

T.C.  
DOKUZ EYLUL UNIVERSITY  
IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

**EFFECT OF SELECTED RECOMBINANT  
PROTEINS ON EFFEROCYTOSIS  
MECHANISMS**

ASLI BABAYAKALI

MOLECULAR BIOLOGY AND GENETICS  
MASTER'S PROGRAM

**MASTER OF SCIENCE THESIS**

**IZMIR-2024**

**THESIS CODE:** DEU.IBG.MSc-2022850011

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Advisor: Assoc. Prof. M.Kasım Diril  
Co-Advisor: Prof. Dr. Mehmet Öztürk

(This study was supported by TUBITAK Project 122S045)

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T.C.  
DOKUZ EYLÜL ÜNİVERSİTESİ

İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ



## YÜKSEK LİSANS TEZ SAVUNMA SINAVI TUTANAĞI

Dokuz Eylül Üniversitesi İzmir Uluslararası Biyotıp ve Genom Enstitüsü Genom Bilimleri ve Moleküler Biyoteknoloji Anabilim Dalı Moleküler Biyoloji Ve Genetik Yüksek Lisans Programı 2022850011 numaralı öğrencisi Aslı Babayakalı, “Efferositoz mekanizmaları üzerine seçilmiş rekombinant proteinlerin etkisi” konulu Yüksek Lisans tezini 16.08.2024 tarihinde yapılan savunma sınavı sonucunda başarılı olmuştur.

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## **LIST OF ABBREVIATIONS**

**Apaf-1:** Apoptotic protease activating factor 1

**APCs:** Antigen presenting cells

**ApoBD:** Apoptotic bodies

**BAD:** Bcl-2 associated agonist of cell death

**BAK:** Bcl-2 antagonist killer 1

**BAX:** Bcl-2-associated X protein

**CD47:** Cluster of differentiation 47

**CRT:** Calreticulin

**CX3CL1:** C-X3-C motif chemokine ligand 1

**DMSO:** Dimethyl sulfoxide

**ECL:** Enhanced chemiluminescence

**ELISA:** Enzyme linked immunosorbent assay

**ER:** Endoplasmic reticulum

**FADD:** Fas-associated death domain

**FasL:** FAS ligand

**FBS:** Fetal bovine serum

**Gas6:** Growth arrest specific 6

**GFP:** Green fluorescent protein

**IAP:** Integrin-associated protein

**ICAM3:** Intercellular adhesion molecule 3

**IgSF:** Immunoglobulin-super family

**ILT2:** Immunoglobulin-like transcript 2

**ITIM:** Immunoreceptor tyrosine-based inhibitory motif

**LB:** Luria broth

**LFA-1:** Lymphocyte function associated antigen 1

**LILRB1:** Leukocyte immunoglobulin-like receptor subfamily B member 1

**LPC:** Lysophosphatidylcholine

**LRP1:** LDL-receptor related protein 1

**Mcl-1:** Myeloid cell leukemia 1  
**MFGE8:** Milk fat globule epidermal growth factor 8 protein  
**MHC:** Major histocompatibility complex  
**MOMP:** Mitochondrial outer membrane permeabilization  
**NKT:** Natural killer T cells  
**PC:** Phosphatidylcholine  
**PE:** Phosphatidylethanolamine  
**PFA:** Paraformaldehyde  
**PROS1:** Protein S  
**PS:** Phosphatidylserine  
**S1P:** Sphingosine-1-phosphate  
**SDS:** Sodium dodecyl sulfate  
**SHP-1:** Src homology-1 containing phosphatases  
**SHP-2:** Src homology-2 containing phosphatases  
**SIRP- $\alpha$ :** Signal regulatory protein  $\alpha$   
**SM:** Sphingomyelin  
**TIM-4:** T-cell immunoglobulin and mucin domain-containing 4  
**TRADD:** Tumor necrosis factor receptor type 1 associated death domain protein  
**WBC:** White blood cells

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I would like to dedicate my thesis to my lovely grandfather Kemalettin also known as Sebahattin in his small village. I wish you were here...



*to my family*  
*aieme...*

## **ABSTRACT**

### **Effect of Selected Recombinant Proteins on Efferocytosis Mechanisms**

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Billions of aged, senescent, damaged, or unwanted cells in the human body are regularly renewed. This process is primarily carried out through apoptosis. Phagocytes, including macrophages, neutrophils, and dendritic cells, play a crucial role in eliminating cells that have undergone apoptosis, a process known as efferocytosis. Efferocytosis is derived from the Greek word "effere," meaning "to carry to the grave," and is guided by "eat me" and "don't eat me" signals displayed on the cell surface. These signals are recognized by receptors on phagocytes. Proper regulation of efferocytosis is vital for maintaining immune balance and preventing secondary necrosis, which can lead to inflammation and immune-related disorders.

In our study, we aimed to design a chimeric recombinant protein, Sirpα-Fc, that binds to CD47 on apoptotic cells. Additionally, we evaluated commercially available proteins Gas6 and MFGE8, which bind to phosphatidylserine (PS) on apoptotic cells, to determine whether they enhance phagocytosis. To test these hypotheses, apoptosis was induced in the 4T1 mouse cancer cell line, and which were subsequently co-cultured with RAW 264.7 mouse macrophages. As a result, we found that Sirpα-Fc, either alone or in combination with MFGE8 and Gas6, significantly affected phagocytosis percentages, showing a statistically significant impact.

**Keywords:** Efferocytosis, recombinant protein production, apoptosis, Sirpα, Gas6, MFGE8

## **ÖZET**

### **Eferositoz Mekanizmaları Üzerine Seçilmiş Rekombinant Proteinlerin Etkisi**

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İnsan vücudundaki milyarlarca yaşlanmış, senesense uğramış, hasar görmüş veya istenmeyen hücreler düzenli olarak yenilenir; bu süreç başlıca apoptoz yoluyla gerçekleştirilir. Fagositler; makrofajlar, nötrofiller ve dendritik hücreler dahil olmak üzere, apoptoz sonucu oluşan hücreleri ortadan kaldırmada önemli bir rol oynar ve bu işlem eferositoz olarak adlandırılır. Eferositoz, Yunanca "ölüyü mezara taşımak" anlamına gelen *effere* kelimesinden türetilmiştir ve hücre yüzeyinde sergilenen "beni ye" ve "beni yeme" sinyalleri tarafından yönlendirilir. Bu sinyaller fagositlerdeki reseptörler tarafından tanınır. Eferositozun uygun şekilde düzenlenmesi, bağışıklık dengesini korumak ve inflamasyon ile bağışıklıkla ilgili bozukluklara yol açabilecek ikincil nekrozu önlemek için hayati önem taşır.

Çalışmamızda, apoptoza uğramış hücrelerdeki CD47'ye bağlanan bir kimerik rekombinant protein olan Sirpα-Fc proteini tasarlamayı hedefledik. Ek olarak, apoptoza uğramış hücrelerdeki fosfatidilserin'e (PS) bağlanan ve fagositozu artırıp artırmadığını öğrenebilmek için ticari olarak temin edilebilen Gas6 ve MFGE8 proteinlerini değerlendirdik. Bu hipotezleri test etmek amacıyla, 4T1 fare kanser hücre hattında apoptoz indüklendi ve bu hücreler RAW 264.7 fare makrofajları ile birlikte kültüre alındı. Sonuç olarak Sirpα-Fc'nin tek başına veya MFGE8 ve Gas6 ile kombinasyon halinde fagositoz yüzdelere etki ederek istatistiksel olarak anlamlı bir etki gösterdiği gösterilmiştir.

**Anahtar kelimeler:** Eferositoz, rekombinant protein üretimi, apoptoz, Sirpα, Gas6, MFGE8

## **1. INTRODUCTION AND AIM**

Efferocytosis refers to the process by which apoptotic cells are efficiently cleared by phagocytes, primarily macrophages. Proper efferocytosis is critical to maintaining tissue homeostasis and preventing inflammation. Dysregulation of this process can result from various mechanisms, including impaired phagocytic activity of macrophages or disruptions in the recognition of apoptotic cells by phagocytic receptors. Apoptotic cells display a variety of 'eat-me' signals, such as phosphatidylserine, as well as 'don't eat-me' signals that modulate their recognition by phagocytes like macrophages, dendritic cells, and neutrophils. Among these phagocytes, macrophages have been extensively studied for their role in efferocytosis. As a result, we conducted an in-depth investigation into the specific signals involved in the efferocytosis of apoptotic cells by macrophages.

CD47 is a key 'don't eat me' signal expressed on the surface of cells, which inhibits phagocytosis by binding to its receptor, Signal Regulatory Protein Alpha (Sirpa), on macrophages. In this study, we aimed to produce a chimeric recombinant protein, Sirpa-Fc, designed to specifically bind the extracellular domain of CD47 on target cells. Our construct involved the fusion of the mouse Sirpa domain to the N-terminus of mouse IgG-Fc. Comparative in vitro studies were performed using Sirpa-Fc and an IgG-Fc control. We hypothesized that by introducing Sirpa-Fc, the interaction between CD47 on apoptotic cells and Sirpa on macrophages would be altered, potentially modulating the rate of phagocytosis.

In addition, we explored the use of commercial mouse Gas6 and MFGE8 proteins, which act as bridging molecules that facilitate the recognition of phosphatidylserine on apoptotic cells by macrophages. Gas6 is known to bind tyrosine kinase receptors, including AXL, Mer, and Tyro3, expressed on macrophages, while MFGE8 interacts with  $\alpha v \beta 3$  and  $\alpha v \beta 5$  integrins on these cells.

We hypothesized that the application of Sirpa-Fc, Gas6 and MFGE8 proteins in co-culture experiments could enhance the phagocytosis of apoptotic cells. Efferocytosis experiments were conducted using the 4T1 mouse breast cancer cell line and RAW 264.7 mouse macrophage cell line. 4T1 cells were used as targets and RAW 264.7 cells were used as effector cells to evaluate the effects of Sirpa-Fc, Gas6, and MFGE8 on macrophage mediated clearance of apoptotic cells.



## **2. GENERAL INFORMATION**

### **2.1. Apoptosis as programmed cell death**

Apoptosis is a sort of programmed cell death characterized by the efficient removal of damaged or aged cells that occur during development or exposing to a toxic chemical, material, or radiation (Nagata & Tanaka, 2017 ; Diamantis et al., 2008). Apoptosis is known as 'immunologically silent' since it does not create any inflammatory response within the tissue or area (Elmore, 2007). There is an important organization between the pro-apoptotic and anti-apoptotic protein regulators in healthy cells and tissues (O'Brien & Kirby, 2008). If this organization has faded due to some factors that can damage the cells, it causes uncontrolled cell proliferation, malformations, and cancer development (Wong, 2011).

Apoptosis carries out its action via caspases, which are cysteine proteases and belong to the protease enzyme family (Cohen, 1997). Caspases have a role in the destruction of cells by enzymatic cleavage of their proteins, depending on different signaling pathways. Cysteine in their active site cleaves target protein only after an aspartic acid residue (Blatt & Glick, 2001). Caspases that have a role in apoptosis are categorized into two subclasses by their mechanism of action. Briefly, Caspase -2, -8, and -9 are known as initiator caspases, and caspase -3, -6, and -7 are known as executioner caspases (McIlwain & Berger & Mak, 2013).

Initiator caspases exist in their inactive form in the cytosol, which are monomeric procaspases. Later they need to be dimerized for activation with the help of the Fas-associated death domain (FADD), an adapter protein (Boatright et al., 2003). This term is known as 'proximity-induced dimerization' (Salvesen & Dixit, 1999).

Executioner caspases are produced as inactive dimers and they need to be cleaved from their protease domains for proper activation. Activated initiator caspases cleave these inactive procaspase dimers of executioner caspases (Riedl & Shi, 2004). Once activated, one executioner caspase is capable of cleaving and activating other executioner caspases too, which leads a widespread protein cleavage events that control cell death (Widmann & Gibson & Johnson, 1998).

### 2.1.1. Pathways of apoptosis

Apoptosis can happen in response to cellular or external stimulants. Mainly apoptosis includes two signaling pathways which are the intrinsic pathway and the extrinsic pathway

#### 2.1.1.1. Intrinsic pathway

The intrinsic pathway (see Figure 1) involves MOMP (mitochondrial outer membrane permeabilization) with the release of cytochrome c from the mitochondria (Kharbanda et al., 1997, Kalkavan & Green, 2018). After cytochrome c is released from intracellular space to extracellular space, cytochrome c facilitates the formation of Apaf-1 adaptor protein (Apoptotic protease activating factor 1) (Baliga & Kumar, 2003). Later they recruit initiator caspase-9 and form a large ring complex, which is called 'apoptosome'. Bcl-2 antagonist killer 1 (BAK) and Bcl-2-associated X protein (BAX) create pores in the membrane of mitochondria and this conformation leads to the activation of effector caspases that can degrade cellular cytoskeletal components (Zhang et al., 2017). B-cell lymphoma (Bcl-2) proteins regulate the intrinsic pathway and these proteins can be divided into two different subtypes (Abaza et al., 2022). The ones that positively regulate apoptosis are called "pro-apoptotic" which are mainly, BAX, BAK, and BAD (Bcl-2 associated agonist of cell death), and the ones that negatively regulate apoptosis called "anti-apoptotic" which are Bcl-2, Bcl-xL and Mcl-1 (Myeloid Cell Leukemia 1) (O'Brien & Kirby, 2008).

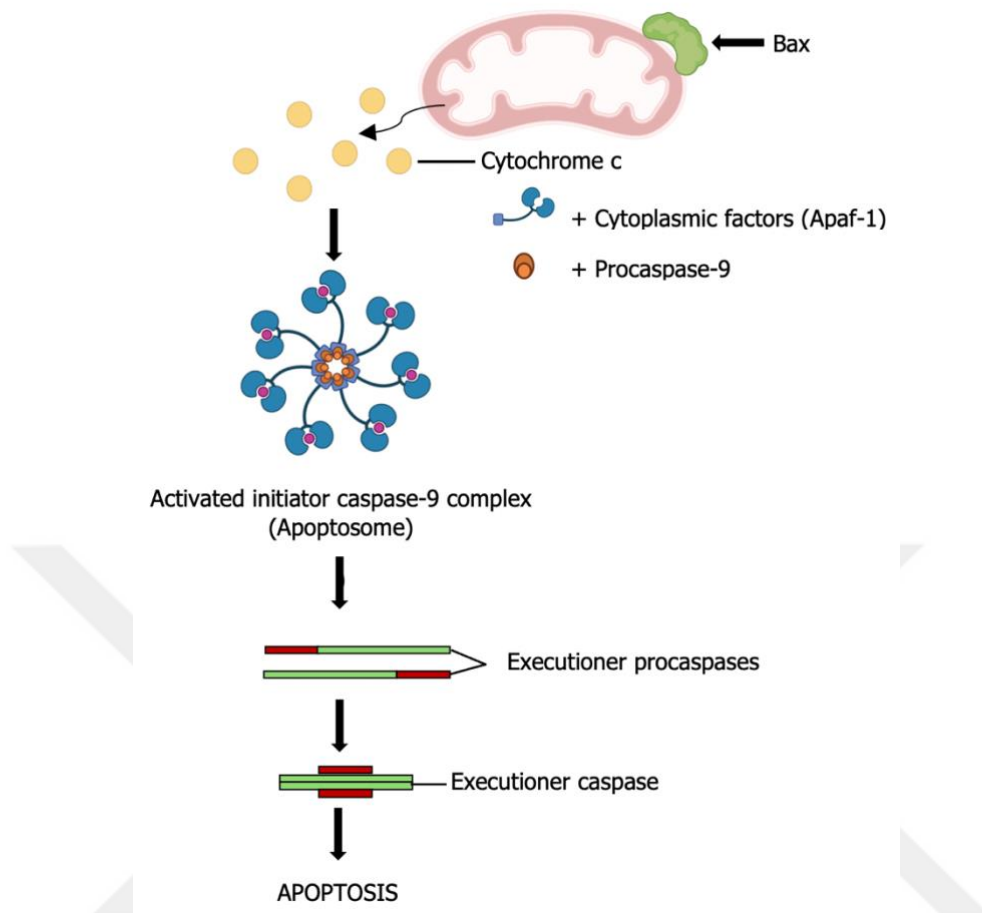


**Figure 1. Intrinsic pathway of apoptosis.**

The intrinsic pathway involves mitochondrial outer membrane permeabilization, leading to the release of cytochrome c, which facilitates apoptosome formation through Apaf-1 and initiator caspase-9, while BAX proteins create mitochondrial membrane pores, ultimately activating effector caspases that degrade cellular components. (Illustrations were created using BioRender)

#### 2.1.1.2. Extrinsic pathway

Extrinsic pathway (see Figure 2) occur due to external stimulants, from outside of the cell. Death receptors which are cell membrane proteins play roles in the extrinsic pathway of apoptosis. For example; FAS ligand (FasL) binding to Fas receptors, tumor necrosis factor-alpha (TNF- $\alpha$ ) binding to TNF- $\alpha$  receptor, and TNF-related apoptosis-inducing ligand (TRAIL) pathway. This binding then triggers oligomerization of

receptors and recruitment of adaptor proteins such as FADD (FAS-associated death domain protein) and TRADD (Tumor necrosis factor receptor type 1 -associated death domain protein). After the interaction, they activate pro-caspases-8 (Ogura et al., 2008).

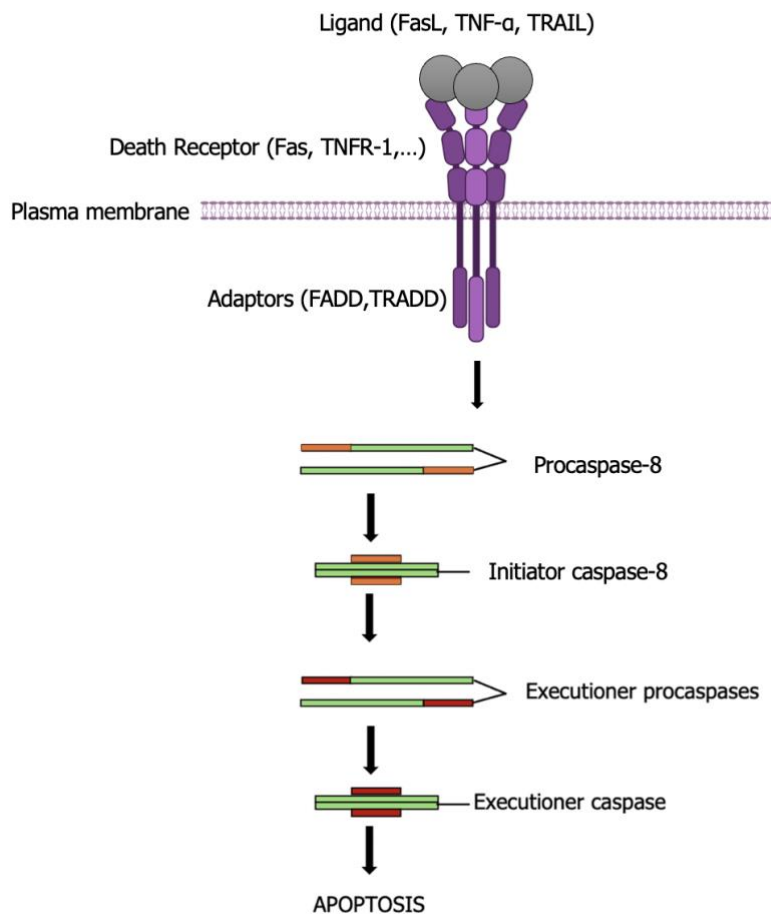


**Figure 2. Extrinsic pathway of apoptosis.**

The extrinsic pathway of apoptosis is triggered by external signals, such as Fas and TNF-α receptors, bind to its ligands FasL or TNF-α, and this leads to receptor oligomerization and recruitment of adaptor proteins which then activate pro-caspases-8. (Illustrations were created using BioRender)

### 2.1.2. Apoptosis mechanisms and steps

Cells undergoing apoptosis release some “find me” signals such as LPC (Lysophosphatidylcholine), S1P (Sphingosine-1-phosphate), and CX3CL1 (C-X3-C Motif

Chemokine Ligand 1) and these signals can be recognized by receptors on phagocytes or through bridging molecules (Li & Li & Fan, 2021; Law et al., 2019). Later on, “eat me” signaling molecules from apoptotic bodies (ApoBD), mainly phosphatidylserine (PS) bind to receptors or bridging molecules and can alter cytoskeletal modifications of phagocytes for appropriate ingestion of ApoBDs (Rosales & Uribe-Querol, 2017). The main steps of apoptosis are shown in Figure 3 and can be summarized as; cell shrinkage, chromatin compensation, and membrane blebbing that occurs when pro-apoptotic signals come to healthy cells, then apoptotic bodies form and these apoptotic bodies are engulfed by phagocytes. In the end, there is no inflammation regarding this phagocytosis process.

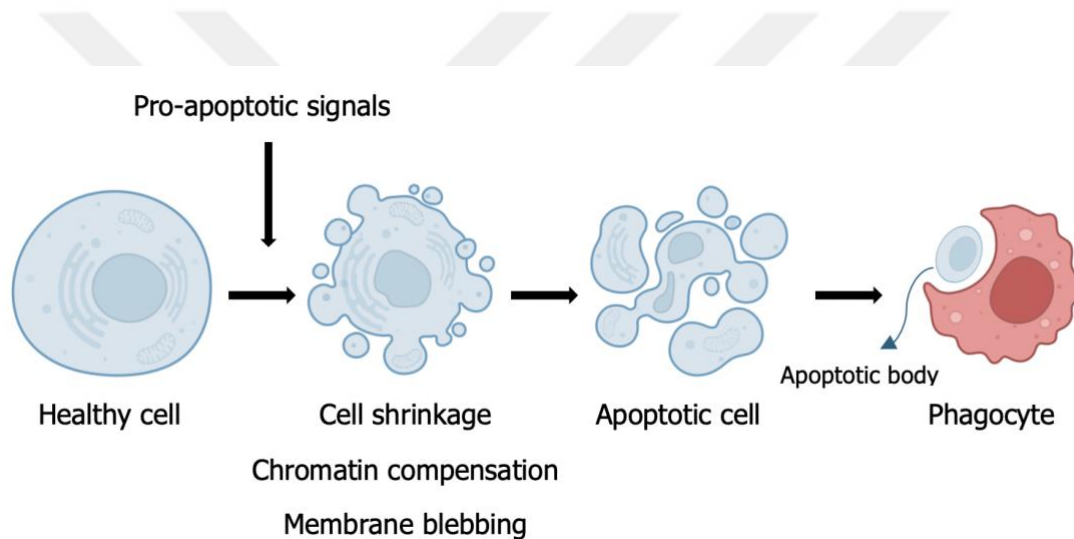


**Figure 3. The main steps of apoptosis.**

Pro-apoptotic signals such as BAX, BAD, BAK have a significant role in apoptosis. They initiate many signaling cascades related to physical changing of the cell just as compensation of chromatins and membrane blebbing. Later apoptotic bodies occur and they cleared by phagocytes. (Illustrations were created using BioRender)

## 2.2. Phagocytosis

Phagoptosis which is cell death by phagocytosis occurs when a live cell is phagocytosed by another cell, such as macrophages, or dendritic cells, and engulfed cells die because of the digestion process inside the engulfing cell (Brown, 2024). Cell death by phagocytosis is not the same thing as efferocytosis, which is the phagocytosis of an apoptotic cell. Because in efferocytosis, also known as 'secondary phagocytosis', phagocytosis contributes to the clearance of the cells, not causing the death of the cell actually (Boada-Romero et al., 2020). Phagocytosis of the cell can be performed by professional phagocytes (macrophages, dendritic cells, neutrophils, monocytes) or non-professional phagocytes (epithelial cells, fibroblasts, endothelial cells) (Uribe-Querol & Rosales, 2020).

Elie Metchnikoff also known as the father of innate immunity, discovered the phagocytosis of dead cells in the 1880s (Gordon, 2016). He examined white blood cells (WBCs) are phagocytosing pathogens and he won the Nobel Prize in Physiology and Medicine in 1908 for that discovery.

Phagocytes recognize their targets with the receptors on their cell surface. With these receptors, eat-me signals, opsonins, or molecular patterns on target cells are recognized directly or indirectly. As an example of indirect recognition, opsonins are extracellular soluble proteins and act as bridging molecules just as GAS6, Protein S, and MFG8 between the target and effector receptors (Hart & Smith & Dransfield, 2004). IgG and IgM antibodies, which are opsonins, activate Fc receptors on phagocytes (Mosser & Zhang, 2011). In animal cells, pathogen phagocytosis mainly occurs with scavenger receptors that can recognize a wide range of polyanionic ligands found on host cells or pathogens (Taban et al., 2022; Jauslin et al., 2021).

## 2.3. Efferocytosis

Normally in the human body around 300 billion cells have been turning over to get rid of some cells. Such as T cells has a high binding affinity to self-peptides, known as negative selection, also damaged, senescent, and aged cells (Kinchen & Ravichandran,

2008; Sender & Milo, 2021). This turnover of cells proceeds with apoptosis and it does not activate or trigger inflammatory responses or inflammatory-mediated immune cell alternations in the area (Ravichandran & Lorenz, 2007). Phagocytes such as macrophages, neutrophils, and dendritic cells play a main role in removing apoptotic cells, known as efferocytosis. It derives from the Greek word 'effere' which means 'carrying the dead to the grave'. The distinction of healthy cells from dying or unwanted ones takes place with the guidance of different signals such as "eat me" and "don't eat me" signals. Cells display these markers or ligands on their surface and later they can be recognized by various receptors on phagocytes. These signals determine if a cell is edible or passing by phagocytes. If the cell undergoes apoptosis, don't eat me signals are down-regulated or presented in an altered structure.

Macrophages play an essential role in the innate immune system such as the secretion of cytokines that can accelerate or decelerate many other immunological reactions or regulation of the engulfment process depending on the activating and inhibitory signals.

Efferocytosis has a vital role in antigen cross-presentation. If the cell is infected and later becomes apoptotic, efferocytosis of these cells provides different sources of foreign antigens to be expressed through MHC class I to cytotoxic T-cells rather than MHC II (Tzelepis et al., 2015). Also, appropriate and rapid efferocytosis is important because if the tidying process is not enough or takes too much time, there is a risk of secondary necrosis which leads an inflammation or immune-related diseases (Lin et al., 2020; Ge & Huang & Yao, 2022).

### 2.3.1. Don't eat me signals on healthy cells

#### 2.3.1.1. CD47 - Sirpa interaction

Cell surface glycoprotein CD47 (Cluster of Differentiation 47) is a well-known "don't eat me" signal as it protects the cells from engulfment. Nearly all living cells display CD47, also known as integrin-associated protein (IAP) protein on their surfaces as a "don't eat me" signal that can bind to its receptor Sirpa- $\alpha$  (Signal regulatory protein  $\alpha$ ) on phagocytes and this interaction successfully inhibits phagocytosis. In several

studies, it has been shown that the cells that lack CD47 are quickly engulfed by macrophages (Yi et al., 2015). In cancer cells, the expression of CD47 is frequently upregulated as a way to evade immune surveillance (Huang et al., 2020). This has sparked interest in investigating the potential of CD47 inhibition or blockade for use in cancer therapy (Takimoto et al., 2019). In studies done with CD47 blocking antibodies, it has been reported that cancer cell growth was slowed down also resulting in tumor elimination in some diseases such as Non-Hodgkin's Lymphoma and brain tumors (Advani et al., 2018; Gholamin et al., 2017). CD47 blockade has potential for treating not just cancer, but also diseases like viral infections and atherosclerosis (Kojima et al., 2016; Cham et al., 2020).

Sirpa protein on the surface of macrophages or dendritic cells interacts with the CD47 on target cells. It is known as an inhibitory receptor due to its multiple intracellular immune tyrosine-based inhibitory motifs (ITIMs). In a related study, it has been shown that macrophages without Sirpa don't show decreased phagocytosis of CD47-bearing targets which suggests that Sirpa is the main receptor for the CD47 signal (Morrissey & Kern & Vale, 2020).

#### 2.3.1.2. MHC I - LILRB1 interaction

On healthy cells, MHC class I (major histocompatibility complex 1) protein can inhibit phagocytosis by binding to LILRB1 (Leukocyte immunoglobulin-like receptor subfamily B member 1) on phagocytes (Barkal et al., 2018). MHC I molecules bind peptides to display them for T cells. LILRB1, also known as ILT2 (Immunoglobulin-Like Transcript 2) is also expressed on a cell surface primarily on immune cells (Hu et al., 2024) and functions as an inhibitory receptor. In many studies, it has been shown that cancer cells can utilize this MHC I/ LILRB1 axis to evade immune surveillance. For that reason, studies that block this interaction gained interest in recent years (Wu et al., 2023).

### 2.3.2. Eat-me signals on apoptotic cells

When a cell undergoes apoptosis, it displays some signals known as 'eat me' signals such as phosphatidylserine, calreticulin, and intercellular adhesion molecule 3 (ICAM3) (see Figure 4). Among them, PS is the most characterized and studied one.

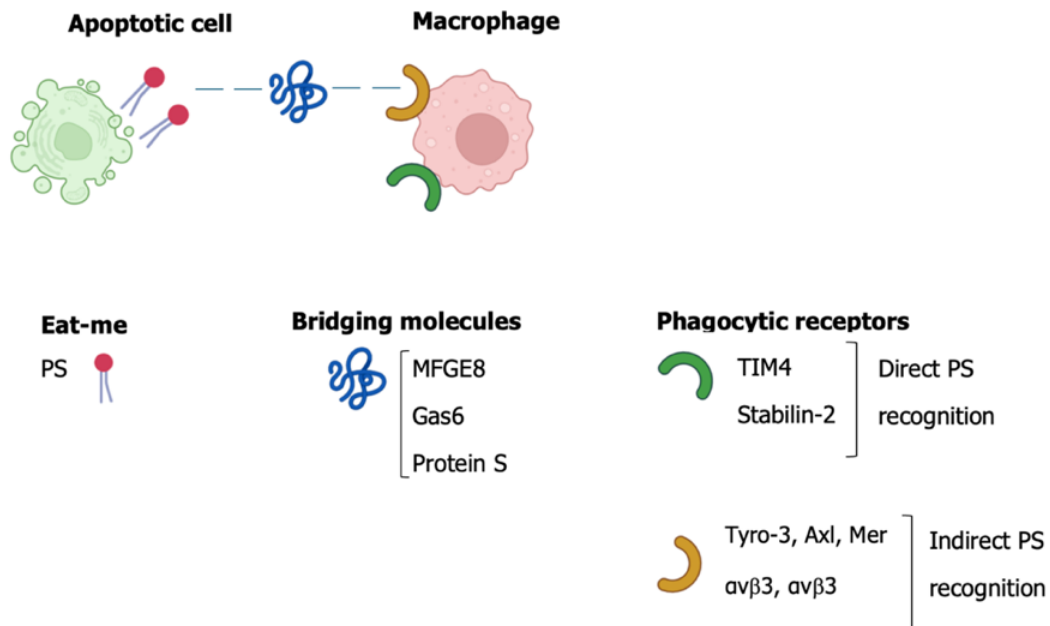


**Figure 4. Eat-me signals on apoptotic cells.**

Phosphatidylserine on apoptotic cells can be recognized directly or indirectly by phagocytes. Bridging molecules which can bind to PS on one side and to receptors on phagocytes on the other side. Because of that they play significant role in indirect recognition of PS by phagocytes. (Illustrations were created using BioRender)

#### 2.3.2.1. Phosphatidylserine

Phosphatidylserine is an anionic phospholipid found on plasma membranes. Normally in healthy cells, PS is held on the cytoplasmic surface of the plasma membrane with the help of flippase, which is an ATP-dependent enzyme (Nagata et al., 2016). Because of that, viable cells establish phospholipid asymmetry, since phosphatidylcholine (PC)

and sphingomyelin (SM) are on the outer leaflet but PS and phosphatidylethanolamine (PE) are in the inner leaflet of the plasma membrane (Fadeel & Xue, 2009). In apoptosis, PS is exposed to the outer leaflet of the membrane by scramblases, which are specific proteins responsible for phospholipid translocation and this process is also calcium-dependent. PS externalization is a marker for a cell undergoing apoptosis and can be recognized by phagocytic receptors found on macrophages (direct recognition) or by soluble bridging molecules such as Gas6 and MFGE8 (indirect recognition). For the studies, fluorescent conjugates of Annexin V, which can bind PS in a calcium-dependent manner, are widely used to detect apoptotic cells (Kari et al., 2022).

### 2.3.3. Direct PS recognition

#### 2.3.3.1. TIM-4

TIM-4 (T-cell immunoglobulin and mucin domain-containing 4) is a receptor found on antigen-presenting cells (APCs) such as macrophages and can bind to PS to promote efferocytosis. Firstly TIM-4 is defined as a protein involved regulation of T-cell proliferation (Meyers et al., 2005). In other studies, it has been also shown that TIM-4 is not only expressed on APCs but also on natural killer T cells (NKT), peritoneal B1 cells, and mast cells (Wong et al., 2010; Zhang et al., 2016; Li et al., 2014). TIM-4 cannot directly transfer phagocytic signals but it is esteemed to be a 'tethered receptor' that supports efferocytosis of apoptotic cells and later other apoptotic signals can interact with them to continue the process (Miyanishi et al., 2007; Wang et al., 2023).

#### 2.3.3.2. Stabilin-2

Stabilin-2, which functions as a scavenger receptor, is shown to recognize PS and has a role in the engulfment of apoptotic cells (D'Souza & Park & Kim, 2013). Unlike TIM-4, stabilin-2 mediates both tethering and also the internalization of tethered dead cells and later stimulates downstream signaling pathways, known as 'tickling' (Park et al., 2008; Park et al., 2019). Stabilin-2 is expressed on macrophages and other phagocytes.

#### 2.3.4. Bridging molecules that play role in indirect PS recognition

##### 2.3.4.1. MFGE8

Milk fat globule-EGF factor 8 protein (MFGE8), also known as lactadherin, is a cell adhesion protein that functions in arteries. Since MFGE8 includes a PS binding domain and because of that it serves as a bridging molecule for its receptors,  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrins on macrophages that allow engulfment of the dying cell (Fuller & Van Eldik, 2008; Borisenko et al., 2004).

##### 2.3.4.2. Gas6

Growth arrest-specific 6 (Gas6) is a vitamin K-dependent soluble protein, and a ligand for all receptor tyrosine kinases Axl, Mer, and Tyro3 found on phagocytes. Just as other vitamin K-dependent proteins, Gas6 contains gamma-carboxyglutamic acid (Gla) domain at N-terminus (Rajotte, Hasanbasic, Blostein, 2008). Gas6-PS binding is  $\text{Ca}^{2+}$  dependent and also vitamin K-dependent gamma-carboxylation of Gas6 is necessary (Geng et al., 2017; Nakano et al., 1997). When Gas6 is bound to PS, TAM (Tyro3/Axl/Mer) receptors expressed on phagocytes get activated and this leads to efferocytosis of dead cells.

##### 2.3.4.3. Protein S

Protein S (PROS1) is a  $\gamma$ -carboxylated vitamin K-dependent protein too just like Gas6. Protein S and Gas6 share partly homolog domain structures but Protein S is able to bind Tyro3 and Mer but not Axl (Tsou et al., 2014). It is first known as a blood coagulation regulator protein but later in the studies, it has been shown that protein S mediates macrophage phagocytosis of apoptotic cells and serves as a bridging molecule between apoptotic cell and phagocyte (Heeb et al., 1994; Anderson et al., 2002).

### 2.3.5. Indirect PS recognition

#### 2.3.5.1. Tyro3/Axl/Mer Receptors

Tyro3/Axl/Mer (TAM) receptors belong to the receptor tyrosine kinases family and they have various roles in many diseases such as affecting cell growth, adhesion, migration in cancer cells, and regulation of coagulation. These receptors are activated by Protein S and Gas6, which are vitamin K-dependent soluble proteins (van der Meer & van der Poll & van 't Veer, 2014). All three TAM receptors are expressed on macrophages and this signaling plays a key role in efferocytosis.

#### 2.3.5.2. $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins

Integrins are multifunctional glycoproteins that play various roles in tumor growth, migration, and invasion as well as differentiation of healthy cells. Integrins are involved in cell to cell or cell to extracellular matrix interactions. They are formed from  $\alpha$  and  $\beta$  subunits. In recent years,  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrins gained interest as they can be used as targets in the oncology area for the treatment of cancers.  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrins are expressed on macrophages and they interact with MFGE8 bridging molecule to promote phagocytosis (Böger et al., 2015).

#### 2.3.2.2. Calreticulin

Calreticulin (CRT) is expressed in many parts of the cell but is mainly located in the endoplasmic reticulum (ER) and has many various roles such as calcium homeostasis, folding of proteins, and regulation of immune systems (Mei et al., 2024). Besides these roles, calreticulin acts as 'eat me' signals on the dying cells. Calreticulin is upregulating on the surface of apoptotic cells and later they externalized, bind, and activate LDL-receptor related protein 1 (LRP1, also known as CD91) on the surface of phagocytic cells (Gardai et al., 2005).

#### 2.3.2.3. Intercellular adhesion molecule 3

Intercellular adhesion molecule 3 (ICAM3) also known as CD50, is a transmembrane glycoprotein and a member of the immunoglobulin-super family (IgSF). ICAM3 is mainly expressed on leukocytes and serves as another 'eat-me' molecule on apoptotic cells (Kristóf et al., 2013). LFA-1 (Lymphocyte function-associated antigen 1) expressed on macrophages can bind ICAM3 and this contributes to efferocytosis of dead cells (Torr et al., 2012).



### **3. MATERIALS AND METHODS**

#### **3.1 Type of the study**

This study includes experimental methods to analyze the effect of recombinant proteins on efferocytosis mechanisms on mouse cancer cell line 4T1.

#### **3.2. Time and location of the study**

All experiments in this study took place at Izmir Tınaztepe University, Galen Research Center between September 2023-August 2024.

#### **3.3. Test systems**

Cell cultures were used as a test system in this study. HEK293T cell was in Ozturk Lab's inventory. RAW264.7 cell line was kindly supplied by Genc Lab and 4T1 cell line by THORLAB in IBG.

#### **3.4. Variables of the study**

Phagocytosis capability of macrophages cocultured with apoptotic target cells with or without recombinant proteins are the variables of the study. The differences in phagocytosis percentage were analyzed in this study.

#### **3.5. Materials**

**Table 1.** List of equipment

| <b>Equipment</b>           | <b>Brand</b> | <b>Catalogue Number</b> |
|----------------------------|--------------|-------------------------|
| CO2 Incubator              | Nuve         | 03.0789                 |
| Biosafety Cabin Class IIA2 | Bilser       | 12101                   |
| Bench-Top Centrifuge       | Nuve         | Z10.NF 400              |
| Magnetic Stirrer           | Miulab       | MU-E164-91137           |
| Mini Centrifuge            | Miulab       | MU-E34B-91548           |
| Cell Aspirator             | Miulab       | MU-E153-91003           |
| Orbital Shaker             | Miulab       | MU-E49-91046            |
| Vortex                     | Miulab       | MU-E88-91147            |
| Light Microscope           | Olympus      | 1C41054                 |
| Analytical Balance         | Radweg       | 684089                  |

|                                        |                 |           |
|----------------------------------------|-----------------|-----------|
| ELISA Reader                           | ChroMate        | 4300-4968 |
| Cytoflex Flow Cytometry                | Beckman Coulter | BE51541   |
| NanoPhotometer                         | Implen          | N60 touch |
| NGC Quest 100<br>Chromatography System | Biorad          | 7880004   |

**Table 2.** List of chemical supplies and kit

| Product Name                                  | Brand             | Catalogue Number |
|-----------------------------------------------|-------------------|------------------|
| <b>Cell Culture</b>                           |                   |                  |
| RPMI-1640                                     | Gibco             | 21875-034        |
| DMEM                                          | Gibco             | 11965-092        |
| Fetal Bovine Serum (FBS)                      | Gibco             | 10270-106        |
| Penicillin Streptomycin                       | Gibco             | 15140-122        |
| Non-Essential Amino Acids Solution (100x)     | Gibco             | 11140-050        |
| %0,25 Trypsin-EDTA (1x)                       | Gibco             | 25200-056        |
| Trypan Blue                                   | Gibco             | 15250-061        |
| Fugene HD                                     | Promega           | E2311            |
| Optipro                                       | Gibco             | 12309-050        |
| Dimethylsulfoxide (DMSO)                      | Sigma             | 276855           |
| Glutamax                                      | Gibco             | 35050-061        |
| Geneticin Selective Antibiotic (G418 Sulfate) | Thermo Scientific | 10131035         |
| Sodium selenite                               | Sigma             | 214485           |
| LPS                                           | Sigma             | L6529            |
| Vybrant Dil Cell Labeling Solution            | Thermo Scientific | V22885           |
| Vybrant DiO Cell Labeling Solution            | Thermo Scientific | V22886           |
| <b>Flow Cytometry</b>                         |                   |                  |
| Annexin V – 7AAD kit                          | Beckman Coulter   | IM3614           |

|                                 |                   |          |
|---------------------------------|-------------------|----------|
| <b>ELISA</b>                    |                   |          |
| TMB Substrate Kit               | Thermo Scientific | 34021    |
| Other                           |                   |          |
| Nucleobond Xtra Midi Kit        | MN                | 740410   |
| Mix and Go Kit                  | Zymo research     | T3001    |
| GeneJET gel isolation kit       | Thermo Scientific | K0691    |
| GeneJET plasmid isolation kit   | Thermo Scientific | K0502    |
| EconoFit Unosphere SuprA column | Biorad            | 12009321 |

**Table 3.** Cell lines used in the study

| Cell Line | Derivation                                    | Organism            | Cell/tissue type      | Growth properties |
|-----------|-----------------------------------------------|---------------------|-----------------------|-------------------|
| 4T1       | tumor-derived cell line from BALB/cfC3H mouse | <i>Mus musculus</i> | Breast; Mammary gland | Adherent          |
| HEK293T   | kidney of a human embryo                      | <i>Homo sapiens</i> | Kidney; Embryo        | Adherent          |
| RAW 264.7 | from a tumor in a BALB/c mouse                | <i>Mus Musculus</i> | Macrophage            | Adherent          |

### 3.6. Methods

#### 3.6.1. Culture of the cell lines

For cell thawing, a vial was taken from liquid nitrogen storage and immediately transferred to a 37°C water bath, where it was left for a couple of minutes until fully thawed. The vial containing 1 mL of freezing medium was then transferred to a falcon tube containing pre-warmed RPMI medium. The cells were centrifuged at 300 x g for 5 minutes at room temperature. To completely remove DMSO, the supernatant was discarded, and the pellet was resuspended in a %10 FBS (fetal bovine serum) complete

medium. For cell counting, 10  $\mu$ L of the resuspended cells were mixed with 10  $\mu$ L of trypan blue and counted using a hemocytometer. For adherent cells, approximately  $0.6 \times 10^5$  cells were seeded in a T25 flask, and  $2 \times 10^6$  cells were seeded in a T75 flask, depending on the further experiment conditions. Freezing media, which consists %90 FBS and %10 DMSO was used for the storage of cells.

### 3.6.2. Design of chimeric proteins

In order to block the CD47 "don't eat me" signal on target cells by Sirpa-Fc, a chimeric protein consisting of the addition of the CD47 binding region of mouse Sirpa on the mouse Fc outline was designed.

The mouse Fc region was designed based on IgG2a and contains the CH2-CH3 domain of the IgG heavy chain between amino acids 98-330 (UniProt ID: P01863). The Sirpa-Fc protein is engineered to be added into the N-terminal region of the structure. Since Sirpa binds to CD47 through the V region located outside the cell, the V module was used for the construction of Sirpa-Fc. For this region, amino acid sequences 7-114 of the Sirpa form obtained from the NOD mouse, which has a higher binding capacity to CD47, were used. Representative structures of Sirpa-Fc chimeric proteins designed to be produced and IgG-Fc structure that will be used as a negative control in the experiments are shown in Figure 5. There is a 6x-histidine tag at the carboxy ends of the proteins.

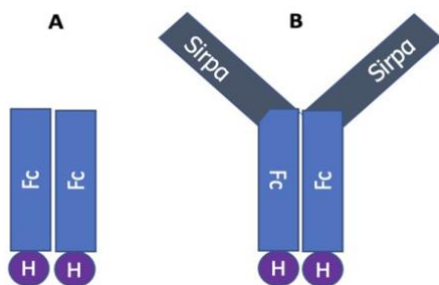


**Figure 5. Structure of chimeric recombinant proteins.**

Illustration on the left (a) represents mouse control IgG-Fc protein. The right construct (b) involves the fusion of the mouse Sirpa domain to the N-terminus of mouse IgG-Fc.

Sirpa-Fc coding region was cloned into the vector containing the V<sub>k</sub>A26 signal peptide. V<sub>k</sub>A26 signal peptide at the N-terminus is for the designed gene to be expressed and secreted outside the cell.

### 3.6.3. Acquisition of plasmid inserts through gene synthesis

The sequences were designed in silico using the SnapGene software, as illustrated in Figure 6, and their synthesis was provided by services from the company. Upon synthesis, the gene sequences were delivered to us within the pUC57 carrier vector. The vectors, provided in lyophilized form, were reconstituted in pure water. To facilitate their use in subsequent experiments, these plasmids were transformed into TOP10 *E. coli* competent cells, which had been previously prepared using the 'Mix & Go' kit from Zymo Research. The transformed bacteria were plated on solid agar containing ampicillin and incubated at 37°C overnight to allow colony formation. Single colonies were then selected and cultured in 5 mL of Luria Broth (LB) medium with ampicillin selection in a shaking incubator. A glycerol stock of each culture was prepared and stored at -80°C, while plasmid isolation was conducted on the remaining cultures using the MN NucleoSpin Plasmid Kit.

To construct a mammalian expression plasmid library, the pDAD2.0 expression vector containing our primary module, IgG2a-Fc, was first assembled. The IgG2a-Fc gene and the target expression vector pDAD2.0, initially carried in the pUC57 vector, were cut with NheI and NotI restriction enzymes. Following digestion, the gene and plasmid fragments were separated on an agarose gel, and the desired fragments were purified using the GeneJET Gel Extraction Kit. Subsequently, the purified gene and plasmid fragments were ligated using the NEB Quick Ligation Kit.

The resulting plasmids, which contain IgG2a-Fc and pDAD2.0 constructs, were transformed into competent bacteria. The bacteria were then plated on solid agar containing ampicillin and incubated overnight at 37°C. The next day, single colonies were selected, cultured in LB medium, and subjected to plasmid isolation using the GeneJET Plasmid Isolation Kit. To confirm the successful integration of IgG2a-Fc into

the pDAD2.0 vector, the candidate plasmids were digested with EcoRV and ClaI restriction enzymes for verification.

After obtaining our main module, the pDAD2.0\_IgG2a-Fc vector, the remaining gene fragment was transferred to the front of the IgG2a-Fc region in the pDAD2.0 vector (between the signal peptide and IgG2a-Fc) as we designed. For these processes, firstly, our pDAD2.0\_IgG2a-Fc main module vector was cut with EcoRV and ApaI enzymes. Similarly, the gene sequence of the chimeric protein Sirpa was excised from the pUC57 vector using the same enzyme pair, EcoRV and ApaI. The cloning steps, including ligation of the excised fragment into the vector, transformation of the plasmids into competent cells, selection of positive clones, and subsequent verification following ampicillin selection, were carried out in a sequential manner, consistent with the procedures used in constructing the pDAD2.0\_IgG2a-Fc main module vector. The plasmid map of Sirpa-Fc were shown in Figure 6.

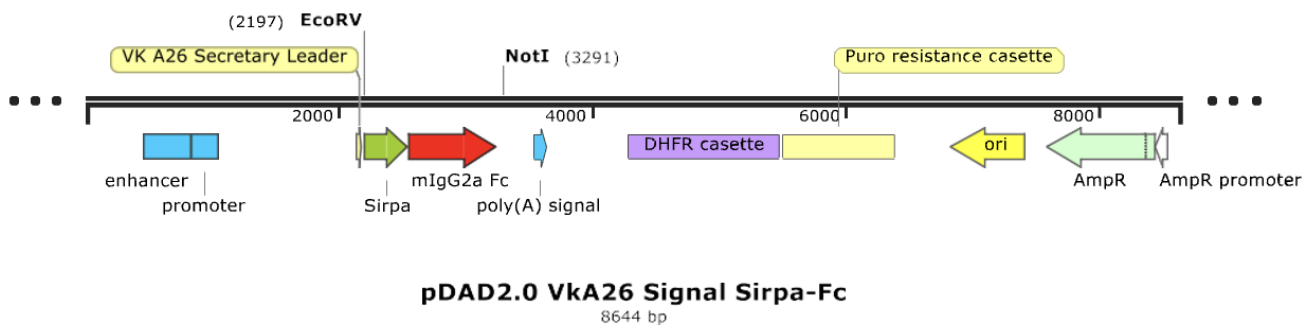


**Figure 6. pDAD2.0 Sirpa-Fc plasmid map.**

The plasmid map illustrates the cloning of the mouse Sirpa domain into the N-terminus of a mouse IgG-Fc construct. This plasmid incorporates the VK A26 secretory leader sequence to ensure proper secretion of the recombinant protein.

#### 3.6.4. Transfection of HEK293 cells with expression vectors

HEK293 cells were transiently transfected with IgG2a-Fc and IgG2a-Sirpa-Fc expression vectors and the relevant protein production was later evaluated by ELISA method. HEK293 cells, were seeded at 1 million cells per well in six well plates one day before transfection. Transfection was performed the following day when the cells reached approximately 70-80% confluency. FuGENE HD mammalian transfection agent (Promega) was used for transfection. In addition to the 2 mammalian expression vectors, a green fluorescent protein (GFP) expressing vector, was included to monitor transfection efficiency and a rabbit IgG producing vector were used as negative control. The instructions of the transfection kit were followed, with 3 µg of plasmid and 9ul of transfection agent in each well. The cells were then incubated for three days to allow for protein expression.

#### 3.6.5. ELISA analysis of proteins produced by HEK293 cells

After three days of expression, the supernatants from transiently transfected HEK293 cells were collected. ELISA was conducted on these protein samples.

96-well plates were coated with goat anti-mouse IgG antibody in carbonate-bicarbonate buffer. The plates were incubated overnight at 4°C. Following incubation, the wells were washed with PBS-T buffer and then blocked with PBS-T buffer containing 1% BSA for 1 hour at room temperature. After discarding the blocking solution, 100 µL of the cell supernatant samples were directly added to the wells. For the secondary antibody control group, PBS-T buffer containing 1% BSA was added. A commercial mouse IgG antibody, diluted in PBS-T buffer with 1% BSA, used as a positive control. After a 1 hour incubation at room temperature, the wells were washed three times with PBS-T buffer. A goat anti-mouse IgG2a HRP-conjugated secondary antibody, diluted in PBS-T buffer containing 1% BSA, was then added to the wells and incubated for 1 hour at room temperature. Following another washing step, TMB substrate solution was added to the wells and incubated for 5 minutes at room

temperature in the dark. The reaction was stopped with a stopping solution, and absorbance was measured at 450 nm using a spectrophotometer.

#### 3.6.6. Production of recombinant proteins

The HEK293T monoclonal cell line obtained during stable transfection of the relevant recombinant protein was cultured in 75 cm<sup>2</sup> cell dishes containing 10% FBS DMEM. Once the cells reached 70-80% confluency, they were washed once with 1x PBS and then incubated with trypsin for 3 minutes to detach the cells. The suspended cells were subsequently divided into seven 10 cm cell culture dishes, each containing 15 mL of DMEM with 10% FBS. The cells were allowed to grow for 2 days until they reached 70-80% confluency.

After reaching the desired density, the medium was removed, and fresh DMEM containing 1% FBS was added to the cells. The cells were then incubated for 5 days to facilitate recombinant protein production. At the end of the incubation period, approximately 100 mL of the conditioned medium, containing the recombinant proteins secreted by the cells, was collected. The collected medium was centrifuged at 300 x g for 5 minutes to remove any cellular debris. The supernatant was then collected for subsequent protein purification.

#### 3.6.7. Purification of recombinant proteins

Collected supernatant was purified by the Protein A affinity chromatography method. Before purification, the collected supernatant was filtered through a 0.22 micron filter. The filtered medium was then mixed with a binding solution at a ratio of at least 1:4 to match the pH and composition of the Protein A binding buffer (20 mM Sodium Phosphate, pH 7.2). The diluted medium was loaded onto a Protein A affinity column at a flow rate of 1 mL/min using the Bio-Rad NGC Quest 100 chromatography system. The column was washed with the binding buffer equivalent to five column volumes to remove non-specifically bound proteins. Recombinant proteins were then eluted from the column by passing an elution buffer (20 mM Sodium Citrate, pH 3.3) through the column at a flow rate of 1 mL/min. Based on the 280 nm absorbance peaks observed

in the chromatogram, fractions suspected to contain recombinant proteins were collected. The pH of these fractions was immediately adjusted to 7.2 by adding 85  $\mu$ L of neutralization solution (1M Tris, pH 9.0). The concentrations of the purified recombinant proteins were determined by measuring absorbance at 280 nm using a spectrophotometer. The recombinant proteins were subsequently desalted and buffer-exchanged into phosphate-buffered saline (PBS) using a desalting column. The final protein samples were stored at -20°C for further studies.

#### 3.6.8. Coomassie blue staining of purified recombinant proteins

For characterization, aliquots from the eluted fractions mixed with laemmli buffer which contains SDS (sodium dodecyl sulfate), glycerol, beta-mercaptoethanol and bromophenol blue and samples denatured at 95°C for 5 minutes and subjected to SDS-PAGE on a 12% polyacrylamide gel, followed by 80 volts conduction for 40 minutes. Coomassie blue staining was performed overnight to visualize the proteins. For destaining, %40 methanol and %10 acetic acid solution was used.

#### 3.6.9. Isolation of GFP plasmids

For labeling the mouse 4T1 cell line with green fluorescent protein (GFP) for subsequent use in efferocytosis experiments, the pCDNA13031 GFP vector was utilized, as shown in Figure 7. This vector was kindly provided by the Atabey Lab at İzmir Tinaztepe University, Galen Research Center. Plasmid purification was carried out as described previously, with the NucleoBond Xtra Midi Kit (MN) being used according to the manufacturer's instructions.



and 3  $\mu$ L FuGENE HD reagent in OptiPRO medium. After a 10-minute incubation, complete medium was added to the wells.

Using this reverse transfection method, approximately 50% transfection efficiency was achieved. The difference in transfection efficiency between the standard transfection protocol and the reverse transfection protocol is shown in Figure 8.

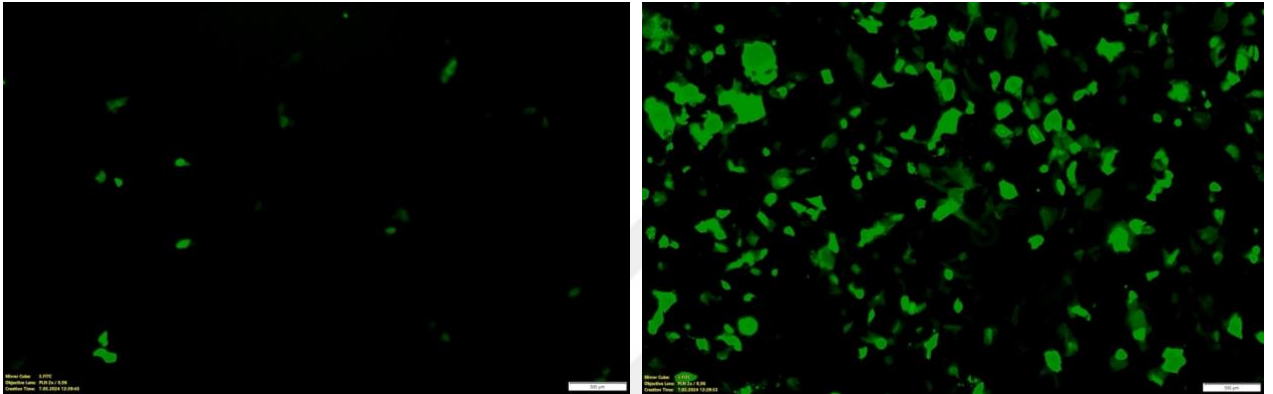


**Figure 8. Transient transfection of 4T1 cells using GFP plasmid.**

Cell images obtained using the standard protocol are shown on the left, while images using the reverse transfection method are displayed on the right. The images were captured with the FITC channel of a fluorescence microscope after 24 hours.

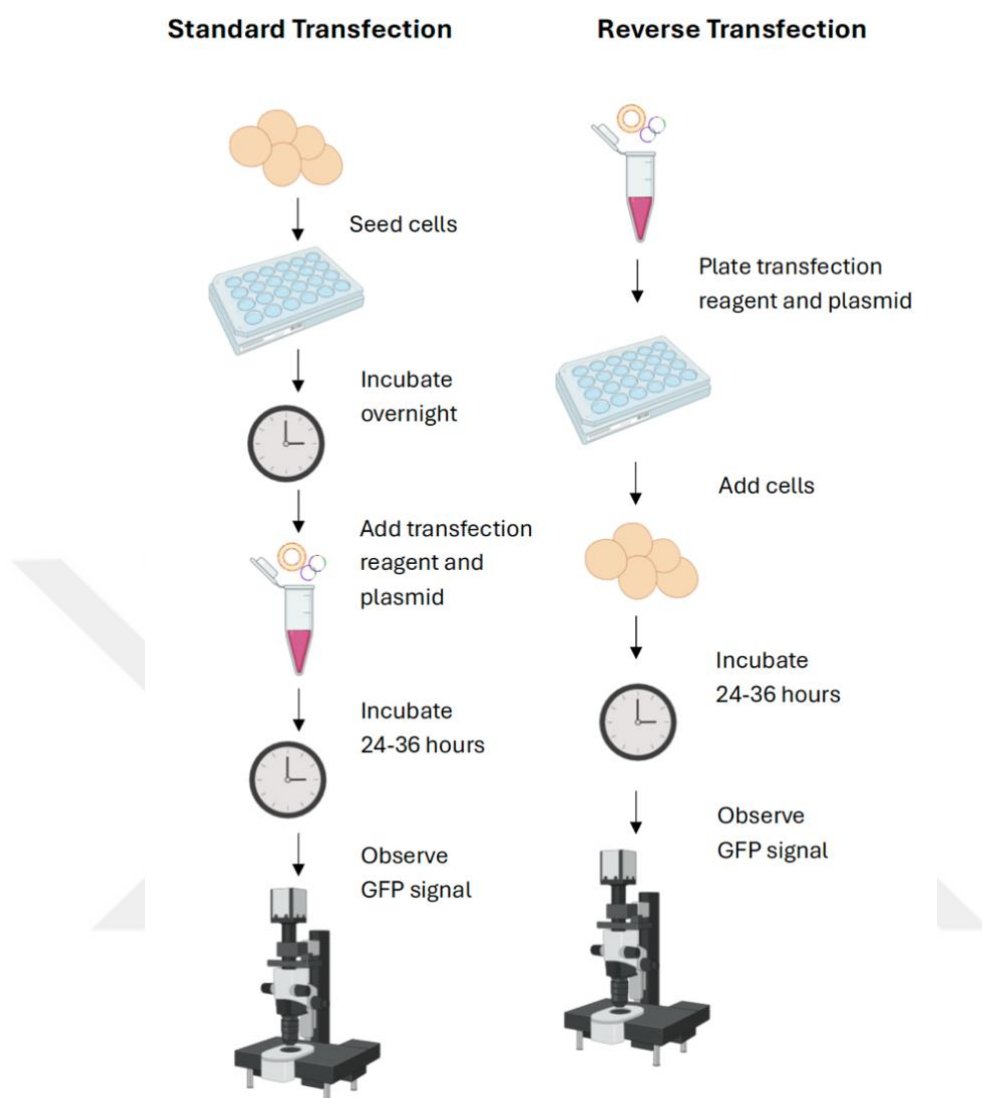


**Figure 9. Standard vs reverse transfection method.**

Demonstration of the differences between two different transfection methods (Illustrations were created using BioRender).

To stabilize the cells after transient transfection, they were incubated with 100 µg/mL geneticin selective antibiotic (G418 sulfate) for approximately one week. Monoclones were then established through single-cell seeding, and stocks were subsequently prepared. Images of the stabilized monoclonal are shown in Figure 10.

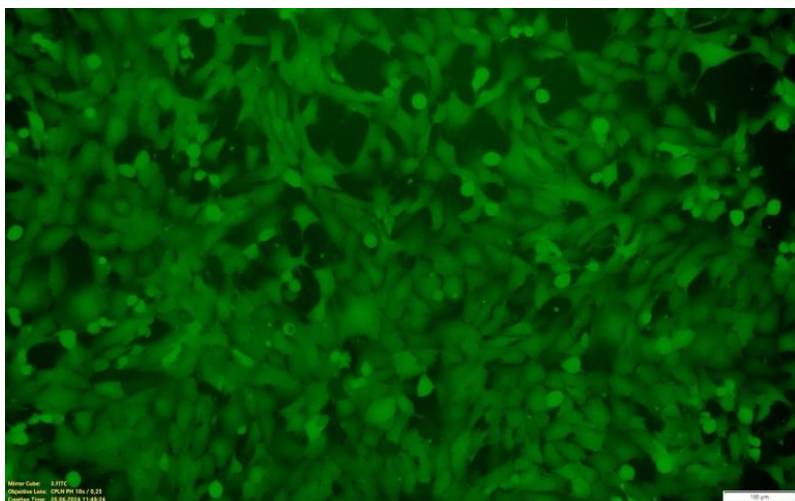


**Figure 10. Stably transfected 4T1 cell**

Demonstration of stable monoclones of 4T1-GFP cells. The images were captured with the FITC channel of a fluorescence microscope.

#### 3.6.11. Induction of apoptosis in 4T1-GFP cells

To be used in efferocytosis experiments, apoptosis was induced using 10  $\mu$ M sodium selenite. A total of  $2 \times 10^5$  cells were seeded in a 12-well plate with 1 mL of complete RPMI medium. Sodium selenite was added to the wells in specified concentrations, while untreated cells without sodium selenite used as controls. After 24 hours of incubation, the wells were examined under a light microscope to compare morphological differences with control cells.

The supernatants were collected into tubes, and the cells were washed once with 1x PBS. To detach the cells, a 1 mM EDTA + PBS solution was used. Detached cells and supernatants were combined in the same tube and centrifuged at  $300 \times g$  for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 100  $\mu$ L PBS. Because these cells were GFP positive, only 7AAD staining was performed and incubated for 15 minutes in the dark, following the manufacturer's instructions. Flow cytometry analyses were then performed. Unstained cells were used for gating.

#### 3.6.12. Evaluation of CD47 levels and Sirpa-Fc binding in 4T1 cells

CD47 expression in 4T1 cells was evaluated by using flow cytometry. For this purpose, a 1 mM EDTA + PBS solution was used to detach cells. Cells were blocked with FACS buffer for around 40 minutes and then incubated with 1 µg/ml anti-mouse CD47 antibody (InVivoMAb) for 1 hour. After washing steps, Alexa Fluor 568 goat anti-mouse IgG (H+L) secondary antibody was incubated for 1 hour with 1:2000 dilution. After washing steps, samples dissolved in FACS buffer and the expression of CD47 in 4T1-GFP cells was evaluated using the PE-CF594 channel.

For Sirpa-Fc binding, after blocking step, 25 µg/ml purified Sirpa-Fc was added to the wells and incubated for one hour. After washing steps, Alexa Fluor 488 goat anti-mouse IgG (H+L) secondary antibody was incubated for 1 hour with 1:2000 dilution and later performed flow cytometry. As a control, 25 µg/ml IgG-Fc was used.

#### 3.6.13. Cell labeling with fluorescent membrane dyes

Before the efferocytosis assay, cells were labeled with fluorescent membrane dyes. Vybrant DiI, an orange-red fluorescent dye, was used to stain macrophages, while target cells were labeled with Vybrant DiO, a green fluorescent dye. Initially, the cells were centrifuged at 300 x g for 5 minutes and washed once with 1x PBS. After counting the cells, the pellet was resuspended in 100 µL of PBS. For each  $1 \times 10^6$  cells, 1 µL of the respective dye was added to the suspension, and the cells were incubated for 20 minutes at 37°C. After labeling, the cells were seeded at optimal densities for further experiments.

#### 3.6.14. Induction of apoptosis in 4T1 cell

Prior to the efferocytosis assays, apoptosis was induced using sodium selenite. A total of  $2 \times 10^5$  cells were seeded in a 12-well plate with 1 mL of complete RPMI medium. Sodium selenite was added to the wells in specified concentrations, while untreated cells without sodium selenite used as controls. After 24 hours of incubation, the wells

were examined under a light microscope to compare morphological differences with control cells.

The supernatants were collected into tubes, and the cells were washed once with 1x PBS. To detach the cells, a 1 mM EDTA + PBS solution was used. Detached cells and supernatants were combined in the same tube and centrifuged at 300 x g for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 100 µL of Annexin V-binding buffer, provided with the Annexin V-FITC/7AAD kit.

To identify apoptotic cells, 5 µL of Annexin V and 10 µL of 7AAD were added to the cell suspension and incubated for 15 minutes in the dark, following the manufacturer's instructions. Flow cytometry analyses were then performed. Unstained cells were used for gating.

#### 3.6.15. Macrophage induction by lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) was used to induce and activate RAW264.7 macrophages. One day prior to the co-culture and efferocytosis experiments, 10 ng/mL LPS was used and the cells were incubated for 24 hours at 37°C in a 5% CO<sub>2</sub> incubator. Morphological changes in the cells were observed using a light microscope.

#### 3.6.16. Co-culture experiments and efferocytosis assays

4T1 cell, mouse breast cancer cell line, were used as target cells in co-culture experiments, and mouse macrophage cell line RAW264.7 were used as effector cells. Co-culture experiments and efferocytosis assays were performed in 96-well plates. After apoptosis was induced in 4T1 cells and RAW264.7 cells were induced by LPS, cells were seeded at a different target/effector ratio in serum-free RPMI medium and incubated overnight. Later the cells were washed and fixed by using %4 paraformaldehyde (PFA) and diluted with PBS for better counting in the next step. The phagocytosis of apoptotic target cells by effector cells was observed using a fluorescence microscope. The effector cells alone (red) and engulfed cells (green+red)

were counted. The percentage of phagocytosis was calculated by the ratio of total green+red cells to total red + (green+red) cells. All efferocytosis experiments were performed in triplicate and each pictures were taken from five different areas of 96 wells. TRITC channel was used for monitoring red-stained macrophage and the FITC channel was used for green-stained 4T1 cells later two channels were combined. Representative images of efferocytosis experiments are shown in Figure 11.

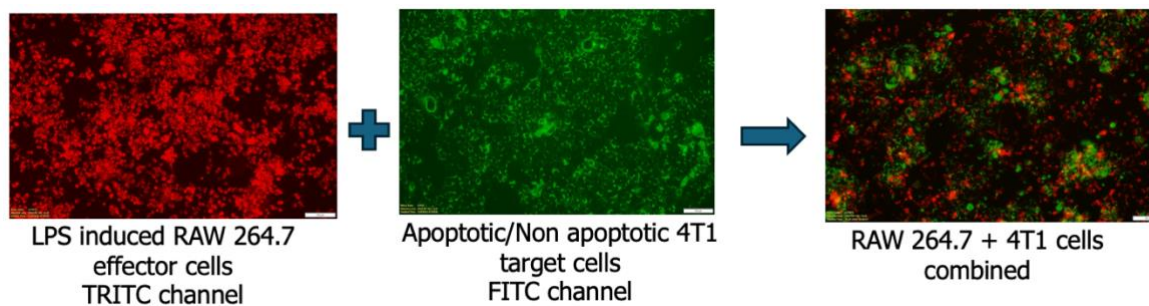


**Figure 11. Representantative images of efferocytosis experiments.**

Macrophages were stained with Vybrant DiI, an orange-red fluorescent dye, while target cells were labeled with Vybrant DiO, a green fluorescent dye. The TRITC channel was used to monitor the red-stained macrophages, while the FITC channel was employed for the green-stained 4T1 cell.

## 4. RESULTS

### 4.1. Recombinant protein production

#### 4.1.1. Validation of gene constructs via restriction enzyme digestion

To validate the gene structures in all constructed vectors, each vector was subjected to restriction enzyme digestion using enzyme pairs distinct from those employed during the cloning process. In this verification step, the digestion patterns simulated in the SnapGene software were compared with the corresponding agarose gel images obtained experimentally. The results confirmed that the vectors were structurally consistent with our expectations (Figure 12).

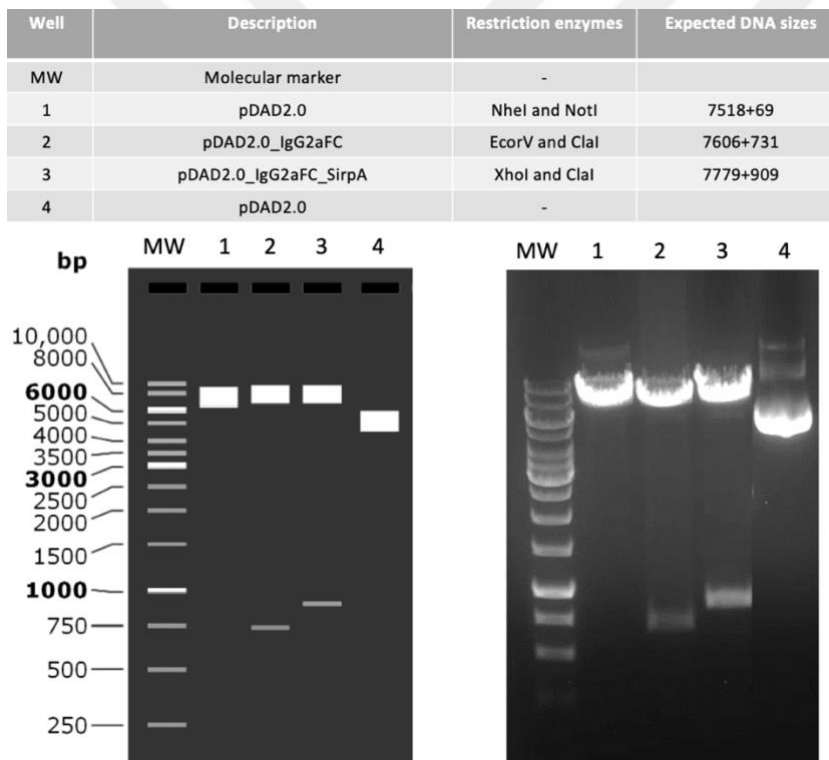


**Figure 12. Validation of gene constructs.**

Gene constructs confirmed via restriction enzyme digestion. During this verification step, the digestion patterns predicted by SnapGene software were matched against the corresponding agarose gel images obtained experimentally.

#### 4.1.2. ELISA analysis of proteins produced by transfected HEK293 cells

To quantify and analyze the expression levels of mouse IgG and Sirpa-Fc proteins, we employed the enzyme linked immunosorbent assay (ELISA). Firstly we determined if the cells secrete IgG proteins. In Figure 13, it was shown that as expected, no signals were obtained from culture medium sample and negative control groups. We received high signal from the Sirpa supernatant and also 1:10 diluted supernatant too. As a positive control, commercial mouse IgG-containing antibody was used.

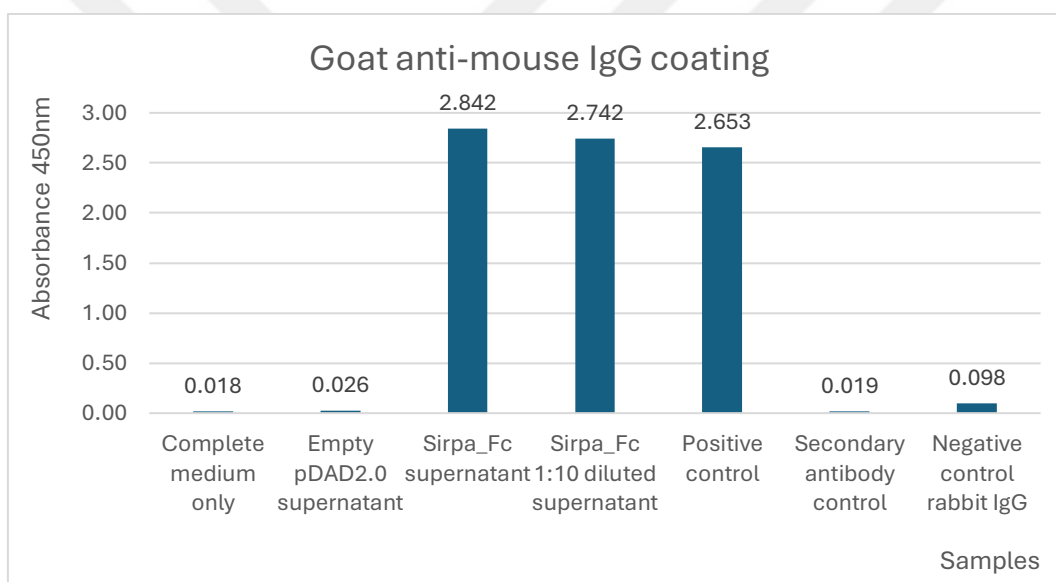


**Figure 13. ELISA analysis of mouse IgG proteins.**

Mouse IgG proteins secreted by transfected cells evaluated by ELISA method. Mouse IgG containing antibody used as a positive control. Absorbance was measured at 450 nm using a spectrophotometer.

Later, Sirpa-Fc was evaluated for its capacity to bind to mouse CD47 protein. For this purpose, commercially bought mouse CD47 protein was used. As shown in Figure 14, no signals were obtained from culture medium sample and negative control groups. HRP-conjugated anti-his antibody against his tag on CD47 used as a positive control.

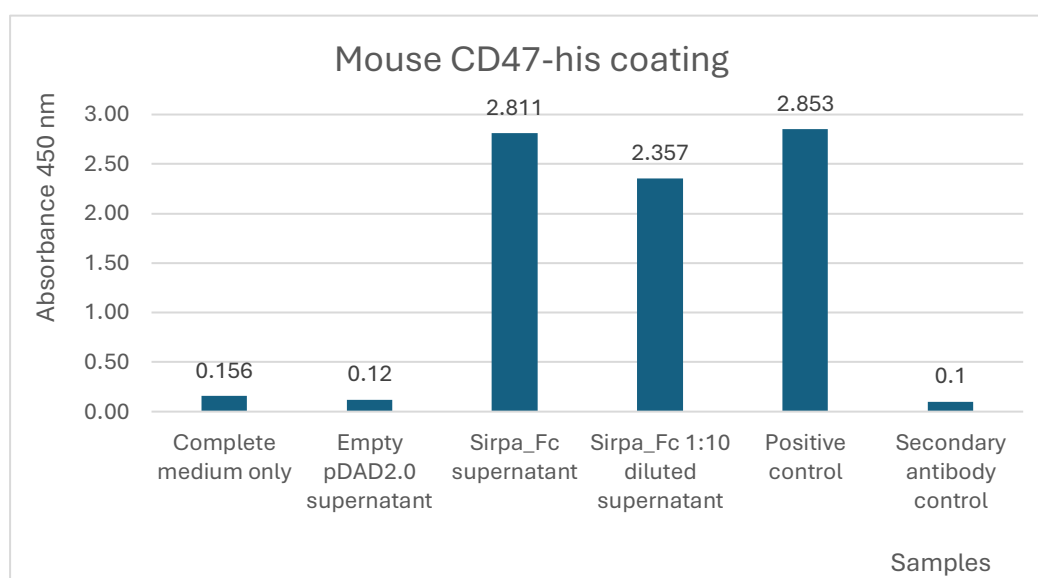


**Figure 14. ELISA analysis of mouse Sirpa-Fc proteins.**

Mouse Sirpa-Fc proteins secreted by transfected cells evaluated by ELISA method. As a positive control, HRP-conjugated anti-his antibody was used. Absorbance was measured at 450 nm using a spectrophotometer.

#### 4.1.3. Purification of recombinant proteins

**-Control-Fc:** 80 mL of medium was collected, and Protein A affinity chromatography was performed and the elutions were analyzed by 12% SDS-PAGE and coomassie staining (Figure 15). The final yield was 0.9 mg of recombinant control-Fc protein, also with over 95% purity.

**-Sirpa-Fc:** A total of 90 mL of medium was collected for purification. Protein A affinity chromatography was conducted using the described method. The eluted fractions were analyzed by 12% SDS-PAGE followed by coomassie staining. After purification, approximately 2.2 mg of Sirpa-Fc with over 95% purity was obtained.

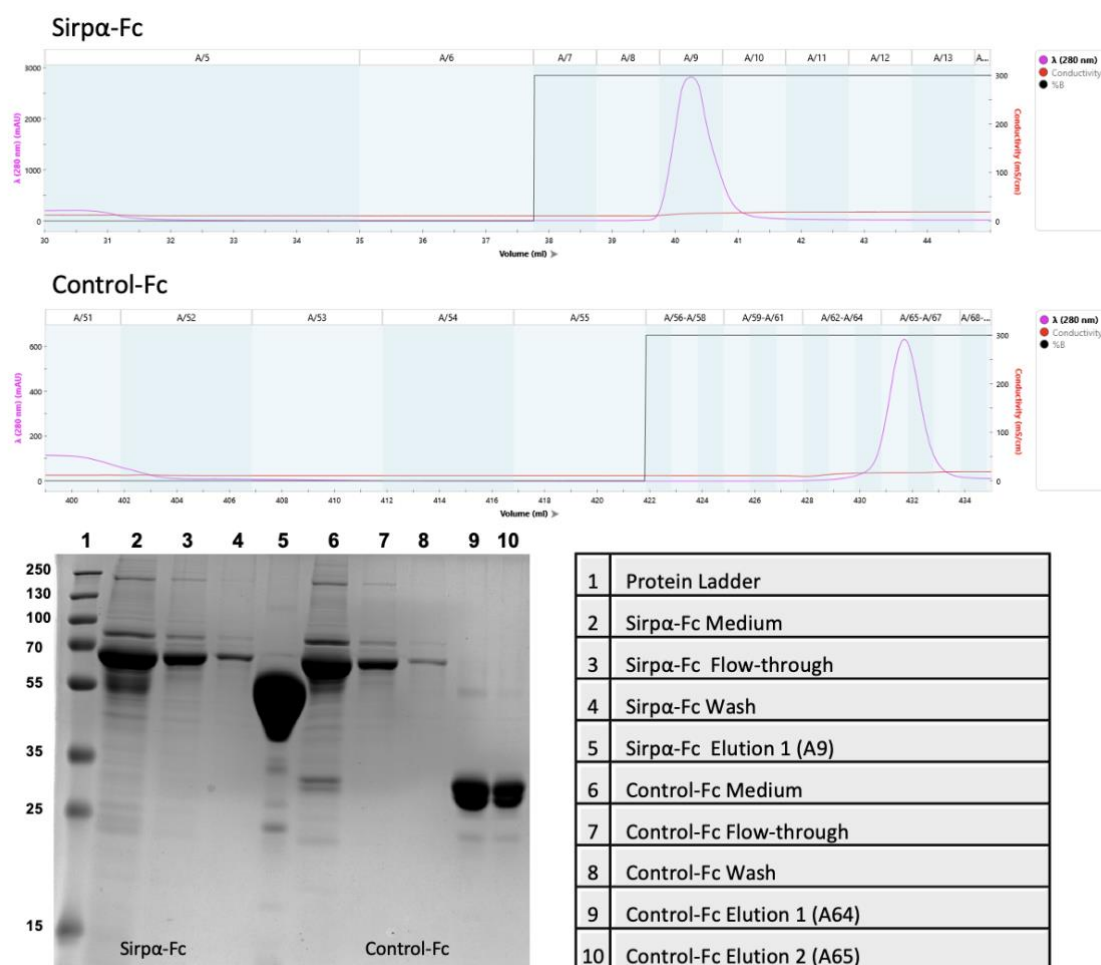


**Figure 15. Purification of recombinant proteins.**

Recombinant proteins were purified by Protein A affinity chromatography. Elutions were analyzed by 12% SDS-PAGE and coomassie blue staining.

#### 4.1.4. Physico-Chemical Characterization of Recombinant Proteins

Before the biological activity tests, the binding kinetics of the inhouse purified mouse Sirpα-Fc protein to commercially available mouse CD47 protein were evaluated using an ELISA assay. Wells coated with mouse CD47 protein at a concentration of 1 µg/mL per well. Serial dilutions of Sirpα-Fc samples were prepared at concentrations of 200 µg/mL, 100 µg/mL, 10 µg/mL, 1 µg/mL, and 0.1 µg/mL. An HRP-conjugated secondary antibody specific for mouse IgG2a was used. Absorbance was measured at 450 nm using a spectrophotometer. The signal obtained from the secondary antibody control

(blank) was subtracted from all measured values to account for background noise. The resulting absorbance values were then normalized between 0 and 1 to facilitate comparison across different concentrations are showing in Figure 16.

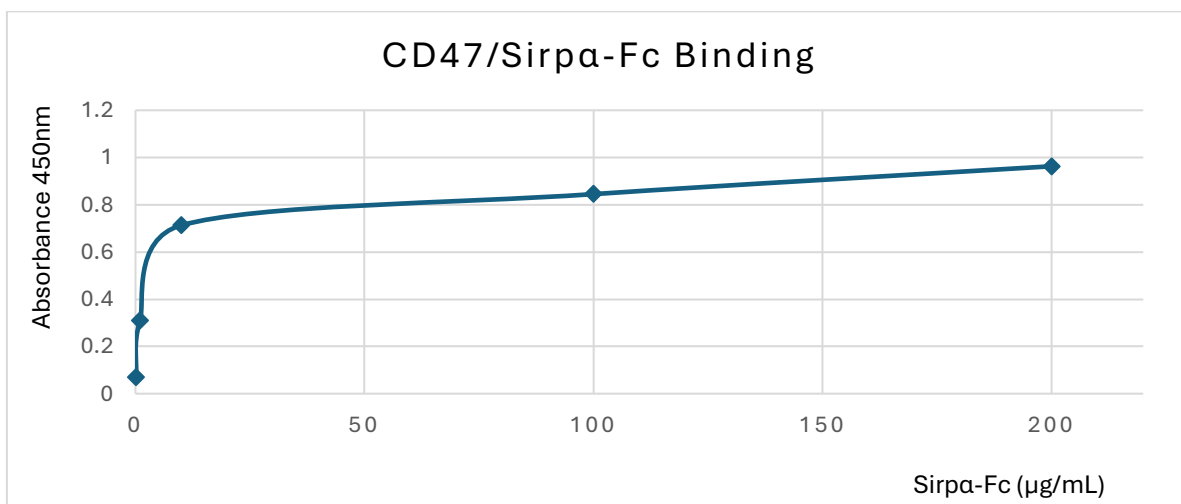


**Figure 16. Physico-chemical characterization of recombinant proteins.**

Binding kinetics of the inhouse purified mouse Sirpa-Fc protein to commercially available mouse CD47 protein were evaluated using an ELISA assay. Absorbance was measured at 450 nm using a spectrophotometer.

**Table 4.** Sirpa-Fc and CD47 binding by ELISA

| Concentration (µg/mL) | Concentration (µM) | Absorbance | Normalized absorbance |
|-----------------------|--------------------|------------|-----------------------|
| 0.1 µg/mL             | 0,0024             | 0,071      | 0                     |
| 1 µg/mL               | 0,024              | 0,31       | 0,26793722            |
| 10 µg/mL              | 0,24               | 0,713      | 0,719730942           |
| 100 µg/mL             | 2,38               | 0,845      | 0,867713004           |
| 200 µg/mL             | 4,76               | 0,963      | 1                     |

Additionally, the theoretical molecular weight of Sirpa-Fc was estimated to be 42 kDa, and protein concentrations added to the wells were calculated in µM. A plot of normalized absorbance versus protein concentration (µM) was generated, and from the logarithmic function derived, the KD50 value for the Sirpa-Fc protein was determined to be 0.1 µM (Figure 17).

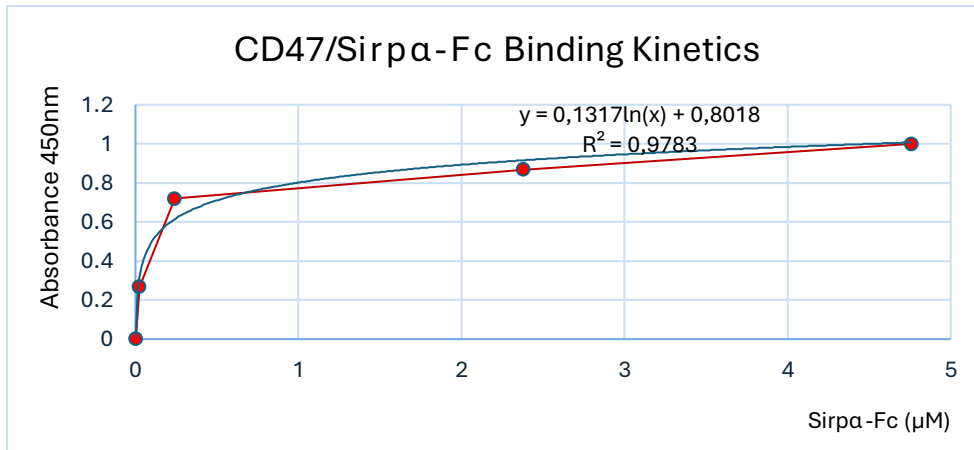


**Figure 17. CD47/ Sirpa-Fc binding kinetics.**

A plot of normalized absorbance versus protein concentration (μM). KD50 value for the Sirpa-Fc protein was calculated to be 0.1 μM.

#### 4.2. Determination of CD47 expression & Sirpa-Fc binding in 4T1 cells

CD47 expression in 4T1-GFP cells was assessed by flow cytometry. For this purpose, anti-mouse CD47 was used as the primary antibody, and Alexa Fluor 568 goat anti-mouse IgG (H+L) was used as the secondary antibody. Given that these cells are GFP-positive, CD47 expression was detected using the PE-CF594 channel. The results are illustrated in Figure 18. Following this, we evaluated the effect of our purified Sirpa-Fc on CD47 binding. As shown in Figure 19, comparison of 25 μg/ml Sirpa-Fc with secondary controls or 25 μg/ml IgG-Fc control revealed approximately 46% binding of Sirpa-Fc to CD47 on the surface of live 4T1 cells.

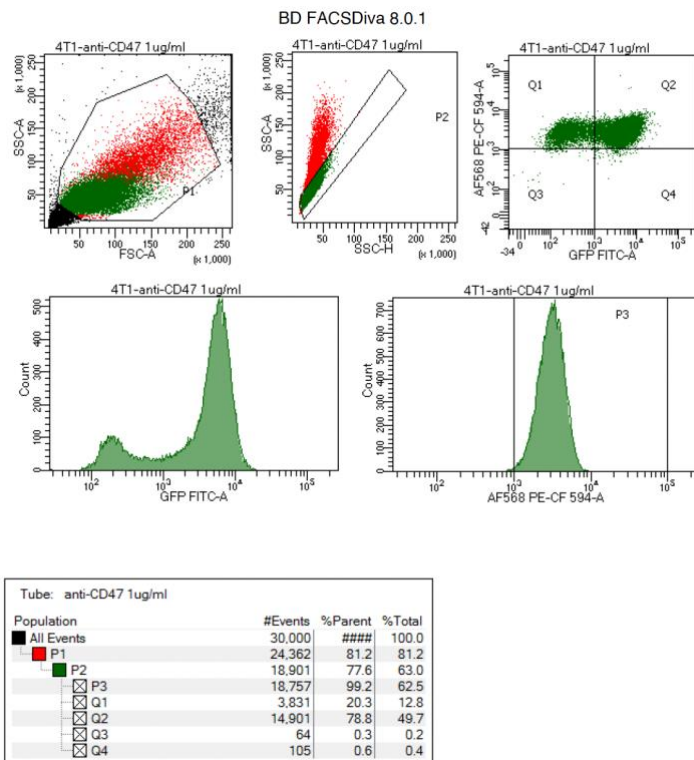


**Figure 18. Determination of CD47 expression levels in 4T1 cells.**

Expression of CD47 in 4T1-GFP cells was evaluated using the PE-CF594 channel via flow cytometry. Unstained cells were used for gating.

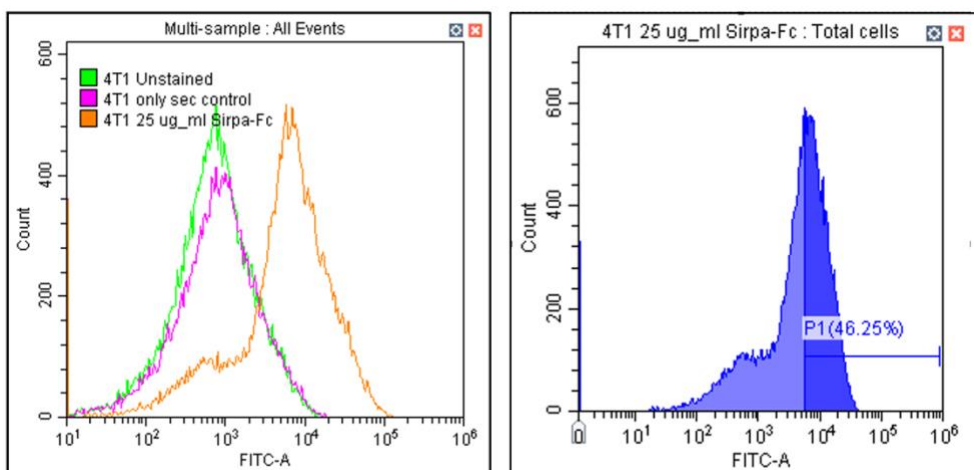


**Figure 19. Sirpa-Fc/CD47 binding in 4T1 cells.**

Binding of 25  $\mu$ g/ml Sirpa-Fc to CD47 in 4T1 cells was evaluated using the FITC channel via flow cytometry. As a control, 25  $\mu$ g/ml IgG-Fc was used. Unstained cells were used for gating.

### 4.3. Induction of apoptosis in 4T1 cells

To be used in efferocytosis experiments, a mouse 4T1 cell line was used as a target cell. For that aim, apoptosis was induced by using sodium selenite, a toxic inorganic salt. Firstly we induce apoptosis in our 4T1-GFP cells by using 10  $\mu$ M sodium selenite and stain them with 7AAD dyes. After flow cytometry analyses, we saw a huge decrease of our GFP positive populations (see Figure 20) in FITC channel after induction of apoptosis. For that reason, we continue our experiments by using untransfected 4T1 cells.

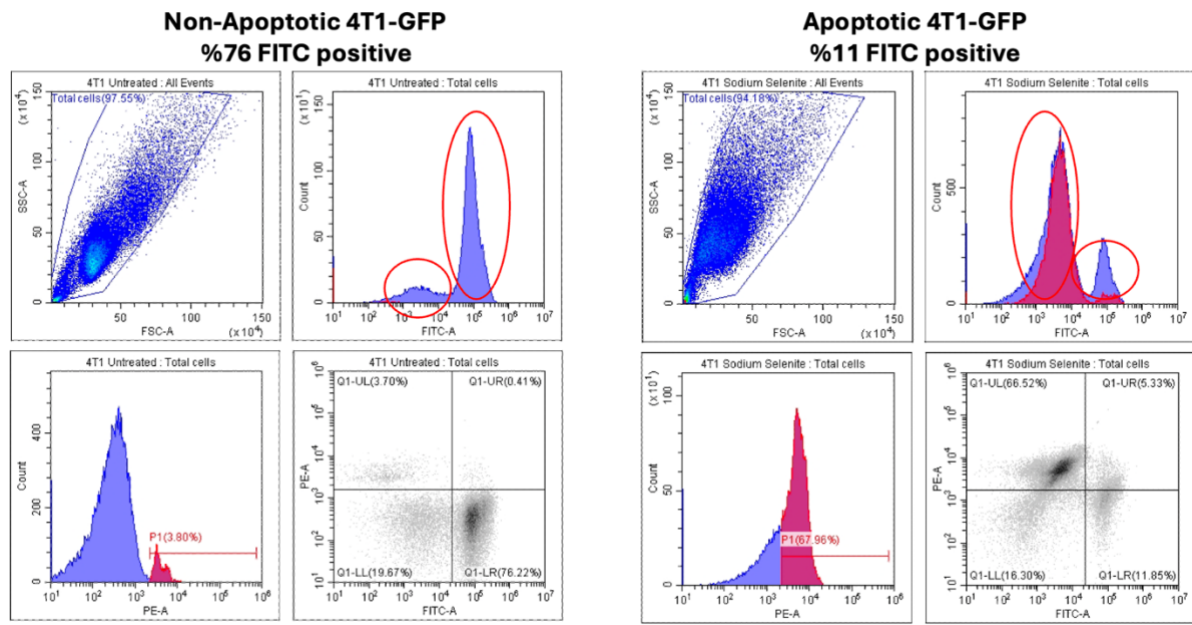


**Figure 20. Induction of apoptosis in 4T1-GFP cells.**

7AAD staining was conducted on both apoptosis-induced (right) and non-induced (left) 4T1-GFP cells using flow cytometry. The FITC channel detected GFP signals from the cells, while the PE channel indicated 7AAD binding.

4T1 cells incubated with various concentrations of sodium selenite; 20  $\mu$ M -10  $\mu$ M – 5  $\mu$ M and 2  $\mu$ M for 24 hours in complete RPMI. After incubation, cells were stained with FITC-conjugated Annexin V and PE-conjugated 7AAD dyes for 15 minutes in the dark. Unstained cells were used for gating in flow cytometry analyses. The results are shown in Figure 21. Both the FITC and PE negative section shows live cells. FITC positive, and

PE negative sections represent early apoptotic cells while both positive sections represent late apoptotic cells. After these results, we decide to continue by using 10  $\mu$ M sodium selenite for further apoptosis experiments.

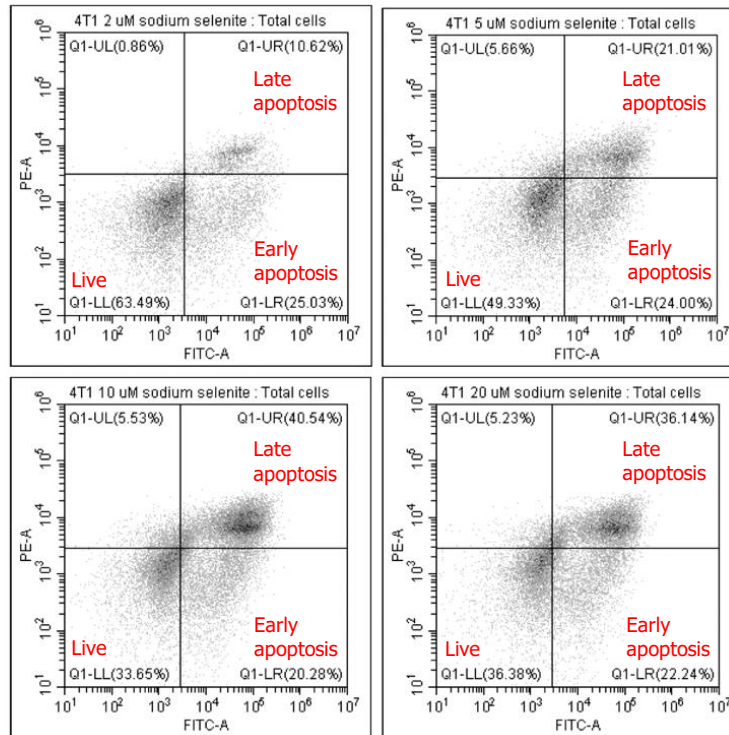


**Figure 21. Induction of apoptosis in 4T1 cells.**

Annexin V-7AAD staining was conducted on apoptosis induced 4T1 cells using 20  $\mu$ M - 10  $\mu$ M – 5  $\mu$ M and 2  $\mu$ M sodium selenite for 24 hours. The FITC channel detected Annexin V binding while the PE channel indicated 7AAD binding.

#### 4.4 Efferocytosis experiments

##### Experiment 1:

To find out the differences in efferocytosis between apoptotic and non-apoptotic conditions, we performed our initial co-culture experiment, without the addition of recombinant proteins, by using a 2:1 target-to-effector cell ratio ( $10 \times 10^4$  target cells:

5x10<sup>4</sup> macrophages). The experiment was performed in triplicate wells for each condition. After apoptosis was induced in 4T1 cells and RAW264.7 cells were induced by 10 ng/mL LPS for 24 hours, cells were seeded at a 2:1 target-to-effector cell ratio in serum-free RPMI medium and incubated overnight. The phagocytosis of apoptotic target cells by effector cells was observed using a fluorescence microscope. Later the cells were washed and fixed by using %4 paraformaldehyde (PFA) and diluted with PBS for better counting in the next step. At least 5 different images from the different areas of the wells were taken. The effector cells alone (red) and engulfed cells (green+red) were counted. The phagocytosis rate was observed to be 12% in non-apoptotic conditions, whereas it increased to 86% under apoptotic conditions. After achieving a high phagocytosis rate in the apoptotic cell group, we attempted to reduce this percentage by adjusting the number of target cells in co-culture experiments. Our aim was to lower the phagocytosis rate to around 30-40% in the control group, without added any proteins, to better observe the effects of recombinant proteins such as Sirpa-Fc, Gas6, and MFGE8 in subsequent experiments. The plate set up is shown in Table 5 and representative combined images of the two groups are shown in Figure 22.

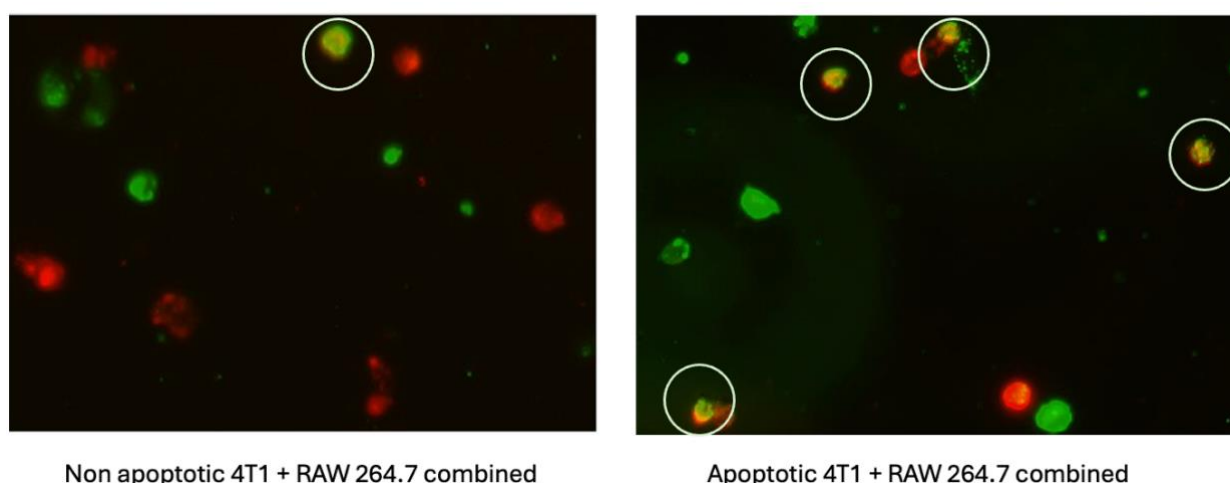


**Figure 22. A representantive image of TRITC-FITC combined wells.**

Macrophages were pre-treated with LPS 24 hours prior to the co-culture experiments. Apoptotic or non-apoptotic 4T1 cells (green) were then co-incubated with RAW 264.7 macrophages (red) in the well for an additional 24 hours. Phagocytosed (orange) cells are circled.

**Table 5.** Efferocytosis in apoptotic and non-apoptotic conditions

| Plate set up                                              | Red cell count<br>(only effector) | Orange/yellow cell count<br>(effector+target) | % Percentage of<br>phagocytosis |
|-----------------------------------------------------------|-----------------------------------|-----------------------------------------------|---------------------------------|
| Apoptotic 4T1 cell +<br>RAW264.7, no protein<br>added     | 15                                | 95                                            | %86                             |
| Non-apoptotic 4T1 cell +<br>RAW264.7, no protein<br>added | 203                               | 18                                            | %12                             |

### Experiment 2:

After we got a high percentage of phagocytosis in our apoptotic cell group (%86) we tried to decrease this percentage by changing the numbers of target cells in co-culture experiments. Our goal was to achieve around %30-40 percent in our no protein-adding group so that in further experiments done by Sirpa-Fc, Gas6, and MFGE8, we could see the effects of recombinant proteins better. After our results from experiment 1, the second experiment was performed with a reduced target cell number, using a 1:2 target-to-effector cell ratio ( $2.5 \times 10^4$  target cells:  $5 \times 10^4$  macrophages). Also we used 50 µg/mL and 10 µg/mL Sirpa-Fc and 50 µg/mL and 10 µg/mL IgG-Fc to observe their effect on apoptotic or non-apoptotic conditions. The experiment was performed in triplicate wells for each condition. Firstly 4T1 target cells were seeded and they incubated with proteins for 30 minutes after LPS-induced effector macrophages were added and co-incubated for 24 hours. Then cells were fixed as described before and diluted with PBS for better counting. Plate set up is shown in Table 6. Unfortunately, the results indicated no significant difference in phagocytosis rates, between the two groups, with percentages around 10% for apoptotic conditions and 9% for non-apoptotic conditions. Because of that issue, we performed experiment 3 to find out optimal target:effector cell ratio.

**Table 6.** Efferocytosis with 1:2 target:effector ratio

| Plate set up                                                               | Red cell count<br>(only effector) | Orange/yellow cell count<br>(effector+target) | % Percentage of<br>phagocytosis |
|----------------------------------------------------------------------------|-----------------------------------|-----------------------------------------------|---------------------------------|
| Apoptotic 4T1 cell +<br>RAW264.7 with 50 µg/mL<br><i>Sirpa</i> protein     | 112                               | 13                                            | %11                             |
| Non-apoptotic 4T1 cell +<br>RAW264.7 with 50 µg/mL<br><i>Sirpa</i> protein | 160                               | 13                                            | %8                              |
| Apoptotic 4T1 cell +<br>RAW264.7 with 10 µg/mL<br><i>Sirpa</i> protein     | 104                               | 12                                            | %10                             |
| Non-apoptotic 4T1 cell +<br>RAW264.7 with 10 µg/mL<br><i>Sirpa</i> protein | 130                               | 12                                            | %8                              |
| Apoptotic 4T1 cell +<br>RAW264.7 with 50 µg/mL<br>IgG-Fc protein           | 101                               | 9                                             | %8                              |
| Non-apoptotic 4T1 cell +<br>RAW264.7 with 50 µg/mL<br>IgG-Fc protein       | 158                               | 9                                             | %5                              |
| Apoptotic 4T1 cell +<br>RAW264.7 with 10 µg/mL<br>IgG-Fc protein           | 109                               | 11                                            | %9                              |
| Non-apoptotic 4T1 cell +<br>RAW264.7 with 10 µg/mL<br>IgG-Fc protein       | 171                               | 10                                            | %5                              |

### Experiment 3:

In experiment 2, when the number of target cells decreases, the phagocytosis percentages in apoptotic and non-apoptotic cell groups are very close to each other. Because of this problem, we performed experiment 3 for the optimisation of target cell:effector cell ratio to achieve around %30-40 phagocytosis percentage in our no protein-adding group. As the results seen in Table 7,  $3.5 \times 10^4$  target cell condition was chosen for the further studies.

**Table 7.** Optimisation of target:effector ratio

| Plate set up                                                       | Red cell count<br>(only effector) | Orange/yellow cell count<br>(effector+target) | % Percentage of<br>phagocytosis |
|--------------------------------------------------------------------|-----------------------------------|-----------------------------------------------|---------------------------------|
| $2.5 \times 10^4$ Apoptotic 4T1<br>cell + $5 \times 10^4$ RAW264.7 | 123                               | 27                                            | 18                              |
| $3.3 \times 10^4$ Apoptotic 4T1<br>cell + $5 \times 10^4$ RAW264.7 | 93                                | 51                                            | 35                              |
| $4.1 \times 10^4$ Apoptotic 4T1<br>cell + $5 \times 10^4$ RAW264.7 | 92                                | 63                                            | 40                              |
| $5 \times 10^4$ Apoptotic 4T1 cell<br>+ $5 \times 10^4$ RAW264.7   | 51                                | 71                                            | 58                              |

### Experiment 4:

As a final efferocytosis assay, we used  $3.5 \times 10^4$  target cell and  $5 \times 10^4$  effector cell. Firstly 4T1 target cells were seeded and they incubated with proteins for 30 minutes after LPS-induced effector macrophages were added and co-incubated for 24 hours. Then cells were fixed as described before and diluted with PBS for better counting. In this study, 100  $\mu\text{g/mL}$  Sirpa-Fc, 5  $\mu\text{g/mL}$  Gas6, and 5  $\mu\text{g/mL}$  MFGE8 recombinant proteins were used to find out if they affect efferocytosis of apoptotic cells by macrophages. Proteins were tested both individually, in pairs, and in triple states.

As a control, 100 µg/mL IgG-Fc protein was used. The conditions are summarized in Table 9. Statistical analysis was performed using ANOVA followed by Tukey's multiple comparison test.

**Table 8.** Efferocytosis using different proteins

| Plate set up                                                                                | Red cell count<br>(only effector) | Orange/yellow cell count<br>(effector+target) | % Percentage of<br>phagocytosis |
|---------------------------------------------------------------------------------------------|-----------------------------------|-----------------------------------------------|---------------------------------|
| Apoptotic 4T1 cell +<br>RAW264.7 <b>no protein</b>                                          | 136                               | 37                                            | %21                             |
| Apoptotic 4T1 cell +<br>RAW264.7 <b>with 100<br/>µg/mL Sirpα protein</b>                    | 117                               | 45                                            | %27                             |
| Apoptotic 4T1 cell +<br>RAW264.7 <b>with 100<br/>µg/mL IgG-Fc protein</b>                   | 140                               | 37                                            | %20                             |
| Apoptotic 4T1 cell +<br>RAW264.7 <b>with 5 µg/mL<br/>Gas6 protein</b>                       | 138                               | 37                                            | %21                             |
| Apoptotic 4T1 cell +<br>RAW264.7 <b>with 5 µg/mL<br/>MFGE8 protein</b>                      | 159                               | 64                                            | %28                             |
| Apoptotic 4T1 cell +<br>RAW264.7 <b>with 100<br/>µg/mL Sirpα + 5 µg/mL<br/>Gas6 protein</b> | 120                               | 59                                            | %32                             |
| Apoptotic 4T1 cell +<br>RAW264.7 <b>with 100<br/>µg/mL Sirpα + 5 µg/mL<br/>MFGE8</b>        | 155                               | 67                                            | %30                             |

|                                                                                                       |     |    |     |
|-------------------------------------------------------------------------------------------------------|-----|----|-----|
| Apoptotic 4T1 cell +<br>RAW264.7 with 5 µg/mL<br>Gas6 protein + 5 µg/mL<br>MFGE8                      | 138 | 46 | %24 |
| Apoptotic 4T1 cell +<br>RAW264.7 with 100<br>µg/mL Sirpα + 5 µg/mL<br>MFGE8 + 5 µg/mL Gas6<br>protein | 132 | 55 | %29 |

According to the results, shown in Figure 23 and Table 9, Sirpα-Fc and MFGE8 alone can able to enhance efferocytosis in apoptotic 4T1 cancer cell line compared to IgG-Fc control, while Gas6 can not. When we look at the comparison of double protein conditions, interestingly it is seen that there is a synergistic effect between the Sirpα-Fc & Gas6 and Sirpα-Fc & MFGE8 group when we compare them with the IgG-Fc group. It is interesting that, while Gas6 alone can not enhance efferocytosis, when Gas6 is cocultured with Sirpα-Fc and Sirpα-Fc & MFGE8, it significantly modulates efferocytosis.

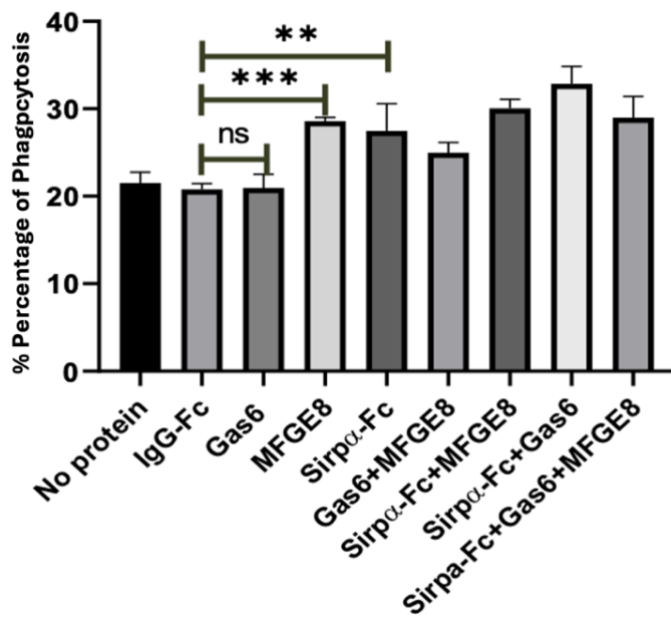


**Figure 23. Percentage of phagocytosis in different protein conditions.**

Statistical analysis was performed using ANOVA followed by Tukey's multiple comparison test.

**Table 9.** Statistical analysis of experiment groups compared with control IgG-Fc

| Tukey's multiple comparisons test       | Significance | Summary | Adjusted P Value |
|-----------------------------------------|--------------|---------|------------------|
| IgG-Fc vs. Gas6                         | No           | ns      | >0.9999          |
| IgG-Fc vs. MFGE8                        | Yes          | ***     | 0,0007           |
| IgG-Fc vs. Sirp $\alpha$ -Fc            | Yes          | **      | 0,0035           |
| IgG-Fc vs. Gas6+MFGE8                   | No           | ns      | 0,1255           |
| IgG-Fc vs. Sirp $\alpha$ -Fc+MFGE8      | Yes          | ****    | <0.0001          |
| IgG-Fc vs. Sirp $\alpha$ -Fc+Gas6       | Yes          | ****    | <0.0001          |
| IgG-Fc vs. Sirp $\alpha$ -Fc+Gas6+MFGE8 | Yes          | ***     | 0,0004           |

## **5. DISCUSSION**

In this study, we engineered a chimeric recombinant protein, Sirpa-Fc, designed to bind to the extracellular domain of CD47. We hypothesized that if this protein could affect in vivo CD47-Sirpa interaction between cancer cells and macrophages, thereby affecting efferocytosis mechanisms when compared to a control recombinant IgG-Fc. Additionally, we probed other recombinant proteins, Gas6 and MFGE8, to test if they have a role in PS recognition on apoptotic cells. These proteins were tested individually and in combination to evaluate their effects on the efferocytosis of apoptotic cells by macrophages.

Our initial results demonstrated successful production and purification of the chimeric recombinant proteins, IgG-Fc and Sirpa-Fc. Furthermore, Sirpa-Fc's specific binding to its ligand, CD47, was confirmed in vitro using ELISA and flow cytometry analyses. For subsequent efferocytosis experiments, 4T1 target cells were stably transfected with a GFP plasmid to facilitate cell identification, while macrophages were planned to be visualized using a PE-conjugated F4/80 antibody. However, challenges arose during the induction of apoptosis, such as the dramatic increase of GFP-negative subpopulations seen in flow cytometry analysis, while we didn't see such a thing in our non-apoptosis group. As a result, we adjusted our approach and choose to label these cells with fluorescent membrane dyes: Vybrant DiO (green) for target cells and Vybrant DiI (red) for effector cells.

We optimized apoptosis induction in 4T1 cells using sodium selenite, determining that a 10  $\mu$ M concentration after 24 hours of incubation was optimal based on flow cytometry analysis of Annexin-V and 7-AAD staining. In our initial co-culture experiment, without the addition of recombinant proteins, we used a 2:1 target-to-effector cell ratio ( $10 \times 10^4$  target cells:  $5 \times 10^4$  macrophages). The phagocytosis rate was observed to be 12% in non-apoptotic conditions, whereas it increased to 86% under apoptotic conditions. Given that this high percentage of phagocytosis could complicate comparisons in subsequent experiments involving recombinant proteins,

we conducted a second experiment with a reduced target cell number, using a 1:2 target-to-effector cell ratio ( $2.5 \times 10^4$  target cells:  $5 \times 10^4$  macrophages). The results indicated no significant difference in phagocytosis rates, with percentages around 10% for apoptotic conditions and 9% for non-apoptotic conditions.

Following further optimization experiments, we decided to proceed with  $3.5 \times 10^4$  target cells to  $5 \times 10^4$  macrophages for subsequent studies. Notably, under these conditions, efferocytosis experiments showed Sirpa-Fc, either alone or in combination with MFGE8 and Gas6, effected phagocytosis percentages, demonstrating a statistically significant effect.

Since previous studies (Weiskopf et al., 2013) showed that engineered Sirpa proteins, does not induce phagocytosis on their own, but with combination with other antibodies, our findings are similar as we see highest percentage of phagocytosis (%35) in our Sirpa-Fc+Gas6 group. But interestingly, in our Gas6 alone group no significant difference was obtained. It suggests that Sirpa-Fc and Gas6 proteins may show synergistic effect.

## **6. CONCLUSION & PERSPECTIVES**

In this study, we engineered and characterized the chimeric recombinant protein Sirpa-Fc, which demonstrated specific binding to its ligand, CD47. We hypothesized that Sirpa-Fc could modulate the CD47-Sirpa interaction between cancer cells and macrophages, thereby influencing efferocytosis mechanisms. Additionally, we explored the use of commercial mouse Gas6 and MFGE8 proteins, which act as bridging molecules that facilitate the recognition of phosphatidylserine on apoptotic cells by macrophages. These proteins were tested individually and in combination to evaluate their effects on the efferocytosis of apoptotic cells by macrophages.

Firstly we evaluated the phagocytosis percentage using both apoptotic and non apoptotic cells cocultured with macrophages. 2:1 target:effector cell ratio was used in initial experiments. After achieving a high phagocytosis rate (86%) in the apoptotic cell group, we attempted to reduce this percentage by adjusting the number of target cells in co-culture experiments. Our aim was to lower the phagocytosis rate to around 30-40% in the control group, without added any proteins, to better observe the effects of recombinant proteins such as Sirpa-Fc, Gas6, and MFGE8 in subsequent experiments. The second experiment was conducted with a reduced target-to-effector cell ratio of 1:2 target:effector cell ratio. However, the results showed no significant difference in phagocytosis rates between the groups, with approximately 10% in the apoptotic condition and 9% in the non-apoptotic condition. Because of this reason, we conducted other experiments to find out optimal target:effector ratio. Later we continue other efferocytosis assays using  $3.5 \times 10^4$  apoptotic 4T1 cell +  $5 \times 10^4$  RAW264.7 cells. 100  $\mu\text{g/mL}$  Sirpa-Fc, 5  $\mu\text{g/mL}$  Gas6, and 5  $\mu\text{g/mL}$  MFGE8 recombinant proteins were used to find out if they affect efferocytosis of apoptotic cells by LPS induced macrophages. Proteins were tested both individually, in pairs, and in triple states.

According to our results, Sirpa-Fc and MFGE8 alone can able to enhance efferocytosis in apoptotic 4T1 cancer cell line compared to IgG-Fc control, while Gas6 can not. When

we look at the comparison of double protein conditions, interestingly it is seen that there is a synergistic effect between the Sirpa-Fc & Gas6 and Sirpa-Fc & MFGE8 group when we compare them with the IgG-Fc group.

The lack of enhancement in phagocytosis with Gas6 alone may be because of its complex and unique biochemical properties.  $\gamma$ -carboxylation of Gas6's Gla domains is crucial for binding to phosphatidylserine (PS), a process that is dependent on the presence of vitamin K. Although the commercial recombinant Gas6 protein used in our experiments was produced under vitamin K conditions, the datasheet notes that its ability to bind PS has not been tested. Additionally, PS binding by Gas6 also requires the presence of  $\text{Ca}^{2+}$ , similar to Annexin V. These factors likely contribute to the observed outcomes in the Gas6 experiment group, affecting the phagocytosis of apoptotic cells.

Our findings can help the therapeutic potential of targeting the CD47-Sirpa axis in cancer treatment. Further investigation is essential to clarify the underlying mechanisms on downstream of TAM receptors and also assess its in vivo applications. While this study focused on efferocytosis using the 4T1 mouse breast cancer cell line, future research should be done to explore the effects of these proteins in other cancer types. The utility of Sirpa-Fc, along with Gas6 and MFGE8, extends beyond oncology and could be relevant in diseases such as atherosclerosis and autoimmune disorders, where impaired efferocytosis plays a role. We believe this research will inform broader therapeutic strategies.

In summary, our study underscores the promise of inhouse purified Sirpa-Fc as a potential approach to enhance efferocytosis in 4T1 mouse cancer line, with significant implications for the improved eradication of malignant cells.

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## 8. APPENDIX

### 8.1. Ethics committee approval



**İZMİR BİYOTIP VE GENOM MERKEZİ**  
**GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR ETİK KURULU (İBG-GOEK)**  
**KARARI**

**Toplantı Tarihi** : 25/08/2023 **Toplantı Günü** : Cuma

**Toplantı Sayısı** : 9 **Toplantı Saati** : 10:00

**Sayın Doç.Dr. Kasım DIRİL,**

**2023-025 Protokol No'lu;** sorumlusu olduğunuz "Efferositoz mekanizmaları üzerine seçilmiş rekombinant proteinlerin etkisi" başlıklı araştırmanın uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

|                                                        |                                                   |
|--------------------------------------------------------|---------------------------------------------------|
| <b>Prof. Dr. H. Alper BAĞRIYANIK</b><br>Başkan         | <b>Prof. Dr. Hilmi ORHAN</b><br>Başkan Yardımcısı |
| <b>Prof. Dr. Sedef AKGÜNGÖR</b><br>Üye (Katılmadı)     | <b>Prof. Dr. Gülgün OKTAY</b><br>Üye (Katılmadı)  |
| <b>Dr. Serap ERKEK OZHAN</b><br>Üye                    | <b>Prof. Dr. Şermin GENÇ</b><br>Üye               |
| <b>Doç. Dr. Canan Aslı YILDIRIM</b><br>Üye (Katılmadı) | <b>Doç. Dr. Can KÜÇÜK</b><br>Üye                  |
| <b>Dr. Öğr. Üyesi Yavuz OKTAY</b><br>Üye               |                                                   |

## 8.2. Curriculum vitae (CV)

### ASLI BABAYAKALI

#### *Contact Information*

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|                |       |
|----------------|-------|
| Address        | Atatu |
| Phone          |       |
| E-mail address |       |

#### *Personal Information*

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|                      |                     |
|----------------------|---------------------|
| Date of Birth/ Place | 28.04.1999/ Nilufer |
| Nationality          | Turkish             |

#### *Languages*

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|                          |
|--------------------------|
| Turkish (Native speaker) |
| English (Advanced)       |
| German (Beginner)        |

#### *Education*

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|           |                                                                                                                                  |
|-----------|----------------------------------------------------------------------------------------------------------------------------------|
| 2022-2024 | M.Sc. in Molecular Biology and Genetics<br>(GPA: 3.93/4.00)<br>Dokuz Eylul University, Izmir Biomedicine and<br>Genome Institute |
| 2017-2021 | B.Sc. in Molecular Biology and Genetics<br>(GPA: 3.32/4.00)<br>Bursa Uludag University, Faculty of Arts & Science                |

#### *Research Experience*

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|-------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| October 2023 - September 2024 | Chimeric Proteins That Trigger Phagocytosis<br>Against Atherosclerosis, 1001 project supported by<br>The Scientific and Technological Research Council<br>of Turkey (TUBITAK). Position: Researcher<br>Supervisor: Prof. Dr. Mehmet Ozturk |
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| February 2022 – October 2023 | Target-Specific Pan-Cancer Therapies (PAN-TER), 1004 project supported by The Scientific and Technological Research Council of Turkey (TUBITAK). Position: Research Staff Member<br>Supervisor: Prof. Dr. Mehmet Ozturk                                                                                    |
| January 2021 – June 2021     | Treatment of Experimental Allergic Encephalomyelitis by Tolerance-Inducing Dendritic Cells Rendered by Multiple Gene Modifications: A Novel Approach for the treatment of MS.<br>Position: Bursary Student funded by Intern Research Scholarship Program (STAR).<br>Supervisor: Prof. Dr. H. Barbaros Oral |

### *Conferences and Workshops*

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| 28-30 September 2023 | International Biotechnology Congress, Istanbul, Turkey.                                          |
| 12-15 July 2023      | UBIKON National Congress of Biology, Izmir, Turkey.                                              |
| 6-7 May 2023         | IZTECH National Molecular Biology and Genetics Student Congress, Izmir, Turkey.                  |
| 27-30 September 2022 | 7 <sup>th</sup> Turkish-US Cytometry Workshop<br>Izmir, Turkey.                                  |
| 15-16 February 2020  | Bogazici University Molecular Biology and Genetics Student Congress, Istanbul, Turkey.           |
| 4-6 October 2019     | Istanbul Technical University Molecular Biology and Genetics Student Congress, Istanbul, Turkey. |
| 1-3 March 2019       | Istanbul University, IUGEN Molecular Biology and Genetics Winter School XVI, Istanbul, Turkey.   |

### *Certifications*

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| October 2022 | Certificate of Experimental Animal Usage for Researchers Animals Testing Committee of Local Ethics Testing Animal Utilization of Dokuz Eylul University, Izmir, Turkey. |
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-Karakas B., Oz O., **Babayakali A.**, Erdoğan A., Kahyaoglu S., Bal E., Ekin U., Tokat F., Ince U., Ozturk M. (2023). Development and clinical validation of Napsin A and p40 monoclonal antibodies for the differential diagnosis of lung cancer. UBIKON National Congress of Biology, 12-15 July 2023, Izmir, Turkey. *(Oral presentation)*

-Karakas B., Oz O., **Babayakali A.**, Erdoğan A., Kahyaoglu S., Bal E., Ekin U., Ozturk M. (2023). Domestic Production of Monoclonal Antibodies Used in Clinical Pathology. International Biotechnology Congress, 28-30 September 2023, Istanbul, Turkey. *(Oral presentation)*



