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Expression And Cell Surface Re-engineering of Thrombomodulin on Macrophages

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EXPRESSION AND CELL SURFACE RE-ENGINEERING OF THROMBOMODULIN ON MACROPHAGES

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ABSTRACT

Thrombomodulin (TM) is a transmembrane glycoprotein that is primarily expressed on the surface of endothelial cells, where it serves as a receptor of thrombin for protein C activation, which regulates coagulation and inflammation. Research has revealed that TM is also expressed in immune cells, including monocytes and macrophages, however, its function is unclear. In this dissertation study, the TM expression in THP-1 monocytes upon their differentiation to macrophages was profiled.

Due to the beneficial roles of TM, the protein has caught the attention to be used as a therapeutic. A recombinant form of TM can be expressed that contains EGF-like domains 4-6 (rTM_{456}) and this form, TM acts as pure anticoagulant while retaining the ability to activate protein C. However, this recombinant form has poor stability *in vivo* when left unmodified. Macrophages can be used as drug and antigen delivery carriers and can be directed to sites of inflammation. Modifying rTM_{456} to allow it to be incorporated into the membrane of macrophages may help with its stability and allow it to be deliver to sites of inflammation. One way to modify cells is lipid fusion by using lipid anchors that insert into the plasma membrane through hydrophobic interactions. In this dissertation, cell surface re-engineering of macrophages with rTM_{456} was investigated *via* a lipid fusion approach.

First, the expression levels of TM were measured on THP-1 monocytes and macrophages. Using confocal microscopy and flow cytometry analysis, it was found that

THP-1 monocytes express more TM on their cell surface compared to macrophages. It was also determined that THP-1 monocytes express more total TM, as determined by western blot and ELISA analysis. Western blot and ELISA data also revealed that monocytes shed significantly more TM into the culture medium when they are undergoing differentiation into macrophages versus resting monocytes. Despite expressing less TM on their cell surface, THP-1 macrophages were able to convert more protein C to activated protein C.

Second, the anchoring efficiency of different lipid anchors for future cell surface re-engineering applications using a lipid fusion approach was investigated. Two different anchors were selected for this study, DSPE-PEG_{2000}-Biotin and Cholesterol-PEG_{2000}-Biotin. To determine anchoring efficiency, RAW 264.7 macrophages were incubated with different concentrations of DSPE-PEG_{2000}-Biotin or Cholesterol-PEG_{2000}-Biotin and tagged using streptavidin-FITC as a probe. Surface anchoring was determined using confocal microscopy. The cholesterol-based anchor showed drastically better incorporation efficiency than DSPE. In addition to incorporation efficiency, the membrane residence time of Cholesterol-PEG_{2000}-Biotin was shown to have a concentration dependent loss of anchor from the cell surface with an overall retention half-life of 1hr.

Last, a cell surface re-engineering strategy was developed for conjugating rTM_{456} to the surface of RAW 264.7 using a lipid fusion approach. Using a sortase A mediated ligation reaction, an azide was incorporated to the C-terminal end of the protein. This azide was used to attach a DBCO-terminated cholesterol anchor using copper free click chemistry. The anchoring efficiency of the afforded rTM-PEG_{2000}-Cholesterol was then tested on RAW 264.7 macrophages. Confocal microscopy analysis showed that the rTM

conjugate could successfully anchor itself into the cell membrane. The insertion also causes little toxicity to the cell.

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ABBREVIATIONS

ABE1	Anion-Binding Exosite 1
APC	Activated Protein C
BSA	Bovine Serum Albumin
CFCC	Copper Free Click Chemistry
CXCR4	CXC Chemokine Receptor 4
DAPI	4',6-diamino-2-phenylindole
DBCO	Dibenzylcyclooctyne
DIFO	3,3'-difluotocyclooctyne
DM	Diabetes Mellitus
DMEM	Dulbeco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DSPE	1,2-Distearoyl-sn-glycero-3-phosphorylethanolamine
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EPCR	Endothelial Protein C Receptor
FBS	Fetal Bovine Serum
FcR	Fc Receptor
FITC	Fluorescein Isothiocyanate
GFP	Green Fluorescent Protein
HI-FBS	Heat Inactivated Fetal Bovine Serum
HMGB1	High-Mobility Group Box 1 Protein
HRP	Horseradish Peroxidase

IgG	Immunoglobulin G
IL-6	Interleukin 6
IL-10	Interleukin 10
IPTG	Isopropyl β-D-thiogalactopyranoside
LPS	Lipopolysaccharide
MODS	Multiple Organ Dysfunction Syndrome
MSC	Mesenchymal Stem Cell
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate Buffered Saline
PE	Pre-Eclampsia
PEG	Polyethylene Glycol
PMA	Phorbol 12-Myristate 13-Acetate
PMSF	Phenylmethylsulfonyl Fluoride
PVDF	Polyvinylidene Fluoride
rhsTM	Recombinant Human Soluble Thrombomodulin
rTM	Recombinant Thrombomodulin
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SrtA	Sortase A
sTM	Soluble TM
TAFI	Thrombin Activatable Fibrinolytic Inhibitor
TBST	Tris-Buffered Saline, Tween-20
TM	Thrombomodulin
TNF-α	Tumor Necrosis Factor Alpha

CHAPTER I

INTRODUCTION

1.1 Thrombomodulin

1.1.1 Discovery and Expression of Thrombomodulin

Thrombomodulin (TM) was discovered in 1981 by Esmon and Owen.[1] The first hint of thrombomodulin came from a 1980 study where a cofactor for antithrombin III was seen on endothelial cells that caused the rapid inactivation of thrombin.[2] TM was later isolated from rabbit lung samples in 1982 by using affinity chromatography using a thrombin-agarose stationary phase.[3]

Since its original identification as an endothelial cell protein, TM has been found on a large variety cells and organs. Cells that have been proven to express TM are macrophages, monocytes, platelets, neutrophils and mesothelial cells.[4–7] In addition to being a membrane bound protein, both active and inactive forms of TM have been found circulating in the blood and in urine.[8] These circulating forms of TM lack the transmembrane domain and are a result of being cleaved, not excreted, from the cell membrane.[9]

1.1.2 Structure of Thrombomodulin

In its complete form, TM is made up of 557 amino acids to give it a total molecular weight of about 74kDa.[10] TM has various functions in the body and these functions can be attributed to the different domains that make up the protein. There are five total domains that make up the total structure of mature TM. Starting from N-terminus these domains are the lectin-like domain (TMD1), epidermal growth factor (EGF)-like domain (TMD2), serine/threonine-rich domain (TMD3), transmembrane domain (TMD4) and a cytosolic tail (TMD5) (Figure 1.1).[10]

The lectin-like domain of TM is similar in structure to C-type lectins but lacks a calcium-binding site. This domain is involved in inflammation, tumor growth and cell adhesion. This domain exerts anti-inflammation actions by binding pro-inflammatory stimuli before they can reach their target. These include lipopolysaccharide (LPS) and high-mobility group box 1 protein.[11,12] For its role in cell adhesion, the lectin-like domain can bind to fibronectin of the extracellular matrix.[13] It has also been demonstrated that cancer cells can downregulate surface TM expression and this can lead to an increase in metastatic potential.[14] In addition, the TMD1 domain also contains *N*-linked glycosylation sites.

The TMD2 domain of TM contains six EGF-like repeats and is the location for TM's anticoagulation and fibrinolysis functions. These functions are allowed by TM's ability to activate protein C, for anticoagulation, and thrombin activatable fibrinolytic inhibitor (TAFI) activation, for fibrinolysis. Both of these processes require thrombin, which requires EGF45 for binding.[15,16] It has been elucidated that the minimum binding for protein C activation is EGF456 while thrombin-activatable fibrinolysis inhibitor (TAFI)

activation requires EGF3456.[17–19] In addition to the coagulation functions of TM, the TMD2 domain of TM has been shown to have mitogenic activity, although the exact repeats needed for this activity is unknown.[20,21]

Next, the TMD3 domain is a serine/threonine-rich domain, which contains attachment sites for chondroitin sulfate.[22,23] The chondroitin sulfate moiety of TM is important for the enhancement of protein C activation by the thrombin-thrombomodulin complex.[24,25] Lastly, it has been demonstrated that this domain can help stabilize TM *in vivo* as recombinant versions of TM that only displayed TMD1 and TMD2 experienced rapid clearance.[26]

The last two domains of TM are the transmembrane domain of TM, TMD4, and the cytosolic tail, TMD5. The TMD4 domain anchors TM to the cell surface and classifies TM as a type I membrane protein.[27] The TMD5 domain is a small cytoplasmic tail region that plays a role in TM's ability to multimerize.[28,29] Mice expressing a mutated form of TM where TMD5 was missing did not shown any significant abnormalities, suggesting that the cytoplasmic tail is not required for normal embryonic growth and overall survival.[30,31]

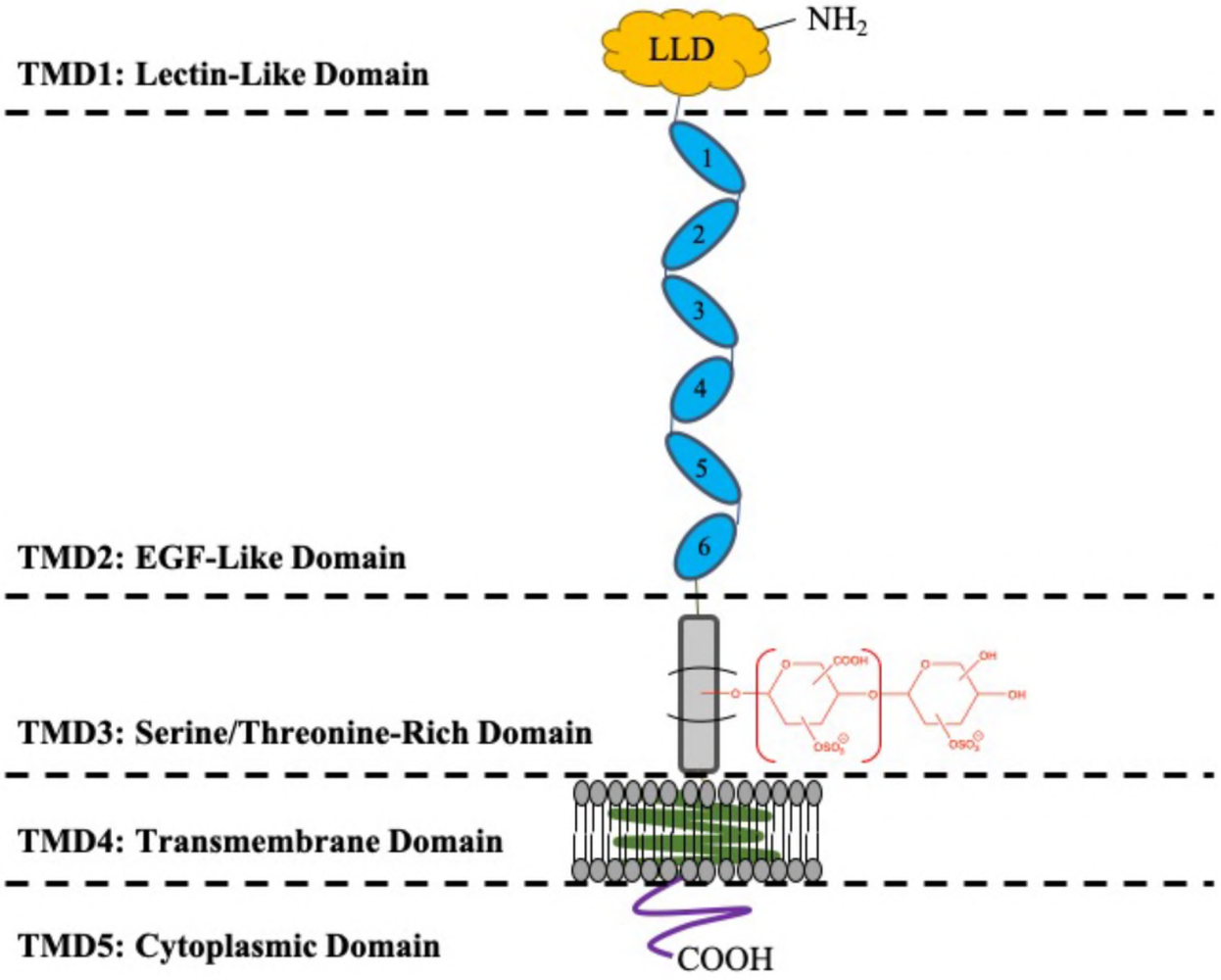

FIGURE 1.1: Structure of Thrombomodulin. Adapted from [45]

1.1.3 Biological Roles of Thrombomodulin

The most recognized function of TM is its participation in the balance of hemostasis. TM can act as both an anticoagulant and procoagulant factor. TM accomplishes its role of an anticoagulant by regulating thrombin. Free circulating thrombin acts as a pro-thrombotic agent. Thrombin exerts this role through activating coagulation factors, such as Factors V and VIII, fibrinogen and platelets.[32] In the presence of TM, thrombin forms a high-affinity complex with TM. When this complex is formed, thrombin's anion-binding exosite (ABE1) is blocked, which is required for thrombin's procoagulant proteolytic activity.[33,34] However, thrombin can now activate protein C, to create activated protein C (APC), circulating in the blood. APC acts as a protease and can inactivate procoagulant factors Va and VIIIa when in the presence of protein S.[35,36]

Opposite its ability to act as an anticoagulant factor, TM can also act as a procoagulant. This ability is executed by TM's ability to activate TAFI, which can slow down the removal of fibrin.[37] TAFI is a carboxypeptidase proenzyme that is produced in the liver.[38,39] When activated by the TM-thrombin complex, activated TAFI (TAFIa) cleaves terminal lysine and arginine residues from fibrin. This process removes positive charged residues from fibrin clots and hinders fibrin's cofactor role in the activation of plasminogen, which ultimately retards clot lysis.[40]

TM can also act as an anti-inflammatory molecule thanks to its lectin-like domain.[41] In addition to the anti-inflammatory properties of the lectin-like domain, TM's ability to activate protein C also contributes to its overall role as an anti-inflammatory molecule. Once activated, APC can inhibit monocytes' and macrophages' abilities to produce of TNF-α and tissue factor.[42,43] APC has been shown to protect and help lessen the effects of

sepsis, a disease associated with hyper-inflammation, in mammals.[44,45] A recombinant form of APC was marketed by Eli Lilly as an approved treatment for sepsis in 2001 but was later withdrawn for efficacy issues in 2011.[46]

TM has also been shown to exhibit anti-proliferation activity. Anti-proliferative activity has been seen with smooth muscle cells, endothelial cells and tumor cells. It has been shown that overexpressing TM in the common femoral artery of rabbits slowed down neointima formation after injury.[47] In endothelial cells, TM was shown to regulate the thrombin-induced ERK pathway, which plays a role in cell proliferation.[48] TM's influence on thrombin-induced proliferation has also been further confirmed in HUVEC cells.[49,50] Recombinant TM containing all extracellular domains has also been shown to inhibit thrombin-induced proliferation in smooth muscle cells and suggests a possible future therapeutic for helping prevent or control restenosis after angioplasty.[51] In tumor cells, down-regulation of TM has been implicated with the tumor's increased ability to proliferation and invade tissue.[52] The lectin-like domain and cytoplasmic domain has been implicated as the domains involved in TM's involvement in tumor progression.[53]

Lastly, TM shows the ability to act as a cytoprotective agent. These cytoprotective activities can either be APC-dependent or APC-independent. The APC produced by the thrombin-thrombomodulin complex mainly exerts its cytoprotective effects by interacting with protease receptor-1 (PAR-1).[54] When bound to endothelial protein C receptor (EPCR), APC can activate PAR-1 which can block p-53 mediated apoptosis and provides endothelial barrier protection.[55,56] APC-independent cytoprotective activity of TM has been explored by treating HUVEC cells with a recombinant form of TM. It was found that rTM induced expression of the antiapoptotic protein myeloid cell leukemia-1 and also

protected against cyclosporine-induced apoptosis.[57]

1.1.4 Thrombomodulin as a Biomarker for Disease

When endothelial cells are injured, TM is found circulating in plasma and urine in a soluble form known as soluble thrombomodulin (sTM).[8] The soluble form of TM does not contain the transmembrane domain due to the site of cleavage by proteolysis or oxidative stress.[58,59] Levels of sTM have been linked to increased mortality and have been correlated to the extent of endothelial cell damage.[60,61] Soluble TM consists of fragments of different molecular weights. The different molecular weights of sTM may vary in diseases that cause endothelial cell damage.[62] The levels of sTM in plasma can be related to inflammation due to different diseases or after trauma. Changes in the levels of sTM can be used as a biological marker for several diseases and traumatic injuries. Further understanding of sTM and the methods used to measure sTM levels could be used to understand its significance and role in other diseases and be used to predict the severity of diseases. Many researchers have now looked at TM as a biomarker for diagnosis and tracking of different diseases. Some of the most commonly studied diseases are pre-eclampsia, diabetes and sepsis.

Pre-Eclampsia (PE) is a condition that complicates a small percentage of pregnancies where there is the onset of non-preexisting hypertension and multiple organ failures.[63,64] Though the etiology of PE has been difficult to fully elucidate, it is accepted that the starting site is the placenta.[65] Despite knowing the initial cause of PE, it has been accepted that endothelial cell injury and dysfunction plays a role in the pathogenic changes in PE.[66,67] TM is present on syncytiotrophoblasts and the endothelium of the vasculature that covers the trophoblastic surface.[68,69] In fact, TM is the main mediator of the

anticoagulant system in the placenta.[70] TM can be cleaved from the endothelium surface by factors such as oxygen radicals and inflammatory proteases.[58] PE patients have displayed an up-regulated antioxidant system and increased oxidative products, suggesting an increase presence of oxygen radicals.[64,71] In addition to PE being a hypercoagulative state, it also is associated with hyper-inflammation.[72,73] With these clinical features in mind, it enforces the idea that TM could be a useful biomarker for the development of or severity of PE.

Overall with PE, levels of TM are increased in the blood compared to normotensive pregnancies.[74–77] While a key feature of PE is hypertension, pregnant patients who are hypertensive do not necessarily have PE but could have another condition, such as gestational hypertension. A 1995 study compared serum TM levels between patients with PE, gestational hypertension, chronic hypertension and without any form of hypertension. Among the groups, only TM levels of PE patients were significantly different, which were elevated.[78] TM levels also increase with each trimester in normal pregnancy, which is made worse in PE complicated pregnancies.[79,80] Another observation was that plasma TM levels began to significantly rise earlier in patients who would later develop PE, by week 24, when compared to pregnancies that were uneventful, by week 32.[81] The rise in TM levels is thought to be mainly due to cleavage from the endothelial surface, which is further supported by the finding that the placentas of PE patients express less TM on their endothelial surfaces versus normotensive patients.[82]

Diabetes Mellitus (DM) is not a singular disease but rather a group of disorders, mainly characterized by hyperglycemia but also is associated with vascular complications.[83–85] Hemostatic dysfunction in DM pushes the balance to a procoagulant

state with lower fibrinolytic activity and platelet malfunction.[86] Endothelial dysfunction is also seen in DM patients and can lead to many of the complications, mainly vascular, associated with diabetes.[87–90] The cause of endothelial dysfunction has multiple factors that include increased oxidative stress and increased pro-inflammatory compounds.[91,92] High glucose levels have also been determined as cause of endothelial damage.[93,94] With both endothelial damage and dysfunction at play in this disease, it is highly likely that TM levels would be raised in diabetic patients.

A great depth of research has been performed into researching how TM levels change in DM. The overall trend is that TM is increased in the biological fluids of both adult and juvenile diabetic patients.[94–99] TM levels were also shown to have a weak positive correlation with disease duration and number of complications.[94,96,99,100] However, no difference was found between type I and type II diabetic patients.[96] It has also been shown that high TM levels were associated with increased risk for all-cause mortality and CVD deaths.[101] It is thought that decreased renal clearance or tubule damage could be the cause of the higher levels of TM seen.[95,102] One study did not find a relation between TM and two markers for tubule damage, uNAG and ub2m, but did postulate that TM could come from glomerular damage as determined by the increase of urinary albumin, a marker for nephropathy.[95]

Sepsis is a leading cause of death for hospitalized patients and the primary cause of death of those suffering from an infection.[103,104] Traditionally sepsis is defined as severe systemic inflammation caused by infection.[105] The uncontrolled spread of inflammation is a critical part of the pathogenesis of septic shock.[106] Recently the definition of sepsis has expanded to include organ dysfunction caused by the dysregulation of the infection

response of the body.[107] Sepsis can then develop into more severe forms including septic shock and multiple organ dysfunction syndrome (MODS).[108] In sepsis both inflammation and coagulation are dysregulated and both crosstalk to influence each other.[109] Inflammation also deregulates the coagulation system with changes to the endothelium being a main cause.[110,111]

Multiple studies have shown a positive correlation between sTM levels and the severity of sepsis in both adult and pediatric patients.[61,112–114] The highest levels of sTM being in patients who experienced organ failure or MODS.[61,112,115,116] Further, patients who died of sepsis had higher levels of plasma TM levels compared to those who did not.[112,113] From these studies it can be inferred that TM can be used to track the severity of sepsis and possibly how the patient's disease will progress.

1.1.5 Therapeutic Potential of Thrombomodulin

Due to its participation in many beneficial biological processes, TM has caught the attention of researchers as a possible therapeutic. The recombinant form of TM known as recombinant human soluble thrombomodulin (rhsTM) or ART-123 has been highly tested as a therapeutic. ART-123 is currently an approved therapeutic in Japan and is indicated for the treatment of disseminated intravascular coagulation (DIC). This recombinant form of TM contains 498 out of the 557 amino acids naturally present in TM. These amino acids make up the lectin-like domain, EGF-like domain and the serine/threonine-rich domain of TM. The transmembrane domain and the cytoplasmic tail are missing in ART-123. The cDNA for rhsTM was constructed by site-directed mutagenesis using CHO cells as a host.[117] ART-123 maintains can still bind thrombin and protein C, allowing it to exert antithrombotic effects similar to that of native, full length TM. Therefore, rhsTM has two

ways in which it can have antithrombotic activity. The first is the direct inhibition of thrombin's procoagulant functions by binding to its active site. Secondly, the binding of thrombin creates the thrombin-thrombomodulin complex, which will facilitate the activation of protein C, which has been described earlier to act as an anticoagulant.

Pre-clinical trial studies have found some promising therapeutic properties of ART-123. Thromboelastography studies have revealed that ART-123 can prevent thrombus formation by inhibiting fibrin formation, fibrinopeptide A production and clot-induced thrombin generation.[118,119] The recombinant protein has also been shown to increase clotting time and block the aggregation of platelets in a dose-dependent manner.[118] Experiments with rat models have shown that ART-123 can block the hematological changes, such as activated partial thromboplastin time, seen in tissue factor and endotoxin induced DIC.[120,121] Lastly, ART-123 was able to reduce thrombosis in arteriovenous shunt in rats without effecting activated partial thromboplastin time.[122]

ART-123 has shown many advantages that make it an intriguing anticoagulant. These include a long half-life, good bioavailability when delivered via subcutaneous injection, less bleeding and an overall broad safety margin. A phase I study was conducted in 2004 in the United States to determine the pharmacokinetics and pharmacodynamics in healthy volunteers. The plasma half-life after subcutaneous injection was 2-3 days, which could lead to ART-123 being an outpatient therapeutic for the prevention of thromboembolism. Overall, ART-123 was found to be safe and did not cause major bleeding.[123] Phase II and III studies performed with patients suffering from DIC showed good clearance of the disease and an increased efficacy over heparin.[124,125] After clinical

testing and approval, Asahi Kasei Pharma of Japan marketed ART-123 as Recomodulin® in 2008.[126]

1.2 Cell Surface Re-Engineering

The cell surface is a stage for many important biological processes including cell signaling, cell-cell adhesion and the uptake of exogenous ligands. This is due to the large variety of biomolecules present including lipids, carbohydrates and proteins.[127–129] The combination of biomolecules present on the cell membrane reflects the functionality of the cell. It has been seen that removing particular molecules from the cell surface can make the cell function less efficiently or lose its function altogether.[130] If one can remove biomolecules to cause a change cellular function, the opposite holds true to add functionality to cells. The introduction of biomolecules to cells can allow cells to regain functions they may have lost or gain functions not native to the cell.[131,132] The large diversity of biomolecules on the cell surface also provides numerous available functional groups for use for conjugation.

1.2.1 Methods for Cell Surface Re-Engineering

Methods for re-engineering the cell surface can fall into two major categories: genetic modifications and chemical modifications. Although not the focus of this research, altering the genetics of a cell has allowed for the introduction of nonnative functional groups and nonnative biomolecules to the cell surface.[133] However, this technique for altering the cell surface is complicated and labor intensive and these drawbacks are what make the chemical techniques so intriguing.

There are three main techniques used to chemically modify the cell surface: (i) metabolic engineering, (ii) direct chemical modification and (iii) liposome/lipid fusion[134] (Figure 1.2). In metabolic engineering, cells are incubated with substrates that have slight variations in their structure. Certain enzymes, such as sialytransferases, can tolerate some changes to the structure of the substrates they work on. The rationale behind this method is that one can alter natural substrates the cell uses to have an unnatural functional group present and as the substrate is processed and the unnatural function group will be displayed on the cell surface. This functional group can then be selectively targeted using a specific chemistry. The most common method of metabolic engineering is introducing modified sialic acid precursors that will eventually be incorporated into the cell's glycoform.[135] It have been shown that enzymes responsible for synthesizing sialic acids can process precursors with N-acyl substituents which result in C-5 substituted sialic acids.[136] The altered sialic acids can present functional groups that can be targeted by specific chemistries or present moieties that can be targeted by antibodies.[137,138] It has also been shown that cells can be incubated with full sialic analogs and the cell with incorporate them into the glycoform of the cell surface.[139] This form of cell surface re-engineering has been used to deliver therapeutics and influence cell adhesion.[140] Although the bulk of metabolic engineering techniques revolves around modifying the glycocalyx of cell, there have been techniques that have accomplished modifying proteins to bear new functional groups. One example is Tirrell and colleagues who used a series of molecular biology techniques to modify a cell surface protein. Here, methionine residues were replaced with azidohomoalanine, which allowed for the introduction of azido functionality to the protein.[141] The main drawback of metabolic engineering versus the other two forms of cell

surface modification is that it is time consuming. Modification using lipid fusion or direct chemical modification can take place in as little as twenty minutes while metabolic engineering can take days to accomplish.[142] Other drawbacks to metabolic engineering are limited incorporation and cellular toxicity.

A second, less common, approach is direct chemical modification of the cell surface. There are two main categories for direct chemical modification. The first set of modifications uses the functional groups already present on the cell surface, such as amines, present on proteins. One group used free amines present on the surface of Lewis Lung Carcinoma cells to add two different toll-like receptor agonists. The study was able to show that the addition of these agonists resulted in an increase in immune stimulation.[143] This type of modification is faster than metabolic engineering in that the addition of the molecule of interest can as little as thirty minutes for the conjugation. The second kind of direct chemical modification uses functional groups already present on the cell surface and then converting them to a functional group that are not native to the cell. This type of direct chemical modification focuses on creating carbonyl groups (aldehydes and ketones) and azide groups. One method for introducing aldehydes is to oxidize sialic acids with sodium periodate. Sodium periodate is able to oxidize one hydroxyl group of a diol and convert it to an aldehyde.[144] This type of modification has been used to modify O-type erythrocytes to allow them to respond to anti-A or anti-B sera.[145] Besides being changed by purely chemical means, functional groups can be transformed by enzymes. One enzyme in particular is sortase A, which cuts in a poly-glycine sequence present on the outside of the cell and ligates a ligand with a LPETG amino acid sequence to the cell. This process has been used to add biotin to a large variety of cell types including yeast and mouse

splenocytes.[146] Although biotin is not a therapeutic agent, this method opens the door for the introduction of therapeutic agents as long as the correct amino acid sequence is present for the ligation to occur. Direct chemical modification is an overall simple concept of modifying the cell surface but is has some drawbacks. The major drawback is the overall lack of specificity during modification. When freely targeting amines, thiols and other functional groups, there is risk of modifying a site that is key to some biological process. In addition, the chemical reaction conditions are often not biocompatible and even with less efficiency and thus limited their practical applications in cell surface re-engineering.

The last type of re-engineering technique used is lipid fusion. Some natural proteins that are located on cell membrane are anchored by lipids versus spanning the whole width of the lipid bilayer like protein channels. Earlier forms of this technique used liposomes to deliver molecules of interest. Some lipids would be linked to a target molecule and complexed with non-linked lipids to create liposomes. Cells could then be incubated with these liposomes and the target molecule would be incorporated into the cell membrane. The cell membrane is mostly made of phospholipids, whose lipophilic tails allow for nonpolar interactions with the lipids of the liposome.[135] Newer techniques incubate cells with individual modified lipids rather than ones incorporated into liposomes.

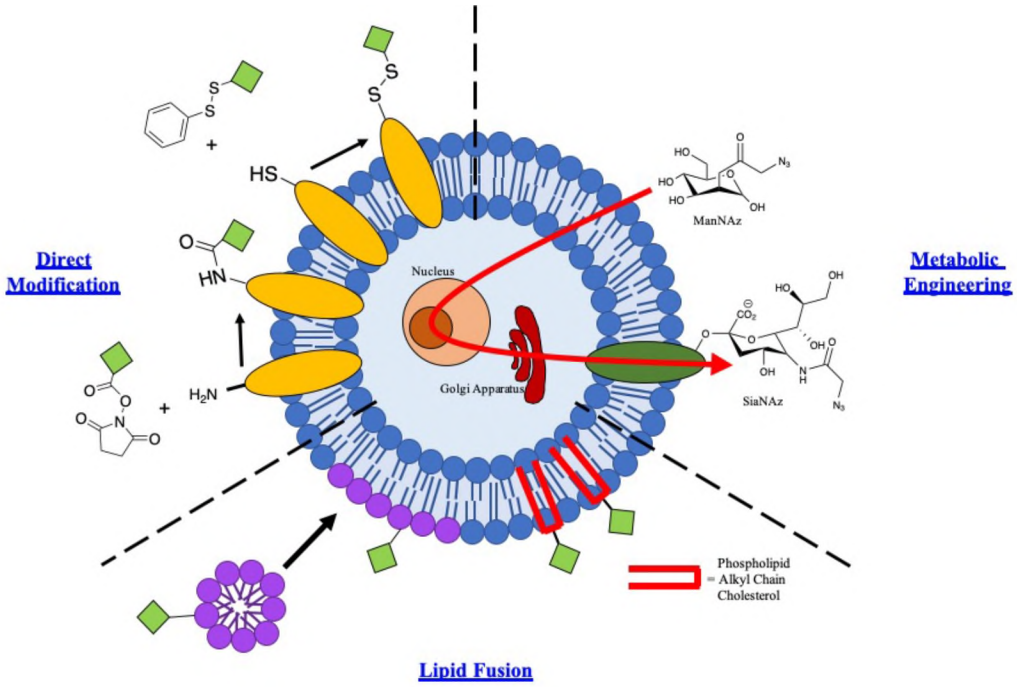

Figure 1.2: Cell Surface Re-Engineering Techniques. Direct chemical modification methods target naturally occurring functional groups such as amines and thiols to introduce molecules to the cell surface. Metabolic engineering methods use modified biomolecular precursors, such as ManNAz, that will be metabolized into larger biomolecules and can later be targeted. Lipid fusion methods deliver cargo to cells using hydrophobic interactions between the plasma membrane and hydrophobic molecules/constructs. Adapted from [134]

1.2.1 Biomedical Applications of Cell Surface Re-Engineering

Changes in the expression patterns of biomolecules present on the cell surface is often associated with changes in cellular functions and possible disease states. Being able to tag and track these changes is important for understanding the progression of diseases and developing therapeutics. One specific subset of biomolecules that change during certain disease states is glycans. Changes in sialoglycoconjugate levels in particular have been linked with tumor metastasis.[147,148] Analysis of surface sialoglycoconjugates is a complicated venture despite the development of a wide variety of techniques.[149,150] Recently, a method has been developed that uses a laser cleavable probe to profile these types of glycans. This method uses a N-azidoacetyl-mannosamine tetraacylated to incorporate azides into surface sialoglycoconjugates. This azide can then be targeted with an alkyne terminated probe, which is this study was pent-4-yn-1-yl(trityl)sulfane. This probe can be cleaved by laser irradiation and releases a carbocation that can be detected by laser desorption/ ionization spectrometry. The concentration of the released carbocation is proportional to the level of surface sialoglycoconjugate expression.[151] This technique can be used to track change in surface glycoconjugate levels or comparison between different samples.

Modifying the cell surface can also be used to elucidate how certain biological processes work. For example, the Bertozzi group has engineered the surface of various cancer cells with sialylated glycopolymers in order to deduce how the increase in sialic acid helps with natural killer (NK) cell evasion. It has been shown that cancer cells can evade detection by shedding ligands that activate NK cells and/or upregulating ligands that block NK activity.[152,153] In addition, upregulation of surface sialic acid levels has been

correlated with NK cell resistance and overall poor prognosis.[154,155] However, the specific mechanism of evasion remains unknown. Using various types of glycopolymers linked to phospholipids, the Bertozzi group was able to determine that Siglec-7 played a role in NK resistance of the decorated cells. Only sialylated glycopolymers were observed to inhibit NK activity, leading to the idea that siglecs, receptors that bind sialic acid containing glycans.[156] This study was able to show how increased levels of surface sialic acid levels can recruit Siglec-7 to the NK synapse site, leading to inhibition of activity.[157]

As mentioned earlier, cells can be modified to present nonnative biomolecules on their cell surface to add new functions to cells. One group introduced Fc receptors (FcRs) to the surface of Jurkat cells. In the human body, FcRs are present on leukocytes and epithelial cells and their main role is to bind immunoglobulin-G (IgG) molecules. The IgG molecules can either be free or bound, for example to a pathogen, and once bound they are subsequently internalized into the cell.[158–160] These internalized molecules can then be destroyed or transported across tissues to a new location.[161] The Peterson group was interested in adding FcR mimics to the cell surface for use for targeted binding and degradation of therapeutically relevant ligands. In their study, they were able to introduce a small FcR mimic to the surface of Jurkat cells, who do not express any type of FcR, using a cholesterol-like anchor. After a simple and efficient modification, Jurkat cells were able to gain the ability to capture and uptake fluorescently labeled IgG molecules. This approach could be used as a therapeutic for certain diseases that results in an increase of IgG in the bloodstream.[162]

Cell therapies have also been utilized for tissue regeneration and repair. One particular area of interest is trying to repair damaged cardiac tissue caused by heart disease.

A particular cell type of interest are mesenchymal stem cells (MSCs), who can release certain factors to aid in wound healing and treatment of cardiac diseases.[163–165] Unmodified MSCs have been tested as possible cell-based therapies but ran into the problem of poor retention of the cells to the target tissues.[166] To help with retention, MSCs have been modified with CXC chemokine receptor 4 (CXCR4). CXCR4 is a receptor for stromal-derived factor-1 (SDF-1), a protein released by injured myocardium.[167] SDF-1 gradients will cause CXCR4+ cells to migrate towards wounds and thus helps directs cell for repair.[168] The MSCs were decorated with DMPE conjugated CXCR4 protein using a hydrophobic anchoring approach. Upon rapid anchoring, decorated MSCs were better able to respond to SDF-1 gradients and could potentially be used as a therapy for tissue regeneration.[169]

Some cell-based therapies have made their way through clinical trials and have been approved by the FDA. Most of these therapies are for the treatment of various cancers. One of the promising strategies for treating cancer is immunotherapy, an approach that stimulates the patient's immune system rather than attacking the tumor outright.[170] Chimeric antigen receptor (CAR) T-cell therapy as leading candidate in immunotherapy. CAR T-cells are produced by genetically engineering T-cells in order for them to express chimeric-antigen receptor. T-cells are transfected with plasmids that will allow the cells to express particular tumor surface antigen receptors.[171] Also, CAR T-cells only bind to tumor antigens that are bound to the surface of cells, allowing for added specificity.[172] Currently two CAR T-cell therapeutics are FDA approved and are indicated for the use for treatment of B-cell lymphoma and lymphoblastic leukemia. These two products are Kymriah® from Novartis and Yescarta® Gilead Sciences and were both approved in 2017.[173]

1.3 Macrophages

1.3.1 Macrophages in General

Macrophages are a class of phagocytic white blood cells found in tissues and display a wide variety of functions. These cellular activities include the removal of dead cells, tissue repair and antigen presentation.[174,175] They originate from blood monocytes whom have migrated to specific tissues, often in response to a stimulus like inflammation or an infection, or from the embryonic stage of the organism.[176,177] The tissues the macrophages settle in dictate their phenotype and function, leading to a large heterogeneity of macrophage species.[178] Macrophages can further be polarized into two general phenotypes, M1 and M2. Polarized macrophages differ in their activity and in the cell surface markers they express. M1 macrophages are considered classically activated macrophages while M2 macrophages are alternatively activated. M1 macrophages are pro-inflammatory and can release pro-inflammatory cytokines such as TNF-α and IL-6. M2 macrophages on the other hand are anti-inflammatory and help with tissue repair.[179] Due to their vast distribution and biological functions, macrophages have garnered the attention of researchers in various fields.

1.3.2 Functions of Macrophages

Macrophages play a pivotal role in innate immunity and apoptotic cell clearance and most importantly in antigen processing and its presentation on cell surface.[180] Macrophages take up antigens from their environment recognized by particular receptors through phagocytosis.[181] These receptors are known as pattern-recognition receptors (PRRs) and bind to antigens known as pathogen-associated molecular patterns

(PAMPs).[182] Some PAMPs include bacterial peptides, lipopolysaccharides and cell wall components.[183] These PAMPs are then presented onto the cell surface using either MHC-1 or MHC-2 molecules, followed by activating T-cells. T-cells begin as "naïve" T-cells and travel through the peripheral circulatory system and through lymphoid tissues. Antigens on antigen presenting cells interact with T-cell receptors (TCR) on the surface of the T-cell. This interaction initiates a cascade of cellular events that eventually leads to T-cell proliferation (activation). These primed T-cells will then circulate through the body and upon re-introduction to the antigen will release cytokines, these cytokines can then further activate the immune cells to induce inflammation or cell removal.[184]

1.3.3 Biomedical Applications of Macrophages

Due to their key roles in immune system and during inflammation, tissue injury, repair processes, macrophages have been explored widely as drug/antigen delivery targets, drug delivery carrier systems and also in transplantation/grafting applications for treatment of many disease conditions.[185,186] Macrophages have been looked at for drug delivery of cancer drugs because they can be recruited by cancer cells and become differentiated into tumor associated macrophages and are then incorporated into the tumor.[187] One group used macrophages as a drug deliverer for doxorubicin and to deliver iron oxide for MR imaging to tumors.[188] Certain macrophages can even pass through the blood brain barrier, which has allowed for the idea that macrophages can even deliver drugs to the brain.[189,190] Macrophages have also been explored to expedite tissue recovery because they are naturally found near damaged tissues.[191] Polarized M1 macrophages have been delivered to damaged muscles and were shown to improve tissue recovery overtime.[192]

Recently, macrophage-mediated programmed cell removal (PrcR) has been confirmed as an important mechanism in diseased and damaged cell elimination before programmed cell death.[193] Therefore, enhancing the selective adhesion of macrophage to cancer cells may be an effective macrophage-mediated anti-cancer therapeutics. Iwasaki and colleagues metabolically engineered macrophages to display methacryloyl functional groups on their surface that can be conjugated by thiol-linked nucleic acid aptamers. These aptamers were shown to bind to protein tyrosine kinase-7, which is overexpressed in cancer cells. Once bound, the macrophages would remove the cancer cell from the system via endocytosis.[194] This has been considered as an effective macrophage-mediated anti-cancer therapeutic.

1.4 Protein Modifications

Due to their importance and diverse biological activities in living systems, proteins have garnered the attention of researchers for use as molecular tools or therapeutics. Besides being studied in their native environment or as isolated biomolecules, proteins can be modified for biotechnology purposes. A common example is using conjugated antibodies for use for detection of antigens in samples. Besides being used for tagging purposes, proteins have been modified in order to increase their therapeutic potential or to be used for cell surface re-engineering purposes. The development of a plethora of broad and site-specific modification strategies has allowed proteins to surge forward as rewarding research platform.

1.4.1 Traditional Protein Modifications

Proteins provide a lucrative platform for modifications due largely in part to their amino acid side chains. The side chains of these amino acids have a large variety of functional groups that can be targeted by ligation strategies. Traditional methods of bioconjugation focus on targeting these functional groups, which include amines, carboxylic acids and thiols. The most commonly targeted amino acids are cysteine and lysine. Cysteine is a favorable target due to its unique chemistry amongst natural amino acids and its relativity low abundance.[195] The sidechain of cysteine contains a thiol, which can be subjected to alkylation and the creation of disulfide bonds.[196]

Lysine is another popular amino acid to target for modification. Despite being more prevalent in proteins, making modifications less selective compared to targeting cysteine, the primary amine on the side chain makes lysine an attractive target. There are many different chemical techniques available to conjugate primary amines, making lysine a favorable target. Some of these reactions include using N-hydroxysuccinimide esters and sulfonyl chloride containing ligands.[197]

Though less common, some of the other amino acids have also been manipulated for conjugation purposes. In the case of tyrosine, another rare amino acid, the phenol group offers a unique group for site-specific modification.[198] Metal-mediated oxidation of the phenol group has allowed for the introduction of biotin and alkynes to proteins.[199] Other reactions that have been used modify tyrosine include using diazonium salts to form azobenzenes and Mannich-type reactions with aldehydes.[198,200] Aspartate and glutamate have carboxyl groups in their side chains, which can be coupled with amines.[197]

Tryptophan has also been targeted but the reaction requires a highly acidic environment that may lead to protein denaturation.[201]

1.4.2 Bioorthogonal Chemistry

Despite the specificity of some of the above-mention protein conjugation strategies, there is one main drawback. These reactions become unselective when more than one available type of amino acid is present. It is possible that one can modify the wrong position on the protein and hinder or even block its activity. A solution to this issue is using a class of reactions known as bioorthogonal chemical reactions. The key point of these reactions is that they do not interfere with normal biological processes. The types of reactions that fall into this category must work in physiological conditions, have quick reactions rates and be nondisruptive to the normal functions of the molecule or cell being modified.[197]

A popular subset of bioorthogonal chemistry is click chemistry (Figure 1.3). Click chemistry reactions have many variations: including cycloadditions, nucleophilic ring openings, non-aldol carbonyl reactions and additions to carbon-carbon multi-bond groups.[202,203] The most widely used variation are the cycloaddition reactions. One of the first instances of this type of reaction was by Huisgen in 1963 who used azides and terminal alkynes. In this reaction, a 1,3-dipolar cycloaddition is conducted to form a 1,2,3-triazole containing endproduct.[204] The reaction however suffered from some drawbacks such as long reaction times and the need for high temperatures. A critical modification to this system came from the introduction of a copper catalyst.[205] This reaction has many advantages over the original including regiospecificity, having a wide solvent tolerance, including being able to be performed in aqueous conditions, and being able to be performed

in a wide variety of temperature settings.[206] This has allowed the reaction to be used for a variety of applications including labeling of viral proteins and activity-based protein profiling.[207,208]

Though a very appealing reaction, there is one main disadvantage of copper-catalyzed click chemistry. The copper needed as a catalyst can be harmful to living systems, including kidney disease and neurological disorders in humans, thus limiting its application for modifying cells.[141,209] Luckily, researchers have been developing variations of the original click reaction to help overcome this barrier. A key design change comes from switching from using a terminal alkyne to an alkyne that is strained. Using cyclooctyne derivatives became a promising lead as it was shown that unsubstituted cyclooctyne could react with phenyl azide rapidly to create a triazole group.[210]

Early uses of substituted cyclooctynes have been shown to readily react with azides but had problems with slow reaction times in the beginning.[211] Later versions of substituted cyclooctynes however have been able to fix this issue and provide more favorable reaction conditions. These include using 3,3-difluorocyclooctyne (DIFO) and dibenzylcyclooctyne (DBCO).[212,213] These strained cyclooctynes have allowed for many applications of click chemistry to living systems *in vitro* and *in vivo*.[214,215]

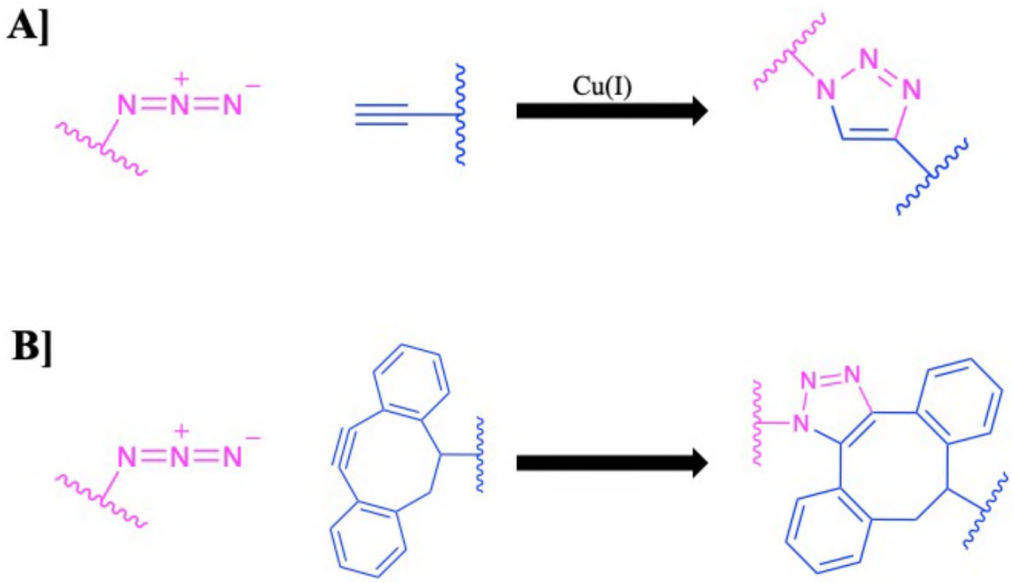

Figure 1.3: Bioorthogonal reactions. A] Copper-catalyzed click chemistry between an azide and terminal alkyne B] Copper-free click chemistry between an azide and dibenzycyclooctyne (DBCO). Adapted from [197]

1.4.3 Enzymatic Modifications and Sortase A

Similar to modifying cell surfaces, enzymes have been used to modify proteins in solution. One of these enzymes is sortase A (SrtA), a transpeptidase expressed by most Gram-positive bacteria.[216–218] Sortase A was first identified as one of the enzymes responsible for the cell wall sorting process seen in bacteria.[219,220] Though initially thought of as a target for bacterial infection, Sortase A has emerged as a useful tool for modifying proteins and cell surfaces.

Sortase A recognizes the five amino acid sequence of LPXTG, where X can be any amino acid except cysteine or tryptophan, when it is in the C-terminal of the protein.[218,221] The thiol group in the active site of the enzyme will attack the carbonyl carbon between the tyrosine and glycine residues forming a thioester intermediate.[222,223] To complete the reaction, small molecules containing one or multiple terminal glycines or proteins containing at least one glycine at its N-terminus will perform nucleophilic attacks on the carbonyl carbon of the thioester to regenerate sortase A and give the desired ligation product (Figure 1.4).[224–226]

The high specificity, wide substrate tolerance and overall straightforwardness of the sortase A reaction has made it an attractive method for protein modifications. An early use of sortase A as a method for protein modification came in 2004 when Mao and colleagues ligated green fluorescent protein (GFP) containing a LPETG motif with small molecules, peptides and proteins. In terms of peptides, they were able to introduce linear, branched and nonnative sequences to GFP efficiently. The successful introduction of nonnative peptides led the group to try adding small molecules using sortase A. They were able to introduce folate using a triglycine derivatized version of the molecule. Lastly, the

GFP-LPETG protein was successfully ligated to GFP containing a N-terminal glycine.[226] This study laid the groundwork for demonstrating the versatility of using sortase A to modify proteins. Recent studies have been able to add PEG, polystyrene beads and more biologically relevant proteins, such as antibody fragments, to proteins.[227,228]

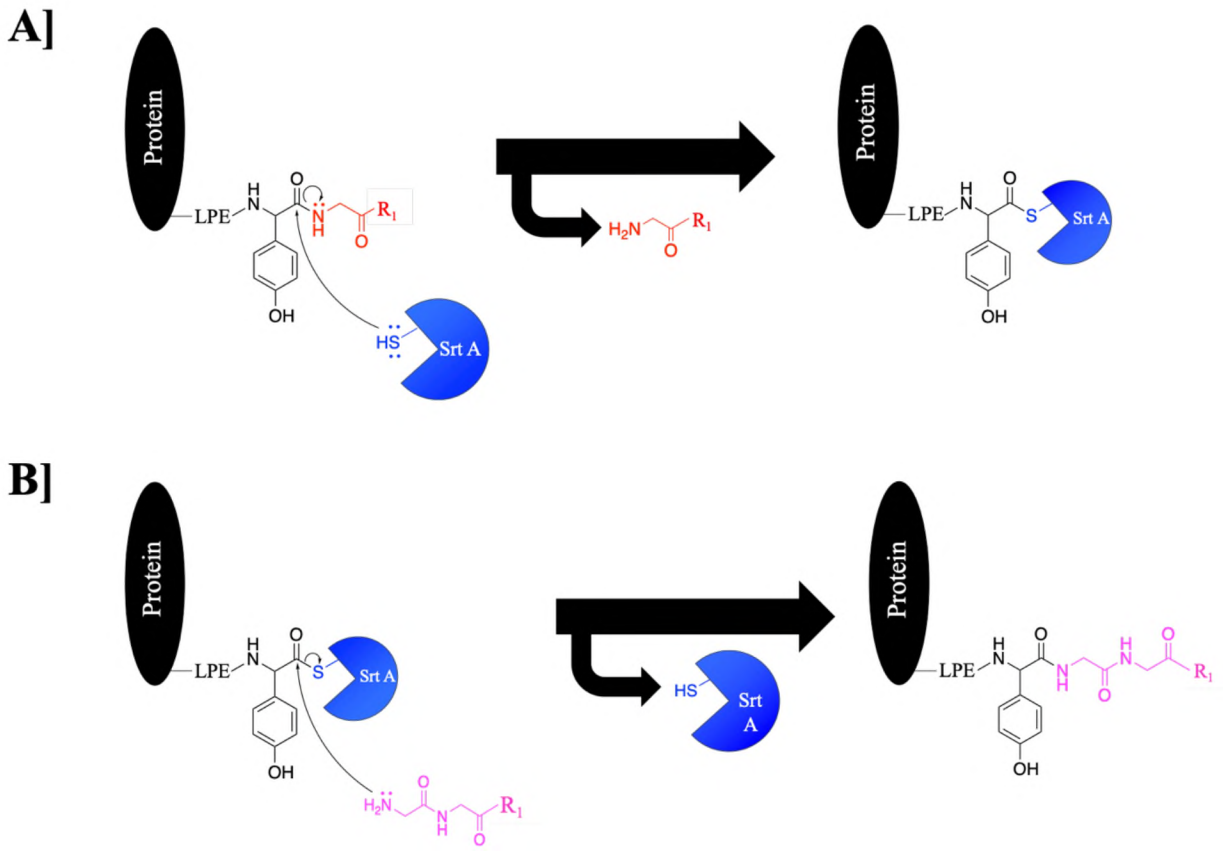

Figure 1.4: Sortase A reaction mechanism. A) Sortase A identifies the LPXTG motif, here X=E, at the C-terminal end of the protein and performs a nucleophilic attack on the carbonyl carbon of tyrosine using a thiol group in its active site. The enzyme becomes covalently attached to the protein, creating a thioester intermediate. B) The terminal amine group of a diglycine containing substrate will perform a nucleophilic attack on the carbonyl carbon of the thioester, regenerating the Sortase A enzyme and producing the desired end product. Adapted from [220].

1.5 Research Rationale

Thrombomodulin is an important protein of biomedical interest due to its biological functions and its essential distribution throughout the body. Though mainly known as an endothelial membrane protein, TM has been found to be expressed by numerous other cell types. Among these cell types are monocytes and macrophages. However, the expression patterns and biological roles of TM on these cells have been poorly studied. Many studies focus on how TM expression is affected when monocytes or macrophages are exposed to certain exogenous molecules. A ground up approach for determining differences in TM expression patterns of monocytes and their differentiated form of macrophages would be useful for determining TM's role in these cells. TM is a glycoprotein, containing such glycosylation moieties as sialic acid and chondroitin sulfate. In endothelial cells, TM exists as two major forms, one with chondroitin sulfate and one lacking the moiety. It is relatively unknown if TM in monocytes and macrophages is glycosylated, if so, how this glycosylation differs between the two cell types and how glycosylation modifications play a role in cellular function. Therefore, studying the expression and glycosylation patterns of TM present on monocytes and macrophages can help elucidate some of its biological functions on these cell types.

Some of TM's biological roles in the body, anticoagulation and antiinflammation, have caught the attention for therapeutic development. TM is also shed from the surface of the endothelium in certain diseases. Losing TM from the local environment can lead to local inflammation and/or a shift to a pro-thrombotic state. It is possible that re-introducing TM to the body can help reverse these issues. Recombinant forms of TM have been developed as possible therapeutics. Most recombinant forms of TM include the

extracellular domains, excluding the transmembrane and cytosolic domains, where the bulk of TM's biological activities take place. Expressing all these domains can pose some possible issues though. TM's EGF-like domain is the site of the protein's anti-coagulation activates. Here, thrombin can bind to help in the activation in protein C, which can act as an anticoagulant enzyme. However, thrombin binding in this domain can also activate TAFI, which will act as a procoagulant enzyme. Luckily, the procoagulant function of TM, while maintaining its anticoagulant activity, can be removed by only expressing EGF-like domains 4-6. EGF-like subdomains 4-6 are the minimal domains needed for protein C activation. This activated protein C can then perform anticoagulant and anti-inflammation processes. Therefore, recombinant TM of EGF-like domains 4-6 will be a choice anticoagulation and anti-inflammation drug development.

Despite its great potential as a therapeutic, recombinant TM has some problems for therapeutic development. Its main issue deals with its short half-life in living systems. This is a common obstacle that protein-based therapeutics have to overcome and multiple strategies have been tested to fix this issue. Several techniques have been developed, including adding PEG moieties and loading therapeutics to liposomes. Using cells as a drug carrier can offer beneficial properties to a protein therapeutic. Cells can provide a more natural environment for proteins, versus them being free in solution or on a synthetic support and can allow for increased stability and activity. Also, some cells can target specific tissues or sites of dysfunction allowing for targeted deliver. Macrophages can be directed towards sites of inflammation, so they can be used to deliver antiinflammation drugs or therapeutics that can treat the primary cause of inflammation. We hypothesize

using macrophages as a drug carrier for rTM as a strategy to increase stability and for possible delivery rTM to disease sites.

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CHAPTER II

THROMBOMODULIN EXPRESSION AND SHEDDING DURING MONOCYTE'S DIFFERENTION TO MACROPHAGE

2.1 Introduction

Thrombomodulin (TM) was first discovered by Esmon and Owen in 1981 as an endothelial cell cofactor for thrombin-mediated protein C activation.[1] Endothelial TM contributes to local hemostatic balance by serving as an essential cofactor for the activation of anticoagulant and anti-inflammatory proteins.[2] TM exerts antithrombotic effects by at least two mechanisms: directly, *via* direct thrombin inhibition and indirectly, *via* accelerating the thrombin-catalyzed local activation of protein C at the site of thrombin generation.[3] When bound to TM on the endothelial surface, thrombin is unable to catalyze fibrin generation or activate platelets, but instead becomes a potent activator of protein C. The activated protein C (APC) is an anticoagulant protease that selectively inactivates coagulation factors Va and VIIIa, providing an essential feedback mechanism to prevent excessive coagulation and inflammation. Further, TM has direct anti-inflammatory activity by suppressing adhesion molecule expression.[4]

Endothelial TM is a type I transmembrane glycoprotein with 557-amino acid residues, which is divided into five domains: *N*-terminal lectin-like domain (TMD1), epidermal growth factor-like (EGF) domain (TMD2), serine/threonine-rich domain containing chondroitin sulfate glycosaminoglycan (TMD3), transmembrane domain (TMD4) and cytoplasmic tail (TMD5).[5] Different domains are responsible for different biological functions of TM.[6]

The expression of TM has been confirmed on various vascular endothelial cells, lymphatic endothelial cells and other cells, such as epithelial cells, mesothelial cells, immune cells and osteoblasts.[7] It has been confirmed that TM is a significant biomolecule involved in many physiological and pathological processes such as coagulation, inflammation, cancer development, embryogenesis and immunoregulation as well.[7] Therefore, TM has attracted tremendous attention from both basic understanding of its structure and biological functions involved in these physiological and pathological processes.

In addition to the above mentioned cell types, TM has also been shown to be expressed in monocytes and macrophages.[8] While the role of TM in this class of cells is still poorly understood, recent research has shed some light in this area. For monocytes, a main focus of research is how TM is influenced by the presence of and interactions with lipopolysaccharide (LPS). Initial studies focused on how LPS influenced the expression of TM. A 1996 study showed that when exposed to LPS, monocytes expressed less TM on their cell surface but an increased number of microparticles that stained positive for TM.[9] More recent studies have looked at the function TM has on monocytes. Again, LPS is a major focus for elucidating the function. It has been shown that the lectin-like domain of

TM interacts with the Lewis Y antigen on LPS and blocks LPS's ability to trigger an inflammatory response.[10] This hinted at the possibility that TM's role on monocytes is inflammation related. Recently it has been found that TM is a component of the CD14/TLR4/MD-2 complex, which plays a role in Gram-negative bacterial based inflammation.[11–16] It was demonstrated that TM facilitates LPS binding to the cell surface to help induce inflammation.[11] In addition to modifying the inflammation response of monocytes, TM also has been shown to be involved in monocyte migration. When TM was knocked down in THP-1 monocytes, their chemotactic response to IL-6 was disrupted.[17] The cytosolic domain on TM was identified as the negative regulator of chemotaxis.[18]

Macrophages display remarkable plasticity that allows them to acquire functionally distinct phenotypes and take part in a large number of physiological and pathological processes.[19] Monocytes are precursors for tissue macrophages, in replenishing resident macrophages under normal states and in response to inflammation signals by quickly moving to the sites of infection and dividing/differentiating to macrophages in the tissues. The differentiation of monocytes into macrophages results in phenotypic changes illustrated by the expression of specific cell surface antigens and the production of either pro- or anti-inflammatory mediators.[20] This differentiation process influences the biological functions of these immune cells and leads to a reorganization of cell surface glycans. Our group and others confirmed that glycan changes occur for the human monocyte THP-1 cell line upon differentiation to macrophages.[21,22] Protein glycosylation plays a critical role in macrophage cell biology, development, inflammation, and immunology.

There are two forms of endothelial TM based on its *O*-glycosylation, one with and one without chondroitin sulfate (CS).[23] The activation of protein C requires thrombin binding to the EGF-like domain and is enhanced by a second site binding to the CS moiety attached to the serine/threonine–rich domain.[24] However, there is no research reported on the *O*-glycosylation of monocyte TM and macrophage TM. Whether the CS moiety of monocyte and macrophage TM participates in their biological function remains unclear.

In the present study the TM expression differences between monocytes and after their differentiation into macrophages was investigated. THP-1 cells have been used extensively as a model monocyte/macrophage.[25–28] These cells also offer several advantages over primary cells that include ease of culturing, a homogenous genetic background and cost effectiveness.[29] Therefore, the THP-1 cell line was used as model monocytes in this study. First the cell surface expression of TM on monocytes and macrophages was determined. Since TM is a transmembrane protein, determining differences in surface expression levels of TM, in addition to total TM levels, is important. Flow cytometry and confocal microscopy studies revealed that monocyte's express a significantly higher amount of TM on their surface compared to their macrophage counterpart. This difference is reflected in total TM levels of the two cell types as well. ELISA and Western Blot analysis of cell lysates showed that THP-1 monocytes express more total TM than macrophages. Next, TM activity of THP-1 monocytes and macrophages was determined by the cells' abilities to activate protein C. Interestingly, macrophages were able to produce more activated protein C (APC) than monocytes. This difference in APC generation might be linked to differences in posttranslational modifications of TM.

Overall, this study showed how monocytes and macrophages express TM differently. Also, it was found that THP-1 monocytes and macrophages show different APC generation abilities. This difference is thought to be due to either differences in post-translational modifications of TM or the presence/up-regulation of a cofactor for protein C activation. These findings can give preliminary insight to how the role of TM may change from monocytes to their differentiation to macrophages.

2.2 Materials and Methods

2.2.1 Materials

All solvents and reagents were purchased from commercial sources and were used as received unless otherwise noted. Phorbol 12-myristate 13-acetate (PMA), heparin, 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Mouse anti-human thrombomodulin antibody, AlexaFL-488 conjugated goat anti-mouse IgG antibody, horseradish peroxidase conjugated goat anti-mouse IgG antibody and Human Thrombomodulin ELISA set were purchased from Abcam (Cambridge, MA). Human protein C, human thrombin and human antithrombin III were purchased from Haematologic Technologies (Essex Junction, VT). BIOPHEN-CS01 was purchased from Aniara (West Chester, OH). Para-formaldehyde was purchased from Electron Microscopy Sciences (Hatfield, PA). Fetal bovine serum (FBS), heat-inactivated fetal bovine serum (HI-FBS), RPMI 1640 medium, penicillin/streptomycin solution and bovine serum albumin (BSA) were purchased from VWR (Radnor, PA). Chondroitin sulfate antibody and SuperSignal™ West Pico PLUS Chemiluminescent Substrate kit were purchased from Thermo Fisher Scientific (Waltham, PA).

2.2.2 Cell Culture

THP-1 monocytes were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Differentiation of monocytes to macrophages was induced by treating cells with 10ng/mL PMA for 48hrs. in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. All cell types were incubated at 37°C and 5% CO_2.

2.2.3 Flow Cytometry Analysis of Surface TM Expression

THP-1 monocytes or differentiated macrophages ($1x10^6$ cells/mL) were fixed with 4% para-formaldehyde for 10min. After fixing, cells were washed three times with cold 1x PBS buffer. Cells were blocked with 5% BSA in 1x PBS buffer for 1hr. at room temperature. Next the blocking solution was removed and cells were treated with a mouse IgG anti-human TM primary antibody (1:1000 dilution in 5% BSA, 1x PBS solution) for 1hr. at room temperature. After incubation, cells were wash three times with cold 1x PBS buffer then treated with an AlexaFL-488 conjugated goat anti-mouse IgG secondary antibody (1:1000 dilution in 5% BSA, 1x PBS solution) and incubated for 1hr. at room temperature. After incubation, cells were washed three times with cold 1x PBS buffer and resuspended in 1x PBS buffer containing 1% BSA and 0.1% NaN_3. Cells were immediately analyzed using a FACSCanto II system (BD Bioscience) for flow cytometry analysis.

2.2.4 Confocal Microscopy Analysis of Surface TM Expression

THP-1 monocytes ($6x10^4$ cells per tube) were fixed with 4% para-formaldehyde for 10min. After fixing, cells were washed three times with 1x PBS buffer and blocked using a 5% BSA, 1x PBS buffer solution. Cells were blocked for 1hr. at room temperature.

After incubation, the blocking solution was removed and cells were incubated with 300μL of a primary antibody solution (1:1000 dilution of mouse IgG anti-human TM antibody in 5% BSA, 1x PBS buffer solution) for 1hr. at room temperature. The antibody solution was removed and the cells were washed three times with 1x PBS buffer. Next cells were incubated with 300μL of a secondary antibody solution (1:1000 dilution of AlexaFL-488 conjugated goat anti-mouse IgG antibody in 5% BSA, 1x PBS buffer solution) for 1hr. at room temperature. After incubation the antibody solution was removed and the cells were washed three times with 1x PBS buffer. Following washing, cells were incubated with 300μL of a 300nM DAPI solution for 1hr. at room temperature. The DAPI solution was then removed and the cell pellet was washed three times with 1x PBS. Cells were pelleted down and the excess solution aspirated off. The cell pellet was then resuspended with 30μL of Prolong Gold Anti-fade reagent and deposited onto a coverslip. The coverslip was placed onto a microscope slide and allowed to cure overnight.

Differentiated macrophages were plated into 6-well plates at a density of $6x10^4$ cells/well where each well contained a glass coverslip. Cells were allowed to incubate overnight to allow for adherence. After overnight incubation cells were washed three times with 1x PBS buffer and fixed with 4% para-formaldehyde for 10min. After fixing, the para-formaldehyde solution was aspirated off and the cells were washed three times with 1x PBS buffer. Coverslips were then blocked with 5% BSA, 1x PBS buffer for 1hr. Blocking solution was aspirated off and 300μL of primary antibody solution was added (1:1000 dilution of mouse IgG anti-human TM antibody in 5% BSA, 1x PBS buffer solution) and incubated for 1hr. at room temperature. The antibody solution was then removed and the coverslips were washed three times with 1x PBS buffer. 300μL of secondary antibody

solution was then added to each coverslip (1:1000 dilution of AlexaFL-488 conjugated goat anti-mouse IgG antibody in 5% BSA, 1x PBS buffer solution) and incubated for 1hr. at room temperature. Excess solution was removed and coverslips were washed three times with 1x PBS. After washing, 300µL of 300nM DAPI solution was added to each coverslip and incubated for 10min. at room temperature. After incubation the DAPI solution was removed and coverslips were washed three times with 1x PBS buffer. Coverslips were mounted to slides by adding 30µL of ProLong™ Gold Anti-fade reagent to the coverslip and placed onto the microscope slide and allowed to cure overnight. Slides were imaged the following day using a Nikon A1RSI Confocal Microscope using a 60x oil objective.

2.2.5 Western Blot Analysis of Total TM Expression of THP-1 Monocytes and Differentiated Macrophages

200µg of total protein from cell lysates were added to each lane and run by SDS-PAGE. Gels were transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 5% milk in tris-buffered saline, tween-20 (TBST) buffer for 1hr. at room temperature. Membranes were incubated with a primary antibody solution (1:1000 dilution of mouse IgG anti-human TM antibody in 5% milk, TBST buffer) overnight at 4°C. After overnight incubation, membranes were washed three times with TBST buffer for 5min. Next, membranes were incubated with a secondary antibody solution (1:1000 dilution of HRP-conjugated goat anti-mouse IgG antibody in 5% milk, TBST buffer) at room temperature for 1hr. Membranes were then washed three times with TBST buffer for 5min. Antibody detection was determined using SuperSignal™ West Pico PLUS Chemiluminescent Substrate and photographic film

2.2.6 ELISA Determination of Total TM Expression of THP-1 Monocytes and Differentiated Macrophages

Total amount of TM from cell lysates were determined by using the Thrombomodulin Human ELISA set (Abcam) with some modifications. Briefly, 40µL of stock capture antibody solution was diluted into 10mL of coating buffer (1x PBS, 0.09% NaN_3) and 100µL of this diluted solution was added to each well of a 96-well ELISA plate. The plates were incubated overnight at 4°C. The next day the capture antibody solution was removed and the plates were washed. To each well, 400µL of washing buffer (1x PBS, 0.05% Tween-20) was added and incubated for 5min., this step is repeated one time. Plates were then blocked with 5% BSA solution and incubated for 2hrs., then washed twice. Next, 100µL of sample or standard was added to each well along with 50µL of diluted detection antibody solution (1x PBS, 1% BSA). Sample solutions contained 100µg total protein each and the concentration range for the standards was 0-20ng/mL TM. These solutions were incubated for 1hr. at room temperature. After incubation, the plates were washed twice then treated with a streptavidin-HRP solution (1x PBS, 1% BSA, 0.1% Tween-20) for 30min. Plates were washed and 100µL of TMB substrate mix was added to each well. The plates were allowed to develop a blue color then read at 620nm.

2.2.7 ELISA Determination of Shed TM of THP-1 Monocytes and Differentiated Macrophages

For THP-1 monocytes, $3x10^5$ cells were seeded into a $100mm^2$ culture dish in 10mL of medium. Cells were allowed to incubate for two days and then the cell medium was collected. For THP-1 macrophages, $1x10^6$ cells were seeded into a $100mm^2$ culture dish in 10mL medium and immediately differentiated using the above-mentioned protocol. After

the differentiation process the medium was collected. Medium samples were concentrated using a 10kDa molecular weight cutoff centrifugal filter. TM levels were measured using the Thrombomodulin Human ELISA set as described earlier, where 200μg of total protein was added to each sample well.

2.2.8 APC Generation Assay

THP-1 monocytes or differentiated macrophages were plated at a density of 60,000 cells/well in a 12-well plate. Cells were incubated with 200nM protein C for 5min. at 37°C. Next, thrombin was added to each well to a final concentration of 8.9mg/mL. The cells mixture was then allowed to incubate for 1 hr. at 37°C while shaking. To terminate the production of APC, 2μL of heparin (10U/mL) and 30μL of antithrombin III (1mg/mL) were added to each well and allowed to incubate for 10min. Plates were centrifuged down and 100μL of medium was taken from each well and transferred to a 96-well plate. To determine APC generation, 50μL of 0.5mM BIOPHEN CS-01 (38) substrate was added to each well and incubated for 20min. at 37°C while shaking. Absorbance was read at 405nm using a multiplate reader.

2.3 Results and Discussion

2.3.1 Flow Cytometry Analysis of Surface TM Expression

First, the surface expression of TM of THP-1 monocytes and THP-1 macrophages was analyzed by flow cytometry. To tag TM protein, a double immunostaining approach was used. First, cells were incubated with a mouse anti-human TM antibody and further tagged using an AlexaFL-488 conjugated goat anti-mouse IgG antibody. After analysis, it was shown that monocytes express significantly higher levels of TM than their macrophage

counterpart. When looking at the histograms of the complete stained cells (Figure 2.1), the monocytes show a greater right shift, indicating a higher fluorescence signal. Since fluorescent signal is proportional to the amount of TM present, this translates to THP-1 monocytes expressing more TM on their cell surfaces compared to THP-1 macrophages. Also, control histograms show that untreated cells, cells treated with the primary antibody only or cells treated with the secondary story do not show any significant fluorescent signal. Comparing mean fluorescent intensities of THP-1 monocytes and macrophages show that macrophages express about 50% less TM than monocytes on their cell surface (Figure 2.2).

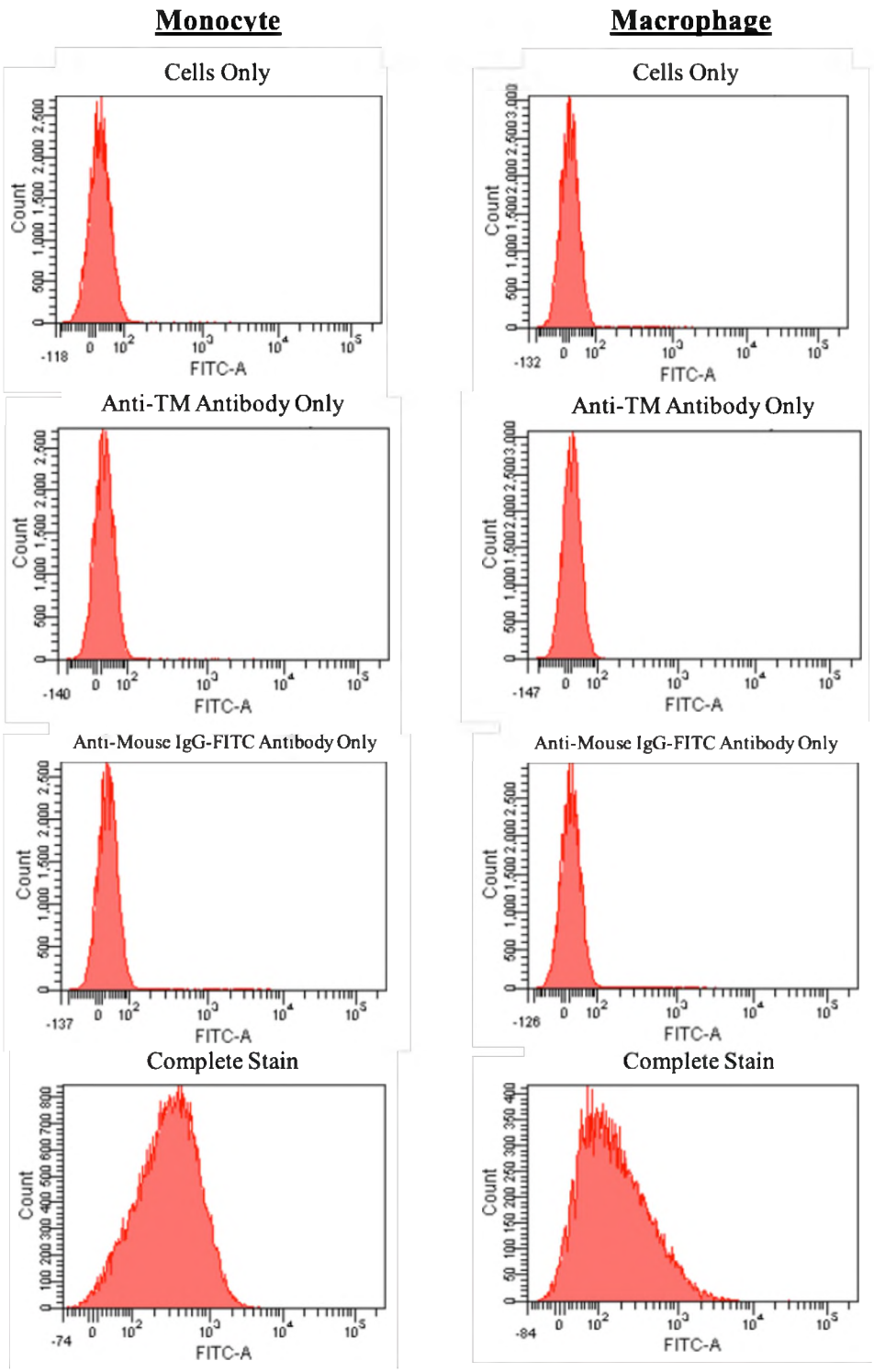

Figure 2.1: Histograms for flow cytometry analysis of surface thrombomodulin expression of THP-1 monocytes and macrophages

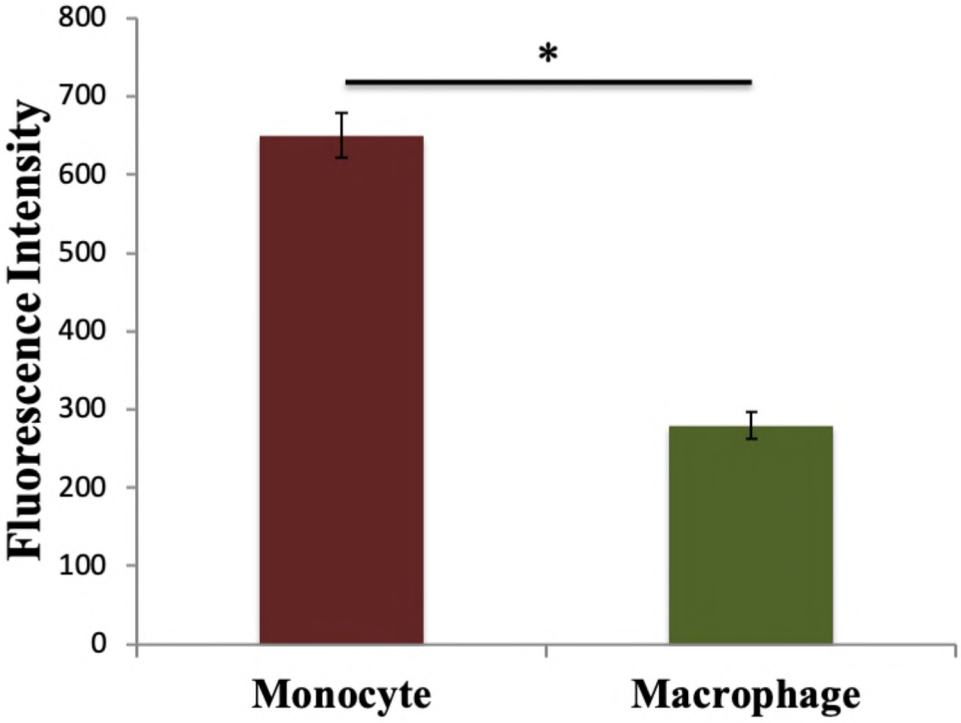

Figure 2.2: Mean fluorescent intensities of surface TM of THP-1 monocytes and macrophages using flow cytometry analysis. Data represents mean +/- standard error of the mean, n=3, P<0.05

2.3.2 Confocal Microscopy Analysis of Surface TM Expression

To confirm the results seen from the flow cytometry study, confocal microscopy analysis of surface levels of TM of THP-1 monocytes and macrophages was performed. TM protein on cells was stained for using a double immunostaining technique. THP-1 monocytes or macrophages were first incubated with an anti-human TM antibody, then followed by an AlexaFL-488 conjugated goat anti-mouse IgG antibody. Cells were further stained with DAPI to visualize the nucleus. After staining, monocytes were mounted onto microscopy slides and analyzed. THP-1 monocytes were shown to have a strong fluorescence signal on their surface (Figure 2.3). In comparison, macrophages showed a significantly fainter fluorescent signal on their cell surface (Figure 2.4). These results confirm that macrophages express less TM on their cell surface compared to their monocyte precursor.

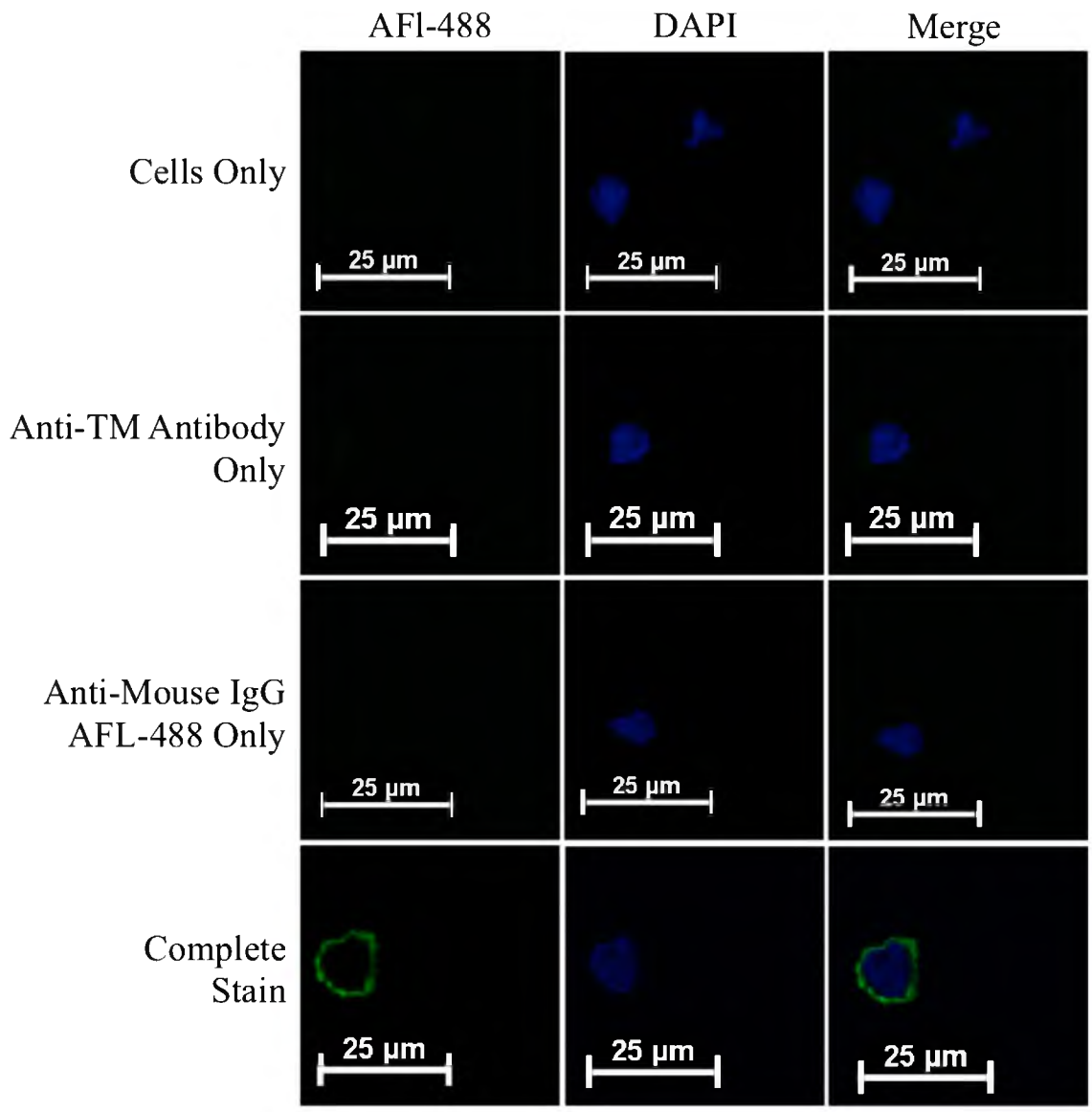

Figure 2.3: Confocal microscopy analysis of surface thrombomodulin expression of THP-1 monocytes

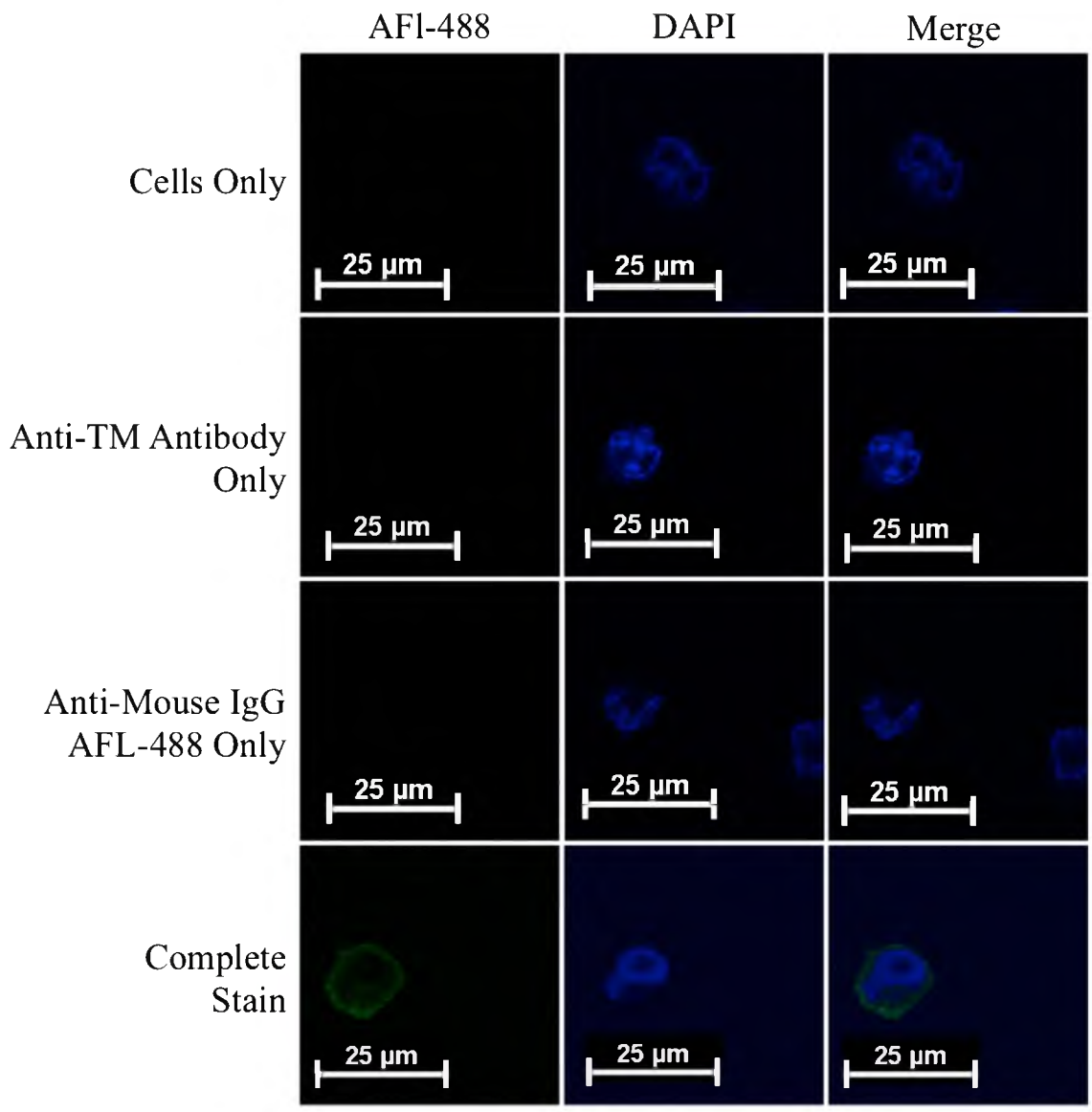

Figure 2.4: Confocal microscopy analysis of surface thrombomodulin expression of macrophages

2.3.3 Western Blot Analysis of Total TM Expression of THP-1 Monocytes and Differentiated Macrophages

To determine if the differences seen in surface TM expression are a result of differences in overall expression, western blot analysis was first performed. Total cell lysates of THP-1 monocytes and macrophages were run using SDS-PAGE and then transferred to a PVDF membrane. TM protein was probed for using a mouse anti-human antibody as a primary antibody and then a goat anti-mouse IgG antibody conjugated with HRP. Membranes were developed using a fluorogenic substrate and photographic film. Comparing band densities of THP-1 monocyte and macrophage cell lysates, it was shown that monocytes show a denser band than macrophages (Figure 2.5). This translates into monocytes expressing more total TM compared to macrophages. When normalizing to β-actin, monocytes were shown to express almost double the amount of TM than macrophages. This result provides reasoning to the differences seen in surface TM levels. Since monocytes express more TM overall, it correlates well that they would also express more TM on their cell surface.

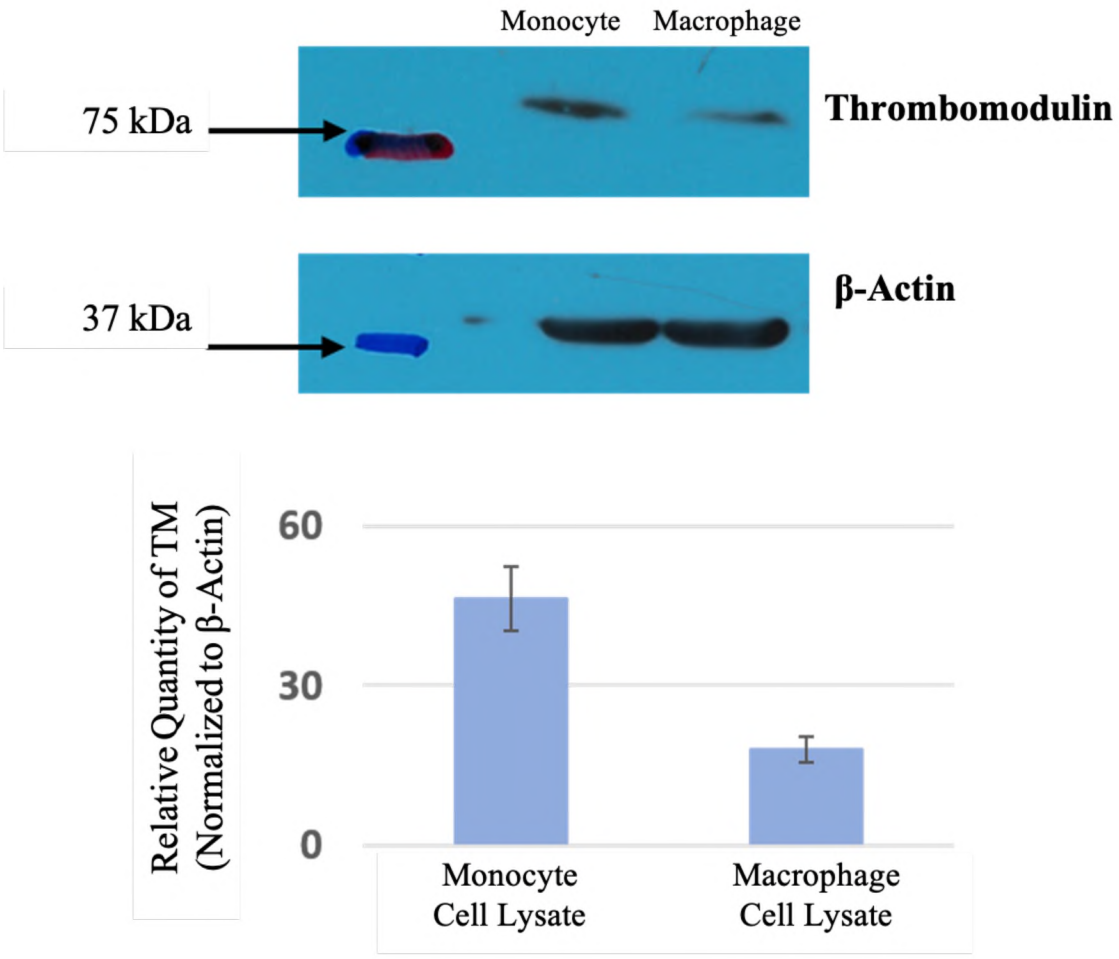

Figure 2.5: Total TM expression of THP-1 monocyte and macrophage cell lysates as determined by western blot. Data represents mean +/- standard deviation, n=3

2.3.4. ELISA Determination of Total TM Expression of THP-1 Monocytes and Differentiated Macrophages

To gain a more quantitative comparison of total TM levels of THP-1 monocytes and macrophages, ELISA analysis of cell lysates was performed. Total cell lysate samples were applied to an ELISA plate containing an anti-human TM capture antibody. After incubation and washing, biotinylated anti-TM detection antibody and HRP conjugated streptavidin solutions were added. The quantity of TM was determined by the addition of a substrate that becomes blue upon conversion by HRP. Similar to the results seen from western blot analysis, THP-1 monocytes expressed more total TM compared to macrophages (Figure 2.6). These results further give reason as to why monocytes express more TM on their cell surfaces compared to their macrophage counterpart.

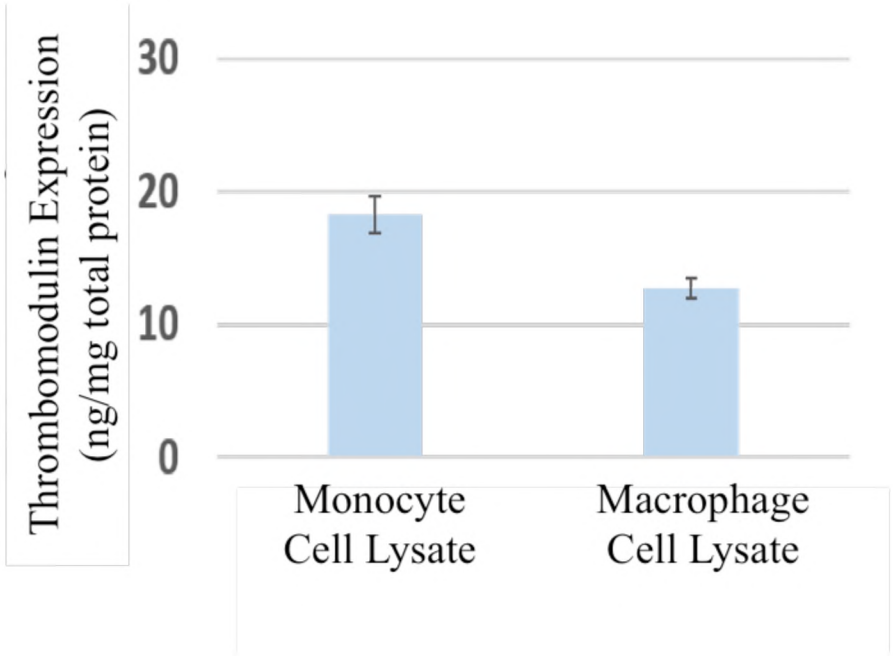

Figure 2.6: Total TM expression of THP-1 monocyte and macrophage cell lysates as determined by ELISA. Data represents mean +/- standard deviation, n=3

2.3.5 ELISA Determination of Shed TM of THP-1 Monocytes and Differentiated Macrophages

In addition to exploring differences in surface and total TM levels, it was of interest to how THP-1 monocytes and macrophages shed TM differently. The amounts of shed TM were determined by a sandwich ELISA technique. Both the capture and detection antibodies were against human TM. The detection antibody was biotinylated and could be tagged using streptavidin, which is conjugated with HRP. The quantity of TM was then determined using a HRP compatible chromogenic substrate. ELISA results showed that macrophages shed significantly more TM during their differentiation from monocytes versus resting monocytes (Figure 2.7). This increase in shedding might also play a role in why macrophages express less TM on their cell surface. During cell differentiation there are a lot of changes that occur, which includes changes to the cell surface. Since macrophages express less TM on their cell surface compared to monocytes, the monocytes have to remove TM from their cell surface during differentiation. One possible route of removal could be direct shedding from the membrane based on these results.

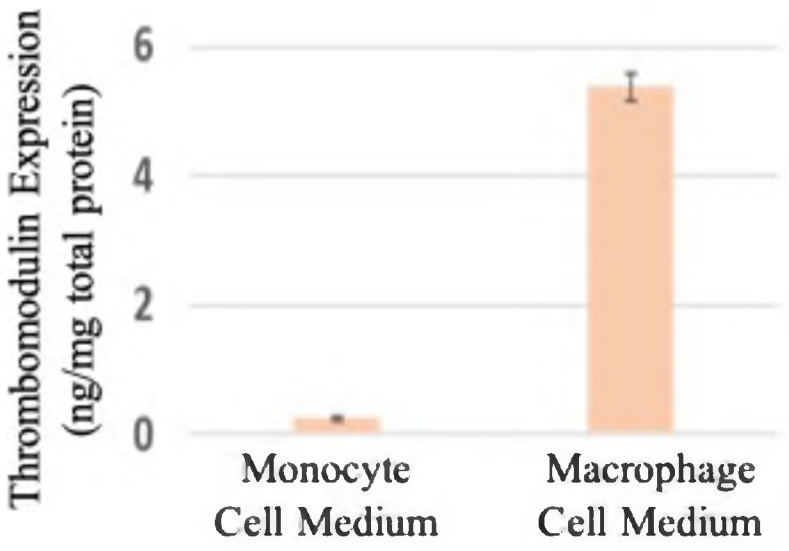

Figure 2.7: Amount of shed TM from THP-1 and differentiated macrophages as determined by ELISA. Data represents mean +/- standard deviation, n=3

2.3.6 APC Generation Assay

One of TM's hallmark activities is its involvement in activating protein C. However, not much is known about how well monocytes and macrophages can activate protein C. To determine the APC generation ability of monocytes and macrophages, cells were incubated with protein C and thrombin and allowed time to generate APC. The amount of APC generated was determined by the conversion of a chromogenic substrate. Earlier results showed that monocytes express more TM on their cell surface compared to macrophages, so it would be expected that they would also have a higher APC generation activity. Surprisingly, the opposite was found in this study. Despite expressing less TM on their cell surface, macrophages were able to generate more APC than monocytes (Figure 2.8).

This finding raises questions as to why this difference in activity is seen. Three reasons have been proposed for the change. First, it could be possible that the TM expressed between the two cell types have large structural differences, such as missing domains. However, when looking back at western blot data (Figure 2.5), the TM bands seen are in the same position. This means that there are no large structural changes between the TM expressed by monocytes and macrophages. Second, macrophages could be expressing a new or increasing cofactor than monocytes that helps activate APC. In endothelial cells, there is a specific receptor that helps bind protein C to allow it to be better activated by the thrombin-thrombomodulin complex. This receptor is the endothelial protein C receptor (EPCR).[30] Notably, EPCR has also been found to be expressed THP-1 monocytes, however the expression of EPCR is unknown in THP-1 macrophages.[31] Third, the post-translational modifications between monocyte and macrophage TM could be different and

may relate to changes in TM's ability to activate protein C. TM is a glycoprotein and contains sites for *N*-type and *O*-type glycosylation.[32–34] Two specific types of glycosylation modifications of TM are the addition of sialic acid and chondroitin sulfate. Previous work in our lab has found that when THP-1 cells differentiate from monocytes to macrophages, their sialylation profile changes. Specifically, sialic acid levels were decreased in THP-1 macrophages compared to monocytes.[21] These changes seen globally could also be reflected at the protein level and have an effect of protein C binding, ultimately leading to how well protein C is activated. Chondroitin sulfate is a glycosaminoglycan found in the serine/threonine-rich domain of TM.[35] It has been demonstrated that chondroitin sulfate enhances thrombin binding to TM and helps the activation of protein C.[35–37] However, when looking again at the western blot data it appears that both forms of TM does not contain chondroitin sulfate. Researchers exploring how the chondroitin sulfate group affects rTM function have seen consistent western blotting patterns between TM with and without chondroitin sulfate. When TM expresses chondroitin sulfate, it shows a smeared band compared to a sharp band seen with TM lacking the moiety[38,39]. The bands seen for THP-1 monocyte and macrophages are sharp with no smearing, indicating they may not contain chondroitin sulfate. However, the TM of the THP-1 monocytes and macrophages could have other changes in glycosylation, such as *N*-glycans with different amounts of sialic acid.

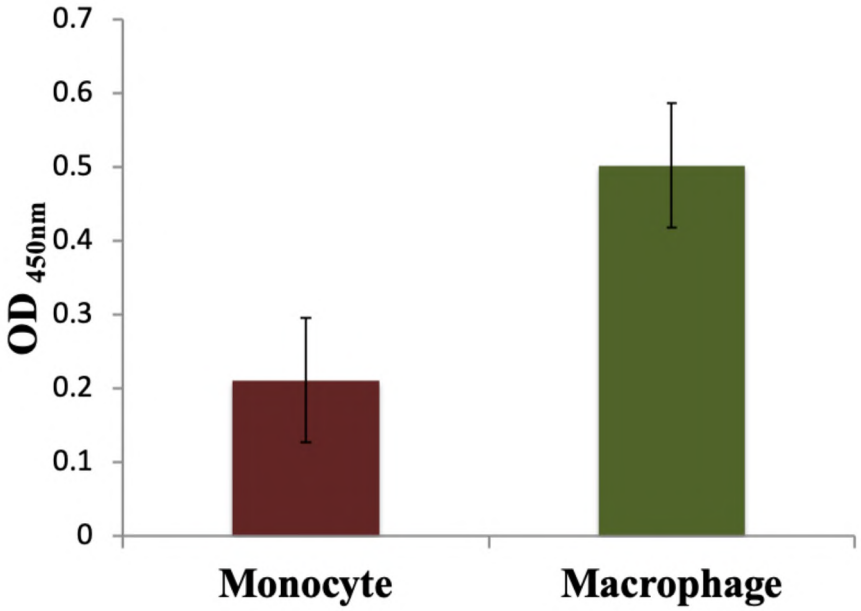

Figure 2.8: Activated protein C (APC) generation of THP-1 monocytes and macrophages. Data represents mean +/- standard deviation, n=3

There are some pitfalls that face this current project. First, the APC assay used relies on the assumption the APC is the only agent that can convert the chromogenic substrate. However, it may be possible that another molecule present could be able to do the same conversion. A second assay to determine APC levels that does not rely on its activity is needed. Looking for APC protein levels directly using western blotting or ELISA techniques would be a reliable approach. Secondly, the cell line used for this study, THP-1 cells, does not overexpress TM. This can pose an issue for future experiments where a large amount of purified TM protein from these cells are needed. Assay protocols may have to be adjusted in order to detect the low levels of TM on these cells.

2.4 Conclusion

In this study, the differences in TM expression, shedding and activity between THP-1 monocytes and macrophages was investigated. Flow cytometry and confocal microscopy analysis showed that THP-1 monocytes express more TM on their cell surfaces than macrophages. Western blot and ELISA results showed that in addition to THP-1 monocytes expressing more surface TM, they also express more total TM. When looking at shedding dynamics between resting monocytes and monocytes differentiating into macrophages, it was seen that a higher amount of TM is shed during differentiation. This may be one contributing factor as to why monocytes express more surface TM compared to their macrophage counterpart. Lastly, the APC generation activity of THP-1 monocytes and macrophages was determined as a measure of overall measure of TM activity. Despite expressing less surface TM, macrophages were shown to be able to generate more APC in a given time versus monocytes. This result raises many questions as to why and how macrophages are able to activate more protein C despite expressing less TM. Differences

in post-translational modifications and other cofactors expressed on THP-1 macrophages could be the reasons for the increase in APC generation activity. Overall, this research confirmed the differences in TM expression, shedding and APC generation between THP-1 monocytes and their macrophages form, indicating their physiological and pathological relevance *in vivo*. Continuing research is well deserved in the future.

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CHAPTER III

CELL SURFACE RE-ENGINEERING WITH ANCHORING LIPIDS WITH DIFFERENT PERFORMANCE

3.1 Introduction

The cell surface is ornamented with a large variety of biomolecule that allow it to serve as a platform for many biological processes. One set of these biomolecules is lipids, which the cell membrane contains a large variety of. There are more than 1,000 unique lipids that compose the membrane of eukaryotic cells but the most common types are glycerophospholipids such as phosphatidylcholine and phosphatidyethanolamine.[1,2] When considering the plasma membrane, sterols and shphingolipids are in higher concentrations due to their ability to pack closer together to help the cell against physical stress.[1] One of these sterols is cholesterol, which helps determine the cell membrane's fluidity and elasticity.[3–5]

With being home to a diverse number of biomolecules, the cell membrane has been looked to as a platform for introducing cargo to cells. These applications include drug delivery, antigen delivery and imaging.[6–8] To accomplish these applications, numerous cell surface re-engineering techniques have been developed. Common chemistry based techniques include direct chemical modification, metabolic engineering and lipid/liposome fusion.[9,10]

Modifications to the cell membrane using lipid-based systems have been explored and have found success. One of these methods of delivery is using free lipids as an anchor to tether molecules of interest to the cell membrane. Lipids that have shown successful incorporation include cholesterol and various phospholipids.[11–13] Free lipids have been able to deliver a wide variety of molecules such as fluorophores, glycopolymers and oligonucleotides.[14–16]

One major role macrophages play is as antigen presenting cells. Macrophages can uptake foreign molecules, process them and then present them on their cell surface.[17] Macrophages have drawn attention as a possible drug delivery system. Using cells, in particular immunocytes such as macrophages, as drug delivery carriers offers some advantages over common techniques. These include targeted transport, improved drug half-lives and decreased toxicity.[18] So far macrophages have been used to deliver many different types of therapeutics including anti-cancer agents and antiretroviral drugs. [19,20]

Recently, our group has shown the potential of using functionalized lipids for cell surface re-engineering applications. Two anchoring lipids, phospholipid (DSPE-PEG_{2000}-DBCO) and cholesterol (CHOL-PEG_{2000}-DBCO) based anchor lipids were synthesized and characterized by NMR spectroscopy. The potential prospect of using DSPE-PEG_{2000}-

DBCO and CHOL-PEG_{2000}-DBCO anchor lipids for cell surface re-engineering was assessed with RAW 264.7 cells as model. Both a one-step and two-step introduction of cargo was assessed using biotin as a model cargo. The successful incorporation of biotinylated anchor lipids was confirmed by confocal microscopy and flow cytometry after specific streptavidin-FITC binding onto the cells. Cholesterol afforded relatively higher cell membrane incorporation efficiency with less internalization compared to the phospholipid as an anchoring lipid. Furthermore, the cytotoxic effect of the synthesized biotinylated anchor lipids on the RAW 264.7 cells was assessed. Thus, this study suggests the possible use of these lipids for potential cell surface re-engineering applications.[21]

In this present study the results of the one-step introduction of biotin to the cell surface of RAW 264.7 cells using either a biotinylated DSPE or cholesterol-based anchor was confirmed (whose structures are shown in Figure 3.1) as well as determine their residence lifetime on the cell membrane. Two types of lipid anchors were investigated in this study, DSPE-PEG_{2000}-Biotin and cholesterol-PEG_{2000}-Biotin. The incorporation efficiencies of the two anchors lipids were determined using different concentrations at a constant incubation time of 20 min. Successful incorporation was determined using a FITC-labeled streptavidin molecule followed by confocal microscopy (Figure 3.2). The cholesterol-based anchor lipid afforded a significantly higher incorporation efficiency compared to the DSPE-based anchor lipid. Due to its higher incorporation efficiency, only the membrane residence time of the cholesterol-based anchor was determined. RAW 264.7 cells were incubated with different concentrations of cholesterol-PEG_{2000}-biotin for 30min. The fluorescent intensities of streptavidin labeled cells were tracked for a 4-hour time period after the initial incubation period by confocal microscopy. It was shown that

cholesterol anchor lipids leave the cell membrane at different rates based on their initial treatment concentration but all have a residence half-life of about 1 hr. Overall, this study shows how different lipid anchors have different incorporation efficiencies into the cell membrane of macrophages and how long these anchors can last on the cell membrane, indicating how long introduced cargo will remain on the cell to exert a particular function.

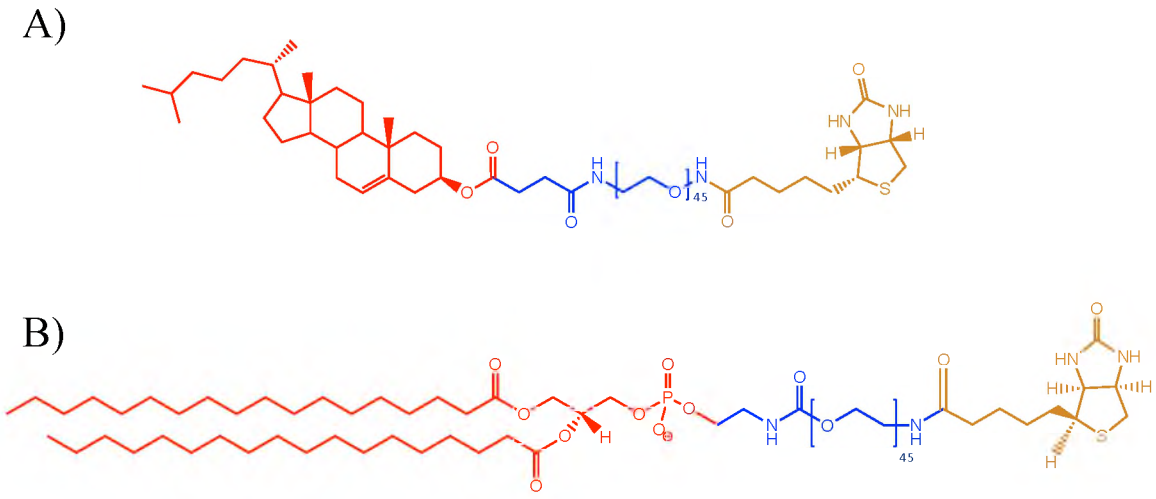

Figure 3.1: Structures of Cholesterol-PEG$_{2000}$-Biotin and DSPE-PEG$_{2000}$-Biotin

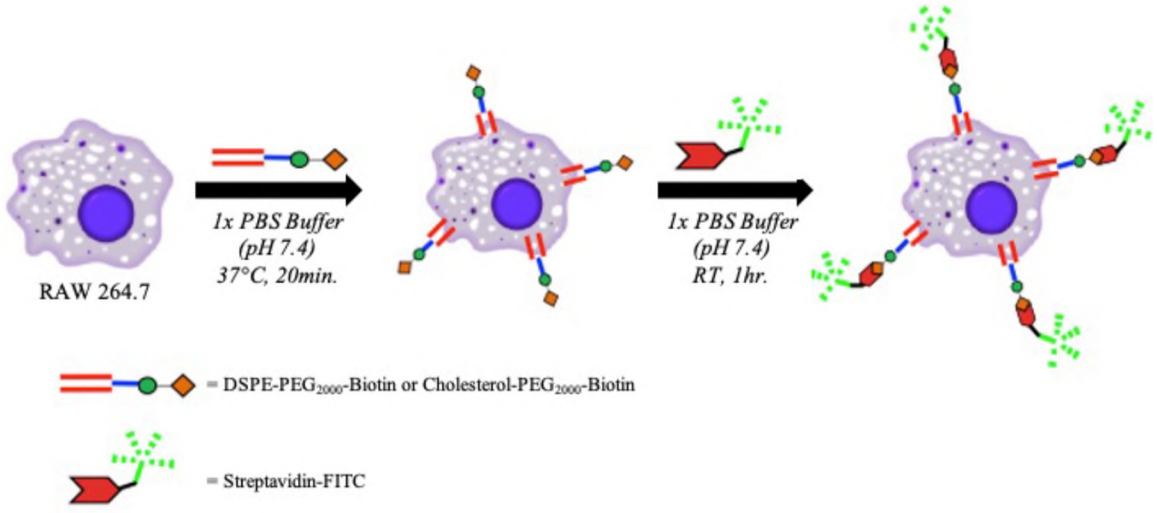

Figure 3.2: Research design of studying different anchoring lipids for cell surface re-engineering applications

3.2 Materials and Methods

3.2.1 Materials

All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Deionized water was used as a solvent in all procedures. DSPE-PEG_{2000}-Biotin and Cholesterol-PEG_{2000}-Biotin were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Streptavidin-fluorescein isothiocyanate (Streptavidin-FITC) were purchased from Biolegend (San Diego, CA). Dulbecco's modified Eagle medium (DMEM) medium and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Life Technologies (Grand Island, NY).

3.2.2 Cell culture

The mouse macrophage cell line RAW264.7 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37 °C in 5% CO_2 conditions. Upon reaching confluency, cells were subcultured by trypsinization with (0.25% trypsin, 0.02% EDTA) for 5min. at 37 °C.

3.2.3 Cell Surface Re-engineering of RAW 264.7 cells with Cholesterol-PEG_{2000}-Biotin and DSPE-PEG_{2000}-Biotin anchor lipids

RAW 264.7 cells were washed three times and seeded into 6-well plates, where each well contained a glass coverslip, and allowed to incubate overnight to allow cells to adhere to the coverslips. Medium was aspirated off and cells were gently washed three times with cold 1x PBS buffer (pH 7.4). Cells were then incubated with a 5, 10, 15, 20, 25 or 30μM Cholesterol-PEG_{2000}-Biotin or a 10, 20, 30, 50, 75 or 100μM DSPE-PEG_{2000}-Biotin solution. The lipid anchors were dissolved in a 1x PBS buffer (pH 7.4), 0.05%

DMSO solution prior to addition. The cells were allowed to incubate for 20min. at 37°C. The lipid anchor solutions were then aspirated off and the cells were washed twice with 1x PBS buffer (pH 7.4). Cells were then fixed with a 4% para-formaldehyde solution and incubated for 10min, at room temperature and in the dark. After the fixing process, cells were washed twice with 1x PBS buffer and treated with 300μL of a streptavidin-FITC solution (0.625μg/mL in 1x PBS) and incubated for 1hr. at room temperature in the dark. The streptavidin solution was then removed from the coverslips were washed by completely submerging them in 1x PBS buffer (pH 7.4) for 5min, twice. Next, cells were incubated with 300μL of a 300nM DAPI solution for 10min. at room temperature in the dark. After incubation, the DAPI solution was removed and the coverslips were washed by submersion in 1x PBS buffer (pH 7.4) for 5min., three times. Lastly, 30μL of ProLong Gold Antifade reagent was added to the cell side of each coverslip and mounted onto microscope slides. The slides were allowed to cure overnight at room temperature prior to analysis by confocal microscopy. The slides were imaged using a Nikon A1RSI Confocal Microscope with a 60x oil objective.

3.2.4 Retention time of biotinylated lipid conjugate (CHOL-PEG$_{2000}$-Biotin) on the cell membrane

RAW 264.7 cells were seeded into 6-well plates with each well containing a glass coverslip. The cells were then left to incubate overnight to allow for attachment to the coverslip. After the overnight incubation, the cell medium was aspirated off and the cells were washed twice with 1x PBS buffer (pH 7.4). Next, cells were incubated with either a 2.5, 5, or 10μM cholesterol-PEG$_{2000}$-biotin solution (total volume 1 mL) for 30min. at

37°C. Control cells were incubated with 1x PBS buffer (pH 7.4) for 30 min. at 37°C. The treatment solution was then aspirated off and the cells were washed twice with 1x PBS buffer (pH 7.4). Fresh DMEM medium was added to each well and cells were placed back into the incubator. After a set time point, cells were removed from incubation, washed, and fixed. Time points were 0, 0.5, 1, 2, and 4hrs. from the removal of the cholesterol solution. Control cells were taken out with time 0hr. cells. When a time point was reached, media was aspirated from the cells and then the cells were washed twice with 1x PBS buffer (pH 7.4). Cells were then fixed with a 4% para-formaldehyde solution for 10min. at room temperature and then washed twice with 1x PBS buffer (pH 7.4). Next, 300µL of a 0.625µg/mL streptavidin-FITC solution was added to each coverslip and let set for 1hr. at room temperature. After streptavidin incubation, coverslips were washed by submerging the coverslips in 1x PBS buffer (pH 7.4) for 5 min. and repeated two times. After washing, 300µL of 300nM DAPI solution was added to each coverslip and let sit for 10min. at room temperature. After incubation, coverslips were washed by submerging the coverslips in 1x PBS buffer (pH 7.4) for 5min., this was repeated three times. Coverslips were mounted to slides by adding 30µL of ProLong™ Gold Anti-fade reagent to each coverslip and cured at room temperature overnight. The slides were imaged using a Nikon A1RSI Confocal Microscope with a 60x oil objective.

3.3 Results and Discussion

This study demonstrated a simple, fast and efficient method to modify the surface of RAW 264.7 cells using free lipid anchors. The different abilities of DSPE and Cholesterol based anchors to insert themselves into the cell membrane were demonstrated.

It was also shown how long the Cholesterol-PEG_{2000}-Biotin anchor could reside on the cell surface of RAW 264.7 macrophages, giving insight to how long a therapeutic cargo could last on the membrane using this method of introduction.

3.3.1 Cell Surface Re-engineering of RAW 264.7 cells with Cholesterol-PEG_{2000}-Biotin and DSPE-PEG_{2000}-Biotin anchor lipids

Confirmation of the different incorporation efficiencies of Cholesterol and DSPE-based anchors was further proven in this study. Using confocal microscopy, Cholesterol-PEG_{2000}-Biotin treated cells labeled with streptavidin-FITC (Figure 3.3) showed greater fluorescent intensities compared to their DSPE counterpart (Figure 3.4). This is in agreement with our group's previous findings. Cells treated with 5μM of the cholesterol anchor solution showed the highest incorporation into the membrane of RAW 264.7 macrophages. Concentrations 10-25μM showed similar fluorescent intensities to each other and were only slightly less bright. Despite this, the cells treated at these concentrations still showed good anchor insertion. However, when the treatment was 30μM, there was noticeable decrease in fluorescence intensity.

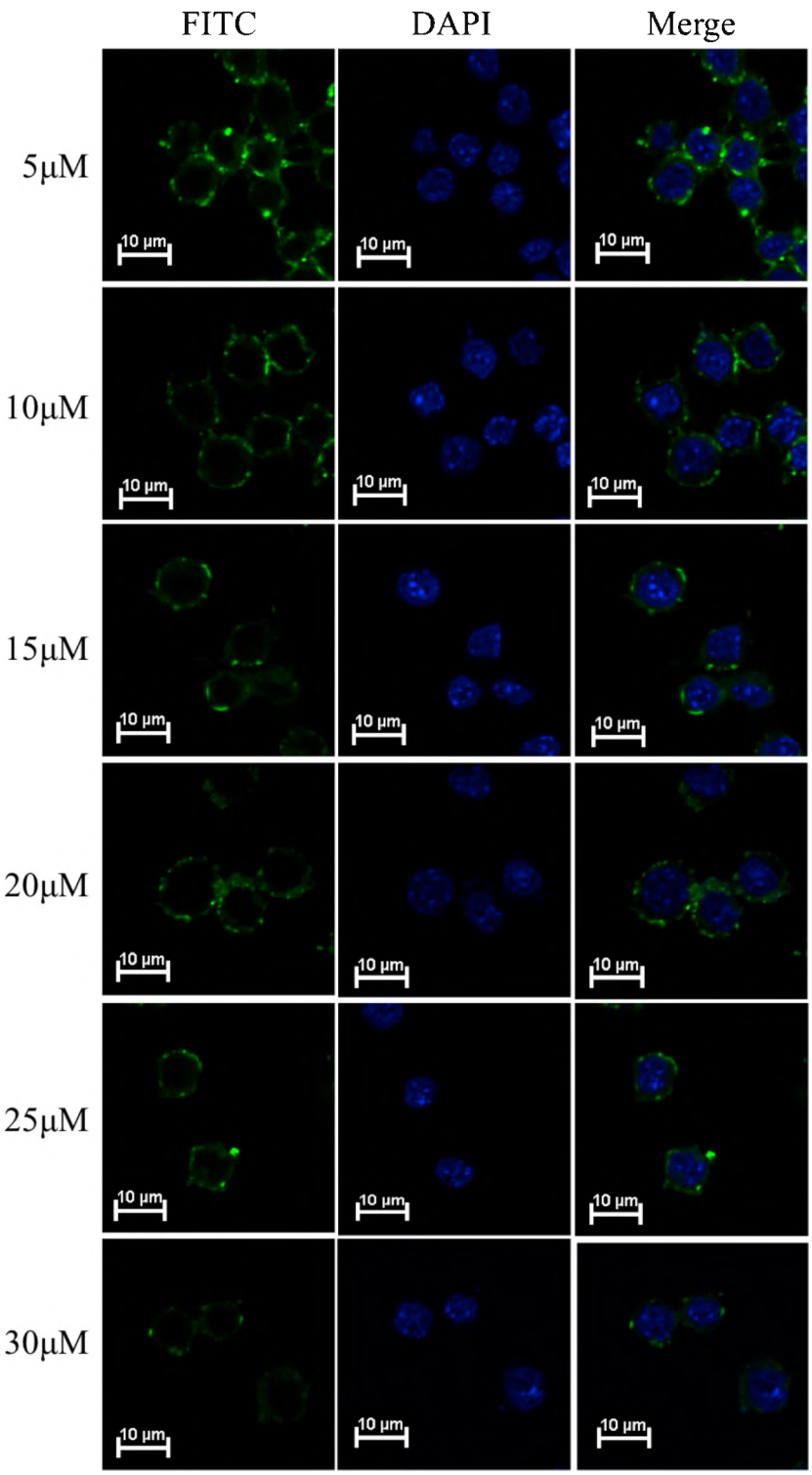

Figure 3.3: Confocal Microscopy images of RAW 264.7 cells treated with different concentrations of Cholesterol-PEG$_{2000}$-Biotin

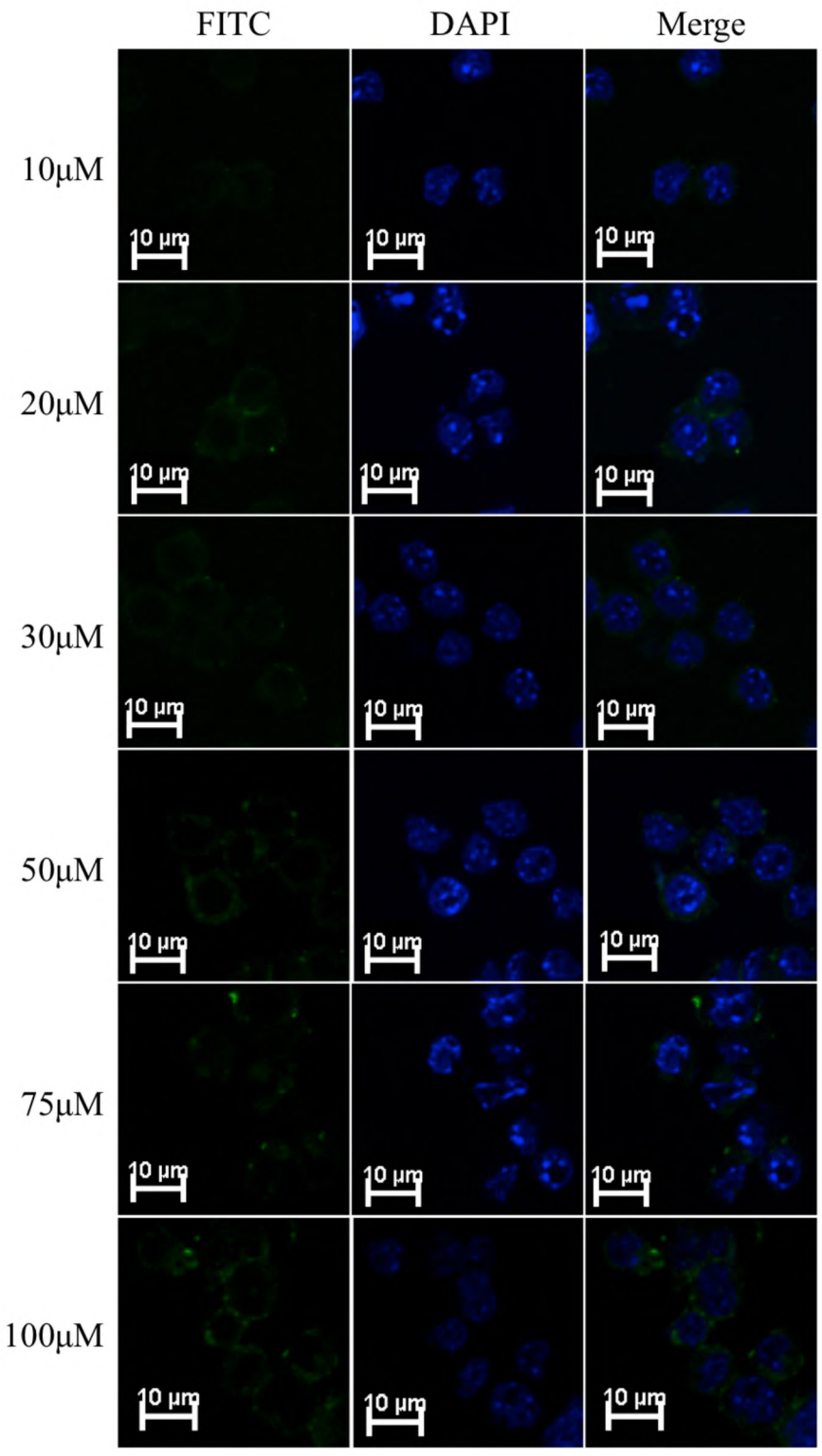

Figure 3.4: Confocal Microscopy images of RAW 264.7 cells treated with different concentrations of DSPE-PEG$_{2000}$-Biotin

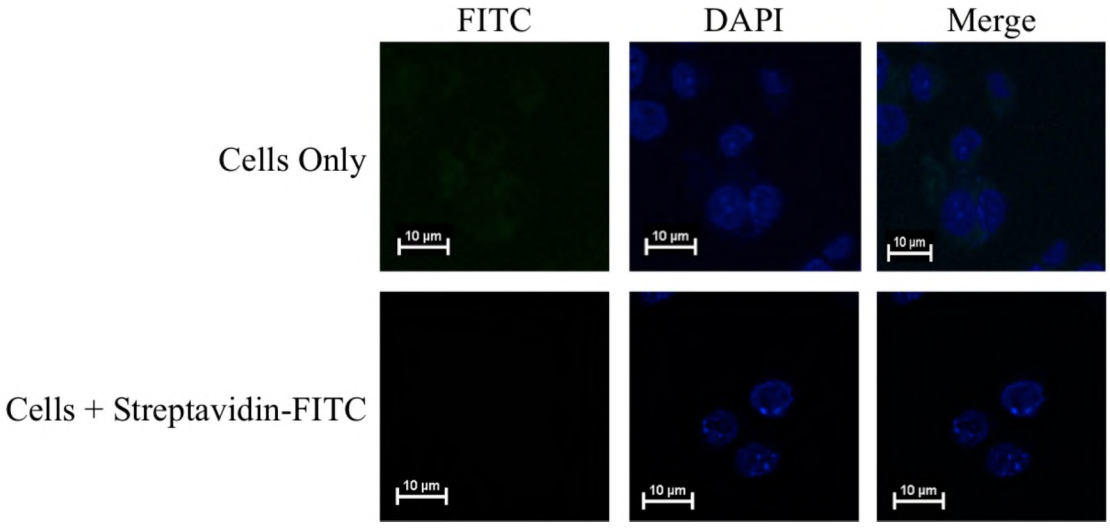

Figure 3.5: Confocal Microscopy images of untreated RAW 264.7 cells and cells treated with streptavidin-FITC only

3.3.2 Retention time of biotinylated lipid conjugate (CHOL-PEG_{2000}-biotin) on the cell membrane

In order for lipid anchors to be good molecules for cell surface modifications, they must reside on the cell surface long enough for the introduced molecule of interest to exert its function. Since the cholesterol-based anchor afforded much higher incorporation efficiency over DSPE, only the cholesterol anchor was looked at for this experiment. RAW 264.7 cells were treated with various concentrations (2.5, 5 and 10μM) of free cholesterol-PEG_{2000}-biotin anchor. Cells were then probed with a FITC labeled streptavidin molecule and analyzed by confocal microscopy (Figure 3.6 and Figure 3.7). Interestingly, the kinetic profile 5 and 10μM treatments show a sharp jump from the time 0hr. measurement and the 0.5hr. measurement before a straightforward decrease in fluorescence intensity (Figure 3.8). There are many phenomena that could explain this observation. Two of these causes center around the use of streptavidin as the probe. The first of these is that streptavidin can bind up to four biotin molecules. At high concentrations of lipid anchor on the cell surface, streptavidin could bind multiple biotin moieties so that the there is not a 1:1 ratio of streptavidin to biotinylated lipid anchor. As the anchors leave the cell surface, the anchors still present may be able to spread out enough to allow for more of a true 1:1 binding of streptavidin to biotin. The second of these phenomena is that the binding of streptavidin could block incoming streptavidin molecules from binding to free biotin on the anchors. Streptavidin is a large molecule and can sterically hinder other molecules from binding. This situation is also relieved when the surface concentration of lipid anchor is reduced. The last situation that could cause the spike in fluorescence intensity is fluorescence quenching. To test whether the above-mentioned signal jump is concentration based, we

tested a low concentration of the lipid anchor (2.5μM). Predictively, the decay in fluorescence intensity at this treatment concentration is straightforward, with no spike in concentration in the early time points. Overall it was found that there is a concentration dependent rate loss of anchors from the cell membrane and the retention half-life of the Cholesterol-PEG_{2000} for residing on the cell surface of RAW 264.7 cells is 1hr.

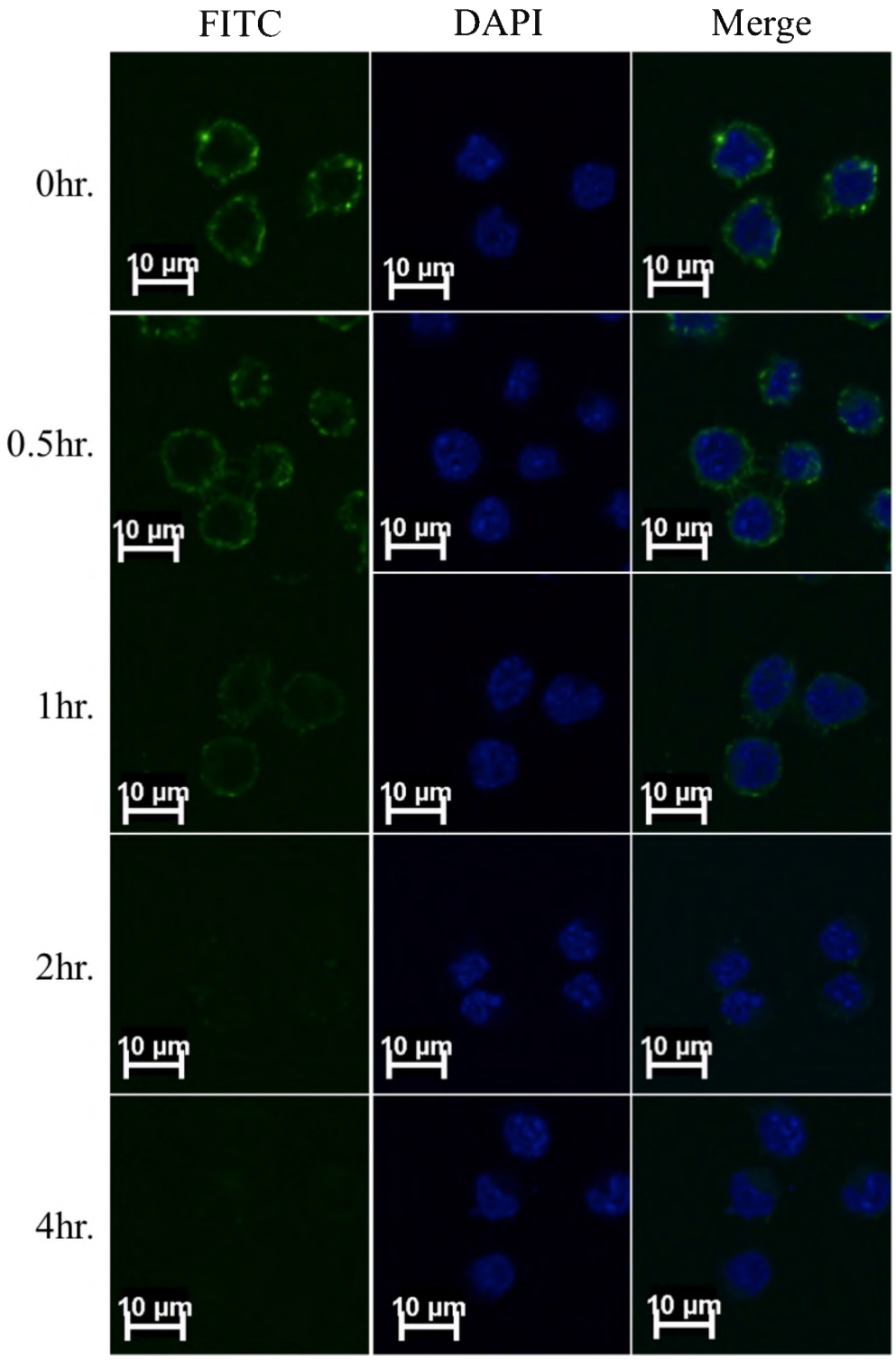

Figure 3.6: Confocal microscopy images of RAW 264.7 cells treated with 2.5µM Cholesterol-PEG$_{2000}$-Biotin and their fluorescence decay overtime

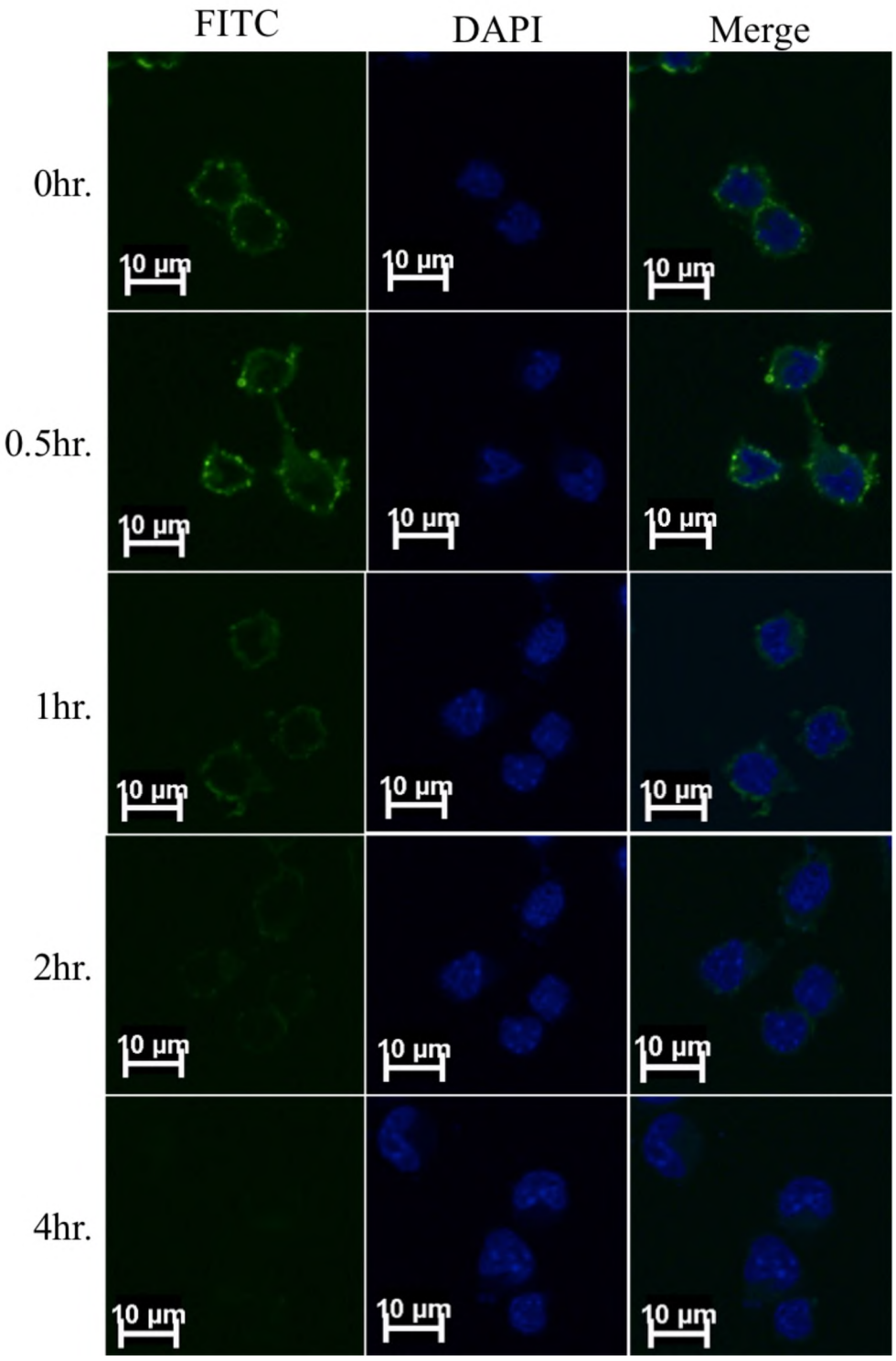

Figure 3.7: Confocal microscopy images of RAW 264.7 cells treated with 5µM Cholesterol-PEG$_{2000}$-Biotin and their fluorescence decay overtime

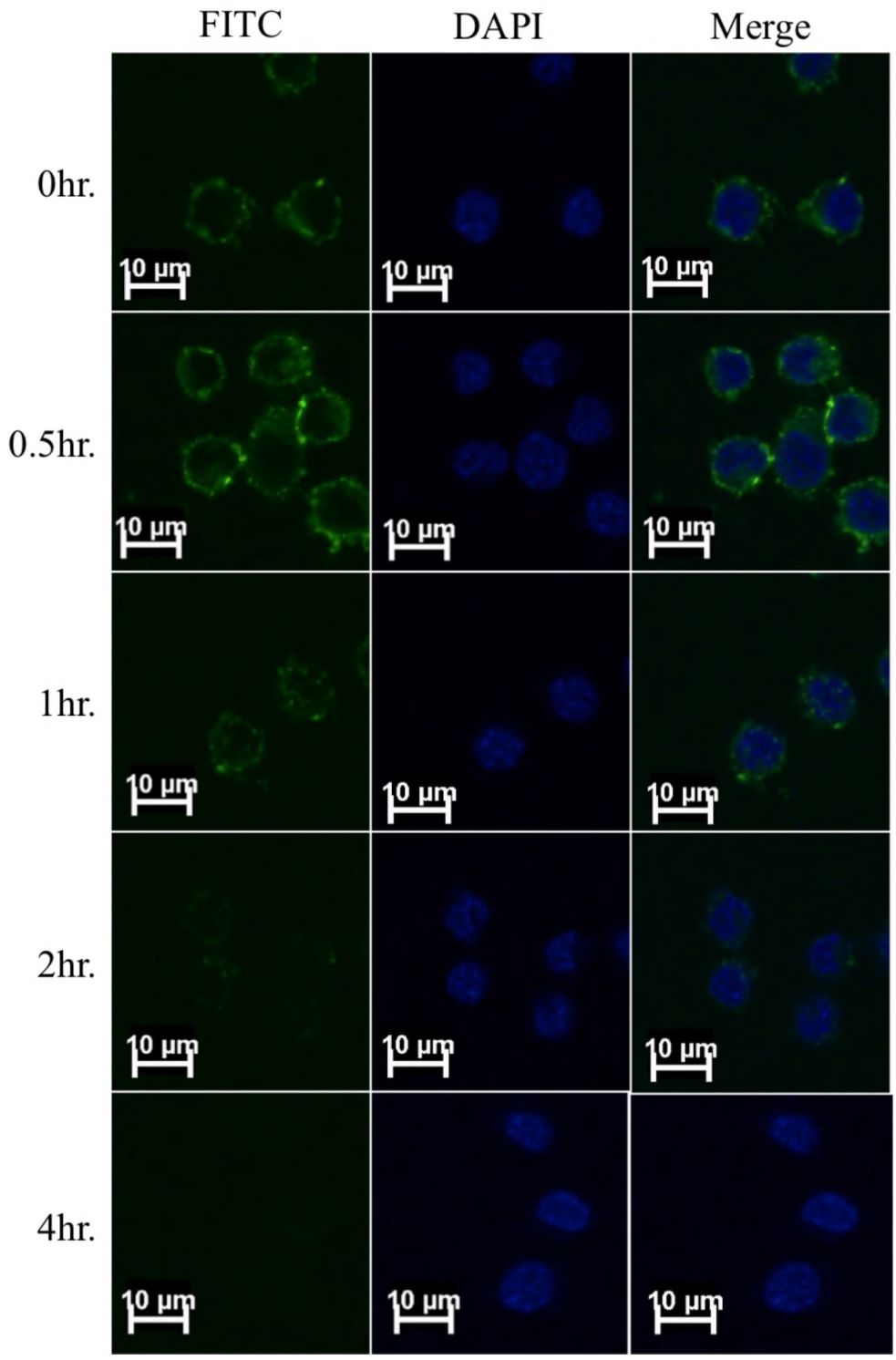

Figure 3.8: Confocal microscopy images of RAW 264.7 cells treated with 10µM Cholesterol-PEG$_{2000}$-Biotin and their fluorescence decay overtime

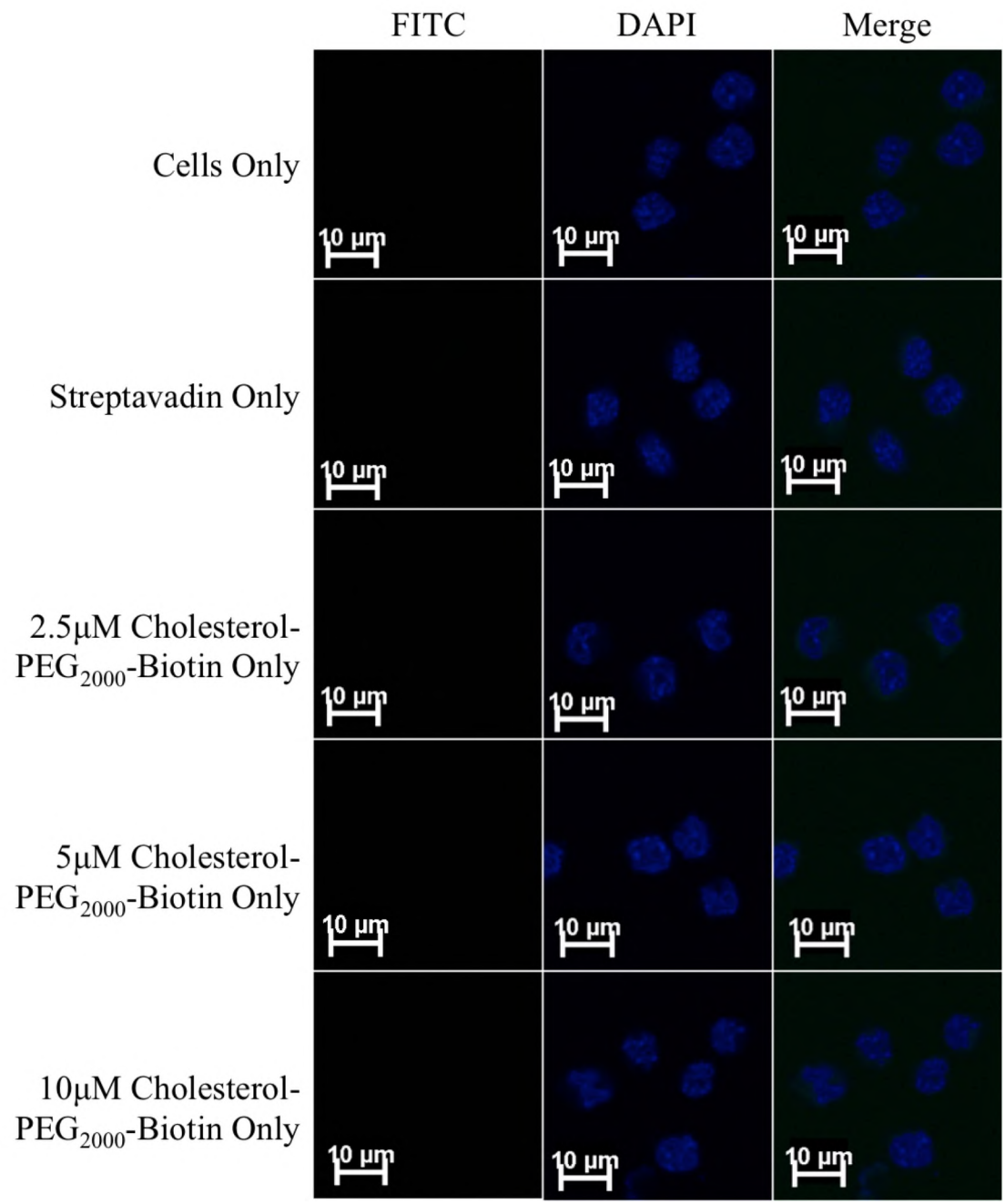

Figure 3.9: Confocal Microscopy images of untreated RAW 264.7 cells, cells treated with streptavidin-FITC only and cells only treated with varying concentrations of Cholesterol-PEG_{2000}-Biotin

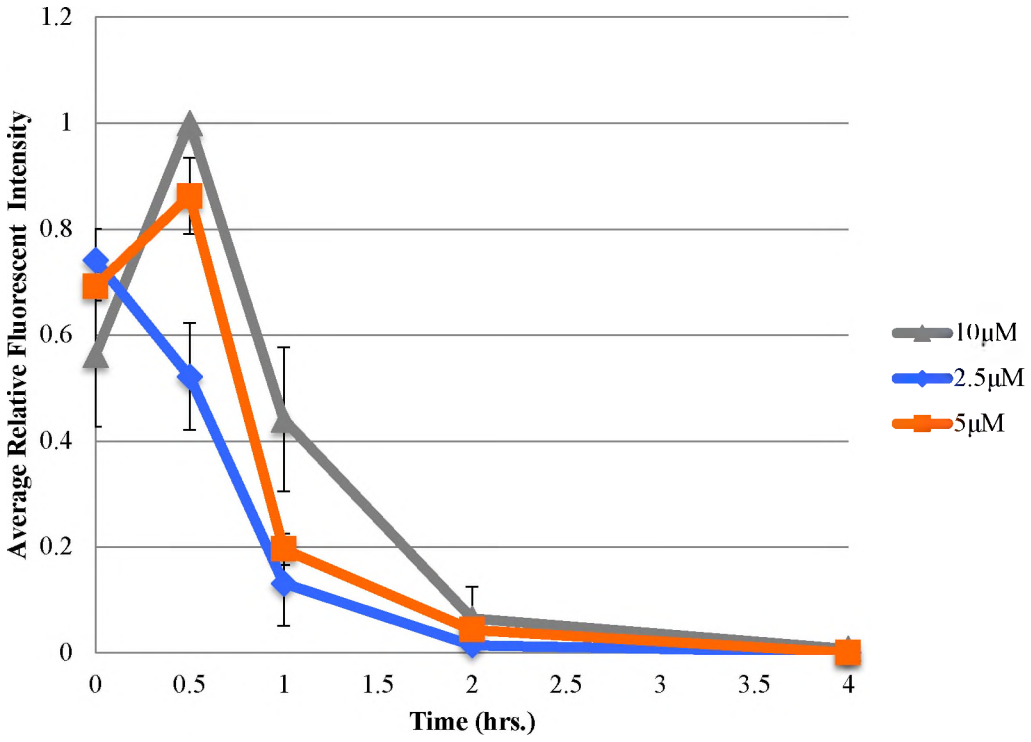

Figure 3.10: Retention time of directly anchored biotinylated cholesterol lipid onto cell surface of RAW 264.7 cells at different concentrations (2.5, 5 and 10 µM) in 1x PBS (pH 7.4) at room temperature. Error bars represent standard deviation, n = 3.

There are some pitfalls that face this current project. For example, it needs to be investigated if the introduction of the anchor lipid to the macrophage cell membrane leads to their activation. Activated macrophages can release cytokines into their surroundings, which could cause unwanted side effects. It is ideal for the modified macrophages to remain in the M0 state.

3.4 Conclusion

In this study, a fast and efficient method for modifying the cell membrane using DSPE and cholesterol-based anchor lipids was demonstrated. Confocal microscopy findings indicated the successful anchoring of cholesterol-PEG_{2000}-biotin and DSPE-PEG_{2000}-biotin into the cell membrane of RAW 264.7 macrophages. However, the cholesterol-based anchor afforded a much more efficient anchoring compared to the DSPE anchor. It was also observed that negligible amounts of the anchor were internalized into the cell. The difference in the incorporation efficiencies between the two types of anchors is most likely due to their structural differences. Cholesterol is uncharged, small and slightly rigid while DSPE is a phospholipid that carries a charge and its lipid chains are more flexible compared to the ring structure of cholesterol. The negative charge of the phosphate head of DSPE can cause repulsion between it and the overall negative charge of the cell membrane due to the phospholipids already present in the membrane. Also, the flexibility of the fatty chains may make it difficult for them to insert into the membrane. Cholesterol on the other hand has no charge, so it does not have to overcome issues relating to charges and is small so it may have an easier time of inserting into the membrane. In addition to incorporation efficiency, membrane residence time of cholesterol was

determined. For higher concentrations of cholesterol, a jump in fluorescence intensity was seen between 0hr. and 0.5hr. timepoints and then a normal decay trend is seen. This issue was resolved when using a lower concentration. This difference was attributed to the use of streptavidin as the probing molecule or fluorescent quenching. These findings combined with those made previously in our lab show the potential of using cholesterol-based anchors for cell surface re-engineering applications.

3.5 References

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CHAPTER IV

CELL SURFACE RE-ENGINEERING OF MACROPHAGES WITH RECOMBINANT THROMBOMODULIN USING LIPID FUSION

4.1 Introduction

Due to its role in a variety of biological processes, thrombomodulin (TM) has been proposed as a possible therapeutic protein. TM has many beneficial activities that are exerted by the protein itself and through the activation of other cofactors such as protein C. Direct activities of TM include acting as an anticoagulant by its ability to bind thrombin, a procoagulant enzyme in its unbound form, and anti-inflammatory activities through its lectin-like domain. TM's EGF-like domain binds thrombin and blocks thrombin's exosite needed to inactivate certain coagulation factors.[1–3] The lectin-like domain of TM protects against inflammation by interfering with complement activation, neutralizing lipopolysaccharide (LPS) and binding to high-mobility group box 1 protein (HMGB1) and promoting its degradation.[4–6] LPS is a pro-inflammatory stimulus displayed on the surface of Gram-positive bacteria and HMGB1 is a inducer of systemic inflammation.[7–9] Thus blocking these molecules can lead to anti-inflammatory effects. TM can exert beneficial

functions through indirect actions too, mainly by its ability to activate protein C. Activated protein C (APC) can inactivate procoagulant factors and block inflammatory cytokine production.[10–12] It is through these activities that TM can act as a possible therapeutic.

Recombinant forms of TM have been developed as possible therapeutics. These forms of TM are often a combination of the first three domains of TM: the lectin-like domain, the EGF-like domain and the serine/threonine-rich domain.[13,14] Since these forms lack the transmembrane domain, they are more soluble in aqueous environments and are often called soluble thrombomodulin (sTM). So far, sTM has shown positive outcomes for the treatment of disseminated intravascular coagulation, preeclampsia and acute respiratory distress syndrome.[15–17] There is currently one sTM therapeutic approved in Japan, ART-123 or Recomodulin ®, and is approved for the treatment of disseminated intravascular coagulation (DIC).[18]

Using proteins as therapeutics has promising implications but they do suffer from some drawbacks. The main issue of protein therapeutics is their stability in the human body. Protein drugs can undergo rapid metabolism and be cleared by the body before they can execute any therapeutic activity.[19] Degradation of proteins is also an issue. Some proteins can become unstable when removed from their natural environment and the high amount of proteolytic enzymes in the blood can also degrade unprotected proteins.[20,21] To overcome these issues, researchers have started to modify or formulate these proteins differently to help with stability issues. These strategies include PEGylation, polymer encapsulation and by adding the protein to a support.[22–24] These supports include liposomes, nanoparticles and cells.[25–27]

Macrophages have caught the attention of researchers as a drug carrier due to its role in a wide variety of biological processes. One key feature of macrophages is their ability to migrate to specific locations in the body. Cells undergoing damage or stress can release chemical signals that attract cells, known as chemokines, to the site of disturbance.[28,29] Inflammation is a common trigger for the release of chemokines. Among these chemokines are a few specific for attracting macrophages, including macrophage migration inhibitory factor and macrophage chemotactic protein-1.[30,31] This ability of being able to be directed to sites of inflammation makes macrophages a potentially useful carrier for anti-inflammatory drugs.[32] Also, since inflammation accompanies a large variety of primary disease conditions, macrophages could be used to deliver therapeutics to resolve the cause of inflammation.

Macrophages are an ideal candidate for cell re-engineering with recombinant TM (rTM). As mentioned above, rTM can act as an anti-inflammatory agent and macrophages can be directed to sites of inflammation. Re-engineering the surface of macrophages with rTM can allow for a delivery system to bring rTM to sites of inflammation. Macrophages have been demonstrated to be useful platforms for cell-based therapies so this extension could be lucrative. Also, studies in Chapter II demonstrated that THP-1 macrophages express less surface TM than their monocyte precursor. Thus, it may be beneficial to re-introduce TM to the surface of macrophages to help with possible loss of function due to the decrease in expression.

Our lab has developed a recombinant form of TM that expresses EGF-like subdomains 4-6 (rTM_{456}). This protein also expresses and N-terminal FLAG tag that is used for identification proposes. At the C-terminus, a LPETG motif was incorporated for

ligation with sortase A reactions and a His_6 tag for purification use.[33] Previous research in our lab has shown the ability to introduce an azide group at the C-terminus of rTM_{456} using a sortase A reaction. This added azide can then be targeted for copper free click chemistry for additional modification to the protein.[34] With this azide group at the C-terminus of rTM_{456} a liposomal formulation was prepared, which showed increased stability of the protein while maintaining its activity.[35,36]

This study aimed to expand the previous work by modifying rTM_{456} with a cholesterol anchor to re-engineer the surface of RAW 264.7 macrophages (Figure 4.1). rTM_{456} was expressed and an azide functional group was incorporated to the C-terminus using a sortase A ligation strategy. The introduction of the azide group allows for subsequent modification with a DBCO-functionalized cholesterol anchor using copper free click chemistry. The successful conjugation and characterization of rTM_{456} resulted in rTM_{456}-PEG_{2000}-Cholesterol. Successful incorporation of the conjugate into the surface of RAW 264.7 cells was confirmed by confocal microscopy using an AlexaFL-488 conjugated anti-FLAG antibody to tag incorporated rTM-conjugate. The incorporation of rTM_{456}-PEG_{2000}-Cholesterol also showed little toxicity toward the decorated cells as shown through MTT assay measurements.

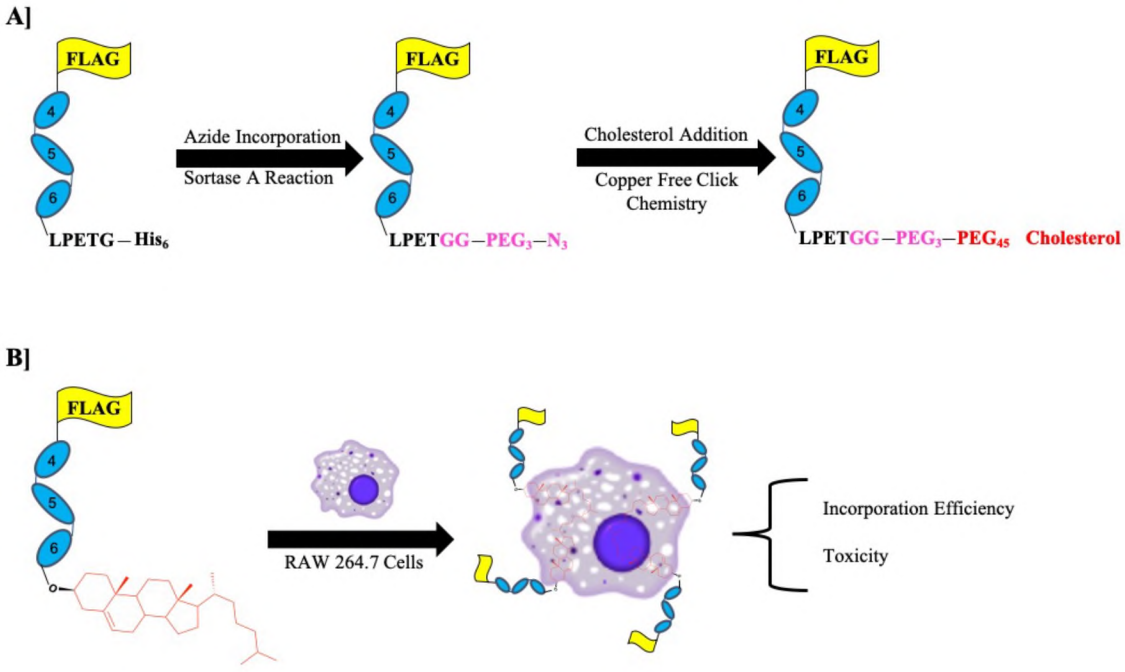

Figure 4.1: Overall research design of cell surface re-engineering of macrophages with cholesterol anchored rTM$_{456}$

4.2 Materials and Methods

4.2.1 Materials

All reagents and materials were used as received from commercial sources unless otherwise noted. Cholesterol-PEG_{2000}-DBCO was purchased from Avanti Polar Lipids (Alabaster, AL). DAPI, Lennox Broth medium, kanamycin sulfate, isopropyl β-D-thiogalactopyranoside (IPTG) and DBCO-Cy5 were purchased from Sigma-Aldrich (St. Louis, MO). The mouse monoclonal antibody against human TM and the goat antibody against mouse IgG conjugated to horseradish peroxidase (HRP) were purchased from Abcam (Cambridge, MA). DMEM medium, fetal bovine serum, penicillin/streptomycin solution, and the mouse monoclonal antibody against FLAG-tag conjugated with AlexaFL-488 were purchased from VWR (Ragnor, PA). DBCO-PEG_3-azide was purchased from Click Chemistry Tools (Scottsdale, AZ). Para-formaldehyde was purchased from Electron Microscopy Sciences (Hatfield, PA). ProLong™ Gold Antifade Reagent, Nickel-NTA agarose, methyl-thiazolyl-tetrasolium (MTT) and SuperSignal™ West Pico PLUS Chemiluminescent Substrate kit rtrs purchased from ThermoFisher Scientific (Waltham, MA).

4.2.2 Cell Culture

The mouse macrophage cell line RAW 264.7 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37 °C in 5% CO_2 conditions. Upon reaching confluency, cells were subcultured by trypsinization with (0.25% trypsin, 0.02% EDTA) for 5min. at 37°C.

4.2.3 Expression, Purification and Characterization of FLAG-rTM_{456}

30μL of *E. coli* B834 (DE3) cells that have been transfected with the expression plasmid for FLAG-TM_{456}, pET28B-FLAG-TM3, (Figure 4.2) were added to 25mL of LB medium with 35mg/L kanamycin and incubated overnight at 37°C with shaking. The overnight culture was then added to 500mL of LB medium with 35m/mL kanamycin sulfate and incubated until the solution an OD_{600} of 0.6 was reached. Induction was then initiated by adding IPTG to a final concentration of 1mM. The bacteria were allowed to incubate for 5hrs. at 37°C with shaking to allow for the overexpression of protein. After incubation, bacteria were pelleted down and the pellet was stored at -80°C for further use.

For purification, cell pellets were resuspended in 40mL of cold lysis buffer (20mM Tris, 150mM NaCl, pH 8.0) containing phenylmethylsulfonyl fluoride (PMSF). Cells were lysed by sonication. The lysed bacterial solution was then centrifuged at 20,000rpm at 4°C for 10min. The resulting supernatant was then loaded into a column packed with Nickel-NTA Agarose. The column was washed with lysis buffer, lysis buffer with 50mM imidazole and lysis buffer with 100mM imidazole. FLAG-TM_{456} was eluted off the column using a 250mM imidazole buffer. Pooled fractions were then concentrated and dialyzed against lysis buffer. The concentration of protein was determined by Bradford assay.

Characterization of the purified protein was accomplished by gel electrophoresis followed by either Coomassie staining or western blotting. For western blotting, membranes were blocked for 1hr. with 5% milk in TBST buffer at room temperature. Membranes were then incubated with mouse anti-human thrombomodulin antibody (1:1000 dilution in 5% milk in TBST buffer) overnight at 4°C. Next, membranes were washed with TBST buffer for 10min, three times. After washing, the membranes were treated with goat anti-mouse IgG HRP antibody (1:1000 dilution in 5% milk in TBST

buffer) for 1hr. at room temperature. After the secondary antibody incubation, the membrane was washed like described earlier. The membrane was developed using the SuperSignal™ West Pico PLUS Chemiluminescent Substrate kit.

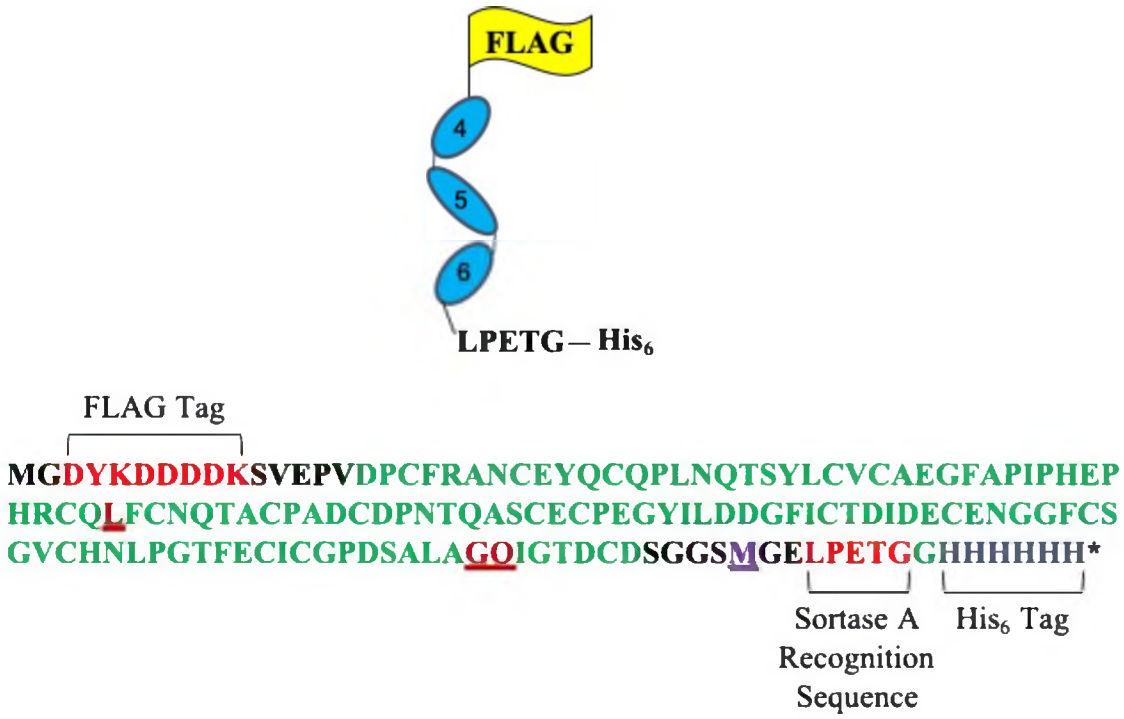

Figure 4.2: Structure and amino acid sequence of rTM_{456}

4.2.4 Expression and Purification of Sortase A

A recombinant form of Sortase A (residues 60-206) containing a C-terminal His_6-tag was expressed from *E. coli* BL21 containing the pET28b-SrtA plasmid. 30μL of stock *E. coli* cells was added to 25mL of LB medium with 35mg/L kanamycin and incubated overnight at 37°C with shaking. After overnight incubation, the overnight culture was transferred to 500mL of LB medium with 35m/mL kanamycin and incubated until the solution an OD_{600} of 0.6 was reached. Induction was then initiated by adding IPTG to a final concentration of 1mM and the bacteria was allowed to incubate for 5hrs. at 37°C with shaking. After incubation, bacteria were pelleted down and the pellet was stored at -80°C for further use.

To purify sortase A, the previously obtained cell pellets were resuspended in 40mL of cold lysis buffer (20mM Tris, 150mM NaCl, pH 8.0) containing PMSF. Cells were then lysed by sonication then centrifuged at 20,000rpm at 4°C for 10min. The supernatant was then loaded into a Nickel-NTA agarose column. Unbound protein was washed off with lysis buffer, lysis buffer with 50mM imidazole and then lysis buffer with 100mM imidazole. Sortase A was eluted off the column using a 250mM imidazole buffer. Pooled fractions were then concentrated and dialyzed against lysis buffer. The concentration of protein was determined by Bradford assay.

4.2.5 Synthesis, Characterization and Sortase A ligation of rTM_{456} to Produce rTM_{456}-azide

To a 2mL tube, 800uL of 0.6mg/mL rTM_{456} solution, 100uL of 0.6mg/mL Sortase A solution, 75μL of 200mM $CaCl_2$ solution, 20μL of 8mg/mL Gly_2-PEG_4-azide and 505μL of reaction buffer (20mM Tris, 150mM NaCl, pH 8.0) was added. The solution was

incubated for 2hrs. at 37°C. Successfully conjugated rTM-azide was purified from the reaction mixture by collecting the flow through fraction of a HisTrap column. Excess Gly_2-PEG_4-azide was removed from the purified solution by using a centrifugal filter with a molecular weight cutoff of 10kDa. The concentration of the pure rTM-azide was determined by Bradford Assay.

The success of the reaction was determined by incubating the purified rTM-azide with a DBCO-conjugated dye followed by gel electrophoresis. Briefly, 10μg of rTM-azide was incubated with 10x molar concentration of DBCO-Cy5 dye for 1hr. in the dark. Samples were then run on a 15% SDS-PAGE Gel and scanned using a fluorescent scanner (Amersham Typhoon, General Electric). The gel was then stained with Coomassie blue.

4.2.6 Synthesis and Characterization of Cholesterol Addition to rTM_{456}-azide

To a glass vial, rTM_{456}-azide solution was incubated with 10 molar excess of Cholesterol-PEG_{2000}-DBCO overnight at room temperature. The reaction solution was then filtered with a 10kDa molecular weight cutoff centrifugal filter to remove unreacted Cholesterol-PEG_{2000}-DBCO. The concentration of rTM-Cholesterol was determined by Bradford Assay. The solutions concentration was then adjusted to 5μM protein. The success of the reaction was determined by SDS-PAGE followed by Coomassie Staining.

4.2.7 Determination of Anchoring Efficiency of rTM_{456}-PEG_{2000}-Cholesterol onto RAW 264.7 Macrophages Using Confocal Microscopy

RAW 264.7 cells were seeded into 6-well plates, where each well contained a glass coverslip, at a density of 15,000cells/well, and incubated overnight to allow the cells to adhere to the coverslip. After overnight incubation, old culture medium was removed and

the cells were washed three times with 1x PBS (pH: 7.4). Cells were then treated with 5μM rTM_{456}-PEG_{2000}-Cholesterol or 1x PBS (total volume 1mL) for control wells for 30min. in the cell incubator. The solutions were then aspirated off the cells were washed three times with 1x PBS. Cells were then fixed with a 4% para-formaldehyde solution for 10min. at room temperature. After fixing, cells were washed three times with 1x PBS. Coverslips were then removed from the wells and moved to racks. For fluorescent detection, cells were treated with 300μL of a mouse anti-FLAG antibody conjugated with FITC (1:1000 dilution in 1x PBS) and incubated for 1hr. at room temperature, in the dark. Coverslips were washed three times by completely submerging them into cold 1xPBS buffer for 5min. Next, 300μL of a 300nM DAPI solution was added to each coverslip. Cells were then incubated for 10min. at room temperature. The coverslips were then washed three times by submerging them in cold 1x PBS buffer for 5min. Microscope slides were then prepared by adding 30μL of Prolong™ Gold Anti-fade reagent to each coverslip and mounting the coverslip to a glass microscope slide. Slides were allowed to cure overnight at room temperature in the dark. The slides were imaged using a Nikon A1RSI Confocal Microscope with a 60x oil objective.

4.2.8 MTT Assay

RAW 264.7 cells were seeded into 96-well plates at a density of $2.5x10^4$ cells per well and incubated for 24hrs. The next day, old medium was removed and cells were gently washed with 1x PBS twice. Cells were then incubated with either serum-free phenol red-free DMEM medium, PBS or 5μM rTM_{456}-Cholesterol (total volume 100μL) for 20min. in the cell incubator. The treatment solutions were then removed and the cells were washed twice with 1x PBS. To each well, 100μL of serum-free phenol red-free medium and 10μL

of 5mg/mL MTT solution was added. Cells were then placed back in the incubator and allowed to incubate for 4hrs. After incubation, 100μL of DMSO was added to each well to solubilize the formed MTT crystals and the plate was read at 570nm using a multiplate reader.

4.3 Results and Discussion

4.3.1 rTM_{456} Expression and Characterization

A recombinant form of TM containing EGF-like domains 4-6 was expressed using *E. coli* as an expression host. This recombinant TM, rTM_{456}, also contains a FLAG tag at its N-terminus that will be later used for tagging. It also contains a LPETG motif and a His_6 tag at the C-terminal end. The LPETG motif can be recognized by sortase A and used to introduce different modifications to the protein, in this study this ligation technique was used to introduce an azide functional group. The His_6 tag was used for purification purposes using a nickel-NTA column. After expression and purification, fractions were run by SDS-PAGE then analyzed by Coomassie Blue staining and western blot. Coomassie Blue staining shows strong bands around 25kDa in elution fractions 1-5 (Figure 4.3). Only rTM_{456} should be eluted in these fractions so seeing bands here is a first indication of successful expression and purification of rTM_{456}. To further validate that this band is for rTM_{456}, western blot analysis using an antibody against TM was employed (Figure 4.4). Positive staining seen in the elution lanes proves that the bands seen in the Coomassie Blue stained gel is TM.

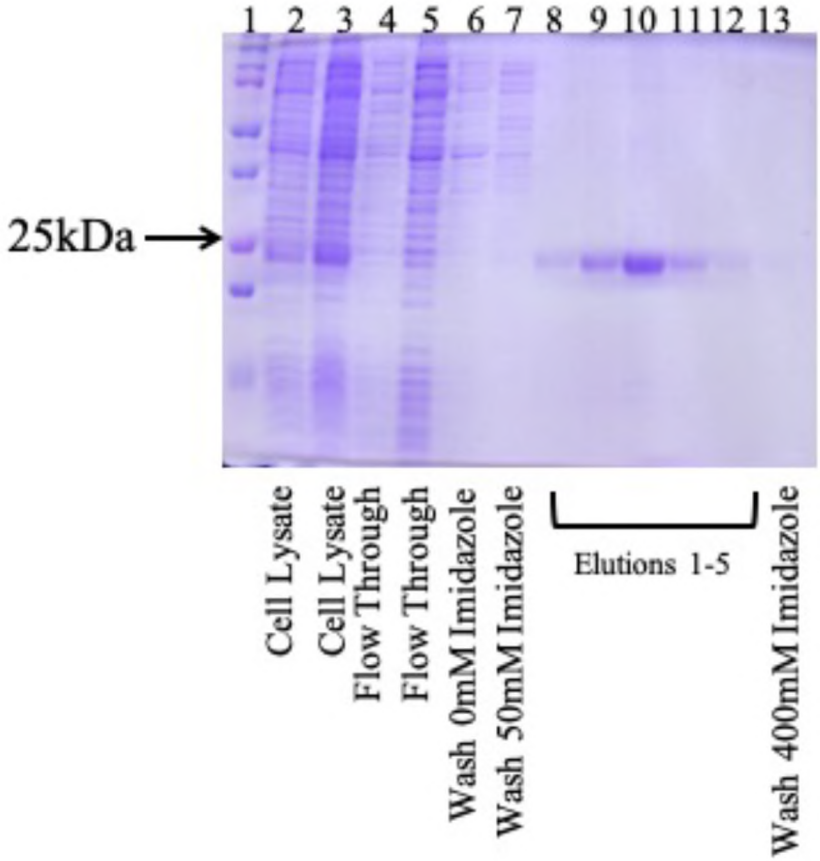

Figure 4.3: Coomassie Blue stained SDS-PAGE gel of rTM_{456} expression products. Lane 1, molecular weight ladder; lanes 2 & 3, *E. coli* cell lysate supernatant; lanes, 4 & 5, flow through fraction; lane 6, 0mM imidazole wash fraction; lane 7, 50mM imidazole wash fraction; lanes 8-12, 250mM elution fractions; lane 13, 400mM imidazole wash fraction

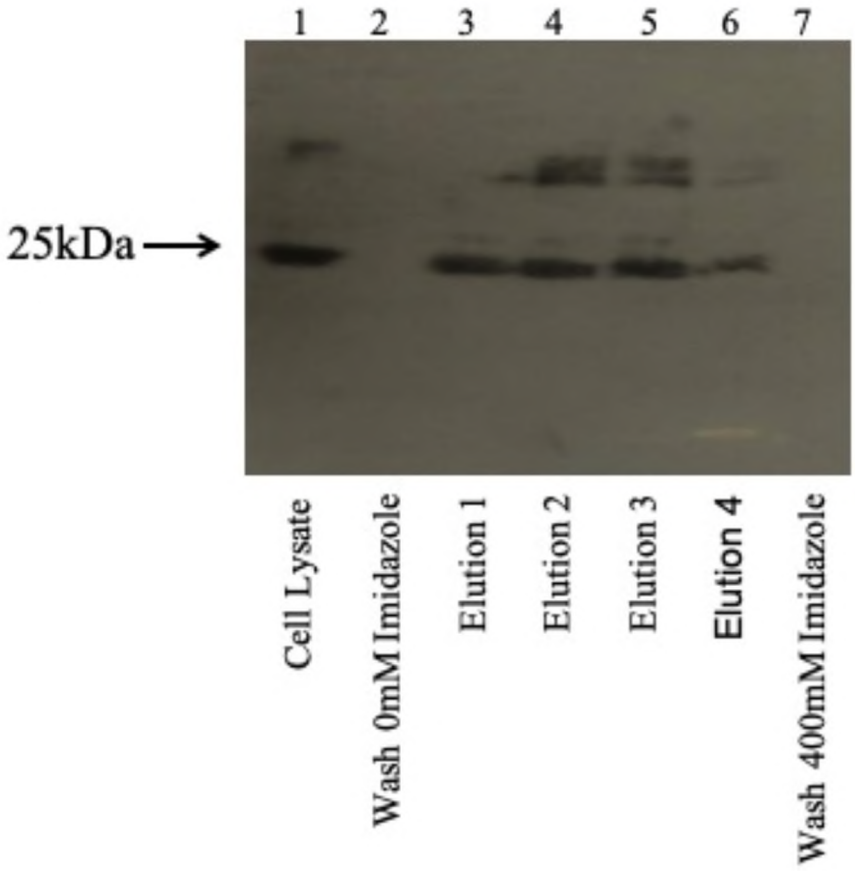

Figure 4.4: Western Blot analysis of rTM_{456} expression products. Lane 1, *E. coli* lysate supernatant; lane 2, 0mM imidazole wash fraction; lanes 3-6, 250mM elution fractions 1-4; lane 7, 400mM imidazole wash fraction

4.3.2 Synthesis and Characterization of rTM_{456}-azide

In order to introduce an azide group into rTM_{456}, sortase A-mediated ligation was employed (Figure 4.5). Sortase A will recognize the LPETG motif at the C-terminal end of the protein and cleave between the tyrosine and glycine residue. This creates an acyl-enzyme intermediate. To remove the ligated sortase A enzyme from rTM_{456}, Gly_2-PEG_3-azide was used as the nucleophile. This diglycine molecule creates a new amide bond with the tyrosine of rTM_{456} to create rTM_{456}-azide. For purification the loss of the His_6 tag of successfully reacted rTM_{456} was taken advantage of and a nickel-NTA column was used to bind unreacted rTM_{456} and sortase A, both of which still have His_6 tags. The flow through portion of the column yields rTM_{456}-azide and excess Gly_2-PEG_3-azide. To remove the excess Gly_2-PEG_3-azide, the solution was run through a 10kDa molecular weight cutoff filter. The resulting concentrated solution only contains rTM_{456}.

To characterize the purified protein, SDS-PAGE was utilized (Figure 4.6). Using Coomassie blue staining alone, it is difficult to confirm if the reaction was successful. The change in molecular weight between the starting and end products of the sortase A ligation reaction are too close and any change in band position is not easily observable. However, the newly incorporated azide group can be tagged with a small fluorescent reporter, here Cy5 was used, linked to a DBCO group. The azide and DBCO functional groups will react via CFCC to produce a fluorescently labeled protein. Before running the samples on the gel, designated samples were treated with DBCO-Cy5 and let set for 1hr. After the samples were run using SDS-PAGE, they were analyzed using a fluorescent scanner. If the azide was successfully introduced into rTM_{456}, it should react with the DBCO-Cy5 dye and show a signal in a position that matches its corresponding band seen in the Coomassie stained

gel. Successful incorporation of the azide group into rTM_{456} was seen by the positive signal seen in the fluorescent scan image of the purified reaction product. This proved that the purified protein from the sortase A ligation reaction is rTM_{456}-azide. A lane which contained unreacted rTM that was incubated with the DBCO-Cy5 dye was also ran. No positive signal was present in this lane, which is expected, and shows that the dye did not nonspecifically binding to rTM_{456} itself.

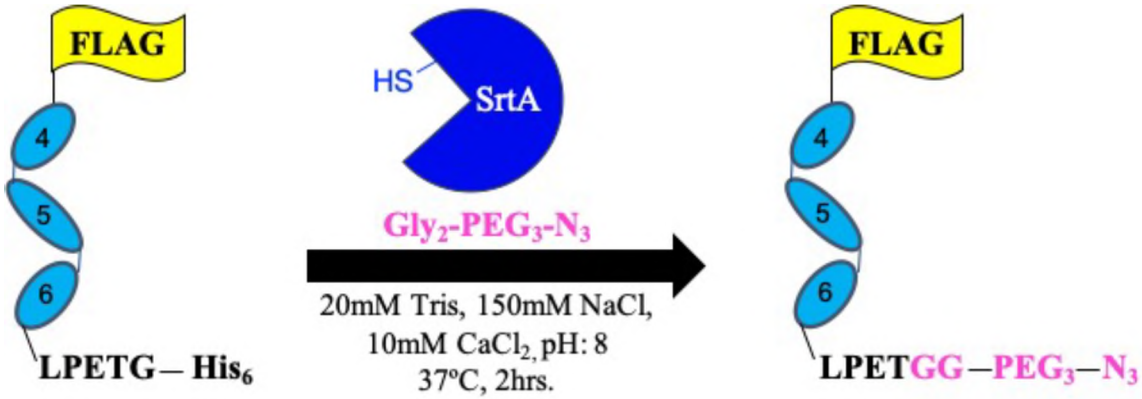

Figure 4.5: Sortase A Ligation Scheme

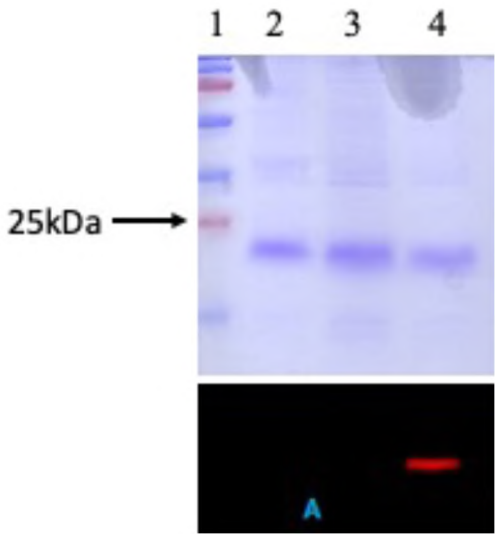

Figure 4.6: SDS-PAGE and fluorescent image of rTM_{456}-azide. Lane 1, molecular weight marker; lane 2, rTM_{456}; lane 3, rTM_{456} plus DBCO-Cy5 dye; lane 4, rTM_{456} conjugated with DBCO-Cy5 dye

4.3.3 Synthesis and Characterization of rTM_{456}-PEG_{2000}-Cholesterol

After successful incorporation of an azide group into our rTM_{456} protein, the lipid anchor can be covalently attached. In this study, a cholesterol anchor was used. The cholesterol anchor used contained a terminal DBCO group, to be used for CFCC, and a long PEG linker, which helped with water solubility of the anchor. The CFCC attachment of the cholesterol anchor to the rTM_{456}-azide protein is simple and straightforward, making it a user-friendly reaction (Figure 4.7). After overnight incubation of the protein and lipid anchor, the reaction was purified using a 10kDa molecular weight cutoff filter to remove excess Cholesterol-PEG_{2000}-DBCO. To characterize the purified product, the samples were analyzed using SDS-PAGE (Figure 4.8). When Cholesterol-PEG_{2000}-DBCO is successfully reacted with rTM_{456}-azide, the is a downshift in band position seen in the SDS-PAGE gel. Normally when adding groups to proteins, thus increasing its overall molecular, one would expect a shift upward in band position. However, a unique phenomenon happens when adding hydrophobic groups.[37] Due to increased binding of SDS to the hydrophobic groups, the protein gains more negative charge and moves farther down the gel.

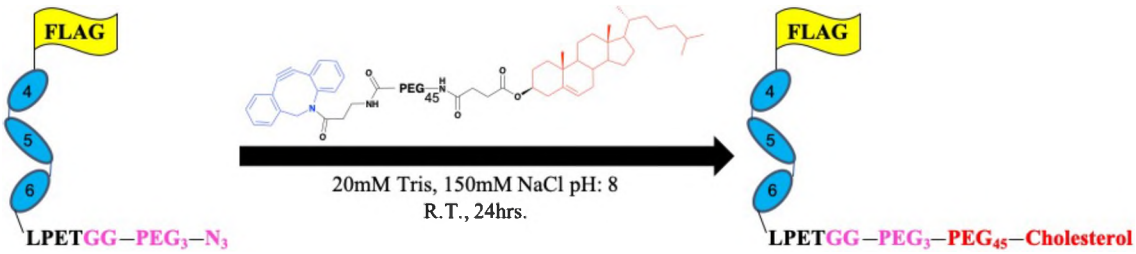

Figure 4.7: Copper free click chemistry addition of DBCO-PEG$_{2000}$-Cholesterol to rTM$_{456}$-azide

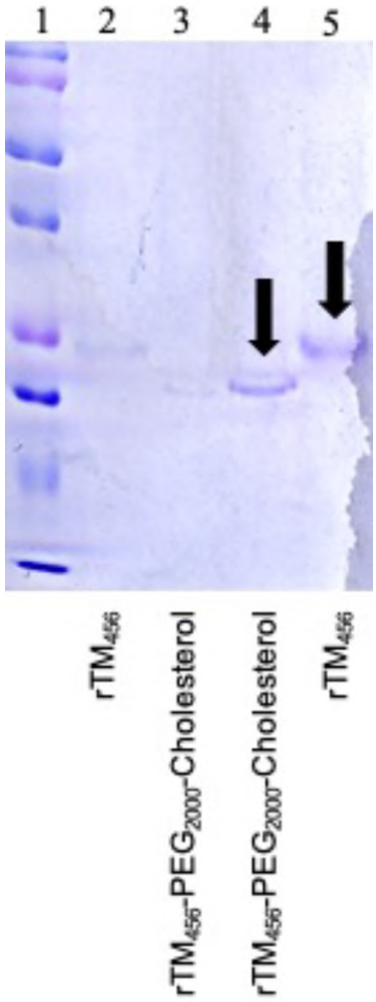

Figure 4.8: Coomassie stained SDS-PAGE gel of rTM_{456}-PEG_{2000}Cholesterol product. Lane 1, molecular weight marker; lane 2, rTM_{456} (5μg); lane 3, rTM_{456}-PEG_{2000}-Cholesterol (μg); lane 4, rTM_{456}-PEG_{2000}-Cholesterol (10μg); lane 5 rTM_{456} (10μg)

4.3.4 Anchoring Efficiency of rTM_{456}-PEG_{2000}-Cholesterol

With the successful synthesis of rTM_{456}-PEG_{2000}-Cholesterol, the first test of the lipidated protein was to assess how well it could incorporate into living cells. The plasma membrane of cells is mostly composed of phospholipids and other types of hydrophobic molecules, including cholesterol. These molecules offer many regions for hydrophobic interactions with hydrophobic anchors. Cholesterol and cholesterol-type anchors has been used to anchor for small molecules and peptides to living cells so it is a good candidate to deliver small proteins to the plasma membrane.[38,39]

To test anchoring efficiency, RAW 264.7 mouse macrophages were treated with a rTM_{456}-PEG_{2000}-Cholesterol solution. To search for successfully incorporated protein, a fluorescently labeled anti-FLAG antibody was used. The rTM_{456} expressed in this study has a FLAG tag at its N-terminus and in this study was used for identification. FLAG motifs are not naturally expressed so this motif is unique to rTM_{456}. Using confocal microscopy, the incorporation of rTM_{456}-PEG_{2000}-Cholesterol was assessed (Figure 4.9). Cells treated with the conjugate showed fluorescent signal on the cell membrane, indicating the successful anchoring of rTM_{456}-PEG_{2000}-Cholesterol into RAW 264.7 cells. Cells whom were not treated with rTM_{456}-PEG_{2000}-Cholesterol were also treated with the anti-FLAG AlexaFl488 antibody to check for nonspecific binding of the detection antibody. When analyzed, no fluorescent signal was seen indicating that antibody is not binding random molecules and requires rTM_{456}-PEG_{2000}-Cholesterol present on the cell surface in order for a positive signal to be seen. Overall, confocal microscopy showed the successful incorporation of rTM_{456}-PEG_{2000}-Cholesterol into the plasma membrane of RAW 264.7 cells.

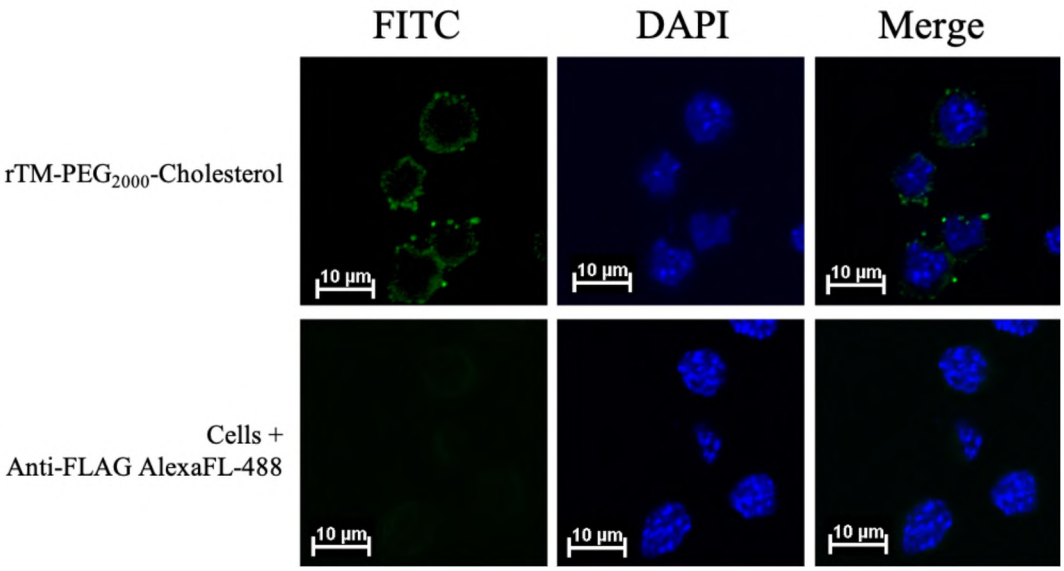

Figure 4.9: Confocal microscopy analysis of RAW 264.7 cells decorated with rTM_{456}-PEG_{2000}-Cholesterol

4.3.5 MTT Assay

To test whether the RAW 264.7 cells were healthy after incorporation of rTM_{456}-PEG_{2000}-Cholesterol, cellular toxicity of the conjugate was determined by MTT assay. After a 20min. treatment time, the cells treated with PBS, the solvent used to keep rTM_{456}-PEG_{2000}-Cholesterol in solution, and 5μM rTM_{456}-PEG_{2000}-Cholesterol showed little toxicity (Figure 4.10). Actually, cells treated with the two treatment conditions were able to convert more MTT into its formazan form when compared to cells treated with medium only. This could be due to increases in mitochondrial function or an increase in proliferation. However, no dramatic toxicity to the cells was seen.

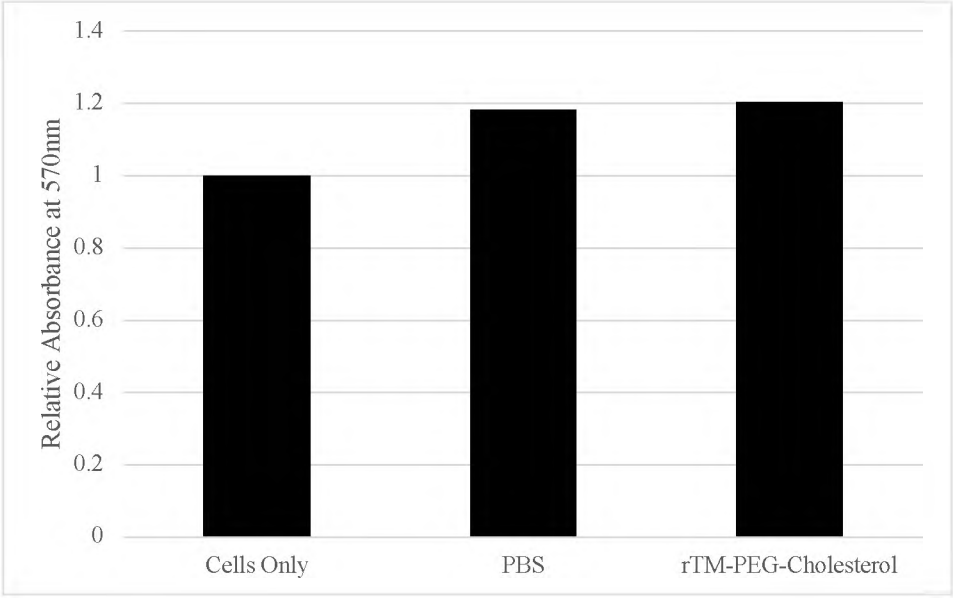

Figure 4.10: MTT assay results of RAW 264.7 cells treated with rTM_{456}-PEG_{2000}-Cholesterol, n=2

4.4 Conclusion

In this study, cell surface re-engineering of RAW 264.7 macrophages with rTM_{456} using lipid fusion was demonstrated. Using a sortase A ligation strategy, an azide functional group was able to be introduced at the C-terminus of rTM_{456}. This azide allowed for the addition of a DBCO-functionalized cholesterol anchor using copper free click chemistry. This cholesterol anchor allowed the protein to be anchored into the plasma membrane of macrophages. After only 30min. of incubation, rTM_{456}-PEG_{2000}-Cholesterol was able to successfully incorporate into the cell surface of RAW 264.7 macrophages, as confirmed by confocal microscopy. Also, the introduction of the conjugate caused little toxicity to the cells. Overall, a conjugation strategy to add a cholesterol anchor to rTM_{456} to allow for its introduction to the cell surface of macrophages *via* lipid fusion was shown. This lipid fusion approach is fast and efficient and could potentially be used as a cell surface re-engineering strategy for modifying other cells with rTM_{456} for therapeutic purposes.

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CHAPTER V

SUMMARY

Thrombomodulin (TM) is a transmembrane protein that is mainly expressed by endothelial cells but also other cells including monocytes and macrophages. Given its distribution throughout the body, TM plays a role in many biological functions such as coagulation, inflammation and cell adhesion. These functions are clearly defined for endothelial TM but remain poorly understood for other cell types including monocytes and macrophages. TM's activities have also garnered attention as a possible therapeutic agent. Recombinantly expressing TM's EGF-like domains 4-6 only offers an anticoagulant protein that retains the ability to activate protein C. However, unmodified recombinant TM (rTM) suffers from poor stability *in vivo*, a common issue with protein therapeutics. To overcome this issue, proteins can be modified and/or conjugated to supports to help protect the protein. Using cells, especially macrophages, as a drug delivery system is a good approach because cells are natural to the human body and can be directed to disease sites. There were two main goals of this research. First was to study the differences in TM expression between monocytes and macrophages. Second was to develop a lipid fusion

based cell surface re-engineering method to deliver rTM_{456} to the surface of macrophages to be used as a cell-based drug delivery system.

First, TM expression levels and APC generation activity of THP-1 monocytes and macrophages were assessed. Flow cytometry and confocal microscopy results show that THP-1 monocytes express more TM on their cell surface than THP-1 macrophages. This can be partially attributed to the fact that monocytes express more total TM than macrophages, as shown by western blot and ELISA analysis. It was also determined that during differentiation to the macrophage phenotype, monocytes shed significantly more TM in cell medium compared to when they are resting. This increase in shedding can also help explain why macrophages express less TM on their cell surface. In addition to expression levels, the ability of THP-1 monocytes and macrophages to activate protein C, a hallmark activity of TM, was assessed. Despite expressing less TM on their cell surface, THP-1 macrophages were able to generate more APC than monocytes. It is thought that this difference is due to possible changes in post-translational modifications, such as glycosylation, or due to the presence of another cofactor that can help generate APC. Overall, this study showed that TM is expressed differently by THP-1 monocytes and macrophages and the ability of these cell to generate APC is also different.

Second, a fast and efficient method for modifying the cell surface with lipid anchors was demonstrated. The anchoring efficiencies of DSPE-PEG_{2000}-Biotin and Cholesterol-PEG_{2000}-Biotin into the membrane of RAW 264.7 cells were determined by confocal microscopy using streptavidin-FITC as a probe. It was shown that the cholesterol-based anchor afforded significantly better incorporation than the DSPE-based anchor. Also, cholesterol showed a concentration dependent membrane residence time. Overall, this

study confirmed the ability of cholesterol to be used as an anchor to re-engineer the surface of RAW 264.7 cells.

Lastly, a lipid fusion technique to anchor rTM_{456} to the cell surface of RAW 264.7 macrophages was demonstrated. An azide group was introduced to the C-terminal end of rTM_{456} using sortase ligation. This azide could then be targeted by copper free click chemistry (CFCC) to introduce a DBCO-PEG_{2000}-Cholesterol anchor. After complete conjugation, the protein conjugate was characterized by SDS-PAGE analysis, in which rTM_{456}-PEG_{2000}-Cholesterol was successfully identified. RAW 264.7 cells were then incubated with the rTM_{456}-PEG_{2000}-Cholesterol conjugate and analyzed by confocal microscopy to determine its successful incorporation. Cells showed fluorescent signal around their border indicating the ability of rTM_{456}-PEG_{2000}-Cholesterol to efficiently insert into the cell membrane. This insertion also caused little toxicity to the macrophages as determined by MTT analysis. Overall, an efficient method for introducing rTM_{456} to the surface of macrophages was developed using a cholesterol anchor for lipid fusion cell surface re-engineering.

CHAPTER VI

FUTURE DIRECTIONS

6.1 Thrombomodulin Expression and Shedding During Monocyte's Differentiation to Macrophage

6.1.1 Determination of Glycosylation Levels of TM of THP-1 Monocytes and Macrophages

It is known that some species of TM are glycosylated. These moieties include sialic acid and chondroitin sulfate[1,2]. However, the glycosylation patterns of TM expressed by monocytes and macrophages and its effect on cellular function is poorly understood. Thus, determining sialic acid and chondroitin sulfate levels of these forms of TM is important. Previously, our lab determined that the global sialyation status changes when monocytes differentiate into macrophages. This includes changes in the proportion of 2,3 to 2,6-linked sialic acid and overall sialic acid amount[3]. These global changes in sialyation may be reflected at the protein levels and may cause differences in TM activity.

To determine differences in glycosylation, TM will be immunoprecipitated from THP-1 monocytes and macrophages. For sialic acid, total levels will be determined by LC-MS. Relative levels of linkages will be determined by lectin-based assays. Lectins are

carbohydrate binding proteins that are very specific for certain carbohydrate linkages. Lectin assays to be utilized are lectin blotting and lectin based ELISAs. For chondroitin sulfate, relative levels will be determined by western blotting and ELISA. Lastly, sialic acid and chondroitin sulfate moieties will be removed from TM, using sialidase and chondroitinase respectively, to see if there is any change in TM activity.

6.1.2 Determination of Levels of Endothelial Protein C Receptor on THP-1 Monocytes and Macrophages

The reason for the increase in APC generation activity of THP-1 macrophages over their monocyte counterpart currently remains unresolved. One possible reason for the difference is that there is another molecule expressed or overexpressed on macrophages that aids in protein C activation. A good candidate is the endothelial protein C receptor (EPCR). In endothelial cells, EPCR binds protein C and positions it in a favorable orientation to be acted upon by the thrombin-thrombomodulin complex[4]. EPCR has been found to be expressed by THP-1 monocytes, but its expression levels in THP-1 macrophages is unknown[5]. Determination of EPCR levels by western blotting, flow cytometry and confocal microscopy can possibly provide insight to the differences in APC generation between the two cell types.

6.2 Cell Surface Re-Engineering with Anchoring Lipids with Different Performance

6.2.1 Testing Anchoring Efficiency and Membrane Residence Time of Different Lipid Anchors

Previously, the anchoring efficiency of Cholesterol-PEG_{2000}-Biotin and DSPE-PEG_{2000}-Biotin was determined. It was shown that the cholesterol anchor was able to anchor more efficiently than the DSPE anchor[6]. Optimizing the conditions for efficient DSPE anchoring should be explored. It may take a longer incubation period to allow for insertion but the DSPE anchor may afford a higher membrane residence time, thus allowing added cargo to stay on the cell surface longer. In addition to optimizing DSPE anchoring conditions, other lipids anchors should be tested to see if they can offer more efficient anchoring methods.

6.3 Cell Surface Re-Engineering of Macrophages with Recombinant Thrombomodulin Using Lipid Fusion

6.3.1 Effect of rTM_{456}-PEG_{2000}-Cholesterol Anchoring on Cellular Toxicity and Activation

Brief testing of cellular toxicity showed that anchoring of rTM_{456}-PEG_{2000}-Cholesterol does not show any immediate cellular toxicity. However, further analysis is needed to ensure the cells remain healthy after modification. Since the rTM_{456} is being anchored physically via cholesterol, there is the possibility of damaging the membrane and causing small openings to form. This leads to cell leakage and eventual cell death. Testing for cell membrane integrity, such as LDH and calcein red assays, will be needed to check for membrane leakage. In addition to damaging the cell membrane, insertion of rTM_{456} into the macrophages could cause activation of the cell. This may cause the cell to release cytokines, which could be harmful to living systems if this application was used *in vivo*. Macrophages can be activated into inflammatory and anti-inflammatory phenotypes[7].

Testing for cytokines, such as TNF-α, IL-6 and IL-10, by ELISA can help establish if the macrophages become activated upon their surface being re-engineered with rTM_{456}-PEG_{2000}-Cholesterol.

6.3.2 APC Generation Activity of rTM_{456}-PEG_{2000}-Cholesterol Decorated RAW 264.7 Macrophages

It was shown that rTM_{456}-PEG_{2000}-Cholesterol can successfully incorporate into the cell membrane of RAW 264.7 cells. To further the idea that this system could be used as a therapeutic, the APC generation ability of RAW 264.7 cells needs to be tested. The newly added rTM_{456} should provide the macrophages with increased ability to generate APC.

6.3.3. Membrane Residence Time of rTM_{456}-PEG_{2000}-Cholesterol

In addition to activity and toxicity effects of rTM_{456}-PEG_{2000}-Cholesterol on RAW 264.7 macrophages, determination of how long the conjugate will remain on the cell surface is important. Measuring membrane residence time can help in determining how long rTM_{456}-PEG_{2000}-Cholesterol can exert its activity. rTM_{456}-PEG_{2000}-Cholesterol needs to remain on the surface an acceptable amount of time for it to be considered a possible therapeutic.

6.4 References

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