publications, we have also found out that there is need for more comprehensive studies to explain the effect of complement system in development of neurodegenerative diseases such as Alzheimer's Disease.

8. CONCLUSION

In summary, in this study, the effect of the complement system component C5a and its antagonist PMX53 in AD pathology was analyzed using an in vitro cell culture model developed using a human neuronal precursor cell line, RenCell VM. Some of the findings were congruent with the literature, while others were conflicting. While the C5aR1 level was predicted to rise, there was no significant increase. When C5a and PMX53 were added together, the C5aR1 level increased contrary to expectations. Existing literature information was also contradictory within itself. While some sources mention a protective and therapeutic effect of C5a, some sources mention its inflammation-increasing effect. Results like these show us that more studies should be done both on the mechanism of action of C5a and on the effects of other antagonists such as PMX205. This study sheds light on the fact that there are still many unknown factors regarding the complement system and Alzheimer's relationship, and that the discovery of these factors may contribute greatly to the treatment and development process of Alzheimer's disease.

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