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Tetraspanins Associated With oxLDL and IgG Mediated Phagocytosis In Human U937 Macrophages

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TETRASPANINS ASSOCIATED WITH oxLDL AND IgG MEDIATED PHAGOCYTOSIS IN HUMAN U937 MACROPHAGES

by

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ABSTRACT

TETRASPANINS ASSOCIATED WITH oxLDL AND IgG MEDIATED PHAGOCYTOSIS IN HUMAN U937 MACROPHAGES

Pardis Pakshir, Master of Science, Molecular Science, Ryerson University, 2013

One of the crucial key targets in treatment of diseases are cell surface proteins, such as receptor complexes, and their associated signaling pathways. The Fc receptor is one of the most important phagocytic receptors of the cells of immune system. The ligand of the Fc gamma receptor is immunoglobulin G (IgG), which triggers the engulfment of foreign molecules coated by antibodies by a process called phagocytosis. A Specialized subset of cells including macrophages engulfs foreign particles by the Fc receptor. Another phagocytic receptor of macrophages is the CD36 receptor, which binds the ligand oxLDL and is known to be involved in the development of atherosclerotic lesions in the arteries. A few members of the Tetraspanin proteins have been found to be associated with theses receptors in macrophages. Tetraspanins may act as "molecular facilitators" grouping specific cell-surface proteins and thus increasing the formation and stability of functional signaling complexes. There is a significant amount of research done on the receptors of the surface of macrophages, however, the proteins associated with these receptors, their potential signaling pathways and the mechanisms involved are not yet fully understood. This thesis aims to investigate the presence and potential functional role of the specific Tetraspanin isoforms in Fc and CD36 mediated phagocytosis. Silencing RNA, quantitative assays of phagocytosis, and laser scanning confocal microscopy were used to test the phagocytic efficiency of macrophages in IgG and oxLDL mediated phagocytosis. Understanding the regulatory roles of Tetraspanins can provide insight into various immune diseases.

ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. John Marshall, for his continued support and belief in me throughout my graduate studies. I consider myself very fortunate to have had the opportunity to work with him. He has thought me not only scientific principles but also life lessons that I will carry with me through the years.

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Thank you.

DEDICATION

Parents sacrifice a lifetime in order to achieve their greatest success, their children's accomplishments. Every success that I have achieved can be directly traced to the sacrifices that my parents and my family have made for me. I know that no matter what I accomplish, it will never measure up to the amount of time and effort that they have spent teaching me, setting up the right circumstances and proving the right tools and environment to create my success.

No matter how many times I utter the words" thank you" and "I appreciate it", it will not begin to compare to the amount of hours that they have worked in order to provide a better opportunity for achievement, it will not compare to the amount of hours they have stayed up late just to call me from the other side of the planet to wish me luck on the eve of my exams. No matter how many thank you notes I write, I know it will not compare to the amount of worry that have lived through, when I have been sick so far away from them.

I know the only way I will ever be able to come close to ensuring that I have earned the opportunity that they have provided for me, is that I must accomplish more than I have, go further than I have and continue on to my dying days; to live every moment of my life, making the most of every minute of every day.

I have stood on the shoulders of my family, and they have been the support and the backbone of my success, and I know I have one single task and one single responsibility that I must accomplish. That one single task is to earn it. I would like to dedicate this thesis to my loving parents Mohammad Reza and Mojgan and my dear beautiful sister Paniz, whose words of encouragement and push for determination has been a source of motivation and inspiration throughout my life.

I couldn't have done it without you. Thank you.

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LIST OF SELECTED ABBREVIATIONS

Ab	Antibody
acLDL	Acetylated low-density lipoprotein
apoB-100	Apolipoprotein B-100
CD36	Cluster of differentiation 36
Cy	Cyanine
Cys	Cysteine
DAG	Diacyl glycerol
DC	Dendritic cell
EC1	Extracellular loop 1
EC2	Extracellular loop 2
EC	Endothelial cells
ERK	Extracellular signal-regulated kinase
FBS	Fetal Bovine serum
FcR	Fragment crystallizable receptor
GFP	Green Florescent protein
HDL	High-density lipoprotein
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HTLV-1	Human T-cell Lymphotropic virus
IFNα/β	Interferon beta and gamma
IgG	Immunoglobulin G
IL-4	Interlukin-4
InsP3	Inosotil 1,4,5- triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal protein kinase
LARC	Live cell affinity receptor chromatography
LC–MS/MS	Liquid chromatography and tandem mass spectrometry
LDL	Low-density lipoprotein
LOX-1	Lectin-type oxidized low-density lipoprotein receptor 1
Lyn	V-yes-1 Yamaguchi sarcoma viral related oncogene homologue
MAPK	Mitogen activated protein kinase
MARCO	Macrophage receptor with collagenous structure
MEKK2	MAPK/ERK kinase kinase 2
MPO	Myeloperoxidase
MS/MS	Tendem mass spectrometry
NO	Nitric oxide
NLDL	Native LDL
oxLDL	Oxidized low-density lipoprotein
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PKC	Protein kinase C
PLA2	Phospholipase A2
PLCγ	Phospholipase Cγ
PMA	Phorbol 12-myristate 13-acetate

PLD	Phospholipase D
PI-3K	Phosphatidyl- inositol 3-kinase
PI(4,5)P2	Phospholipid phosphatidylinositol 4,5-bisphosphate
RLR	Retinoic acid-inducible gene I-like receptor
RPMI	Roswell Park Memorial Institute medium
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SCARA-5	Scavenger receptor A5
SH2	Src Homology 2
SiRNA	Silencing RNA
SRA	Scravenger receptor A
SREBPs	Sterol regulatory element binding proteins
SRCL	Scavenger receptor with C-type lectin
Syk	Spleen tyrosine kinase
TEM	Tetraspanin-enriched Microdomain
TGF	Transforming growth factor
TLRs	Toll like receptors
TSPAN	Tetraspanin
TSR	Thrombospondin repeat
VLDL	Very low-density lipoprotein

INTRODUCTION:

Background

The immune system defends the body against infectious organisms and foreign invaders that can cause various diseases. The immune system is composed of two branches; innate and adaptive immunity. The adaptive immune system is comprised of memory cells such as T lymphocytes and antibody producing B lymphocytes which have receptors that are able to recognize pathogens. The adaptive immune system produces antibodies in response to antigens belonging to a pathogen that has previously infected the body.

The innate immune system is the first line of defense in the body and it consists of cells and proteins that fight microbes or invaders at the site of infection. Cells such as macrophages are able to recognize pathogens, internalize and destroy them. One of the receptors of macrophages is Fc receptor that is the most important phagocytic receptor of the cells of immune system. Fc receptors are involved in antigen recognition and contribute to the protective functions of the immune system. Fc receptors bind to the Fc portion of Immunoglobulin g (IgG). The cells of the adaptive immune system guide the innate immune system by the presenting antibodies that are recognized by the Fc receptor. Therefore, the Fc receptor connects the adaptive and innate responses (Florentinus et al., 2011).

Another phagocytic receptor of the macrophages is CD36, which is a class B scavenger receptor and is known for its capacity to internalize modified low-density lipoproteins (LDL). The uptake of modified lipoproteins by the macrophages results in the deposition of cholesterol ester within cells and the formation of foam cells (Torontoz et al., 1998). The clustering of foam cells eventually leads to the formation of plaques and to the initiation and development of atherosclerotic lesions (Hajjar et al., 1997).

One of the key targets in treatments of diseases is cell surface proteins such as receptor complexes and their associated signaling pathways. A study done by Jankowski, Zhu &

Marshall, 2008 showed that macrophage receptor complexes can be captured from the membrane of live cells by Ligand affinity receptor chromatography (LARC). This is done by presenting the receptor's ligand on a microscopic bead and introducing it to the cells. Further studies on the cell surface receptors and the proteins that associate with them can provide insights into studies of immune responses and many human diseases.

Phagocytosis

A Russian scientist, Elie Metchinkoff, first discovered phagocytosis 100 years ago. Phagocytosis is one of the strategies that cells have developed to internalize particles and solutes. Phagocytosis is referred to as the receptor stimulated process of engulfing particles over 0.5 μm in diameter (Flannagan, Jaumouillé, & Grinstein, 2012).

Phagocytosis is a very crucial component of both the innate and acquired immune system, which is present in organisms ranging from unicellular microorganisms to higher organisms (Flannagan et al., 2012; Garcia-Gardia & Rosales, 2002).

Phagocytosis helps the defense mechanism by internalization and elimination of infectious pathogens. It is known to be involved in development, tissue remodeling, the immune response, and inflammation (Garcia-Gardia & Rosales, 2002).

A special division of cells of the immune system termed professional phagocytes consisting of monocytes/macrophages and neutrophils perform phagocytosis to engulf and ingest pathogens at the inflammation sites.

The binding of the invading pathogen by antibodies is termed opsonization, which warns the cells of the immune system of the upcoming threats. The initiation of phagocytosis is marked by the interaction of the receptors on the surface of phagocytes with their ligands. Upon interaction of the cell surface receptors with their ligands phagocytosis proceeds within, a membrane-bound vacuole, phagosome. The internalization of the pathogen requires the phagocytes to extend pseudopods to encircle the upcoming pathogen, which requires actin (Greenberg & Grinstein, 2002). Actin cytoskeleton remodeling is required at the site of receptor complexes (Aderem & Underhill, 1999). The phagocytes are also shown to provide extra endomembrane in order to accommodate the incoming particle

(Greenberg & Grinstein, 2002). The phagocytosis is completed once the particle is internalized and phagosome is separated from the membrane.

Once the particle is internalized, the phagosome matures and eventually joins with lysosome, one of the components of the endocytic pathway, to form the phagolysosome. Phagolysosomes are very acidic and rich in hydrolases providing the required conditions to degrade the internalized pathogen (Aderem & Underhill, 1999).

The binding of the ligand to the receptor leads to a specific cascade of signaling pathways that vary based on the membrane and cytoskeleton around the ligand receptor complex. The mechanism by which phagocytosis takes place is dependent on actin and is not a clathrin mediated mechanism (Aderem & Underhill, 1999).

Regulating the induction of phagocytosis by macrophages might be a means of therapeutically regulating the immune system. Studying the parameters that affect phagocytic efficiency may lead to improved methods of delivery mechanisms in drug development (Pacheco, White & Sulchel, 2013).

The Fc gamma receptor

One of the known phagocytic receptors involved in antigen recognition by cells of the immune system is Fc receptor (FcR) that binds to the Fc portion of immunoglobulins.

The family of Fc receptors and their ligands, the immunoglobulins, are part of the Ig superfamily (Florentinus et al., 2011; Garcia-Gardia & Rosales, 2002). The activated Fc receptor has been isolated from the surface of live cells and the members of the complex have been successfully identified (Jankowski et al., 2008).

Fc receptors are expressed on many cell types of the immune system. An Fc receptor class exists for every antibody class: FcγR binds IgG, FcαR binds IgA, FcεR binds IgE, FcμR binds IgM and FcδR binds IgD (Flannagan et al, 2012). Three classes of FcγR have been recognized to date: FcγRI, FcγRII, and FcγRIII. Each class of FcγR consists of several individual receptor isoforms.

Upon binding of the ligand to the Fc receptor a series of downstream signaling pathways are initiated. The binding of IgG coated particles to the Fc receptor causes multiple Fc receptors to aggregate in the plane of the cell membrane, which brings the cytosolic

domains of these receptors together (Flannagan et al., 2012). Upon receptor clustering, the cytoplasmic portion of FcγR, which has a specific tyrosine residues termed ITAMs (immunoreceptor tyrosine based activation motifs), gets phosphorylated by a Src tyrosine-kinase family (Flannagan et al., 2012; Swanson, 2004). The phosphorylated Tyrosine residues of the cytoplasmic domain then become docking sites for the SH-2 domain of a tyrosine kinase, Syk. Docking of Syk triggers its phosphorylation (by Src kinases) and its activation leads to the recruitment of additional signaling proteins to the activated FcγR complex (Lu et al., 2011). Syk is known to activate phosphatidyl-ionositol 3-kinase PI3K. Syk is also known to activate phospholipase C (PLC), which cleaves the membrane phospholipid phosphatidylinositol 4,5-bisphosphate PI (4,5) P2 to generate Inositol 1,4,5- triphosphate (InsP3) and diacyl glycerol (DAG) (Flannagan et al., 2012). InsP3 releases Ca^{2+} from intracellular Endoplasmic Reticulum Ca^{2+} stores. The presence of Calcium has been determined to be very important in regulating the phagocytosis of macrophages. Activation of both PLC and PKC requires prior activation of PI3K. DAG and calcium can then go on to activate Protein kinase C (PKC) which goes on to phosphorylate other molecules, leading to different cellular activities (Flannagan et al., 2012; Swanson, 2004; Garcia-Gardia & Rosales, 2002; Greenberg & Grinstein, 2002).

The internalization of particles by Fc receptor is an actin-mediated process. Active GTP-bound forms of Rac and Cdc42 are present at sites of phagosome formation during FcR-mediated phagocytosis (Cox et al, 1997). Rac and Cdc2 bind Wiskott-Aldrich syndrome protein (WASP), which binds to further adaptor proteins such as Ena/vasodilator-stimulated phosphoprotein (VASP) to activate the molecular complex Arp2/3 (Lorenzi et al, 2000). The activation of Arp2/3 complex is known to be crucial step in the polymerization of actin filaments (Garcia-Gardia & Rosales, 2002). The active forms of Cdc42 and Rac also induce an enzyme named PAK1 that can result in inhibition of actin-depolymerizing factor cofilin, by phosphorylating LIM kinase (LIMK) (Edwards & Gill, 1999).

Macrophages Scavenger Receptors

Macrophages have two categories of receptors, which are responsible for discriminating between different pathogens and their own cellular components. One category contains the receptors that recognize pathogens and particles coated with opsonins. The other category contains those receptors that bind ligands endogenous to the pathogen or external pathogen termed, non-opsonic receptors (Aderem & Underhill, 1999).

The macrophage scavenger receptors were first discovered by Brown and Goldstein when studying the accumulation of cholesterol from low-density lipoprotein (LDL) in atherosclerotic plaques (Goldstein et al., 1979). The scavenger receptors are important components of the innate immune system since they bind many different ligands such as microbial pathogen, and host modified molecules. Scavenger receptors are found in myeloid cells and selected endothelial cells and are involved in disease such as Alzheimer's and atherosclerosis (Plüddemann, Neyen & Gordon, 2007). The innate scavenger receptors are divided into 8 classes (Classes A, B, C, D, E, F, G and H) based on their structure homology. Class A consists of SR-AI, SR-AII, MARCO (macrophage receptor with collagenous structure), SCARA-5 (scavenger receptor A5) and SRCL-I/II (Plüddemann et al., 2007; Elomaa et al., 1995; Jian et al., 2006). Class B consists of CD36 and SR/B1. Class C encompasses Drosophila melanogaster scavenger receptor dSR-CI. Class D contains CD68 and Class E contains LOX-1. SREC, SR-PSOX and FEEL1 belong to classes F, G and H respectively (Plüddemann et al., 2007). Class B scavenger receptors particularly CD36 is one of the focuses of this research because of its ability to uptake modified low density lipoproteins and its contribution to the initiation and development of atherosclerosis as discussed further in the introduction.

Oxidized low-density Lipoprotein

Lipoproteins are particles composed of lipids and proteins held together by non-covalent forces. Lipoproteins are categorized based on their density, size and protein composition. Lipoproteins fall into one of the following categories: chylomicrons, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) (Segrest et al., 2001). Low-density lipoprotein is the carrier of cholesterol in the bloodstream and its function is to supply various tissues with adequate levels of cholesterol at all times (Prassl & Laggner, 2008). LDL is highly heterogeneous, differing in size, density and shapes. The average size of the LDL particles is 18 to 25 nm and they are globular in shape (Prassl & Laggner, 2008). The core of the LDL particle is composed of Triglycerides and esterified cholesterol and is confined by a phospholipid membrane, which has a single copy of apolipoprotein B-100 (apoB-100). A Schematic representation of a generic LDL particle is shown in Figure 1 (Murtola et al., 2011).

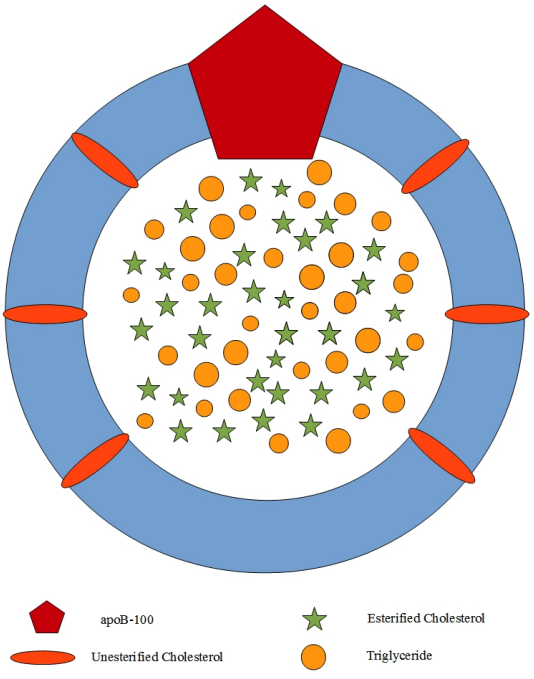


Figure 1. Low-density lipoprotein schematic. Adopted from Murtola et al., 2001.

High levels of LDL accumulating in the arterial walls are associated with the development of cardiovascular diseases. The plaque formation on the wall of vessels can

lead to the narrowing of arteries, rupture, clotting, and ultimately death (Murtola et al., 2011). Low-density lipoprotein can be modified by chemicals and enzymes. Some of these modifications include oxidation, acetylation, enzymatic degradation and lipolysis (Prassl & Laggner, 2008).

Native LDL cannot lead to the formation of foam cells because its uptake by LDL receptors of macrophages is very slow and its receptor down regulates its uptake (Steinberg, 2002). High levels of cholesterol down regulate the LDL receptor on the surface of macrophages through the mechanism of sterol regulatory element binding proteins (SREBPs), which are transcriptional factors responsible for regulating genes involved in cholesterol production and uptake (Horton et al., 2002; Yoshida & Kisgui, 2010; Kim et al., 1996; Brown & Goldstein, 1977). The contribution of native LDL to the formation of foam cells is by modifications to its structure such as oxidation, which makes it recognizable by the scavenger receptors of the macrophages (Horton et al., 2002). The cellular uptake of modified LDL is done by scavenger receptors of classes B/E. The uptake of LDL is dependent on its structure and the proper conformational orientation of the Apolipoprotein B 100 located on the surface of the particle. Therefore, the size and shape are two important factors of the physiological functions of LDL (Prassl & Laggner, 2008).

The damaged endothelial cells of the walls of arteries recruit macrophages at the site of inflammation in the presence of high levels of LDL. An inflammatory stimulus causes endothelial cells (EC) and macrophages to produce oxidative products. These oxidative products can cause the conversion of LDL into oxidized LDL (Febbraio et al., 2001). LDL can be oxidized enzymatically or non-enzymatically. LDL is known to be oxidized in two stages. At the first stage, LDL is partially oxidized by minor oxidations of apolipoprotein B100. This modification is named minimally oxidized LDL, which still has the ability to bind the LDL receptor (Yoshida & Kisugi, 2010). However, further oxidation of LDL leads to oxidized LDL, which is now recognized by the scavenger receptors (Yoshida & Kisugi, 2010). Free radicals, metal ions such as iron and copper and oxidative agents, produced by the inflammatory stimulation of endothelial cells (EC) and macrophages, such as nitric oxide (NO) and reactive oxygen species can lead to

oxidation of LDL. Oxidative enzymes such as Lipoxygenase and Myeloperoxidase can also oxidize LDL (Yoshida & Kisugi, 2010).

oxLDL is recognized and internalized by CD36 scavenger receptors as ligands. The mechanism by which oxLDL up-regulates CD36 involves activation of the transcription factor, PPARγ (peroxisome proliferator activated receptor-γ) (Nicholson et al., 2011). PPARγ is an important regulator of the expression of CD36 (Nagy et al., 1998; Febbraio et al., 2001). Upon internalization of oxLDL by the CD36 receptor, the PPARγ transcriptional factor is produced and transferred to the nucleus resulting in production of PPARγ itself and binding to the promoter of CD36 gene, increasing its expression (Torontoz et al., 1998; Febbraio et al., 2001). The thiazolidinedione class of anti-diabetic drugs is recognized as ligand for PPARγ and thus can result in increased CD36 expression (Torontoz & Nagy, 1999). The increase in the expression of CD36, results in a positive feedback loop. The more CD36 receptors on the surface of macrophages mean more uptake of oxLDL, which can cause the accumulation of cholesterol ester by macrophages resulting in the formation of foam cells (Febbraio et al., 2001). The foam cells tend to form clusters and are known to have low mobility, large vacuolated shapes and secrete pro-inflammatory cytokines and reactive oxygen species (ROS) (Bobryshev, 2006). The foam cells represent monocyte/macrophages that have taken up modified forms of lipoproteins rather than the native LDL (Boullier et al., 2013).

oxLDL is composed of two components, the lipid and the protein (apoB) parts. The two components of the oxLDL have been shown to compete with each other to bind to a common ligand-binding site of macrophage scavenger receptors. An 80% inhibition of intact oxLDL internalization has been recorded in the presence of lipid and protein moieties of oxLDL (Boullier et al., 2103). The oxidized phospholipids of the oxLDL have been shown to significantly contribute to binding of the oxLDL to the scavenger receptor (Boullier et al., 2103).

CD36 structure

CD36 receptor is another receptor of interest in this study. CD36 is an 88-kDa (mass with glycosylations) transmembrane glycoprotein and is expressed on macrophages as well as platelets, adipocytes and some epithelial and endothelial cells (Hajjar et al., 1997: Plüddemann et al, 2007; Terpstra et al., 2000). It is well established that CD36 is involved in many pathways such as cardiovascular diseases, parasitology, cancer biology, Alzheimer's disease, stroke, diabetes and many more (Silverstein and Febberaio, 2007). CD36 receptors along with SR/B1 are the two members of class B scavenger receptors which are known to form dimers and multimers (Plüddemann et al, 2007).

CD36 receptor binds a variety of ligands such as anionic phospholipids, apoptotic cells, fatty acids and thrombospondin (Rigotti et al., 1995; Ren et al., 1995; Nicholson et al., 2011). The CD36 receptors are linked to the uptake of apoptotic bodies by recognizing the phosphatidylserine on the surface of apoptotic cells (Savil et al, 1991; Fadok et al, 1998; Febbraio & Silverstain, 2007).

One of the most studied ligands of the CD36 receptor is oxLDL. The accumulation of oxLDL leads to an increase in the production of CD36, which is linked to foam cell development in atherosclerosis (Hajjar et al., 1997). It is not only the CD36 receptor that can internalize oxLDL, but also SRA-1, SRA-2, SRA-3, MARCO, CD36, SR-B1, CD68/ macrosialin, and LOX-1 receptors are known to uptake oxLDL (Witztum et al., 1991).

The first ligand to be discovered for scavenger receptors was acetylated LDL which is not produced under normal physiological conditions, and the natural ligand was not known until it was shown that oxidized LDL is the ligand for these receptors (Kodama, et al., 1988; Rohrer et al., 1990; Parthasarathy et al., 1986).

CD36 is also involved in negatively regulating angiogenesis in vascular endothelial cells by its ability to be the receptor for thrombospondin-1 (Silverstein & Febbraio, 2009; Silverstein et al., 1992; Asch et al., 1987).

In a study done by Hajjar et al (1997) murine macrophage cell lines incubated with acetylated low-density lipoprotein for 2 hours and oxidized low-density lipoprotein for 4 hours exhibited an increase in the level of CD36 expression. The level of expression of

CD36 is dependent on multiple factors such as the presence of cytokines and growth factors as well as differentiation level of macrophages (Hajjar et al., 1997).

Subjects with macrophages that lack CD36 display less efficient uptake of oxLDL compared to the subject with normal macrophages. This study highlights the importance of CD36 in the internalization of oxLDL (Boullier et al., 2000; Nozaki et al., 1995). In a study done by Endemann et al (1993) the blockage of CD36 binding sites with antibodies such as OKM5, was followed by less internalization of oxLDL in PMA treated monocyte cell line (THP-1) (Endemann et al., 1993). It is widely thought that CD36 is regulated by its own ligand oxLDL. This was shown in a study where cells incubated in medium containing oxLDL showed higher levels of CD36 compared to cells incubated in medium only (Nakagawa et al., 1998).

Structure of CD36

The structure of the CD36 receptor consists of two short intracellular domains, which are 5-7 and 11-13 amino acids respectively. It also has an extracellular loop (84 to 204 amino acids) with multiple glycosylation sites, six conserved Cysteine residues, giving rise to three disulfide bridges (Cys243- Cys311, Cys272-Cys333, and Cys313-Cys322), and two ligand binding sites (Silverstein & Febbraio, 2009 ; Rac et al., 2007; Febbraio et al., 2001). The structure of CD36 receptor is shown in Figure 2.

The extracellular loop is the location where the binding of the ligands takes place (Puente Navazo et al., 1996). The glycosylation of the CD36 receptor on the extracellular loop gives the protein protection from proteases such as lysosome at the sites of inflammation or tissue damage (Febbraio et al., 2001). There are two binding domains in the extracellular loop of CD36 receptor as shown in Figure 2, one is for binding proteins that contain thrombospondin repeat (TSR) domains and the other ligand-binding site is for oxidized lipids (Silverstein & Febbraio, 2009; Plüddemann et al, 2007; Rac et al., 2007; Febbraio et al., 2001). The C and N terminal tails of the receptor contain palmitoylation sites. The C terminal tail of CD36 receptor is important in downstream signaling pathways and is involved in the uptake of the oxLDL (Hajjar et al., 1997: Plüddemann et al, 2007).

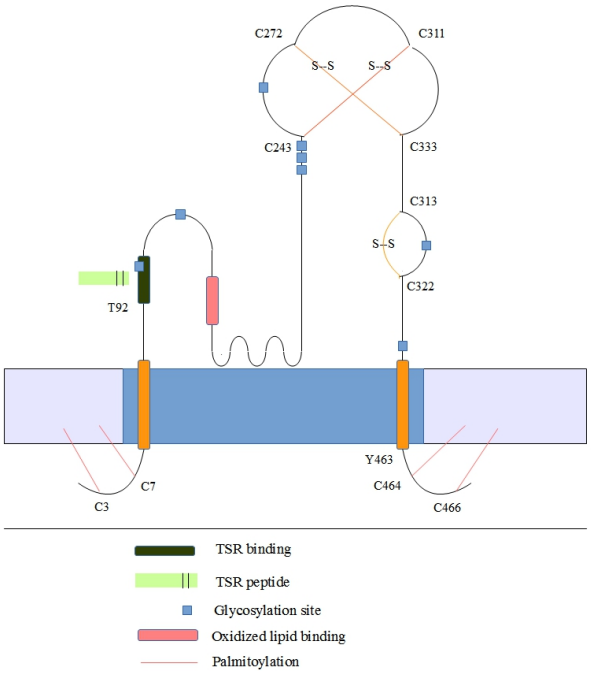


Figure 2. Schematic representation of the CD36 structure. Adopted from Silverstein et al., 2009.

Localization of CD36 in the plasma membrane

CD36 is located in specialized cholesterol-rich membrane micro-domains of macrophages, therefore, this localization may suggest interactions between this receptor and members of Tetraspanin family as well as integrins as shown in Figure 3. In addition, the presence of the receptor on the surface of macrophages and monocytes signifies its role in phagocytosis and inflammatory responses (Silverstein & Febbraio, 2009 ; Sabill & Hogg, 1992).

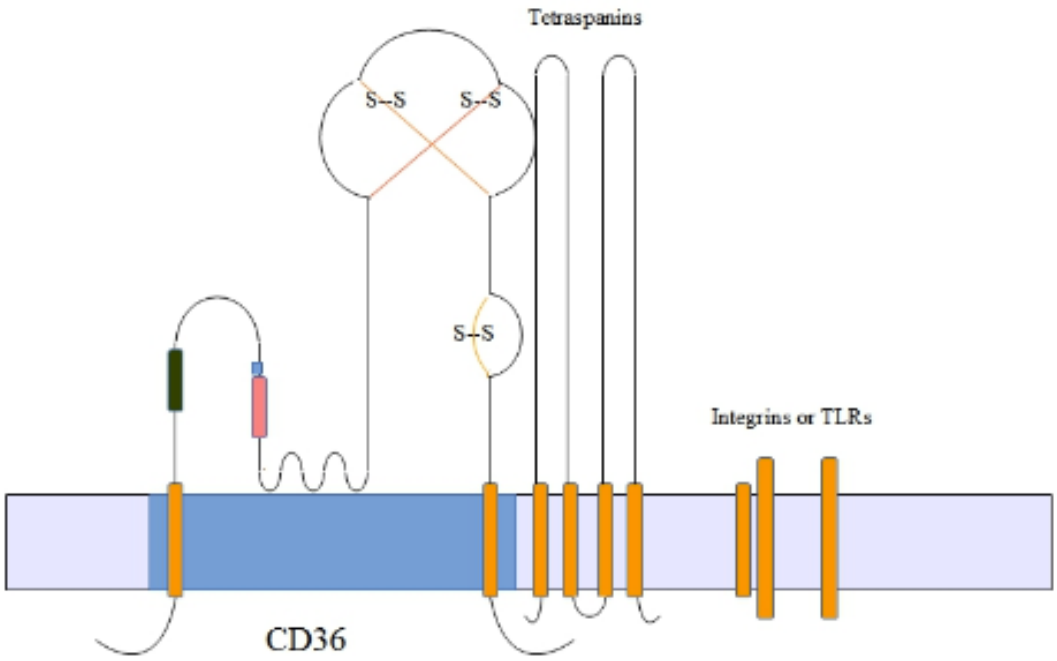


Figure 3. Interaction of CD36 with Tetraspanins and Integrins in specific cell membrane microdomains. Adapted from Silverstein et al., 2009.

There is evidence of Tetraspanins interacting with scavenger receptor. A study done by Bartosch et al (2003) showed that Hepatitis C virus entry into the cells requires a co-receptor complex consisting of Tetraspanin CD81 and SR-B1 class B Scavenger Receptor. CD36 physically associates with Tetraspanin CD9 and $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins in C32 melanoma cells through its extracellular domain (Thorne et al., 2000). On the surface of platelets scavenger receptor CD36 is known to form association with Tetraspanin CD9 and integrins αIIbβ3 and $\alpha_6\beta$ to form a membrane complex (Miao et al., 2001).

CD36 signaling

The immediate events following the uptake of oxLDL by CD36 are poorly understood. Most of the research has been done at the transcriptional levels and signaling pathways that either up regulate or down regulate the receptor. The ligand of the CD36 receptor binds to and is internalized by signaling pathways that are not very well understood, however it is known that Src-family kinases, Jnk and Rho family GTPases are some of the key players in the internalization of the ligands of CD36 (Heit et al., 2013). oxLDL particles are internalized through a lipid raft pathway that is different from the clathrin-mediated or caveolin internalization pathways (Zeng et al., 2003). CD36 is shown to cluster upon binding of oxLDL, which initiates downstream signaling events. A study done by Collins et al (2009) showed that CD36 internalization is an actin dependent process and requires the activation of Src family kinases as well as Rac and cdc42. Different choices of experimental systems may explain why different studies propose different mechanism for the early events followed by the binding of oxLDL to its receptor (Collins et al., 2009). It is widely thought that CD36 uptakes its ligands by two possible mechanisms. One is the pathway that leads to the internalization of lipids from oxLDL that activate nuclear receptors such as PPARγ and the other is the common signaling cascade generated upon ligand binding (Nicholson et al, 2011). Upon oxLDL binding, the changes in the transcriptional factors lead to the up-regulation of the CD36 gene. The transcriptional factor PPARγ joins the retinoid X receptor (RXR) and the PPARγ:RXR to form a complex that binds to the elements on the promoter of the CD36 gene. The binding of this complex to the CD36 gene results in an increase in CD36 expression (Rahaman et al., 2006; Collot-Teizeira et al., 2007).

A study done by Rahaman et al (2006) showed that when oxLDL is bound to its receptor CD36, MAP kinases JNK-1 and JNK-2 were phosphorylated and activated. The carboxyl terminal intracellular domain of the C36 receptor is the site where signaling molecules or adaptor proteins are diverted to their functions (Rahaman et al., 2006; Shaw et al., 1990). Src-family Lyn was also shown to be a part of the pathway that leads to the internalization of oxLDL as blocking the Src-kinase stopped foam cell generation (Rahaman et al., 2006).

It is now known that kinases such as JNK are required for raft-mediated internalization of particles, therefore it can be thought that JNK is a key player in the uptake of oxLDL by CD36 (Rahaman et al., 2006; Pelkmans et al., 2005).

Protein kinase C (PKC) is also known to be involved in the activation of the PPARγ transcription factor therefore, can be linked to CD36 uptake of its ligand (Eng et al., 2000; Zibara et al., 2000). Moreover, Protein Kinase B is linked to CD36 signaling by its ability to up-regulate the CD36 by stimulating the CD36 promoter as well as a PPAR-gamma element-driven reporter gene (Munteanu et al., 2006). The over-expression of PKB can result in an increase in CD36 expression in macrophages. Thiazolidinedione class of anti-diabetic drugs is also known to be another regulator of CD36 receptor by regulating PPARγ (Torontoz et al., 1998).

The CD36 receptor signals by association with co-receptors and accessory molecules that vary for different ligands (Heit et al., 2013). A study done by Heit et al (2013) shows that CD36 receptor complex contains molecules such as β1 and β2 integrins, Tetraspanin CD9 and CD81, which are known to link CD36 to immune receptor tyrosine activation motif (ITAM) cytoplasmic portion of Fc gamma receptor. The coupling of CD36 to the ITAM results in the internalization of CD36's ligands via Src and Syk kinases (Heit et al., 2013). This study suggests a functional association between the CD36 receptor and FcR, by showing that CD36 relies on FcR to internalize its ligands.

CD36 expression is improved in reaction to cytokine IL-4 and oxLDL by a mechanism involving Protein Kinase C and PPAR-gamma (Feng et al., 2000). Transforming growth factor (TGF1/2) can also modify the induction levels of CD36 by activating MAP kinase and inactivating PPAR transcriptional factor in PMA differentiated THP-1 macrophages (Han et al., 2000; Acton et al., 1996).

Atherosclerosis and The role of CD36 in Atherosclerosis

Atherosclerosis is a condition where the arteries become thickened and hardened. The thickening can lead to the narrowing of the vessels, which is partly due to the build up of plaques (Collot-Teizeira et al., 2007). The blood vessels affected by the thickening and reduced blood supply include the aorta, coronary, carotid and cerebral arteries (Eij et al., 2006). The atherosclerotic plaques (atheroma) are formed from the macrophage-derived foam cells (Eij et al., 2006). Atherosclerosis is one of the leading causes of death in industrialized countries. Individuals with high blood cholesterol levels, high blood pressure, diabetes, obesity, inactive lifestyles and smokers are at the highest risks to develop atherosclerosis. These risks can contribute to the initiation and progress of the lesions by disrupting lipid regulatory and inflammatory pathways (Eij et al., 2006).

At the first stage of development of Atherosclerosis, the dysfunctional endothelial cells (EC) engage the monocytes from the blood stream by activated adhesion molecules and cytokines (Collot-Teizeira et al., 2007). The CD36 receptor is linked to the atherosclerotic lesion development by its ability to uptake oxLDL by macrophages that are later differentiated into foam cells (Collot-Teizeira et al., 2007). Due to the important role of CD36 in atherogenisis, it is critical to study the factors that may influence its expression in macrophages, to present the development of atherosclerosis in the arterial walls (Nakagawa et al., 1998). oxLDL uptake leads to the generation of inflammatory cytokines such as TNFα/β, IL-1 β, IL-6, and interferon beta and gamma (IFNα/β). The level of these cytokines are significantly reduced in the absence of CD36 from macrophages (Janabi et al., 2000).

Dual knockdown of CD36 and scavenger receptor A I/II (SRA) in mice show 75%-90% decrease in lipoprotein uptake and degradation. This strain of mice was obtained by intercrossing mice that lack SR-A or CD36, producing animals lacking both receptors. The knockout of both CD36 and SRA results in no better protection from murine atherosclerotic lesion development than absence of CD36 alone. These findings support the commonly accepted hypothesis that CD36 plays a major role in the pathology of atherosclerosis and its blockage can be protective in the atherosclerotic lesion development (Febbraio et al., 2000; Kunjathoor et al., 2002; Kuchibhotla et al., 2008).

Modulation of scavenger receptors by lipids may potentially impact on the accumulation of cholesteryl esters in macrophages during atherosclerosis (Han et al., 1997).

Tetraspanins

A few members of Tetraspanin protein family were among the proteins that were found to be associated with the receptors of macrophages. This thesis will investigate six isoforms of the Tetraspanin family. Tetraspanins are a broadly expressed superfamily of trans-membrane hydrophobic proteins with over 30 members found in humans and with homologues conserved through distantly related species (Table 1). Tetraspanins are present a variety of organisms such as Caenorhabditis elegans, Drosophila, fungi, plants and vertebrates (Yanez et al., 2009). The tissue distribution of this Tetraspanins is heterogeneous. Although Tetraspanins are very abundant and are found in the membranes of a variety of cells, their individual functions are not very well known. The function of Tetraspanins is still under investigation; however, their key function seems to be in the formation of large membrane micro-domains (also termed the Tetraspanin web or TEMs). By the formation of these membrane domains, Tetraspanins provide a platform for other members of the cell membrane to interact (Yunta & Lazo, 2003). The association of cell membrane components is crucial in the function of cell membrane of eukaryotic cells (Espenel et al., 2008). Tetraspanins are known to be involved in a variety of events including cellular signaling, migration, adhesion, fusion, cytoskeletal reorganization, and proliferation (Martin et al., 2005).

Table 1. List of known members of Tetraspanin family. * indicates Tetraspanins used in this study. Adopted from Yáñez-Mó et al., 2009

Protein Name	Other names
TSPAN1	Net1, TM4-C
TSPAN2 *	
TSPAN3 *	TM4-A, TM4SF8
TSPAN4	NAG2, TM4SF7

TSPAN5	NET4, TM4SF9
TSPAN6 *	TM4SF6, T245
TSPAN7	TM4SF2, A15, CCG-B7, CD231M,MXS1, TALLA-1
TSPAN8 *	Co-029, D6.1, TM4SF4
TSPAN9	NET-5, TNE5
TSPAN10 *	OCULOSPANIN
TSPAN11	CD151-like
TSPAN12	NET-2, TM4SF12
TSPAN13	NET-6, TM4SF13
TSPAN14	TM4SF14, DC-TM4F2
TSPAN15	NET-7, TM4SF15
TSPAN16	TM4-B, TM-8, TM4SF16
TSPAN17	FBX23, TM4SF17
TSPAN18	
TSPAN19	
TSPAN20	Uroplakin 1b, UP1b, UPK1B
TSPAN21	Uroplakin 1a, UP1a, UPK1A
TSPAN22	RDS, Peripherin, AOFMD, AVMD, Nmf193, PRPH, RP7, Rd-2
TSPAN23	ROM1, ROSP1
TSPAN24	CD151, gp27, MER2,PETA-3, RAPH, SFA-1
TSPAN25	CD53, MOX-44, OX-44
TSPAN26	CD37, GP52-40
TSPAN27	CD82, KAI1, 4F9, C32, GR15, IA4, R2, SAR2, ST6
TSPAN28	CD81, TAPA-1, S5.7
TSPAN29	CD9, 5H9, BA2, BTCC-1, DRAP-27, GIG-2,MIC-3, MRP-1, P24
TSPAN30	CD63, LAMP3, ME490
TSPAN31 *	SAS
TSPAN32	ART1, PHEMX, TSSC6
TSPAN33	Penumbra

Structural features of Tetraspanins:

This study aims to investigate the possible functional role of Tetraspanins in phagocytosis mediated by receptors of macrophages. Therefore, understanding the structure of the proteins of this family can be very helpful. Tetraspanins cross the cell membrane four times creating four transmembrane domains with two extra-cytoplasmic regions of unequal sizes, a small extracellular loop (EC1) containing 20 – 28 amino acids and a large extracellular loop (EC2) containing 76 – 131 amino acids flanked by short N and C terminal cytoplasmic tails (Figure 4) (Helmer, 2005). The large extracellular loop has two parts, one is the conserved region containing three alpha helices A, B and E and a variable region (Helmer, 2005). The variable region of the EC2 contains a conserved CCG motif and two other conserved cysteine residues that form the two intra-molecular disulphide bonds as shown in figure 4. Some Tetraspanins have two additional cysteine residues giving rise to an extra disuphide bond (Helmer, 2005).

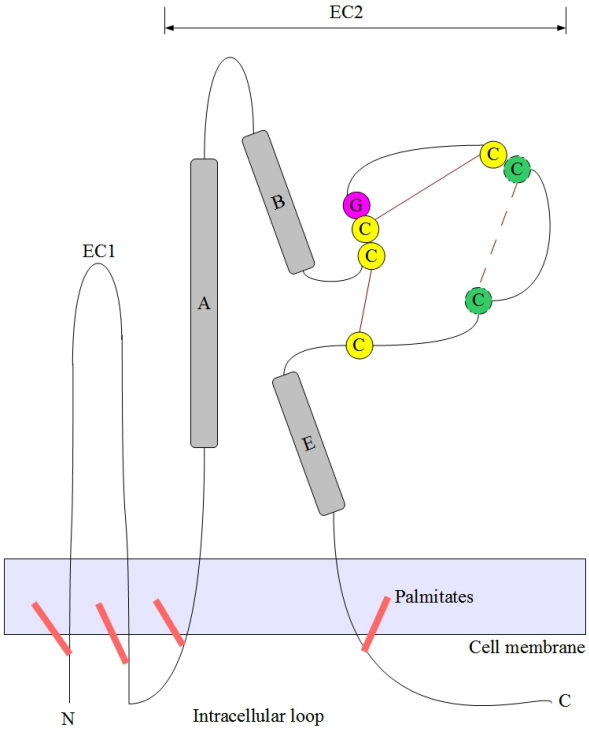


Figure 4. Schematic representation of a generic Tetraspanin. Adopted from Helmer, 2005.

Intracellular and the juxtamembrane regions of Tetraspanins contain several conserved palmitolation sites. (Yanez et al, 2009). Palmitoylation is the covalent attachment of fatty acids, such as palmitic acid, to cysteine and less frequently to serine and threonine residues of proteins, which are usually membrane proteins.

Palmitoylation of cysteine residues has a significant effect on the function of the members of Tetraspanin family. Palmitoylation has been shown to be involved in the formation of associations between Tetraspanins and their partners as well as regulation of interactions with lipid rafts. (Charrin et al., 2002). The palmitoylation of CD9 for instance contributes to the interaction with other Tetraspanins, and in particular to the higher stability of the CD9/CD81 interaction (Charrin et al., 2002).

The size of the protein core of all Tetraspanins is 20-50 kDa (Helmer, 2003; Yunta & Lazo, 2003). Tetraspanins act primarily as novel adapter proteins to facilitate the interaction of associated molecules in the Tetraspanin web since they do not have intrinsic enzymatic activity and signaling motifs in their structure (Martin et al., 2005).

Tetraspanin web:

The large extracellular loop (EC2 region) imparts specificity and allows the identification of a protein as a member of this family. Tetraspanins can also be grouped based on the structure of the large extracellular loop. The EC2 region is the site where the interaction of Tetraspanin with non-Tetraspanin partners takes place (Yunta & Lazo, 2003). The most studied interactions of Tetraspanins are known to occur between different members of the family (Tarrant et al, 2003). An example of interaction between members of Tetraspanin family is the interaction of CD9 and CD51 in human endothelial cells (Barreiro et al., 2008; Levy & Shoham, 2005).

Tetraspanins interact with other types of proteins such as integrins, membrane receptors, and intracellular signaling molecules as well as with other Tetraspanins, thus forming a large complex, which can be combined with other Tetraspanin complexes to act as a membrane micro-domain (Boucheix & Rubinstein, 2001). There are 840 possible ways in which Tetraspanins can interact with other Tetraspanins and with the associated proteins (Tarrant et al, 2003). The flexibility of members of this family to make associations with other proteins and molecules enables them to take part in many different biological roles. One of the roles of Tetraspanins appears to be modifying signals initiated by membrane receptors, by directly or indirectly affecting the intracellular signaling (Berditchevski, 2001).

Both lipid rafts and TEM provide platforms for interaction of various cell membrane proteins; however, they are structurally different. The lipid rafts are disintegrated following cholesterol reduction while TEM are more resistant to the absence of cholesterol (Charrin et al, 2003; Yanez et al., 2009; Espenel et al., 2008). Another structural difference between these two is the absence of lipid raft proteins such as GPI-anchored proteins and caveolin in TEM (Yanez et al., 2009).

Architecture of Tetraspanin domain:

TEMs are known to be very dynamic structures and are constructed from a series of interactions (Martin et al., 2005). The primary interactions, facilitated by the large

extracellular loop, involve direct interaction of Tetraspanins and their partners. The secondary interactions include Tetraspanin-Tetraspanin interactions that enable indirect interaction of primary Tetraspanin complexes. The secondary interactions are done via trans-membrane and/or intracellular regions (Martin et al., 2005; Min, Wang, Sun & Kong, 2006). Tertiary interactions are the weakest complexes and involve the interactions between secondary complexes leading to large signaling domains (Martin et al., 2005).

Tetraspanins are known to be involved in many diseases. For instance, X-linked mental retardation is linked to a mutation of Tetraspanins TM4SF2, also called TALLA-1 (Zemni et al., 2000). The absence of Tetraspanin CD9 in the cells of prostate cancer is known to lead to the increased level of progression of this type of cancer (Wang et al., 2007).

Studies have linked Tetraspanins to viral infections (Martin et al., 2005). For instance, CD151 is known to up-regulate HTLV-1 (Human T-cell Lymphotropic virus) infection and is also expressed in colon and lung cancers (Ashman, L et al., 1997; Sincokc et al., 1997).

Members of Tetraspanin are known to play roles in immunity. For example, CD19-CD81-CD21 complexes are known to play a major role during B cell stimulation. The absence of all 3 types (CD19, CD81 and CD21) in mice show having defective antibody responses to the same type of T cell–dependent antigen (Maecker & Levy 1997; Rickert et al. 1995)

A Study by Kaji et al. (2001) showed that mouse Tetraspanin CD9 is functionally associated with Fc gamma receptors and it is able to regulate signals for phagocytosis and inflammatory responses. In this study, rat anti-CD9 mAb induced the tyrosine phosphorylation of proteins such as Syk as well as cell accumulation, which suggests that CD9 interaction with FcR is required for Fc receptor's proper functioning. Tetraspanin CD82 is involved in the activation of human monocyte cell line U937 (Lebel-Binay, Lagaudriere, Fradelizi & Conjeaud, 1995). It is shown that the association of Fc receptor and CD82 results in an increase in intracellular calcium levels (Lebel-Binay et al., 1995). Another evidence of involvement of Tetraspanins in regulating the fc receptors is the study done by Felming et al, (1997), which suggests that Tetraspanins CD81 negatively

regulates FceRI by the demonstration that antibody against CD81 could inhibit signaling through the FceRI.

A major feature of the members of the Tetraspanin family in vivo is a certain degree of functional redundancy in their functions. A study done by Wright, Moseley and Vanspriel (2004) shows a similar effect of knockouts of CD151, CD81, and CD37 in a particular cell line, suggesting that tetraspanins can either compensate for the absence of the members of the family or they have a very high degree of functional similarities. Another evidence for this hypothesis is the study done by Kaji, Miyazaki and Kudo (2002) which shows that when CD9, responsible for infertility of mice females is knocked out, CD81 expression can compensate for the deficiencies in sperm-egg fusion.

RATIONAL AND CENTRAL HYPOTHESIS

The effect of many proteins at the cell surface receptor complexes as activators or inhibitors and their possible involvement in the downstream signaling pathways are not very well understood. I hypothesize that if Tetraspanins are required for the function of innate immune receptors, then Tetraspanins should be specifically detected with receptors by mass spectrometry. Furthermore, Tetraspanins should be required for the receptors function and could be tested by silencing RNA. Confocal microscopy, silencing RNA and quantifying assays of phagocytosis are performed to study the possible functional role of Tetraspanins in the oxLDL and IgG mediated phagocytosis. Understanding the regulatory roles of Tetraspanins can provide insight into various immune diseases.

MATERIALS AND METHODS

Reagents

Cell culture

Human U937 cells obtained from the American Type culture Collections (ATCC, Manassas, VA, USA) were used is this study and cultured in 37° C at 5% PRMI 1640 medium with L-glutamine (Mediatech, Manassas, VA, USA) with 10% fetal bovine serum (EFBS) (Invitrogen, Carlsbad, CA, USA).

Silencing RNA

Silencing RNA against Tetraspanins 2, 3, 6, 8, 10 and 31 were purchased from Sigma-Aldrich-Proligo (St. Louis, MO, USA) and were used in this study for silencing RNA transfection experiments. The experiment was performed to knock down the proteins to study the phagocytosis of U937 cells in the absence of that particular protein.

Antibodies

Monoclonal antibody Mouse anti-human CD63 from BD pharmegin (Franklin lakes, NJ, USA) was generously provided by Dr. Sergio Grinstein and was used in this study. Secondary antibody Donkey anti mouse was also provided by Dr. Sergio Grinstein.

Experimental procedures

Confirmation of Proteins Identified by SEQUEST and Fc-Ligand Specific Protein Manual Selection

The carefully isolated proteins associated with the macrophage receptors were determined by liquid chromatography and tandem mass spectrometry (LC–MS/MS) (Jankowski et al., 2008). The MS/MS data were then searched against a non-redundant library of proteins using SEQUEST. A Manual examination of the proteins and peptides identified by LARC along with the controls was then performed to ensure that the proteins detected by mass spectrometry were specifically associated with the Fc receptor–ligand complex. This was done to eliminate false positives. The proteins were compared with the control beads incubated with crude lysates or growth medium.

In order to study the role of and to confirm that proteins identified by LARC were truly associated with the macrophages receptor complexes of U937 cells, confocal microscopy, silencing RNA and quantifying assays of phagocytosis were performed.

Silencing RNA transfection

U937 cells were seeded onto (2.5cm) sterile microscope cover slips and placed in (3.5 cm diameter) 6-well plates. U937 cells were plated to approximately 25% confluency in the 6 well plates and were grown for 72 h before removing the old medium and replacing it with new 10% FBS RPMI medium. PMA was added to a final concentration of 100 nM per well in order for monocytes to differentiate. PMA results in the differentiation of monocytes through activation of protein kinase C (Yoshimi et al., 1986).

PMA treated cells were then transfected by short interfering double-stranded RNA (siRNA) using X-tremeGENE siRNA transfection reagent (Roche, Indianapolis, IN, USA). The X-tremeGENE siRNA transfection reagent siRNA was diluted with serum-free medium. The siRNAs against Tetraspanin proteins were also diluted with the serum free medium. The two dilutions were then mixed and incubated for 15 to 20 min. Following the incubation time, the transfection reagent-siRNA complex was added to

PMA treated cells. The cells were then incubated with the complex for 72 hr. In the transfection experiment, scrambled siRNA was used as the negative control while no treatment was used for the positive control.

Bead internalization assay

Following the siRNA transfection experiment, the phagocytosis assay was performed in order to quantify the phagocytic activity of the cells. The cells were incubated with oxLDL and IgG coated micro-beads and examined by florescent microscopy.

○ *Preparation of ligand coated micro-beads*

1 mg of human IgG was dissolved in 1 ml of water. In each well, 1 μl of 2 μm polystyrene beads (Bangs Labratories Inc, Fisher, IN, USA), from a 10% (v/v) suspension stock of approximately 2.185-E10 microbeads per milliliter, was dispersed in 400 μl of phosphate-buffered saline (PBS) and incubated with 2 μl of the IgG solution for 30 min. For the oxLDL assay, 2 μl of oxLDL was added to 400 μl of PBS and incubated with 1 μl of beads per well. The IgG-bead and oxLDL-bead complexes were then washed with 500 μl PBS three times to remove any unbound beads followed each time by pelleting beads in a micro-centrifuge at 14,000 RMP. The oxLDL and IgG bead complexes were then re-suspended in 200 μl PBS and added to the 6-well plate dishes.

○ *Fixing and blocking of the cells*

The cover slips were then washed with ice-cold PBS. The cells were fixed with 4% paraformaldehyde for 20 min. Fixing was terminated by adding PBS with 5% Glycine followed by blocking using PBS with 5% skim milk powder and 1% donkey serum.

○ *Antibody staining of cells*

The cover-slips exposed to oxLDL-coated beads were then incubated with anti-oxidized human lipoprotein antibody (1:25) for 30 min followed by secondary cy5-conjugated donkey anti rabbit antibody (1:10,000 -1:20,000) for 30 min in dark. Cover-slips exposed to IgG-bead complexes were incubated with cy5-conjugated donkey anti human antibody

(1:10,000 -1:20,000) for 30 min in dark. Cover-slips in both cases were then washed three times with PBS and mounted on microscopic slides with fluorescence mounting medium (Dako, Carpentaria, CA, USA).

The internalization of oxLDL-coated microspheres was studied in two additional buffers. After the cells were grown in RPMI media for 72 hr, as discussed above, they were incubated with oxLDL-bead complexes in Hepes buffer for 2 and 4 hr, and in RPMI medium without fetal bovine serum for 4 hr. This step was then followed by the aforementioned fixing and blocking methods.

Confocal Microscopy Imaging

Images of cells binding ligand-coated beads were recorded on a Zeiss LSM 510 Meta Confocal Microscope with Ar 488-nm and He/Ne 543-nm lasers with 40x objective lens and analyzed using Zeiss LSM Image Browser.

The beads were then counted using Image J software where the number of the outside beads as well as the total number of beads for each cell was recorded on excel sheet. The number of beads inside the cells was calculated by subtracting the number of beads outside the cell from the total number of beads. The phagocytosis efficiency of the cells was then calculated by dividing the number of inside beads by the total number of beads.

Immunofluorescent Staining

Permeabilized and non permeabilized U937-PMA treated cells were stained with Mouse anti-human CD63 to confirm the localization of tetraspanin CD63. Cells were seeded onto 2.5 cm cover-slips as mentioned above and incubated with oxLDL and IgG coated beads for 4 and 2 hr respectively. Cells were the fixed with 3% paraformaldehyde at room temperature (RT) for 20 min, blocked with 1% donkey serum for 30 min and permeabilized with 0.01% Triton at RT for 30. The cells were incubated with anti-oxidized human lipoprotein antibody followed by cy5 conjugated Donkey anti-rabbit for oxLDL coverslips and with cy5-conjugated donkey anti human antibody for IgG

coverslips in order to visualize the outside beads. The cells were then incubated with primary mAb mentioned above for 1 h at 1:25 concentration, followed by staining with a secondary antibody at 1:1000 concentration in dark for 1 hr. As a control, permeabilized and non permeabilized U937 cells were incubated with secondary antibody alone. After each antibody incubation, the cells were washed three times with PBS. The coverslips were washed five times at the end before affixing them to microscopic slides with mounting medium. For immunofluorescent analysis, the cells were examined with Zeiss LSM 510 Meta Confocal Microscope.

RESULTS

Manual inspection of Tetraspanin proteins identified by LARC along with the controls was performed in order to select the potential candidates that are involved with the uptake of oxLDL and IgG from this family. Following careful considerations, Tetraspanins 2,3,6,8,10 and 31 were selected from the list of all proteins detected by mass spectrometry.

Table 2 shows the Tetraspanin proteins identified by mass spectrometry with the number of peptide hits for each of the conditions. LARC targeted both CD36 and Fc receptors and the proteins associated with both receptors were identified. In the table below the Larc-oxldl column shows the number of times that the following Tetraspanins were identified when oxLDL coated beads were incubated with U937 cells. The column named Larc-oxldl-media beads shows the number of times the proteins were seen in the media in which the oxldl-coated beads were incubated with the cells. The column Larc-oxldl-control shows the number of times that the mentioned proteins are seen in oxLDL coated-beads applied to crude homogenates. The Larc-IgG column represents the proteins seen when IgG-coated beads were incubated with live cells. Larc-IgG control shows the proteins seen in IgG-coated beads incubated with crude homogenates. Larc-untreated beads shows the number of times the proteins were seen when cells were incubated with untreated beads. The Larc-uncoated beads extract shows uncoated beads incubated in the crude homogenates. The proteins with the high number of hits in the LARC IgG and LARC oxLDL compared to the controls were picked as potential candidates for this study.

LARC spectrometry was performed as described (Jankowski et al, 2008) and the following data was obtained. Each number represents a peptide from the appropriate Tetraspanin indicated in each column.

Table 2- Tetraspanin peptides detected by mass spectrometry-

Gene sybmol	LARC -oxldl	LARC -oxldl-media-beads	LARC -oxldl-control	LARC -anti CD36-IgA	LARC -anti CD36-IgA-control.	LARC -IgG	LARC-IgG-Controls	LARC-untreated beads	LARC-Uncoated Beads-Extract
TSPAN2	32	29	0	17	8	20	8	0	9
TSPAN3	48	45	1	19	6	16	7	1	27
TSPAN6	86	36	0	72	36	26	20	0	27
TSPAN8	33	29	0	9	7	4	4	4	3
TSPAN10	150	145	0	61	45	31	44	2	49
TSPAN31	181	133	1	53	36	25	22	4	60
TSPAN11	272	200	0	60	20	20	8	0	9
TSPAN32	181	133	1	72	19	16	7	1	27
TSPAN17	37	28	0	12	8	21	11	3	10
TSPAN7	11	6	0	9	2	21	11	3	10
TSPAN11	77	24	0	23	11	21	11	18	25

Silencing RNA transfection coupled to beads internalization assay

○ *IgG-coated beads internalization assay in growth media*

Silencing RNA was used against Tetraspanins 2, 3, 6, 8, 10 and 31. A total of three independent experiments were done to study the functional requirements of Tetraspanins in the Fc receptor mediated phagocytosis in U937 cells. Figure 5 shows the mean of

phagocytic efficiency of U937 cells incubated with IgG coated beads in growth media with serum for three independent experiments.

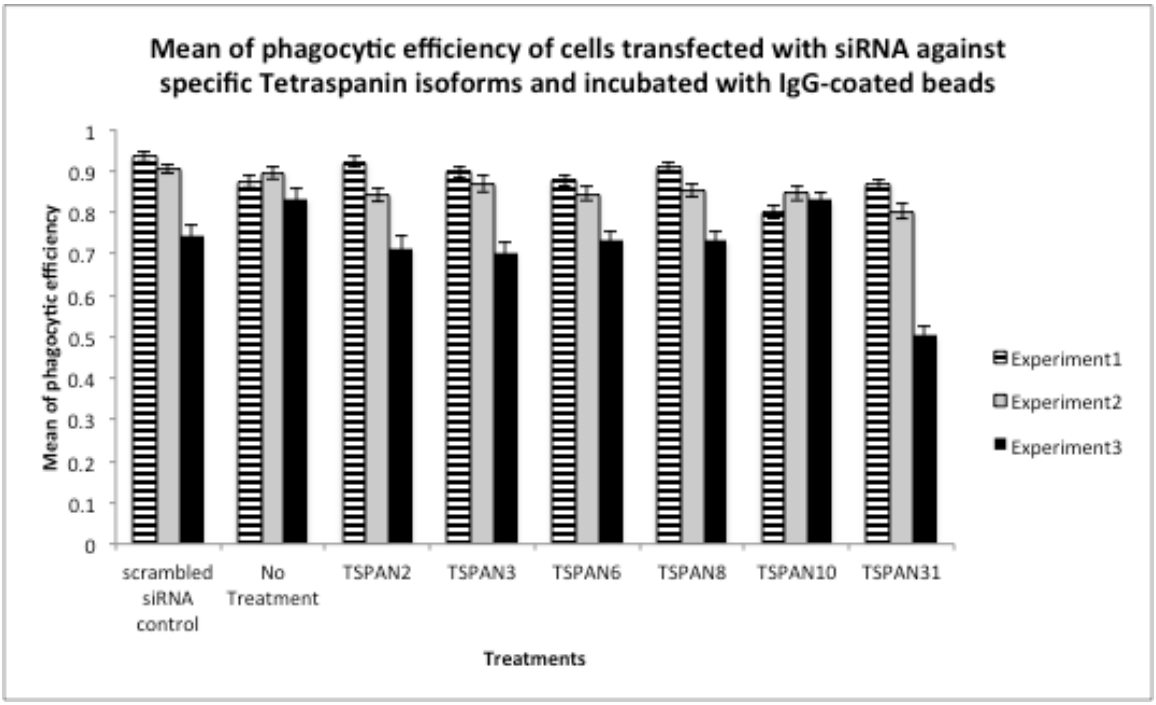


Figure 5- Mean of the phagocytic efficiency of U937 cells incubated with IgG-coated beads in growth media with serum for three independent experiments

Each bar represents the mean of the phagocytic efficiency of the cells (n=300 cells for each treatment). Cells were differentiated for 3 days with 100nM PMA, transfected with siRNA against Tetraspanins 2,3,6,8,10 and 31, and incubated with IgG-coated beads in growth media with 10% FBS for 2 hr. Scrambled siRNA served as a control in the transfection experiments along with cells with no treatment. Cells were then fixed and blocked and stained with cy5-conjugated donkey anti IgG antibody. Following confocal microscopy imaging, the quantification of the beads was then performed by counting the outside and total number of beads using Image J software. The number of internalized beads was counted for each cell and divided by the total number of beads for that cell to obtain the phagocytic efficiency. The average of phagocytic efficiency of each treatment was calculated and used to plot this figure.

Figure 6 shows the average of all three experiments done on the IgG-mediated phagocytosis of the cells in the absence of Tetraspanins 2,3,6,8,10 and 31. The statistical analysis of results of these three experiments is shown in Appendix D-F.

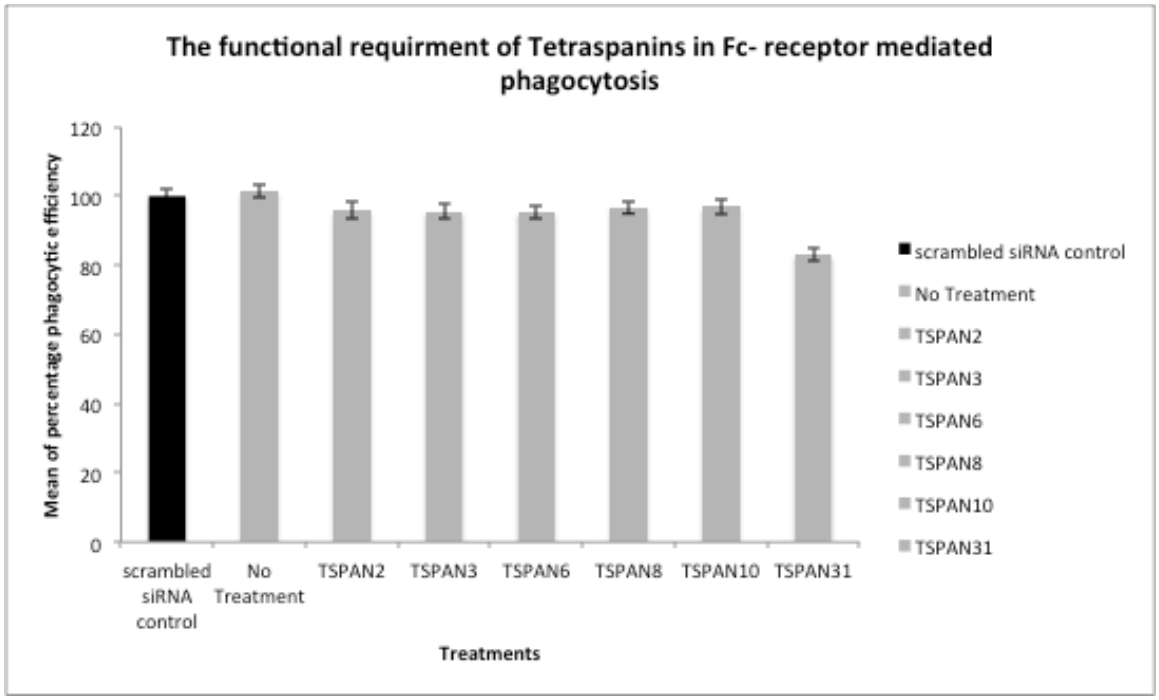


Figure 6- Reduction of mRNA for Tetraspanins 2, 3, 6, 8, 10 and 31 in U937 cells reduces the level of phagocytosis relative to controls

Each bar represents the average of three independent experiments and shows the overall mean of percentage phagocytic efficiency of the treatments cells (n=300 cells for each treatment). Cells were differentiated for 3 days with 100nM PMA, transfected with siRNA against Tetraspanins 2,3,6,8,10 and 31, and incubated with growth media with 10% FBS for 2 hr. Cells were then fixed and blocked and stained with cy5-conjugated donkey anti IgG antibody. The quantification of the beads was then performed by counting the outside and total number of beads using Image J software. The mean of internalized beads and the phagocytosis efficiency was then calculated for each experiment.

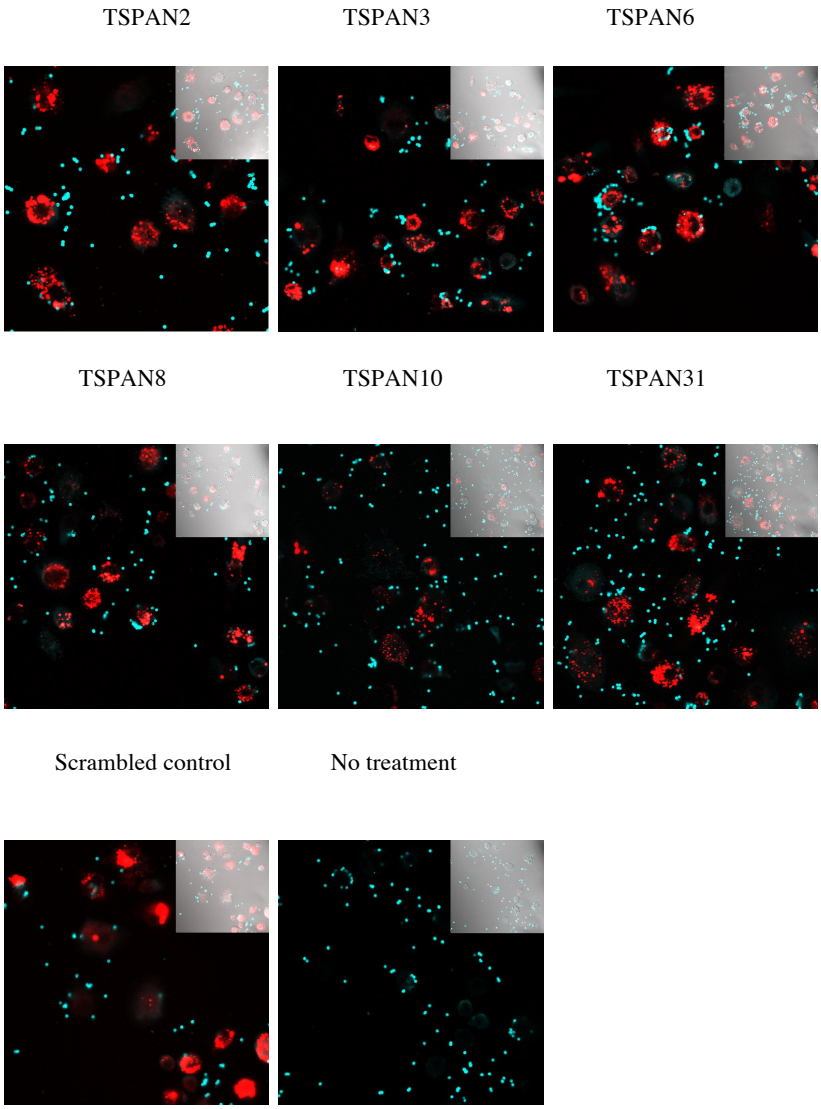


Figure 7- Confocal Microscopy images of the Internalization of IgG-coated beads by U937 cells treated with siRNA against Tetraspanins 2, 3, 6, 8, 10 and 31.

U937 cells were differentiated by 100 nM PMA for three days. The PMA-treated cells were then transfected with cy3-conjugated siRNA against Tetraspanins 2, 3, 6, 8, 10 and 31. The transfected cells were then incubated with IgG coated beads in growth media for

2 hr. The cells were then fixed, blocked and the beads were treated with cy5-conjugated donkey anti-IgG secondary antibody. The blue channel represents the outside beads, and the red channel shows the transfected cells.

- ***oxLDL-coated beads internalization assay in different incubation buffers***

Silencing RNA transfection of U937 cells against Tetraspanins 2,3,6,8,10 and 31 were also done to study the internalization of oxLDL by macrophages. In order to study the role of Tetraspanins in oxLDL-mediated phagocytosis, silencing RNA was used against tetraspanins 2,3,6,8,10 and 31. A total of four independent experiments were done. The internalization of oxLDL-coated microspheres was studied in four different conditions as shown in Figure 8. After the cells were grown in RPMI media for 72 hr, as discussed previously, they were incubated with oxLDL-bead complexes in growth media for 4 hr, in Hepes buffer for 2 and 4 hr, and in RPMI medium without fetal bovine serum for 4 hr. Figure 8 shows the mean of phagocytic efficiency of oxLDL-coated beads internalization by U937 cells for four independent experiments with different incubation conditions.

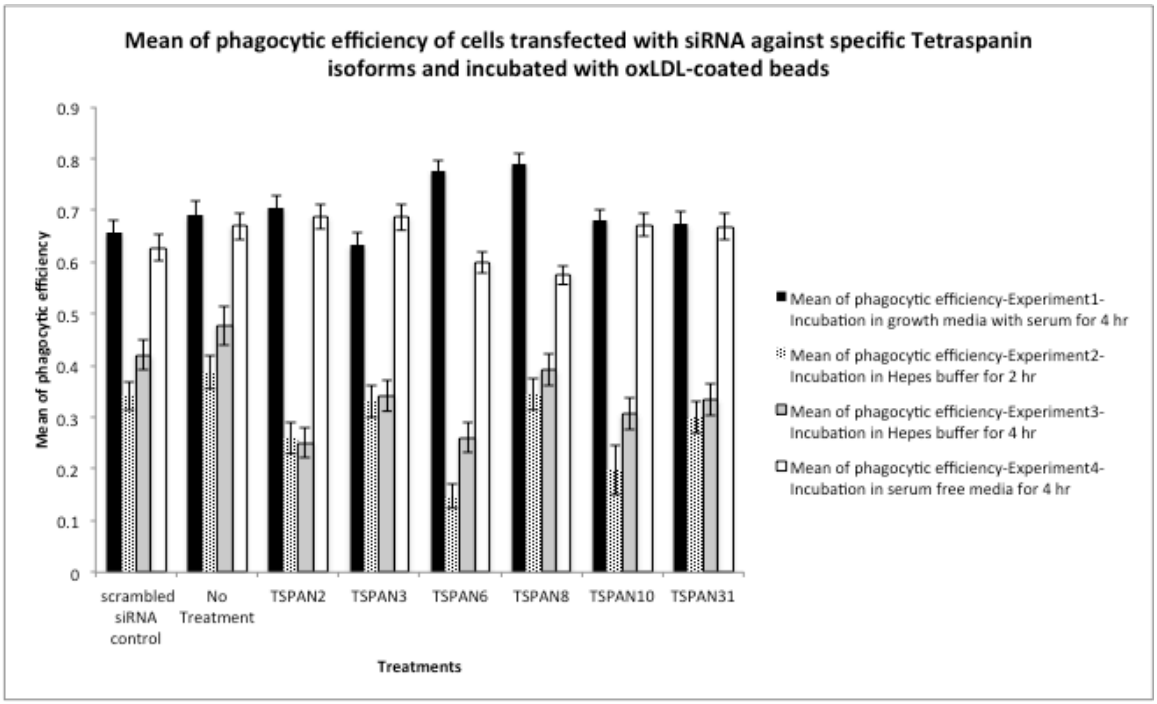


Figure 8- Mean of the phagocytic efficiency of U937 cells incubated with oxLDL-coated beads in four different incubation conditions

Each bar represents the mean of the phagocytic efficiency of U937 cells for each of the experiments (n=150 cells for each treatment). Cells were differentiated in 100 nM PMA for three days. The PMA-treated cells were then transfected with cy3- conjugated siRNA against Tetraspanins 2,3,6,8,10 and 31. Scrambled siRNA served as a control in this study along with cells with no treatments. The transfected cells were then incubated with oxLDL-coated beads in growth media for 2 hr, Hepes buffer for 2 and 4 hr, and in serum free media for 4 hr. The cells were then fixed by a solution of 4% paraformaldehyde, and blocked by donkey serum and skim milk. They were then stained by rabbit anti oxLDL antibody, followed by incubation cy5 conjugated donkey anti-rabbit antibody to visualize non-internalized beads. The phagocytic efficiency of cells was the calculated for each transfection treatment.

- ***oxLDL-coated beads internalization assay in Hepes buffer for 4 hr***

Following statistical analysis of the data for oxLDL-mediated phagocytosis of U937 cells in all four experiments (Appendix G-J), the incubation in Hepes buffer for 4 hr showed more significant difference among treatments than other incubation conditions. Therefore, another experiment was done on oxLDL-mediated phagocytosis of U937 cells transected against Tetraspanins 2,6,10 and 31 in Hepes buffer for 4 hrs (Appendix K, L ,I). The knock down of the Tetraspanins showed a significant effect on the phagocytosis efficiency of cells. Figure 9 shows the effect of Tetraspanin 2,6,10 and 31 knock down on the phagocytosis of oxLDL-coated beads for three independent experiments.

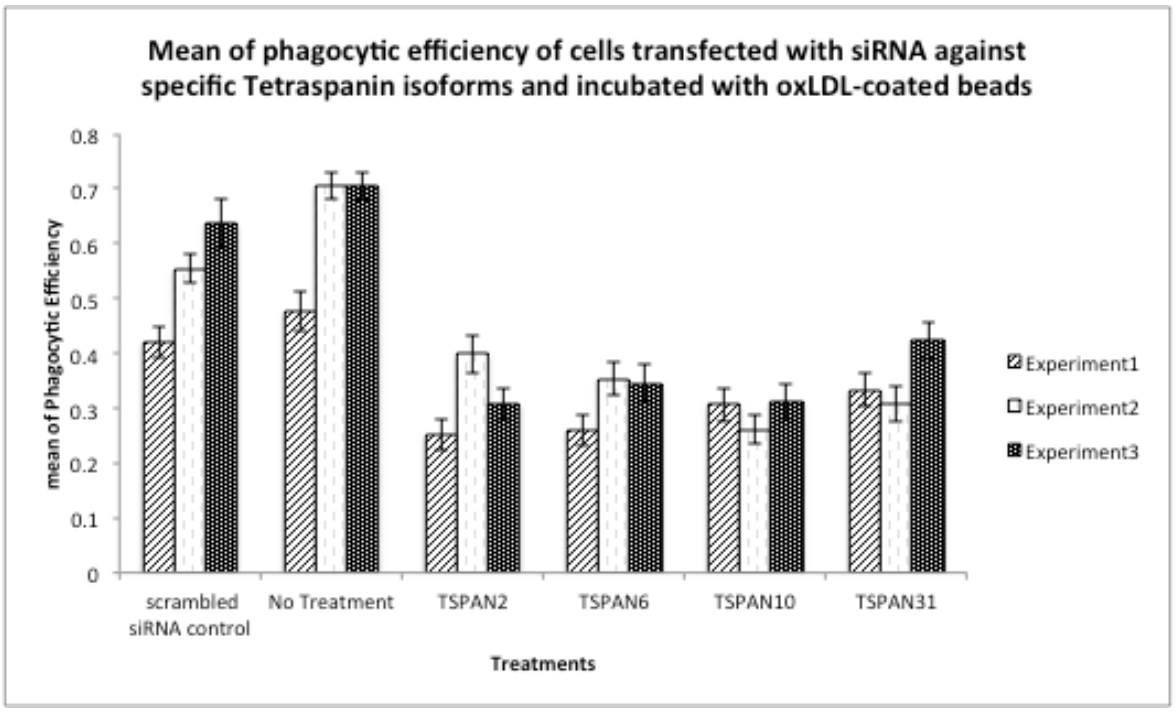


Figure 9- Mean of the phagocytic efficiency of U937 cells incubated with oxLDL-coated beads incubated in Hepes for 4 hr

Each bar represents the mean of the phagocytic efficiency of U937 cells for each of the experiments (n=150 cells for each treatment). Cells were differentiated in 100 nM PMA for three days. The PMA-treated cells were then transfected with cy3- conjugated siRNA against Tetraspanins 2,6,10 and 31. Scrambeled siRNA served as a control in this study along with cells with no treatments. The transfected cells were then incubated with oxLDL-coated beads in Hepes buffer for 4 hr. The cells were then fixed by a solution of 4% paraformaldehyde, and blocked by donkey serum and skim milk. They were then stained by rabbit anti oxLDL antibody, followed by incubation cy5 conjugated donkey anti-rabbit antibody to visualize non-internalized beads.

Figure 10 shows the effect of Tetraspanins 2,6,10 and 31 knockdowns on the phagocytic efficiency of U937 cells to uptake oxLDL-coated microspheres.

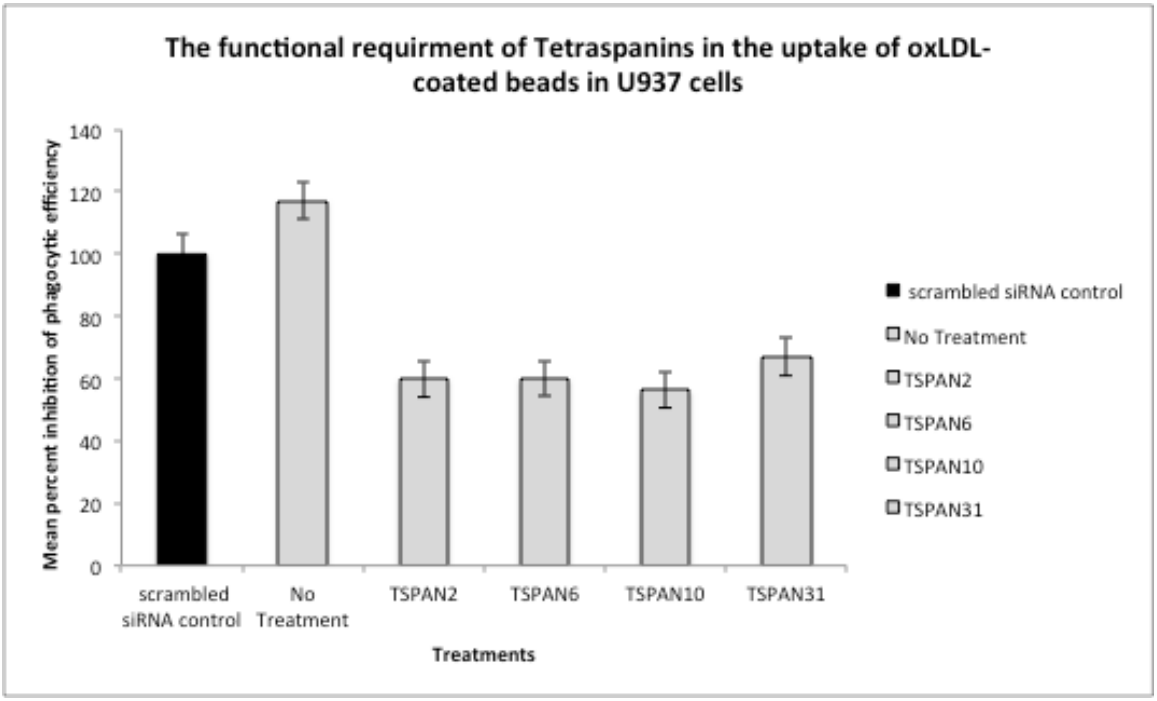


Figure 10- Treatment of U937 cells with siRNA targeting the Tetraspanin molecules reduced their phagocytic efficiency relative to controls

Each bar represents the mean of percentage phagocytic efficiency of transfected cells for three experiments (n=150 cells for each treatment). U937 cells were differentiated in 100 nM PMA for three days. The PMA-treated cells were then transfected with cy3-conjugated siRNA against Tetraspanins 2,6,10 and 31. The transfected cells were later incubated with oxLDL-coated beads in hepes buffer for 4 hr. The cells were then fixed by a solution of 4% paraformaldehyde, and blocked by donkey serum and skim milk. They were then stained with rabbit anti oxLDL antibody, followed by incubation cy5 conjugated donkey anti-rabbit antibody to visualize non-internalized beads. The number of internalized beads as well as the total number of beads was then counted using image J software.

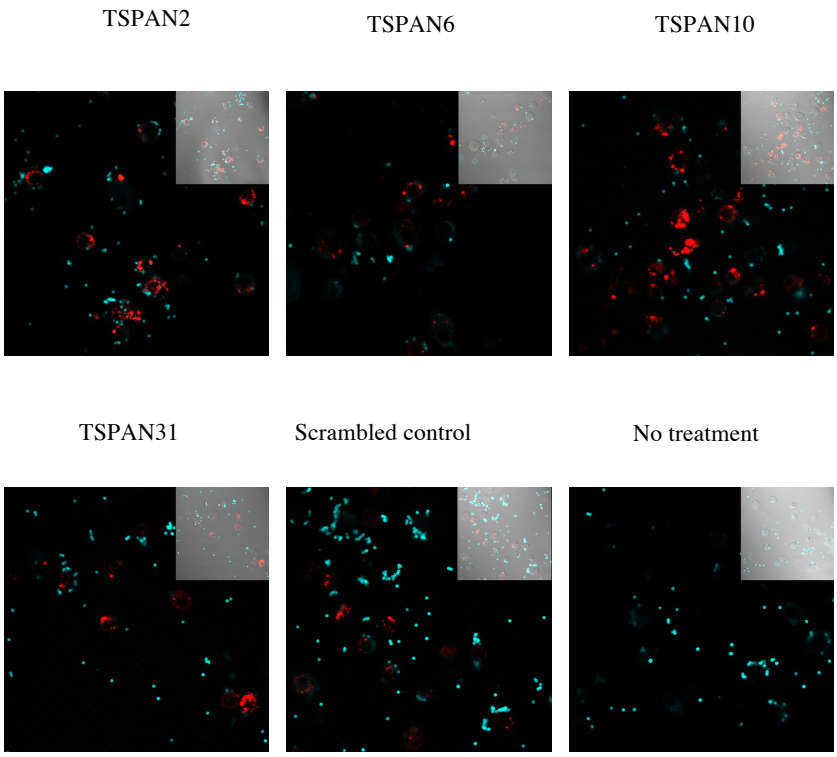


Figure 11- Confocal Microscopy images of the Internalization of oxLDL-coated beads by U937 cells treated with siRNA against Tetraspanins 2, 6, 10 and 31.

U937 cells were differentiated in 100 nM PMA for three days. The PMA-treated cells were then transfected with cy3-conjugated siRNA against Tetraspanins 2, 6, 10 and 31. The transfected cells were then incubated with oxLDL-coated beads. The cells were then fixed, blocked and the beads were stained by rabbit anti oxLDL antibody, followed by incubation with cy5 conjugated donkey anti-rabbit antibody. The blue channel represents the outside beads, and the red channel shows transfection of the cells.

Immunoflorescent staining

Antibody staining was done in order to determine the presence and distribution of Tetraspanin CD63 (tspan30) in macrophages. Mouse anti human CD63 was used in the Immunoflorescent staining of permeablized and non permeablized U937 cells (figure12-13).

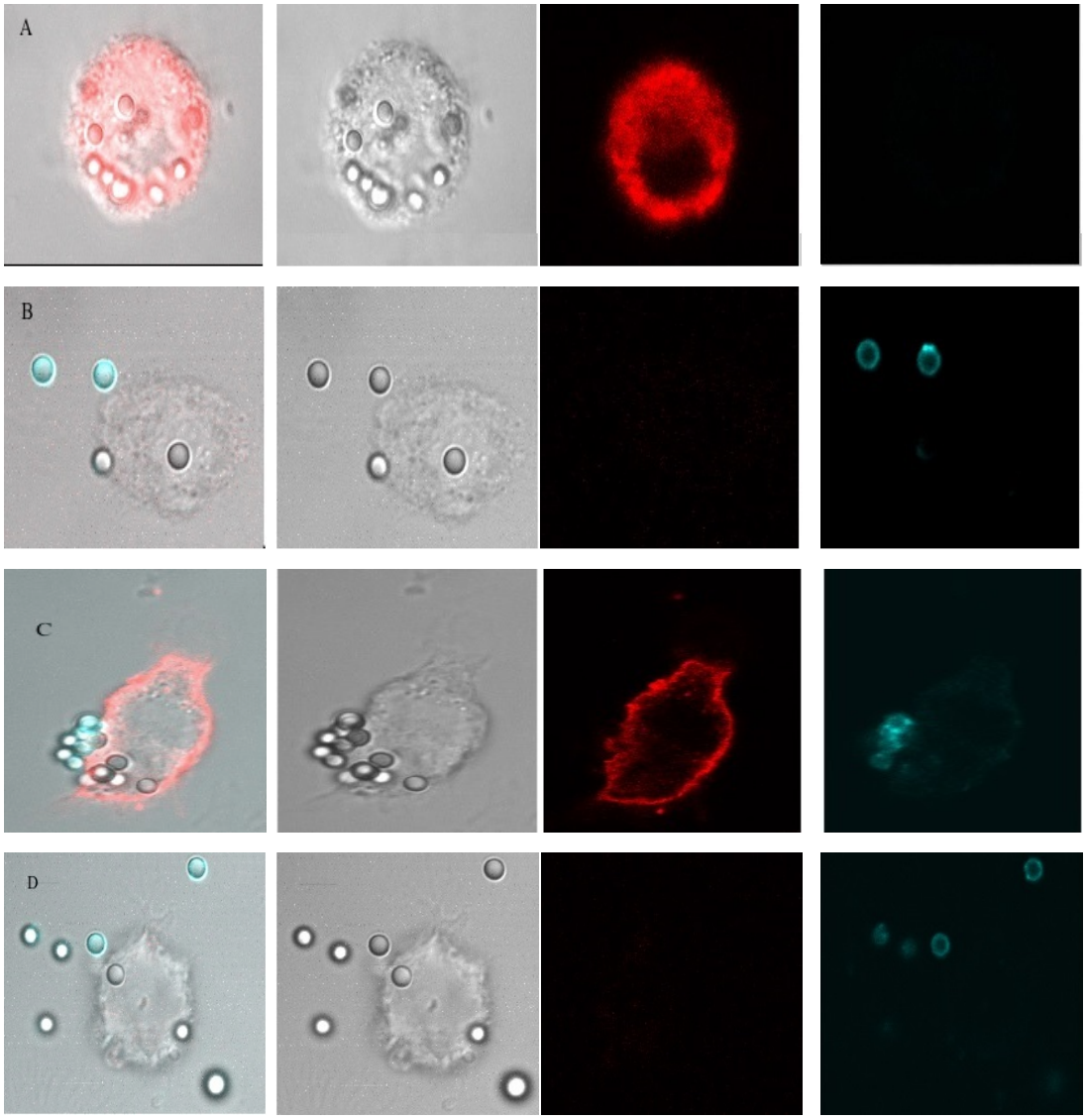


Figure 12- Immunoflorescent staining of U937 cells, incubated with oxLDL beads, with Mouse anti human CD63 antibody with or without a 30 minute 0.1% Triton X-100 permeablization step.

Row A. Permeablized U937 cells incubated with oxLDL and stained with Mouse anti human CD63 antibody and donkey anti Mouse secondary antibody- Row B. .

Permeablized U937 cells incubated with oxLDL and stained with donkey anti Mouse secondary antibody only Row C- Non-permeablized U937 cells incubated with oxLDL and stained with Mouse anti human CD63 antibody and donkey anti Mouse secondary antibody. Row D- Non-permeablized U937 cells incubated with oxLDL and stained with donkey anti Mouse secondary antibody only.

U937-PMA treated cells were incubated with oxLDL coated beads in serum free media for 4 hr. The cells were then fixed, blocked and stained with rabbit anti oxLDL antibody, followed by incubation cy5 conjugated donkey anti-rabbit antibody to visualize non-internalized beads. This was followed with or without a 30 minute 0.1% Triton X-100 permeablization step. The cells were stained with Mouse anti human CD63 antibody followed by staining with donkey anti Mouse secondary antibody in order to visualize CD63. The blue channel shows the outside beads and the red channel represents the CD63 localization.

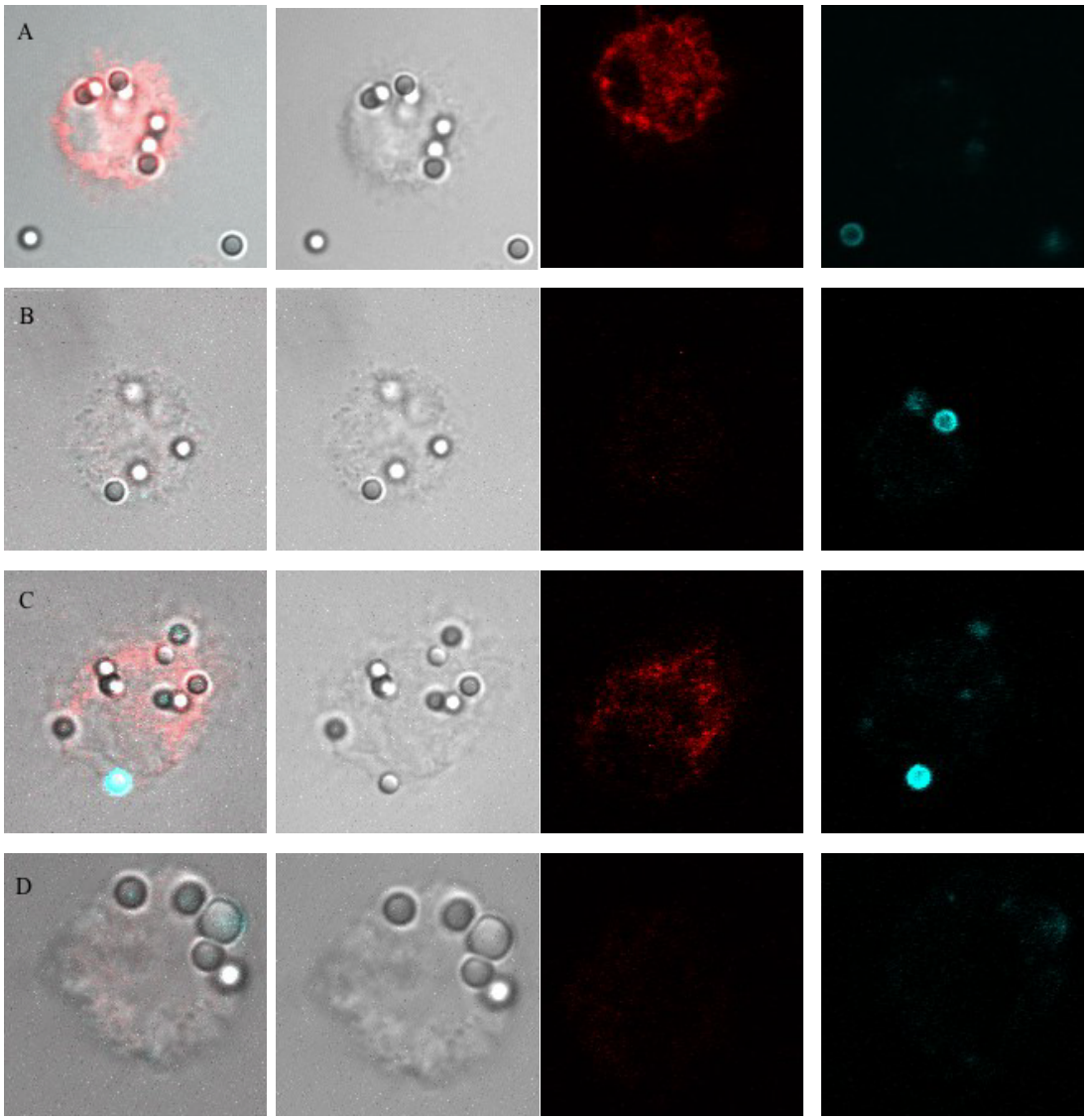


Figure 13- Immunoflorescent staining of U937 cells, incubated with IgG beads, with Mouse anti human CD63 antibody with or without a 30 minute 0.1% Triton X-100 permeablization step.

Row A. Permeablized U937 cells incubated with IgG and stained with Mouse anti human CD63 antibody and donkey anti Mouse secondary antibody- Row B. Permeablized U937 cells were incubated with IgG and stained with donkey anti Mouse secondary antibody only- Row C. Non-permeablized U937 cells incubated with IgG and stained with Mouse anti human CD63 antibody and donkey anti Mouse secondary antibody- Row D. Non-permeablized U937 cells incubated with IgG and stained with donkey anti Mouse secondary antibody only.

U937-PMA treated cells were incubated with IgG coated beads in growth media for 2 hr. The cells were then fixed, blocked and stained with cy5-conjugated donkey anti IgG secondary antibody to visualize non-internalized beads. This was followed with or without a 30 minute 0.1% Triton X-100 permeablization step. The cells were stained with Mouse anti human CD63 antibody followed by stating with donkey anti Mouse secondary antibody in order to visualize CD63. The blue channel shows the outside beads and the red channel represents the CD63 localization.

Discussion

Mass spectrometry sensitivity and specificity

Ligand Affinity Receptor Chromatography (LARC) may be used to successfully capture receptor complexes from the surface of live cells and to provide the detailed identification of receptor complex members by LC–MS/MS. By the LARC method many components of the cellular pathways associated with the phagocytic receptors of macrophages can be directly analyzed by mass spectrometry. Controls for non-specific binding from the homogenate or the media are crucially required to interpret the LARC experiment. Certain Tetraspanin proteins were detected in the receptor ligands with much greater frequency than from the beads incubated with crude homogenate. From this observation we conclude that Tetraspanins may be specifically associated with the oxLDL receptor complex. Thus the significant correlation scores, and the presence of at least three different tryptic peptides together with the comparison to control experiments for non-specific binding, minimized the possibility of false positive acceptance of Tetraspanin proteins. Analysis of the proteins identified by mass spectrometry by LARC shows the association of members of Tetraspanin family with the receptors responsible for uptake of IgG and oxLDL. We conclude that tetraspanins 2,3,6,8,10 and 31 were among the proteins detected by LARC to be specifically associated with macrophages receptors. Hence the biophysical method of mass spectrometry detected the specific presence of Tetraspanins at the activated phagocytic receptor complex. U937 cells line was used in the analysis of this thesis because the same line was used in the LARC experiment and there is a significant amount of studies done on this cell line.

Biochemical confirmations

The independent biochemical techniques of siRNA and immunological staining were performed to confirm the presence of Tetraspanins in the macrophages receptor complexes.

- ***Role of Tetraspanins in IgG mediated phagocytosis***

Silencing RNA experiments may demonstrate a possible functional requirement for Tetraspanins in phagocytic receptors of macrophages. The Fc receptor is one of the very well studied phagocytic receptors of the immune system. It is known to bind the Fc region of Immunoglobulin G. The binding of the Fc receptor to its ligand initiates a series of receptor clustering and downstream signaling pathways. A functional role for Tetraspanins 2,3,6,8,10 and 31 in the Fc receptor complex was tested using siRNA experiments. These Tetraspanins were among the proteins detected by LARC to be associated with the Fc receptor. Analysis of the results obtained from the experiments on the IgG-mediated phagocytosis of U937 cells showed that the knock down of Tetraspanin 31 has a significant effect ($F=11.38$, $Pr < 0.0001$) on the internalization of IgG coated beads by U937 cells. PMA treated U937 cells seem to be less efficient in up taking IgG-coated beads when Tetraspanin 31 is absent. Tetraspanin 31 is also known as SAS. The SAS gene is known to be amplified in human sarcomas (Jankowski et al., 1995). However, its role in immunity is currently unclear. We conclude that tetraspanin 31 might be directly or indirectly binding to the Fc receptor in the micro-domain that it forms on the surface of macrophages surrounding the activated receptor and may play a role in Fc mediated phagocytosis.

- ***Role of Tetraspanins in oxLDL mediated phagocytosis***

oxLDL mediated phagocytosis by macrophages scavenger receptors was another focus of this study. CD36 is the receptor responsible for the uptake of oxLDL by macrophages that later differentiate into foam cells (Collot-Teizeira et al., 2007).

The oxidation of low-density lipoprotein (LDL) in the arterial wall gives rise to the atherosclerotic lesion formation, partly by the uptake of oxLDL by scavenger receptors (Endemann et al., 1993). The oxLDL-mediated phagocytosis was tested under four different conditions in this study. To determine the best incubation condition, the incubation of oxLDL-coated beads with cells was done in growth media for 2hr, Hepes buffer for 2 and 4 hr and in serum free media for 4 hr. We concluded that incubation in Hepes buffer for 4 hr is the optimal condition to study the possible role of Tetraspanins in

oxLDL-mediated phagocytosis without the confounding effect of serum. In serum free experimental buffer the analysis of the results of siRNA experiments showed that knock down of Tetraspanins 2,6,10 and 31 has a significant effect ($F=21.17$, $p < 0.0001$) on the phagocytic efficiency of U937 cells to uptake oxLDL coated beads. The use of the STRING algorithm indicated potential structural or functional interactions of scavenger receptors of macrophages with Tetraspanin 2,6,10 and 31. The individual functions of Tetraspanisn 2,6,10 and 31 are unclear and not much research has been done on these specific isoforms. However Tetraspanins are known to be involved in the endocytosis of oxLDL and may act as part of a system that adapts CD36 to the Fc receptor (Heit el a., 2013). In the Rat, Tetraspanin 2 is involved in signaling in oligodendrocytes differentiation. Tspan2 along with CD81 and an integrin protein were identified by immuneprecipitation to be expressed in developing oligodendrocytes (Birling et al., 1999; Terada et al, 2002).

Tspan6 is involved in the retinoic acid-inducible gene I-like receptor-mediated immune responses, which plays a crucial role in the protection of the body from RNA virus infections (Wang et al, 2012). These receptors along with Toll like receptors (TLRs) and NOD like receptors (NLRs) are members of pattern recognition receptors of the innate immune system that identify pathogen-associated molecular patterns (PAMPs) (Ding, 2012).

Tspan6 works as an inhibitor of RLR-mediated immune signaling since the knockdown of this protein results in an increase in the level of RLR-mediate immune response (Wang et al, 2012). The multiple functions of Tetraspanins in many different receptor driven cellular processes may indicate that Tetraspanins act as generic adaptors between receptors to help stabilize receptor supramolecular complexes. However, further work will need to be done to clarify this further.

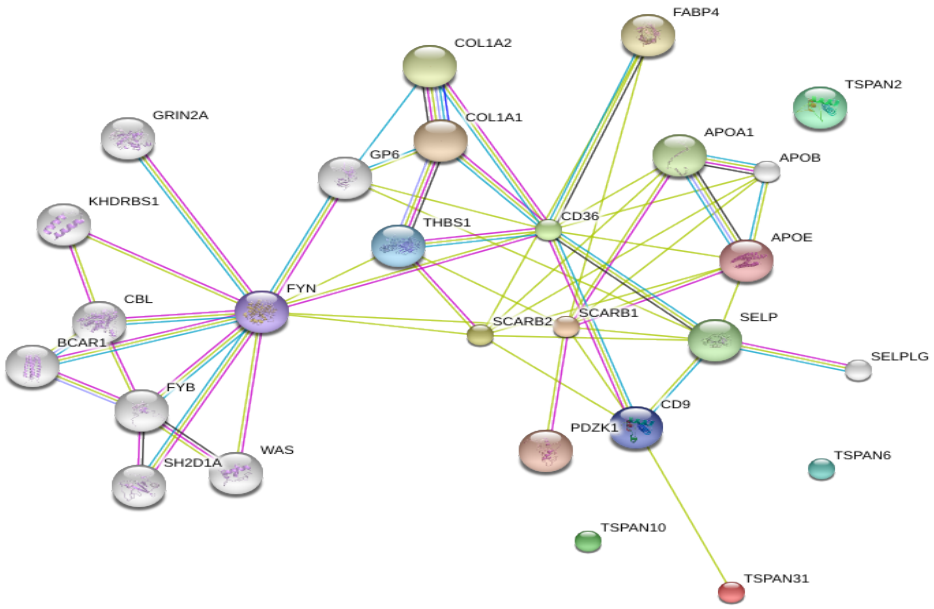


Figure 14- Protein-protein interaction map of Tetraspanins 2,6,10 and 31 and scavenger receptors of macrophages - Generated by STRING9.0 developed by Szklarczyk et al., 2010

○ *Immunoflorescent staining of Tetraspanins*

Immunoflorescent staining was performed to examine the presence and distribution of Tetraspanin CD63 as a representative of Tetraspanin family.

Tetraspanin CD63 also known as TSPAN30, LAMP3 and ME490 is known to be highly glycosylated and involved in many pathways. CD63 interacts with many partners such as MHC class II molecules, integrins, phosphatidylinositol 4-kinase and Tetraspanins like CD82 (Kuijpers et al., 1991; Duffield et al., 2007). CD63 is involved in Weibel-Palade bodies of endothelial cells (Vischer & Wagner, 1993), and its expression elevates after activation of platelets, neutrophils and basophils (Kuijpers et al., 1991). CD63 was also shown to be involved in the activation of T cell (Hemler, 2005; Pfistershammer et al., 2004). Tetraspanin CD63 is present on secretory vesicles, late endosomes and lysosomes as well as the surface of cell membrane and it cycles between these components (Duffield et al., 2007). CD63 was found to immuneprecipitate with the gastric H,K-ATPase β subunit in parietal cells (Duffield et al., 2007). In this context CD63 acts as a helper in intracellular trafficking from the cell surface to the late endosome-lysosomal compartment in order to aid internalizing HKβ (Duffield et al., 2007). CD63 has

Tyrosine-based motifs on its C-terminus which enables it to play roles in endocytosis and lysosomal targeting by interacting with various adaptor proteins (Boucheix & Rubinstein, 2001; Robinson & Bonifacino, 2001). AP-2 and AP-3 are among the adaptor proteins that CD63 interacts with and since these two are involved in clathrin mediated endocytosis, it is hypothesized that CD63 is involved in internalization of particles associated with the membrane (Rous et al., 2002; Lin et al., 2008). Tetraspanin CD63 is known to associate with two integrins $\alpha_3\beta_1$ and $\alpha_6\beta_{1,}$ which are functionally linked to the scavenger receptor CD36 in human Melanoma cells (Thorne et al., 2000).

The localization of Tetraspanin CD63 was studied in both IgG and oxLDL mediated phagocytosis in u937 cells. In permeablized macrophages incubated with IgG beads, CD63 was distributed evenly throughout the cells, however not so much on the cell membrane. Non-permeablized U937 cells incubated with IgG showed a low level of expression on the cell surface. CD63 was distributed consistently across the permeablized U937 cells incubated with oxLDL beads. The non-permeablized cells incubated with oxLDL only showed exofacial localization. These results may indicate that CD63 is more abundant in macrophages when up taking oxLDL compared to IgG. The immuneflorescence staining experiment shows the presence of Tetraspanins in the phagocytic cup upon internalization of oxLDL-coated beads.

A possible function of Tetraspanins in the oxLDL and IgG mediated phagocytosis

Silencing RNA experiments coupled with an Immunoflorence staining technique identified a number of Tetraspanins as associated with the oxLDL and IgG mediated phagocytosis. According to the study done by Heit et al (2013) the CD36 receptor complex contains molecules such as β1 and β2 integrins and two members of Tetraspanin family, CD9 and CD81, which are known to link CD36 to the immunoreceptor tyrosine activation motif (ITAM) cytoplasmic portion of Fc gamma receptor. This study shows that the coupling of CD36 to the ITAM of the Fc receptor results in the internalization of CD36's ligands via Src and Syk kinases (Heit et al., 2013). In agreement with the previous work we showed here for the first time the identity of novel isoforms of tetraspanins that play apparent role in the Fc and oxLDL receptor complex formation.

A Novel extracellular role for Tetraspanins

Mass spectrometry shows the presence of Tetraspanins in the media in which cells are being incubated with oxLDL-coated beads. This finding may indicate that Tetraspanins are being secreted by macrophages. Tetraspanins are also shown to be highly abundant in blood peptides (Unpublished data). The confirmation of the presence of Tetraspanins in the phagocytic receptor of macrophages by three independent biochemical methods along with the presence of these proteins in the incubation media and their high abundance in blood, can lead to a novel hypothesis on the function of Tetraspanins. Tetraspanins have previously been proposed to function by diffusing laterally within the plane of cell membrane that might help adapt co-receptors or prey molecules to the phagocytic receptors (Heit et al., 2013). The abundance of secreted Tetraspanins may indicate that Tetraspanins may play a role similar in concept to ionophore or siderphores that diffuse away from macrophages to help carry prey molecules back to the phagocytic receptors on the cell surface. Thus the presence of tetraspanins in the cell mediate and blood may indicate that this complex family of receptor interaction molecules may play novel roles in phagocytosis and the innate immune response mot previously considered, although further evidences are required to establish the mechanism involved.

CONCLUSION AND FUTURE OBJECTIVES

Despite the high abundance of Tetraspanins, their individual functions have not been very well studied. Biophysical and biochemical analysis successfully showed the presence of members of Tetraspanin family at the site of the activated phagocytic receptor complex.

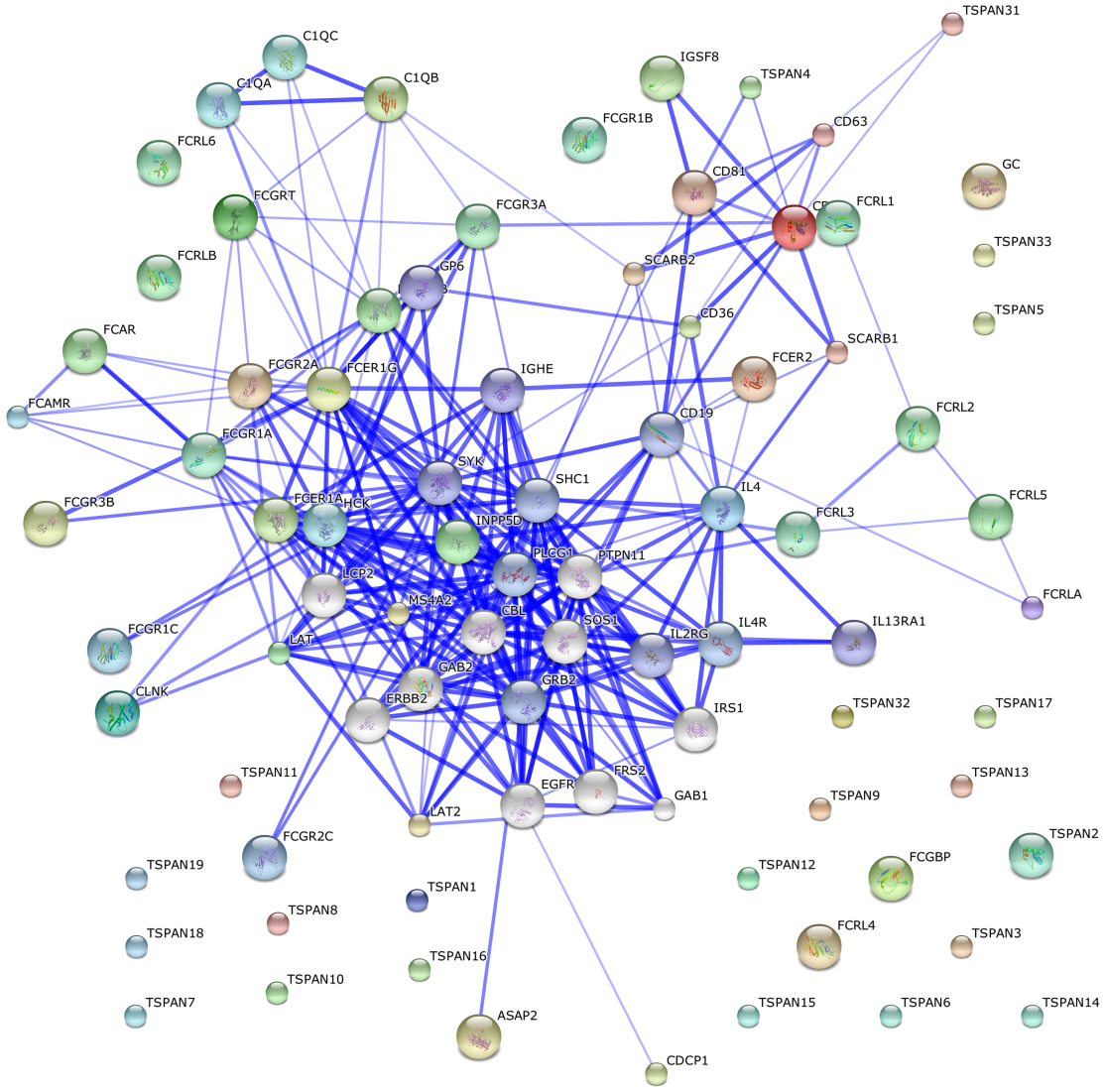
Bead internalization assays were used to assay phagocytic receptor function and showed that Tetraspanins may play a role in phagocytosis by innate immune cells. This study showed that the effect of Tetraspanins on IgG versus oxLDL-mediated phagocytosis can be quantified and confirmed the inhibitory effect of Tetraspanins 2, 6, 10 and 31 on oxLDL-coated beads internalization by U937 cells. The knockdown of Tetraspanin 2, 6, 10 and 31 lowered the ability of cells to uptake oxLDL-coated beads. The knockdown of Tetraspanin 31 also lowered the phagocytic efficiency of cells to internalize IgG-coated beads by U937 cells.

It is of great importance to develop techniques to experimentally differentiate and to distinguish the roles of specific proteins to the oxLDL versus IgG mediated phagocytosis. There may be important therapeutic benefits to inhibiting the uptake of oxLDL by scavenger receptors without interrupting the beneficial clearance of bacteria by Fc receptors. This study shows that the novel biophysical and biochemical employed here can reveal the differences between IgG and oxLDL bead internalization and clearly indicates that the IgG and oxLDL mediated phagocytosis utilized different regulatory mechanisms.

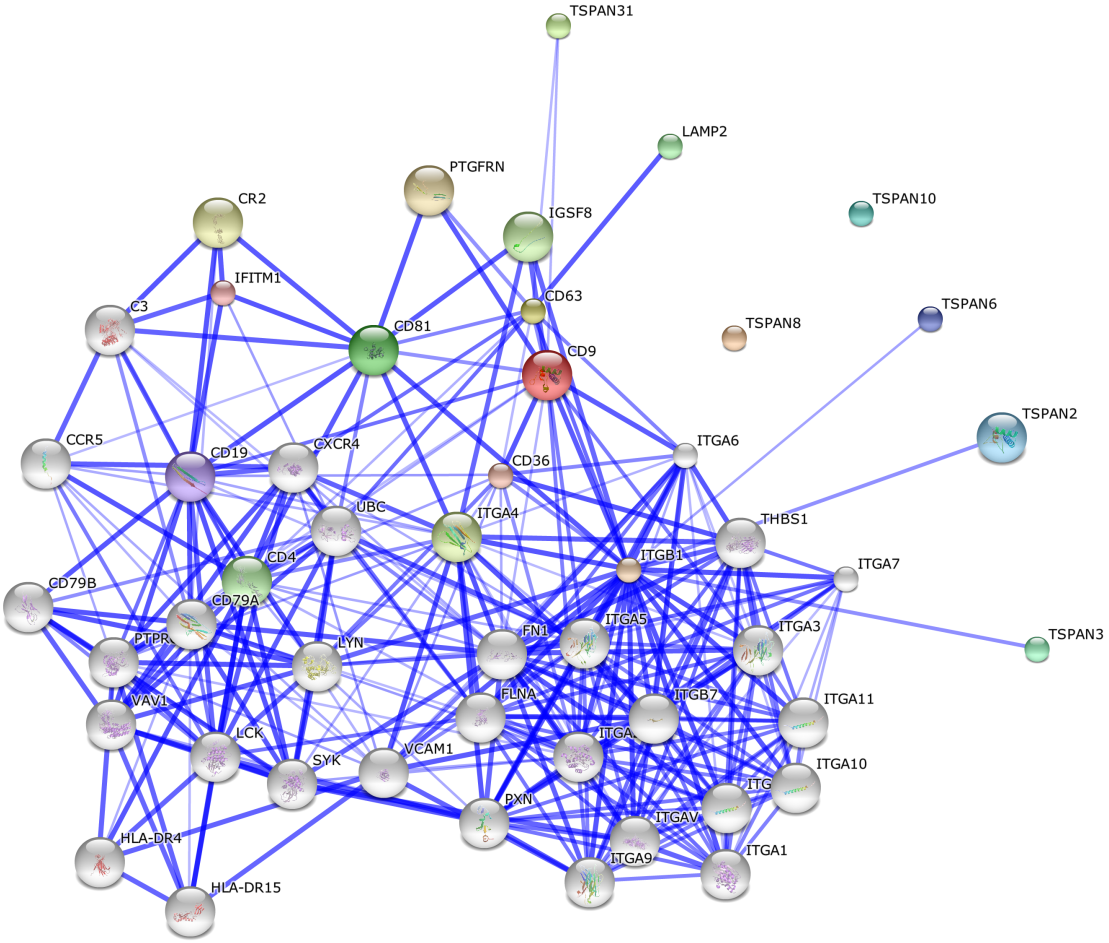
In future studies, GFP constructs as well as immunoflorescent staining can be further used to determine the presence of Tetraspanin 31 in the Fc receptor complex and Tetraspanins 2,6,10 and 31 at the site of oxLDL internalization and to confirm the knockdowns. It is very important to identify as many members of the Tetraspanin family as possible in the macrophage receptor complexes since understanding the regulatory roles of Tetraspanins can provide insight into various immune diseases.

APPENDIX

Appendix A- Protein-protein interaction network map of memebrs of Tetraspanins with Fc and CD36 receptor- Predicted by STRING9.0 developed by Szklarczyk et al., 2010

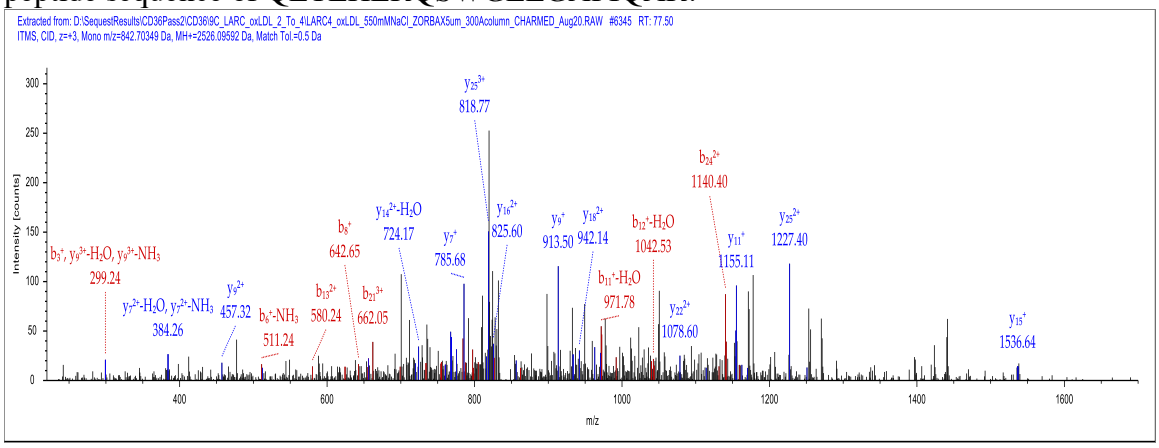


Appendix B- Protein-protein interaction map of Tetraspanins discussed in this study- Generated by STRING9.0 developed by Szklarczyk et al., 2010

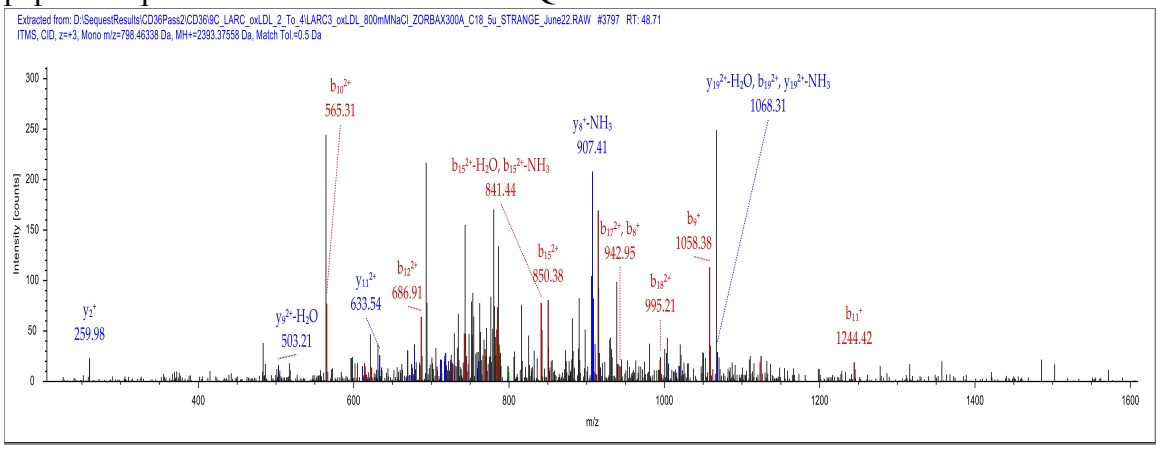


Appendix C-Mass spectrometry detection of Tetraspanins 2,3,6,8,10 and 31

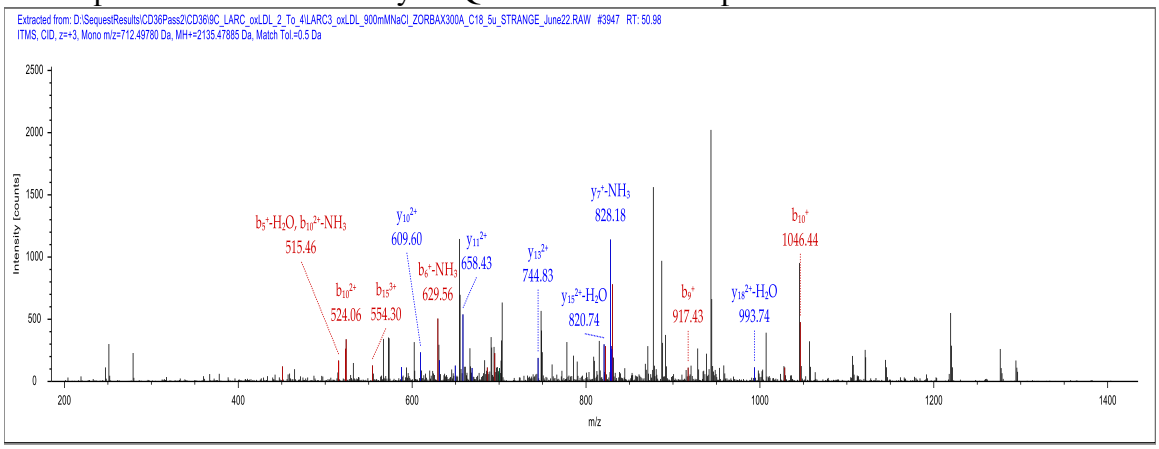
Detention of Tetraspanin 10 by a linear trap at nanomolar concentration. The MS/MS depicting the MH+ 2526.09 Da fragment and peptide fragmentation details of tetraspanin 10. The protein was identified by SEQUEST with a total protein count 526 and single peptide sequence of QLTEHLRQSWGLLGAPIQAR.



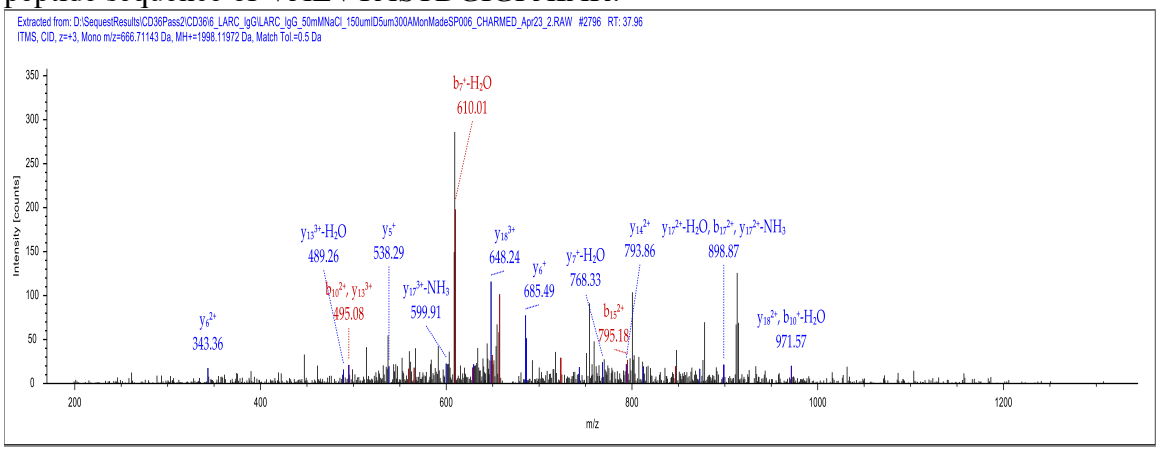
Detention of Tetraspanin 6 by a linear trap at nanomolar concentration. The MS/MS depicting the MH+ 2393.37 Da fragment and peptide fragmentation details of tetraspanin 6. The protein was identified by SEQUEST with a total protein count 437 and single peptide sequence of SEKVKVIDVTVPLQCLVK.



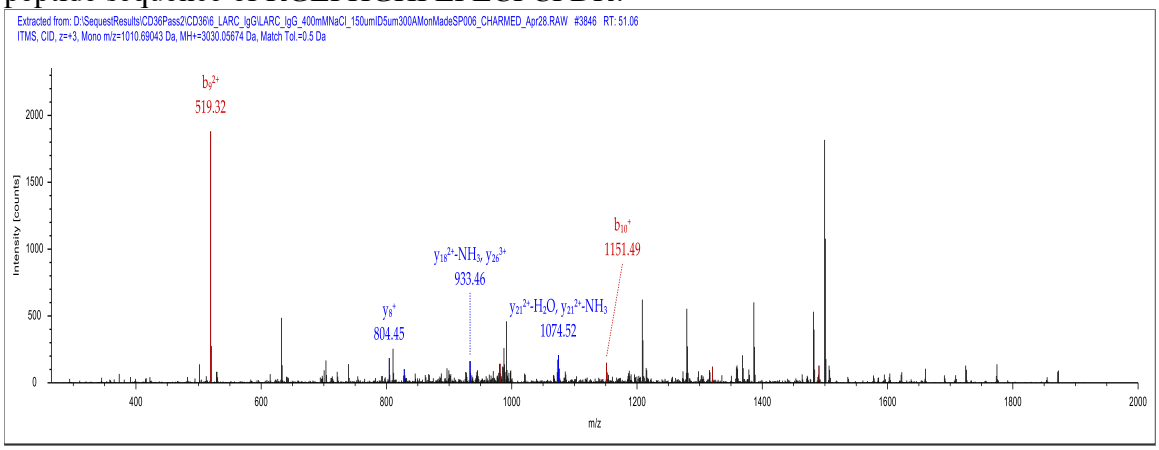
Detention of Tetraspanin 3 by a linear trap at nanomolar concentration. The MS/MS depicting the MH+ 2135.47 Da fragment and peptide fragmentation details of tetraspanin 3. The protein was identified by SEQUEST with a total protein count 129



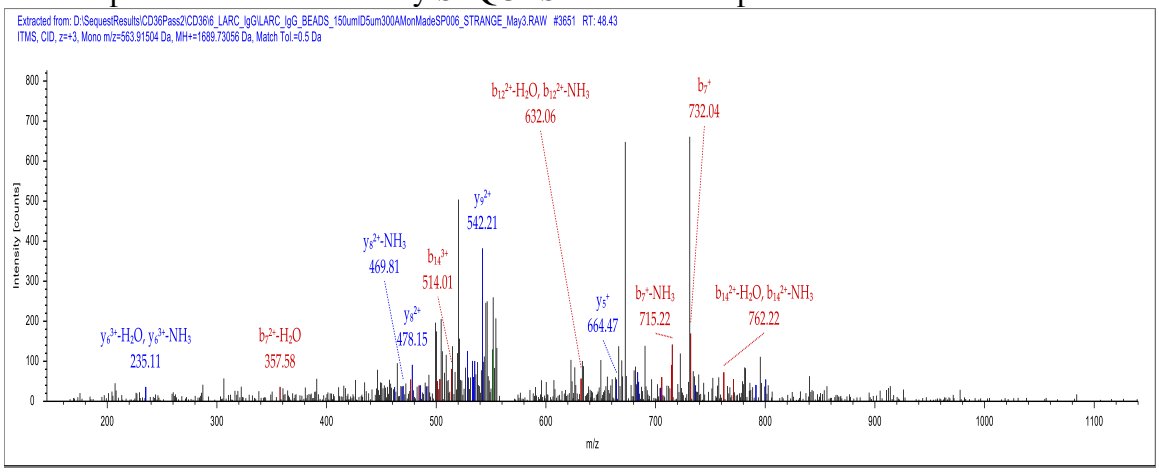
Detention of Tetraspanin 2 by a linear trap at nanomolar concentration. The MS/MS depicting the MH+ 1998.11 Da fragment and peptide fragmentation details of tetraspanin 2. The protein was identified by SEQUEST with a total protein count 98 and single peptide sequence of VALVTASTDGIGFAIAR.



Detention of Tetraspanin 8 by a linear trap at nanomolar concentration. The MS/MS depicting the MH+ 3030.05 Da fragment and peptide fragmentation details of tetraspanin 8. The protein was identified by SEQUEST with a total protein count 168 and single peptide sequence of RGLPHGHPLPLCPCFDR.



Detention of Tetraspanin 31 by a linear trap at nanomolar concentration. The MS/MS depicting the MH+ 1689.73 Da fragment and peptide fragmentation details of tetraspanin 31. The protein was identified by SEQUEST with a total protein count 285.



Appendix D- Statistical analysis of IgG-coated beads internalization of U937 cells transfected with siRNA against Tetraspanins 2,3,6,8,10 and 31- Experiment one

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
Tspan	8	Negative Control Tetraspanin 10 Tetraspanin 2 Tetraspanin 3 Tetraspanin 31 Tetraspanin 6 Tetraspanin 8 scrambled control

Number of Observations Read	1402
Number of Observations Used	1402

The GLM Procedure

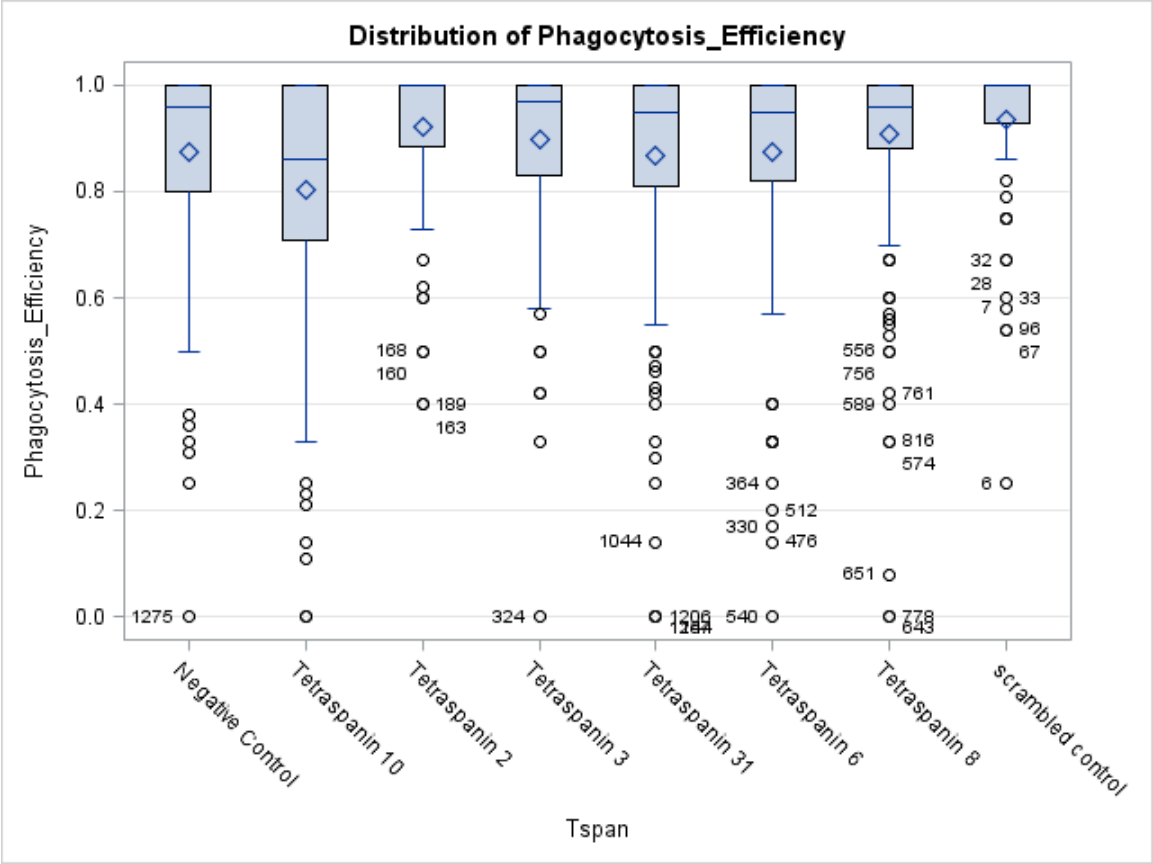
Dependent Variable: Phagocytosis_Efficiency

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1.84380632	0.26340090	8.59	<.0001
Error	1394	42.76488476	0.03067782		
Corrected Total	1401	44.60869108			

R-Square	Coeff Var	Root MSE	Phagocytosis_Efficiency Mean
0.041333	19.86085	0.175151	0.881890

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Tspan	7	1.84380632	0.26340090	8.59	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Tspan	7	1.84380632	0.26340090	8.59	<.0001



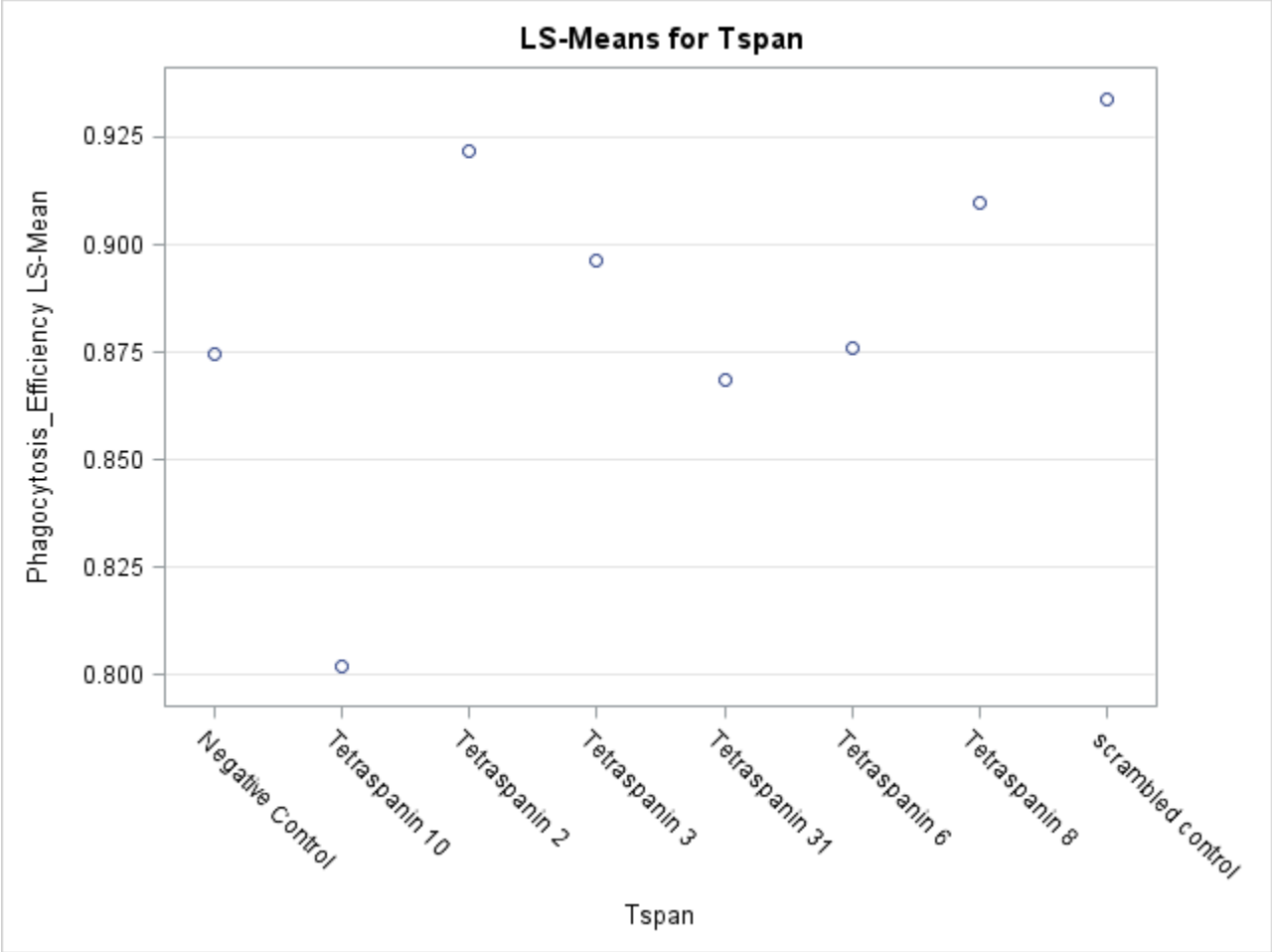
The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

Tspan	Phagocytosis_Efficiency LSMEAN	LSMEAN Number
Negative Control	0.87473684	1
Tetraspanin 10	0.80216374	2
Tetraspanin 2	0.92190000	3
Tetraspanin 3	0.89652893	4
Tetraspanin 31	0.86859438	5
Tetraspanin 6	0.87590698	6
Tetraspanin 8	0.90965035	7
scrambled control	0.93379630	8

Least Squares Means for effect Tspan
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Phagocytosis_Efficiency

i/j	1	2	3	4	5	6	7	8
1		0.0051	0.4210	0.9713	1.0000	1.0000	0.4918	0.1297
2	0.0051		<.0001	0.0002	0.0035	0.0011	<.0001	<.0001
3	0.4210	<.0001		0.9625	0.1676	0.3711	0.9989	0.9997
4	0.9713	0.0002	0.9625		0.8389	0.9689	0.9972	0.7460
5	1.0000	0.0035	0.1676	0.8389		0.9998	0.1220	0.0276
6	1.0000	0.0011	0.3711	0.9689	0.9998		0.3932	0.0952
7	0.4918	<.0001	0.9989	0.9972	0.1220	0.3932		0.9259
8	0.1297	<.0001	0.9997	0.7460	0.0276	0.0952	0.9259	

The highlighted values show pairs of means that are significantly different.



Appendix E- Statistical Analysis of IgG-coated beads internalization by U937 cells transfected with siRNA against Tetraspanins 2,3,6,8,10 and 32- Experiment 2

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
tspan	8	Negative control Tetraspanin 10 Tetraspanin 2 Tetraspanin 3 Tetraspanin 31 Tetraspanin 6 Tetraspanin 8 scrambled control

Number of Observations Read	2271
Number of Observations Used	2252

The GLM Procedure

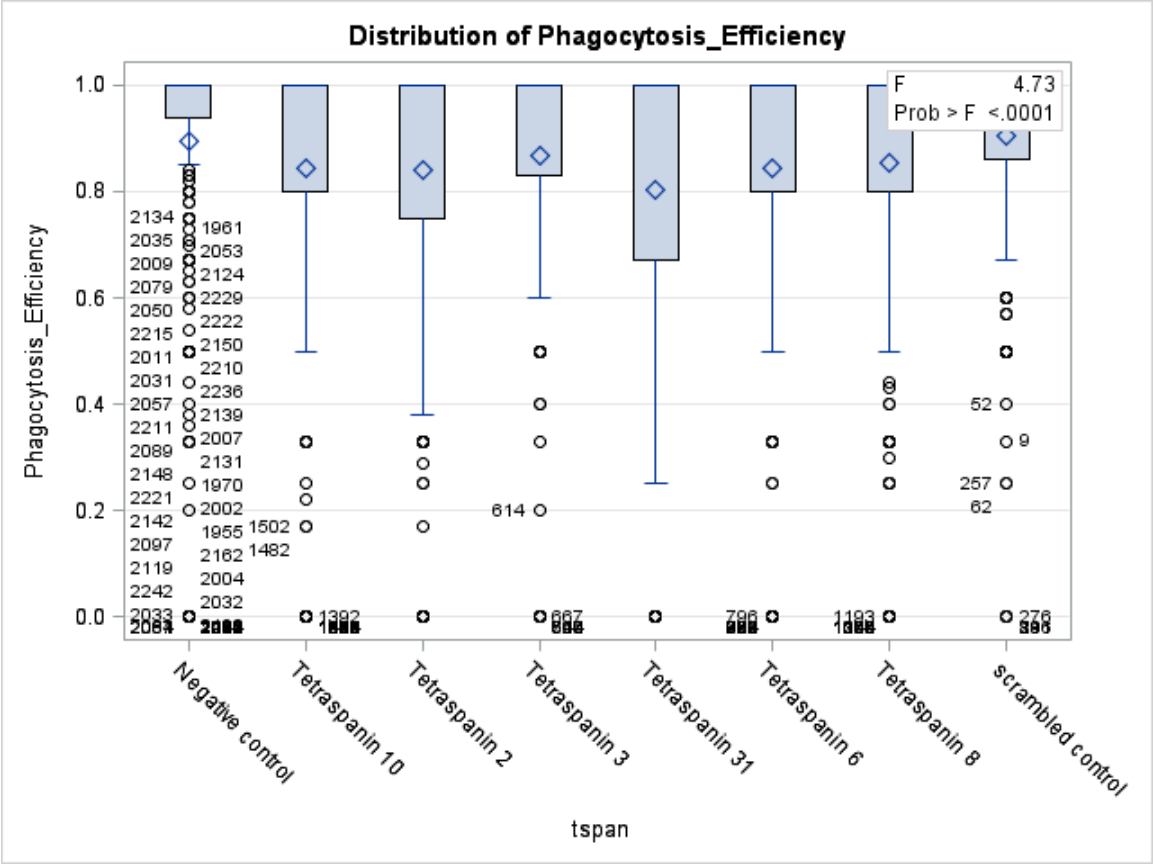
Dependent Variable: Phagocytosis_Efficiency

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	2.1879420	0.3125631	4.73	<.0001
Error	2244	148.3974705	0.0661308		
Corrected Total	2251	150.5854126			

R-Square	Coeff Var	Root MSE	Phagocytosis_Efficiency Mean
0.014530	30.03730	0.257159	0.856132

Source	DF	Type I SS	Mean Square	F Value	Pr > F
tspan	7	2.18794203	0.31256315	4.73	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
tspan	7	2.18794203	0.31256315	4.73	<.0001



The SAS System

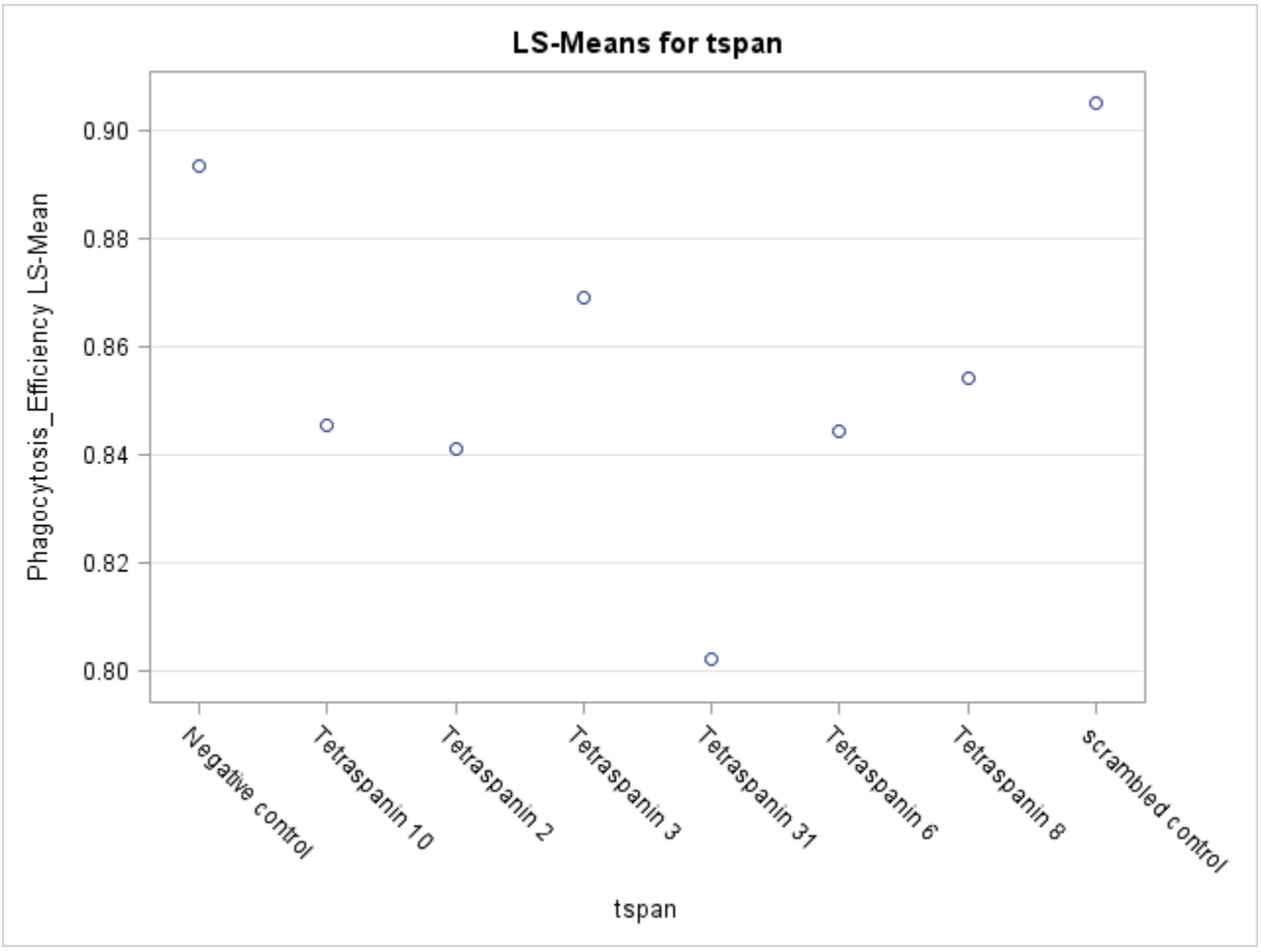
The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

tspan	Phagocytosis_Efficiency LSMEAN	LSMEAN Number
Negative control	0.89360000	1
Tetraspanin 10	0.84536667	2
Tetraspanin 2	0.84100334	3
Tetraspanin 3	0.86922078	4
Tetraspanin 31	0.80213333	5
Tetraspanin 6	0.84454849	6
Tetraspanin 8	0.85430000	7
scrambled control	0.90516667	8

Least Squares Means for effect tspan
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Phagocytosis_Efficiency

i/j	1	2	3	4	5	6	7	8
1		0.2955	0.1946	0.9802	0.0004	0.2754	0.5707	0.9994
2	0.2955		1.0000	0.9826	0.4422	1.0000	0.9999	0.0841
3	0.1946	1.0000		0.9556	0.5859	1.0000	0.9984	0.0472
4	0.9802	0.9826	0.9556		0.1451	0.9789	0.9991	0.8529
5	0.0004	0.4422	0.5859	0.1451		0.4695	0.2025	<.0001
6	0.2754	1.0000	1.0000	0.9789	0.4695		0.9998	0.0762
7	0.5707	0.9999	0.9984	0.9991	0.2025	0.9998		0.2308
8	0.9994	0.0841	0.0472	0.8529	<.0001	0.0762	0.2308	

The highlighted values show pairs of means that are significantly different.



Appendix F- Statistical analysis of IgG-coated beads by U937 cells transfected with siRNA against Tetraspanins 2,3,6.8.10 and 31- Experiment 3

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
tspan	8	Negative control Tetraspanin 10 Tetraspanin 2 Tetraspanin 3 Tetraspanin 31 Tetraspanin 6 Tetraspanin 8 scrambled control

Number of Observations Read	1187
Number of Observations Used	1164

The GLM Procedure

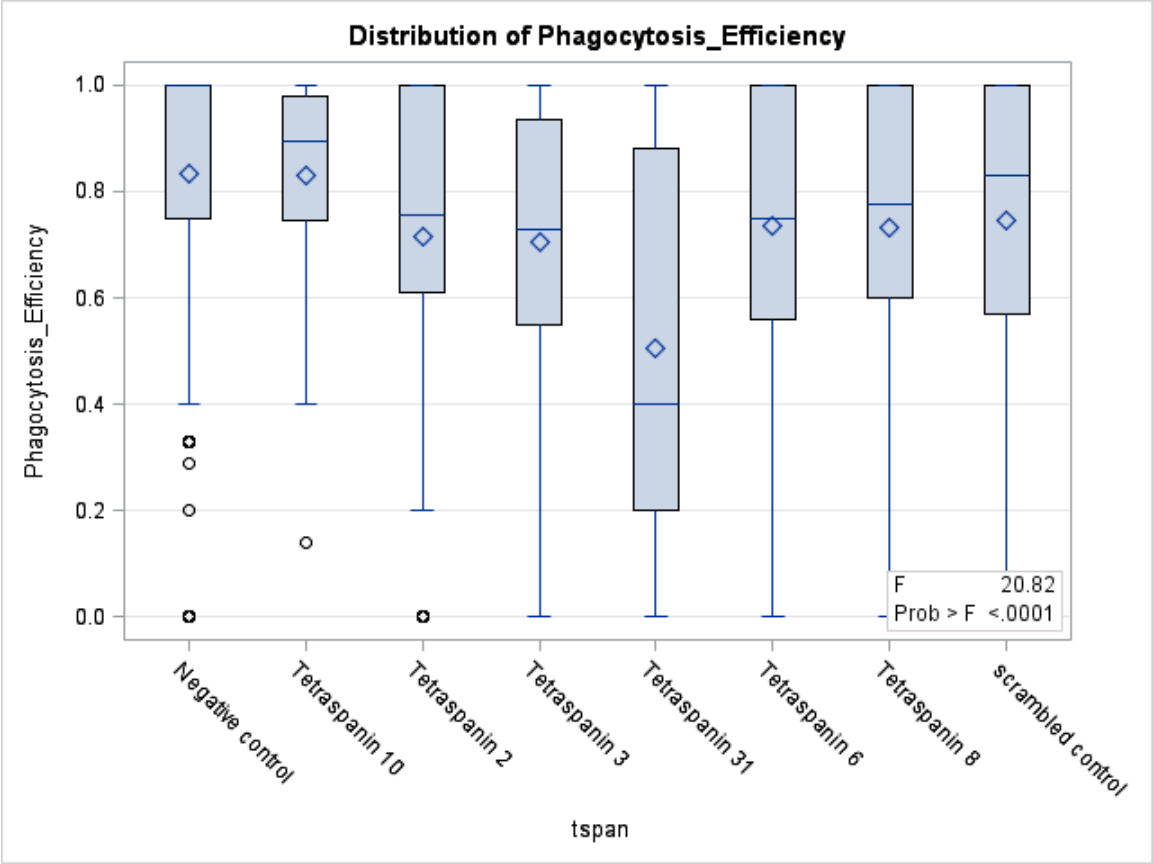
Dependent Variable: Phagocytosis_Efficiency

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	11.3404920	1.6200703	20.82	<.0001
Error	1156	89.9346251	0.0777981		
Corrected Total	1163	101.2751172			

R-Square	Coeff Var	Root MSE	Phagocytosis_Efficiency Mean
0.111977	38.66090	0.278923	0.721460

Source	DF	Type I SS	Mean Square	F Value	Pr > F
tspan	7	11.34049205	1.62007029	20.82	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
tspan	7	11.34049205	1.62007029	20.82	<.0001



The SAS System

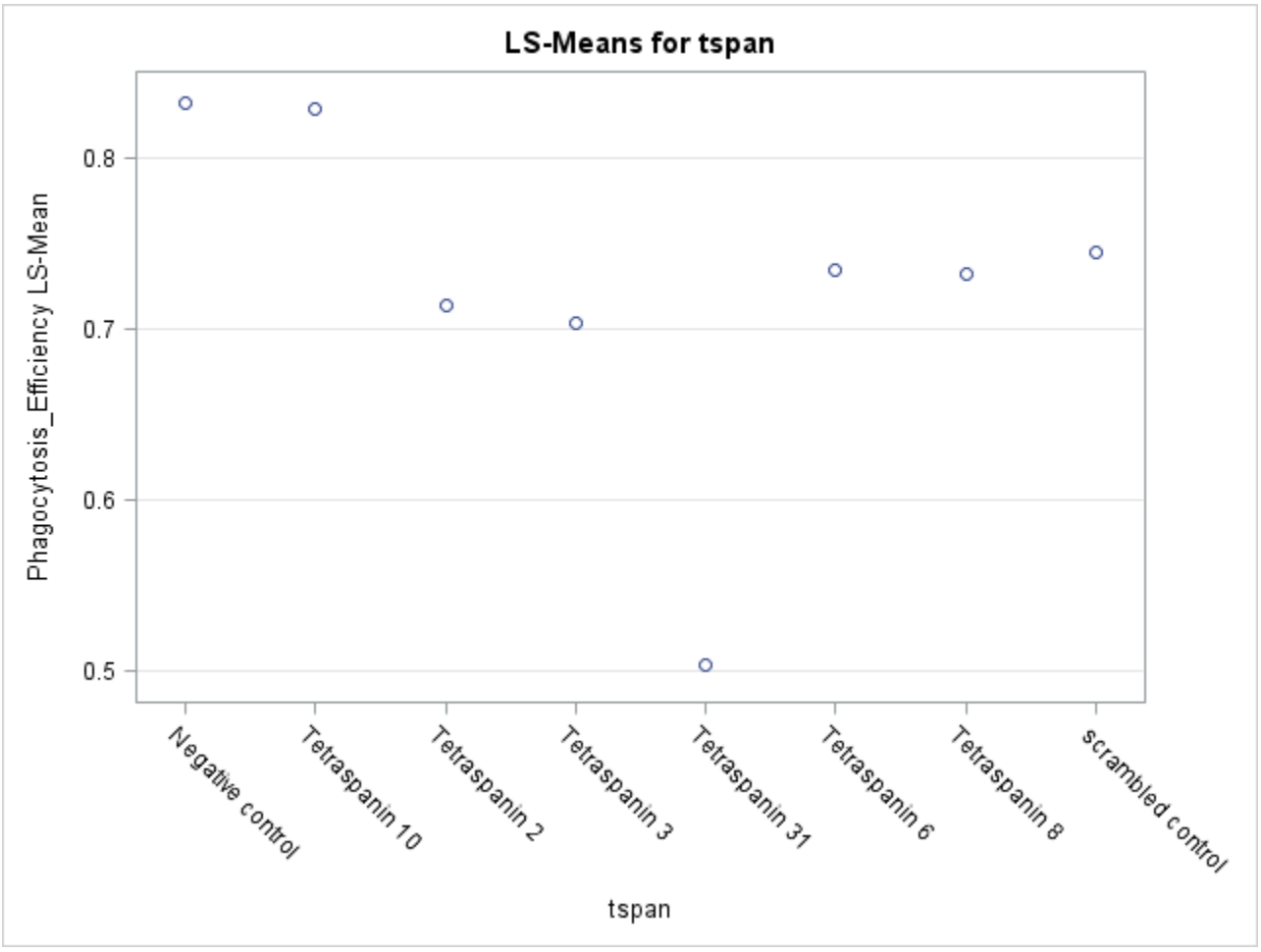
The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

tspan	Phagocytosis_Efficiency LSMEAN	LSMEAN Number
Negative control	0.83255230	1
Tetraspanin 10	0.82942308	2
Tetraspanin 2	0.71402778	3
Tetraspanin 3	0.70364865	4
Tetraspanin 31	0.50397516	5
Tetraspanin 6	0.73424460	6
Tetraspanin 8	0.73273333	7
scrambled control	0.74503817	8

Least Squares Means for effect tspan
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Phagocytosis_Efficiency

i/j	1	2	3	4	5	6	7	8
1		1.0000	0.0015	0.0003	<.0001	0.0219	0.0142	0.0763
2	1.0000		0.1730	0.0966	<.0001	0.4160	0.3808	0.5887
3	0.0015	0.1730		1.0000	<.0001	0.9988	0.9992	0.9841
4	0.0003	0.0966	1.0000		<.0001	0.9833	0.9861	0.9208
5	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001
6	0.0219	0.4160	0.9988	0.9833	<.0001		1.0000	1.0000
7	0.0142	0.3808	0.9992	0.9861	<.0001	1.0000		1.0000
8	0.0763	0.5887	0.9841	0.9208	<.0001	1.0000	1.0000	

The highlighted values show pairs of means that are significantly different.



Appendix G- Statistical analysis of oxLDL-coated internalization of U937 cells incubated in growth media for 4 hours

The GLM Procedure

Class Level Information

Class	Levels	Values
tspan	9	Tetraspanin 10 Tetraspanin 6 Tetraspanin 8 Tetraspanin10 negative control scrambled control tetraspanin 31 tetraspanin2 tetraspanin3

Number of Observations Read	1103
Number of Observations Used	1103

The GLM Procedure

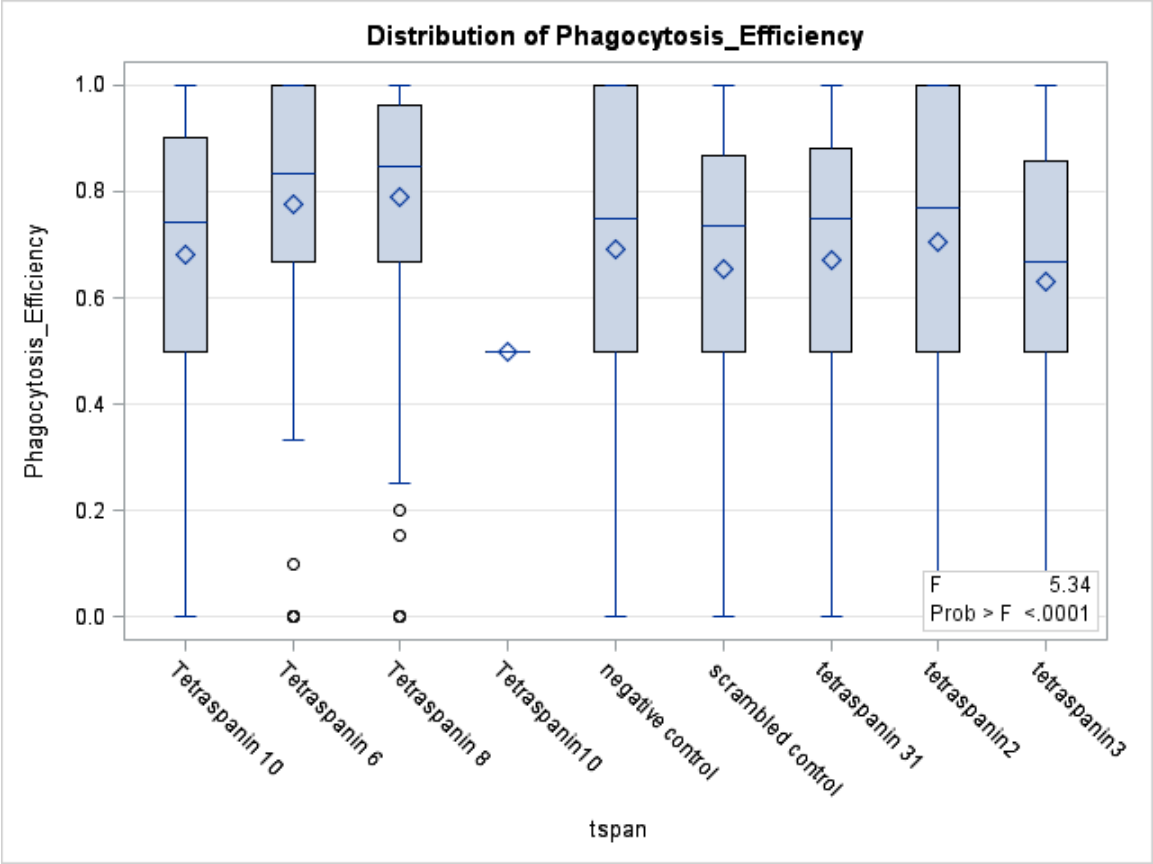
Dependent Variable: Phagocytosis_Efficiency

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	3.24351239	0.40543905	5.34	<.0001
Error	1094	83.00863430	0.07587627		
Corrected Total	1102	86.25214669			

R-Square	Coeff Var	Root MSE	Phagocytosis_Efficiency Mean
0.037605	39.29748	0.275456	0.700952

Source	DF	Type I SS	Mean Square	F Value	Pr > F
tspan	8	3.24351239	0.40543905	5.34	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
tspan	8	3.24351239	0.40543905	5.34	<.0001



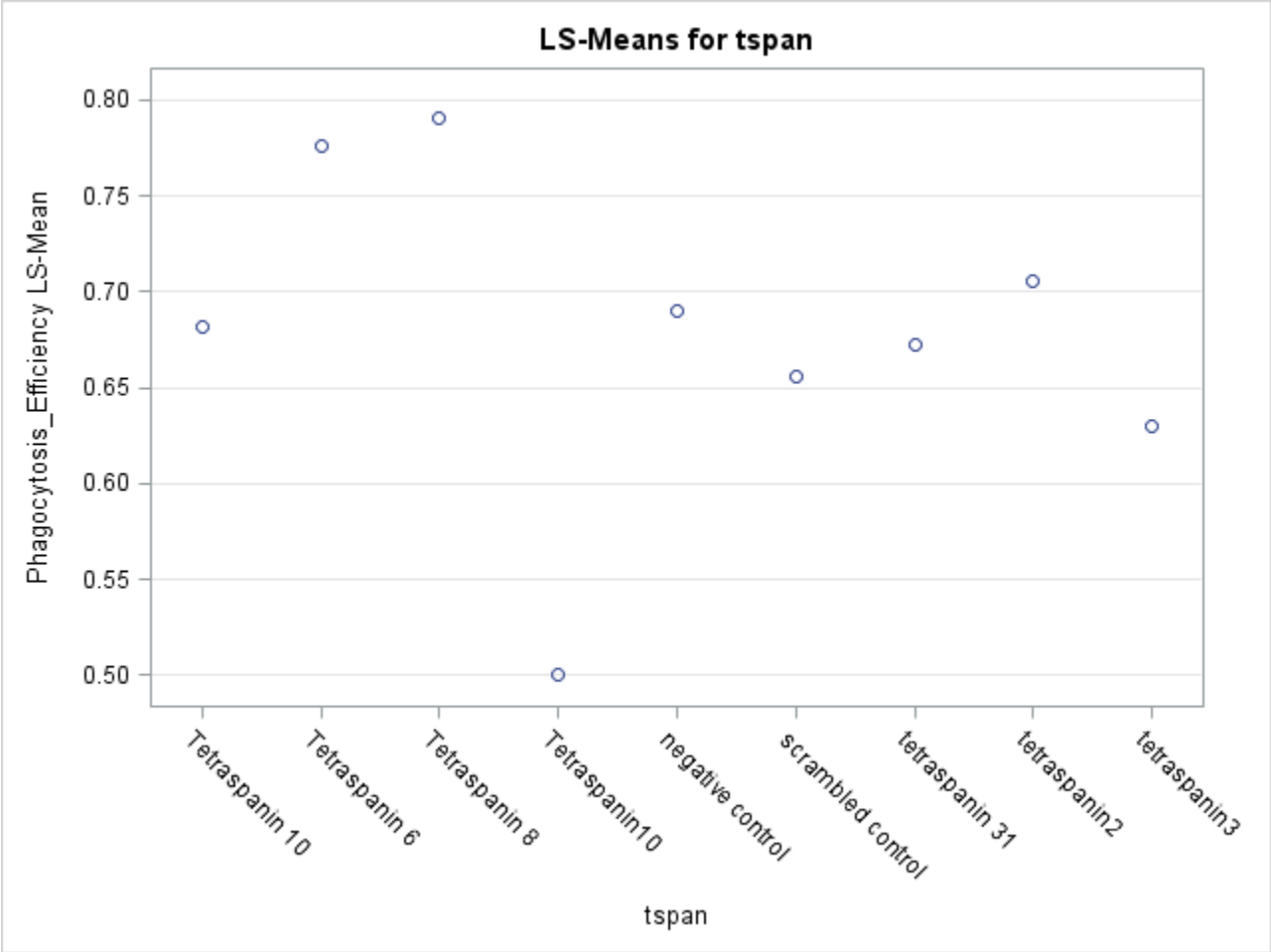
The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

tspan	Phagocytosis_Efficiency LSMEAN	LSMEAN Number
Tetraspanin 10	0.68207756	1
Tetraspanin 6	0.77622144	2
Tetraspanin 8	0.79062276	3
Tetraspanin10	0.50000000	4
negative control	0.69029581	5
scrambled control	0.65577286	6
tetraspanin 31	0.67287456	7
tetraspanin2	0.70544556	8
tetraspanin3	0.62937160	9

Least Squares Means for effect tspan
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Phagocytosis_Efficiency

i/j	1	2	3	4	5	6	7	8	9
1		0.0904	0.0192	0.9992	1.0000	0.9964	1.0000	0.9984	0.7725
2	0.0904		1.0000	0.9860	0.2446	0.0080	0.0850	0.4277	0.0002
3	0.0192	1.0000		0.9806	0.0793	0.0010	0.0208	0.1620	<.0001
4	0.9992	0.9860	0.9806		0.9989	0.9998	0.9995	0.9981	0.9999
5	1.0000	0.2446	0.0793	0.9989		0.9856	0.9999	1.0000	0.6897
6	0.9964	0.0080	0.0010	0.9998	0.9856		0.9999	0.8388	0.9963
7	1.0000	0.0850	0.0208	0.9995	0.9999	0.9999		0.9911	0.9446
8	0.9984	0.4277	0.1620	0.9981	1.0000	0.8388	0.9911		0.2962
9	0.7725	0.0002	<.0001	0.9999	0.6897	0.9963	0.9446	0.2962	

The highlighted values show pairs of means that are significantly different.



Appendix H- Statistical analysis of oxLDL-coated internalization of U937 cells incubated in Hepes buffer for 2 hr

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
tspan	7	Negative control Tetraspanin 10 Tetraspanin 31 Tetraspanin 8 Tetraspanin2 Tetraspanin6 scrambled control

Number of Observations Read	767
Number of Observations Used	767

The GLM Procedure

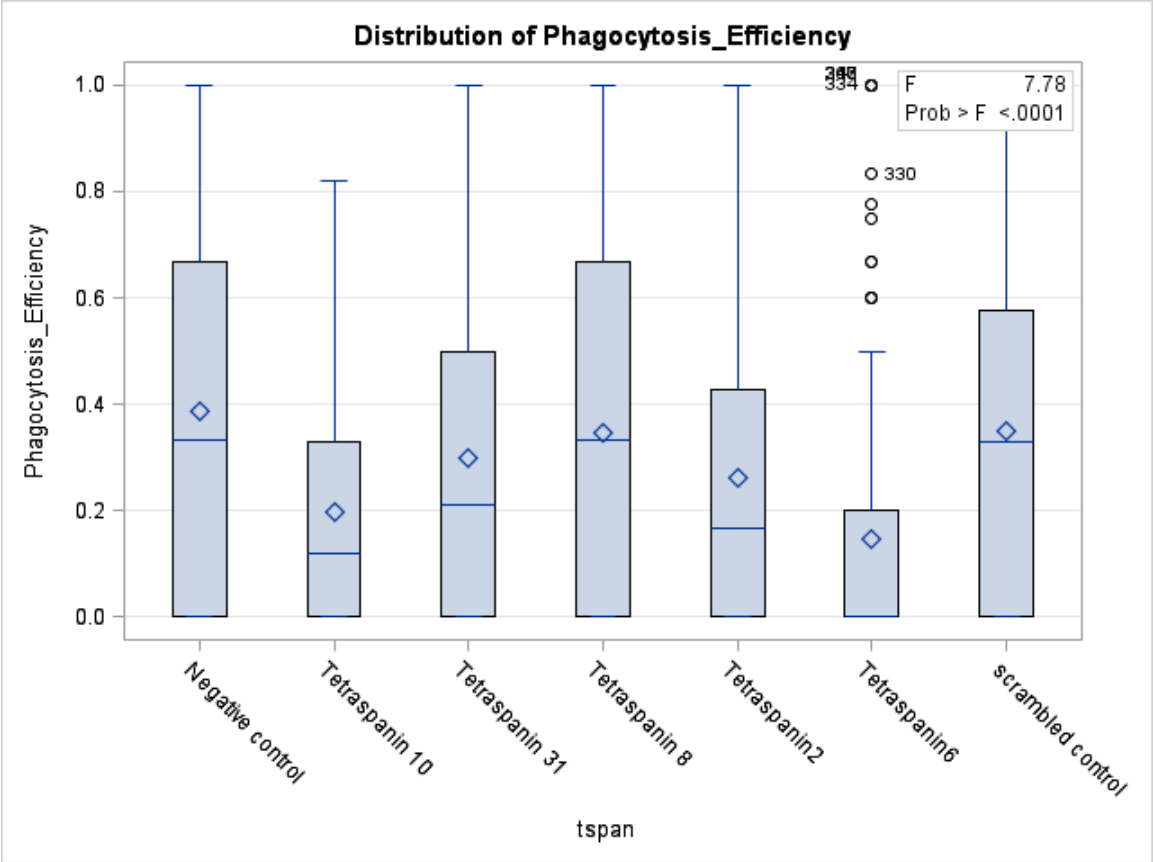
Dependent Variable: Phagocytosis_Efficiency

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	4.91268029	0.81878005	7.78	<.0001
Error	760	80.01973039	0.10528912		
Corrected Total	766	84.93241068			

R-Square	Coeff Var	Root MSE	Phagocytosis_Efficiency Mean
0.057842	108.4707	0.324483	0.299143

Source	DF	Type I SS	Mean Square	F Value	Pr > F
tspan	6	4.91268029	0.81878005	7.78	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
tspan	6	4.91268029	0.81878005	7.78	<.0001



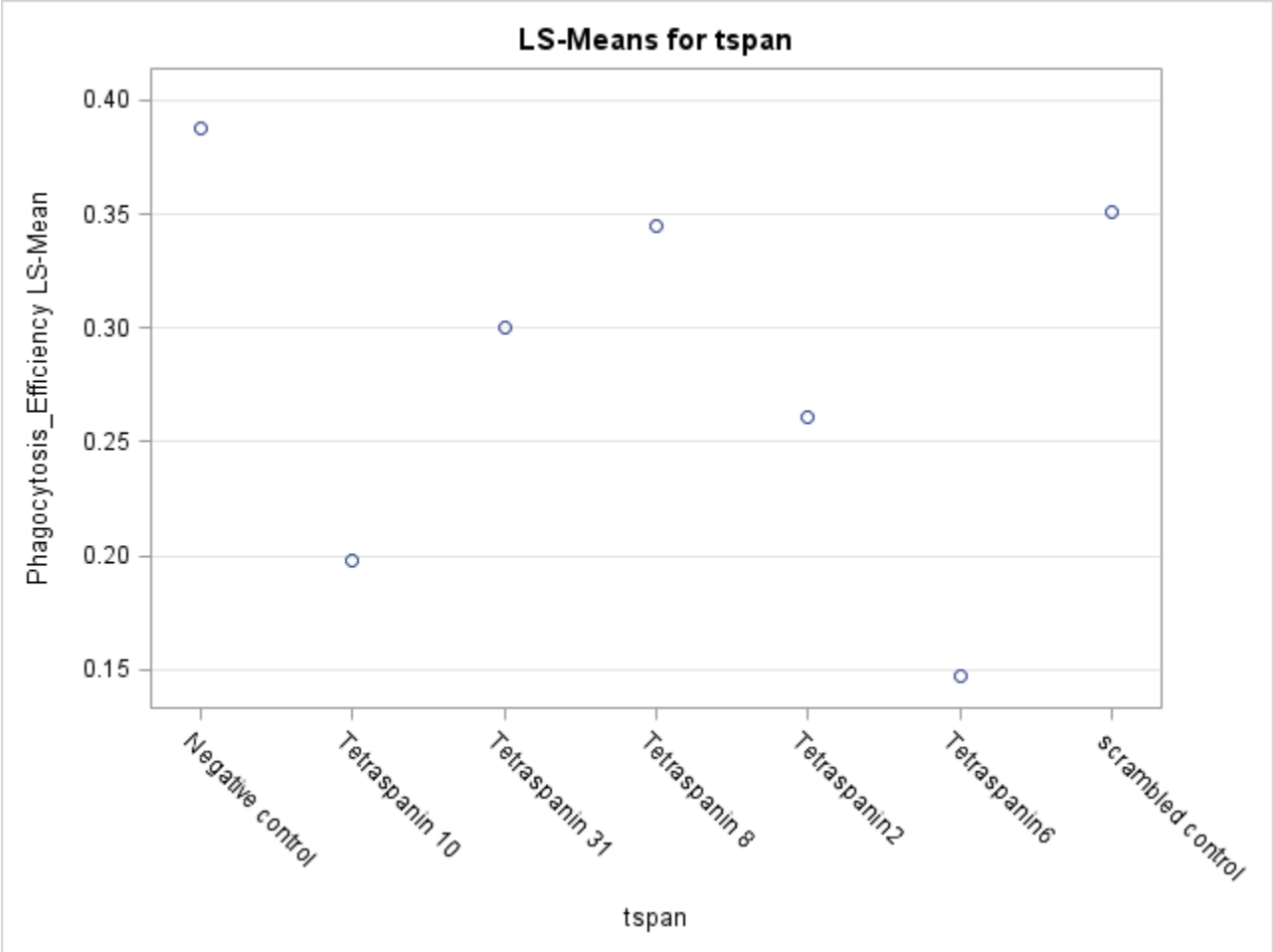
The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

tspan	Phagocytosis_Efficiency LSMEAN	LSMEAN Number
Negative control	0.38782023	1
Tetraspanin 10	0.19785714	2
Tetraspanin 31	0.30014881	3
Tetraspanin 8	0.34496160	4
Tetraspanin2	0.26069186	5
Tetraspanin6	0.14691585	6
scrambled control	0.35098765	7

Least Squares Means for effect tspan
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Phagocytosis_Efficiency

i/j	1	2	3	4	5	6	7
1		0.0753	0.3468	0.9381	0.0548	<.0001	0.9666
2	0.0753		0.7466	0.3090	0.9720	0.9894	0.2526
3	0.3468	0.7466		0.9330	0.9746	0.0055	0.8717
4	0.9381	0.3090	0.9330		0.4514	<.0001	1.0000
5	0.0548	0.9720	0.9746	0.4514		0.1335	0.3389
6	<.0001	0.9894	0.0055	<.0001	0.1335		<.0001
7	0.9666	0.2526	0.8717	1.0000	0.3389	<.0001	

The highlighted values show pairs of means that are significantly different.



Appendix I- Statistical analysis of oxLDL-coated internalization of U937 cells incubated in Hepes buffer for 4 hr

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
tspan	8	Negative control Scrambled control Tetraspanin 10 Tetraspanin 2 Tetraspanin 3 Tetraspanin 31 Tetraspanin 6 Tetraspanin 8

Number of Observations Read	991
Number of Observations Used	991

The GLM Procedure

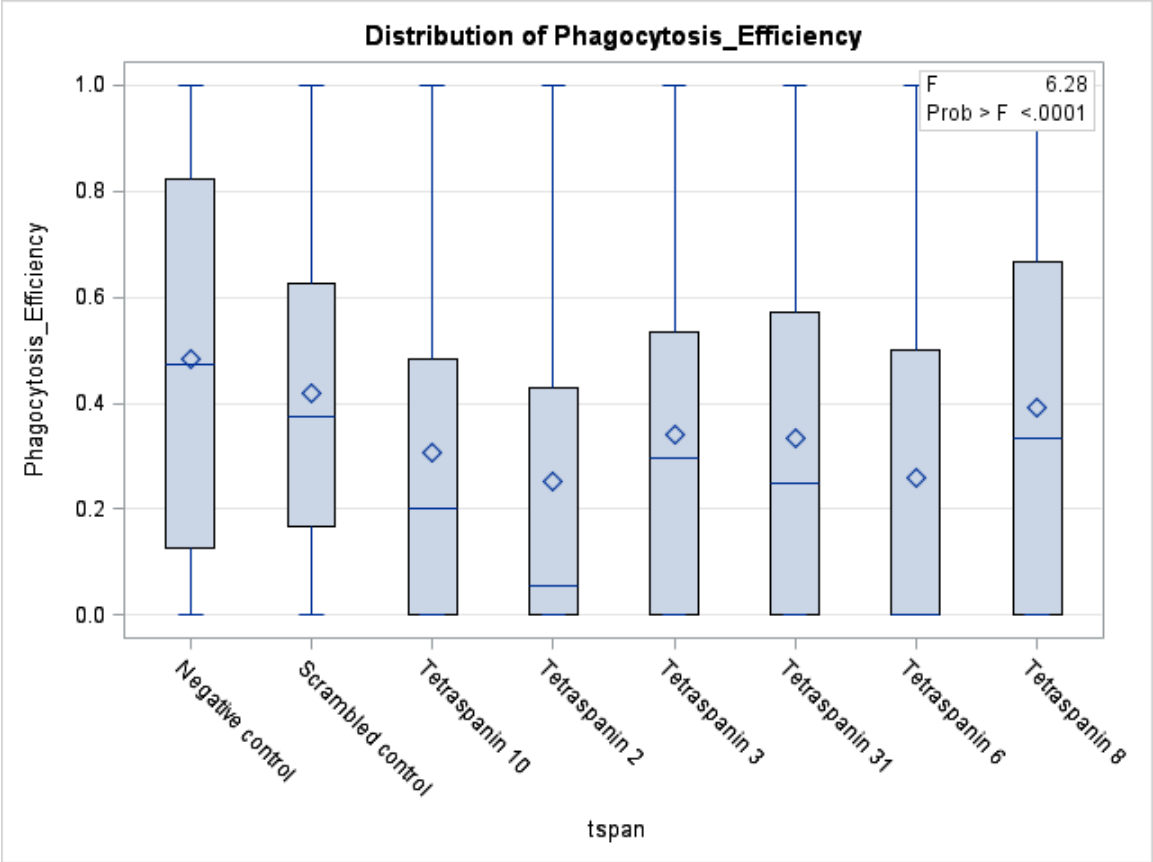
Dependent Variable: Phagocytosis_Efficiency

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	5.0681700	0.7240243	6.28	<.0001
Error	983	113.2422529	0.1152007		
Corrected Total	990	118.3104229			

R-Square	Coeff Var	Root MSE	Phagocytosis_Efficiency Mean
0.042838	98.38726	0.339412	0.344976

Source	DF	Type I SS	Mean Square	F Value	Pr > F
tspan	7	5.06816999	0.72402428	6.28	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
tspan	7	5.06816999	0.72402428	6.28	<.0001



The SAS System

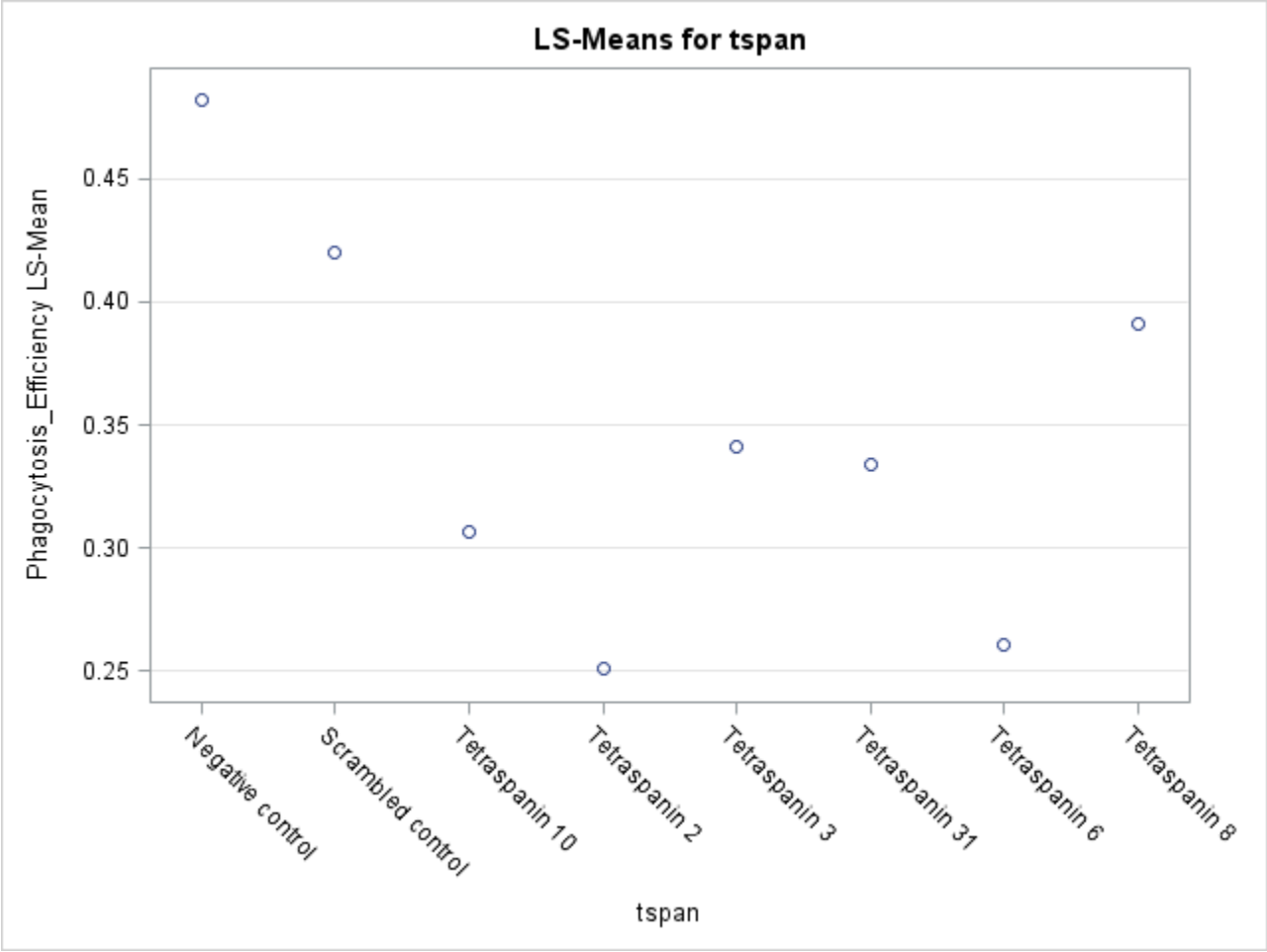
The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

tspan	Phagocytosis_Efficiency LSMEAN	LSMEAN Number
Negative control	0.48218090	1
Scrambled control	0.42028862	2
Tetraspanin 10	0.30625232	3
Tetraspanin 2	0.25076804	4
Tetraspanin 3	0.34131614	5
Tetraspanin 31	0.33358428	6
Tetraspanin 6	0.26075041	7
Tetraspanin 8	0.39113076	8

Least Squares Means for effect tspan
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Phagocytosis_Efficiency

i/j	1	2	3	4	5	6	7	8
1		0.8783	0.0030	<.0001	0.0424	0.0337	<.0001	0.4616
2	0.8783		0.1259	0.0022	0.5755	0.4960	0.0042	0.9970
3	0.0030	0.1259		0.9001	0.9912	0.9985	0.9603	0.4465
4	<.0001	0.0022	0.9001		0.4049	0.5698	1.0000	0.0196
5	0.0424	0.5755	0.9912	0.4049		1.0000	0.5392	0.9305
6	0.0337	0.4960	0.9985	0.5698	1.0000		0.7033	0.8817
7	<.0001	0.0042	0.9603	1.0000	0.5392	0.7033		0.0347
8	0.4616	0.9970	0.4465	0.0196	0.9305	0.8817	0.0347	

The highlighted values show pairs of means that are significantly different.



Appendix J- Statistical analysis of oxLDL-coated internalization of U937 cells incubated in growth media with no serum for 4 hr

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
tspan	8	Negative control Scrambled control Tetraspanin 10 Tetraspanin 2 Tetraspanin 3 Tetraspanin 31 Tetraspanin 6 Tetraspanin 8

Number of Observations Read	978
Number of Observations Used	978

The GLM Procedure

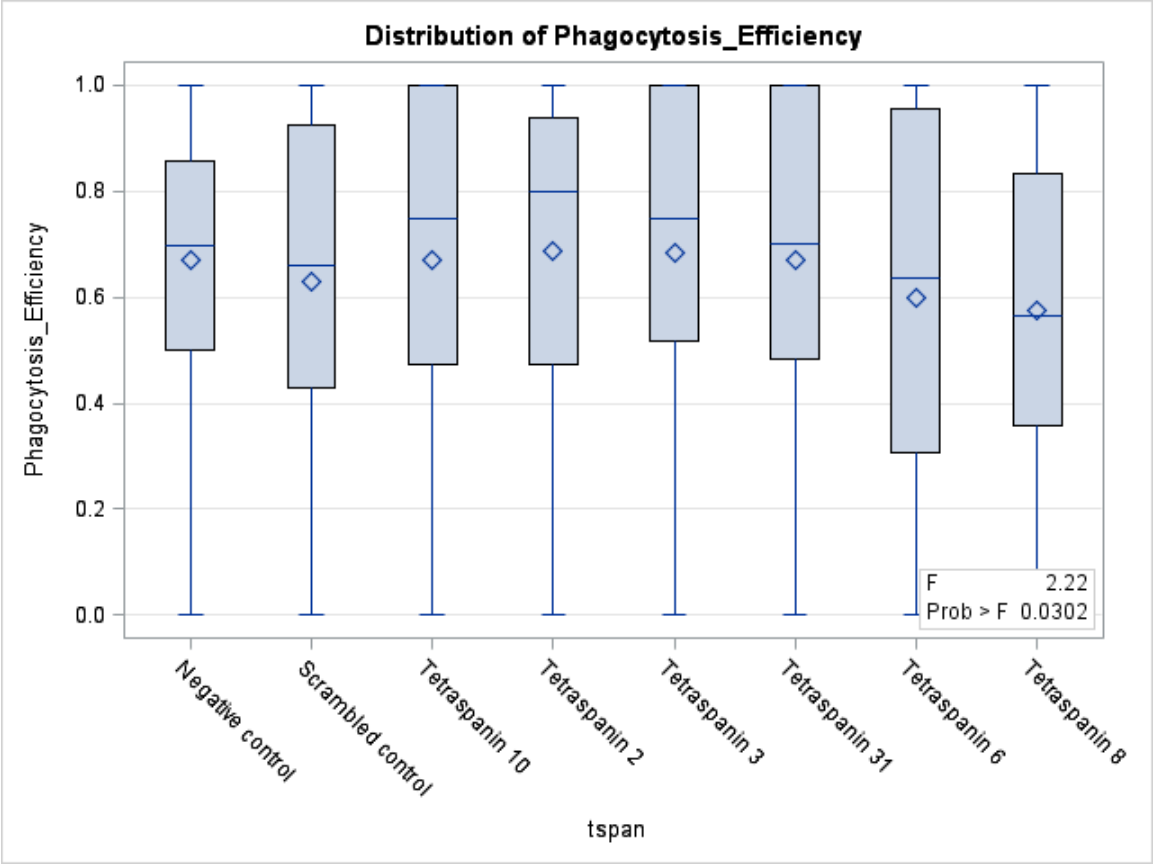
Dependent Variable: Phagocytosis_Efficiency

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1.49790141	0.21398592	2.22	0.0302
Error	970	93.31139220	0.09619731		
Corrected Total	977	94.80929361			

R-Square	Coeff Var	Root MSE	Phagocytosis_Efficiency Mean
0.015799	47.81166	0.310157	0.648706

Source	DF	Type I SS	Mean Square	F Value	Pr > F
tspan	7	1.49790141	0.21398592	2.22	0.0302

Source	DF	Type III SS	Mean Square	F Value	Pr > F
tspan	7	1.49790141	0.21398592	2.22	0.0302



The SAS System

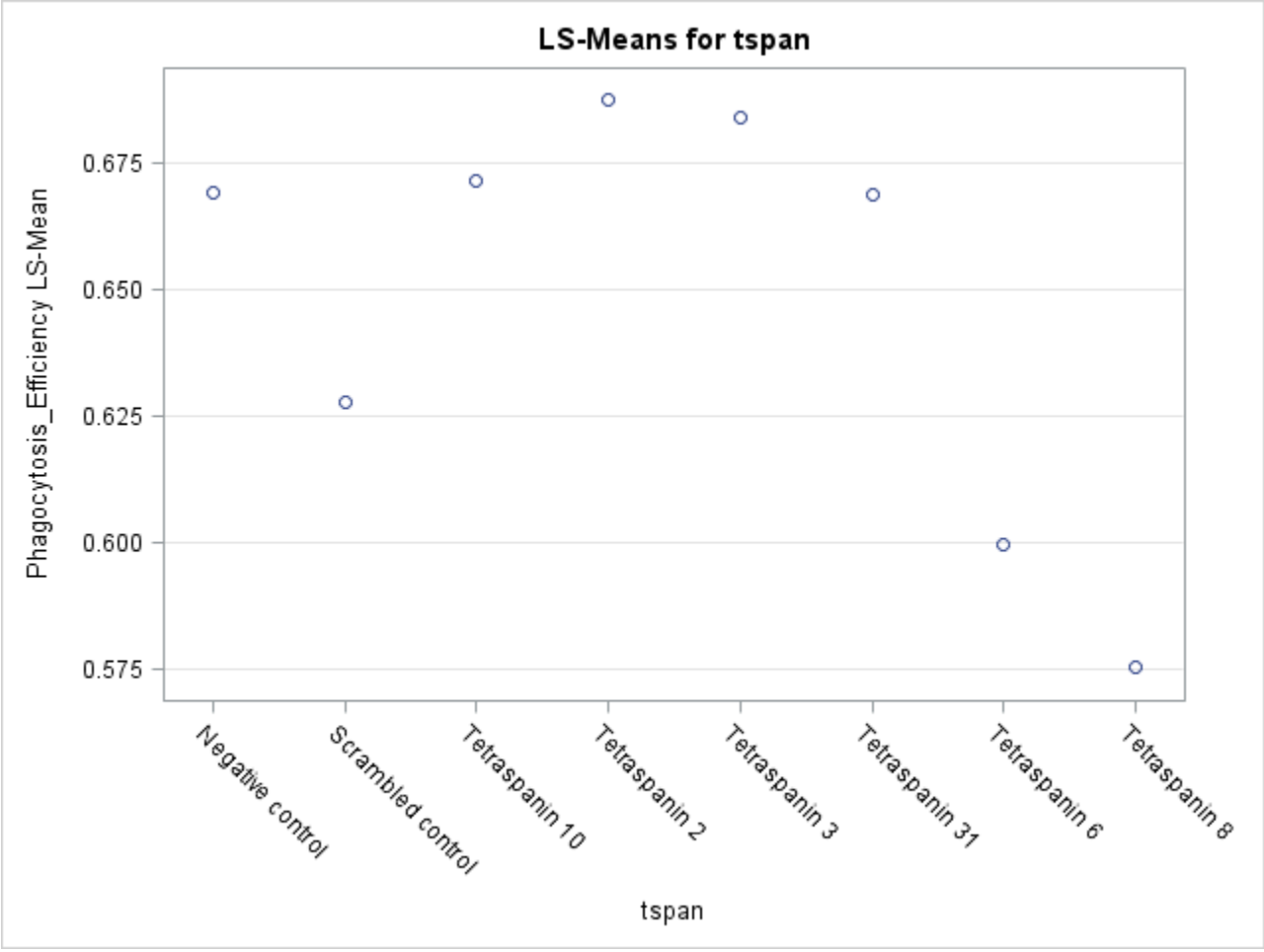
The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

tspan	Phagocytosis_Efficiency LSMEAN	LSMEAN Number
Negative control	0.66930500	1
Scrambled control	0.62777596	2
Tetraspanin 10	0.67167734	3
Tetraspanin 2	0.68773284	4
Tetraspanin 3	0.68403900	5
Tetraspanin 31	0.66874343	6
Tetraspanin 6	0.59956447	7
Tetraspanin 8	0.57541376	8

Least Squares Means for effect tspan
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Phagocytosis_Efficiency

i/j	1	2	3	4	5	6	7	8
1		0.9642	1.0000	0.9998	1.0000	1.0000	0.6823	0.2341
2	0.9642		0.9562	0.7938	0.8525	0.9654	0.9974	0.8845
3	1.0000	0.9562		0.9999	1.0000	1.0000	0.6632	0.2267
4	0.9998	0.7938	0.9999		1.0000	0.9997	0.3851	0.0790
5	1.0000	0.8525	1.0000	1.0000		0.9999	0.4619	0.1123
6	1.0000	0.9654	1.0000	0.9997	0.9999		0.6836	0.2323
7	0.6823	0.9974	0.6632	0.3851	0.4619	0.6836		0.9990
8	0.2341	0.8845	0.2267	0.0790	0.1123	0.2323	0.9990	

The highlighted values show pairs of means that are significantly different.



Appendix K-Statistical analysis of oxLDL-coated internalization of U937 cells incubated in Hepes buffer for 4 hr- Experiment two

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
tspan	6	Negative control Positive control tetraspanin10 tetraspanin2 tetraspanin31 tetraspanin6

Number of Observations Read	681
Number of Observations Used	681

The GLM Procedure

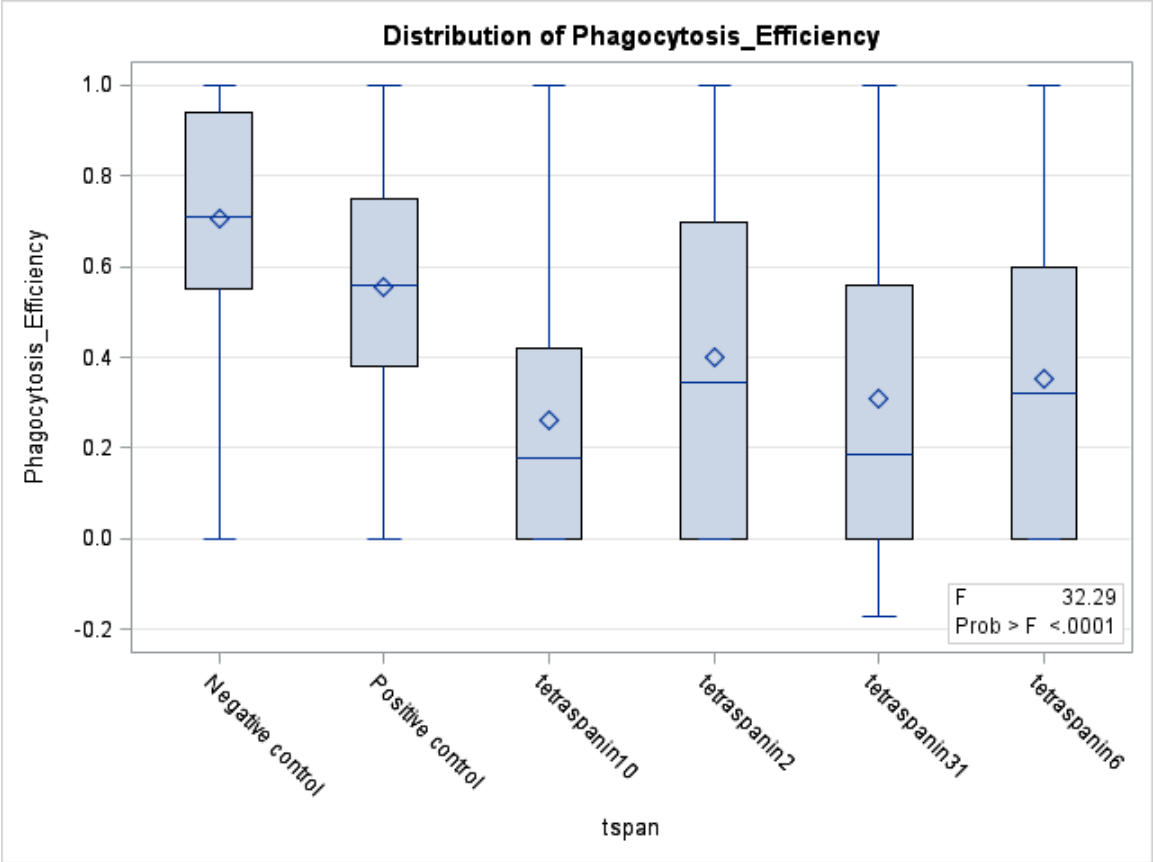
Dependent Variable: Phagocytosis_Efficiency

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	15.59175310	3.11835062	32.29	<.0001
Error	675	65.19363574	0.09658316		
Corrected Total	680	80.78538884			

R-Square	Coeff Var	Root MSE	Phagocytosis_Efficiency Mean
0.193002	73.84252	0.310778	0.420866

Source	DF	Type I SS	Mean Square	F Value	Pr > F
tspan	5	15.59175310	3.11835062	32.29	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
tspan	5	15.59175310	3.11835062	32.29	<.0001



The SAS System

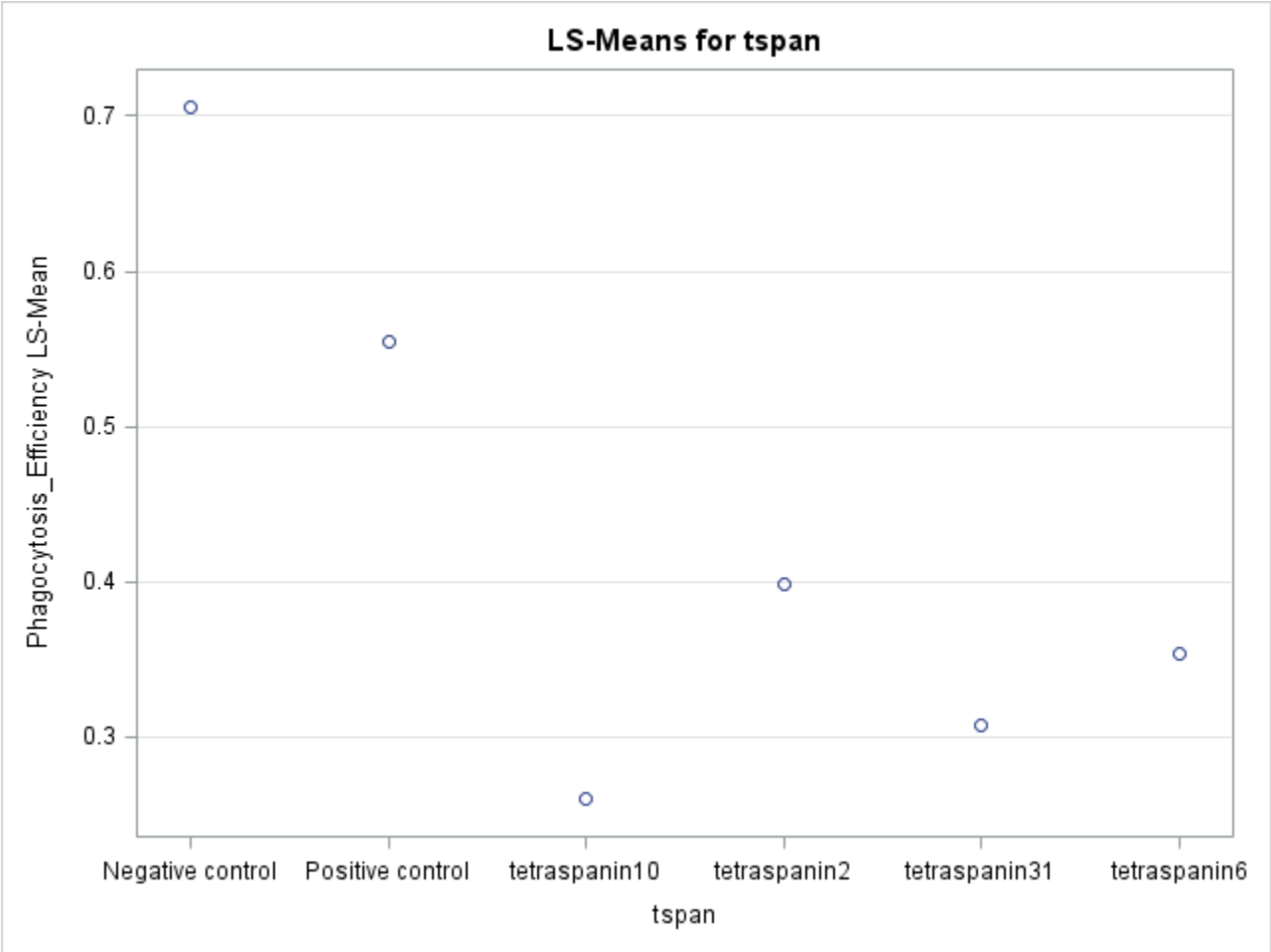
The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

tspan	Phagocytosis_Efficiency LSMEAN	LSMEAN Number
Negative control	0.70584158	1
Positive control	0.55429825	2
tetraspanin10	0.26008000	3
tetraspanin2	0.39870000	4
tetraspanin31	0.30728814	5
tetraspanin6	0.35357724	6

Least Squares Means for effect tspan
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Phagocytosis_Efficiency

i/j	1	2	3	4	5	6
1		0.0051	<.0001	<.0001	<.0001	<.0001
2	0.0051		<.0001	0.0038	<.0001	<.0001
3	<.0001	<.0001		0.0120	0.8448	0.1688
4	<.0001	0.0038	0.0120		0.2561	0.8900
5	<.0001	<.0001	0.8448	0.2561		0.8575
6	<.0001	<.0001	0.1688	0.8900	0.8575	

The highlighted values show pairs of means that are significantly different.



Appendix L- Statistical analysis of oxLDL-coated internalization of U937 cells incubated in Hepes buffer for 4 hr- Experiment three

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
tspan	7	Negative control Scrambled control Tetraspanin10 Tetraspanin2 Tetraspanin31 Tetraspanin6 tetraspanin6

Number of Observations Read	592
Number of Observations Used	592

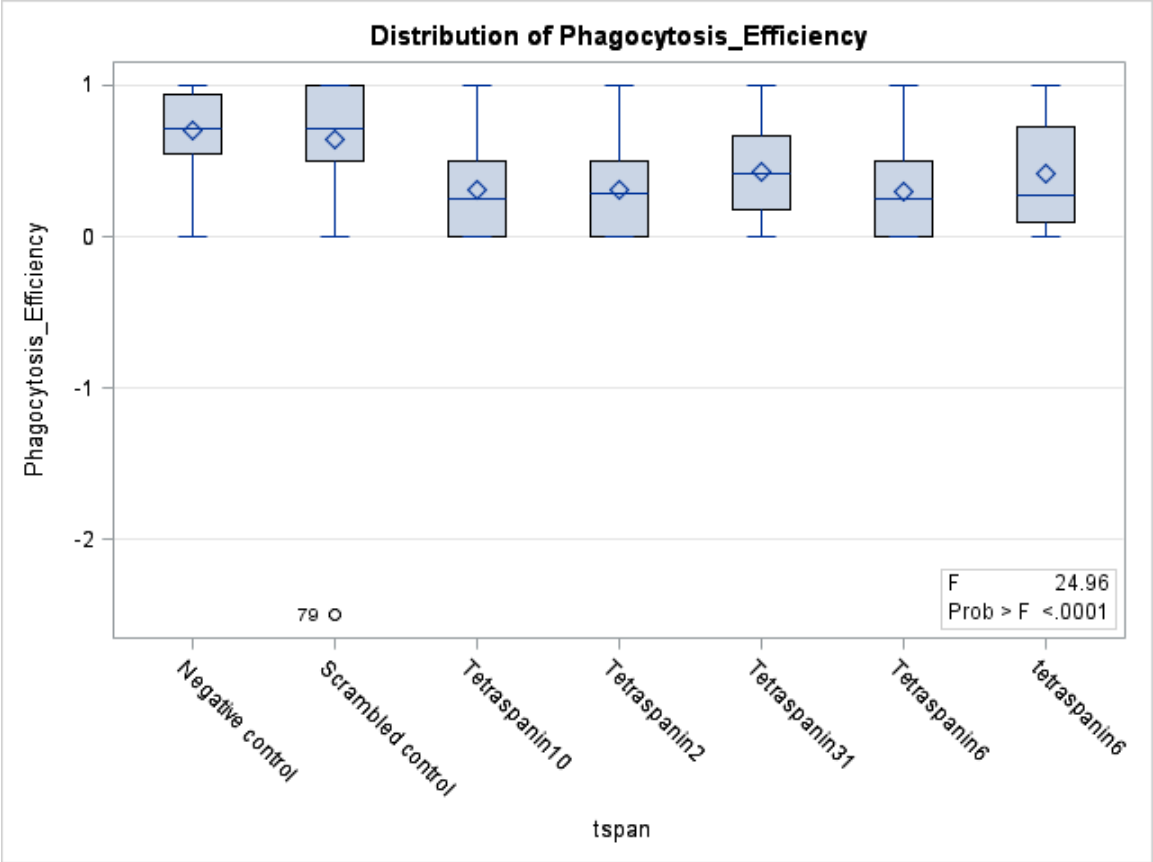
Dependent Variable: Phagocytosis_Efficiency

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	16.00294510	2.66715752	24.96	<.0001
Error	585	62.49976987	0.10683721		
Corrected Total	591	78.50271498			

R-Square	Coeff Var	Root MSE	Phagocytosis_Efficiency Mean
0.203852	72.39729	0.326860	0.451480

Source	DF	Type I SS	Mean Square	F Value	Pr > F
tspan	6	16.00294510	2.66715752	24.96	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
tspan	6	16.00294510	2.66715752	24.96	<.0001



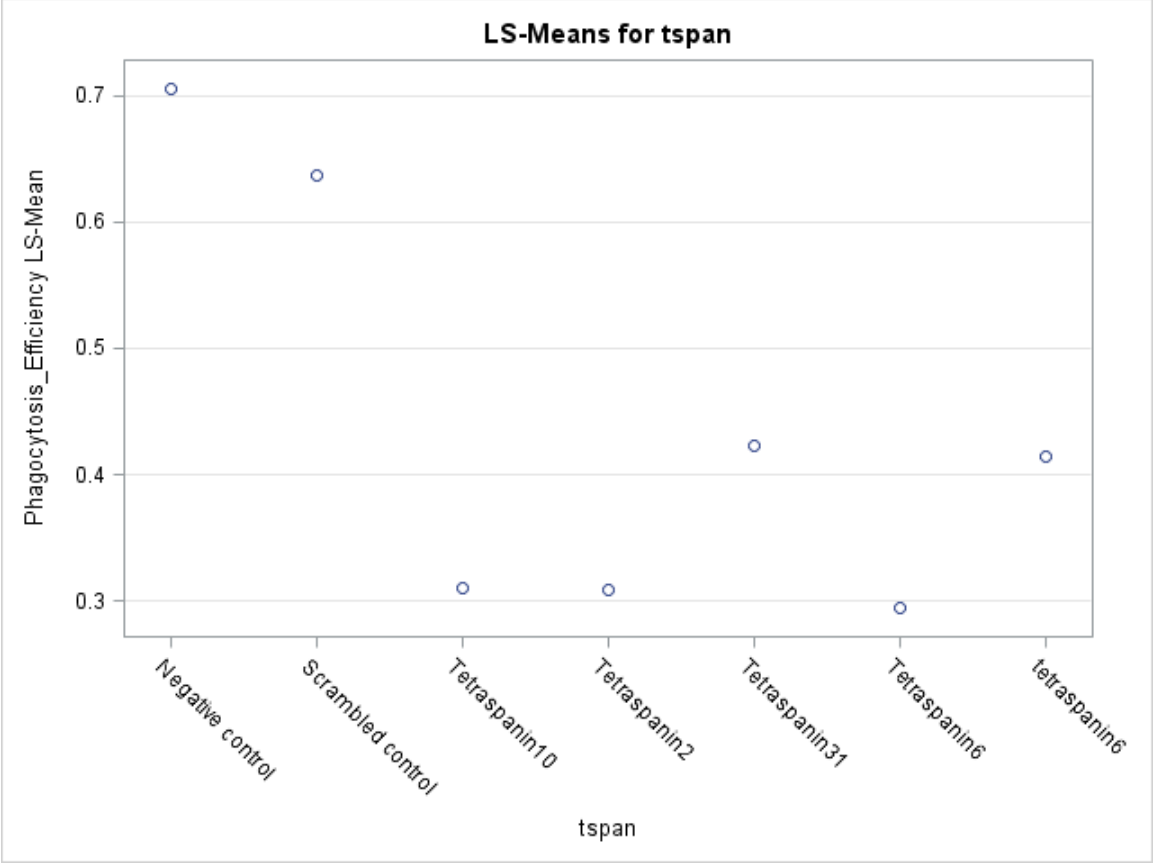
The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

tspan	Phagocytosis_Efficiency LSMEAN	LSMEAN Number
Negative control	0.70551613	1
Scrambled control	0.63716209	2
Tetraspanin10	0.31051659	3
Tetraspanin2	0.30853282	4
Tetraspanin31	0.42291538	5
Tetraspanin6	0.29402589	6
tetraspanin6	0.41397875	7

Least Squares Means for effect tspan
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Phagocytosis_Efficiency

i/j	1	2	3	4	5	6	7
1		0.7575	<.0001	<.0001	<.0001	<.0001	0.0168
2	0.7575		<.0001	<.0001	<.0001	<.0001	0.1491
3	<.0001	<.0001		1.0000	0.2027	0.9999	0.9047
4	<.0001	<.0001	1.0000		0.1753	0.9999	0.8952
5	<.0001	<.0001	0.2027	0.1753		0.1134	1.0000
6	<.0001	<.0001	0.9999	0.9999	0.1134		0.8308
7	0.0168	0.1491	0.9047	0.8952	1.0000	0.8308	

The highlighted values show pairs of means that are significantly different.



Appendix M- Mean of phagocytic efficiency of cells, standard deviation and standard error of the means for IgG mediated phagocytosis experiments

Treatments	Experiment 1			Experiment 2			Experiment 3		
	Mean	Standard deviation	Standard error of the mean	Mean	Standard deviation	Standard error of the mean	Mean	Standard deviation	Standard error of the mean
Scrambled siRNA control	0.934	0.121	0.012	0.905	0.185	0.011	0.745	0.291	0.025
No treatment	0.875	0.185	0.015	0.894	0.233	0.013	0.833	0.274	0.023
Tspan2	0.921	0.140	0.014	0.841	0.253	0.015	0.714	0.283	0.031
Tspan3	0.897	0.166	0.015	0.869	0.250	0.020	0.704	0.356	0.022
Tspan6	0.876	0.185	0.013	0.845	0.275	0.016	0.734	0.233	0.020
Tspan8	0.909	0.152	0.009	0.853	0.253	0.015	0.733	0.249	0.020
Tspan10	0.802	0.217	0.017	0.845	0.288	0.017	0.829	0.186	0.020
Tspan31	0.868	0.188	0.012	0.802	0.296	0.017	0.504	0.503	0.019

Appendix N- Mean of phagocytic efficiency of cells, standard deviation and standard error of the means for oxLDL mediated phagocytosis experiments

Treatments	Experiment 1			Experiment 2			Experiment 3		
	Mean	Standard deviation	Standard error of the mean	Mean	Standard deviation	Standard error of the mean	Mean	Standard deviation	Standard error of the mean
Scrambled siRNA control	0.420	0.309	0.030	0.554	0.286	0.027	0.637	0.444	0.045
No treatment	0.477	0.250	0.038	0.706	0.259	0.026	0.706	0.259	0.026
Tspan2	0.251	0.312	0.029	0.399	0.350	0.035	0.309	0.298	0.029
Tspan6	0.261	0.359	0.029	0.354	0.326	0.029	0.345	0.338	0.034
Tspan10	0.306	0.335	0.030	0.260	0.293	0.026	0.312	0.307	0.032
Tspan31	0.334	0.306	0.032	0.307	0.339	0.031	0.423	0.327	0.033

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