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**INTEGRINS SPECIFICALLY ASSOCIATED WITH
THE ACTIVATED PHAGOCYtic RECEPTOR COMPLEXES FROM
HUMAN U937 MACROPHAGES AND THE INTEGRINS OF HUMAN BLOOD**

by

Jaimie Dufresne

B.Sc., Ryerson University, 2013

A thesis presented to Ryerson University
in partial fulfillment of the requirements for the degree of Master of Science
in the Program of Molecular Science

Toronto, Ontario, Canada, 2013

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Master of Science, Molecular Science, Ryerson University, 2013

ABSTRACT

Receptor complexes are a key locus in the treatment of disease and pain but remain difficult to isolate. The Fc receptor binds to IgG presented on micro particles and triggers phagocytosis that is required for the engulfment of bacteria and non-self, pathogen molecules. Circulating LDL may be converted to oxLDL that is recognized by scavenger or other innate receptors that trigger the production of free radicals and phagocytic engulfment. The Fc receptor complex was captured using its cognate ligand IgG bound to 2 micron polystyrene beads from live cells by live cell affinity receptor chromatography (LARC). Similarly, oxLDL was used to coat micro beads to isolate the oxLDL-scavenger receptor complexes. The ligand coated beads were presented to live human macrophage U937 cells and sampled over the course of cell binding and engulfment in saline experimental medium. The receptor complexes were isolated by disruption with a French press and sucrose gradient centrifugation. The receptor complexes were separated using a salt and acetonitrile gradient prior to digestion with trypsin. The peptides were analyzed by LC-ESI MS/MS using a linear ion trap (Thermo) with the SEQUEST algorithm. In addition ligand affinity chromatography was performed using IgG, oxLDL or anti CD36 microbeads incubated with crude extracts. Controls included uncoated, or ligand coated, beads incubated in crude extract and/or used experimental medium. Certain integrins were shown to be specifically associated with IgG versus oxLDL and/or control treatments. The functional requirement for these integrins was tested using silencing RNA and quantitative assays of phagocytosis using laser scanning confocal microscopy. For the first time we show a small but statistically significant role for integrins in IgG-Fc γ receptor mediated phagocytosis.

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LIST OF SELECTED ABBREVIATIONS

PAMPs	pathogen-associated molecular patterns
LPS	lipopolysaccharide
IgG	immunoglobulin G
CR3	complement component iC3b
Da	Dalton
ESI	electro spray ionization
LARC	live-cell affinity receptor chromatography
MS	mass spectrometry
MS/MS	tandem mass spectrometry
PBS	phosphate buffered saline
NCBI	National Center for Biotechnology Information
PMA	phorbol ester
PNM	polymorphonuclear leukocytes a.k.a. neutrophil granulocytes
RCF	relative centrifugal force
Tris	tris(hydroxymethyl)aminomethane.
ADAMS	a disintegrin and metalloprotease
BSP	bone sialic protein
CCN3	an extracellular matrix protein
COMP	cartilage oligomeric matrix protein
Cyr61	cysteine-rich protein 61
LAP-TGF-β	TGF- β latency-associated peptide
iC3b	inactivated complement component 3
PECAM-1	platelet and endothelial cell adhesion molecule 1
uPA	urokinase

uPAR urokinase receptor

VEGF vascular endothelial growth factor

vWF von Willebrand Factor

oxLDL oxidized low density lipoprotein

CD36 cluster differentiation

ITG integrin

RATIONALE

Integrins are cell-surface adhesion molecules that play an important role in transducing mechanical cues from the extracellular environment into rearrangements of the actin cytoskeleton. These integrin-mediated structural rearrangements aid in cell spreading, cell-cell adhesion, cell and tissue shape, transendothelial migration of leukocytes and the formation of immunological synapses for antigen presentation. Distinct integrins have also been identified as playing important roles in multiple types of phagocytosis including the engulfment of apoptotic bodies, opsonized pathogens and as pathogen associated molecular pattern receptors. Recent studies have suggested that CD36, a critical receptor in the accumulation of oxidized LDL (oxLDL) by macrophages in the arterial intima and the transformation of macrophages into foam cells resulting in plaque formations and ultimately cardiovascular disease. CD36 may be co-opting the cells phagocytic machinery through the Fcγ receptor with the help of integrins and tetraspanins (Heit et al., 2013). If this is the case, a comparative study of both IgG-FcR mediated phagocytosis and oxLDL-scavenger receptor mediated phagocytosis could help identify integrins involved in the engulfment of oxLDL but not IgG opsonized pathogens. Multiple inhibitory antibodies against integrin subunits and heterodimers are currently used as therapies against diseases associated with thrombotic and immune disorders (Byron et al., 2009). Thus, integrins associated with the phagocytic internalization of oxLDL and not IgG mediated phagocytosis could be a viable treatment target for cardiovascular disease since they should not compromise immune function. In addition to receptor complexes, integrins in blood was performed in addition to the analysis of integrins in phagocytic receptor complexes. Endogenous proteins that end up in blood are either excreted into blood via exosomes (Loose et al., 2009) or spilled into blood from lysed cells (Kennedy, 2001). Many low molecular weight peptides found in blood via mass spectrometry have been shown to be biologically significant (Marshall et al., 2004). Human plasma is very complex and requires multiple fractionations steps in order to be adequately characterized. This study also compares the integrins present in normal human plasma as well as disease samples including breast and ovarian cancer in order to determine whether differences in integrin peptide hits can be detected between diseased states. Whether these changes in integrin expression are a result of acute phase illness or are specific to the disease in question would require a matrix of multiple disease types.

CHAPTER 1: INTRODUCTION

Receptor Complexes

Receptor complexes are a main target for the understanding and treatment of human diseases. Nearly one third of our genes encode membrane proteins and more than 60% of drug therapies target cell surface receptors (Christopoulos, 2002). Isolating and identifying the proteins present in activated receptor complexes is exceptionally challenging because of their associations with the plasma membrane. In addition, complex samples can be difficult to resolve using mass spectrometry if not adequately separated.

Receptor complex assembly requires precise modulation of factors that are not yet completely understood such as membrane potentials, membrane curvature, and tensile forces created by interactions between the cytoskeleton and extracellular matrix. Therefore, it is important to try to isolate receptor complexes assembled in their native cellular environments and to use classical biochemistry to adequately separate these proteins in space and time so that they may be accurately identified.

1.1 INTEGRINS

Structure and function

Integrins are important receptors and co-receptors for many biological processes. Integrins, first characterized in 1986 by Tamkun et al., are a family of transmembrane cell surface glycoproteins made up of heterodimers of one alpha and one beta subunit. There are 18 alpha and 8 beta integrins that combine to form 24 distinct non-covalently bound combinations in humans (Luo et al., 2007) as shown in Figure 1.1.

Integrins are adhesion molecules expressed in unique profiles on all nucleated cells and in most metazoans (Berton, 1999). Integrins have relatively large extracellular domains that connect the extracellular matrix (ECM) to the structural elements of the cytoskeleton via smaller cytoplasmic

domains. The one exception to this is integrin $\beta 4$, which has an unusually long cytoplasmic tail. Extracellular ligand recognition depends on both the alpha and beta subunits which bind relatively short motifs that typically contain an acidic amino acid (Arnaout et al., 2002). Integrins also form cell-cell adhesions via counter receptors on other cells known as intracellular adhesion molecules (ICAMS). Connections to the ECM impart structure to individual cells which supports the development and organization of specific tissue types. Integrins are important intermediaries between external cues providing tensile strength and mechanical stress which can mediate cellular responses. Integrins form part of multiple actin associated adhesive structures such as focal adhesions, podosomes and invadopodia (Geiger et al., 2011).

Figure 1.1: Known combinations of integrin heterodimers in found in humans

Most integrin heterodimers can bind to multiple extracellular matrix proteins, cell surface proteins and soluble ligands as shown in Figure 1.2. Integrins also play a part in laying down various components of the extracellular matrix. Integrin heterodimers possess distinct roles, ligands and functions throughout the lifetime of an organism, especially in its early development as shown in double mouse knockout studies.

Table 1.1. Known ligands of integrin heterodimers

Integrin heterodimer	Ligand
$\alpha 1\beta 1$	Laminin, collagen
$\alpha 2\beta 1$	Laminin, collagen, thrombospondin , E-cadherin, tenascin
$\alpha 3\beta 1$	Laminin, thrombospondin , uPAR
$\alpha 4\beta 1$	Thrombospondin , MAdCAM-1, VCAM-1, fibronectin, osteopontin, ADAM, ICAM-4
$\alpha 5\beta 1$	Fibronectin, osteopontin, fibrillin, thrombospondin , ADAM, COMP, L1
$\alpha 6\beta 1$	Laminin, thrombospondin , ADAM, Cyr61
$\alpha 7\beta 1$	Laminin
$\alpha 8\beta 1$	Tenascin, fibronectin, osteopontin, vitronectin, LAP-TGF- β , nephronectin
$\alpha 9\beta 1$	Tenascin, VCAM-1, osteopontin, uPAR, plasmin, angiostatin, ADAM [25], VEGF-C, VEGF-D
$\alpha 10\beta 1$	Laminin, collagen
$\alpha 11\beta 1$	Collagen
$\alpha V\beta 1$	LAP-TGF- β , fibronectin, osteopontin, L1
$\alpha L\beta 2$	ICAM, ICAM-4
$\alpha M\beta 2$	ICAM, iC3b, factor X, fibrinogen, ICAM-4, heparin
$\alpha X\beta 2$	ICAM, iC3b, fibrinogen, ICAM-4, heparin, collagen
$\alpha D\beta 2$	ICAM, VCAM-1, fibrinogen, fibronectin, vitronectin, Cyr61, plasminogen
$\alpha IIb\beta 3$	Fibrinogen, thrombospondin, fibronectin, vitronectin, vWF, Cyr61, ICAM-4, L1, CD40 ligand
$\alpha V\beta 3$	Fibrinogen, vitronectin, vWF, thrombospondin , fibrillin, tenascin, PECAM-1, fibronectin, osteopontin, BSP, MFG-E8, ADAM-15, COMP, Cyr61, ICAM-4, MMP, FGF-2, uPA, uPAR, L1, angiostatin, plasmin, cardiotoxin, LAP-TGF- β , Del-1
$\alpha 6\beta 4$	Laminin
$\alpha V\beta 5$	Osteopontin, BSP, vitronectin, CCN3, LAP-TGF- β
$\alpha V\beta 6$	LAP-TGF- β , fibronectin, osteopontin, ADAM
$\alpha 4\beta 7$	MAdCAM-1, VCAM-1, fibronectin, osteopontin
$\alpha E\beta 7$	E-cadherin
$\alpha V\beta 8$	LAP-TGF- β

Table replicated from (Tadaka, 2007) Bold integrins: known integrin heterodimers combinations expressed in leucocytes.

Integrin regulation

Integrins can be regulated in multiple ways including conformational changes that modify the integrin's affinity for its ligand, changes in avidity, and mechanisms that control integrin recycling. Integrins can exist in a low affinity state (inactive), a high affinity state (primed) and an activated or ligand bound state. The first crystal structure at 3.1 Å resolution of inactive $\alpha V\beta 3$ integrin obtained by Xiong et al. in 2001 showed that the integrin α and β subunits form two long legs and join to form a head group whose

Figure 1.2: First crystal structure of inactive integrin heterodimer $\alpha V\beta 3$ elucidated by Xiong et al., 2001

role is to bind the extracellular ligand. In the inactive or closed conformation the “legs” are bent and the head group faces downward toward the plasma membrane (Xiong et al., 2001). It has been shown however that this bent conformation likely does not affect the binding of smaller ligands (Askari et al. 2009). In the primed state, the legs straighten at a domain known as the knee which is the leg’s articulation point. Once primed, the integrin head group faces upward. A second crystal structure of an integrin ($\alpha IIb\beta 3$) in the active conformation by Xiao et al. in 2004 revealed that binding of the ligand (in this case three variants of the natural fibrinogen ligand

used to treat clotting of the coronary artery) to the BI domain produced a conformational change at the hybrid domain or neck where the beta “leg” swung approximately 60° away from the ligand binding domain. The swing of the beta leg away from the α subunit may open up cytoplasmic binding regions resulting in downstream signalling (Xiao et al., 2004).

Integrins are known to interact with large numbers of proteins in focal adhesion sites. Integrins have no intrinsic enzymatic activity but can activate signalling pathways by co-clustering with kinases such as integrin linked kinases and various adaptor proteins in focal adhesion complexes. Integrins are bidirectional signalling molecules where inside out signalling alters the integrin’s binding affinity for its extracellular ligand and outside-in signalling mediates cellular responses such as cell spreading, migration or proliferation.

The alpha integrin leg is made up of three domains: calf-1, calf-2 and a thigh domain which all support the seven-bladed β propeller head. The β propeller has allosteric calcium binding regions on the underside of the propeller blades that affect ligand binding (Humphries et al. 2003; Oxvig and Springer 1998). Nine of the eighteen alpha integrins have an αI domain that forms a Rossman fold made up of five β -sheets and seven α -helices and contain a metal-ion-dependent

adhesion site (Lee et al. 1995). Alpha integrins that can bind collagen have an extra α C helix (Emsley et al. 1997)

The integrin β subunit is made up of a plexin-sempahorin-integrin (PSI) domain, a hybrid domain, a β I domain containing a MIDAS and an adjacent to MIDAS (ADMIDAS) domain that inhibits ligand binding when bound to Ca^{2+} as well as four cysteine-rich epidermal growth factor repeats. All β integrins except for β 4 all have short homologous tails, and are capable of binding phosphotyrosine-binding (PTB) domains via NPX/Y motifs. β 1 has two NPX/Y domains that can recruit adaptor proteins as well as clathrin. In clathrin mediated endocytosis β 1 acts as a sorting signal for cargo proteins (Maginnis et al., 2008). β 4 has an unusually long cytoplasmic tail. Another important exception is that while most integrins connect to the actin cytoskeleton, α 6 β 4 links to the intermediate filament system (Nievers et al., 1999). Intracellular integrin activating proteins that bind the beta tail include: talin 1 and 2 and kindlin 1, 2 and 3 (Larjava et al. 2008; Senetar et al. 2007). Beta tails can also be negatively regulated by filamin A (Kiema et al. 2006).

A large number of integrin antibodies have been shown to inhibit and stimulate integrin function. Some monoclonal antibody inhibitors such as ReoPro (abciximab) are used as drug therapies. ReoPro reduces platelet aggregation by preventing α IIb β 3 from binding fibrinogen resulting in a reduction of blood formation (Mackman, 2008). RePro is used to treat unstable angina. Integrins have been linked to many human diseases associated with abnormal development, blood clotting, angiogenesis, cancer, inflammation and inherited immunodeficiency disorders. Integrins are also important to the innate and adaptive immune system and play an integral role in phagocytosis.

1.2 PHAGOCYTOSIS

Phagocytosis; first observed in 1884 by Ilya Ilyich Metchnikoff, is the receptor mediated engulfment of particles greater than 0.5 μm in size (Metchnikoff, 1884). The pseudopodia of phagocytic cells are made up of filamentous actin that probe their surroundings for prey ligands on large foreign particles, pathogens and apoptotic bodies. Low affinity phagocytic predator receptors bind their prey ligands sequentially in a zipper like fashion mediating the reorganization of the of the actin cytoskeleton. The force required to enclose the cell membrane around these particles is generated by ATP hydrolysis as myosin molecular motors drive along these actin filaments (Araki et al., 2003; Swanson et al., 1999; Harris et al., 2002).

Phagocytic receptors are classified into three different classes: pattern recognition receptors, opsonic and apoptotic corpse receptors (Flannagan et al., 2012). The phagocytosis of pathogens is carried out exclusively by professional phagocytes and is an important part of both innate and adaptive immunity. Professional phagocytes are the first line of defence in innate immunity and take up foreign bodies including bacteria and fungi. Professional phagocytes differentiate from myeloid progenitors; this class of cells includes neutrophils, dendritic cells and macrophages.

Pattern recognition receptors bind directly to pathogens by recognizing pathogen associated molecular patterns (PAMPs). Opsonic receptors bind pathogens through an intermediate or bridging molecule. The Fc gamma receptor, the best known opsonic receptor binds pathogen coated in its intermediate IgG. Fc γ receptor signalling is initiated by the phosphorylation of both tyrosines in the ITAM domain which seems to occur as a result of receptors clustering and possible interactions with Src family kinases such as Lyn, Fgr and Hck (Crowley, 1997). Thus, speed of lateral diffusion is important for phagocytosis as well as the speed of recycling. Phosphorylation of the ITAM domain recruits the tyrosine kinase Syk which has two SH2 domains that bind to phosphorylated tyrosines and also have the potential to autophosphorylate other clustering receptors. Rho family GTPases such as Rac1, Rac2 and Cdc42 are essential for driving actin assembly and cytoskeleton modification (Lerm et al., 2007). Cdc42 also seems to play a role in phagosome maturation (Lerm et al., 2007). Rac1 localizes throughout the phagocytic cup whereas Cdc42 localizes to the leading edge of the forming cup. Many other

proteins have been identified in the downstream signaling cascade. A comprehensive list of 288 proteins associated with the Fc receptor-actin cytoskeleton supramolecular complex formed in human neutrophils was produced in 2008 by ligand affinity chromatography and mass spectrometry (Jankowski et al.).

The extracellular domains of the Fc gamma receptor isotypes have several differences. FcR γ I has three IgG binding domains compared to the rest of the Fc γ R types and has greater affinity for IgG in general (Russell, 2009). The cytosolic domains each have a combination of two or four ITAM domains while hFc γ IIb1 and 2b2 each have one ITIM domain. Fc γ RIIb2 localizes to clathrin-coated pits but Fc γ RIIb1 does not, and both tend to confer inhibitory ITAM based signals (Miettinen et al, 1989).

Only professional phagocytes are known to engulf pathogens. However, cell types without endogenous Fc γ receptors such as COS-1 have been transfected with Fc γ and have been used as a model system to study Fc γ isoforms (Indik et al., 1995). Many pathogens have strategies for avoiding opsonins and phagocytic receptors or coopting these proteins in order to gain entry into the cell. Some pairs of integrins including α M β 2, α V β 2 and α 5 β 1 have been classified as opsonic receptors (Shimaoka, 2011).

Apoptotic Receptors

Fibroblasts, endothelial and epithelial cells are also capable of phagocytosis, however these cells do not engulf microorganisms but rather they clear the apoptotic bodies produced from cells undergoing programmed cell death. Hence phagocytosis plays an important housekeeping role by clearing apoptotic bodies which is required for tissue remodelling and homeostasis (Flannagan et al., 2012). Apoptotic corpse receptors bind molecules expressed uniquely on dying cells such as phosphatidylserine which flips from the inside of the plasma membrane leaflet to the outside and is a ligand for multiple apoptotic corpse receptors. Two integrin heterodimers have been identified as apoptotic corpse receptors: α V β 3 and α V β 5 (Flannagan et al., 2012).

The phagocytosis of pathogens and apoptotic bodies leads to very different signalling outcomes hence there should be distinct differences in the composition of the resultant receptor complexes. The phagocytosis of foreign bodies for example, leads to the release of pro-inflammatory cytokines such as INF- γ , the attraction of lymphoid cells, and the presentation of engulfed particles, while the activation of receptors that bind apoptotic bodies releases anti-inflammatory mediators such as IL-4 and IL-10 protecting surrounding tissues from damage (Kishore, 2000). Professional phagocytes are also capable of ingesting apoptotic bodies, however when they do so, they release anti-inflammatory molecules. Signalling and gene expression varies between the engulfment of apoptotic bodies and pathogen engulfment. It is likely that multiple phagocytic receptors are engaged simultaneously and act synergistically, with multiple downstream signalling events occurring concurrently and in an integrated manner.

1.3 PHAGOCYTOSIS OF oxLDL BY SCAVENGER RECEPTORS

Phagocytosis of Oxidized LDL by scavenger receptors

The comparison of the integrins involved in IgG – Fc receptor mediated phagocytosis versus those in oxLDL–scavenger receptor mediated phagocytosis may have far reaching consequences for the understanding and treatment of cardiovascular disease. Cardiovascular disease is now the leading cause of death in most developed nations (Dahlof, 2010) accounting for 16.7 million deaths globally in 2002 (Lloyd-Jones, 2010). The underlying pathology of most cardiovascular disease is atherosclerosis. Atherosclerosis is a chronic inflammatory disease resulting in the build-up of plaque on the arterial intima causing a narrowing and hardening of the arteries. Macrophages internalize oxidized low density lipoprotein (oxLDL) via scavenger receptors such as CD36 until they become lipid laden foam cells (Platt& Gordon, 2001) (Silverstein and Febbraio, 2000). Foam cells get trapped on lesions of the arterial intima making up arterial plaques. Foam cells produce reactive oxygen species (ROS) and inflammatory cytokines perpetuating an inflammatory state by oxidizing more lipids and attracting more immune cells (Taubman et al., 1997). Plaque regression has been shown to result from foam cell clearance and emigration back into the lymph nodes (Llodra et al., 2004)

Pro-atherogenic apolipoprotein E knockout mouse models have been used to study atherogenesis and to identify the receptors responsible for oxLDL uptake. Apolipoprotein E mouse knockouts result in hyperlipidemia and a shift from high density to low density lipoproteins on regular mouse feed. Apoe plays an important role in the transport and metabolism of lipids.

Febbraio and colleagues, showed that CD36 is likely a main scavenger receptor in oxLDL uptake since CD36 null mice in an apoE null background show greater inhibition of lesion formation than their apoE-null counterparts (Febbraio et al., 2000) However, a contradictory study by Moore et al., argues that CD36-null mice were not protected against the formation of atherosclerotic lesions (2005) Macrophages expressing CD36 are important for achieving homeostasis, by recognizing pathogen associated molecular patterns and helping to clear pathogens, apoptotic cells, and oxidized lipoproteins.

CD36 Structure and Function

CD36 is member of the class B scavenger receptor family encoded by Chr7 q11.2. The class B scavenger receptors include scavenger receptor B type 1 (SR-BI) and lysosomal integral membrane protein II (LIMP-II). CD36 is a membrane protein that is primarily expressed on a wide variety of cell types including monocytes, mononuclear phagocytes, hepatocytes, adipocytes, platelets and some epithelia (Febbraio et al., 2001). CD36 is mostly extracellular with only two short cytoplasmic tails that can be palmitoylated (Gruarin et al., 2000). CD36 has many N-linked glycosylations which likely protect the protein from degradation.

CD36 has two membrane spanning domains, one at the carboxy-terminus made up of 27 hydrophobic amino acids and the second located at the amino-terminus which contains a cleavable signalling peptide (likely that it spans the membrane). CD36 is a multiligand receptor capable of binding thrombospondin-1, collagen I and IV, oxidized LDL, long-chain fatty acids, anionic phospholipids. CD36's binding affinity for thrombospondin -1 and collagen likely depends on the phosphorylation of threonine 92 (Asch et al., 1993). CD36 has a high affinity binding site for thrombospondin-1 located between amino acids 93 and 120. The affinity of the thrombospondin binding site is likely modulated by the amino acid sequence between amino acids 139 to 155. CD36 has two binding regions for oxLDL; one high affinity binding region

between amino acids 120 and 155 and a second lower affinity site between 28 to 93. CD36 colocalizes in caveolae with caveolin-1 (Lisanti et al., 1994) and likely plays a role in trafficking cholesterol by non-clathrin mediated endocytosis.

CD36 also regulates angiogenesis in concert with thrombospondin-1. CD36 is associated with susceptibility to coronary heart disease (Ma et al., 2004), susceptibility to malaria and platelet glycoprotein IV deficiency in humans. Some evidence suggests that CD36 may also play a role in cell adhesion. As previously stated, CD36 has been linked to the uptake of oxidized LDL, however it has no recognizable signalling motifs in the cytoplasmic tails to activate the phagocytic machinery. It has been postulated that CD36 forms a complex with integrin B1, B2, tetraspanin CD9 and/or CD81 and then links to the Fc gamma receptor (Heit et al., 2013). CD36 may be able to signal the src family kinases and syk through the ITAM domains of Fc gamma receptor.

It is possible that oxLDL may form aggregates that activate macrophages leading to a phagocytic response. The response to oxLDL may require a different receptor supramolecular complex compared to IgG.

1.4 MASS SPECTROMETRY FOR ELUCIDATING RECEPTOR COMPLEXES

Ligand affinity receptor chromatography

Live cell affinity receptor chromatography (LARC) is an established technique devised by Marshall, Zhu and Jankowski used to identify the members of an activated receptor complex by presenting ligand coated beads to live cells (Jankowski et al., 2008). In 2008, LARC was successfully used to identify a set of 288 non-redundant proteins associated with the Fc receptor complex. This list contains a large proportion of proteins known to be associated with the Fc receptor complex as well as other proteins that were previously unknown such as RhoG (Jankowski, 2008). The requirement for RhoG in Fc γ receptor mediated phagocytosis was

independently confirmed by Tzircotis et al. in 2011. Crkl1 was also identified by Jankowski and confirmed by Grinstein and colleagues in 2007.

In this system, the cognant ligand of a receptor, is used to coat 2um polystyrene beads. Ligand coated beads are presented to the cells and allowed to bind the receptor, causing activation and formation of the activated receptor complex. The cell membrane is then disrupted by physical compression with a French press in media containing a eukaryotic protease inhibitor cocktail and the beads carrying the ligand associated proteins from the activated receptor complex are isolated by sucrose gradient ultracentrifugation. This is followed by a salt step gradient, extraction in buffered organic solvents and several other extractions for soluble and insoluble extracts.

Fifteen fractions in total of the proteolysed proteins are subsequently analysed by liquid chromatography and mass spectrometry (LC-ESI-MS/MS). The collected parent and fragment masses are then identified by searches against a non-redundant protein library of proteins mapped to known parent and fragment masses using search algorithms such as SEQUEST. Multiple controls are run in the same fashion to account for non-specific binding by presenting beads to crushed cell lysates and presenting naked and ligand coated beads to growth media. This is the method that was used to identify the integrins in both IgG-Fc receptor mediated phagocytosis and oxLDL-scavenger receptor mediated phagocytosis.

The first isolation of integrin receptor complexes

The first quantitative analysis by mass spectrometry directly targeting integrin receptor complexes activated by presenting live cells with ligand coated beads was conducted in 2010 by Humphries et al. In this method, fibronectin coated beads and VCAM-1 coated beads were presented to live cells, activating the formation of Fibronectin- $\alpha 5\beta 1$ and VCAM-1- $\alpha 4\beta 1$ receptor complexes respectively. The receptor complexes were stabilized by cross-linking with DTBP. Detergents and sonication was used to isolate the receptor complexes. The cross linkers were cleaved and the resultant samples were run on SDS PAGE for protein separation and then digested with trypsin. The samples were then run on LC-MS/MS. The peptides were then

identified, quantified and analysed using cluster analysis to produce an interaction network model (Humphries et al., 2010).

Essential differences between LARC and the Humphries Method

The LARC and Humphries methods differ in several important ways. First no cross-linker is used in the LARC method in order to avoid additional non-specific binding events between the cross linkers and proteins not associated with the receptor complex. A series of controls to account for all possible types of nonspecific binding are subtracted from the LARC data. These include non-specific binding of growth media and crude cell lysates to both naked and ligand coated beads.

LARC uses a sucrose gradient in order to separate polystyrene beads instead of magnetic beads and sonication which were used by Humphries. LARC avoids the use of harsh detergents in favor of step extractions in physiological concentrations of salts in an attempt to keep receptor complexes intact.

Many of the challenges associated with traditional methods for determining protein interaction partners may be resolved in part by LARC which combines mass spectrometry with traditional protein separation techniques to extract receptor complexes from live cells and uses crude cell lysates and other non-specific binding controls.

Central Hypothesis

Integrins may have different roles in IgG versus oxLDL mediated phagocytosis. It may be possible to analyze receptors such as integrins by mass spectrometry and confirm the results by independent methods such as siRNA knockdowns of integrin mRNAs, phagocytosis assays, immunofluorescence and confocal microscopy.

CHAPTER 2: MATERIALS AND METHODS

Materials

The microbeads were obtained from Bang's beads (Fishers IN, USA). The solvents were of optical grade and obtained from Caledon Laboratories (Georgetown ON, Canada).

The LTQ ion trap was obtained from Thermo Fisher Scientific (Thermo Electron Corporation, Waltham MA, USA). The HPLC was from Agilent Technologies (Santa Clara CA, USA).

Protein sequencing grade trypsin was obtained from Roche (Mississauga ON, Canada). The human IgG ligand and all other buffers, salts and reagents were obtained from Sigma–Aldrich (St. Louis MO, USA). Donkey Anti Rabbit oxLDL antibodies were obtained from Millipore and mouse anti human integrin $\beta 1$ and $\alpha 3$ antibodies were obtained from Oncogene. Small interfering RNA was obtained by Sigma-Aldrich and Xtreme Gene transfection reagent was obtained by Roche. The 510 Meta-laser confocal microscope was obtained from Carl Zeiss (Jenna, Germany).

Cell culture

U937 human macrophage cells were grown in RDPMI medium with 10% fetal bovine serum (FBS) in 75 cm² flasks to 50% confluence. The U937 cell line was originally extracted from the pleural effusion of a 37 year old Caucasian male with histiocytic lymphoma in 1974 (Harris et al., 1985).

Preparation of oxLDL

Oxidized LDL was extracted from EDTA treated plasma derived from the blood of a fasting patient followed by ultracentrifugation. EDTA was then dialyzed against PBS with 0.5 mM EDTA at 4°C. Isolated LDL was allowed to oxidize at 37°C for 18 h in PBS with 5 μ M CuSO₄. Oxidation was terminated with 0.3 mM EDTA at pH 8.5 and 40 μ M butylated hydroxytoluene. Oxidized LDL was dialyzed at 4°C against PBS with 0.5 mM EDTA and then sterilized by membrane filtration and stored at 4°C in the dark (Shen et al., 2008).

Preparation of IgG coated beads

10uL of 10 mg/mL human IgG dissolved in PBS was combined with 10uL of 2 um polystyrene beads from a 10% (v/v) suspension of a 2.185-E10 microbeads per milliliter stock solution (Bang's Laboratories, Fishers, IN, USA) and diluted in 500 uL of 1 x phosphate buffered saline (PBS) and incubated for 30 min prior to washing three times after pelleting IgG coated beads at 12,000 relative centrifugal force (RCF), and removing supernatant containing unbound IgG. IgG coated beads were re-suspended in 500uL of PBS.

Preparation of oxLDL coated beads

The same procedure was used to produce the oxLDL coated beads as the IgG coated beads however the oxLDL coated beads were incubated in the dark due to light sensitivity.

Live-cell affinity receptor chromatography

100uL of ligand coated beads were prepared as described and added to live U937 cell cultures grown in 75 cm² flasks and allowed to bind to the surface of live cells suspended in HEPES buffer on ice for 30 minutes. Free beads were washed away three times using ice-cold HEPES. In cold conditions, the ligand coated beads were not internalized but instead remained on the surface of the cells bound to their respective receptors. Cells were warmed for 0, 2.5, 5, 7.5, and 10 min at 37°C to activate the receptors before quenching again with ice-cold medium. The cell membranes were then disrupted using a French press containing multiple eukaryotic protease inhibitors. The ligand coated microbeads associated with receptor complexes were isolated using sucrose gradient in 1 ml of sample volume using a centrifuge according to (Beckman). Crude lysate of U937 macrophages was also incubated, passed through the French press with an equivalent number of 2um polystyrene beads without ligands as a control.

Disruption of Cells by French Press

Cells manually loaded with 10 mLs of cell suspension or crude cell extract were disrupted using a precooled French press (FA-078A, Thermo Electron, Waltham, MA, USA) at 400 psi and left to equilibrate across the cell suspension for 5 min. Cells were disrupted by gently opening the exit valve and forcing the cell suspension through the exit needle. The crushed cell lysates were placed on ice and disruption was confirmed under light microscope at 40X magnification.

Extraction of Proteins from Receptor Complexes

Beads were isolated in a sucrose gradient by ultracentrifugation as described by Burkhardt and collected in a 1mL fraction (Burkhardt et al., 1996). Salt soluble proteins were extracted using a salt step gradient in 200uL of 150, 200, 250, 300, 350, 450, 500, 550, 600, 700, 800, 900, 1000 mM NaCl. The protein eluted in each salt fraction was measured using the Dumbroff/Gosh method and digested with trypsin prior to LC-MS/MS. Insoluble proteins were extracted in 70% acetonitrile. 100ug of protein from each fraction was digested in Tris (pH 8.8) in 1M urea and 5% acetonitrile and 1ug of trypsin and incubated at 37°C for 12 hrs. Fractions were analysed by liquid chromatography mass spectrometry (LC-ESI-MS/MS) alongside the controls which are described in Table 2.1.

Table 2.1: Controls run in the LARC experiment to observe enrichment of proteins where the Fc receptor is activated by IgG

Controls	Treatment
Live anti CD36-IgA	Anti CD36 conjugated with IgA coated beads presented to live cells
Crude anti CD36-IgA	Anti CD36 conjugated with IgA coated beads incubated with crude homogenates
Live anti CD36-IgG	Anti CD36 conjugated with IgG coated beads presented to live cells
Crude anti CD36-IgG	Anti CD36 conjugated with IgA coated beads incubated with crude homogenates
Live IgG	IgG coated beads presented to live cells
Crude IgG	IgG coated beads incubated with crude homogenates
Media IgG	IgG coated beads incubated in media
Live oxLDL	oxLDL coated beads presented to live cells
Crude oxLDL	oxLDL coated beads presented to crude homogenates
Media oxLDL	oxLDL coated beads incubated in media
Naked Beads Crude	Naked beads incubated with crude homogenates
Naked Beads Live Cells	Naked beads presented to live cells

Peptide correlation

LARC and control spectra were correlated together and searched against a database subset derived from the National Center for Biotechnology Information (NCBI) nr database. The MS/MS data files from the LC–ESI–ion trap runs were searched against the nm library using the SEQUEST algorithm.

Use of LARC data to select integrins enriched in the IgG-Fcγ and oxLDL-scavenger receptor complexes

The frequency distribution of the various integrins in the LARC experiments compared to controls described in table 2.1 showed different frequency distributions with corrections made for the 14 integrins compared (Benjaminin and Hochberg). The identified integrins were tested for their functional roles in phagocytosis using siRNA directed against each integrin combined with phagocytosis assays.

Role for LARC identified proteins examined using siRNA against integrins

Cells from a 70% confluent cell culture of U937 cells were transferred onto 25mm cover slips in six well dishes in 3 mL of RDPMI media with 10% fetal bovine serum and 200nM phorbol myristate acetate (PMA) and incubated for 72 hours at 37°C. At 72 hours the media was replaced and 5uL of extreme gene transfection reagent (XGTR) and 5uL of each double stranded RNA for silencing was diluted to a final volume of 50uL with serum free media, the diluted siRNA and XGTR were mixed and incubated for 15 minutes at room temperature before being applied to each well. Each plate contained a silencing RNA control and a second control with no treatment and four different integrin knockouts. Cells were incubated for another 72 hours before phagocytosis assays were conducted.

Phagocytosis Assay

Media was changed on the seventh day prior to phagocytosis assays with IgG and oxLDL coated beads. Beads were coated as previously described. oxLDL coated beads were incubated with cells for four hours at 37°C with cells in serum free RPMI media. For assays with IgG coated beads, cells were incubated for 2 hours in RPMI and 10% FBS. The rate of phagocytosis differs

between IgG and oxLDL coated beads. Incubations periods were adjusted to achieve adequate ratios of signal to noise. Cells were fixed in 4% paraformaldehyde for 30 minutes washed and then quenched in 5% w/v glycine in PBS. Outside beads were stained prior to incubation while antibody staining for identified integrins was conducted after permeabilization with 0.1% TritonX-100 in PBS. Cells were mounted with Dako.

Confocal Images

Images were recorded by 510 meta laser confocal microscope with Ar 488-nm and He/Ne 543-nm lasers (Carl Zeiss Microscopes, Jenna, Germany) under 40X magnification for bead counting. 100X magnification was used for antibody staining of integrin $\alpha 3$ and integrin $\beta 1$. Total and outside beads were counted manually using ImageJ.

Figure 2.1: Summary of method used to capture the Fc receptor complexes formed in U937 cells, identify the proteins forming the complex and determine potential integrin candidates for small interfering RNA screens

Analysis of Normal, Breast and Ovarian Cancer Human Blood samples by Organic Differential Extraction

Samples were manipulated at room temperature but were kept on ice when not in use. Samples were kept in plastic 1.5 mL epindorff tubes and manipulated with plastic pipette tips for extraction of solvents and re-suspension. After manipulation, samples were stored at -80°C.

Precipitation in Organic Solvent and Sequential Extraction Based on Polarity and Stability

Five volumes of acetonitrile was added to 200uL of human plasma causing it to precipitate out of solution at room temperature. The sample was resuspended, centrifuged at 12,000 RCF for one minute and the supernatant was extracted. The pellet was re-suspended sequentially in 200uL of 70, 60, 50, 40, 30, 20 and 10 percent solvent followed by water and then 5% formic acid. After each re-suspension, the sample was centrifuged at 12,000 RCF for one minute. The extraction of supernatant at each stage produced a separation of peptides based on polarity. Each fraction was assayed using a Bradford assay and Dumbroff Dotblot. The experiment was executed twice with two different organic solvents; first with acetonitrile and then with a 1:1:2 mixture of acetonitrile, methanol and water. This experiment was also conducted with serum. Each sample was dried down and stored in the solid phase prior to re-suspension in 5% formic acid, refined and applied to a C18 reversed phase column. Peptides were analyzed by liquid chromatography with electrospray ionization followed by tandem mass spectrometry (LC-ESI-MS/MS).

Analysis of Normal, Breast and Ovarian Cancer Human Plasma samples

Five 200uL samples of human plasma for each normal, breast and ovarian cancer were extracted and analysed as previously described using acetonitrile as the organic solvent. Peptide hits were compared between the normal and diseases states using chi-square tests.

CHAPTER 3: RESULTS

Comparison of IgG-Fc and oxLDL-Scavenger receptor complexes identified by Ligand Affinity Receptor Chromatography

In order to compare the receptor complexes formed in U937 cells after binding to either oxLDL coated beads and IgG coated beads, a ligand affinity receptor complex experiment was performed. The fractionated complexes digested with trypsin were analyzed by LC-MS/MS as described in the materials and methods. Four repeats of all LARC experiments and their respective controls were run and collapsed into a distinct set of accession numbers and associated peptide hits. The integrin subset of this dataset is shown in Table 3.1. For nearly all integrins, the live cell ligand affinity experiments produced a higher number of peptide hits compared to controls where ligand coated beads were presented to crude homogenates. The data indicates a higher level of complexity and organization in these samples as previously demonstrated in similar experiments on the analysis of the Fc receptor compared to controls (Jankowski, 2008). The LARC data shows that more of the receptor complex stays intact with a greater diversity of peptides when assembled from live cells than from crude homogenates.

Table 3.1: Comparison of integrins in IgG-Fcγ receptor complex and oxLDL-scavenger receptor complexes identified by ligand affinity chromatography

Gene Symbol	Total Protein ID Count	Live anti CD36-IgA	Crude anti CD36-IgA	Live anti CD36-IgG	Crude anti CD36-IgG	Live IgG	Crude IgG	media IgG	Live oxLDL	crude oxLDL	media oxLDL	live naked	crude naked
ITGA1	8360	918	710	331	202	334	311	10	1585	1074	4	13	452
ITGA2	5114	521	398	203	161	279	132	9	816	823	0	31	282
ITGA3	9975	1119	773	295	213	533	368	60	1569	1590	11	109	540
ITGA4	8504	1025	896	386	159	429	320	58	1338	977	20	37	744
ITGA5	14586	1760	1289	610	517	337	546	90	2275	2101	57	127	807
ITGA6	31401	3655	3648	1297	1255	956	1176	43	5043	3989	0	319	1651
ITGA7	43361	6321	4477	2132	1696	1432	1540	198	6292	5502	69	333	2216
ITGA8	18360	2339	1850	893	654	351	400	39	2908	2296	39	183	1078
ITGA9	9659	1040	843	505	266	285	469	12	1563	1353	4	74	537
ITGA10	5693	650	435	201	134	253	223	12	1094	741	0	36	243
ITGA11	20906	2480	2096	922	689	798	865	77	3740	2372	25	53	1232
ITGAD	11586	1196	1222	360	277	416	409	36	1727	1576	29	97	684
ITGAE	5886	868	543	285	187	246	270	19	989	593	8	26	376
ITGAIIB	3444	339	203	77	95	144	151	38	673	483	11	55	136
ITGAL	8859	864	679	293	210	583	352	62	1295	1466	52	31	476
ITGAM	15594	1612	1219	726	432	712	699	52	2667	2170	0	56	825
ITGAV	13007	1045	1037	477	269	627	623	65	2260	1742	24	71	601
ITGAX	4640	510	228	211	82	258	177	25	863	622	21	17	266
ITGB1	14084	1993	1111	670	204	540	469	125	2610	1641	61	106	567
ITGB2	18140	2149	1533	691	558	658	686	20	3365	2276	9	53	985
ITGB3	5041	378	357	147	48	358	234	32	1067	606	16	50	236
ITGB4	27754	3103	2273	1213	969	1223	1136	105	4531	3584	24	471	1646
ITGB5	5981	750	465	319	284	285	173	7	984	725	0	28	382
ITGB6	3302	353	186	192	101	234	155	19	586	375	6	34	151
ITGB7	2537	227	271	132	86	109	110	15	487	288	0	0	131
ITGB8	3839	358	217	138	89	142	176	13	676	591	0	100	142

Ligand affinity receptor chromatography experiments conducted by my predecessors.

Table 3.2: The integrins identified by ligand affinity receptor chromatography associated with each IgG-Fc γ and oxLDL-scavenger receptor complexes compared to peptides extracted from naked beads incubated in crude cell extracts using a chi-square test.

Integrin	Naked beads in crude cell extract	IgG beads presented to live cells	Chi squared test (columns 1 and 2)	oxLDL beads presented to live cells	Chi squared test (columns 1 and 4)
ITGA1	452	334	30.81	1585	2840.02
ITGA2	282	279	0.03	816	1011.19
ITGA3	540	533	0.09	1569	1960.82
ITGA4	744	429	133.37	1338	474.24
ITGA5	807	337	273.73	2275	2670.41
ITGA6	1651	956	292.57	5043	6968.91
ITGA7	2216	1432	277.37	6292	7497.19
ITGA8	1078	351	490.29	2908	3106.59
ITGA9	537	285	118.26	1563	1960.29
ITGA10	243	253	0.41	1094	2980.25
ITGA11	1232	798	152.89	3740	5105.57
ITGAD	684	416	105.01	1727	1590.42
ITGAE	376	246	44.95	989	999.39
ITGAIIB	136	144	0.47	673	2120.36
ITGAL	476	583	24.05	1295	1409.16
ITGAM	825	712	15.48	2667	4112.68
ITGAV	601	627	1.13	2260	4579.50
ITGAX	266	258	0.24	863	1339.88
ITGB1	567	540	1.29	2610	7361.29
ITGB2	985	658	108.56	3365	5750.66
ITGB3	236	358	63.07	1067	2926.11
ITGB4	1646	1223	108.71	4531	5056.64
ITGB5	382	285	24.63	984	948.70
ITGB6	151	234	45.62	586	1253.14
ITGB7	131	109	3.69	487	967.45
ITGB8	142	142	0	676	2008.14

Integrins chosen manually to test for functional role in phagocytosis are indicated by bold type.

A comparison of the integrins in both IgG and oxLDL mediated receptor complexes formed in live cells showed a greater number of peptide hits for all integrins where oxLDL coated beads were presented to cells compared to IgG coated beads. It is possible that the integrins have a greater affinity in general for complexes that form as a result of the scavenger receptors that bind to oxidized LDL compared to IgG.

It could be argued that oxLDL coated beads simply bind a greater number of non-specific proteins in general than their IgG coated counterparts. However, controls show that there are no

major differences in integrin peptide hits between IgG coated beads and oxLDL coated beads presented to media. No ligand coated beads for either oxLDL or IgG had more than 200 hits in media and only three had peptide hits over 100 and all three came from IgG coated beads rather than oxLDL coated beads. Thus, there is an overall enrichment of integrin content in oxLDL-scavenger receptor complexes versus those formed in response to IgG. How and why this enrichment occurs and whether or not there is a functional role for this integrin enrichment in oxLDL mediated phagocytosis versus IgG is currently unknown.

Additional controls were performed that included naked beads presented to either live cells or crude cell extracts. In order to account for non-specific binding to the bead itself naked beads presented to live cells showed very few integrin peptide hits indicating that non-specific binding to the beads is quite low. Naked beads presented to crude extracts however had much greater numbers of associated integrins. This indicates that integrins and their co-receptors and binding partners are not adhering to the polystyrene beads when the cells are intact. When naked beads are exposed to crushed homogenates, integrins are binding the beads in much greater numbers. This is to be expected since presumably integrins are released when cells are lysed.

LARC was also used to selectively target CD36 without activating the Fc γ receptor by coating beads with anti-CD36 immunoglobulin A (IgA) and compared to LARC experiments run with anti-CD36 –IgG coated beads. The integrin peptide hits observed in anti-CD36-IgA were much higher than those anti-CD36 –IgG in live cells which indicates that CD36 is likely playing an important role in phagocytosis. The peptide hits for the anti-CD36 IgA LARC experiment were comparable to the LARC experiment run with oxLDL coated beads. A more comprehensive comparison of the entirety of both datasets would be required to determine if there is enough information to say that CD36 is the main scavenger receptor responsible for oxLDL uptake. Here we show for the first time the integrin isoforms associated with the CD36, IgG and oxLDL receptor complexes.

Figure 3.1a: Examples of MS/MS spectra. Integrin peptides identified by ligand affinity chromatography and LC-MS/MS where IgG coated beads were presented to live human neutrophils (integrins $\alpha 3$, $\alpha 4$, $\alpha 7$, αD , αM , αX , $\beta 2$ and $\beta 4$ labelled below each spectra with identified amino acid sequence)

Figure 3.1b: Examples of MS/MS spectra. Integrin peptides identified by ligand affinity chromatography and LC-MS/MS where IgG coated beads where presented to live human neutrophils (integrins $\alpha 2$, $\alpha 6$, αL , and $\beta 1$)

The frequency distribution of the various integrins in the LARC experiments compared to that of extracts mixed with uncoated beads found that 14 integrins: $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\alpha 7$, $\alpha 11$, αD , αL , αM , αX , $\beta 1$, $\beta 2$, $\beta 4$ showed different frequency distributions compared to the untreated controls after correction for the 26 integrins compared (Benjaminin and Hochberg).

Expected Binding Partners for Identified Integrins

A network model was created for ITGA6 which was identified as having a different frequency distribution compared to controls for Fc-IgG and oxLDL-scavenger receptor complexes. This network diagram summarizes known binding partners for ITGA6 and was searched against databases for the IgG and oxLDL ligand affinity chromatography experiments to confirm the LARC system and to identify any differences between both phagocytic receptor complexes.

Figure 3.2: Protein interaction map of Itga6 and known associated proteins identified by STRING (2013) Evidence of protein-protein interactions is based on Green: Neighborhood, Red: Gene fusion, Dark blue: Co-occurrence, Black: Co-expression, Pink: Experiments, Light blue: Databases, Yellow: Text mining; Purple, Homology.

As described by the interaction network model, $\alpha 6$ binds to two integrin beta subunits ITGB1 and ITGB4 forming two separate integrin heterodimers. The $\alpha 6\beta 1$ integrin heterodimer is known to bind the following extracellular matrix proteins: Laminin, thrombospondin, cysteine-rich protein 61 (Cyr61) and a disintegrin and metalloprotease (ADAM) (Tadaka, 2007).

Thrombospondin-1 is an important adhesive glycoprotein that is a ligand for multiple integrins as well as scavenger receptor CD36 and mediates cell-cell and cell-extracellular matrix interactions. $\alpha 6\beta 4$ is only known to bind laminin (LAMA5) in the extracellular matrix, which is important for attachment, migration and tissue organization during embryonic development (Takada, 2007). $\alpha 6\beta 4$ is known to bind plectin in the formation of hemidesmosomes which anchor epithelial cells to basement membranes (de Pereda et al., 2009). Plectin binds intermediate filaments and links them with microtubules and microfilaments. Plectin may also be involved in the regulation of the cytoskeleton intermediate filament network (de Pereda et al., 2009). Collagen type XVII $\alpha 1$ (COL17A1) may be important for hemidesmosome integrity as well. CD151 is required for the proper assembly of basement membranes in the kidney. Fibronectin binds a wide variety of molecules including various extracellular matrix proteins and cell surface proteins, actin and DNA. Growth factor receptor-bound protein 2 (GRB2) is an adaptor protein found between growth factor receptors and the Ras pathway (Lowenstein et al., 1992). Many of these integrin binding proteins were found in the mass spectrometry data in samples from live cells but not controls.

Figure 3.3a: Images shown are U937 cells responding to IgG coated beads. Laser confocal microscopy images of U937 cells were treated with double stranded RNA directed against the integrin shown and then treated with coated beads to assess phagocytosis (small interfering RNA labelled with Cy3, Cy5 used to label beads not engulfed by human macrophages)

Figure 3.3b: Images shown are U937 cells responding to IgG coated beads. Laser confocal microscopy images of U937 cells were treated with double stranded RNA directed against the integrin shown and then treated with beads to assess phagocytosis (small interfering RNA labelled with Cy3, Cy5 used to label beads not engulfed by human macrophages)

A functional role for integrins in oxLDL mediated phagocytosis

The functional role for each of the fourteen integrins identified as being involved in phagocytosis was assessed in silencing RNA experiments in U937 human macrophages presented with IgG coated beads and oxLDL coated beads. Controls consisted of an siRNA control to account for any effect of the small interfering RNAs and a control with no treatment. The experiment was repeated three times with IgG coated beads in media with serum and once for oxLDL coated beads in media without serum. Phagocytosis of IgG coated beads was most effected by

knockdowns of the mRNA of integrins $\alpha 1$, αM , and $\beta 1$ as shown in Figure 4.1. However, the knockdown of each of the target integrins seemed to have a general inhibitory effect on IgG mediated phagocytosis, although one way ANOVA identified no significant difference between any of the treatments when $\alpha = 0.05$. Here we show for the first time that some integrins have a small but statistically significant role in the IgG mediated phagocytic engulfment of foreign particles.

Figure 3.4: siRNA directed against integrins affects phagocytosis of IgG coated beads.

Percent inhibition of phagocytosis was calculated using the total number of inside beads for three repetitions of phagocytosis assays where U937 cells were treated with double stranded mRNA cells against various integrins and presented with IgG coated beads for 2 hours in RPMI media with 10% fetal bovine serum.

Figure 3.5: Treatment of U937 cells with siRNA affects phagocytosis of oxLDL treated beads. First repetition showing the mean of internalized beads when incubated for 4 hours in serum free media.

Figure 3.6: Treatment of U937 cells with siRNA affects phagocytosis of oxLDL treated beads. First repetition showing the mean of internalized beads when incubated for 4 hours in serum free media.

The knockdown of integrins identified through LARC generally inhibited IgG receptor mediated phagocytosis. The knockdown of integrins in oxLDL receptor mediated phagocytosis seemed to have a stimulatory effect on phagocytosis in the case of ITGB4, ITGAD, ITGAX and especially ITGA6. The knockdown of ITGA6 had the greatest stimulatory effect and ITGA3 had the greatest inhibitory effect on oxLDL mediated phagocytosis. These findings are only preliminary and must be replicated. Of greatest importance, the novel data present here for the first time indicates that the knockdown of specific integrins that play a role in self-recognition between normal human cells and proteins, releases the inhibition of phagocytosis of human oxLDL particles and perhaps indicates the self- recognition of human apolipoproteins by integrins may play a role in governing the decision to engulf a foreign particle.

Visualization of Integrin $\beta 1$ and $\alpha 3$ in oxLDL-scavenger receptor mediated phagocytosis

Antibody staining was used to visualize the localization of $\beta 1$ integrins during oxLDL-scavenger receptor mediated phagocytosis in U937 cells after four hours of incubation with oxLDL coated beads in RPMI media without serum. Staining was conducted with permeabilization of the cells. Integrin $\beta 1$ seemed to be evenly distributed on the surface of the cells and especially in the actin dependent pseudopodia. The antibody staining for ITGA3 showed different localization in IgG and oxLDL as shown in Figure 3.8.

Figure 3.7: Phagocytosis assay of U937 cells incubated with oxLDL coated beads for 4 hours in serum free media with outside beads stained with Cy2 and $\beta 1$ integrins stained with Dy488

Figure 3.8a: (Left) Phagocytosis assay of U937 cells incubated with oxLDL coated beads for 4 hours in serum free media with outside beads stained with Cy2 and $\alpha 3$ integrins stained with Dy488

Figure 3.8b: (right) Phagocytosis assay of U937 cells incubated with IgG coated beads for 2 hours in media and serum with outside beads stained with Cy2 and $\alpha 3$ integrins stained with Dy488

CHAPTER 4: THE INTEGRIN PEPTIDES OF NORMAL HUMAN BLOOD AND THE BLOOD OF PATIENTS WITH BREAST AND OVARIAN CANCER

To determine if differences could be detected in the number of integrin peptide fragments in normal human blood versus blood from patients in a diseased state, plasma samples were fractionated by an organic differential extraction and analysed by LC-MS/MS as described in the materials and methods.

The peptides of human plasma were obtained by precipitating all plasma blood samples in 90% acetonitrile followed by the selective extraction of low molecular mass peptides in 10 fractions by sequentially increasingly polar mixtures of acetonitrile and water. Acetonitrile and methanol was also used as the extraction solvent however acetonitrile alone was superior. The separation of peptides is demonstrated by Dumbroff Dotblot in Figure 3.10.

Figure 4.1: Quantification of protein content at each step of the differential organic extraction performed on normal human plasma using a Dumbroff dot blot (mg/mL). (Rows 1 and 2: BSA standards; Rows 3 and 4: extraction with acetonitrile; rows 5 and 6: extraction with 1:1 acetonitrile and methanol)

Each sample was dried down and stored in the solid phase prior to re-suspension in 5% formic acid, refined and applied to a C18 reversed phase column. Peptides were analyzed by liquid chromatography with electrospray ionization followed by tandem mass spectrometry (LC-ESI-

MS/MS). Using this method, the peptides from normal human blood and breast and ovarian cancer samples were reproducibly detected. The TIC traces and base peak chromatographs are shown in Figure 5.1 for normal human plasma. The total ion current (TIC) chromatograms show the sum of the ion intensities for the range of masses being detected over an 80 minute gradient. For each TIC there is an obvious difference between the signal of the sample that elutes over the course of the gradient and the background noise shown at the beginning and end of the TIC.

The base peak chromatograms show only the ion intensity for the most intense peak at each point in time. The base peak chromatograms show good separation of the peaks in space and time and are an indication of the success of the separation technique used.

Figure 4.2: Total ion current (TIC), and base peak chromatographs for the sequential organic differential extraction of peptides from normal blood in 90, 70, 60, 50, 40, 30, 20, 10 and 0% ACN followed by 5% formic acid and subject to ESI-LC-MS/MS (base peak: left, TIC: right)

Peptides derived from the normal human plasma samples included signal transduction molecules such as kinases, G-proteins and their adapter and regulatory proteins. Also, peptides from interleukins, chemokines, cytokines and peptides from their soluble or membrane bound receptors were observed. The integrin subset of the peptides of normal human blood is presented in table 5.1. Thus far, only one integrin, integrin alpha 9 (ITGA9) has been identified from the tryptic peptides of blood by mass spectrometry by Daniel Chan and colleagues with 2 peptide hits (Hanash et al., 2005). In five normal human plasma samples of 200uL each, 3,417 integrin peptide hits were observed with all alpha and beta subunits represented.

Figure 4.3: Known and predicted protein-protein interaction model for normal plasma and serum using STRING 9.0.

Table 4.1 List of Integrin peptide hits observed in normal, breast cancer and ovarian human plasma samples. Chi square test used to compare normal and disease samples

Integrin Gene Symbol	Normal Human Plasma	Breast Cancer	Chi squared test columns 1 & 2	Ovarian Cancer	Chi squared test columns 1 & 3
ITGA1	41	85	47.21	58	7.05
ITGA2	65	28	21.06	24	25.86
ITGA3	144	133	0.84	152	0.44
ITGA4	93	202	127.75	88	0.27
ITGA5	190	202	0.76	115	29.61
ITGA6	265	340	21.23	464	149.44
ITGA7	113	454	1029.04	445	975.43
ITGA8	202	199	0.04	116	36.61
ITGA9	54	38	4.74	68	3.63
ITGA10	108	81	6.75	215	106.01
ITGA11	216	173	8.56	212	0.07
ITGAD	129	246	106.12	259	131.01
ITGAE	19	16	0.47	18	0.05
ITGAILb	0	49	Undefined	260	Undefined
ITGAL	26	100	210.62	148	572.46
ITGAM	150	214	27.31	233	45.93
ITGAV	26	181	924.04	207	1260.04
ITGAX	20	7	8.45	132	627.2
ITGB	503	620	27.21	1196	954.77
ITGB1	149	310	173.97	1199	7399.33
ITGB2	144	106	10.03	88	21.78
ITGB3	81	120	18.78	238	304.31
ITGB4	418	766	289.72	905	567.39
ITGB5	125	118	0.39	88	10.95
ITGB6	45	30	5	59	4.36
ITGB7	27	2	23.15	28	0.04
ITGB8	17	38	25.94	26	4.76

Gene symbol in bold where chi square test is above 500 for comparison of peptide hits in disease samples compared to controls

Integrins are known to have low pico molar levels of expression and they were observed quite reliably in normal human blood. They were also observed in ovarian and breast cancer as well as heart attack and sepsis samples where some preliminary differences can be seen between each dataset and their respective controls (data not shown). The ion and fragment ion intensity values from all of these peptides and their MS/MS fragments can be recorded in a structure query

language (SQL) database, and the ion intensity values statistically analyzed between treatments using the statistical analysis system (SAS). In addition to the integrins observed many other novel blood peptides from parent proteins that show some homology to proteins with significant biological functions were discovered.

CHAPTER 5: DISCUSSION

The aim of this research was to determine if LARC could be used to identify the integrins of the oxLDL-scavenger and IgG-Fc γ receptor complexes (LARC previously obtained by the Marshall lab) and to determine if a functional role could be demonstrated for the integrins identified in each type of ligand mediated phagocytosis. This was approached using double stranded RNA and phagocytosis assays where oxLDL coated beads and IgG coated beads were presented to U937 cells. Three repetitions of the experiment were conducted where IgG coated beads were presented to U937 cells. The mean phagocytic efficiencies of the three repetitions was statistically analysed by ANOVA. Each repetition was compared using either the phagocytic efficiency or the total number of inside beads by multiple comparison. Knockdown of integrin mRNAs showed significant effects on phagocytic efficiency when cells were presented with either IgG or oxLDL coated beads.

Experimental Design

The experimental design of presenting ligand coated beads to live cells or crude homogenates in order to capture and compare activated receptor complexes, is a sufficient and reasonable means to address the difficult problem of analysing insoluble receptor complexes provided that controls for the nonspecific binding of naked and ligand coated beads presented to crude homogenates and media are taken into account.

Integrins captured and identified by LARC had the highest signal to noise ratios when compared to controls of crude homogenates indicating higher levels of complexity when complexes are formed on the surface of live cells as demonstrated in a similar previous study of the Fc receptor in RAW cells (Jankowski, 2008). As expected, the lowest numbers of peptide hits for all integrins was observed in controls where the naked beads and ligand coated beads were presented to media alone.

Specificity

Many peptides per integrin were identified in both the LARC data and in normal human plasma. Thus, the chance of a false positive result is very low (Zhu et al., 2011). Models of random spectra, noise and random libraries confirm that the chance of type I error is low where the ratio of peptides to proteins is high (Zhu et al., 2011). The capture of the Fc receptor complex by LARC in RAW cells, human neutrophils and now in U937 human macrophages has shown strong agreement with known associated proteins derived by other biochemical means (Janowski, 2008) (Florentinas, 2011). Many unique peptides only found in particular integrins were observed, however many peptides common to multiple integrins were also identified thus there is some ambiguity in the result.

Sensitivity

The limit of sensitivity for the detection of peptides in complex samples was estimated to be in the nanograms per mL range in 2009 (Tucholska). With regard to this study, integrins are known to be expressed on the surface of cells in picomolar amounts and so it can be concluded that the sensitivity in this experiment achieved was at least in the picomolar range.

Mass spectrometry

Not only were integrins detected in the LARC experiments but the String model predicting the binding partners and associated proteins to ITGA6 were also observed in the LARC experiments but not in controls consistent with the validity of mass spectrometry. Peptides to protein ratios are not a true measure of quantification. It must be possible to directly quantify proteins by intensity values since the data is random, independent and normal in the case of electrospray.

However, these intensity values are not available and preliminary observations indicates that nano-spray intensity distribution are not normal compared to electrospray. Hence the peptide to protein data presented here is a reasonable metric to compare the proteins discovered and can be analyzed by Chi square analysis (Zhu et al., 2011).

Independent biochemical and cellular methods

Upon comparison, the integrins detected by LARC in the IgG-Fc receptor complex versus the oxLDL-scavenger receptor complex showed some interesting differences. Knockdowns of the mRNAs corresponding to integrins identified by the LARC procedure showed a general decrease in phagocytic inhibition in Fc-IgG receptor mediated phagocytosis and a general increase in oxLDL scavenger receptor mediated phagocytosis. However, the oxLDL ligand coated phagocytosis assays must be repeated to confirm the result. Of all the double stranded mRNA integrin knockdowns, integrin alpha 6 seemed to have the largest effect on phagocytosis and may act as an inhibitor of oxLDL mediated phagocytosis. In the first repeat, the silencing RNA control the mean of internalized beads was $5.21 \text{ SE} \pm 0.55$ and the mean for the ITGA6 knockdown was $10.78 \text{ SE} \pm 1.14$. Integrin beta 4 also seems to have an inhibitory effect on oxLDL mediated phagocytosis with the second highest mean of internalized beads of $7.82 \text{ SE} \pm 0.82$ when knocked down. It is logical that both of these integrins should act together since integrin alpha 6 is the only known integrin to bind with integrin $\beta 4$. Integrin $\alpha 6$ is only known to bind with one other beta subunit which is ITG $\beta 1$. The knockdown of integrin $\alpha 3$ has the greatest inhibitory effect on phagocytosis with a mean of $4.78 \text{ SE} \pm 0.42$ internalized beads per cell. Interestingly, a study by Thorne et al., demonstrated a physical and functional relationship between integrins $\alpha 6\beta 1$, $\alpha 3\beta 1$ and CD36 in melanoma cells by co-immunoprecipitation, immunoblotting and demonstrated co-localization by confocal microscopy (Thorne et al., 2000). Another study conducted in platelets also confirmed an association of CD36 with integrin $\alpha 6\beta 1$ and additionally integrin $\alpha \text{IIb}\beta 3$ and tetraspanin CD9 by immunoprecipitation with anti-CD36 antibodies and mass spectrometry (Miao et al., 2001). Anti- integrin monoclonal antibodies are currently being used to treat unstable angina by blocking platelet aggregation through integrin $\beta 3$. If further studies confirm a role for integrin $\alpha 6$ as an inhibitor of oxLDL mediated phagocytosis by human macrophages a stimulatory monoclonal antibody such as monoclonal antibody could be a viable treatment for atherosclerosis.

CHAPTER 6: CONCLUSION AND FURTHER WORKS

Conclusion

In conclusion, it is possible to isolate receptor complexes with specific isoforms of their integrin co-receptors. Furthermore, it is possible to detect functionally important integrins by comparative receptor complex studies using LARC and that multiple ligands can be used in multiple experiments to solve a particular problem given all appropriate measures are taken to account for nonspecific binding.

Overall, this experimental design more completely addresses the problems associated with nonspecific binding than in previous studies undertaken by Janksowki, Florentinus and Humphries. In particular, the antiCD36-IgA and antiCD36-IgG were an important additional control/experiment which allowed for the comparison of oxLDL receptor mediated phagocytosis and the resultant receptor complexes that formed as a result of specific binding to CD36 with and without the activation of the Fc receptor through IgG.

The significant effect of some of these integrins in phagocytosis was consistent with the interpretation that that mass spectrometry correctly identified and enumerated the relative levels of the protein in complex samples derived from live receptor complexes.

Most importantly, here we have shown for the first time the integrin isoforms that associate with the CD36, IgG and oxLDL receptor complexes. Also for the first time we show for that some integrins have a small but statistically significant role in the IgG mediated phagocytic engulfment of foreign particles.

Of greatest importance, the novel data obtained indicates that the knockdown of specific integrins that play a role in self-recognition between normal human cells and proteins, releases the inhibition of phagocytosis human oxLDL particles and perhaps indicates that self-recognition of human apolipoproteins by integrins plays a role in governing the decision to engulf a foreign particle.

Further work

Of all the drug therapies that target cell surface receptors, GPCRs are the most highly targeted, coming from a pool of roughly 200 known GPCRs. However, according to new developments in the human genome, there are likely another 400 GPCRs to mine as potential drug targets (Christopoulos, 2002).

We might use these integrins for comparison of other co-receptors including GPCRs and innate immune receptors discovered in the same experiments. There is already evidence to support the link between GPCRs and integrin activation. This work could result in the discovery of important new therapeutics for previously unknown GCPR targets.

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Figure 7.1: Distribution of data for three repetitions of mean phagocytized IgG coated beads for various integrin knockdowns observing phagocytosis assays with siRNA controls and no treatment controls where ANOVA was used to obtain an F value of 0.18 and $p=0.9989$

Figure 7.2: IgG-Rep1: Distribution of phagocytized IgG coated beads amongst various integrins knockdowns and controls with square root transformation of data to achieve a normal distribution.

Least Squares Means for effect integrin_knockdown Pr > t for H0: LSMean(i)=LSMean(j) Dependent Variable: inside_beads														
i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1		0.9881	1.0000	1.0000	<.0001	0.0682	0.9727	0.8588	0.0140	0.2846	1.0000	0.7157	0.1001	<.0001
2	0.9881		0.9819	0.9929	0.0340	0.8908	0.3825	0.1981	0.0004	0.0199	0.9920	0.1352	0.9220	0.0094
3	1.0000	0.9819		1.0000	0.0002	0.1039	0.9983	0.9783	0.1033	0.6367	1.0000	0.9171	0.1386	<.0001
4	1.0000	0.9929	1.0000		0.0002	0.1342	0.9914	0.9381	0.0507	0.4740	1.0000	0.8352	0.1769	<.0001
5	<.0001	0.0340	0.0002	0.0002		0.9068	<.0001	<.0001	<.0001	<.0001	0.0009	<.0001	0.9033	1.0000
6	0.0682	0.8908	0.1039	0.1342	0.9068		0.0018	0.0005	<.0001	<.0001	0.1895	0.0005	1.0000	0.7179
7	0.9727	0.3825	0.9983	0.9914	<.0001	0.0018		1.0000	0.7329	0.9976	0.9989	1.0000	0.0031	<.0001
8	0.8588	0.1981	0.9783	0.9381	<.0001	0.0005	1.0000		0.9297	1.0000	0.9858	1.0000	0.0009	<.0001
9	0.0140	0.0004	0.1033	0.0507	<.0001	<.0001	0.7329	0.9297		0.9998	0.1660	0.9972	<.0001	<.0001
10	0.2846	0.0199	0.6367	0.4740	<.0001	<.0001	0.9976	1.0000	0.9998		0.7203	1.0000	<.0001	<.0001
11	1.0000	0.9920	1.0000	1.0000	0.0009	0.1895	0.9989	0.9858	0.1660	0.7203		0.9395	0.2346	0.0002
12	0.7157	0.1352	0.9171	0.8352	<.0001	0.0005	1.0000	1.0000	0.9972	1.0000	0.9395		0.0008	<.0001
13	0.1001	0.9220	0.1386	0.1769	0.9033	1.0000	0.0031	0.0009	<.0001	<.0001	0.2346	0.0008		0.7166
14	<.0001	0.0094	<.0001	<.0001	1.0000	0.7179	<.0001	<.0001	<.0001	<.0001	0.0002	<.0001	0.7166	

Figure 7.3: IgG-Rep1: Tukey-Kramer's test used to determine which pairwise comparisons between each of the screened integrin knockdowns were significantly different from the scrambled siRNA control in terms of internalized beads

Figure 7.4: IgG-Rep2: Distribution of phagocytized IgG coated beads amongst various integrins knockdowns and controls

Least Squares Means for effect integrin_knockdown Pr > t for H0: LSMean(i)=LSMean(j) Dependent Variable: inside_beads															
i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		1.0000	0.9265	0.9999	0.9998	1.0000	1.0000	1.0000	0.9991	1.0000	1.0000	0.9856	0.9943	1.0000	0.3875
2	1.0000		0.9712	1.0000	0.9999	1.0000	1.0000	1.0000	0.9997	1.0000	1.0000	0.9946	0.9982	1.0000	0.6160
3	0.9265	0.9712		1.0000	1.0000	0.9959	0.9794	0.9998	1.0000	0.5905	0.9354	1.0000	1.0000	0.9989	1.0000
4	0.9999	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	0.9837	0.9996	1.0000	1.0000	1.0000	0.9904
5	0.9998	0.9999	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	0.9830	0.9995	1.0000	1.0000	1.0000	0.9955
6	1.0000	1.0000	0.9959	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	0.9995	0.9999	1.0000	0.8364
7	1.0000	1.0000	0.9794	1.0000	1.0000	1.0000		1.0000	1.0000	0.9999	1.0000	0.9974	0.9994	1.0000	0.5799
8	1.0000	1.0000	0.9998	1.0000	1.0000	1.0000	1.0000		1.0000	0.9991	1.0000	1.0000	1.0000	1.0000	0.9432
9	0.9991	0.9997	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		0.9419	0.9981	1.0000	1.0000	1.0000	0.9892
10	1.0000	1.0000	0.5905	0.9837	0.9830	1.0000	0.9999	0.9991	0.9419		1.0000	0.8232	0.8757	0.9940	0.0929
11	1.0000	1.0000	0.9354	0.9996	0.9995	1.0000	1.0000	1.0000	0.9981	1.0000		0.9825	0.9922	1.0000	0.5230
12	0.9856	0.9946	1.0000	1.0000	1.0000	0.9995	0.9974	1.0000	1.0000	0.8232	0.9825		1.0000	1.0000	0.9999
13	0.9943	0.9982	1.0000	1.0000	1.0000	0.9999	0.9994	1.0000	1.0000	0.8757	0.9922	1.0000		1.0000	0.9990
14	1.0000	1.0000	0.9989	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.9940	1.0000	1.0000	1.0000		0.8372
15	0.3875	0.6160	1.0000	0.9904	0.9955	0.8364	0.5799	0.9432	0.9892	0.0929	0.5230	0.9999	0.9990	0.8372	

Figure 7.5: IgG –Rep2: Tukey-Kramer’s test used to determine which pairwise comparisons between each of the screened integrin knockdowns were significantly different from the scrambled siRNA control in terms of internalized beads

Figure 7.6: IgG-Rep3: Distribution of phagocytized IgG coated beads amongst various integrins knockdowns and controls

Least Squares Means for effect integrin_knockdown Pr > t for H0: LSMean(i)=LSMean(j) Dependent Variable: inside_beads															
i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		0.6454	1.0000	1.0000	0.9995	1.0000	1.0000	0.9996	0.9562	0.0869	0.9608	1.0000	1.0000	0.0033	0.9139
2	0.6454		0.9051	0.7984	0.9942	0.9048	0.9065	0.9992	1.0000	0.9981	1.0000	0.9670	0.8684	0.7449	1.0000
3	1.0000	0.9051		1.0000	1.0000	1.0000	1.0000	1.0000	0.9988	0.2150	0.9989	1.0000	1.0000	0.0092	0.9919
4	1.0000	0.7984	1.0000		1.0000	1.0000	1.0000	1.0000	0.9879	0.1618	0.9889	1.0000	1.0000	0.0091	0.9640
5	0.9995	0.9942	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	0.5212	1.0000	1.0000	1.0000	0.0520	0.9998
6	1.0000	0.9048	1.0000	1.0000	1.0000		1.0000	1.0000	0.9987	0.2194	0.9988	1.0000	1.0000	0.0101	0.9916
7	1.0000	0.9065	1.0000	1.0000	1.0000	1.0000		1.0000	0.9988	0.2192	0.9989	1.0000	1.0000	0.0098	0.9919
8	0.9996	0.9992	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	0.7579	1.0000	1.0000	1.0000	0.1967	1.0000
9	0.9562	1.0000	0.9988	0.9879	1.0000	0.9987	0.9988	1.0000		0.8962	1.0000	0.9999	0.9976	0.2646	1.0000
10	0.0869	0.9981	0.2150	0.1618	0.5212	0.2194	0.2192	0.7579	0.8962		0.9360	0.3534	0.1680	1.0000	0.9991
11	0.9608	1.0000	0.9989	0.9889	1.0000	0.9988	0.9989	1.0000	1.0000	0.9360		0.9999	0.9979	0.3731	1.0000
12	1.0000	0.9670	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.9999	0.3534	0.9999		1.0000	0.0254	0.9981
13	1.0000	0.8684	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.9976	0.1680	0.9979	1.0000		0.0054	0.9878
14	0.0033	0.7449	0.0092	0.0091	0.0520	0.0101	0.0098	0.1967	0.2646	1.0000	0.3731	0.0254	0.0054		0.8955
15	0.9139	1.0000	0.9919	0.9640	0.9998	0.9916	0.9919	1.0000	1.0000	0.9991	1.0000	0.9981	0.9878	0.8955	

Figure 7.7: IgG-Rep3: Tukey-Kramer's test used to determine which pairwise comparisons between each of the screened integrin knockdowns were significantly different from the scrambled siRNA control in terms of internalized beads

Figure 7. 8: oxLDL-Rep1: Distribution of phagocytized oxLDL coated beads amongst various integrins knockdowns and controls

Least Squares Means for effect integrin_knockdown Pr > t for H0: LSMean(i)=LSMean(j) Dependent Variable: inside_beads													
i/j	1	2	3	4	5	6	7	8	9	10	11	12	13
1		1.0000	0.8888	1.0000	<.0001	0.9998	1.0000	1.0000	1.0000	0.9934	0.3732	0.7945	1.0000
2	1.0000		0.8206	1.0000	<.0001	1.0000	1.0000	1.0000	1.0000	0.9723	0.8836	0.9910	0.9994
3	0.8888	0.8206		0.9745	<.0001	0.5726	0.6444	0.9765	0.7993	1.0000	0.0119	0.0744	0.9998
4	1.0000	1.0000	0.9745		<.0001	0.9999	1.0000	1.0000	1.0000	0.9991	0.5709	0.8864	1.0000
5	<.0001	<.0001	<.0001	<.0001		0.0003	<.0001	<.0001	<.0001	<.0001	0.0440	0.0110	<.0001
6	0.9998	1.0000	0.5726	0.9999	0.0003		1.0000	0.9996	1.0000	0.8648	0.9755	0.9996	0.9884
7	1.0000	1.0000	0.6444	1.0000	<.0001	1.0000		1.0000	1.0000	0.9128	0.9138	0.9959	0.9957
8	1.0000	1.0000	0.9765	1.0000	<.0001	0.9996	1.0000		1.0000	0.9994	0.4781	0.8360	1.0000
9	1.0000	1.0000	0.7993	1.0000	<.0001	1.0000	1.0000	1.0000		0.9683	0.8562	0.9877	0.9993
10	0.9934	0.9723	1.0000	0.9991	<.0001	0.8648	0.9128	0.9994	0.9683		0.0662	0.2552	1.0000
11	0.3732	0.8836	0.0119	0.5709	0.0440	0.9755	0.9138	0.4781	0.8562	0.0662		1.0000	0.2810
12	0.7945	0.9910	0.0744	0.8864	0.0110	0.9996	0.9959	0.8360	0.9877	0.2552	1.0000		0.6232
13	1.0000	0.9994	0.9998	1.0000	<.0001	0.9884	0.9957	1.0000	0.9993	1.0000	0.2810	0.6232	

Figure 7.9: oxLDL-Rep1 Tukey-Kramer's test used to determine which pairwise comparisons between each of the screened integrin samples were significantly different from the scrambled siRNA control

Least Squares Means for effect Integrin Pr > t for H0: LSMean(i)=LSMean(j) Dependent Variable: inside_beads															
i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		1.0000	1.0000	0.5642	0.9601	1.0000	0.8344	0.8855	0.9997	0.7740	0.4666	0.9982	0.9634	1.0000	1.0000
2	1.0000		1.0000	0.6239	0.9927	1.0000	0.8571	0.8968	0.9995	0.7901	0.6989	0.9975	0.9644	1.0000	1.0000
3	1.0000	1.0000		0.9287	0.9435	0.9997	0.9900	0.9938	1.0000	0.9686	0.4840	1.0000	0.9991	1.0000	0.9997
4	0.5642	0.6239	0.9287		0.0639	0.3147	1.0000	1.0000	0.9988	1.0000	0.0059	0.9999	1.0000	0.9840	0.3226
5	0.9601	0.9927	0.9435	0.0639		1.0000	0.1584	0.2053	0.6787	0.1511	0.9999	0.5884	0.3228	0.8740	1.0000
6	1.0000	1.0000	0.9997	0.3147	1.0000		0.5576	0.6285	0.9730	0.4983	0.9683	0.9443	0.7797	0.9975	1.0000
7	0.8344	0.8571	0.9900	1.0000	0.1584	0.5576		1.0000	1.0000	1.0000	0.0185	1.0000	1.0000	0.9990	0.5631
8	0.8855	0.8968	0.9938	1.0000	0.2053	0.6285	1.0000		1.0000	1.0000	0.0274	1.0000	1.0000	0.9994	0.6318
9	0.9997	0.9995	1.0000	0.9988	0.6787	0.9730	1.0000	1.0000		0.9996	0.1889	1.0000	1.0000	1.0000	0.9719
10	0.7740	0.7901	0.9686	1.0000	0.1511	0.4983	1.0000	1.0000	0.9996		0.0206	1.0000	1.0000	0.9936	0.5012
11	0.4666	0.6989	0.4840	0.0059	0.9999	0.9683	0.0185	0.0274	0.1889	0.0206		0.1458	0.0524	0.3636	0.9763
12	0.9982	0.9975	1.0000	0.9999	0.5884	0.9443	1.0000	1.0000	1.0000	1.0000	0.1458		1.0000	1.0000	0.9429
13	0.9634	0.9644	0.9991	1.0000	0.3228	0.7797	1.0000	1.0000	1.0000	1.0000	0.0524	1.0000		1.0000	0.7803
14	1.0000	1.0000	1.0000	0.9840	0.8740	0.9975	0.9990	0.9994	1.0000	0.9936	0.3636	1.0000	1.0000		0.9973
15	1.0000	1.0000	0.9997	0.3226	1.0000	1.0000	0.5631	0.6318	0.9719	0.5012	0.9763	0.9429	0.7803	0.9973	

Figure 7.10: oxLDL-Rep2 Tukey-Kramer's test used to determine which pairwise comparisons between each of the means on engulfed beads per cell where the mean of the inside beads was equal to or greater than 1

Figure 7.12: Mean percent inhibition for three repetitions of engulfed IgG coated beads by U937 human macrophages with knockdowns of candidate integrins by small interfering RNAs

TREATMENT	Average	Standard Error
No Treatment	110.43	15.56445002
Scram siRNA	100.00	0
A1	71.73	9.04284672
A2	76.03	6.728844945
A3	79.82	18.33837125
A4	83.66	15.07276211
A6	91.96	9.861278578
A7	71.95	11.62567586
A11	80.09	22.57343052
B1	70.97	15.21796209
B2	73.08	7.615893566
B4	76.02	20.19205574
AX	89.33	28.40160041
AL	74.27	16.03563805
AD	75.34	13.13748191
AM	69.63	15.79624507

Figure 7.13: Mean of internalized oxLDL coated beads by U937 human macrophages with knockdowns of candidate integrins by small interfering RNAs

Integrin	Mean	Std dev	Std error
No Treatment	7.79	7.026168	0.740623
Scram	5.6	5.21812	0.550038
Itga1	4.8	5.291687	0.557793
Itga2	6.6	6.102114	0.643219
Itga3	4.78	3.996413	0.421259
Itga4	6.1	6.252231	0.659043
Itga6	11.35	10.76787	1.135033
Itga7	6.9	5.558635	0.585932
Itga11	6.95	6.590239	0.694672
Itgb1	6.56	6.830975	0.720048
Itgb2	5.17	4.148483	0.437288
Itgb4	8.24	7.815593	0.823836
ItgaD	7.89	7.961716	0.839239
ItgaL	6	5.814441	0.612896
ItgaM	6.06	6.023454	0.634928
ItgaX	7.48	7.344731	0.774203

Figure 7.14: Mean of internalized oxLDL coated beads by U937 human macrophages with knockdowns of candidate integrins by small interfering RNAs (second repetition)

Integrin	mean	std error
no treatment	10.7171717	0.918672
siRNA ctrl	12.6372549	1.131586
A1	12.2781955	1.172925
A2	11.7391304	1.061791
A3	11.0917431	1.123267
A4	8.4485981	0.588527
A6	13.7291667	1.580548
A7	12.587156	1.47353
A11	10.44	0.759435
B1	15.1923077	2.068834
B2	9.923913	0.840226
B4	9.3939394	1.004711
AD	9	0.817551
AL	9.0408163	0.75271
AM	10.2	0.975249
AX	8.4383562	0.951044