DOCTOR OF PHILOSOPHY

Characterization of Transglutaminase 2 in macrophage clearance of apoptotic cells

Vinod Nadella

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Aston University

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Thesis Summary

Removal of dead or diseased cells is crucial feature of apoptosis for managing many biological processes such as tissue remodelling, tissue homeostasis and resolution and control of immune responses throughout life. Tissue transglutaminase (TG2) is a protein cross-linking enzyme that has been implicated in apoptotic cell clearance but also mediates many important cell functions including cell adhesion, migration and monocyte-macrophage differentiation. Cell surface-associated TG2 regulates cell adhesion and migration, via its association with receptors such as syndecan-4, β1 and β3 integrin. Whilst defective apoptotic cell clearance has been described in TG2-deficient mice, the precise extracellular role of TG2 in apoptotic cell clearance remains ill-defined. This thesis addresses macrophage TG2 in cell corpse clearance.

TG2 expression (cytosolic and cell surface) in human macrophages was revealed and data demonstrate that loss of TG2 activity through the use of inhibitors of function, including cell-impermeable inhibitors significantly inhibit the ability of macrophages to clear apoptotic cells (AC). This includes reduced macrophage recruitment to and binding of apoptotic cells. Association studies reveal TG2-syndecan-4 interaction through heparan sulphate side chains, and knockdown of syndecan-4 reduces cell surface TG2 activity and apoptotic cell clearance. Furthermore, inhibition of TG2 activity reduces crosslinking of CD44, reported to augment AC clearance. Thus it defines for the first time a role for TG2 activity at the cell surface of human macrophages in multiple stages of AC clearance and proposed that TG2, in association with heparan sulphates, may exert its effect on AC clearance via crosslinking of CD44.

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Abbreviations

ABC1……..ATP binding-cassette-transporter 1

AC……...Apoptotic cell

ACAMPs…….Apoptotic cell-associated molecular patterns

ANT1……..Adenine nucleotide translocator-1

APS…….Ammonium persulphate

ATP…….Adenosine triphosphate

BAI1……Brain-specific angiogenesis inhibitor 1

BMP……Bone morphogenic protein

BTC…….Biotinylated cadaverine

BSA…….Bovine serum albumin

CARD…….Caspase Recruitment domain

CD……...Celiac disease

CMFDA……..5-chloromethylfluorescein diacetate

CNS…….Central nervous system

CO_2.....Carbon dioxide

CRP…….C-reactive protein

cRPMI…….Complete RPMI

DAPI……4', 6-diamidion-2-phenylindole, dihydrochloride

dH_2O……Distilled water

DAMPs……Damage-associated molecular patterns

DISC……Death inducing signalling complex

DMSO…….Dimethylsuphoxide

DNA……Deoxyribonucleic acid

DON……6-diazo-5-oxonorleucine

DS……Double stimulated (VD3/PMA)

ECL…….Enhanced chemiluminescence

ECM……Extracellular matrix

EDTA……..Ethylenediaminetetra-acetic acid disodium salt

ER……...Endoplasmic reticulum

FCS…….Foetal calf serum

FITC……Flourescein isotheiocyanate

GAG……Glycosaminoglycan

GAS-6 …….Growth arrest-specific 6

GDP…….Guanosine diphosphate

GTP…….Guanosine triphosphate

HA……...Hyaluronic acid

HMDMØ…….Human monocyte-derived macrophages

HRP…….Horseradish peroxidise

IBD……..Inflammatory bowel disease

ICAM-3……...Intercellular adhesion molecule 3

ICE……..Interleukin-1β-converting enzyme

IL…….Interleukin

IG……Immunoglobulin

IGF……..Insulin-like growth factor

IGFBP-3……..Insulin-like growth factor binding protein-3

LDL…….Low-density lipoprotein

LFA-1……..Lymphocyte function associated antigen-1

LOX-1…… Lectin-like oxLDL receptor

LPC…….Lysophospahtidylcholine

LPS……. Lipopolysaccharide

LRP…….LDL-receptor-related protein

mAb……Monoclonal antibody

MDA……...Malondialdehyde

MDC……Monodansylcadaverine

Mer……. Tyrosine-kinase receptor

MFG-E8……. Milk fat globule epidermal growth factor

MMP……Matrix metalloproteinases

MODY…….Maturity-onset diabetes of young

MSC…….Mesenchymal stem cells

NCX1……...Sodium calcium exchanger 1

NF-κB……. Nuclear factor kappa-light-chain-enhancer of activated B cells

NGS…….Normal goat serum

NHX1……..Sodium-proton exchanger 1

NHS…….Normal human serum

NOD……Nucleotide-binding oligomerization domain

OD……..Optical density

OPD…….o-phenylenediamine dihydrochloride

Ox-LDL……Oxidised low-density lipoprotein

PAGE……...Polyacrylamide gel electrophoresis

PAMPs…….Pathogen associated molecular patterns

PARP……Poly (ADP-ribose) polymerase

PBS……..Phosphate-buffered saline

PBS-T…….. PBS containing tween

PC…….Phosphatidylcholine

PCD……..Programmed cell death

PDAC……..Pancreatic ductal adenocarcinoma

PDI……...Protein disulphide isomerase

PE…….Phycoerythrin

PI……. Propidium iodide

PKC……. Protein kinase C

PMA…… Phorbol 12-myristate 13-acetate

PMN…….Polymorphonuclear leukocytes

PMSF……Phenylmethanesulphonyl fluoride

PRR……..Pattern recognition receptor

PS…….Phosphatidylserine

PSR……..Phosphatidylserine receptor

RAGE……. Receptor for advanced glycation end-products

RBC…….Red blood cells

rhTG2……...Recombinant human transglutaminase 2

RPMI……Roswell Park Memorial Institute

S1P……...Sphingosine-1-phosphate

SDS……..Sodium dodecyl sulphate

sfRPMI…….Serum-free RPMI

shRNA…….Small hairpin ribonucleic acid

siRNA……..Small interfering ribonucleic acid

SIRPα……..Signal-regulatory protein α

SLE……..Systemic lupus erythematosus

SRe……...Sarcoplasmic reticulum

SR……Scavenger receptors

SDC4……...Syndecan-4

TEMED……N,N,N’,N’-Tetramethylethylenediamine

TG……Transglutaminase

TGF-β……..Transforming growth-factor β

THP-1/DS……Double stimulated THP-1

TIAM-4……T-cell immunoglobulin and mucin-containing molecule 4

TLR4……Toll-like receptor-4

TMEM 16F……..Transmembrane protein 16F

TNF-α……...Tumour necrosis factor

TNFR……...Tumour necrosis factor receptor

Tris……...Tris(hydroxymethyl)methylamine

Tween 20…… Polyoxyethylene sorbitan monolaurate

UVB…….Ultra violet B

VD3……..Dihydroxyvitamin D3

WT……Wild Type

Chapter 1

Introduction

Chapter 1:

1. Introduction

Adult tissue homeostasis is a tightly-regulated balance between the cell generating-effect of mitosis (cell differentiation or division) and cell loss, as development is not only the production of new cells but also the deletion of outmoded cells (Hammar and Mottet, 1971). There is a constant cell turnover in the body and excess cells are generated as a part of normal development or tissue maintenance. However, few of these cells are fit enough to survive and the rest are unwanted cells. Similarly, aged, infected or irreparably damaged cells are other types of unwanted cells generated in multi-cellular organisms. For proper development of the body and tissue maintenance, removal of these unwanted or damaged cells is essential (Wyllie, 1992). One such example, highlighting the need for the removal of unwanted cells, is clearing the inter-digital webs during limb modelling (Figure 1) (Hume, 2008). This loss of cells, in the process of development and in maintaining tissue homeostasis, follows a sequence of well-programmed events. Kerr *et al*. (1972) for the first time coined the term 'apoptosis' a Greek word to the well programmed events, to describe this mode of cell loss as like 'falling of leaves'. These dying cells are quickly recognized and removed by phagocytes, the ultimate goal of the apoptotic program.

1.1 Apoptosis: A safe cell removal commitment

Apoptosis is a complex, genetically-controlled, highly-conserved process of physiological cell death within multi cellular organisms (Lockshin and Zakeri, 2004). An estimated 10^9 cells per day undergo apoptosis in the human body (Elliott and Ravichandran, 2010). Apoptosis is a vital and an elementary on-going biological phenomenon in various biological processes like growth and differentiation, tissue remodelling and immunological development and control (Bowen, 1993). Apoptosis acts in single cells in response to defined stimuli and proceeds via a genetically-encoded cell suicide machinery, and hence is called programmed cell death (PCD) (Oppenheim et al., 1990, Oppenheim et al., 2001).

A classic example of apoptosis is the death of mature neutrophils in the resolution of acute inflammation (Savill et al., 1989). Neutrophils mediate the first line of defence against invading pathogens and tissue injury. They are recruited to a site of infection and play a central role in the innate immune system by killing and phagocytosing invading pathogens. They produce reactive oxygen intermediates and proteolytic enzymes thereby neutralizing the

Figure 1 Clearing of dying cells in developing footpad during embryonic development. Infiltration of cyan fluorescent protein-5 expressing embryonic macrophages in 'MacBlue' mice in response to apoptosis, and progressive clearance of inter digital webs.
Figure taken from (Hume, 2008).

offending insult and provoking resolution of inflammation. This prolonged mechanism of action against pathogens is also capable of inflicting damage to the surrounding tissue and can induce chronic inflammation (Nathan, 2006, Weiss, 1989). In the process of resolving inflammation, neutrophils undergo constitutive apoptosis once the pathogens are removed (Savill et al., 2002, Nathan and Ding, 2010). Neutrophils are short-lived among leukocytes in the circulation and mature neutrophils undergo constitutive apoptosis (Luo and Loison, 2008, Fox et al., 2010), and are later removed by phagocytes, which is essential for resolution of inflammation. Other common examples where apoptosis plays a significant role includes cell loss during embryonic tissue modelling, tissue degeneration during hormone ablation, removal of infected and senescent cells and normal tissue turnover (Gerschenson and Rotello, 1992, Wyllie, 1992, Wyllie, 1993), deterioration of the prostate following castration (Kerr and Searle, 1973), and cancer (Wong, 2011, Kerr et al., 1972).

Defective apoptosis plays a pivotal role in the pathology of many diseases, either due to excessive apoptosis as in case of degenerative diseases or due to too little apoptosis as in cancer. Defective apoptosis may result in polydactyly (Knudsen and Kochhar, 1981, Bynum, 1991). Deep pre-axial mesodermal PCD abolishment has been considered to be the starting point for the manifestation of the pre-axial polydactyly. Bone morphogenic protein (BMP) signalling inhibition in mice developed extensive limb soft tissue syndactyly and post-axial

polydactyly. The post-axial extra digit in mice limb is also consistent with the role for BMPs in regulating apoptosis (Guha et al. 2002). Defective apoptosis also results in interdigital webbing (Zakeri and Ahuja, 1994) and as a result, programmed cell death or apoptosis in simple can be established as a central architect in the processes of animal development (Potten and Wilson, 2004). Similarly, defective apoptotic cell clearance has been linked to autoimmune conditions and developmental abnormalities (Nagata et al., 2010).

1.1.1 Necrosis

Fundamentally, apoptosis maintains the integrity of the plasma membrane (Babiychuk et al., 2011). Unlike apoptosis, necrosis is a form of cell death that is unprogrammed and accidental, characteristic of increased cell volume, swelling of organelles and loss of plasma membrane integrity (Edinger and Thompson, 2004, Proskuryakov et al., 2003). It is a disorganized breakdown of cells leading to cell membrane rupture, release of intracellular contents and pro-inflammatory consequences (Lockshin and Zakeri, 2004). Necrosis in simple is a passive and damaging form of cell death (Fietta, 2006). However, un-cleared apoptotic cells may undergo post-apoptotic changes (secondary necrosis), which show membrane permeability even while remaining essentially intact leading to necrotic like responses and autoantibody production in response to exposed antigens (Fadok et al., 2001).

1.1.2 Apoptotic machinery

Much of our understanding on the machinery of apoptosis stems from the genetic analysis of the nematode *Caenorhabditis elegans.* Four principal genes *CED-3*, *CED-4*, *CED-9* and *EGL-1* were identified in the regulation of cell death (Meier and Evan, 1998). The inactive pro-form of *CED-3* is activated by the ATPase *CED-4* thus initiating the death process. However, *CED-9* interference with *CED-4* interrupts *CED-3* oligomerization in living cells, while *EGL-1* sequesters *CED-9* to allow death process in a *CED-3*/*CED-4* dependent manner (Conradt and Horvitz, 1998). In resemblance to *C. elegans*, a similar cell death regulating gene family have been evolved in vertebrates. Mammalian caspases, a family of cysteine proteases synthesized as proenzymes have been identified as major players in apoptosis (Assuncao and Linden, 2004) and are similar to *CED-3* (Thornberry and Lazebnik, 1998). Mammalian Apaf-1, Nod-1 (CARD-4), Nod-2, the members of a protein family containing caspase recruitment domain (CARD) linked to a nucleotide-binding domain which regulate apoptosis and/or NF-κB activation are *C.elegans CED-4* homologues. Similarly, the

mammalian *Bcl-2* gene family including anti-apoptotic and pro-apoptotic mediators are *CED-9* and *EGL-1* equivalents.

Initiated by external and internal stimuli, apoptosis is triggered through two signalling pathways namely the extrinsic caspase-8 dominant or/and the intrinsic caspase-9 dominant pathways. Caspases are a family of cysteine proteases synthesized as pro-enzymes and have been identified as major players in apoptosis (Assuncao and Linden, 2004). Activation of caspases has been reported to proceed following a variety of apoptotic stimuli (Mehmet, 2000). Engagement of cell surface death receptors such as TNFR, Fas and TLR will stimulate apoptosis via caspase-8 activation (Lavrik and Krammer, 2012). Ligand binding leads to receptor clustering ultimately forming a death-inducing signalling complex (DISC) (Ashkinazi, 2002). On the other hand, apoptotic promoting stimuli such as DNA damage, endoplasmic reticulum (ER) stress, loss of cell survival factors and heterodimerization between anti-apoptotic (*Bcl-2* and *Bcl-xL*) and pro-apoptotic (*Bax, Bak, Bad*) *Bcl-2* family members triggers caspase-9 activation via mitochondrial cytochrome c signals (Kayagaki et al., 2011). Cytochrome c is a pro-apoptotic signal molecule that activates the caspase cascade and induces apoptosis. Both pathways further rely on activation of effector caspase-3, -6 and -7 to co-ordinate cell apoptosis (Schiller et al., 2008, Fesik, 2000). However, non-caspases like calpains, cathepsins and granzymes mediated cell death have also been documented (Johnson, 2000).

1.3 Death leads to the grave: A two stage process

Threats to cellular integrity trigger the apoptotic programme, a process that is active and continuous throughout life. Apoptosis is programmed in two discrete but mutually inter-linked stages; active death and disposal of dead cell corpses (Kerr et al., 1972).

1.3.1 Active death

Apoptosis is a safe cell removal process that prevents plasma membrane rupture and thereby preventing release of intracellular macromolecules (Savill and Fadok, 2000). Regardless of cell type, classic morphological features of apoptotic cells includes; loss of attachment, cell shrinkage, plasma membrane integrity, and membrane blebbing. The series of biochemical events that are triggered with the onset and activation of the apoptosis programme includes; chromosomal DNA fragmentation mediated by activated endonucleases, nuclear chromatin condensation, loss of mitochondrial membrane potential and leakage of mitochondrial

contents leading to a decrease in intracellular pH, and the activation of a family of cysteine proteases called caspases (Desagher and Martinou, 2000). Another major characteristic of apoptotic cells is its extensive protein cross-linking which is known to be achieved by transglutaminase expression and activation (Nemes et al., 1996). TG2-dependent crosslinking of proteins and formation of protective protenaceous shells will prevent the leakage of harmful cell content from the AC.

One of the major classic features of apoptosis programme is the breakup of dying cells into small apoptotic bodies, a process known as blebbing. Membrane blebbing has been well documented to participate in many cellular activities such as cell spreading, migration, cytokinesis and apoptosis (Charras, 2008). Cytoskeletal forces (Mills et al., 1998, Mills et al., 1999) and caspase-mediated activation of Rho effector protein ROCK 1 (Rho kinase 1) together with phosphorylation of myosin II light chain, a family of ATP-dependent motor proteins responsible for actin-based motility, mediates membrane blebbing during apoptosis (Coleman et al., 2001, Coleman and Olson, 2002). Membrane proteins, sodium-proton exchanger 1 (NHE1) and sodium-calcium exchanger 1 (NCX1) are known to mediate opposing functions in regulating membrane blebbing and permeability (Yi et al., 2012). Inhibition of NCX1 enhances cell blebbing as a result of NHE1 induced intracellular sodium accumulation, suggesting that sodium influx by NHE1 can act as a driving force for membrane blebbing. These timely changes result in relocation and packing of condensed chromatin and fragmented nuclei and other toxic cellular contents into membrane-bound vesicles. These events and alterations brought about by the apoptotic programme advertise the status of the cell, and mediate their own clearance. In most cases, modified molecules are clustered in membrane blebs (Coleman et al., 2001). Release of these apoptotic blebs as microparticles was recognised to attract phagocytes to apoptotic cells while generating a chemotactic gradient (Torr et al., 2012). Some of the best characterized chemoattractants include the lipids lysophosphosphatidylcholine (LPC) and sphingosine-1-phosphate (S1P), the nucleotides, ATP and UTP, the classical protein chemokines, CX_3CL1 (fractalkine) and CCL2 (MCP-1) and immunoglobulin super-family (IgSF) member intercellular-adhesion molecule (ICAM-3). All these molecules appear to act through phagocyte-associated G-protein-coupled receptors. Other phagocyte chemoattractant proteins released from apoptotic cells include the covalent dimer of ribosomal protein S19, endothelial monocyte-activating

polypeptide II (EMAP II), thrombospondin 1, TGFβ and annexin I. (Gregory & Pound, 2010; Peter et al., 2010).

1.3.2 Disposal of the dead

The second stage of the apoptotic programme is the safe disposal of the dead by either non-professional (fibroblasts, epithelial cells, endothelial cells) or professional (macrophages) phagocytes. Irrespective of the type of cell death (physiological or pathological), dead cell clearance is considered to be the most important and complex stage in the apoptotic programme (Devitt and Marshall, 2011). Disruption of the delicate balance between cell proliferation, cell death and cell clearance leads to a variety of diseases. With apoptosis being such an integral part of normal cell development, tissue homeostasis, immune and inflammatory responses, abnormalities in the cell death processes results in autoimmune and inflammatory diseases (Savill and Fadok, 2000, Nagata et al., 2010).

1.3.2.1 Apoptotic cell clearance

Apoptotic cells are recognised and cleared before they are 'fully dead' and before their plasma membrane integrity is lost (Reddien et al., 2001). Apoptotic cells are recognised by the innate immune system by presenting recognisable molecular markers. Several biochemical changes on cells undergoing apoptosis have been identified and are known to induce recognition by phagocytes. Apoptotic cell surface changes will generate new structures which act as 'eat-me' signals to attract phagocytes and aid in their own clearance.

1.3.2.2 'Eat-me': Apoptotic cell self-gear

Apoptotic cells present a number of 'eat-me' signals on their surface which will be detected by receptors on phagocytes (Devitt and Marshall, 2011). Loss of membrane phospholipid asymmetry and exposure of anionic phospholipid phosphatidylserine (PS) (Fadok et al., 1992, Fadok et al., 1993, Savill et al., 1993, Fadok et al., 2001), expression of intercellular adhesion molecule 3 (ICAM-3) on apoptotic B-cells (Moffatt et al., 1999, Torr et al., 2012) and surface sugars (Morris et al., 1984, Azuma et al., 2000, Meesmann et al., 2010), exposure of intracellular proteins such as calreticulin (Gardai et al., 2005, Obeid et al., 2007) and Annexin I (Arur et al., 2003) are well documented to mediate AC clearance.

1.3.2.2.1 Phosphatidylserine (PS)

PS is a phospholipid component confined to the inner leaflet of the plasma membrane in viable cells. Loss of phospholipid asymmetry during programmed cell death results in redistribution of phosphatidylserine (PS) to the outer leaflet of the plasma membrane in a caspase-dependent manner (Nagata et al., 2010). Interestingly, PS exposure occurs very early during the apoptotic programme. PS is one of the best characterised 'eat-me' signals that aid in AC recognition by phagocytes (Fadok et al., 1992, Fadok et al., 1993, Martin et al., 1996, Krahling et al., 1999, Fadok et al., 2001, Verhoven et al., 1995). Inactivation of the aminophospholipid translocase, which confines PS to the inner leaflet of the plasma membrane and activation of non-specific lipid flippase, mediates PS exposure (Krahling et al., 1999). ATP binding cassette transporter (ABC1), a member of the superfamily of ATP binding cassette membrane transporters and also a *C. elegans* protein *CED*-7 homologue, has been implicated in the rearrangement of phospholipids, as loss of ABC1 inhibited membrane phospholipid rearrangement and exposure of PS (Hamon et al., 2000). Activation of phospholipid scramblase, a protein that mediates non-specific bidirectional movement of membrane phospholipids has been reported to be involved in PS externalization in apoptotic cells (Verhoven et al., 1995, Verhoven et al., 1999, Frasch et al., 2000). Segawa *et al.* (2011) has shown that lymphoma cells transformed with transmembrane protein 16F (TMEM 16F), a membrane protein with calcium-dependent phospholipid scramblase activity have exposed a high level of PS compared to that observed on AC. Similarly, blocking PS using specific mAbs inhibited phagocytosis of apoptotic cells *in vitro* (Chang et al., 1999). Moreover, inhibitors of interleukin-1β-converting enzyme (ICE) (Caspase-1) prevented apoptosis and PS externalisation in etoposide-treated human monocyte leukaemia U937 cells suggesting that PS externalisation is a downstream event of caspase protease activation during apoptosis and an important event in apoptotic cell clearance (Naito et al., 1997).

However, viable cells forced to expose PS via TMEM 16F expression are not engulfed suggesting that PS on its own cannot mediate AC (Segawa et al., 2011) or PS modification is needed for identification, as oxidized PS is detected on the surface of apoptotic cells (Kagan et al., 2002) or known to act in conjugation with other 'eat-me' signals such as calreticulin and annexin I, which were shown to colocalize with PS on apoptotic cells and enhance uptake (Arur et al., 2003, Gardai et al., 2005). Recognition of PS is mediated by PS receptors on phagocyte like CD36 (Savill et al., 1992), CD68 (Sambrano and Steinberg, 1995), Oxidized

low-density lipoprotein particle 1 (LOX1)(Oka et al., 1998), T-cell immunoglobulin- and mucin-domain-containing molecule (Tiam-4) (Miyanishi et al., 2007), Brain-specific angiogenesis inhibitor 1 (BAI1) (Park et al., 2007), Stablin-2 (Park et al., 2008), MFG-E8 (Savill et al., 1990, Hanayama et al., 2004), Growth arrest-specific 6 (Gas6) (Scott et al., 2001) and the most recently described receptor for advance glycation end products (RAGE) (He et al., 2011).

1.3.2.2.2 ICAM-3

Intercellular adhesion molecule 3 (ICAM-3; CD50) is a highly glycosylated human leukocyte Ig-superfamily member, and an important ligand for lymphocyte function associated antigen-1 (LFA-1) integrin in the initiation of immune responses in viable cells (Cordell et al., 1994). ICAM3 functions as an adhesion molecule while functioning as signal-transducer involved in immune response. ICAM-3 functions as a signalling molecule in resting T cells for initial activation step through CD3 (Hernandez-Caselles et al., 1993, Berney et al., 1999). Following induction of apoptosis, ICAM-3 undergoes functional modification to act as an 'eat-me' signal. Apoptotic cell-associated ICAM-3 recognition by macrophages was demonstrated on both on leukocytes and non-leukocytes (following transfection with exogenous ICAM-3) (Moffatt et al., 1999). Moffatt *et al*. (1999) have also showed that treating apoptotic B cells with blocking mAb against ICAM-3 inhibited their interaction with macrophage. However, no inhibition in interaction is evident upon treating macrophages with blocking mAb against ICAM-3 suggesting that apoptotic cell–associated ICAM-3 mediates recognition by macrophages. Following apoptosis, ICAM-3 is found to be released in apoptotic cell-derived microparticles allowing apoptotic cell recognition by phagocytes (Torr et al., 2012). ICAM-3 accumulation in the engulfing portals of macrophages phagocytosing apoptotic neutrophils in association with LFA-1 is also reported (Kristof et al., 2013).

1.3.2.2.3 Calreticulin

Calreticulin, with high calcium binding and storage function is present in most viable cells. It is found in the endoplasmic reticulum (ER)/Sarcoplasmic reticulum (SRe), localized to the nucleus and is also found on the surface of mammalian cells (Sadasivan et al., 1996, Arosa et al., 1999, Gardai et al., 2005). Mostly, calreticulin acts as a chaperone in the ER. However, during apoptosis, its cell surface expression is up-regulated as a result of ER stress (Panaretakis et al., 2009). Once externalized, calreticulin is known to associate with surface

protein, PS or complement C1q on target cells and mediate clearance of apoptotic cells via activating well conserved LDL-receptor-related protein (LRP, also known as CD91) on the phagocyte (Paidassi et al., 2011, Gardai et al., 2005) in conjugation with loss of CD47 (integrin-associated protein, IAP), a recognised 'don't eat-me' signal (Chao et al., 2010). Moreover, calreticulin is also known to congregate together with PS in the process of apoptotic cell removal (Gardai et al., 2005).

1.3.2.2.4 Cell surface sugar changes: Sialic acid

The cell surface is characterised by a projecting sugar coat which is covalently linked to membrane proteins and lipids. Early studies by Duvall et al. (1985) have shown that cell surface sugars act as regulators of cell-cell and cell-matrix interactions while confirming that macrophage bind AC in preference to non-AC by a carbohydrate-dependent mechanism. They have shown that sugars inhibit macrophage binding to apoptotic thymocytes in a dose-dependent manner. Loss of cell surface sugars during the process of apoptosis will bring about cell surface charge changes which may act as recognition signals for their clearance by phagocytes in a charge-sensitive mechanism (Savill et al., 1989). Well-studied members of cell-surface sugars are N-actyl neuraminic acids (sialic acids), a highly negatively charged nine carbon monosaccharides whose biosynthesis is catalysed by sialytransferases (Varki, 1993). Sialic acids act as 'don't-eat me' signals, thereby preventing complement C3b and C1q binding and subsequent phagocytosis induction (Linnartz et al., 2012). Loss of sialic acid residues during apoptosis or removing sialic acid via neuraminidase treatment, as well as binding sialic acids on the cell surface with sugar-binding proteins called lectins, in turn acts as an 'eat-me' signal as a result of changes in surface glycosylation and triggers cell clearance (Meesmann et al., 2010).

1.3.2.3 'Eat-me not' signals

Is it quite evident that viable cells express 'eat-me' signals but are masked by 'eat-me not' signals, allowing phagocytes to distinguish between live and dead cells (Elliott and Ravichandran, 2010). Integrin-associated membrane protein CD47 is expressed on the surface of viable cells preventing their recognition and uptake by phagocytes by activating the receptor signal-regulatory protein α (SIRPα) (Tada et al., 2003, Gardai et al., 2005). Re-distribution of CD47 during apoptosis triggers cell clearance via exposing 'eat-me' signals namely PS and/or calreticulin. Similarly, platelet-endothelial cell adhesion molecule-1

(PECAM-1/CD31), oppose viable cell ingestion by phagocytes via transmitting detachment signals, loss of which during apoptosis results in AC uptake (Brown et al., 2002). Shedding of complement regulator CD46 in an ADAM10 mediated manner is seen upon exposure to UVB or staurosporine resulting in AC clearance (Elward, 2003, Elward et al., 2005, Hakulinen and Keski-Oja, 2006).

1.3.2.4 'Find-me' signals: Scent of dying cells

Before a macrophage can remove an apoptotic cell, it must find it. Along with several 'eat-me' and 'eat-me-not' signals which differentiate viable cells from AC and regulating their clearance, several other apoptotic changes bring about 'find-me' signals. These 'find-me' signals establish a chemotactic gradient which guides monocyte extravasation to the close proximity of dying cells. Membrane blebbing and released apoptotic bodies carrying surface markers act as 'find-me' signals generating a chemotactic gradient and induce macrophage chemotaxis (Torr et al., 2012). Phagocytes, via receptors, sense these 'find-me' signals in the process of detecting the dying cells at the earliest stages of apoptosis. Some of the 'Find-me' signals produced in a caspase-dependent manner include triphosphate nucleotides (ATP/UTP) (Elliott et al., 2009), phospholipids like lysophospahtidylcholine (LPC) and sphingosine-1-phosphate (S1P) (Gude et al., 2008) and the chemokine CX3CL1 (fractalkine) (Truman et al., 2008).

1.3.2.4.1 Fractalkine (CX3CL1)

Fractalkine has been established as the only classical chemokine 'find-me' signal identified (Peter et al., 2010). A soluble fragment of fractalkine which serves as an intercellular adhesion molecule on the plasma membrane is released during apoptosis in a caspase dependent manner and acts as chemoattractant (Truman et al., 2008). CX3CR1, the receptor for fractalkine on monocytes sense the chemokine and induce monocyte migration both *in vitro* and *in vivo* and mice lacking fractalkine show defective monocyte migration (Truman et al., 2008). However, expression of fractalkine is limited to few cell types and is more specific for Burkitt's lymphoma or germinal centre B cells (Truman et al., 2008).

1.3.2.4.2 LPS

LPS was the first discovered "find me" signal. LPS is released from AC by the caspase-3-dependent activation of phospholipase A2, resulting in the conversion of phosphatidylcholine

to LPS (Lauber et al. 2003). LPS stimulated macrophage chemotaxis is thought to via the G-protein-coupled receptor G2A (Peter et al. 2008). However, the concentration of LPS required to stimulate macrophage chemotaxis reported to be quite high. Moreover, LPS in circulation being higher than the levels released by AC, LPS is considered an unlikely candidate as a chemotatic mediator (Nagata et al. 2010).

1.3.2.4.3 Nucleotides ATP and UTP

Extracellular nucleotides such as ATP and UTP, released as a consequence of cell damage have been identified as chemotaxins for various human immune cells (Elliott et al. 2009). Release of these nucleotides is mediated via pannexin channels opened during apoptosis in a caspase-dependent manner. These nucleotides are sensed by G-protein-coupled seven-membrane-spanning receptors P2Y2 on the immune cells leading to monocyte chemotaxis. However, as they are readily degraded by extracellular nucleotides, they may act as chemoattractants to tissue resident macrophages rather than serving as long-range "find me" signals (Ravichandran, 2010).

1.3.2.5 'Stay-Away' or 'Keep-Out' signals

None of the find-me signals are specific for monocytes alone and also recruit neutrophils (Chen et al., 2006). However, the AC clearance process recruits minimal neutrophils and is recognised as a non-immunogenic and non-inflammatory process (Savill et al., 2002). Bournazou *et al.,* (2009) for the first time observed iron-binding glycoprotein lectoferrin (LTF) as a potent inhibitor of neutrophil migration while showing no effect on monocyte migration facilitating non-inflammatory AC clearance. Such keep-out signals may ensure clearance of AC remains non-inflammatory.

1.3.3 Phagocytic clearance of AC

Phagocytosis accompanies cell death. Ilya llyich Metchnikoff's classic experiment in the 1880s describing the phagocytic event by amoeboid cells in starfish established the link between cell engulfment and host defence. Ever since Metchnikoff first described phagocytosis, this spectacular event has been well recognized as a vital function of immune cells.

1.3.3.1 Mechanism of AC clearance

Apoptosis is well recognized as a healthy process and dying cells are engulfed by various phagocytes of the body. It is the rapid removal process by phagocytes, before the cells undergo necrosis that makes the whole programme of phagocytosis a well-studied mechanism. Phagocytosis is a complex cellular event and includes a series of events in the process of apoptotic cell clearance. These can be divided into recognition, tethering, signalling and phagocytosis (Gregory and Devitt, 2004) (Figure. 2). Recognition is the initial and most important step in the clearance of apoptotic cells and begins intercellular interaction which leads to tethering, the second stage of the apoptotic programme. Tethering leads to signalling events, which thereby initiate the production of anti-inflammatory mediators and other responses e.g. finely controlled rearrangement of the actin cytoskeleton where phosphoinositide signalling is believed to play an important role mediated by Rho family GTPases (Cox et al., 1997, Caron and Hall, 1998). Signalling events leading to remodelling of the actin cytoskeleton supports the extensions of pseudopodia ultimately result in phagocytosis (confinement of the apoptotic cell or body within the phagocyte).

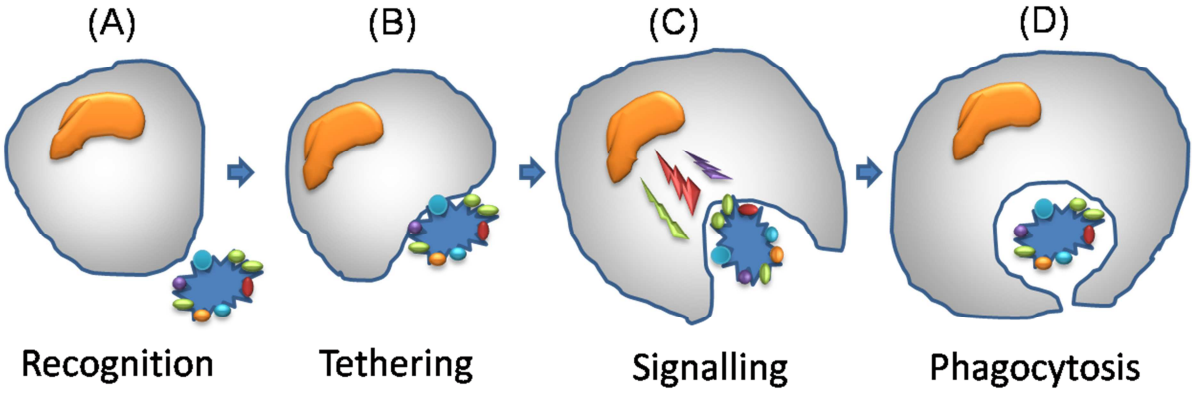


Figure 2: Phases of interaction between apoptotic cell and macrophage. ***A***, Recognition events lead to ***B***, Tethering events which facilitate the initiation of ***C***, two way signalling between the macrophage and apoptotic cell leading to initiation of engulfment and production of anti-inflammatory mediators. This finally results in the ultimate event ***D***, phagocytosis.

1.3.3.2 Professional Phagocytosis: For safe disposal

The apparent purpose of apoptosis is safe cell disposal eliciting no inflammatory response. In spite of available phagocytes like endothelial cells, fibroblasts and dendric cells,

macrophages are considered as professional phagocytes for their efficiency, capacity and ubiquity. Macrophages are the multifunctional mononuclear cells distributed throughout the mammalian organs and are destined to mediate immune response and tissue homeostasis (Chow et al., 2011). Monocytes originate in the bone marrow and are then released into the peripheral blood stream showing morphological heterogeneity such as variability in size, granularity and nuclear morphology (Volkman and Gowans, 1965). They are phenotypically variable as different monocyte subsets occur and distinct phenotypic subsets can be studied by distinct surface receptors e.g. CD14, CX3CR1. Circulating monocytes in the blood stream, in response to local microenvironment gets activated either classically (M1; associated with pro-inflammatory phenotype) or alternately (M2; associated with anti-inflammatory phenotype) and differentiate into macrophages (Gordon and Martinez, 2010). Similarly, the biological functions of the activated macrophage are many and these cells migrate to sites of inflammation where they encounter pathogens and degrade them. As active scavengers, macrophages possess a markedly enhanced ability to clear and degrade intracellular pathogens and unwanted or dead cells during immune response to infection. Macrophages are key cells of the innate immune system and provide defence against harmful and pathogenic foreign materials through phagocytic and microbicidal activities. Moreover, they are specialised to suppress inflammation and promote repair by secreting anti-inflammatory transforming growth factor-β (TGF-β) (Fadok et al., 1998) and prostaglandin E2 (Huynh et al., 2002) while supressing proinflammatory mediators, including tumour necrotic factor α (TNFα), interleukins (IL) -1 and 8 (Fadok et al., 1998).

Apoptotic cell clearance is the ultimate and crucial phase in the process of apoptosis and, moreover the most complex phase involving a wide range of apoptotic cell-derived ligands (section 1.2.1) to receptors on macrophages and soluble bridging molecules. Devitt & Marshall (2011) have reviewed molecules implicated in coordinating the process of macrophage-apoptotic cell interaction. From the immunological perspectives, as mentioned earlier, generation of “eat-me” signals by apoptotic cells assist in initiating the early phases of engulfment by both professional (macrophages & dendritic cells) and non-professional phagocytes (epithelial cells and fibroblasts) (Savill and Fadok, 2000, Gregory and Devitt, 2004). Macrophages, through the evolutionarily conserved innate immune receptors respond non-specifically to stimuli and aid in uptaking the apoptotic cells (Janeway, 1992). The

removal of apoptotic bodies by macrophages should be measured not as just mere waste disposal process but as immunomodulatory (Voll et al., 1997).

1.3.3.3 Pattern recognition receptors (PRR)

As proposed by Charles Janeway, (1992), the innate immune system uses a diverse range of receptors namely pattern recognition receptors (PRRs) which recognise evolutionarily conserved core components, namely pathogen-associated molecular patterns (PAMP) to bind microbes (Medzhitov, 2007), apoptotic cell-associated molecular patterns (ACAMP) to bind apoptotic bodies (Savill et al., 2002) and endogenous stress signals termed damage-associated molecular patterns (DAMP). These are defined based on their specificity to recognize and bind above mentioned specific molecular moieties. They can be either membrane-associated PRR e.g. Toll like receptors or secreted PRR e.g. complement receptors, collectins. These PRR can be either endocytic receptors or signalling receptors.

1.3.3.3.1 Tethering and Endocytic receptors

Tethering and Endocytic receptors exist on the macrophage surface, mediating recognition and uptake of AC. Several receptors have been documented to date including CD14 (Devitt et al., 1998, Devitt et al., 2003, Devitt et al., 2004), vitronectin receptor, $\alpha_v\beta_3$ (Savill et al., 1990, Dupuy and Caron, 2008), mannose receptor, CD36 (Savill et al., 1992) and ATP-binding cassette transporter, ABC-1 (Luciani and Chimini, 1996). Several scavenger receptors including CD68, CD91, lectin-like oxLDL receptor (LOX-1), SRA1 and SRB1 recognise oxidised lipids on the surface of apoptotic cells (Ogden et al., 2001, Peiser et al., 2002, Nakata et al., 1999, Murphy et al., 2006).

1.3.3.3.1.1 CD14

CD14 is preferentially expressed in monocytes and macrophages and its expression is upregulated during monocyte to macrophage differentiation, hence it is considered a marker of macrophage differentiation (Daigneault et al., 2010). It exists both as membrane-anchored and soluble forms (Pugin et al., 1998, Fearns et al., 1995). CD14 is known to bind microbial ligands including peptidoglycans, lipotectonic acid and, most notably, LPS on the surface of Gram negative bacterial cells and aid in their clearance (Wright et al., 1990). Glycosylphosphatidylinositol-anchored LPS receptor, CD14 involvement in AC clearance was demonstrated with increased phagocytic capacity of COS cells following CD14 transfection (Devitt et al., 1998, Devitt et al., 2004). However, due to a lack of a cytosolic

signalling domain, membrane bound CD14 acts as a tethering receptor (Devitt et al., 2004) while interacting with other signalling partners like MD-2 and Toll like receptor-4 (TLR4) to convene ligand-dependent responses (Calvano et al., 2003, Shimazu et al., 1999).

1.3.3.3.1.2 Scavenger receptors

Scavenger receptors (SR) are a family of structurally-diverse receptors with broad ligand specificity for low-density lipoprotein (LDL), PS and polyanionic compounds (Krieger and Herz, 1994, Pearson, 1996). SR are expressed by macrophages, dendritic cells and certain endothelial cells and play an important role in clearance of a diverse array of modified host molecules and AC (Peiser et al., 2002). Scavenger receptors that bind oxidation-modified lipid ligands act as receptors for apoptotic cells implying that similar modifications occur in the process of apoptosis. CD36 in collaboration with $\alpha_v\beta_3$ and the bridging molecule thrombospondin mediate apoptotic neutrophil clearance (Savill et al., 1990, Savill et al., 1992).

1.3.3.3.2 Signalling receptors

The intracellular signalling cascades triggered by the PRRs lead to transcriptional expression of inflammatory mediators and thus coordinating the elimination of infected cells and pathogens. The Toll family of receptors play a central role in the initiation of cellular innate immune responses. Toll-like receptors (Aderem and Underhill, 1999, Gallego et al., 2011) and Nucleotide-binding oligomerization domain (NOD)-like receptor (Chen et al., 2009) mediate downstream or upstream signalling events pre or post phagocyte interaction with AC. NOD-like receptors work in co-operation with Toll-like receptors in regulating inflammatory and apoptotic responses.

1.3.3.4 Bridging molecules: hooks to grave

Along with self-decorated molecular markers on AC and highly specific PRR on macrophages, several other intermediate molecules play a crucial role in mediating phagocytic clearance of AC, acting as bridging molecules which can decorate an Ac and facilitate its engulfment by macrophages. Some of the well characterised bridging molecules are the MFG-E8, Gas6, and Protein S.

1.3.3.4.1 MFG-E8

Milk-fat globule EGF factor-8 (MFG-E8) is known to bridge apoptotic cell exposed PS and phagocyte vitronectin receptor (Hanayama et al., 2004) mediating Rac1-dependent phagocytosis (Wu et al., 2006). MFG-E8 bears a binding domain for PS and an Arginine-Glycine-Aspartic acid (RGD) motif enabling its binding to integrins. High $\alpha_v\beta_3$ integrin expressing NIH 3T3 transfectants upon addition of MFG-E8 engulfed AC (Hanayama et al., 2002). Similarly, MFG-E8 deficient mice developed splenomegaly and suffered from glomerulonephritis as a result of autoimmunity (Hanayama et al., 2004) and lupus-like autoimmunity associated with AC accumulation *in vivo* (Peng and Elkon, 2011).

1.3.3.4.2 Gas6 & Protein S

Vitamin K-dependent serum anti-coagulant factor protein, protein S and its structural homologue Growth arrest state 6 (Gas6) are together known to bind PS on AC and mediate their clearance. They are known ligands of Tyro-3, Axl and Mer (TAM) receptors tyrosine kinases which represent an anti-inflammatory phagocytosis system (Hall et al., 2005).

Others bridging molecules include thrombospondin which is known to bridge macrophages and neutrophils via $\alpha_v\beta_3$ and CD36 synergy (Savill et al., 1992). C1q, (the first component of complement) a member of collectin family of classic complement cascade, is known to bind CD91-associated calreticulin on macrophage for AC (Ogden et al., 2001). Mice deficient for C1q have shown defective clearance of dying cells in kidney (Taylor et al., 2000). Complement factor H, a major regulator of complement system, binds cellular debris via malondialdehyde (MDA; a lipid peroxidation product) epitopes on AC and mediate their uptake by macrophages while regulating MDA-induced proinflammatory effects in mice *in vivo* (Weismann et al., 2011).

Taken together, different recognition ligands on apoptotic cells e.g. phosphatidylserine (PS), the ER protein calreticulin; bridging molecules like MFG-E8, thrombospondin and Gas6; phagocyte receptors like CD14, integrins and kinases; contributing to the recognition, binding and signalling, leading to uptake of the dying cell by the phagocyte in a complex process involving cascade of events reflecting the need and biological significance of the apoptotic cell clearance. In addition, protein cross-linking proteins such as transglutaminases were also found a play a prominent role, in apoptosis (Fesus et al., 1987) and macrophage phagocytosis

(Szondy et al., 2003) with a loss of TG2 causing impaired apoptotic cell engulfment (Laura Falasca, 2005).

1.4 Transglutaminases

Transglutaminases are a family of enzymes with varied biological functions. They were first reported in guinea pig liver by Clarke et al. (Clarke et al., 1957). However, Mycek *et al*. (1959) for the very first time in his scientific report used the official name 'Transglutaminase' to describe this enzyme as a protein modifier by catalysing an acyl transfer reaction between γ-carboxamide group of peptide-bound glutamine and ε-amino group of peptide-bound lysine (or a polyamine) resulting in formation of ε-(γ-glutamyl)lysine isopeptide bond, a protein cross-linking (Mycek et al., 1959, Griffin et al., 2002). This bond is highly resistant to proteolysis and denaturants resulting in stable and rigid protein complexes and with this property, they are considered efficient biological cross linking agents (Chau et al., 2005).

Transglutaminases are widely expressed in nature and are well documented in microorganisms (Kanaji et al., 1993), plants (Della Mea et al., 2004), invertebrates (Mehta et al., 1992) and vertebrates (Zhang and Masui, 1997; Grenard *et al*., 2001). Similarly, transglutaminases are expressed in different human organs and cells, and are also able to exert all their attributed functions within the same tissue (Griffin et al., 2002). Apart from their transamidating function, they are also noted for other enzymatic and non-enzymatic functions making them unique as multifunctional proteins.

1.4.1 Family of Transglutaminases

To date, nine different members of the transglutaminases (TGs) family have been identified at the genomic level in mammals (Table 1). These are the keratinocyte transglutaminase (TG1), tissue transglutaminase (TG2), epidermal transglutaminase (TG3), prostrate transglutaminase (TG4), transglutaminases X, Y, Z (TG5, TG6, TG7), Plasma factor XIIIa and the non-catalytic erythrocyte band 4.2 (Griffin et al., 2002). Having a similarity in gene strauctur, protein primary structure, three-dimensional folding and catalytic mechanism, all transglutaminase genes seems to be evolved through gene duplication from a common ancestral gene (Grenard et al., 2001). These nine TG and TG-like genes are clustered on five different chromosomes. Apart from erythrocyte band 4.2, all transglutaminase members are catalytically active and are related to papain-like cysteine proteases (a large family of

peptide-cleaving enzymes) sharing a similar catalytic triad 'Cys-His-Asp' or 'Cys-His-Asn' (Griffin et al., 2002).

- The circulating **plasma Factor XIII** is a fibrin stabilizing factor with its catalytic A subunits (Factor XIIIA) and carrier B subunits (Factor XIIIB) encoded by the genes F13A1 and F13B on the human chromosome 6 p24-p25. Following cleavage by thrombin to factor XIIIA, it becomes activated, exposing its cysteine binding site in a Ca^{2+} -dependent manner acting on fibrin to form ε-(γ-glutamyl)lysine cross-linking forming a stable, insoluble clot (Lorand and Graham, 2003). Factor XIIIA may also serve as a protein disulphide isomerase in promoting platelet adhesion (Lahav et al., 2013). It is unique in its role among all transglutaminases and its deficiency leads to delayed wound healing and tissue repair (Schroeder and Kohler, 2013);

- The **keratinocyte transglutaminase** (TG1; encoded by the gene *TGM1* in the human chromosome 14q11.2) is epidermal specific. TG1 is the largest of TG family (~106kD) and upon cleavage yields fragments of 10, 33 and 67kDa (Kim et al., 1995). Though majorly available in plasma membrane bound form, it is also available in soluble form in the cytosol. It exists as a zymogen (an inactive enzyme precursor that requires a biochemical change such as hydrolysis or a conformational change to reveal its active site for it to become active enzyme) and is involved in keratinocyte terminal differentiation following activation. TG1 is known to be expressed in the wound site much before the infiltration of leucocytes, implying that TG1 is essential during very early stages of wound repair and also in preparation for the remodelling of stratum corneum. TG1 plays a crucial role in cell envelope formation (Kim et al., 1995). Abnormal keratinisation and impaired skin barrier function leading to weakened neonatal adaptation and death, were reported in TG1 null mice (Matsuki et al., 1998);

- The **tissue transglutaminase** (TG2; encoded by the gene *TGM2* in the human chromosome 20q11.2-q12) is a ubiquitously expressed isoform, existing both intra and extracellularly with varied biological functions like cell adhesion, migration, differentiation, transmembrane signalling, ECM organization, cell survival and apoptosis. (Protein of interest and discussed in detail in section 1.4.2);

- The **epidermal transglutaminase** (TG3; encoded by *TGM3* on human chromosome 20q11-12) is involved in keratinocyte differentiation and hair follicle maturation. In contrast to TG1, it mediates intramolecular activity (Kim et al., 1995) with a known pathological role in Dermatitis herpetiformis in coeliac patients (Rose et al., 2009). Kim et al. demonstrated abundant expression of TG3 along with TG1 and TG6 in the central nervous system (CNS) (Kim et al., 1999);

- The **prostate transglutaminase** (TG4; encoded by the gene *TGM4* in the human chromosome 3 p21.33-p22) with structural similarity to other transglutaminases. TG4 expression is evident in prostatic epithelia in rodents and humans (Dubbink et al., 1998). Similar to TG2 members, TG4 also demonstrates transamidase and GTPase activities (Spina et al., 1999). It is known to play a crucial role in rodent fertility by masking the immunogenicity of sperm cells in the female genital tract, thus hindering immune responses (Mukherjee et al., 1983). It is also a marker for invasive prostate cancer cells and a novel target of prostrate related diseases in humans (Dubbink et al., 1996, Davies et al., 2007);

- **Transglutaminase 5** (TG5; encoded by *TGM5* on human chromosome 15q15.2) was first characterised by Aeschlimann *et al*. (1998) and is widely expressed by epithelial cells. TG5 is homologous to TG2 and TG3 in structure and function (Aeschlimann et al., 1998, Candi et al., 2002). TG5 demonstrates both transamidase and GTPase activities. Its Ca^{2+}-mediated cross-linking activity in epidermis is documented by Cassidy *et al*. (Cassidy et al., 2005);

- **Transglutaminase 6** (TG6; encoded by the gene *TGM6* in the human chromosome 20q11-15) is expressed by neurons in the central nervous system (CNS) (Grenard et al., 2001, Thomas et al., 2013). TG6 is implicated in gluten ataxia with high levels of IgA antibodies in patient's serum against TG6. (Wang et al., 2010a, Hadjivassiliou et al., 2008, Cascella et al., 2012);

- **Transglutaminase 7** (TG7; encoded by the gene *TGM7* in the human chromosome 15q15.2) is ubiquitous in expression with high degree of TGase activity and Ca^{2+} conserved binding sites on the core domain of transglutaminase, sharing the same chromosome 15 along with TG5 and band 4.2. It is been recently identified and little is known about this isoenzyme (Grenard et al., 2001).

- **Erythrocyte Band 4.2** (Band 4.2; encoded by the gene *EPB4.2* in the human chromosome 15 in bands q15 to q21) is the only catalytically inactive member of transglutaminase family with alanine substitution for active-site cysteine, thus strictly a TG structural protein. Band 4.2 is a major constituent of the red blood cell (RBC) membrane cytoskeleton and known to interact with other membrane proteins like ankyrin, cytoplasmic domain of band 3, and spectrin (Korsgren and Cohen, 1988) and thus contributes to membrane integrity maintenance. Band 4.2 is important for normal erythrocyte function since patients with band 4.2 deficient erythrocytes are anaemic (Korsgren and Cohen, 1991). Its deficiency is reported with erythrocyte fragility, haemolytic anaemia and hereditary spherocytosis (Cohen et al., 1993). Via interacting with CD47, it interacts with Rh factor of RBC and assist in cell survival (Satchwell et al., 2009).

Table 1. Members of Transglutaminase Family

Protein	Alternative Name	Gene/Gene location	Tissue expression	Function	Reference
Factor X111A	Plasma transglutaminase	F13A1/Chromosome 6 p24-p25	Astrocytes, chondrocytes, dendritic cells, platelets	Blood clotting, bone growth, ECM stabilization, wound healing,	(Lorand and Graham, 2003, Schroeder and Kohler, 2013)
TG1	TG_K, Keratinocyte transglutaminase, Transglutaminase type 1	TGM1/Chromosome 14 q11.2	keratinocytes	Epidermal keratinocytes differentiation and cell envelope formation	(Kim et al., 1995, Matsuki et al., 1998)
TG2	Transglutaminase type 2, tissue transglutaminase, TGc, G_h, $G_{\alpha h}$	TGM2/Chromosome 20 q11.2-q12	Ubiquitous	Cell adhesion, cell differentiation, ECM stabilization, cell survival, apoptosis, transmembrane signalling,	(Griffin et al., 2002, Telci et al., 2008, Collighan and Griffin, 2009)
TG3	Transglutaminase type 3, TG_E, epidermal	TGM3/Chromosome 20 q11-12	Squamous epithelium	Cornified envelope formation, Hair follicle maturation	(Rose et al., 2009)

	transglutaminase				
TG4	Transglutaminase type 4, Prostate transglutaminase, TG_P,	TGM4/Chromosome 3 p22-p21.33	Prostate	Reproduction, semen coagulation in rodents	(Dubbink et al., 1998, Dubbink et al., 1996, Davies et al., 2007)
TG5	Transglutaminase type 5, TG_X,	TGM5/Chromosome 15 q15.2		Keratinocytes differentiation and cornified envelope formation	(Aeschlimann et al., 1998, Candi et al., 2002, Cassidy et al., 2005)
TG6	Transglutaminase type 6, TG_Y,	TGM6/Chromosome 20 q11-15	Skin epidermis	Development and motor function	(Stamnaes et al., 2010) Fukui *et al*., 2013
TG7	Transglutaminase type 7, TG_Z,	TGM7/Chromosome 15 q15.2	Ubiquitous	Not characterized	(Grenard et al., 2001)
Band 4.2	Erythrocyte Band 4.2, protein 4.2, pallidin	EPB42/Chromosome 15	Erythrocytes	Maintain membrane integrity	(Korsgren and Cohen, 1988, Satchwell et al., 2009)

1.4.2 Transglutaminase 2

Transglutaminase 2 (TG2, tissue transglutaminase, transglutaminase C, tTG, Gαh) is the most diverse and ubiquitous member of the transglutaminase family, and one of the most well studied (Collighan and Griffin, 2009) and biologically characterised, multifunctional molecules (Aeschlimann and Thomazy, 2000). TG2 in mammalian tissue (Thomazy and Fesus, 1989) is distributed, expressed and localised in different tissues and in different parts of the cell. Endothelial cells, fibroblasts, monocyte-derived macrophages, astrocytes, neurons, smooth muscle cells, osteoblasts, hepatocytes as well as a number of organ-specific cell types show consistent expression of the protein (Aeschlimann et al., 1993, Thomazy and Fesus, 1989). TG2 expression is known to be regulated by cytokines, peptide growth factors, retinoids and steroid hormones (Lorand and Graham, 2003). Both at mRNA and protein level, retinoic acid induced TG2 expression is documented *in vitro* and *in vivo* (Defacque et al., 1995).

1.4.2.1 Structure of TG2

As revealed from cDNA sequence studies, TG2 is a monomeric protein with ~ 685 amino acids, with molecular weight of ~80kDa and is encoded by *TGM2* on human chromosome 20q11-12. *TGM2* is composed of 13 exons separated by 12 introns (Grenard et al., 2001). It encodes 4 distinct domains, namely the N-terminal β-sandwich domain (residues 1-138) containing the fibronectin and integrin binding sites; the central core domain (139-471) with α-helices and β-sheets, containing the substrate binding pocket and catalytic triad 'Cys277-His335-Asp358' for mediating acyl-transfer reaction; conserved tryptophan vital for catalytic activity; and two C-terminal β-barrel domains, one with a binding pocket for GTP and interaction sites with the α1B adrenergic receptor, and the other with phospholipase Cδ1 interaction site (Chen and Mehta, 1999) (Figure 3). X-ray crystallography studies revealed the nucleotide binding pockets of TG2 associated with GTP/GDP binding and include residues 476-482 and 580-583 (Begg et al., 2006).

The β-sandwich domain on its N-terminus mediates TG2 interaction with fibronectin with a very high affinity, which is thought to contribute to regulated cell adhesion (Akimov et al., 2000) and TG2 externalization (Balklava et al., 2002). Hang et al. (2005) have identified a new fibronectin binding site with amino acid residues 88-106 and shown that Asp94 and Asp97 residues are critical for fibronectin binding via their mutation to Alanine. The central

core domain carries the catalytic triad, conserved tryptophan and Ca^{2+} binding site. Several potential Ca^{2+} binding sites have been proposed (Kiraly et al., 2009). The two β-barrels carrying the GTP/GDP binding site and the phospholipase C binding site on the C-terminal domain which binds activate phospholipase C (PLC) δ1, a key player in signal transduction processes (Murthy et al., 1999). Nakaoka et al. (1994) demonstrated TG2 GTPase activity and its ability to function as a G-protein. It is the GTP-bound enzyme which is in the inactive, closed conformation and Ca^{2+} binding open conformation which is active, suggesting the transition from acyl-transferase activity to GTPase activity leading to a major conformation change in the structure of the molecule (Pinkas et al., 2007).

A.

B.

Figure 3: Schematic representation of TG2 Structure and conformation. ***A***, Skeletal structure and functional domain of TG2. N-terminal β-sandwich with amino acid residue 88-106 for fibronectin binding; heparin-binding site, catalytic triad Cys277, H335, D358 (Cysteine, Histidine, Aspartic acid), Try241 (Tryptophan) which is essential for catalytic activity and Ca^{2+} binding site on the core domain; GTP/GDP binding site and phospholipase C binding site carrying β-barrels. ***B***, Three dimensional ribbon structure of TG2 in both closed and open conformations. The distinct domains of the human TG2 molecule crystalized in two isoforms upon binding with GDP are TG2 inhibitor. The N-terminal β-sandwich domain shown in blue, the central core domain in green and the C-terminal β-barrels (β1 and β2) were shown in yellow and red. (I) GDP-bound TG2 leading to closed conformation and (II) TG2 inhibitor bound at the catalytic site in the open conformation.
Figure with slight modifications from Pinkas *et al*., (2007)

1.4.2.2 TG2 localisation and cellular distribution

TG2 is predominantly an intracellular protein, but can be membrane-associated well as following externalisation to the cell surface by an undefined mechanism and where it becomes associated with the extracellular matrix (Upchurch et al., 1991, Aeschlimann et al., 1995, Verderio et al., 1998, Griffin et al., 2002, Collighan and Griffin, 2009). Though predominantly a cytosolic protein that lacks a hydrophobic leader sequence, it is a protein with a non-ER/Golgi-dependent externalization mechanism. TG2 is also known to translocate to the nucleus via importin-α-3 protein (Lesort et al., 1998, Peng et al., 1999), where it exist in a chromatin-bound form (Lesort et al., 1998). TG2 gets associated with importin-α-3 in the cytoplasm and gets dissociated once in the nucleus (Peng et al., 1999). In the nucleus, TG2 functions as a G-protein or in presence of Ca^{2+} as a transamidase and is known to cross-link proteins such as histones and the SP1 transcription factor (Keresztessy et al., 2006). Similarly, glutamate stimulation and calcium ionophore-A mediated nuclear translocation of TG2 have also been studied (Mann et al., 2006). With no N-terminal mitochondrial targeting signal in TG2, it is also found in association with mitochondria (Piacentini et al., 2002). Electron microscopy studies revealed that 90-95% of the mitochondrial TG2 is localized on the mitochondrial outer membrane and inner membrane space (Park et al., 2010). TG2 is known to translocate to the plasma membrane in association with β-integrin (Akimov and Belkin, 2001, Mangala et al., 2007) which has been considered as a possible cell surface externalisation agent (Mehta et al., 2006). Its high affinity for extracellular fibronectin and syndecan-4 (Scarpellini et al., 2009, Wang et al., 2012) were also found to be mediators of TG2 cell surface externalization.

Another possible means of TG2 externalization is by recycling endosomes (Zemskov et al., 2011) where TG2 is known to associate with various cytoplasmic early, late, recycling endosomes and lysosomes. It was proposed that newly synthesized cytoplasmic TG2 may target the perinuclear endosomes instead of being directed to the Golgi/ER-dependent pathway resulting in TG2 externalization or cell surface TG2 where it gets internalized into clathrin-coated pits and recruited into early endosomes in a caveolin-dependent manner. This unconventional pathway purely depends on either cytosol TG2 tethering to phosphoinositides on the early endosome via its phospholipid binding site …(590)**K**I**R**ILGEP**K**Q**RKK**(602)… on the C-terminal domain (or, cell surface TG2 recruitment to early endosome following internalization) and subsequent tight binding to endosomal membrane proteins (Roth 2004).

It is thought to follow the long loop of the endosome recycling pathway which is regulated by varied proteins destined to control endosome recycling and finally externalization. Once externalized, TG2 is either retained on the cell surface or deposited into the ECM where it is known to interact with ECM proteins and plays a major role in ECM remodelling (Aeschlimann et al., 1995).

1.4.2.3 Regulation of TG2 activity

TG2 activity is mainly centred in the catalytic triad consisting of a cysteine (C277), histidine (H335), and aspartate (D358). Along with a conserved tryptophan (W241), stabilizes the transition state and all four residues are essential for catalysis (Micanovic et al., 1994, Murthy et al., 2002, Liu et al., 2002, Iismaa et al., 2003). Mutations at any of these sites renders inactivation of the transamidation function of TG2 (Murthy et al., 2002, Tucholski and Johnson, 2002).

Activity of TG2 in the intracellular environment was reported to be regulated both by Ca^{2+} and GTP/GDP levels. The cross linking activity of TG2 will be enhanced in presence of high levels Ca^{2+} (Verderio et al., 2004). TG2 can bind and hydrolyse GTP which links intracellular TG2 to a major signalling pathway, in which TG2 functions as a signal transduction GTP-binding protein, Gαh, transmitting outside signals downstream to cytoplasmic targets such as phospholipase Cδ (PLCδ) (Murthy et al., 1999). Based on the levels of Ca^{2+} and GTP in the cell, TG2 is proposed to have ‘closed’ and ‘open’ conformations with notably distinct features (Pinkas et al., 2007). Under normal conditions, TG2 exits as a latent protein in a ‘closed’ GTP/GDP-bound conformation under low Ca^{2+} conditions. With the onset of stress signals or loss of Ca^{2+} homeostasis, TG2 undergoes conformational change from ‘closed’ to ‘open’. This exposes the catalytic triad on the core domain (Liu et al., 2002), where the Cys277 forms a thioester bond in transamidation and Tyr241 is essential for this process (Chen and Mehta, 1999).

GTP is the control switch of transamidation, acting as a reversible non-competitive inhibitor of the TG2 transamidation activity. In the GDP bound form, TG2 attains a more compact, ‘closed’ conformation state where the two C-terminal β-barrels overlap a significant surface area of the catalytic core domain, effectively blocking substrate access to the active site. Site directed mutational analysis at the position 516 (Y516), replacing tyrosine with phenylalanine (Y516F), highlighted the importance of tyrosine in attaining the closed conformation. The

presence of high levels of Ca^{2+} renders TG2 to attain an open conformation (Casadio et al., 1999, Mariani et al., 2000) that exposes the TG2 active site and aids in TG2 activation (Begg et al., 2006, Pinkas et al., 2007). The calcium binding protein calreticulin is known to inhibit GTP binding and transglutaminase activity of TG2 (Feng et al., 1999, Lee et al., 2003) where it appears to be a tight regulator of G protein and transglutaminase functions. However, TG2 is known to have higher affinity for the guanine nucleotides than for Ca^{2+} (Datta et al., 2006). Therefore, the cells transamidating activity of TG2 is usually latent and gets activated depending on the need and circumstances prevailing within the system, such as apoptosis differentiation or stress. In addition to GTP, nitrosylation of the active site cysteine is also known to inactivate TG2 (Lai et al., 2001), while on the other hand, sphingosylphosphocholine can serve to reduce the Ca^{2+} requirement for intracellular TG2 activity (Lai et al., 1997).

1.4.2.4 TG2 is a multifunctional protein

TG2 is an enzyme with wide range of biological functions (Nurminskaya and Belkin, 2012, Belkin, 2011, Wang and Griffin, 2012, Lorand and Graham, 2003, Park et al., 2010). Transamidation and GTP-binding are two well-known biological functions of TG2 (Fesus and Piacentini, 2002). Unlike the majority of members of the transglutaminase family, TG2 binds and hydrolyses GTP in a magnesium-regulated manner (Lee et al., 1989). In addition to its transamidation activity, TG2 binds and hydrolyses GTP and is involved in regulating signal transduction and acts as a G-protein, transducing signals from α1-adrenergic receptors to phospholipase C-δ1 (Chen and Mehta, 1999). However, TG2 is known to play several other enzymatic and non-enzymatic functions as shown in figure 4 (Aeschlimann and Paulsson, 1994, Chen and Mehta, 1999, Griffin et al., 2002, Lorand and Graham, 2003, Zemskov et al., 2006).

TG2 participates in a plethora of other biological processes like extracellular matrix stabilization, by forming a complex with fibronectin and collagen which aids in wound healing, inhibition of tumour angiogenesis, and bone remodelling (Turner and Lorand, 1989). It also functions as an adaptor protein in facilitating cell adhesion to fibronectin via integrins (Akimov et al., 2000) and syndecans (Wang et al., 2010b) aiding cell motility and in tissue mineralization. It is known to possess an intracellular serine/threonine kinase activity where it is involved in phosphorylation of insulin-like growth factor (IGF) binding protein-3 (IGFBP-

3) in breast cancer cell membranes (Mishra and Murphy, 2006). TG2 possesses extracellular Ca^{2+}-independent protein disulphide isomerase activity (PDI), not inhibited by nucleotides (Hasegawa et al., 2003). PDIs catalyse the formation of disulphide bonds within the protein to facilitate folding into correct conformations. TG2 PDI activity is catalysed by a completely different domain used for transamidation reaction as alkylation of cysteine had no effect on PDI while its TG2 activity was completely abolished. Given that inactive TG2 mediating transamidation in high concentrations of nucleotides and in low Ca^{2+}, it is suggested that TG2 acts as PDI in the cytosol. PDI has potential activity in mitochondria as cardiomyocytes and skeletal muscle cells of TG2 knockout mice exhibited decreased ATP production due to an absence of disulphide bridges provided by TG2 PDI activity (Mastroberardino et al., 2006) resulting in destabilized respiratory complexes. The mitochondrial protein, adenine nucleotide translocator-1 (ANT1) is proposed to be a substrate for PDI activity (Malorni et al., 2009).

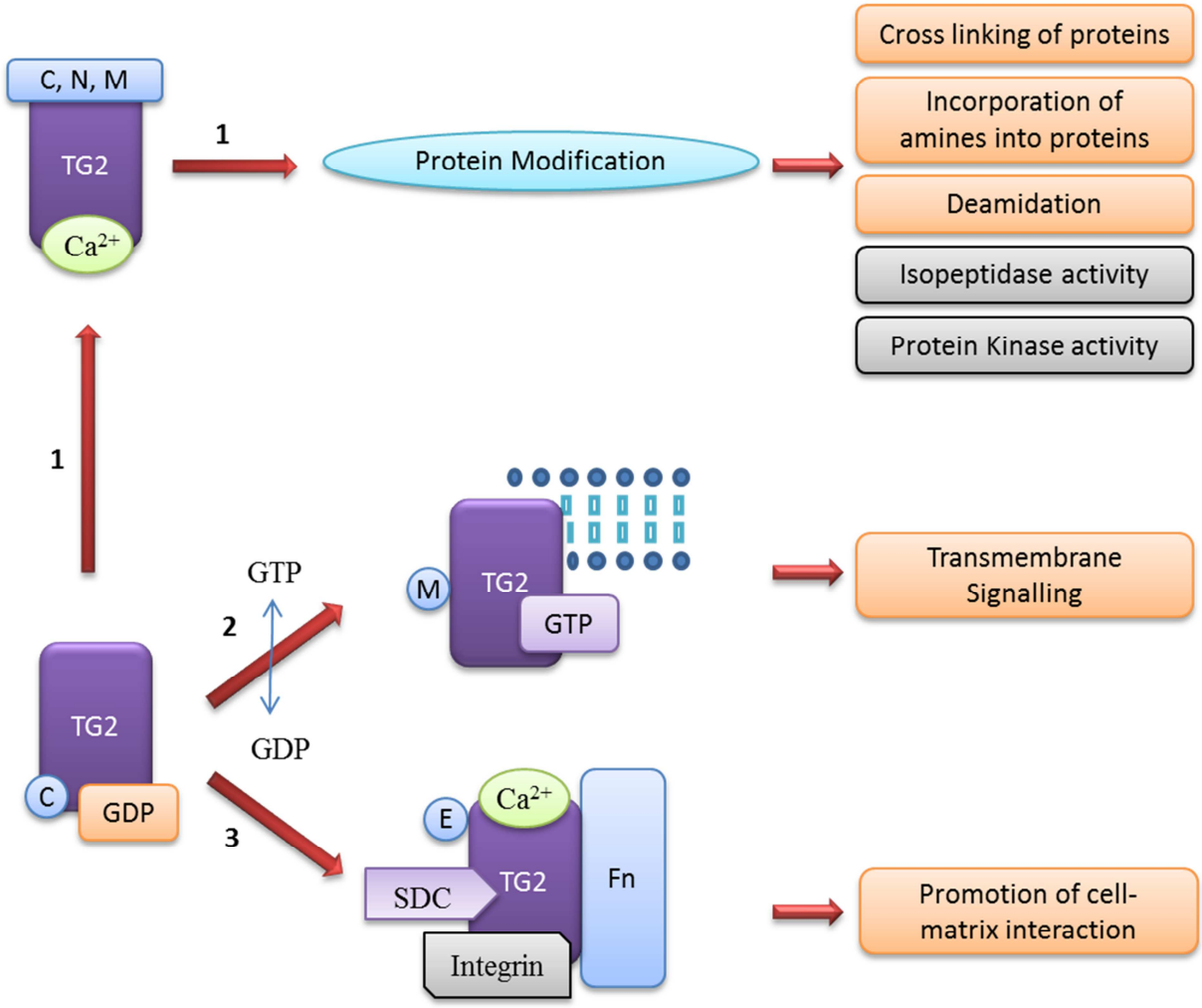


Figure 4. Multifunctional TG2. 1. In a Ca^{2+} dependent manner, TG2 catalyses protein cross linking reaction between γ-carboxamide groups of protein bound glutamine and ε-amino group of protein bound lysine or polyamine and deamidation of peptide glutamines. Through its isopeptide activity, it hydrolyse γ:ε isopeptides in Ca^{2+} independent manner and also known to have protein kinase activity. 2. In its GTP bound form, it activates the phospholipase (PLC-1δ) and mediates signal transduction. 3. Moreover, it acts as an integrin and Syndecan (SDC) bound adhesion co-receptor for fibronectin (Fn) in the extracellular matrix. (C, N, and M stands for cytoplasm, nucleus and membrane respectively).

TG2 is known to play a prominent role in programmed cell death and necrosis (Fesus et al., 1987, Nicholas et al., 2003). TG2 is one of the few genes induced during apoptosis *in vivo* (Szegezdi et al., 2000). Loss of Ca^{2+} homeostasis with apoptosis induction, leads to TG2 activation and formation of cross-linked protein scaffolds. This function of TG2 in cell death assists in maintaining dead cell membrane integrity, thereby preventing release of harmful cell components to the extracellular environment. However, TG2 is also known to mitigate apoptosis via its GTP binding activity, via activating NF-κB, as seen in several cancer cell lines (Mann et al., 2006, Cao et al., 2008). Loss of GTP-binding activity reverses TG2's protective role to a death signal (Datta et al., 2007) indicating TG2 can play a dual role depending on other parameters in programmed cell death (Park et al., 2010). Though TG2 seems to have a strong link with the *in vivo* apoptosis programme, its up-regulation under *in vitro* conditions is not detected in many cells highlighting the need of specific environmental conditions available *in vivo* (Szegezdi et al., 2000). Importantly, in mice model system, TG2 is studied in macrophage phagocytosis (Szondy et al., 2003) suggesting that loss of TG2 *in vivo* results in impaired apoptotic cell engulfment (Falasca et al., 2005). TG2 is proposed to act as an integrin β3 co-receptor for AC engulfment process (Akimov et al., 2000) and also identified as a requirement for an efficient phagocytic portal formation (Toth et al., 2009). $TG2^{-/-}$ macrophages bear a proinflammatory phenotype contributing to develop autoimmunity. However, all the functions attributed to TG2 are apparently dictated by its cellular localization, interaction with other proteins and binding to cofactors (Zemskov et al., 2006, Lorand and Graham, 2003, Griffin et al., 2002).

1.4.2.5 Physiological role of TG2

Being known for multiple functionality in the family of transglutaminases, accumulating evidence to date has demonstrated well-defined cellular roles for TG2 which include apoptosis and necrosis (Fesus et al., 1987, Nicholas et al., 2003), cell adhesion and migration (Akimov and Belkin, 2001, Verderio et al., 2004, Zemskov et al., 2006, van Strien et al., 2011, Wang et al., 2011), cell growth and differentiation (Schroff et al., 1981), Ca^{2+} mediated stimulus secretion coupling (Bungay et al., 1986) and receptor mediated endocytosis/phagocytosis (Davies et al., 1980, Abe et al., 2000).

1.4.2.5.1 TG2 in cell survival and apoptosis

Several investigations to date revealed the supposed role of TG2 in cell death and survival (Fesus and Szondy, 2005, Verma and Mehta, 2007, Park et al., 2010). Depending on the cell type and type of signals, TG2 can either promote or inhibit the cell death process (Piacentini et al., 2002, Fesus and Szondy, 2005, Szondy et al., 2006, Datta et al., 2007, Cao et al., 2008). Apoptosis is a fundamental biological function in maintaining tissue homeostasis. Fesus (1992) identified the TG2 gene to be closely related to those death executing genes. However, currently its dual role is widely acknowledged. Under normal conditions, TG2 activity is tightly regulated by GTP activity. Majority of data support the notion that transamidation by TG2 can facilitate and inhibit apoptosis, while the GTP-bound form of the enzyme generally protects cells against death (Fesus and Szondy, 2005). With extreme stress conditions and depending on the Ca^{2+} influx, TG2 mediates the formation of cross-linked protein scaffolds in the dead cell (Smethurst and Griffin, 1996). This stabilisation assists in maintaining dead cell integrity thereby preventing release of harmful cell ingredients in to the extracellular environment. TG2 transamidating activity was found to be the primary biochemical function involved in the physiological regulation of apoptosis as embryonic fibroblasts obtained from TG2 knock-out mice when reconstituted with wild-type TG2 resulted in a significant exacerbation of caspase 3 activity and Poly (ADP-ribose) polymerase (PARP) cleavage, a caspase-cleaved domain while no such outcome is evident upon reconstitution with transamidation inactive C277S mutant (Rossin et al., 2012). TG2-induced apoptosis was shown to associate with release of apoptosis-inducing factor (AIF) from mitochondria leading to its translocation to the nucleus and subsequent apoptosis of pancreatic ductal adenocarcinoma (PDAC) cells in caspase-independent manner (Fok and Mehta, 2007). However, studied by Yoo et al. (2012) have shown that TG2 promotes both caspase-dependent and caspase-independent apoptosis via the calpain/Bax protein signalling pathway. Though TG2 is strongly linked to the *in vivo* apoptosis programme (Fesus et al., 1996, Szondy et al., 1997, Szegezdi et al., 2000), from the apoptotic cell point of view, the expression of TG2 cannot always be detected during the induction of *in vitro* apoptosis (Fesus et al., 1996, Szegezdi et al., 2000) suggesting that factors required for TG2 induction *in vivo* are present only in the tissue environment.

In contrast, TG2 expression is known to trigger pro-survival pathways via constitutive activation of FAK and its downstream PI3K/Akt1 (Verma and Mehta, 2007). Intracellular

TG2 is testified as an anti-apoptotic effector in breast cancer, melanoma and pancreatic cancer cell types (Mann et al., 2006). TG2 is reported to mediate pro-apoptotic activity by inhibition of capase-3 and -9 through depletion of Bax in HEK293 cells (Cho et al., 2010) and in hypoxic cancer cells (Jang et al., 2010). The pro-apoptotic protein Bax on the mitochondrial membrane, which is also a binding partner for TG2 acts as a key target of TG2 induced cross-linking (Rodolfo et al., 2004). However, TG2 is also known to mitigate apoptosis via its GTP binding activity as seen in several cancer cell lines via activation of NF-κB (Mann et al., 2006, Cao et al., 2008) and, with loss in GTP binding activity reverts its protective role to the death signal (Datta et al., 2007). Likewise, loss of TG2 resulted in a defective cell cycle leading to apoptosis in endothelial cells (Nadalutti et al., 2011). Taken together, TG2 play a dual role in determining the cell fate which may depend on cell type, stimuli and concentrations of intracellular Ca^{2+} and GTP.

1.4.2.5.2 TG2 in cell adhesion and migration

Earlier studies by Gentile et al in fibroblasts suggested TG2 involvement in cell adhesion following observing their resistance to detachment by trypsin and slower migration (Gentile et al., 1992). Further studies to date (Wang and Griffin, 2013, Wang et al., 2011, van Strien et al., 2011) have established TG2 as a pro-adhesive and pro-migratory protein basing on its ability to collaborate with ECM adhesion receptors namely, β1/β3/β5 integrins and syndecan-4 and thus promoting receptor clustering and mediating inside-out signalling via activating FAK, RhoA, PKCα. Similarly, TGFβ mediated cell surface TG2 up-regulation enhances epithelial cell adhesion and migration on fibronectin (Priglinger et al., 2004). However more recent work has shown that the extracellular crosslinking activity of TG2 is involved in S100A4-related cell migration involving a syndecan-4 and β1 integrin co-signalling pathway (Wang and Griffin, 2013). Syndecan-4 facilitates a TG2-fibronectin complex mediating integrin dependent cell adhesion and TG2, Syndecan-4 knockout studies revealed their interdependence in cell adhesion and migration (Wang et al., 2011). Auto antibodies against the TG2-fibronectin heterocomplex blocked epithelial cells attachment via interfering with the heparan sulphate binding side, highlighting the role of TG2 in celiac disease pathology (Teesalu et al., 2012). The pro-invasive element of breast and ovarian cancer cells is regulated by the cell surface TG2 in cell motility (Mangala et al., 2007). Work by van Strien *et al*., (2001) have implicated cell surface integrin bound TG2 with fibronectin on activated astrocytes in multiple sclerosis lesion recruitment, leading to lesion progression. However,

loss of cell migration with increased adhesion is reported with over expression of TG2 (Balklava et al., 2002). Similarly, as a novel therapeutic strategy for improving attachment, mesenchymal stem cells (MSC) were genetically engineered to overexpress TG2 to enhance cell attachment and survival post implant (Song et al., 2007). A 33.1% enhanced adhesiveness is seen in TG2 transfected MSCs compared to control MSCs. Similarly, more pronounced systolic and diastolic cardiac function is evident in TG2-MSC therapy of infracted myocardium compared to MSCs alone (Song et al., 2007). Taken together, TG2 plays a crucial role in cell adhesion and migration and acting accordingly to regulate its expression could be a therapeutic solution in many human conditions.

1.4.2.5.3 TG2 in cell growth and differentiation

Birckbichler and Patterson (1978) initially reported involvement of TG2 in cell differentiation and proliferation suggesting that cells with low TG2 proliferate more rapidly than cells with high TG2. Similarly, TG2 inhibitors like cystamine enhanced some of the proliferation markers with simultaneous reduction in intracellular ε-(γ-glutamyl) lysine (Birckbichler et al., 1981). By using transamidation functional mutants (C277S) which still posseses some GTPase activity transfected into a metastatic hamster fibrosarcoma cell line MetB, a delayed S phase to G2/M progression was shown in the cell cycle (Mian et al., 1995). This suggests that the GTP bound form of TG2 may act as a molecular switch between cell proliferation and differentiation.

TG2 is expressed in a wide array of cells involved in immunity and inflammation including lymphocytes, neutrophils and monocytes (Mohan et al., 2003). Interestingly, TG2's role in macrophages is well documented with higher levels of TG2 expression in macrophages following differentiation (Schroff et al., 1981). Of all the members of the TGs, TG2 and factor XIII are known to get expressed by monocytes and macrophages. Upon monocyte maturation to the macrophage, up regulation of TG2 and markedly down regulation of factor XIII takes place (Seiving et al., 1991) suggesting a profound interrelation between the two TG members while establishing TG2 as a marker for macrophage differentiation. It has been demonstrated that the expression of TG2 increases drastically during monocyte trans-endothelial migration and differentiation into macrophages (Murtaugh et al., 1983, Akimov and Belkin, 2001, Thomas-Ecker et al., 2007).

1.4.2.5.4 TG2 in receptor mediated endocytosis

Via cross-linking the receptor with ligand or by stabilising the membrane for receptor aggregation, TG2 was reported to play a prominent role in mediating receptor mediated endocytosis. Davies et al. reported that by inhibiting TG2 activity using TG2 specific amine competitive inhibitors like cystamine, methylamine and dansylcadaverine, this can inhibit receptor mediated endocytosis (Davies et al., 1980). Concentrations of cystamine that inhibited macrophage TG2, also inhibited Fc receptor mediated endocytosis of red cells by guinea pig peritoneal macrophages (Davies and Murtaugh, 1984). Enhanced macrophage phagocytosis with increased TG2 activity is reported (Murtaugh et al., 1983) and loss of TG2 results in impaired apoptotic cell clearance (Szondy et al., 2003).

1.4.2.5.5 TG2 in Disease

TG2 is known to be expressed normally at low levels in many different tissues and predominantly exists inside the cell in the catalytically inactive closed conformation. TG2 when implicated in chronic diseases can be classified under three main categories: inflammatory diseases such as wound healing, fibrosis, tissue repair and autoimmune conditions; chronic degenerative diseases; and malignant diseases. Under pathological conditions, it gets activated transiently in response to innate immune signals and plays a general protective and stabilizing role in cells and tissue. However, abnormal activation or deactivation of TG2 is linked with different kinds of pathologies. Abnormal increases of TG2 expression in autoimmune inflammatory myopathies such as celiac disease (Briani et al., 2008, Molberg et al., 2000), liver cirrhosis & fibrosis (Mirza et al., 1997, Elli et al., 2009), type 1 diabetes (Bernassola et al., 2002), rheumatoid arthritis (Picarelli et al., 2003), dermatitis herpetiformis (Dieterich et al., 1999); renal scarring (Johnson et al., 2003) are well documented.

TG2 also play a pathological role in neurodegenerative diseases such as Parkinson's disease (Junn et al., 2003, Andringa et al., 2004), Huntington's disease and Alzheimer disease (Kim et al., 1999, Citron et al., 2001) and amyotrophic lateral sclerosis. It is also well established as one of the molecular triggers in thrombosis, monkey model neuro-AIDS by microarray analysis (Roberts et al., 2003), and cancer (Mangala et al., 2007, Mangala and Mehta, 2005, Verma and Mehta, 2007).

1.4.2.5.5.1 TG2 in Coeliac disease

Several lines of evidence have suggested that TG2 is one of the disease relevant proteins encountered in the coeliac gut. It is known to catalyse specific deamination of dietary peptides in the small intestines of coeliac disease (CD) patients which affects about 1-2% of the population including adults and children. Gluten sensitive enteropathy or CD is a multifactorial chronic disease caused by a permanent intolerance to ingested wheat gluten or similar proteins from barley and rye. CD is an autoimmune mediated pathology characterised by damage of the intestinal epithelium resulting from abnormal intestinal adaptive and innate immune responses to dietary gluten proteins in the diet (Jabri and Sollid, 2009). Patients develop IgA antibodies against TG2 mediated deamidated gluten epitopes and autoantibodies against TG2 in CD. Elevated levels of the enzyme were noticed in jejunal biopsies from the CD patients suggesting involvement of TG2 in the pathogenesis of CD (Bruce et al., 1985). TG2 activity was also shown to be associated with generating deamidated gluten peptides that trigger a T-cell mediated reaction in the small intestine of CD individuals. Conversion of glutamine residues in gluten derived peptides into glutamic acid has been shown to be catalysed by TG2. Ciccocioppo *et al*. (2010) revealed a role of TG2-specific T-cells mediated immune response in the pathogenesis of the disease.

1.4.2.5.5.2 TG2 in inflammation and Cancer

Abnormal activation or deactivation of TG2 is a common trait of several inflammatory diseases. Cytokines like TGFβ, Interluekin 1 (IL-1) regulate TG2 expression in keratinocytes and in dermal fibroblast and increase cell adhesion (Priglinger et al., 2004), while TNF-α enhances TG2 synthesis in liver cells via activation of IκBα phosphorylation. TG2 is known to be involved in enhanced inflammation by NF-κB which is considered as a master switch for inflammation (Lee et al., 2004). An increase in TG2 activity is associated with NF-κB activation where TG2 is known to aggravate inflammation by activating the NF-κB cascade. Carrying the binding motif for NF-κB on its promoter (Mirza et al., 1997), TG2 induces the activation of NF-κB by stimulating cross-linking and subsequent degradation of its inhibitory subunit α (IκBα). This cross-linking results in dissociation of NF-κB and translocation to the nucleus (Lee et al., 2004) where it is capable of up-regulating a host of inflammatory genes, including inducible nitric-oxide synthase and tumor necrosis factor α (TNF α) and even promotes expression of anti-apoptotic proteins such as *Bcl-xL* and *BFL1* (Lee et al., 2004, Mann et al., 2006).

TG2 is implicated in cancer progression while sharing similarities with the inflammatory responses (Mantovani et al., 2008). Conflicting outcomes with respect to up- and down-regulation in TG2 expression have been reported with its down-regulation in primary tumors, in tumor progression and its up-regulation in secondary metastatic tumors giving resistance to chemotherapy (Verma and Mehta, 2007, Kotsakis and Griffin, 2007). Jiang et al.(2003) have identified TG2 as one of the amplified genes either specifically or differentially in human lung cancer models. Via a mechanism involving RGD-independent cell adhesion process involving Syndecan-4 and fibronectin, it has been reported that TG2 can mediate cell rescue from anoikis (Verderio et al., 2003). On the contrary, its enzymatic cross-linking activity of the surrounding matrix in primary tumours can prevent tumour progression and metastasis by preventing angiogenesis (Jones et al., 2006).

$TG2^{-/-}$ mice revealed that TG2 has a protective role against cardiac ischemia (Szondy et al., 2006), while its detrimental role is shown in renal ischemia with reduced inflammation occurring in the $TG2^{-/-}$ mice (Kim et al., 2010) revealing its cell specific effects in ischemic pathology. Elevated levels of intracellular Ca^{2+} which is a TG2 activator can result in the formation of β-crystalin dimers in rabbit eye upon treating eyes with calcium ionophore, ionomycin, resembling those formed *in vitro* by incubation of TG2 with β-crystalline suggesting TG2 could be a major contributor to cataractogenesis (Sanderson et al., 2000).

Oxidative stress induced TGF-β activation of the Smad3 signalling pathway resulting in TG2 mediated cross-linking and aggregation of lens proteins is also reported (Shin et al., 2008). Lee et al. (2012) have reported that the activation of TG2 is involved in the formation of cataract in the eye of rats and in human lens epithelial cell line (HLE-B3) induced by sodium selenite (Na_2SeO_3) and is suppressed upon treating with TG2 inhibitor cystamine. Similarly, treatment with cystamine was found to have anti-inflammatory effect in the rat model of inflammatory bowel disease (IBD) suggesting the involvement of TG2 in the pathology of IBD (Elli et al., 2011).

Inflammatory cytokines like IL-1, TNFα, which are involved in atherogenesis, are known triggers of TG2 expression and/or its activity, in the macrophage. Moreover, abundant expression of TG2 in macrophages in atherosclerotic lesions (Boisvert et al., 2006) and its association with smooth muscle cells in established atherosclerotic lesion is well documented (Haroon et al., 2001) while TG2 plays no obvious role in Ox-LDL uptake or degradation

(Boisvert et al., 2006). TG2 cross-linking of Lp(a), a subclass of low density lipoproteins, is reported in the inner and middle layers of aortas bearing atherosclerotic lesions of cholesterol fed rabbit. $TG2^{-/-}$ mice showed no defective vascular structure but displayed atherosclerotic lesion expansion and weak plaque formation leading to sustained inflammation via defective anti-inflammatory clearance of apoptotic cells (Boisvert et al., 2006). Interestingly, in bone marrow donor cells TG2 plays a protective role by limiting atherosclerotic lesion size via TG2 modulation of macrophage function (Boisvert et al., 2006).

Macrophages infiltrate the site of infection and contribute to the degree of inflammation by generating TGF-β, which in turn increases TG2 expression via its response element in the TGM2 gene promoter (Ritter and Davies, 1998). Specifically, its release is regulated by macrophage recognition of phosphatidylserine on apoptotic cell which is an early step in the process of phagocytosis (Fadok et al., 1992). In turn, TG2 on the other hand will activate matrix bound TGFβ via cross-linking latent the large TGFβ-binding protein-1 (LTBP-1). This cycle of events with macrophage recruitment at sites of inflammation stimulates phagocytosis of apoptotic cells and suppresses further proinflammatory mediator production (Griffin et al., 2002, Telci and Griffin, 2006) suggesting a positive feedback loop between TG2 and TGFβ. TG2 null mice fail to activate TGF-β, have delayed clearance of apoptotic cells and have evidence of tissue inflammation and autoimmunity (Szondy et al., 2003).

1.4.2.6 TG2 knockout studies

Initial reports from the TG2 knockout mice studies revealed them to have a normal phenotype (De Laurenzi and Melino, 2001). However, several subsequent studies have highlighted a number of delicate phenotypes in TG2 null mice e.g. impaired glucose stimulated insulin secretion, a phenotype resembling that of maturity-onset diabetes of young (MODY), suggesting a physiological role for TG2 in glucose tolerance (Bernassola et al., 2002); defective rate of mitochondrial ATP synthesis resulting in alteration of the respiratory chains with consequences on the global production of ATP in tissues (Szondy et al., 2006).

From the immunity and inflammation point of view, several cases of abnormal phenotypes like an impaired capability of apoptotic cell clearance and deregulated cytokine production by macrophages leading to autoimmunity such as systemic lupus erythematosus is reported (Szondy et al., 2003; Falasca et al., 2005; Sarang et al., 2009). Szondy et al. have shown that

apoptosis could be induced in TG2-/- mice as induction of thymic apoptosis by dexamethasone, anti-CD3 mAb or γ-irradiation shown to initiate thymocytes death showing a significant increase in annexin V+ apoptotic cells. However, defective clearance of those apoptotic cells was also shown suggesting requirement of TG2 for AC clearance. Similarly, defective clearance of AC in the liver of TG2-/- mice upon inducing the apoptosis with PbNO3 was also shown (Szondy et al., 2003). Reduced rate of phagocytosis by $TG2^{-/-}$ mice was suggested to be a deficiency in activation of TGF-β1 (a known immunoregulatory cytokine that down regulate inflammatory response), as $TG2^{-/-}$ macrophages when co-cultured with AC in presence of conditioned medium derived from WT macrophages efficiently ingested AC (Szondy et al., 2003). Lack of TG2 also prevented the production of active TGF-β1 in macrophages exposed to AC (Szondy et al., 2003). Moreover, the phenomenon of rapid decrease in proinflammatory cytokine release following phagocytosis of AC is impaired in the absence of TG2 (Falasca et al., 2005). Other abnormal phenotypes include, hyperactive B-cell proliferation (Szondy et al., 2003), poor response to chemical wound healing (Sarang et al., 2005, Nardacci et al., 2003), impaired apoptotic cell-induced integrin β3 signalling leading to RhoG and Rac1 activation have been well documented (Toth et al., 2009). TG2 is known to bind integrin β3, a known phagocytic receptor, in the process of phagocytic cup formation. However, accumulation of integrin β3 around the AC and subsequent signalling is impaired in $TG2^{-/-}$ macrophages (Toth et al., 2009). Taken together, data generated form the TG2 knockout mice studies has highlighted the important role of the enzyme TG2 in cell death, cell clearance and inflammation.

1.5 TG2 inhibitors

Genetic knockout, technique in which one of an organism's genes are made inoperative, which is primarily used to understand the role of a specific gene are often difficult to apply to cell cultures and is time consuming. On the other hand, genetic knockout responses in animal models are often explained by the compensatory mechanisms leading to complicated interpretations. Moreover, absence of a protein may show a different phenotype compared to that of its inhibition such as macrophages from a subline of TG2 null mice express high levels of integrin β3 and show enhanced integrin β3 signalling and thus partially compensate for the loss of TG2 in apoptotic cell clearance. Looking the technical advantages of inhibitor usage and availability, different TG2 inhibitors are used to assess the role of TG2 in Ac clearance by human macrophages.

TG2 expression in different cell types and its involvement in different biological settings are well documented. To block its activity and to understand its role in different pathological states, several TG2 inhibitors were developed. Basing on their mode of action, these inhibitors are classified into three different classes namely, competitive amine substrates, reversible and irreversible inhibitors.

- **Competitive amine substrates:** These inhibitors do not completely abolish TG2 activity, but rather inhibit it by competing with natural amine substrates, such as protein-bound lysine residues, in the transamidating reaction. As a result, transamidation occurs continuously, however, the isopeptide bond is formed between the protein bound natural glutamine and the competitive amine substrates rather than natural glutamine and natural amine (Siegel and Khosla, 2007). Some of the commonly used competitive amine substrates are monodansylcadaverine, putrescine, and cystamine. Because of their commercial availability, chemical stability, non-toxicity, these are the most widely used amine competitive TG2 inhibitors (Karpuj et al., 2002).

- **Reversible inhibitors:** These inhibitors inhibit TG2 activity by simply blocking subtract access to active site without covalently modifying the enzyme (Siegel and Khosla, 2007). Some of the commonly used reversible inhibitors are GTP and GDP (Lai et al., 1998); GTP analogues such as GTPγS (Lai et al., 1998), and divalent metal ions like Zn^{2+} which competes with Ca^{2+} for metal binding site in TG2 (Aeschlimann and Paulsson, 1994).

- **Irreversible inhibitors:** These inhibitors are also called as suicidal inhibitors and are the most widely studied TG2 inhibitors in recent times. These prevent activity by covalently modifying the enzyme and preventing substrate binding. These are designed to target the active site cysteine using chemical functional group that are reactive in the presence of a nucleophilic atom, and form relatively stable chemical bonds after reacting (Siegel and Khosla, 2007). Commonly used structurally simple irreversible TG2 inhibitors are iodoacetamide (Macedo et al., 2000), 6-diazo-5-oxonorleucine (DON), sulfonium methylketones (Pliura et al., 1992), N-benzyloxycarbonyl-l-phenylalanyl-6-dimethylsulfonium-5-oxo-L-norleucine and 1,3-

dimethyl-2[(oxopropyl)thio]imidazolium (Alcock et al., 2011) and 4-Aminopiperidine (Prime et al., 2012).

In the current study, along with the commercially available Monodansylcedaverine (amine competitive substrate) and Z-DON (irreversible inhibitor), cell permeable TG2 inhibitor 1, 3-dimethyl-2-imidazolium derived R283 and cell impermeable peptidic TG2 inhibitor R294 and R281 which were synthesized at Aston University, UK (Griffin et al., 2008) were used. R281, R283, R294 and Z-DON were designed to target the active site cysteine in the catalytic tried of the catalytic core domain of the TG2 and known to interfere with the transamidation activity. In terms of inhibitors specificity, human monocytes and macrophages were known to express only two structurally related transglutaminases, TG2 and the A subunit of factor XIII (FXIIIA) of which upregulation of TG2 expression and a reduction in biosynthesis and surface expression of FXIIIA drastically during monocyte differentiation into macrophages is reported (Akimov & Belkin, 2001) suggesting that TG2 inhibitors are specific to TG2 and act on no other TG in human macrophages.

1.6 Aims and Objectives

Apoptotic cell (AC) clearance by macrophages (MØ) has been implicated as a vital programme in terms of immunological modulation. There has been a remarkable study to date to know how apoptotic bodies reach their graves in phagocytes. Moreover, there has been an increasing interest in the mechanisms that underlie the rapid recognition and engulfment of apoptotic cells. TG2 is known to play a prominent role in the above process and several TG2 knock-out studies have highlighted its need in the apoptosis programme. However, the role of cell surface TG2, especially in the context of MØ phagocytosis of AC is yet to be defined. Moreover, there is no study to date aimed at describing the role of TG2 in human macrophage functionality. With increasing interest in understanding the clearance of AC, in this context, this project aims to analyse and characterize the role of TG2 in human MØ function with particular focus on the process of apoptotic cell clearance.

The specific aims of this project are

1) To establish THP-1/MØ as a model system for studying MØ phagocytosis in terms of characterizing the role of TG2.

2) To characterize possible effects of both cell permeable and impermeable TG2 inhibitors on MØ clearance of AC and MØ migration to AC

3) To look into the effect of TG2 inhibitors on TG2 activity/conformation changes leading to alterations in MØ function

4) To look for possible TG2 binding partners in mediating MØ clearance of AC

5) To characterize the knockdown effects of TG2 and its binding partners in MØ clearance of AC

6) To look at possible mechanism involved in TG2 mediated MØ clearance of AC

Results from this study will dissect the role of TG2 in MØ function and provide clues for therapy in inflammatory or autoimmune diseases.

Chapter 2
Materials and Methods

Chapter 2

2. Materials and Methods

2.1 Materials

2.1.1 Antibodies

Table 2. List of Antibodies

Primary Antibodies			
Antigen	**Host Species**	**Clone**	**Company**
TG2 (CUB 7402)	Mouse	Monoclonal	Neomarks, Pierce, UK
TG2 (TG100)	Mouse	Monoclonal	Neomarks, Pierce, UK
TG2	Rabbit	Polyclonal	Neomarks, Pierce, UK
β3 integrin	Rabbit	Polyclonal	Cell signalling, USA
Isotype control IgG1/κ (MOPC21)	Mouse	Monoclonal	Sigma-Aldrich, UK
Syndecan-4	Mouse	Monoclonal	Santa Cruz Biotechnology, UK
Syndecan-4	Rabbit	Polyclonal	Invitrogen, UK
CD44	Mouse	Monoclonal	Cell Signalling, UK
PKCα	Mouse	Monoclonal	Santa Cruz, UK
Vinculin	Mouse	Monoclonal	Sigma-Aldrich, UK
α-Tubulin	Mouse	Monoclonal	Sigma-Aldrich, UK
β-Actin	Rabbit	Polyclonal	Abcam, UK

Secondary Antibodies		
	Host Species	**Company**
Goat anti-mouse	FITC	Sigma-Aldrich, UK
Goat anti-mouse	PE	Sigma-Aldrich, UK
Goat anti-mouse	HRP	Sigma-Aldrich, UK
Goat anti-rabbit	FITC	Sigma-Aldrich, UK
Goat anti-rabbit	PE	Sigma-Aldrich, UK
Goat anti-rabbit	HRP	Sigma-Aldrich, UK

2.1.2 Equipment

Chemotaxis Dunn CTG chamber, DCC100, **Hawksley and Sons LTD, West Sussex, UK**

Confocal Microscopy, **Leica Microsystems, UK**

Cryo-freezing container NALGENE, **Fisher, Loughborough,UK**

Eppendorf Centrifuge 5810R, **DJB Labcare Ltd, Buckinghamshire, UK**

Flow cytometer Quanta SC, **Beckman Coulter High Wycombe, UK**

Flow cytometric analysis software VenturiOne, **Dinnington, Sheffield, UK**

Haemocytometer Double cell standard, **Camlab Ltd, Cambridge, UK**
Inverted fluorescence microscope Zeiss Axiovert 200M, **Hertfordshire, UK**

Minigel Vertical Electrophorisis Apparatus and Western Blot wet transfer system, **Bio-Rad, Hemel Hempstead, UK**

Plate reader EL800, **BioTek Potton, Bedfordshire**, **UK**

Spectrophotometer Model DU-7, **Beckman Instrument (UK) Ltd, High Wycombe, UK**

Chromata-vue C71 light box and UVX radiometer. **UV-P Inc., Upland, CA, USA**

ImageJ, http://rsbweb.nih.gov.ij

Migration and Chemotaxis tool, http://ibidi.com/xtproducts/en/Software-and-Image-Analysis/Manual-Image-Analysis/Chemotaxis-and-Migration-Tool

2.1.3 Reagents

All general laboratory consumables were purchased from Sigma Aldrich (Poole, Dorset, UK), unless otherwise specified.

Other consumables were purchased from following suppliers.

Macrophage medium. **Invitrogen Crop., Paisley, UK**

RPMI 1640 medium, Foetal calf serum (FCS), L-Glutamine, Pencillin & Streptomycin. **PAA Laboratories, Yeovil, Somerset, UK**

Bio-Rad protein assay kit**. Bio-Rad, Hemel Hempstead, UK**

ECL Chemilunimescence development kit. **Amersham Parmacia Biotech, UK**

Human Syndecan-4 targeting siRNAs and TG2 targeting siRNAs, global inactive control siRNA and HiPerfect Transfection Reagent. **Qiagen, UK**

Lipofectamine, **Invitrogen, UK**

Molecular marker, Pre-Stained Rec protein ladder, BP3603-500/EZ-RUN. **Fisher Scientific, UK**

Monodansylcadaverine, heparinase II, chondroitinase and PMA. **Sigma-Aldrich, UK**

P1 peptide (NPKFLKNAGRDCSRRSS) and scrambled control peptide P1s (FNRADLKPRCGSSNKSR). **Peptide Protein Research, UK**

Percoll, **GE Healthcare, UK**

PKCα inhibitor Go6976, human plasma fibronectin and Genecticin (G418 sulphate). **Calbiochem, Nottingham, UK**

Purified guinea pig liver transglutaminase (gplTG) biotinylated-cadaverine (BTC) and Z-DON (Z-DON-Val-Pro-Leu-OMe). **Zedira, Germany**

Sepharose-A & G bead slurry. **GE Healthcare, UK**

TG2 inhibitors R281, R283 & R294, **Synthesized at Aston University, UK (Griffin et al., 2008)**

VD3. **Biomol, Exeter, UK**

Vectashield mounting medium. **Vector Laboratories, UK**

2.2 Methods

2.2.1 Tissue Culture

2.2.1.1 Cell Culture reagents

All tissue culture reagents were purchased from PAA Laboratories Ltd. (Yeovil, Somerset, UK) unless otherwise specified.

2.2.1.2 Cell culture media and solutions

2.2.1.2.1 Cell culture medium (Complete RPMI, cRPMI): Cells were cultured in RPMI 1640 medium containing 10% (v/v) foetal calf serum (FCS), 2mM L-glutamine, penicillin (100U/ml) and streptomycin (100µg/ml).

2.2.1.2.2 Macrophage medium: Monocytes derived from blood were cultured in macrophage medium

2.2.1.2.3 Macrophage-Apoptotic cell interaction assay medium: serum-free RPMI 1640 containing 0.2% (w/v) BSA

2.2.1.2.4 Cell freezing medium: 10% (v/v) Dimethyl sulphoxide (DMSO) in FCS.

2.2.1.2.5 Cell lysis buffer (1% v/v): Nonidet, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1mM benzamidine, 1mM NaF, 1mM Na_3VO_4, 0.1mM phenylmethylsulfonyl fluoride (PMSF) and 1% (v/v) protein inhibitor cocktail.

2.2.1.2.6 Binding buffer: 10mM HEPES pH 7.4, 150mM NaCl, 2.5mM $CaCl_2$

2.2.1.2.7 PMA: 250µM stock made up in absolute ethanol

2.2.1.2.8 Dihydroxyvitamin D3 (VD3): 100µM stock made up by using RPMI+10% FCS following solubilisation in DMSO.

2.2.2 Cell lines

THP-1: Human monocytic leukaemic cell lines.

Mutu: EBV-positive Burkitt lymphoma cell line (Gregory et al., 1991)

Jurkat: human T cell leukaemia cell line (American Type Culture Collection; ATCC).

2.2.3 Cell Culture:

THP1, Mutu and Jurkat cells were cultured in RPMI supplemented with 10% (v/v) foetal calf serum, 1% (v/v) P/S and 2µM L-glutamine (cRPMI) and maintained at 37^{o}C in 5% CO_2 humidified incubator. All the cell lines used were sub-cultured at an interval of 48-72 hours by feeding the cells with fresh culture medium.

2.2.4 Cell Freezing:

Cell cultures with high viability were harvested and following centrifugation, cell pellets were re-suspended in freezing medium. Cells were then pipetted in 1ml aliquots into cryovials and were placed in -80^{o}C to allow slow freezing. Cryovials were finally transferred to liquid nitrogen container for long term storage.

2.2.5 Cell Thawing:

Cryovials of cells stored in liquid nitrogen were removed and immediately thawed at 37^{o}C in a water bath. 10 ml pre-warmed growth medium was added drop wise and finally cells were transferred into a 15ml sterile tube and centrifugation at 300xg to remove cryoprotectants. Following centrifugation, cells were re-suspended into cell culture medium and transferred to cell culture flasks.

2.2.6 Cell concentration determination:

10µl of cells in either suspension culture or adherent cells (following treating with 5mM EDTA and subsequent washing with 1XPBS) are gently placed in between the grated haemocytometer and cover slip. All the cells in 4 separate fields were counted through a

bench top microscope. The average number of cells obtained per field was multiplied by 10^4, to give the cell concentration (cells/ml).

2.2.7 THP-1 differentiation to macrophage-like cells:

THP-1 cells (human myelonocytic) were differentiated into macrophage-like cells by treating the cells with either 250nM phorbol 13-myristate12-acetate (PMA; 250µM stock made up in absolute ethanol) or 100nM dihydroxyvitamin D3 (100µM stock made up by using RPMI+10% FCS following solubilisation in DMSO) or both (double stimulated, DS) for 48/72h at 37^{o}C in 5% (v/v) CO2 incubator.

2.2.8 Preparation of Human Monocyte-Derived Macrophages

2.2.8.1 Isolation of leucocytes from blood

Monocytes were isolated from citrated blood of healthy volunteers by dextran sedimentation and Percoll fractionation. Briefly, freshly drawn blood in 50ml polypropylene centrifugation tube containing 3.8% sodium citrate is centrifuged at 350g for 20min with brake off. For every 10ml of blood, the cell pellet obtained was mixed with 5ml of dextran (6% dextran w/v in 0.9% w/v Nacl, autoclaved) and gently mixed to make uniform suspension. Tubes were left undisturbed for 1hr to allow RBC to sediment under gravity. The supernatant containing leucocytes are transferred to a 50ml tube with an excess of serum-free RPMI supplemented with penicillin and streptomycin (sfRPMI P/S), and are centrifuged at 220g for 6min at 4^{o}C. Pelleted leucocytes are re-suspended in 1ml RPMI P/S.

2.2.8.2 Preparation of 68% (v/v) Percoll

Isotonic Percoll stock (normally defined as 100%) was made by adding 1/10th volume of 10XPBS to Percoll received from supplier (GE Healthcare). Initially, 50ml of isotonic Percoll stock solution was made by adding 5ml of 10XPBS to 45ml of Percoll. From the 100% stock solution, 10ml working stock of 68% Percoll was prepared by adding 6.8ml of 100% Percoll stock to 3.2ml of 1XPBS.

2.2.8.3 Separation of mononuclear cells on Percoll gradient

1ml of leucocytes in 1ml RPMI P/S was layered carefully on top of 5ml, 68% (v/v) Percoll, in 15ml conical polypropylene tube. Tubes were centrifuged at 700g for 20min with brake off. The buffy coat obtained was the layer of mononuclear cells and carefully collected using

pasteur pipette. Mononuclear cells are re-suspended in excess of sfRPMI P/S and again centrifuged at 500g for 5min to remove any traces of Percoll which goes into supernatant. The cell pellet was re-suspended in MØ medium and, following cell counting (section 2.2.6), cells were seeded at an appropriate density in culture dishes and by feeding them with pre-warmed fresh MØ medium on day 2 and day 4, cells were cultured at 37°C in 5% (v/v) CO_2 incubator for 7-8 days to differentiate into human monocyte-derived MØ.

2.3 Phagocyte-Apoptotic cells interaction assay

2.3.1 Apoptotic cell generation:

In the current study, Mutu and Jurkat cells lines were used as apoptotic cell models. Cells at an appropriate cell density were exposed to UV-B irradiation at varying doses 25-200mJ/cm^2 as appropriate using a Chromata-vue C71 light box and UVX radiometer (UV-P Inc., Upland, CA, USA) and incubated for 16h at 37^{o}C in 5% (v/v) CO_2 humidified incubator to allow apoptosis to proceed (Torr et al., 2012).

Apoptosis was analysed by (a) labelling cells with annexin V (FITC)/ propidium iodide (PI) in binding buffer and detecting characteristic changes via light scatter using flow cytometry; and (b) by staining cells after they were fixed in 1% (v/v) formaldehyde with 4,6-diamidino-2-phenylinole (DAPI, Sigma, 250ng/ml) or by staining cells with acridine orange and observed using epifluorescence microscopy to detect nuclear changes and scoring them as a percentage of apoptotic cells per total number of cells counted. Cells that had undergone apoptosis by exposure to 100mJ/cm2 UV radiation following incubation for 16hrs were used for phagocytic interaction assays.

2.3.1.1 Annexin V/Propidium Iodide Staining:

An early event in apoptosis is the flipping of phosphatidylserine (PS) from the inner leaflet of the plasma membrane to the outside surface. Annexin V binds specifically to PS and labelled Annexin V in a Ca^{2+} dependent manner and can be used detect apoptotic cells. Propidium Iodide (PI) is used in conjunction with labelled Annexin V as the cell membrane integrity excludes PI in viable and apoptotic cells, whereas necrotic cells are permeable to PI. In late apoptotic and necrotic cells, the integrity of the plasma and nuclear membranes decreases, allowing PI to pass through the membranes, intercalate into nucleic acids. Thus, dual parameter FACS analysis allows distinguishing viable, apoptotic and necrotic cells.

UV irradiated Mutu BL cells were stained with annexin V-FITC (eBiosciences Ltd, Hatfield, UK). Briefly, cells were washed and re-suspended in binding buffer (10mM HEPES pH 7.4, 150mM NaCl, 2.5 mM $CaCl_2$) containing annexin V-FITC (1µl per $2x10^5$ cells) for 15min on ice. Following washing once with binding buffer, cells were diluted with 1ml binding buffer to which PI is added to a final concentration of 20µg/ml. Samples were analysed immediately on a Quanta SC flow cytometer (Beckman Coulter).

2.3.1.2 Acridine Orange Staining:

Acridine orange is a nucleic acid selective fluorescent cationic dye used to visualize nuclear changes and apoptotic body formation those are characteristic of apoptosis.

For quantitative analyses, cells were stained with acridine orange (Sigma, 10µg/ml) and were enumerated as a percentage of apoptotic cells per total number of cells counted per sample using Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss Ltd, Welwyn Garden city, Fredley, Staffordshire, UK) and Hamamstsu Orca camera driven by Volocity (Perkin-Elmer, Cambridge, UK).

2.3.2 Assays of Phagocyte interaction with apoptotic cells

Interaction (binding and phagocytosis) of phagocytes (THP-1 derived MØ, HMDMØ) with apoptotic Mutu was carried in 24 well plates as described by (Devitt et al., 2004). Briefly, phagocytes and AC at a ratio of 1:100 were co-cultured for 1h at 37°C in RMPI containing 0.2% (w/v) bovine serum albumin. Unbound apoptotic cells were removed by extensive washing with PBS and cells were fixed with 1% (v/v) formaldehyde in PBS. Cells were stained with DiffQuik II (Medion Diagnostics GmbH, Dudingen, Switzerland) and cells scored by microscopy for the percentage of macrophages interacting with apoptotic cells. At least 200 macrophages were scored in each of triplicate wells.

2.3.2.1 MØ treatment with TG2 inhibitors

All TG2 inhibitors were initially solubilised in serum-free RPMI supplemented with penicillin and streptomycin. THP-1/MØ or HMDMØ were initially washed with 1X PBS to remove traces of spent medium and were treated with TG2 inhibitors (R281, R283, R294, Z-Don) and incubated for 1hr at 37˚C in 5% (v/v) CO_2 humidified incubator. Following 1hr incubation, medium was aspirated and cells washed thrice in 1X PBS before cells were co-cultured with AC as detailed in section 2.3.2.

2.3.2.2 Phagocyte binding/tethering assay:

MØ, either treated or untreated with TG2 inhibitors as described in section 2.3.2.2, were co-cultured with AC at room temperature (20°C), a temperature non-permissive for phagocytosis (Torr *et al*. 2012). Following incubation for 1hr, unbound AC were removed by washing and the cells were fixed, stained and scored.

2.4 Toxicity studies

2.4.1 Nuclear staining with DAPI:

THP-1/DS were treated with TG2 inhibitors (R281, R283, R294) at a concentration of 500µM in serum-free RPMI and incubated for 1 hour to 16 hours. Following incubation, cells were washed with PBS, fixed with 1% (v/v) formaldehyde in PBS and finally mounted with hardfix with DAPI (VectorLabs). Cells were later imaged to check for any possible toxic effects using phasecontrast-fluorescence microscope.

2.4.2 XTT assay

THP-1/MØ seeded in 96-well plates were treated with different concentrations of TG2 inhibitors (5, 50, 500 µg/ml). Following incubation for 1hr, medium was aspirated and cells were washed with 1XPBS. 30µl of sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitor) benzeme sulphonic acid hydrate (XTT) reagents mixture was added into each well and incubated for a further 4 h. The absorbance was read at 490nm and 750nm using a Spectrafluor plate reader. Results were expressed as absorbance at (490nm-750nm).

2.5 Immunofluorescence staining of cells

To detect cell surface proteins by indirect immunofluorescence, cells at $5x10^4$ per well on 4 well glass slides (Hendley, Essex, UK) were blocked with 3% (w/v) BSA in PBS, pH 7.4 for 30 min on ice. Following washes with PBS, cells were incubated with primary monoclonal antibodies in serum-free RPMI (1:100 dilution) for 2h on ice. Cell washing with PBS, pH 7.4 to remove unbound Ab was followed by incubation with specific secondary antibody conjugated with either FITC or PE (1:100 dilution) for 2h on ice. Stained cells were washed twice with PBS, pH 7.4, fixed with 1% w/v formaldehyde in PBS, pH 7.4 and were mounted with Vectashield mountant with DAPI (Vector Laboratories) and examined using confocal microscopy (Leica Microsystems).

2.6 Detection of protein expression by Western blotting

2.6.1. Preparation of total cell lysates

Adherent cells were washed once with ice cold PBS, pH 7.4 following aspirating the spent medium and were lysed with 50µl of lysis buffer (50mM Tris-HCl, pH 7.4, 30mM NaCl, 1% (v/v) NP-40, 1mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 0.1 mM PMSF and 1% (v/v) protein inhibitor cocktail (Sigma–Aldrich, UK)) and the lysates were collected with a scraper. For non-adherent cells, cells were collected by centrifuging at 300g for 3 min at 4^oC. The obtained cell pellet was washed once with ice-cold PBS (pH 7.4) and finally lysed with 50µl of lysis buffer with gentle pipetting. Cell lysates were incubated on ice for 30min and clarified by centrifugation at 300g for 3 min at 4^oC to remove debris. Samples were stored at -80^oC until used.

2.6.2. Estimation of protein concentration (Lowry's assay method)

Protein concentrations of the total cell lysates were estimated before loading samples for PAGE and western blotting. The protein content of the cellular extracts was determined using a commercial kit from Bio-Rad based on the Lowry method (Lowry et al., 1951).

The Lowry's method utilizes phenol reagent of Folin and Ciocalteau. This is essentially phosphotungstic phosphomolybdic acid which can be reduced by phenols and many other substances with phenolic rings to 'molybdenum blue'. Proteins reduce phenol reagent, which may be used therefore for their determination.

Standard protein solutions (BSA) ranging from 0.1-1mg/ml were loaded at 5µl/well and 1µl of cell extract diluted with 4µl of dH_2O, were added to wells of a microtitre plate in triplicates. To the standard protein and cell extract samples, 25µl of Reagent A, followed by 200µl of Reagent B were added and incubated for 15 min at room temperature. The absorbance values were recorded at 750nm using a SpectraFluor plate reader to produce the calibration graph. From the protein standard graph, protein concentrations of the cell extracts was estimated.

2.6.3. Sodium Dodecyl- Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Separation of charged molecules under the influence of an applied electric field forms the basis of SDS-PAGE. Protein separation by SDS-PAGE can be used to estimate relative molecular mass, the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions.

2.6.3.1 Preparation of protein extracts for SDS-PAGE

Protein extracts containing known amounts of total protein were solubilised in an equal volume of 2× strength Laemmli buffer (125mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (v/v) SDS, 10% (v/v) 2-β-mercaptoethanol and 0.004% bromophenol blue) (Laemmli et al., 1970) and were denatured at 95^{o}C for 5 min. Denatured protein samples were stored at -20^{o}C until use or resolved by the SDS-PAGE method.

2.6.3.2 Preparation of stacking and resolving gels for SDS-PAGE

Polyacrylamide gels consisting of 3% (w/v) stacking gel and varying percentages of resolving gel were prepared. The stacking gel was prepared using 0.2M Tris in 0.2% (w/v) SDS stock solution, pH 6.8 and the resolving gel contained 0.75M Tris in 0.2% (w/v) SDS stock solution, pH 8.8. The polymerisation is initiated by the addition of freshly prepared 10% (w/v) ammonium persulphate and N,N,N',N'-Tetramethylethylenediamine (TEMED). The recipe of stacking and resolving gels and varying concentrations of each recipe with varying percentages of gels are tabulated in Table 2. Resolving gels were allowed to polymerise for approximately 1hr under a layer of isopropanol to provide an even surface while getting rid of air bubbles. Once the gels were set, isopropanol was washed off and the edges were blotted dry using filter paper. Stacking gels were prepared according to the recipe shown (Table 3), layering the gel solution on top of resolving gel while taking care not to leave any air bubbles. Well comb is inserted and the gels are allowed to polymerise for 30-45min.

Table 3. The recipe for polyacrylamide gels

Resolving GEL Ingradients @ 2gels of 1.5mm thickness (ml)								
Stock solutions	Final acrylamide concentration in the seperating gel (%)							
	5	6	7	7.5	8	9	10	12
30% acrylamide/ 0.8% bisacrylamide	2.812	3.375	3.9375	4.218	4.5	5.062	5.625	6.75
4xTris.Cl/SDS, pH 8.8	5.625	5.625	5.625	5.625	5.625	5.625	5.625	5.625
H_20	14.06	13.125	12.937	12.656	12.375	11.815	11.25	10.125
10% APS	0.075	0.075	0.075	0.075	0.075	0.075	0.075	0.075
TEMED	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015
Stacking GEL Ingradients @ 2gels of 1.5mm thickness (ml)								
Acryl amide (40%)	0.7312							
Tris HCl	1.875							
H_2O	4.818							
10% APS	0.0375							
TEMED	0.0075							

2.6.3.3 Electrophoresis

Following gel polymerisation, gel plates were placed in the gel tank, combs were gently removed from stacking gels and the gel tank is filled with tris-glycine running buffer pH 8.5 (25mM Tris, 192mM glycine, and 0.1% (w/v) SDS). Wells were gently washed with running buffer to remove air bubbles and protein samples (with a maximum of 50µg of protein) were loaded into each well. Pre-stained molecular weight markers were loaded in one well to track protein movement. Electrophoresis was performed at 90V through the stacking gel and at 120V through the resolving gel until the marker reached the bottom of the resolving gel.

2.6.4 Western blotting of proteins from polyacrylamide gels

Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes (Gelman Biosciences, UK) using a Bio-Rad wet blot system. The protein gel from the cast was carefully removed, equilibrated in ice-cold transfer buffer along with fibre pads, blotting papers and nitrocellulose membrane. The protein gel overlapped with nitrocellulose membrane was sandwiched between blotting paper which was further sandwiched with fibre pads. Care was taken to remove air bubbles by rolling a glass rod gently on the surface of the fibre pads. The sandwiched membrane was inserted into the

blotting cassette which is further assembled in blotting apparatus facing the protein facing side on the membrane towards anode electrode. The apparatus was also filled with a frozen ice container to prevent over heating of the system and the apparatus was filled with a litre of ice cold transfer buffer. The electro transfer of the proteins was then performed at a constant 200mA for 2 hours.

Protein transfer was confirmed by the transfer of high weight pre-stained molecular markers on the nitrocellulose membrane. Following transfer, the membrane was washed with TBS-Tween (pH 7.4) incubated in the blocking solution, 5% (w/v) skimmed milk powder in TBS-Tween (pH 7.4) with gentle agitation for 1 hour at room temperature to prevent the non-specific binding of antibodies in later steps.

2.6.5. Immuno-development of Western blots

Following blocking of the membrane with the blocking solution, membranes were incubated with the appropriate primary antibody diluted in a blocking buffer with gentle agitation at 4ºC overnight. Blots were then washed thrice for 20 min in wash buffer (1XTBS-Tween, pH 7.4) with agitation. Membranes were then later incubated with the suitable secondary antibody conjugated with Horseradish Peroxidase (HRP) for 2h at room temperature under agitation. Following incubation with the secondary antibody, another set of washes was performed with 1XTBS-Tween and finally rinsed with PBS.

Amersham ECL Chemiluminescence system kit was used for immune-detection of blots, where the two reagents A and B are mixed in equal proportion in which the nitrocellulose membranes are incubated for 1 min. Following incubation, the membranes were drained, wrapped in cling film and placed in an autoradiography cassette. A photographic film was exposed to the membrane for a varying length of time (depending on the intensity of the signal). Later, the film was developed and fixed using GBX developer and fixer (Sigma, UK). The film was finally washed and air-dried.

2.7 Membrane Stripping

To ensure equal protein loading or to re-probe the membrane for any other specific proteins, primary and secondary antibodies with which a membrane was previously probed were removed by stripping the membranes with stripping buffer (100mM 2-mercaptoethanol, 2% (w/v) SDS, 50mM Tris-HCl, pH 6.7). The membrane was incubated in stripping buffer at

50ºC for 30 minutes by agitating occasionally. The membrane was washed in 1XTBS-Tween (v/v), pH 7.4, thrice for twenty minutes each and blocked in the blocking buffer for 1 hour at room temperature. The immune-development was later performed with relevant antibodies followed by immune detection.

2.8 Detection of cell surface protein by biotinylation

Biotinylation is the process of attaching biotin to proteins and other macromolecules via targeting specific functional groups including primary amines. The extraordinary affinity of avidin for biotin is one of the strongest known non-covalent interactions and this interaction is exploited to detect and purify proteins. Biotinylation of cell surface protein was performed using EZ-link Sulfo-NHS-Biotin (Pierce) as described (Wang et al., 2010).

Briefly, cells were rinsed with ice-cold PBS (pH 8.0) and were labelled with 0.8µM sulfo-NHS-LC-biotin dissolved in PBS (pH 8.0) at 4°C for 20 min. Following washing with 50mM Tris–HCl, pH 8.0, cells were lysed in 1% (w/v) SDS in PBS (pH 8.0) with benzonase at 1:1000 for 30 minutes on ice. Protein concentrations were determined and cell lysates containing 600µg of protein equivalent (following centrifugation at 13,000g for 20 minutes to remove non-broken cells) were incubated with 50µl NeutrAvidin-Agarose resin beads at 4°C overnight on a rotating platform. Following washing with PBS, pH 8.0, resin bead-bound biotin labelled cell surface proteins were extracted by boiling with 2× strength Laemmli buffer at 95^{o}C for 5 min. Following centrifugation, supernatants were collected and samples analysed by western blotting.

2.9 Co-immunoprecipitation

Co-immunoprecipitation, to detect the interaction between proteins, was carried out as previously described (Wang et al., 2012). Briefly, 150µg of protein equivalent cell lysates were cleared by using protein-A-Sepharose beads. Approximately 0.5µg of appropriate antibody was added to pre-cleared cell lysates and incubated for another 90min at 4^{o}C on a rocking platform to form immunocomplexes with the respective protein of interest. Immunocomplexes were pulled down by incubating with protein-A-Sepharose beads and were subsequently collected via boiling in 30µl of 2× strength Laemmli buffer. Immunoprecipitated proteins were detected via western blotting by using specific antibodies.

2.10 Detection of cell surface TG2 activity via biotin-cadaverine incorporation into fibronectin

Measurement of TG2 activity via biotin cadaverine incorporation into fibronectin was carried out as described (Scarpellini et al., 2009, Wang et al., 2012) with minor modifications.

2.10.1. Coating plates with Fibronectin

Wells of a microtitre plate were pre-coated with 50µl of 5µg/ml fibronectin in wash buffer (50mM Tris-HCl, pH 7.4) overnight at 4°C.

2.10.2. Detection of TG2 activity

Following rinsing wells with 50mM Tris-HCl (pH 7.4) and blocking with 3% (w/v) BSA in 50mM Tris-HCl (pH 7.4) for 30min at 37°C, cells were seeded to wells at $2x10^4$ per well in serum-free medium containing 0.132mM biotin X-cadaverine. Wells with 100ng of gplTG with 10mM Ca^{2+} or 10mM EDTA were used as positive and negative controls, respectively. Following incubation for 2 h at 37°C, the reaction was terminated by addition of 2mM EDTA in PBS, pH 7.4 and the cells were removed by 0.1% (w/v) deoxycholate in 2mM EDTA in PBS, pH 7.4.

Following rinsing wells with wash buffer and blocking with 3% (w/v) BSA in 50mM Tris-HCl, pH 7.4 for 30min at 37°C, biotin-cadaverine incorporated into fibronectin was detected by incubating at 37°C for 1 h with blocking buffer conjugated with HRP-conjugated Extr-Avidin (1:1000; Sigma-Alrich, UK). Following washing with wash buffer, development of reaction is initiated by using OPD (Sigma). Colour development was terminated by addition of 50 µl of 2.5M H_2SO_4 and the absorbance was measured at 450nm using spectrafluor plate reader.

2.11 Analysis of protein kinase C α translocation to membrane

THP-1/MØ were washed with 1XPBS, pH 7.4 and were gently scraped into ice-cold homogenisation buffer (10nM EDTA, 1mM NaF, 1mM Na3VO4, 50nM okadaic acid, 0.1mM PMSF, 1% (v/v) protein inhibitor cocktail, and 50mM Tris-HCl, pH 7.4). Cells were subjected to repeated freeze-thaw cycles at -170^{o}C and 37^{o}C, and centrifuged at 300g for 10min to pellet the nuclei and other cell debris. Protein concentration of the cell homogenates was estimated as described in section 2.6.2. 150µg of protein equivalent supernatant was further centrifuged at 20,000g for 20min to separate cytosol and membrane fractions. The

resulting supernatants were considered to be cytoplasmic fractions while the pellet, re-suspended in 30µl of homogenisation buffer, was considered as the membrane fraction. Both the fractions were dissolved in equal volume of 2× strength Laemmli buffer and the probed-developed with mouse monoclonal anti-PKCα and with anti-mouse secondary antibody conjugated with Horseradish Peroxidase (HRP) (Section 2.6.5)

2.12 Transient transfection

Transient transfection is most commonly used molecular technique to investigate the short-term impact of alterations in gene and protein expressions. When cells were transfected with long double stranded RNA (dsRNA), they block the expression of endogenous genes in sequence specific manner. The dsRNA are recognised and cleaved by dicer which is a member of RNAase 3 family of dsRNA specific endonucleases. Cleavage by dicer will create short ds RNA's that are characterised by two nucleotide long 3' over hangs which are called siRNA's. These siRNA's will form a ribonucleoprotein complex called RISC or RNAi silencing complex which includes an unidentified nuclease nicknamed slicer. RISC first mediates the unwinding the siRNA duplex. A single stranded siRNA that is coupled to RISC then binds to a target mRNA in a sequence specific manner and slicer activity enable the RISC to cleave mRNA. Cleaved mRNA is recognised by the cell and destroyed thus preventing translation occurring and silencing the expression of the gene from which the mRNA is transcribed. However, as the nucleic acid sequence is doesn't integrate in to the host cell genome unlike stable transfection, the effect of the targeted gene knockdown will be temporary.

2.12.1 siRNA Transfection

In order to silence the expression of TG2 or syndecan-4 in THP-1/MØ, four different siRNAs targeting human syndecan-4 and another four different siRNAs targeting TG2 or non-silencing control siRNA (Qiagen) were used to inhibit/'knockdown' the expression of each protein. 1mmol lyophilized siRNA was dissolved in 100µl of sterile RNase-free water to obtain a 10µM solution and stored at -20ºC. Transfection was carried out according to the manufacturer's protocol with slight modifications. Briefly, $5X10^5$ THP-1 cells in 2.3ml cRPMI were seeded to 6-well plate. Simultaneously, a complex mixture of siRNA to reach a final concentration of 150ng in 100µl of serum-free RPMI and HiPerfect transfection reagent was prepared and incubated for 10min at room temperature. siRNA transfection reagent

mixture was then added drop-wise to the THP-1 cells in medium with gentle swirling to ensure uniform distribution of the transfection mixture. Following 48h of incubation with siRNA, THP-1 cells were double-stimulated to differentiate to macrophage-like cells. Following incubation for another 48h, whole cell lysates were used to analyse the level of protein expression by western blotting.

Alternatively, $5x10^5$ THP-1 cells seeded to 6-well plates were double stimulated to differentiate to macrophage-like cells. Following 24h of differentiation, cells were transfected with siRNAs using HiPerfect transfection reagent (Qiagen). Following 48h of incubation with siRNA, THP-1 cells were double stimulated to differentiate to macrophage-like cells. Following incubation for another 48h, whole cell lysates were used to analyse the level of protein expression by western blotting.

2.13 Chemotaxis and migration studies

MØ migration towards AC was studied using a Dunn chemotaxis chamber (Hawkley DCC100) in conjunction with time-lapse microscopy (Chaubey et al., 2011). THP-1/DS cells treated as appropriate with cell-impermeable TG2 inhibitor R281 (1h in serum-free medium) were detached into 5mM EDTA at 37^{o}C and reseeded to plastic cover slip in serum-free RPMI. Following incubation at 37^{o}C for 20 min, the coverslip was inverted on a chemotaxis chamber and sealed in position, with chemoattractants placed in the outer circular well and RPMI 1640 medium as control in the inner well. Cell migration was recorded using time-lapse microscopy (Zeiss Axiovert 200M controlled by Improvision Volocity software) focussing THP-1/DS cells on the bridge between the outer and inner circular rings and cell migration either towards chemo attractants (as a representative of macrophage directional migration) or random migration was quantified using ImageJ software.

2.14 Statistical analysis

Data are expressed as the mean ± S.E.M for at least three independent experiments ($n \geq 3$) each undertaken in triplicate and were undertaken using InStat (GraphPad, La Jolla, CA, USA). Statistical analysis of results was undertaken using one-way analysis of variance (ANOVA) using a post-test depending on the requirement.

Chapter 3

THP-1 macrophage model system

Chapter 3

Results 1: THP-1 macrophage model system

3.1 Introduction

Removal of dead or diseased cells is crucial for many biological processes like tissue remodelling, maintaining tissue homeostasis and for resolution of immune responses. With an estimated loss of more than 10^9 cells per day (Elliott et al., 2009), the need for efficient removal is obligatory. Phagocytosis is a physiologically-ancient and an extremely complex process known to play a crucial role in processes such as host defence against foreign materials, infectious agents and diseased cells (Savill et al., 2002). In response to stimuli circulating monocytes in the bloodstream migrate to a site of infection or inflammation where they differentiate to macrophages and clear all corpses.

Macrophages are multifunctional, professional phagocytes which are equipped with a wide range of specialized and complex receptors that mediate tethering and efficient ingestion of dead and diseased cells (Savill et al., 2002, Devitt and Marshall, 2011). Macrophages recognise, bind and internalise target cells while mediating anti-inflammatory responses (Fadok et al., 1998). They interact with pathogens, via their evolutionary conserved PAMPs (Janeway, 1992, Janeway, 2001). However, macrophages are well characterised as professional phagocytes of apoptotic cells which present ACAMPs ligands sharing structural similarities with PAMPs (Tennant et al., 2013). Using multiple receptor-ligand pairs and bridging molecules that are recognised by CD14 and other patter-recognition molecules (Devitt and Marshall, 2011), macrophages actively clear dead and diseased cells, and resolve inflammation via producing anti-inflammatory mediators (Savill et al., 2002).

3.2 THP-1 derived macrophages

In the present study, human monocytic leukaemia cell line; THP-1 (Tsuchiya et al., 1980), is used for macrophage functional studies as this cell model provides a valuable tool for undertaking many experiments in a controllable system. Following external stimulation with phorbol ester or vitamin D3, these THP-1 cells differentiate to macrophage-like cells while expressing adhesion molecules like ICAMs and VCAMs (Thomas et al., 2013). Using three differentiation strategies using phorbol-13-acetate (PMA), 1, 25-dihydroxyvitamin D3 (VD3)

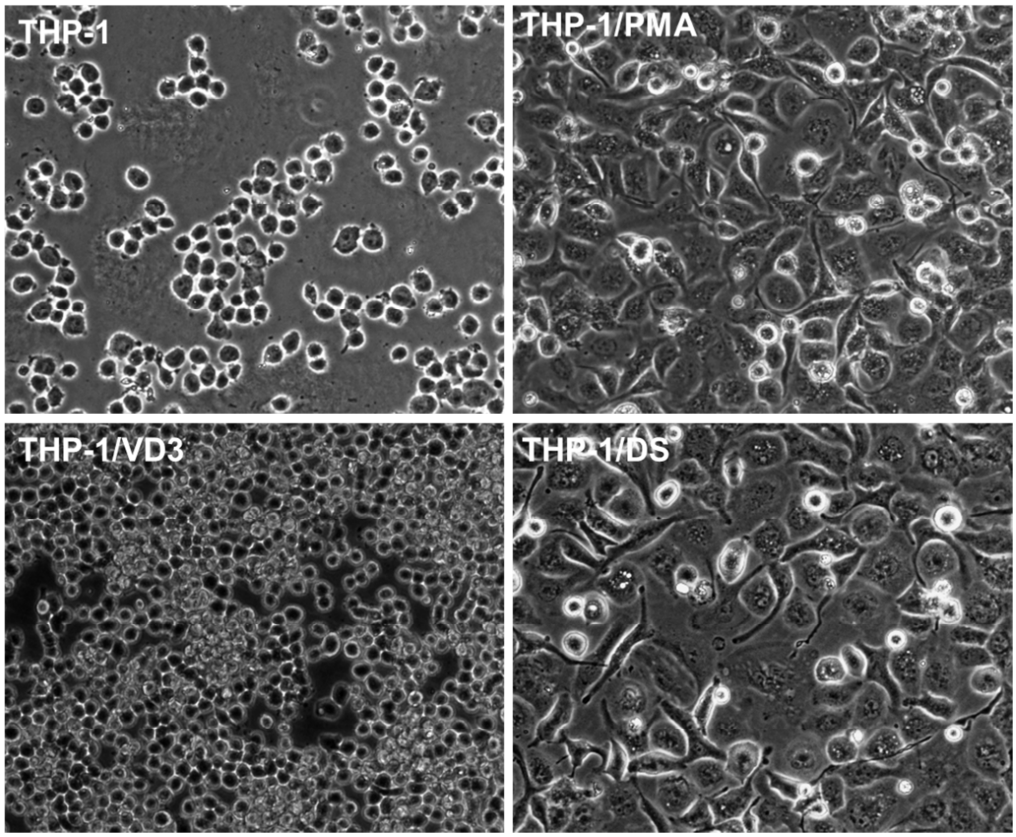
(Murao et al., 1983) or a combination of both (double stimulation; DS), THP-1 were differentiated for 48h and the resultant macrophage-like cells were characterised for macrophage phenotype e.g. change in morphology, adherence and function with their apoptotic cell binding ability.

3.2.1 Phenotypic characteristics of THP-1/MØ

THP-1 cells, following differentiation to macrophage-like cells, exhibit distinct morphological changes compared to undifferentiated cells (Figure 5A). THP-1/PMA and THP-1/DS revealed extensive morphological changes with respect to size, shape and spreading compared to VD3 stimulated and unstimulated cells. PMA and DS induced cells to spread producing irregular shaped bodies (Figure 5B). Simultaneously, a consistent change in nuclear to cytoplasm is evident (Figure. 5B). Moreover, both PMA and DS cell types were strongly adherent to plastic and resistant to washings with either culture medium or PBS. Cells required treatment with EDTA and vigorous washing to strip PMA or DS cells into suspension. On the other hand, VD3 stimulated THP-1 showed few signs of alteration and still continued to possess THP-1 like morphology in terms of size, shape and adhesion. THP-1 and THP-1/VD3 looked semi-adherent and can be removed with simple washings with a pipette (Figure 5A). However, THP-1/VD3 cells exhibit changes in their surface proteins and express lots of CD14 and thus become responsive to LPS (Thomas et al., 2013).

Similarly, flow cytometic analysis revealed differences in cell volume and granularity between differentiated and undifferentiated THP-1 cells (Figure 6A). THP-1/PMA and THP-1/DS revealed an increase in electronic volume and side scatter, suggesting an increase in cell size and granularity. An increase in cell size is a sign of increased number of organelles like mitochondria and secretory vesicles implying that cells have undergone strict internal changes to act as active effector cells (Daigneault et al., 2010). Also differentiation of THP-1 caused an increase in autofluorescence an indication for cytoplasmic inclusions such as protein–bound oxidised lipids, mitochondria, lysosomes rich in hydrolytic enzymes (Havenith et al., 1993, Daigneault et al., 2010) (Figure 6B).

A



B

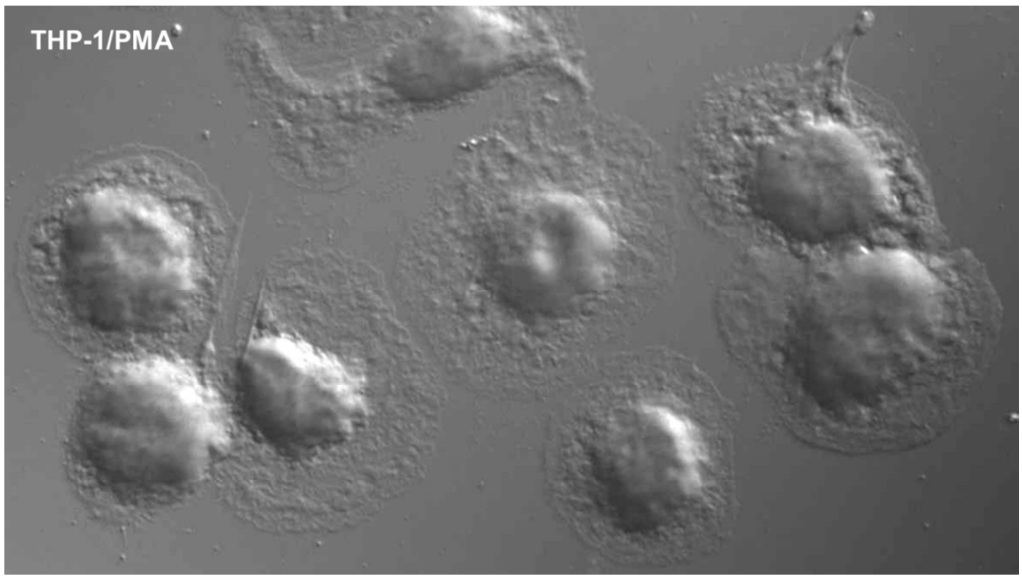
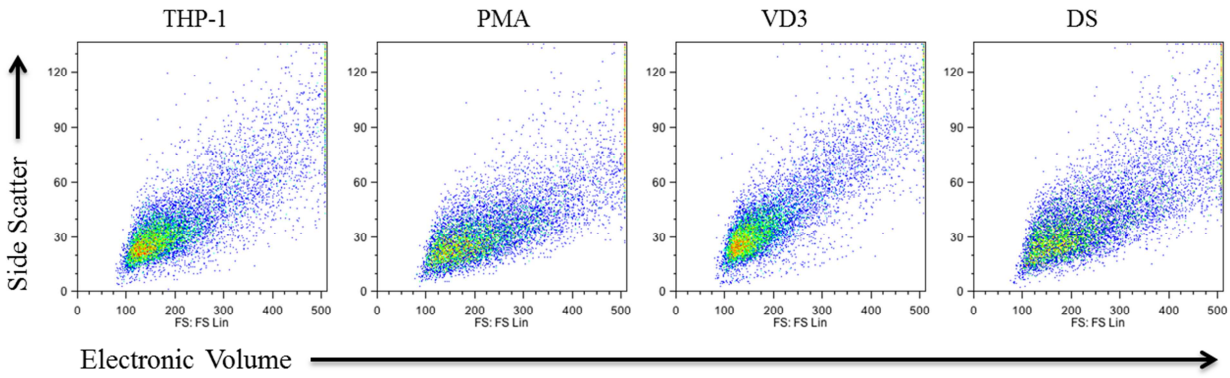


Figure 5. Microscopic analysis of THP-1/MØ morphology. *A.* THP-1 cells, seeded to tissue culture dishes, were treated with either 250nM PMA, 100nM VD3, or both (DS). Untreated cells were maintained as a control. Following 48h differentiation, cells were analysed by light microscopy. ***B***, (63x) DIC image of THP-1/PMA showing clear MØ adhesion and spreading.

A. Volume and Granularity



B. Auto-fluorescence

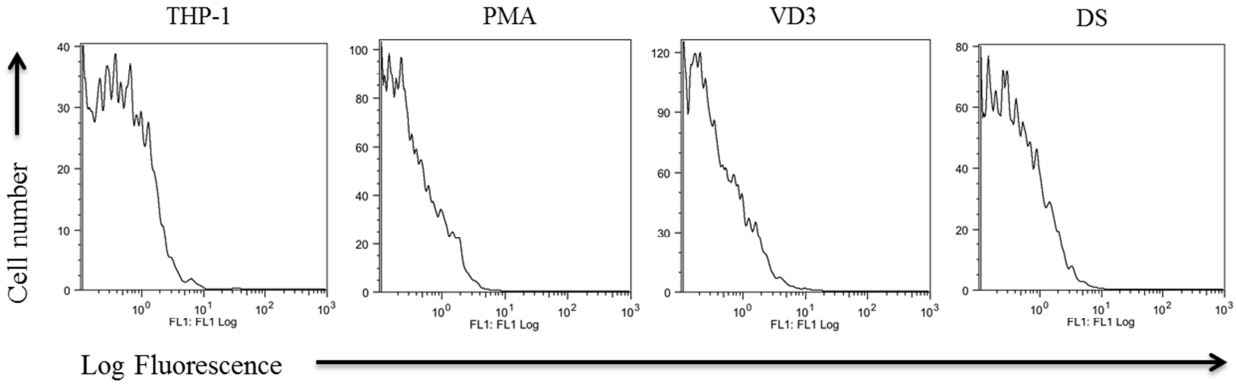


Figure 6. Flow cytometric analysis of THP-1/MØ morphology. THP-1 cells seeded to tissue culture dishes were treated with 250nM PMA, 100nM VD3, or both (DS). Untreated cells were maintained as a control. Following 48h differentiation, cells were stripped off the plastic surface using 5mM EDTA and were analysed by flow cytometry. ***A***, Electronic volume (EV) against side scatter (SS) plots were generated from 5000 events and ***B***, histograms of auto-fluorescence at 530nm with log scale on x-axis and event number on y-axis. Results shown are the representative of three independent experiments.

However, contradictory results were observed with respect to cell density in THP-1 and THP-1/MØ. A substantial increase in cell number during 48h of cell differentiations is seen in THP-1 and THP-1/VD3 consistent with cell division. Following another 24h culture resulted in overcrowding of cells. However, with THP-1/PMA and THP-1/DS, no change in cell number was evident. This suggests that PMA treatment inhibited the proliferating capacity of the cells exhibiting terminal differentiation while THP-1/VD3 still continues to grow like untreated THP-1.

Similarly, both PMA and VD3 differentiating agents were known to up-regulate the expression of Protein kinase C (PKC) isoenzymes, a family of protein kinase enzymes involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of protein bound serine and threonine amino acid residues. PKC activation in turn induces a greater degree of differentiation in THP-1 cells (Schwende et al., 2006). However, PMA but not VD3 mediated differentiation resulted in PKCα isoenzyme translocation to the membrane fractions (Figure 7) suggesting that both differentiating agents induce different signalling pathways resulting different degree of differentiation.

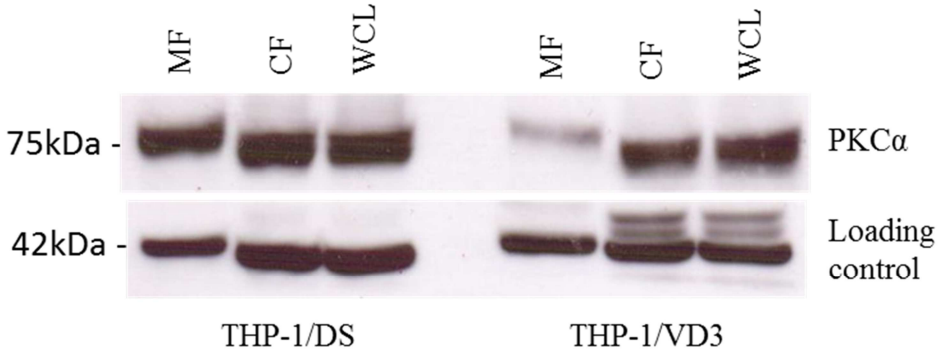


Figure 7: PKCα expression and membrane translocation in THP-1 differentiated macrophages. 50µg protein equivalent whole cell lysates and membrane and cytoplasmic fractionations from 50µg protein equivalent whole cell lysates of THP-1/MØ (THP-1/VD3 and THP-1/DS) were subjected to SDS-PAGE and the membranes following western blotting were probed with mouse monoclonal PKCα antibody. Equal loading was verified by probing with anti-actin antibody on stripped membranes. MF: Membrane fractionation; CF: Cytoplasmic fractionation: WCL: Whole cell lysate.

3.2.2 Functional Characteristics of THP-1/MØ

MØ are well known for their ability to interact with AC and are considered professional phagocytes. In light of this, the ability of our panel of THP-1/MØ with apoptotic cells was analysed following apoptotic cell generation

3.2.2.1 Apoptotic cell generation

In the current study, a human B cell line was used as an apoptotic cell model. Apoptosis was induced by exposing cells to UV (312nm) using Chromata-vue C71 light box and UVX radiometer (UV-P Inc., Upland, CA, USA) and incubated overnight to allow apoptosis to proceed (Torr et al., 2012). Apoptosis was analysed either by staining cells with acridine orange to detect nuclear changes using fluorescence microscopy (Figure 8A) and scoring

them as a percentage of apoptotic cells per total number of cells counted (Figure 8B), or by labelling cells with annexin V (FITC)/ propidium iodide (PI) in binding buffer and detecting characteristic changes via light scatter using flow cytometry (Figure 8C) (Dive et al., 1992).

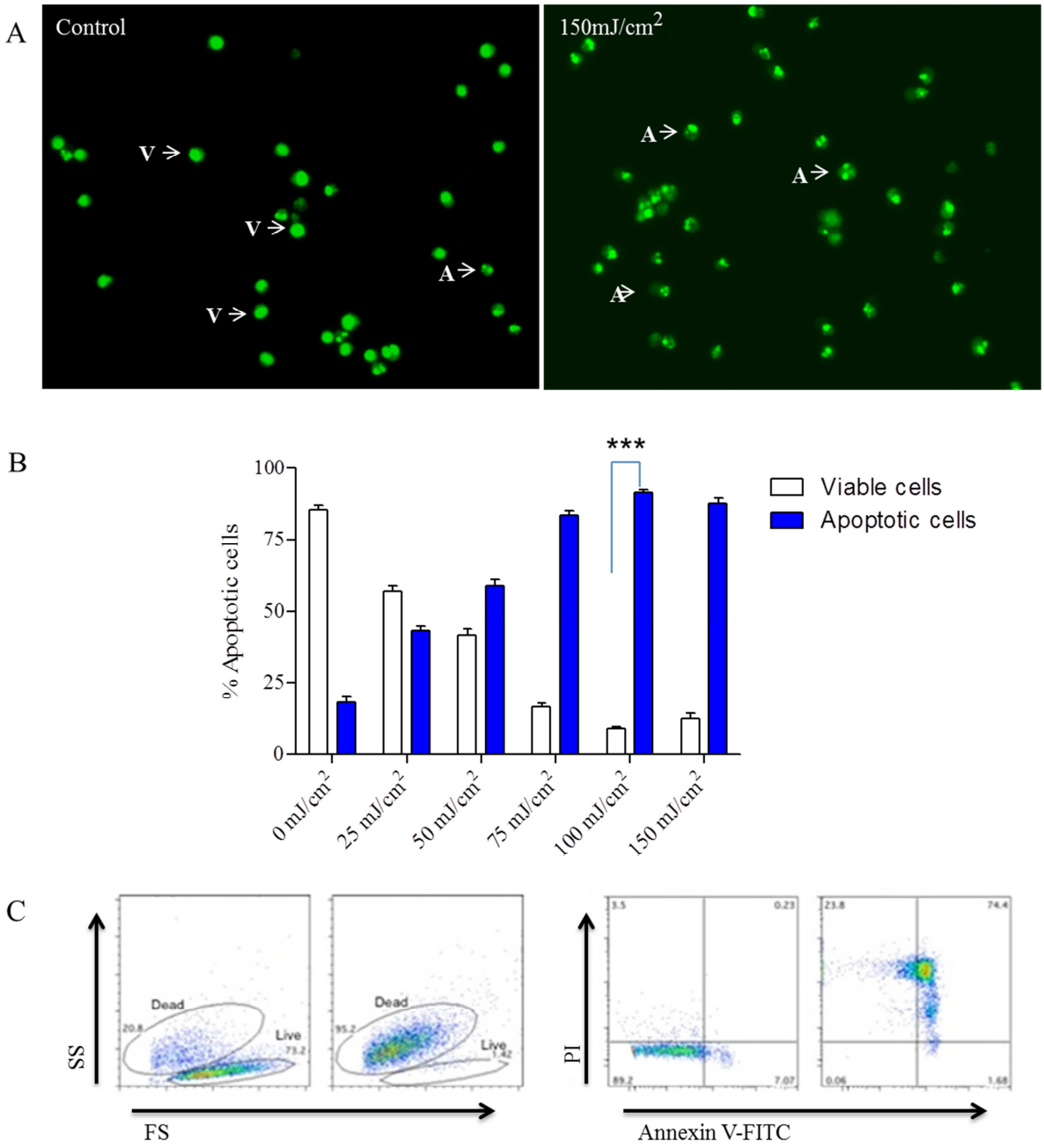


Figure 8. Apoptosis induction and analysis. ***A***, Mutu were induced to undergo apoptosis by exposing to indicated doses of UV. Following overnight incubation, cells were stained with acridine orange to reveal nuclear changes arrow heads point to viable (V) and apoptotic (A) cells, and ***B***, the percentage of cells underwent apoptosis was quantified. ***C***, Flow cytometric analysis of UV induced cells (100mJ/cm^2) compared and viable (non-induced) Mutu. FS and SS analyse (left hand panels) and annexin V/PI stain plots (right hand side) are shown. Apoptotic cells show low forward scatter and high side scatter, characteristic of apoptosis. The results shown are the mean ± S.E.M of a representative of three independent experiments done in triplicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** P<0.001).

As seen from figure 8A, nuclear changes representing classic signs of apoptosis were visualised. As seen from figure 8B, UV induced death in a dose dependent manner. Mutu exposure to 100mJ/cm^2 UV radiation followed by incubation for 16hrs provided a robust induction of apoptosis to maximal levels and thus was chosen for all future phagocytic interaction (binding and phagocytosis) assays.

3.2.2.2 THP-1/MØ-AC interaction assay

To assess the ability of our panel of THP-1-derived MØ cells to interact with apoptotic cells, THP-1 cells or THP-1/MØ were co-cultured with UV-induced apoptotic Mutu in 24 well plates. Human monocyte derived macrophages (HMDMØ) (Section 2.2.8) were used as a known positive control.

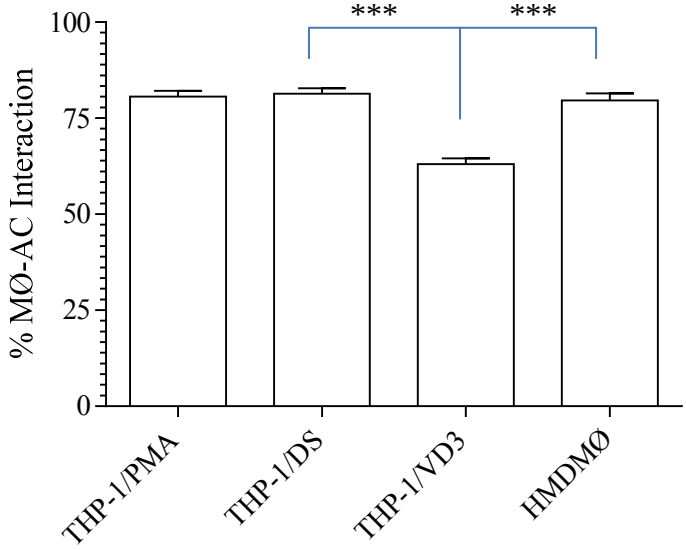


Figure 9. THP-1/MØ interaction with AC. THP-1/MØ (PMA, VD3 or DS) or primary human monocyte derived macrophages (HMDMØ) were co-cultured with apoptotic Mutu for 1h at 37^{o}C in 5% CO_2. Unbound apoptotic cells were washed off and the cells fixed with 1% w/v formaldehyde. Cells were stained with diff quick II and cells were scored as a percentage of macrophages interacting with AC using light microscope. Data shown is the mean ± S.E.M for n≥3 independent experiments done in triplicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** P<0.001).

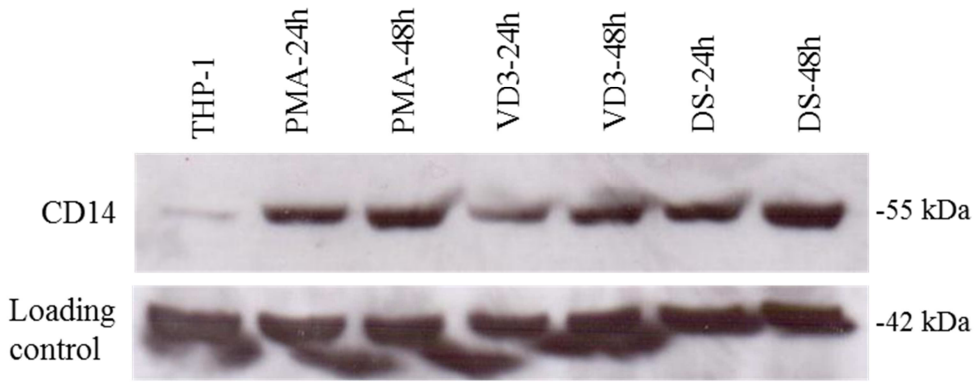
HMDMØ bind lots more AC per MØ. In comparison with HMDMØ all THP-1/MØ showed interaction with AC (Figure 9). However THP-1/PMA and THP-1/DS showed significantly higher level of interaction compared to THP-1/VD3. Moreover both THP-1/PMA and THP-1/DS exhibited high phagocytic capacity by binding with many apoptotic cells (phagocytic

index), ranging from 1-5 per cell, suggesting PMA stimulated THP-1 are more efficient phagocytic cells.

3.3 CD14 expression in THP-1/MØ

CD14 is a well-studied macrophage differentiation marker; its expression in the panel of THP-1/MØ is assessed. CD14 expression in differentiating macrophages at different time point was analysed by western blotting using mouse monoclonal antibody 63D3 for CD14 (Devitt et al., 1998). Whole cell lysates of THP-1/DS and THP-1/VD3 at 24h and 48h of differentiation in comparison with undifferentiated THP-1 were subjected to SDS-PAGE and the membranes, following western blotting, were probed with established mouse monoclonal 63D3 antibody for CD14 (Figure 10A). Similarly, cell surface CD14 expression was assessed using indirect immunofluorescence staining of THP-1 cells and THP-1 derived MØ models with established mouse monoclonal 63D3 antibody for CD14, followed by flowcytometric analysis (Figure 10B).

A



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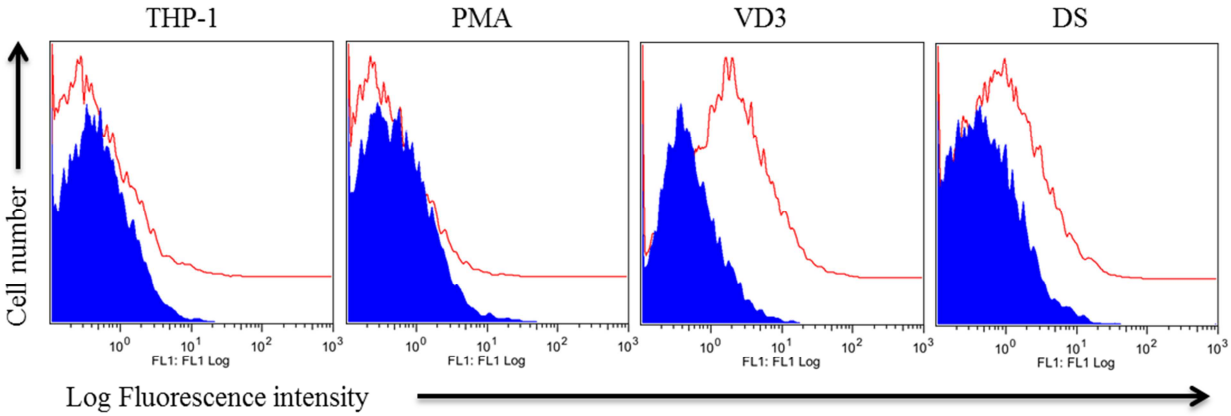


Figure 10. CD14 expression in differentiated THP-1 cells over time. A. 50µg protein equivalent whole cell lysates of THP-1/MØ (THP-1/PMA,THP-1/VD3 and THP-1/DS differentiated for 24h and 48h were subjected to SDS-PAGE and the membranes following western blotting were probed with mouse monoclonal 63D3 antibody for CD14. Undifferentiated THP-1 cells were used as control. Equal loading was verified by probing with anti-actin antibody on stripped membranes. **B. Cell surface expression of CD14 in THP-1 derived macrophages.** THP-1 and THP-1/MØ differentiated for 48h were subjected for indirect immunofluorescence staining for surface expression of CD14 with the established monoclonal antibody 63D3 (Red) or MOPC21, isotype control (Blue) and were analysed using VenturiOne analysis software. Data shown is the representative of three independent experiments.

As seen from figure 10A, detectable CD14 expression is expressed by all three THP-1/MØ models and enhanced CD14 expression is evident with THP-1 differentiation to macrophage-like cells. THP-1/PMA and THP-1/DS expressed high levels of CD14 compared to THP-1/VD3. Moreover, time-dependent expression of CD14 during the course of THP-1 differentiation to macrophage-like cells is evident. However, as seen from figure 10B, cell surface expression of CD14 is more pronounced in THP-1/VD3 compared to THP-1/PMA, contrary to whole cell expression as seen from western blot analysis. This is in line with Scwende et al. (1996), Daigneault et al.(2010), and Thomas et al. (2013) who showed high cell surface CD14 expression in THP-1/VD3 compared to THP-1/PMA by flowcytometric analysis.

3.3.1 CD14 dependent AC clearance

Earlier studies by Devitt et al (1998) using HMDMØ implicated CD14 in the clearance of AC and further confirmed CD14 as a tethering receptor (Devitt et al., 2004). To examine the involvement of CD14 in AC clearance by our panel of THP-1-derived macrophages, a MØ-AC interaction assay was conducted both in presence and absence of anti-CD14 monoclonal antibodies 61D3 (a known blocker of AC interaction with MØ) and 63D3 (an isotype matched, non-blocker) (Devitt et al., 1998) (Figure 11)

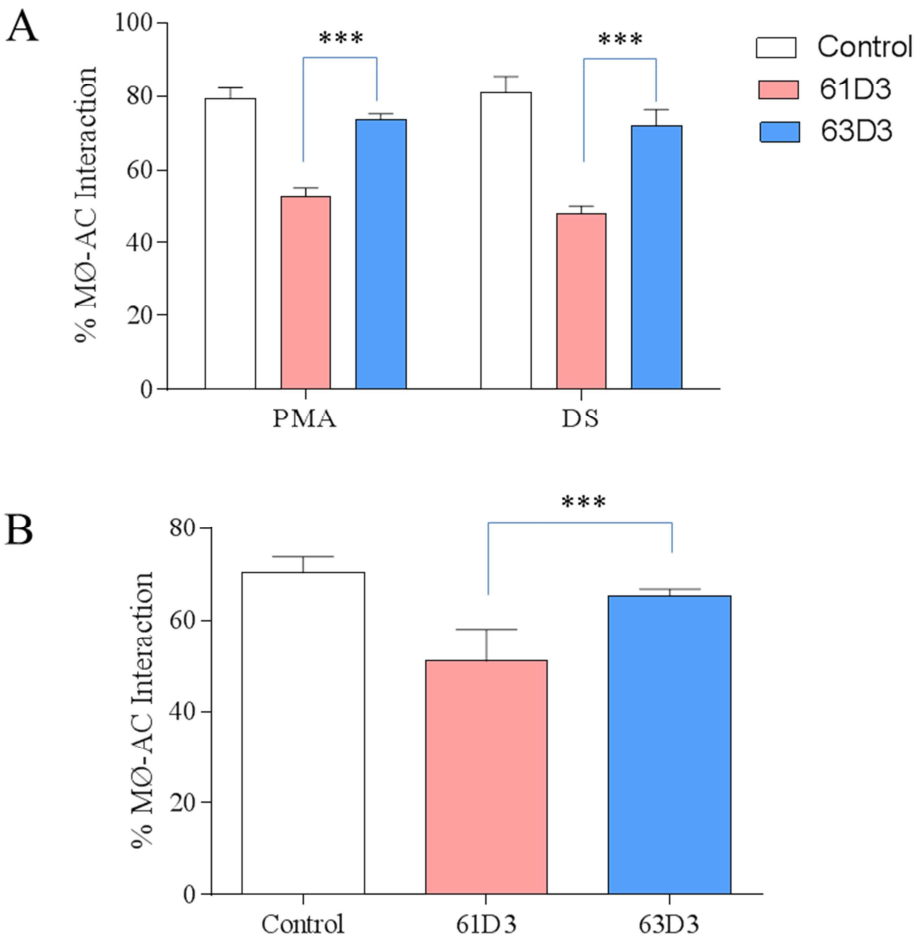


Figure 11. CD14-dependent AC clearance. A. THP-1 cells induced to differentiate with PMA and PMA+VD3 (DS) were co-cultured with apoptotic Mutu for 1h at 37^{o}C both in presence and absence of monoclonal antibodies 61D3, 63D3 or no Ab-control**.** Unbound apoptotic cells were removed by washing and THP-1 cells were fixed with 1% (w/v) formaldehyde. Cells were stained with diff quick II and cells were scored as a percent of macrophages interacting with AC by light microscopy. **B**. while MØ-AC was carried using THP-1 cells induced to differentiate with VD3 which showed less adhesion compared to THP-1 cells induced to differentiate with PMA and DS with minor changes in the protocol in terms of washing. Data shown is mean ±SEM for n≥3 independent experiments done in triplicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** P<0.001).

Irrespective of CD14 expression in PMA or VD3 differentiated, all three THP-1 derived macrophages used CD14 to clear AC with a varying degree of CD14 dependence. 61D3 blocked interaction between MØ and AC in all three THP-1/MØ. CD14 antibody 63D3 that

lacks blocking activity, though it is known to react strongly with CD14 in ELISA and on western blot (Devitt et al., 1998) was used as a known negative control. Though earlier studies by Scwende *et al*. (1996); Daigneault *et al*. (2010), have proposed that THP-1/PMA doesn't express CD14, it is quite evident from western blot analysis (Figure 10A) and MØ-AC interaction assay (Figure 11) that PMA differentiated THP-1 does express CD14 and make use of it to clear AC.

3.4 TG2 expression in THP-1/MØ models

Transglutaminases, a family of Ca^{2+} dependent acyl transferases catalyse covalent bond formation between lysine and glutamine- residues in target proteins leading to their post translational modifications (Griffin et al., 2002). Transglutaminase 2 (TG2) is known to be involved in monocyte extravasation to sites of inflammation and its expression is upregulated during monocyte differentiation to macrophage (Murtaugh et al., 1983). TG2 has been proposed to be involved in the process of MØ clearance of AC (Szondy et al., 2003). As, characterizing the role of TG2 in MØ function is the core aim of this thesis, initially the TG2 expression profile in the panel of THP-1 derived MØ was analysed by western blotting.

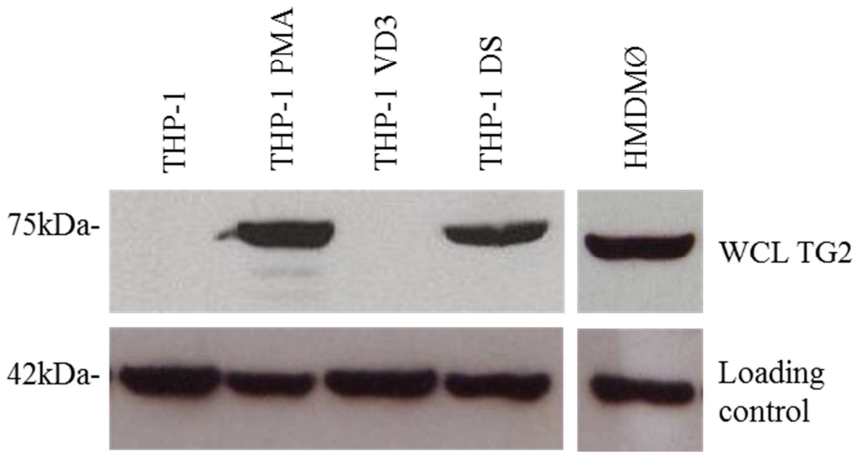


Figure 12. TG2 expression in MØ models. 50µg protein equivalent whole cell lysates of THP-1 and THP-1-derived MØ along with HMDMØ were analysed by western blotting. Membranes were probed using monoclonal anti-TG2 antibody TG100 and mAb binding was revealed by HRP-conjugated anti-mouse secondary antibody. Equal loading was verified by probing with anti-actin antibody on stripped membranes. Blot shown is representative of four independent experiments.

A detectable level of TG2 is expressed in THP-1/PMA, THP-1/DS and in HMDMØ, the most adherent MØ types. However, no detectable level of TG2 expression is seen in THP-1 and THP-1/VD3, the least adherent types suggesting that THP-1/VD3 stimulation may take a different signalling pathway with respect to TG2 expression when compared to phorbol ester mediated signalling. This may be reflected in the degree of differentiation.

To make a comparison of TG2 expression in THP-1 cells throughout their differentiation period with PMA differentiated or with PMA/VD3 (TG2 expressing) TG2 expression in PMA and DS differentiated THP-1 at different time points during differentiation was analysed by western blotting (Figure 13).

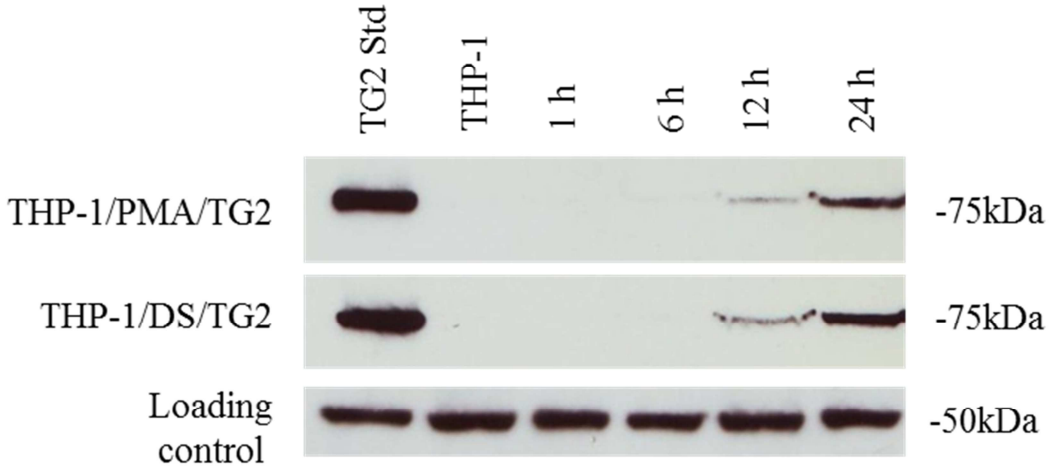


Figure 13. TG2 expression profile in differentiating THP-1 cells. 50µg protein equivalent whole cell lysates of THP-1/PMA and THP-1/DS during different time points of their differentiation were analysed by western blotting. Membranes were probed using monoclonal anti-TG2 antibody TG100 and mAb binding was revealed by HRP conjugated anti-mouse secondary antibody. Equal loading was verified by probing with anti-tubulin antibody on stripped membranes..

Detectable levels of TG2 expression are seen in both THP-1/PMA and THP-1/DS from 6-12hr of differentiation following stimuli (Figure 13).

TG2 is a ubiquitously-expressed isoform, existing intra and extracellularly with varied biological functions. Though TG2 is predominantly localised in cytoplasm, by an unconventional pathway it is known to be externalised where it interacts with cell surface and matrix-bound proteins (Aeschlimann et al., 1995, Griffin et al., 2002, Collighan and Griffin, 2009). With this in mind, TG2 cell surface expression in our panel of THP-1-derived MØ was analysed via biotinylation of cell surface proteins. Consistent with whole cell TG2 expression (Figure 12) THP-1/PMA and THP-1/DS strongly expressed TG2 at the cell surface (Figure 14A). Similarly, cell surface expression of TG2 in THP-1/DS is clearly revealed with indirect immunofluorescence microscopy (Figure 14B).

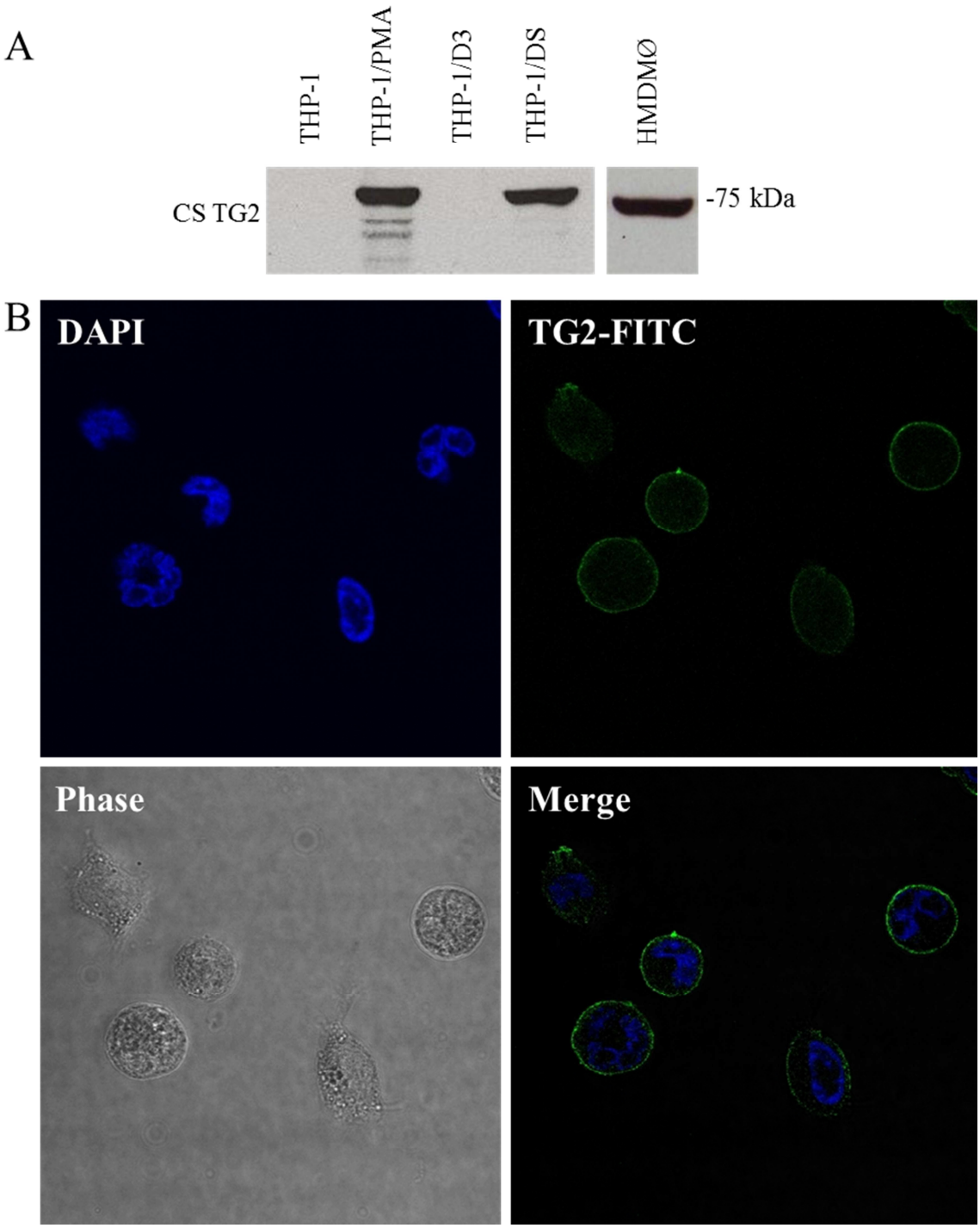


Figure 14. Cell surface expression of TG2 in THP-1/MØ. ***A,*** Cell surface biotinylated and neutravidin purified surface proteins of THP-1, THP-1 MØ and HMDMØ were analysed by western blotting. Membranes were probed using monoclonal anti-TG2 antibody TG100 and were revealed by HRP conjugated anti-mouse secondary antibody. ***B***, THP-1/DS were immunostained with monoclonal anti-TG2 antibody (CUB7402), which was revealed by FITC-congugated secondary antibody. Nuclei were stained with DAPI. Specimens were fixed with 1% w/v formaldehyde following antibody staining and imaged using confocal microscopy for cell surface TG2 expression. Blot shown is the representative of four different independent experiments.

3.5 TG2 expression in an apoptotic cell model

Apoptosis is a fundamental biological function in maintaining tissue homeostasis (Hammar and Mottet, 1971). Several investigations to date revealed the supposed role of TG2 in cell death and survival (Fesus and Szondy, 2005, Verma and Mehta, 2007, Park et al., 2010). Under normal conditions, TG2 activity is tightly-regulated by GTP activity. However, under extreme stress conditions and depending on the Ca^{2+} influx, TG2 activation is no longer inhibited (Iismaa et al., 2009) and this mediates cross-linked protein scaffolding in dead cells (Smethurst and Griffin, 1996). This stabilisation assists in maintaining dead cell integrity thereby preventing release of harmful cell components in to the extracellular environment. In line with the above information, TG2 expression in Mutu cells (used as a source of apoptotic cells) were also analysed for TG2 expression. Interestingly, neither viable nor apoptotic cells/apoptotic bodies of Mutu showed detectable levels of TG2 expression by western blot analysis (Figure 15).

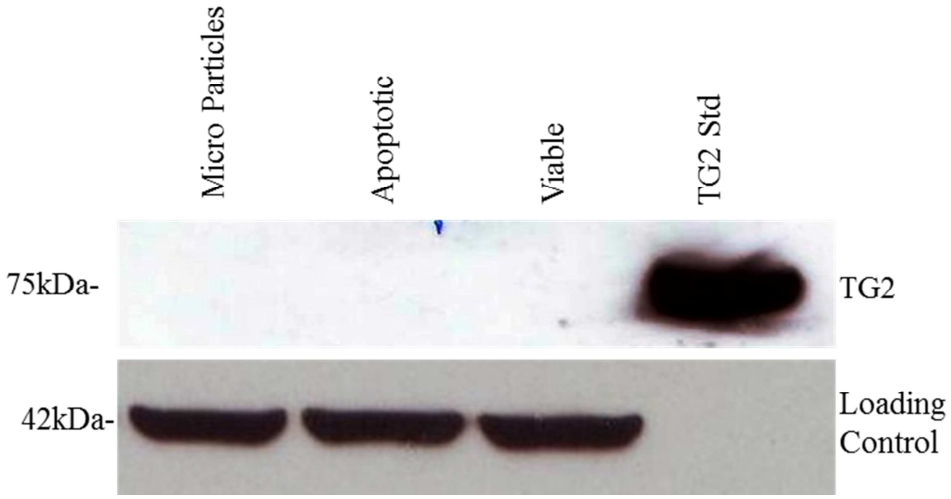


Figure 15. TG2 expression in Mutu B cells. 50µg protein equivalent cell lysates of viable, apoptotic cells and apoptotic bodies (micro particles) of Mutu were analysed by western blotting. Membranes were probed using monoclonal anti-TG2 antibody TG100 and mAb binding was revealed by HRP-conjugated anti-mouse secondary antibody. Equal loading was verified by probing with anti-actin antibody on stripped membranes. Blot shown is the representative of three independent experiments.

3.6 Discussion

Phagocytosis of AC by professional macrophages or other amateur phagocytes is considered to be the ultimate step in the process of apoptosis (Kerr et al., 1972). Defective clearance of AC by MØ is known to result in chronic inflammatory and autoimmune disease. Mechanisms by which apoptotic cells can be recognized and safely removed without triggering immune response have been of great interest for several years. Apoptotic cells express different cell surface changes which allow their recognition by macrophages, while macrophages on the other hand express several specific receptors and bind to the recognition signal directly or indirectly. Bridging molecules may facilitate the indirect binding. As a whole, an array of apoptotic cell-associated molecules, macrophage receptors and soluble bridging molecules work in a well-synchronized manner for rapid clearance of apoptotic cells (Savill et al., 2002, Devitt and Marshall, 2011).

In the present study THP-1, a human monocytic leukaemia cell line was used as a macrophage model system which, following stimulation differentiates to macrophage-like cells. THP-1 is a widely used cell line to model human macrophage function (Schwende et al., 1996, Tominaga et al., 1998, Daigneault et al., 2010, Auwerx, 1991). THP-1 cells are known to behave more like native monocytes compared to other cell lines like U937, HL-60, KG-1 or HEL (Auwerx, 1991, Daigneault et al., 2010). THP-1 differentiation to macrophage like cells was induced by phorbol -1,3-acetate (PMA), 1,25-dihydroxyvitamin D3 (VD3) or both (PMA+VD3) for 48h. Both PMA and VD3 differentiation agents were known to upregulate the expression of PKC isoenzymes but only PMA treatment will result in PKC isoenzyme translocation to membrane, which could be achieved by varied differentiation signalling pathway.

THP-1 cell differentiation resulted in macrophage-like cells characterised by morphological changes and their ability to interact with apoptotic cells, which is a well-established functional characteristic of monocyte-derived macrophages (Devitt et al., 2003). PMA on its own or double stimulation induced characteristic morphological changes in THP-1/PMA and THP-1/DS: increases in cell size, cell volume, granularity, cell adhesion, cell spreading and formation of lamellipodia-like structures. However, VD3-mediated differentiation induced few morphological changes as they retained the appearance of undifferentiated THP-1 cells. Moreover, THP-1 and VD3 differentiated THP-1 continue to proliferate over the period of

differentiation while use of PMA on its own or in conjugation with VD3 had a profound effect on regulating cell density. This is consistent with these cells having a more immature monocytic phenotype when compared to the PMA or DS cells, that both showed reduced division and possible cell fusion (Miyamoto et al., 2012). It might be suggested that THP-1/PMA and THP-1/DS are macrophages as they are terminally differentiated cells. A slight reduction in cell number is observed with PMA differentiation which could be either because of triggered cell adhesion property or cell fusion (Miyamoto et al., 2012). Signalling processes mediated by STAT6 and DAP12, or loss of STAT1-induced cell fusion resulting in multinucleated giant cells (Miyamoto et al., 2012). Similarly cell-cell adhesion, mediated by E-cadherin induces fusion which is in consistent with our cell spreading observation which may lead to cell-cell adhesion (Helming and Gordon, 2009). However, Mystkowska & Sawicki, (1987) have shown that the effect of PMA on mouse embryos is not due to cell fusion but inhibition of cytokinesis before karyokinesis suggesting PMA treatment affected cell division and cell proliferation and there is no real loss of cells by cell death (Mystkowska and Sawicki, 1987).

Similarly, CD14 (one of the best-characterised macrophage markers) was up regulated during THP-1 differentiation to MØ-like cells in all three THP-1/MØ models confirming macrophage identity in the panel of THP-1/MØ irrespective of varying morphological differences. Moreover, MØ-AC interaction assay further confirmed phagocytic ability of THP-1/MØ. Cell surface expression of CD14 is more pronounced in THP-1/VD3 compared to THP-1/PMA and is in line with earlier studies by Thomas *et al.* (2013). At the same time, studies by Scwende *et al.,* (1996) and Daigneault *et al*., (2010) proposed that THP-1/PMA do not express CD14, but it is evident here from western blot analysis (Figure 10A) and MØ-AC interaction assay that PMA differentiated THP-1 do express CD14 and make use of it to clear AC establishing THP-1/PMA, THP-1/VD3 and THP-1/DS as useful MØ model system. However, the differences in these results are not fully understood.

As a platform to the core aim of this thesis, TG2 expression profiles in the panel of THP-1-derived MØ was analysed by western blotting. A detectable level of TG2 is expressed in the whole cell lysates of THP-1/PMA and THP-1/DS. Via surface biotinylation and by indirect immunofluorescence, TG2 expression on the MØ cell surface is clearly revealed. However, THP-1 and THP-1/VD3 expressed no detectable TG2 expression, suggesting that VD3

derived THP-1/MØ continue to be more like undifferentiated THP-1 in terms of TG2 expression. Lack of TG2 expression in THP-1/VD3 is also consistent with morphological changes following differentiation where THP-1/VD3 showed no changes in morphology as like THP-1/PMA and THP-1/DS. Similarly, no detectable TG2 expression is seen following induction of apoptosis in Mutu cells. These results highlight THP-1/PMA and THP-1/DS cells as powerful models to address the function of TG2 at the cell surface of macrophages. This has not been addressed to date.

Chapter 4

Loss of TG2 activity affected MØ interaction with AC

Chapter 4

Result 2: Loss of TG2 activity affected MØ interaction with AC

4.1 Introduction

Diverse physiological and pathological processes initiate cell apoptosis, and the final stage in the apoptotic programme is the removal of apoptotic cells (Henson and Hume, 2006). Prompt removal of apoptotic cells is essential, failure of which results in release of self-antigens leading to several autoimmune diseases (Michlewska et al., 2007).

Transglutaminases are a family of Ca^{2+} and thiol- dependent acyl transferases well known for their ability to catalyse covalent bond formation between γ-carboxamide groups of peptide bound glutamine and ε-amino groups of peptide bound lysine (or, a poly amine) (Griffin et al., 2002). TG2 is a unique and well-studied member of the transglutaminase family (Collighan and Griffin, 2009) with multiple functions (Fesus and Piacentini, 2002, Aeschlimann and Thomazy, 2000). In addition to its well established cross-linking function, it also possesses GTPase activity, deamination activity, protein disulphide isomerase activity and protein kinase activity. TG2 is predominantly expressed in the cytoplasm, but via a yet to be defined mechanism; it gets translocated to the nucleus (via interacting with importin-α3) (Peng et al., 1999), mitochondria (Piacentini et al., 2002) and even to the cell surface where it is known to interact with extracellular matrix proteins. It functions as an adaptor protein in facilitating cell adhesion to fibronectin via integrins (Akimov et al., 2000) and even aids in integrin clustering and signalling (Janiak et al., 2006).

TG2 involvement in MØ and in MØ function has been studied. It is known to be involved in monocyte extravasation to sites of inflammation and even its expression is upregulated during monocyte-MØ differentiation (Murtaugh et al., 1983). Most importantly, TG2 is well recognized in macrophage phagocytosis of apoptotic cells (Szondy et al., 2003) while in vivo loss of TG2 results in impaired AC engulfment (Szondy et al., 2003, Falasca et al., 2005). MØ infiltrate sites of infection and contribute to the inflammatory response through the production of cytokines. One such cytokine is TGF-β1 which is required for TG2 induction via its response element in the *TGM2* gene promoter (Ritter and Davies, 1998) thus promoting phagocytosis (Fadok et al., 1993). Moreover, Toth *et al.* (2009) confirmed that TG2 is needed for efficient phagocytic portal formation. Similarly, Hodrea *et al*. (2010)

proposed that TG2 is expressed and active on the MØ cell surface. However, the function of cell surface TG2 in AC clearance is not known.

Based on our understanding of TG2 involvement in macrophage phagocytic function from the mouse model work, this chapter dissects the function of TG2 in human macrophages by using the human monocytic leukaemia cell line, THP-1 while simultaneously working on the human blood monocyte-derived macrophages. Using site-directed irreversible transglutaminase inhibitors 1, 3-dimethyl-2-imidazolium derivative R283 (cell permeable), peptidic inhibitors R281 and R294 (Cell impermeable) which were synthesized at Aston University (Griffin et al., 2008) along with commercially available Z-DON (*Z-DON-Val-Pro-Leu-OMe*), which are designed to target the active cysteine residue in the catalytic triad of the TG2 core domain, the work presented here will assess whether the role of TG2 in apoptotic cell clearance is activity or conformation-dependent; whether it is involved in other phases of apoptotic cell clearance (e.g. attraction, binding and/or phagocytosis) and whether cell surface TG2 is functional.

4.2 TG2 inhibitors reduce THP-1/MØ interaction with AC

To confirm the role of TG2 in MØ clearance of apoptotic cells, three different THP-1/MØ models were generated by stimulating with PMA (THP-1/PMA), 1,25-dihydroxyvitamin D3 (THP-1/VD3) or by both PMA and VD3 (THP-1/DS). THP-1/PMA and THP-1/DS were known to express TG2 while no detectable levels of TG2 expression are seen in THP-1/VD3 (Figure 12). After 48h of differentiation, cells were washed and treated with either cell permeable (R283 & Z-DON) or cell impermeable (R281 & R294), irreversible TG2 inhibitors at 500µM in RMPI containing 0.2% (w/v) bovine serum albumin for 1h (Griffin et al., 2008). Cells were washed with fresh culture medium and were co-cultured with apoptotic Mutu for 1h at 37^{o}C. Unbound AC were removed by washing, macrophages were fixed with 1% w/v formaldehyde, stained with Diff quik II and scored as a percentage of macrophages interacting (i.e. tethering or phagocytosing) with AC using light microscopy (Figure 16).

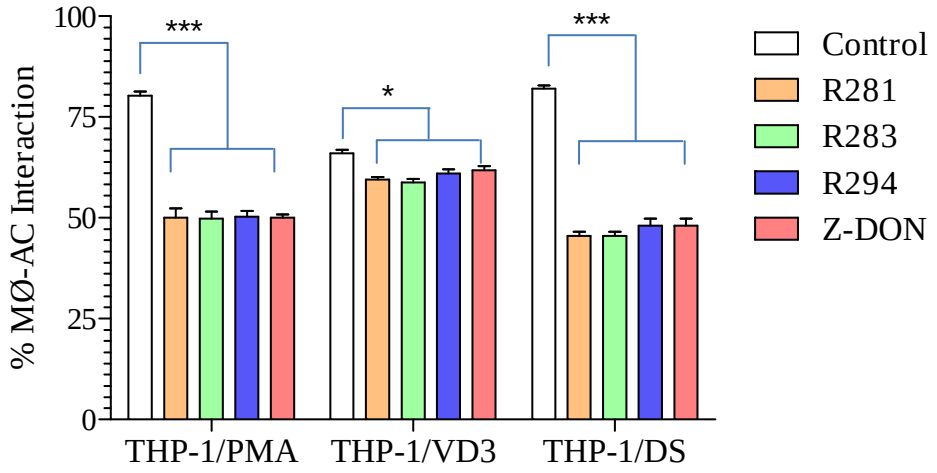


Figure 16. TG2 inhibitors affects THP-1/MØ clearance of AC. THP-1cells stimulated to differentiate with VD3 (THP-1/VD3); PMA (THP-1/PMA) or both (THP-1/DS) were treated with the indicated TG2 inhibitors for 1h at 37^{o}C. R281, R283 and R294 were used at 500µM and Z-DON at 100µM final concentrations. Inhibitor-treated cells were washed and co-cultured with apoptotic Mutu for 1hr. Unbound AC were removed by washing, macrophages were fixed with 1% w/v formaldehyde, stained with Diff quik II and scored as the percentage of macrophages interacting with AC using light microscopy. Atleast 200 MØ were scored in each replicate well. Data shown is mean ±SEM for n≥3 independent experiments done in triplicates. Statistical analysis was conducted using ANOVA followed by Dunnett's post-test. (*P<0.05*** *P*<0.001)

During 1h period of MØ-AC co-culture, THP-1/PMA and THP-1/DS untreated with TG2 inhibitors showed ~80% of MØ interacting with at least one AC, whilst a significantly lower number of THP-1/VD3 interacted with AC. Upon treatment with TG2 inhibitors, a profound inhibition in MØ-AC interaction is seen in THP-1/PMA and THP-1/DS, while no such significant reduction in interaction is seen with THP-1/VD3 in comparison to its control and this is in consistent with TG2 expression (Figure 14). This suggests a likely different differentiation phenotype in THP-1/VD3 unlike THP-1/PMA and THP-1/DS with respect to TG2 expression. All four TG2 inhibitors including the cell impermeable inhibitors R281 and R294 reduced MØ-AC interaction in those MØ that expressed TG2 (Figure 12) suggesting that TG2 is mediating at least some of its actions at the cell surface. Moreover, as seen from figure 17, a dose-dependent TG2 inhibitor activity is seen in all three THP-1 macrophage models which are more pronounced in TG2 expressing THP-1/PMA and THP-1/DS.

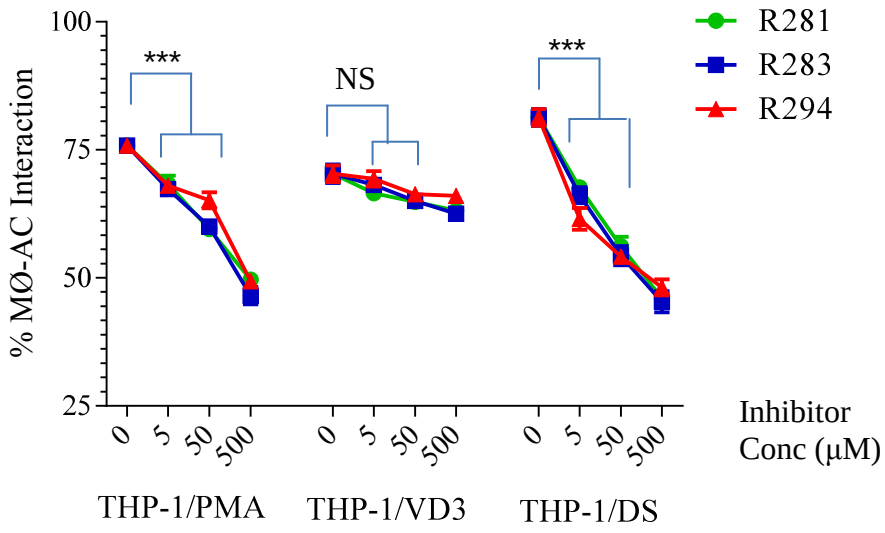
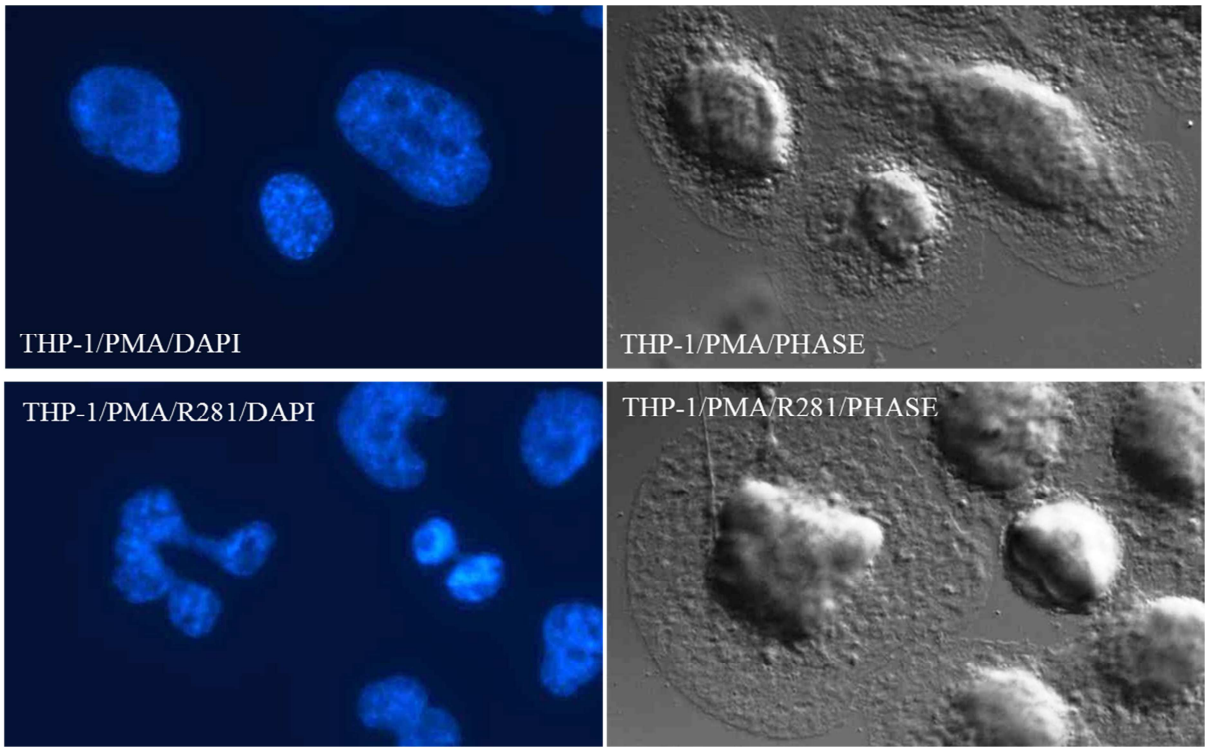


Figure 17. TG2 inhibitors can reduce MØ-AC interaction in a dose-dependent manner. THP-1/MØ were treated with the indicated concentrations of cell impermeable TG2 inhibitor R281 and cell permeable TG2 inhibitors R283 and R294 to check the dose dependency effect of TG2 inhibitor on MØ-AC interaction. Inhibitor treated THP-1/MØ were co-cultured with apoptotic Mutu for 1h at 37^{o}C. Unbound AC were washed, macrophages were fixed with 1% w/v formaldehyde, stained with Diff quik II and scored as a percentage of macrophages interacting with AC using light microscopy. Atleast 200 MØ were scored in each replicate well. Data shown is mean ±SEM for n≥3 independent experiments done in triplicates. Statistical analysis was conducted using ANOVA followed by Dunnett's post-test. (*** P<0.001; NS: Non significant)

With respect to THP-1/VD3, though there is a significant reduction in interaction in the presence of TG2 inhibitors at higher concentrations, no such significant reduction in interaction was evident at lower concentrations. Moreover, any significant reduction in interaction seen with THP-1/VD3 is significantly much lower than that seen with THP-1/PMA and THP-1/DS at a similar TG2 inhibitor concentration. The slight insignificant variations with varying inhibitor concentrations in comparison to control could be because of small levels of TG2 not easily detected by Western blotting; an effect on other TG family members in the cells, or a toxic effect experienced by cells with TG2 inhibitors being used at such a high concentration. If toxicity is an issue causing the slight effects in presence of TG2 inhibitors in THP-1/VD3, a similar toxic effect may also be in THP-1/PMA and THP-1/DS, contributing to a reduction in MØ-AC interaction. To address any possible toxicity effect on MØ due to TG2 inhibitors, cells were treated with inhibitors and checked for nuclear (using DAPI) and other morphological changes in TG2-expressing THP-1/DS and cell viability

using the XTT assay. The XTT assay is based on the conversion of water soluble 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazo-lium hydroxide (XTT) reagent to an orange coloured formazan by actively respiring cells. Mitochondrial succinoxidase and cytochrome P450 systems, as well as flavoprotein oxidases are considered to be primarily involved in the conversation of XTT to formazan (Altman, 1976, Roehm et al., 1991).

A



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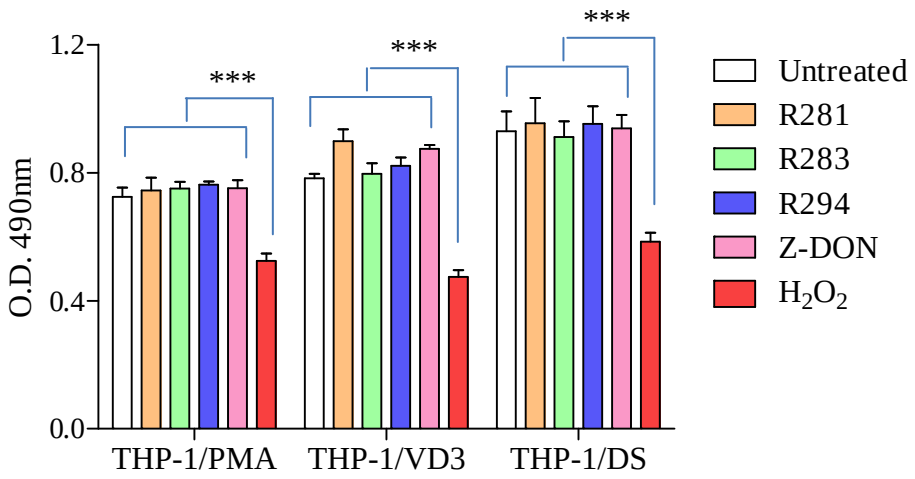

Figure 18. TG2 inhibitors exert no toxic effect on THP-1/MØ. ***A,*** THP-1/DS treated with or without cell impermeable TG2 inhibitor R281 at 500µM final concentration for 1hr. Following washing, cells were stained with DAPI, fixed with 1% w/v formaldehyde and imaged using fluorescence microscopy. ***B***, THP-1 derived macrophage-like cells were treated with both cell impermeable (R281, R294) and cell permeable (R283, Z-DON) TG2 inhibitors for 1h and subsequently incubated with XTT reagent for 4h. Colour development, a representative of cell viability was read at 490nm. Data shown is mean ±SEM for n=3 independent experiments done in triplicates. Statistical analysis was conducted using ANOVA followed by Dunnett's post-test (*** $P<0.001$).

As seen from nuclear staining (Figure 18A), no change in nuclear morphology is noted with R281 treatment and as evident from phase images, the treated cells look healthy with respect to cell adhesion and spreading. Yet, TG2 inhibitor treated THP-1/MØ noted to have multiple nuclei compared to untreated THP-1/MØ which could be a possible cell fusion phenomenon common in MØ. However, Mystkowska & Sawicki, (1987) have shown that PMA treatment inhibits cytokinesis before karyokinesis preventing cell division using mouse embryo models. Similarly, the cell viability test using the XTT reagent showed no evidence of cell death with any of the TG2 inhibitor used on either of the THP-1/MØ model but clearly revealed hydrogen peroxide at 200μM-induced cell death in all models.

4.3 TG2 inhibitors inhibited THP-1/MØ binding to AC.

Following AC recognition, the second stage in the AC clearance mechanism is binding which initiates signalling events leading to AC engulfment. So, to determine the effect of TG2 inhibitors on macrophage binding to AC, THP-1/MØ following treatment with TG2 inhibitors were co-cultured with apoptotic Mutu at 20^{o}C which will facilitate just binding of MØ to AC (Figure 19).

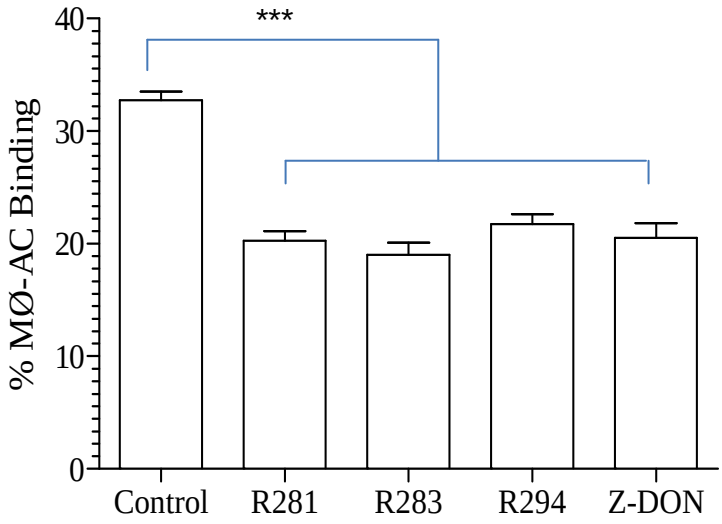

Figure 19. TG2 inhibitors affected THP-1/MØ binding to AC. TG2 inhibitor treated THP-1/DS were co-cultured with apoptotic Mutu for 1h at 20^{o}C in 5% CO_2. Unbound AC were washed off and macrophages were fixed with 1% w/v formaldehyde. Cells were stained with diff quik II and cells were scored as a percentage of macrophages interacting with AC using light microscope. Atleast 200 MØ were scored in each replicate well. Data shown is the mean ± S.E.M for n=3 independent experiments done in triplicates. Statistical analysis was conducted using ANOVA followed by Dunnetts post-test (*** P<0.001).

In contrast to data from interaction (binding and phagocytosis) assays, (Figure 16 & 17) where approximately 80% of macrophages interacted with AC, only approximately 35% of phagocyte showed binding to AC at this reduced temperature (Figure 19). Importantly, TG2 inhibitors exerted a significant reduction in MØ binding to AC as seen in inhibitor-treated MØ and inhibition of cell tethering was shown by all the TG2 inhibitors tested. Data from figure 16 and figure 19 suggests that TG2 plays a prominent role both in macrophage binding to and subsequent phagocytosis of AC and moreover it suggests that TG2 at the macrophage cell surface is responsible for, at least, part of this effect.

4.4 Inhibition of TG2 reduces interaction of HMDMØ with AC

To check the effect of TG2 inhibitors on in vitro differentiated HMDMØ clearance of AC, isolated monocytes, following differentiation for 7-8 days were treated separately with all four TG2 inhibitors for 1hr and co-cultured with apoptotic Mutu for another 1hr. As seen from figure 20, TG2 inhibitors showed a similar pattern of inhibition in HMDMØ-AC interaction

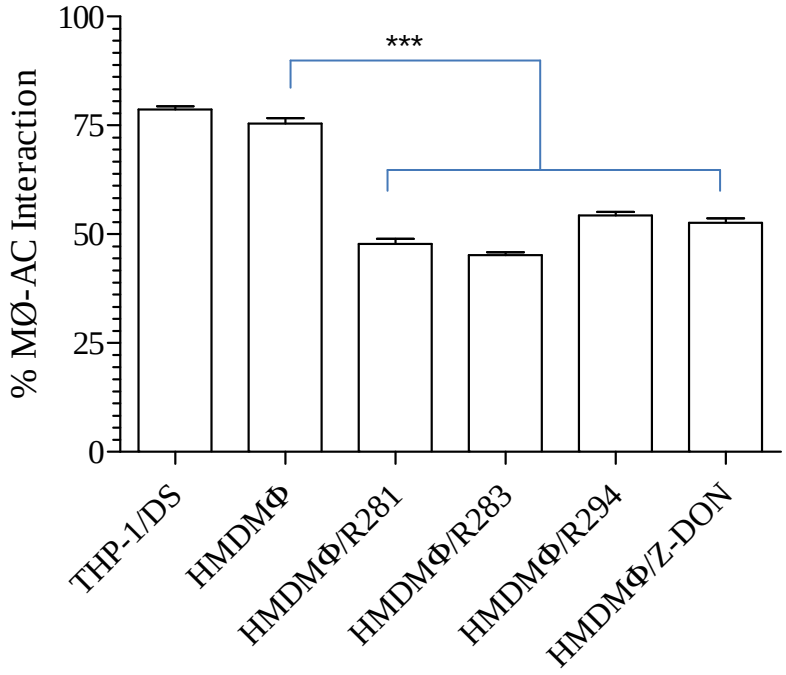

Figure 20. Inhibition of TG2 reduces interaction of HMDMØ with AC. TG2 inhibitor treated HMDMØ were co-cultured with apoptotic Mutu for 1h at 37^{o}C in 5% CO_2. Unbound AC were washed off and macrophages were fixed with 1% w/v formaldehyde. Cells were stained with diff quik II and cells were scored as a percentage of MØ interacting with AC using light microscope. Atleast 200 MØ were scored in each replicate well. Data shown is the mean ± S.E.M for n≥3 independent experiments done in triplicates. Statistical analysis was conducted using ANOVA followed by Dunnetts post-test (*** P<0.001).

All four TG2 inhibitors reduced HMDMØ interaction with AC. Similar interaction patterns were seen in both THP-1/MØ (Figure 16 & 17) and HMDMØ in the presence of TG2 inhibitors suggesting that THP-1/MØ are good model of HMDMØ in terms of phagocytic function and TG2 expression. Moreover, it clearly implies that TG2 plays a crucial role in mediating MØ-AC interaction loss of which will impair phagocytic function.

Similarly, the effect of TG2 inhibitors on macrophage eating yeast (*Candida albicans*) (Keppler-Ross et al., 2010), *E.coli* (Fluorescent K-12 strain) or resin beads (Gilberti et al., 2008) was also tested. Clearance efficiency of *E.coli*, latex beads or yeast cells by both THP-1/DS and THP-1/DS/R281 was carried out by replacing AC (Figure 21) in a standard interaction assay.

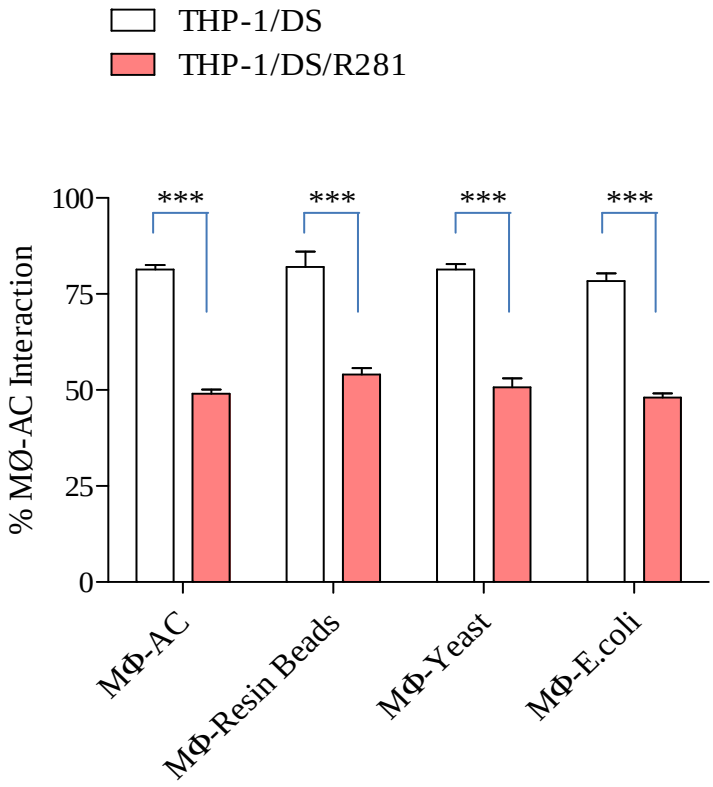

Figure 21. TG2 inhibitors reduced AC replacements up-take by THP-1/DS. THP-1/DS±R281 were co-cultured with latex resin beads, yeast and fluorescent labelled *E.coli* for 1h at 37^{o}C in 5% $CO_{2.}$ Unbound latex beads, yeast or *E.coli* were washed off and macrophages were fixed with 1% formaldehyde, stained with Diff quik II and scored as a percentage of macrophages interacting with resin beads, yeast or E.coli using fluorescence microscopy. THP-1/DS co-cultured with AC was maintained as known positive control. Atleast 200 MØ were scored in each replicate well. Data shown is the mean ± S.E.M for n=3 independent experiments done in triplicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** P<0.001).

Clearance efficiency of resin beads, yeast or *E.coli* by TG2 inhibitor R281 treated THP-1/DS was impaired compared to untreated THP-1/DS as also seen with AC. Taken together, it is evident that TG2 plays a common role in MØ phagocytosis of AC and other immune stimulatory particles and their clearance is reduced in presence of TG2 inhibitors.

4.5 MØ TG2 is active for AC clearance

The results presented above suggest that TG2 inhibitors inhibit both MØ binding to and MØ interaction with AC in both THP-1/MØ and HMDMØ. This indicates a TG2 requirement in mediating MØ clearance of AC. However, it is not clear whether inhibition of MØ-TG2 or AC-TG2 (or both) promotes MØ-AC interaction. In order to understand the target of TG2 inhibitors and to check the effect of TG2 inhibitors on individual cell types with respect to MØ-AC interaction, an interaction assay was performed where MØ alone treated or AC alone were treated with TG2 inhibitors for 1hr prior to co-culture with AC. The results of these assays are shown in figure 22.

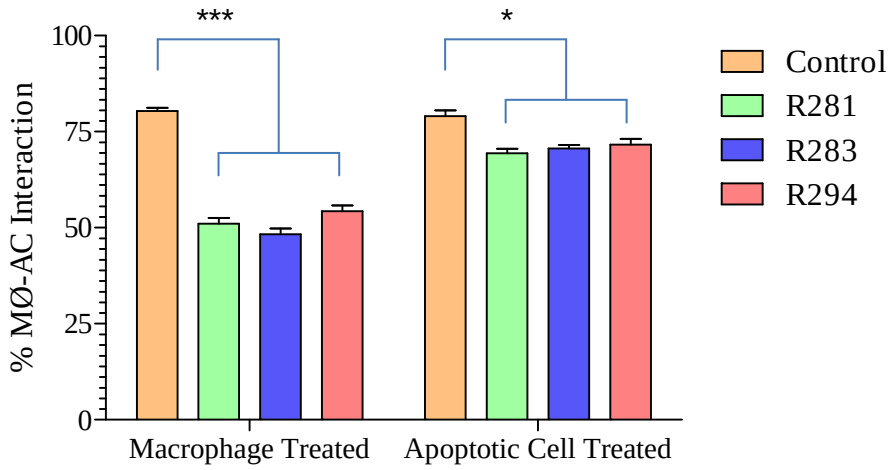

Figure 22. MØ TG2 is crucial for mediating MØ-AC interaction. THP-1/DS and apoptotic mutu were treated with the indicated TG2 inhibitors for 1hr and subsequently co-cultured for 1hr at 37^{o}C. Unbound AC were removed by washing, macrophages were fixed with 1% w/v formaldehyde, stained with Diff quik II and scored as the percentage of macrophages interacting with AC using light microscopy. Atleast 200 MØ were scored in each replicate well. Data shown is mean ±SEM for n≥3 independent experiments done in triplicates. Statistical analysis was conducted using ANOVA followed by Dunnett's post-test (* P<0.05; *** P<0.001).

MØ alone treated with TG2 inhibitors but not AC alone treated resulted in a significant reduction in MØ-AC interaction (Figure 22), implying that the TG2 inhibitor affects MØ-TG2 and is critical in facilitating AC clearance. This is in agreement with TG2 expression studies that suggest Mutu cells do not express TG2 (Figure 15).

It is evident that in both THP-1/MØ (Figure 16) and HMDMØ (Figure 20), TG2 has got a vital role to play and loss of which will affect their phagocytic function resulting in impaired AC clearance. Additionally, MØ-TG2 is central in the above process (Figure 22).

4.6 TG2 knockdown in macrophages results in reduced ability to interact with AC

TG2 inhibitor studies suggest that TG2 plays a vital role in MØ-AC interaction. To confirm this suggestion by another approach, TG2 knockdown was undertaken to assess the effect on MØ-AC interaction. Initially transient transfection using siRNA to knockdown TG2 was undertaken. THP-1/DS were transfected with four different siRNA targeting human TG2 using HiPerfect transfecting reagent as per the manufacturer's instructions. Global inactive control siRNA was used as a control. Following 48h of transfection, whole cell lysates of TG2 siRNA transfected THP-1/DS were analysed for TG2 expression by western blotting. However, no knockdown in TG2 expression was seen with any of the TG2 siRNA. With a slight modification in the existing protocol, THP-1 cells following only 24h stimulation with PMA/VD3 to differentiate towards MØ were transfected for another 48hrs (taking the overall differentiation time to 72h). Following washing, whole cell lysates were analysed by western blotting to check the level of TG2 knockdown. As seen from figure 23, TG2 siRNA#1 and to a lesser degree siRNA#6 showed significant knockdown in TG2 expression in comparison to inactive control siRNA and loading control.

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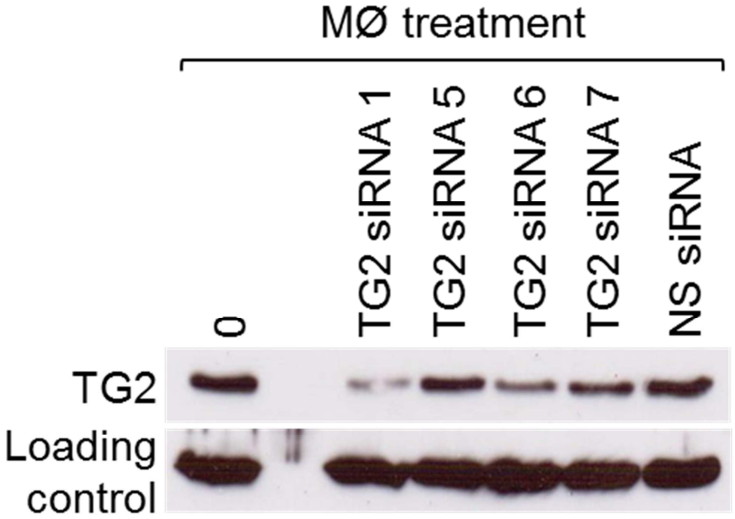

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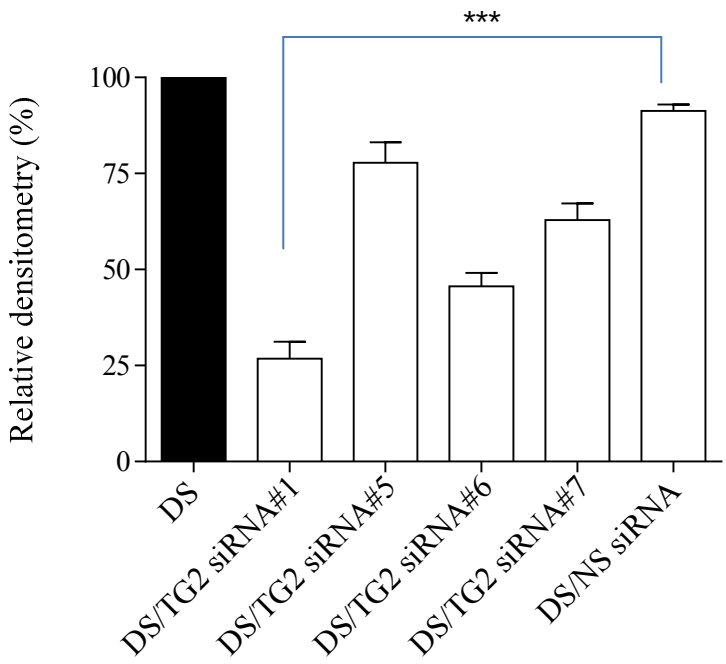


Figure 23. THP-1/DS TG2 Knockdown. **A**, Four different siRNA targeting human TG2 were used to transfect THP-1/DS using HiPerfect transfecting reagent. 24h post-differentation, cells were transfected with siRNA for another 48hrs. THP-1/DS transfected with NS siRNA was used as control. Whole cell lysates were analysed by western blot analysis. Equal loading was verified by probing with anti-actin antibody on stripped membranes. **B,** Densitometric analysis of TG2 expression knockdown in THP-1/DS using ImageJ. Densitometry values relative to the loading control were calculated and are shown as a % of DS. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** $P<0.001$).

Using siRNA#1 which showed a significant knockdown in TG2 expression (Figure 23A), MØ-AC interaction assays were carried out while using NS siRNA transfected cells as control (Figure 24).

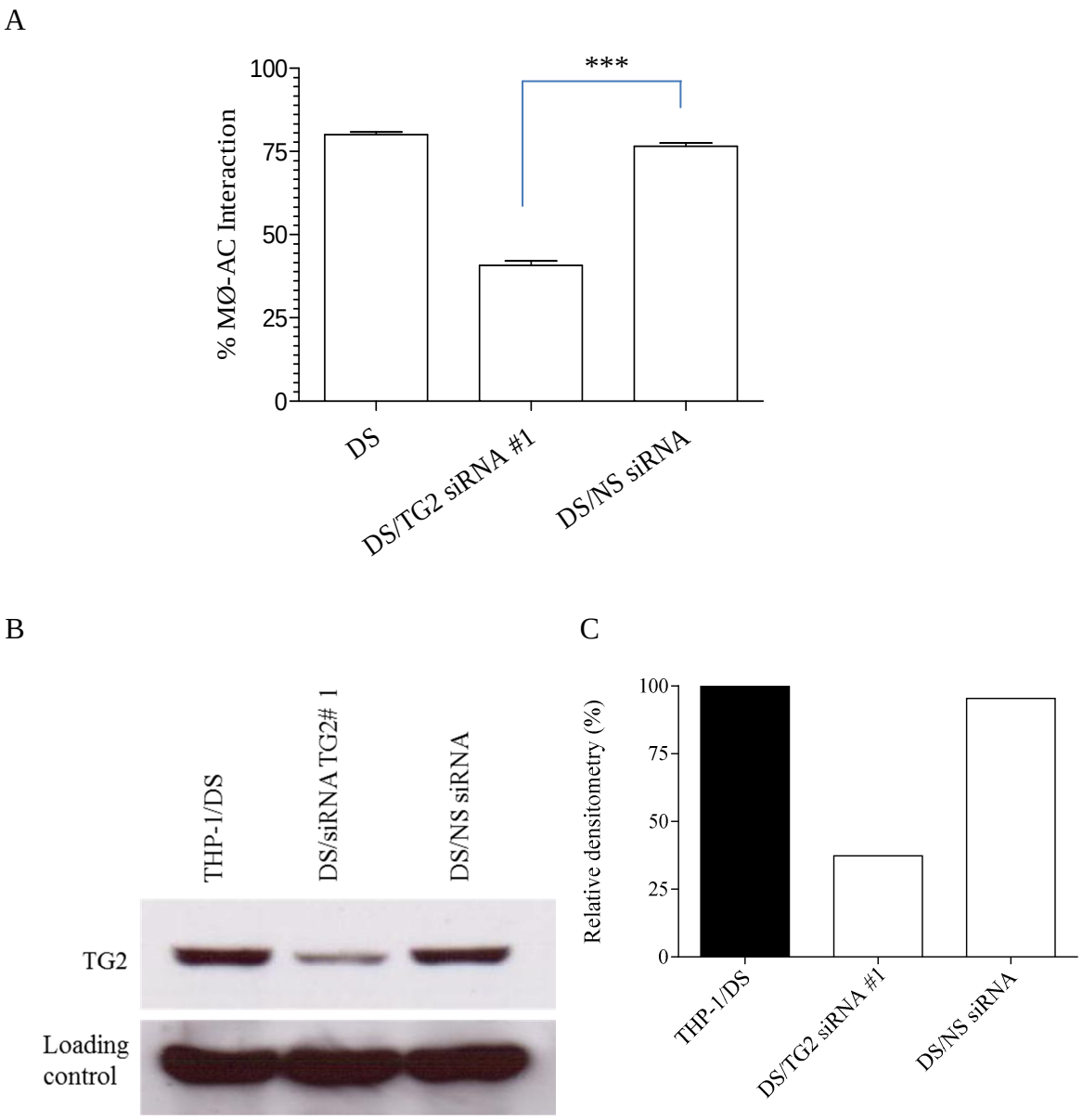


Figure 24. TG2 knockdown reduces MØ-AC interaction. **A**. TG2-siRNA#1 transfected THP-1/DS were co-cultured with apoptotic Mutu for 1h at 37^{o}C. Unbound AC were removed by washing, macrophages were fixed with 1% w/v formaldehyde, stained with Diff quik II and scored as the percentage of macrophages interacting with AC using light microscopy. Atleast 200 MØ were scored in each replicate well. Data shown is mean ±SEM for n=3 independent experiments. THP-1/DS transfected with NS siRNA was used as controls. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** P<0.001). **B**. Representative western blot showing TG2 knockdown upon transfecting with siRNA TG2#1 using HiPerfect transfecting reagent in the left panel. siRNA#1 targeting human TG2 is used to transfect THP-1/DS using HiPerfect transfecting reagent. 24h post-differentation, cells were transfected with siRNA for another 48hrs. THP-1/DS transfected with NS siRNA was used as control. Whole cell lysates were analysed by western blot analysis. Equal loading was verified by probing with anti-actin antibody on stripped membranes. **C**. Densitometric analysis of TG2 expression knockdown in THP-1/DS by ImageJ. Densitometry values relative to the loading control were calculated and shown as a % of DS in the blot shown in B.

Interestingly, in parallel with TG2 inhibitor studies, TG2 knockdown in THP-1/DS resulted in a significant reduction in interaction between macrophages and AC in comparison to NS siRNA transfected cells (Figure 24). Taken together, both the approaches indicate the importance of TG2 in this process. With a substantial reduction in MØ-AC interaction with TG2 knockdon, the additional TG2 inhibitor effect on TG2 knockdown THP-1/DS was tested (Figure 25).

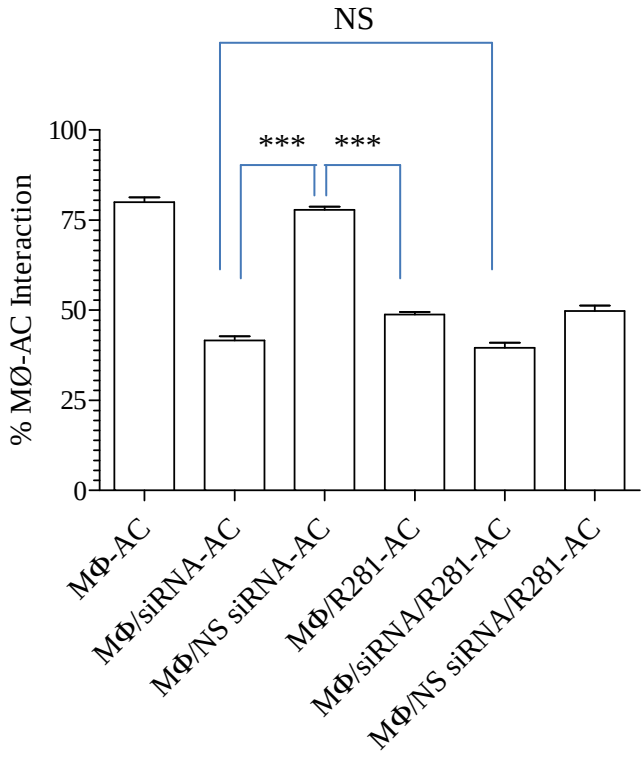


Figure 25. TG2 siRNA/R281 double inhibiting effect. THP-1/DS and TG2 knockdown THP-1/DS/TG2 siRNA #1 treated with and without TG2 inhibitor R281 were co-cultured with apoptotic Mutu for 1h at 37^{o}C. Unbound AC were removed by washing, macrophages were fixed with 1% w/v formaldehyde, stained with Diff quik II and scored as the percentage of macrophages interacting with AC using light microscopy. Atleast 200 MØ were scored in each replicate well. THP-1/DS transfected with NS siRNA were used as controls. Data shown is mean ±SEM for n≥3 independent experiments. Data shown is mean ±SEM for n≥3 independent experiments. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** P<0.001; NS: Not significant).

As seen from figure 25, there was not a significant difference in MØ-AC interaction when TG2 knockdown THP-1/DS were treated with cell impermeable TG2 inhibitor R281. This further confirms that cell surface TG2 is needed for clearance of AC by human MØ.

4.7 Cell surface TG2 protein crosslinking activity mediates MØ-AC interaction

TG2 on the MØ cell surface is found to be required for AC clearance as loss of TG2 results in reduced MØ clearance of AC. It is known that the site-directed TG2 irreversible inhibitors used to check TG2 involvement in MØ-AC interaction block the transamidating activity by covalently modifying the enzyme and prevent substrate binding (Siegel and Khosla, 2007). However they may also exert their effect by altering TG2 conformation. Such changes to TG2 could affect binding of TG2 to its high affinity ligands such as heparan sulphates (Wang et al., 2010). So, to address this issue and understand whether loss in TG2 activity or change in conformation of the molecule was contributing to the effect on AC clearance, an amine competitive inhibitor, monodansylcadaverine (MDC) was used which also blocks protein cross-linking by competing with natural amine substrate such as protein bound lysine residue without changing the TG2 conformation (Siegel and Khosla, 2007). MØ-AC interaction assays were carried using THP-1/DS treated with MDC and the results were shown in figure 26.

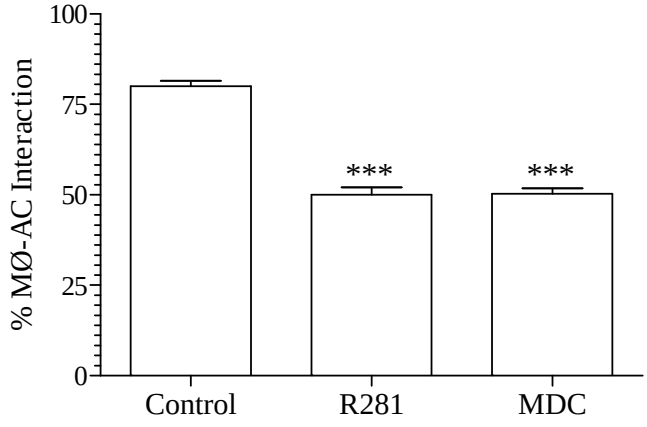


Figure 26. TG2 activity is key for mediating MØ-AC interaction. THP-1/DS were treated with monodansylcadaverine (MDC) for 1h at 37^{o}C. Inhibitor treated cells were washed and co-cultured with apoptotic Mutu for 1hr. Unbound AC were removed by washing, macrophages were fixed with 1% w/v formaldehyde, stained with Diff quik II and scored as the percentage of macrophages interacting with AC using light microscopy. Atleast 200 MØ were scored in each replicate well. THP-1/DS/R281 were used a positive control. Data shown is mean ±SEM for n≥3 independent experiments. Statistical analysis was conducted using ANOVA followed by Dunnetts post-test (*** P<0.001).

MDC reduces MØ-AC interaction to the same degree as the irreversible cell impermeable inhibitors R281 suggesting alterations to TG2 activity rather than conformation is responsible for altered AC clearance (Figure 26). Given that the results suggests, the activity of cell surface TG2 is important in AC clearance, the presence of in situ cell surface TG2 activity in live cells was demonstrated (Figure 27), using a plate-based assay via biotin cadaverin incorporation into fibronectin for 2h (Scarpellini et al., 2009, Wang et al., 2012).

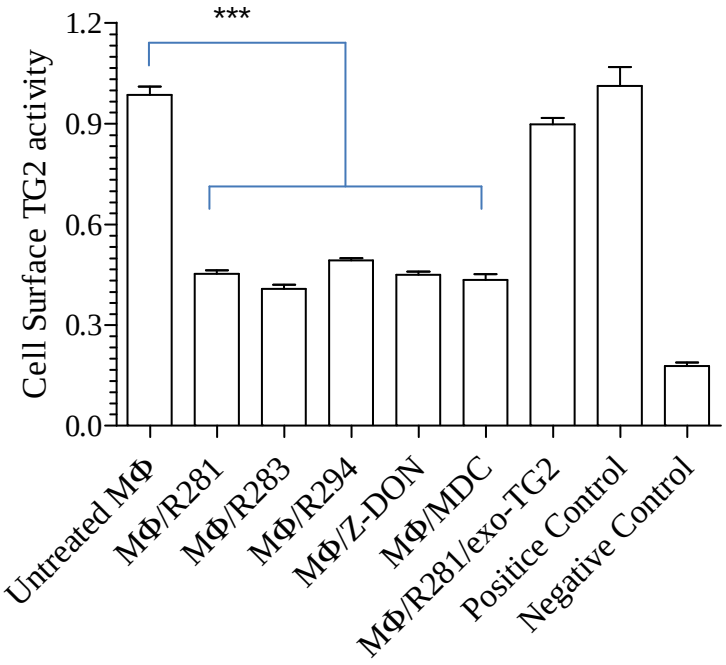


Figure 27. TG2 inhibitors altered THP-1/DS cell surface TG2 activity. Cell surface TG2 activity was tested by assessing biotinylated cadaverin incorporation into fibronectin. THP-1/DS treated with or without TG2 inhibitors (R281, R283 & R294 at 500µM and Z-DON & MDC at 100 µM) were seeded on fibronectin-coated plates and incubated in the presence of biotin-X-cadaverine at 37^{o}C. Fibronectin incorporated TG2 is conjugated with extravidine peroxidase and colour development following treatment with 3,3',5,5'-Tetramethyl benzidine in DMSO representing cell surface TG2 activity is expressed as mean absorbance at 450nm. TG2 is added exogenously to THP-1/DS/R281 and incubated for 20min before the cells seeded on fibronectin coated plates. Guinea pig liver TG in serum free medium containing biotin-X-cadaverine and 10mM EDTA are used as known positive and negative controls. Data shown is mean ±SEM for n≥3 independent experiments in triplicates. Statistical analysis was conducted using ANOVA followed by Dunnett's post-test (*** P<0.001).

Compared with TG2 activity by using purified TG2 or TG2 activity shown by THP-1/DS on their own, a significantly lower level of TG2 transamidation activity was found at the surface of THP-1/DS upon treatment with either irreversible or amine competitive inhibitors (Figure

27). This suggests that TG2 inhibitors acted on altering MØ cell surface TG2 activity, loss of which resulted in reduced MØ-AC interaction. Notably, adding purified active TG2 to R281 treated THP-1/DS resulted in partial compensation for the loss of TG2 activity in inhibitor treated MØ suggesting that exogenous TG2 could compensate for the loss of MØ-AC interaction. Following this, cell surface TG2 activity in TG2 knockdown THP-1/DS was also tested (Figure 28).

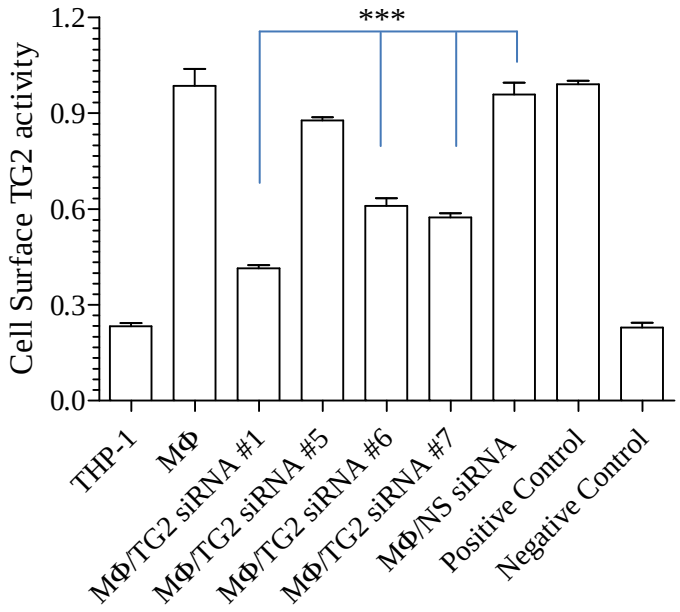


Figure 28. siRNA knockdown of TG2 results in loss of cell surface TG2 activity. Cell surface TG2 activity was tested via biotin cadaverin incorporation into fibronectin. TG2 knockdown THP-1/DS were seeded on fibronectin coated plates and incubated in presence of biotin-X-cadaverine. Fibronectin incorporated TG2 is conjugated with extravidine peroxide and colour development following treatment with 3,3',5,5'-Tetramethyl benzidine in DMSO representing cell surface TG2 activity is expressed as mean absorbance at 450nm. A significant loss of cell surface TG2 activity is seen in presence of both irreversible and amine competitive TG2 inhibitors. Guinea pig liver TG in serum free medium containing biotin-X-cadaverine and 10mM EDTA are used as known positive and negative controls. Data shown is mean ±SEM for n≥3 independent experiments done in triplicates. Statistical analysis was conducted using ANOVA followed by Dunnett's post-test (*** P<0.001).

TG2 knockdown in THP-1/DS resulted in loss of cell surface TG2 activity (Figure 28) and with each siRNA used, a similar pattern of activity loss is seen which is in line with reduced TG2 expression with TG2 knockdown (Figure 23) implying that loss of TG2 results in reduced cell surface TG2 expression which is reflected in reduced cell surface TG2 activity.

To further strengthen our observations, THP-1/DS were treated with a mouse monoclonal antibody capable of blocking TG2 activity (D11D12; GB patent filing 1209096.5). Mouse IgG1-κ monoclonal-MoPC21 isotype control antibody treated THP-1/DS was used as a control (Figure 29).

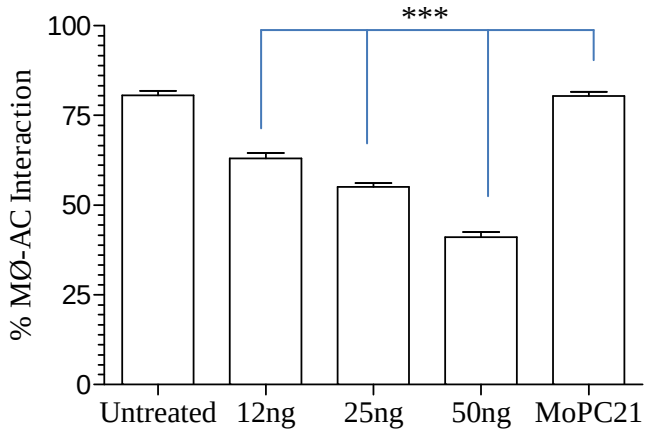


Figure 29. TG2 activity-blocking antibody reduces MØ-AC interaction. THP-1/DS treated with mouse monoclonal TG2 activating blocking antibody (D11D12) at concentrations of 12, 25 and 50ng/ml and MoPC21 at 50ng/ml were co-cultured with apoptotic Mutu for 1h at 37^{o}C. Unbound AC were removed by washing, macrophages were fixed with 1% w/v formaldehyde, stained with Diff quik II and scored as the percentage of macrophages interacting with AC using light microscopy. Atleast 200 MØ were scored in each replicate well. IgG1 isotype control antibody treated THP-1/DS were used as control. Data shown is mean ±SEM for n=3 independent experiments done in triplicates. Statistical analysis was conducted using ANOVA followed by Dunnett's post-test (*** P<0.001).

Treating MØ with a TG2 activity-blocking antibody inhibited MØ-AC interaction in a dose-dependent manner (Figure 29) reconfirming the critical role being played by transamidating activity at the cell surface in mediating MØ-AC interaction.

4.8 Exogenously added active TG2 partially compensates both TG2 inhibitor and TG2 knockdown effects on MØ-AC interaction

As evident from cell surface TG2 activity assays (Figure 27), exogenously added active TG2 partially compensates for the loss of TG2 in R281-treated THP-1/DS. Taking this into consideration, MØ-AC interaction assay was done with both TG2 inhibitor treated and TG2 knockdown THP-1/DS while adding back both active and inactive TG2 (Figure 30). This was undertaken to strengthen the need for MØ cell surface TG2 activity in AC clearance.

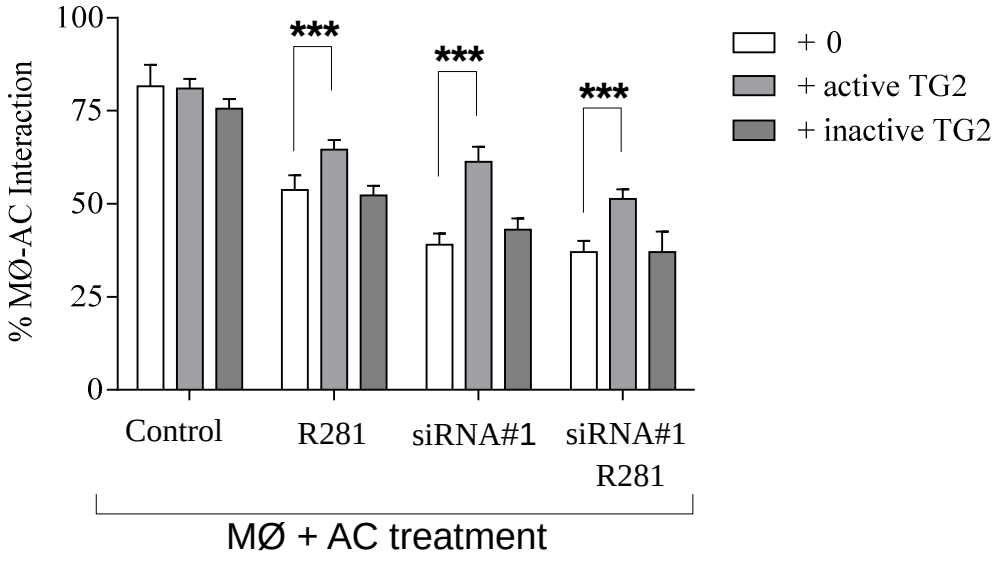


Figure 30. Exogenously added active TG2 compensates for loss of MØ-AC interaction by TG2 inhibitor R281 and by TG2 knockdown. THP-1/DS, THP-1/DS/R281 and THP-1/DS/TG2 siRNA#1, treated with or without active and inactive TG2 for 20min were co-cultured with apoptotic Mutu for 1h at 37^{o}C. Unbound AC were removed by washing, macrophages were fixed with 1% w/v formaldehyde, stained with Diff quik II and scored as the percentage of macrophages interacting with AC using light microscopy. Atleast 200 MØ were scored in each replicate well. Data shown is mean ±SEM for n≥3 independent experiments done in triplicates. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** P<0.001).

Under regular conditions, THP-1/DS have shown ~80% interaction with AC. Treatment with TG2 inhibitor (R281) or knocking down TG2 by siRNA results in reduced interaction between MØ and AC to ~45-55%. However, TG2 inhibitor treatment on TG2 knockdown MØ (double treatment) by siRNA together showed no further reduction in AC clearance suggesting TG2 activity on the MØ cell surface exerts most, if not all of its cell surface effects on AC clearance. Upon adding active TG2 exogenously to either TG2 inhibitor treated MØ's or TG2 knockdown MØ or MØ with double treatment, a partial compensation to reduction in interaction is seen (~15-20%) with no effect by inactive TG2, again suggesting that active TG2 on MØ cell surface is needed for AC clearance (Figure 30).

4.9 TG2 inhibitors reduce MØ migration towards chemo-attractants

Cell migration is an important event in the process of phagocytosis. MØ sense 'find-me' signals such as triphosphate nucleotides (ATP/UTP) (Elliott et al., 2009), phospholipids like lysophosphatidylcholine and sphingosine-1-phosphate (Lauber et al., 2003) and chemokine CX3CL1 (fractalkine) (Truman et al., 2008) released by AC. Membrane blebbing is a characteristic feature of apoptosis and released apoptotic bodies carrying surface markers act as 'find-me' signals generating a chemotactic gradient that induces macrophage chemotaxis (Torr et al., 2012). Cell attachment, spreading and involvement of different 'outside-in' and 'inside-out' signalling events leading to cytoskeletal rearrangements, altogether form a complex of cellular events contributing to cell migration (Abram and Lowell, 2009). Though the cross-linking activity of TG2 is not required for cell adhesion (Akimov et al., 2000), as non-TG2 expressing THP-1 are semi-adhesive, its role in cell spreading (Stephens et al., 2004), focal adhesion formation (Verma et al., 2008), cytoskeleton remodelling (Janiak et al., 2006) in association with fibronectin (Akimov et al., 2000) and signalling through integrins are well documented. Previous studies by Balajthy et al. (2006) have shown impaired and extravasation migration by neutrophils in $TG2^{-/-}$ mice. Here the role of TG2 in MØ migration was assessed.

MØ migration in response to AC derived-attractants may be defined as the first crucial in vivo event in the process of AC clearance. To determine the effect of TG2 inhibitors on MØ migration to AC, untreated THP-1/DS cells or cells treated with TG2 inhibitor R281 were analysed by Dunn chemotaxis chamber assays for their ability to migrate towards AC placed in the outer circular well (Figure 31). THP-1/DS placed in the bridging area between the outer and inner circular wells were focussed at a particular area and cells directional migrating towards chemo attractants in the outer well or cells random circular migration irrespective of chemo attraction was analysed.

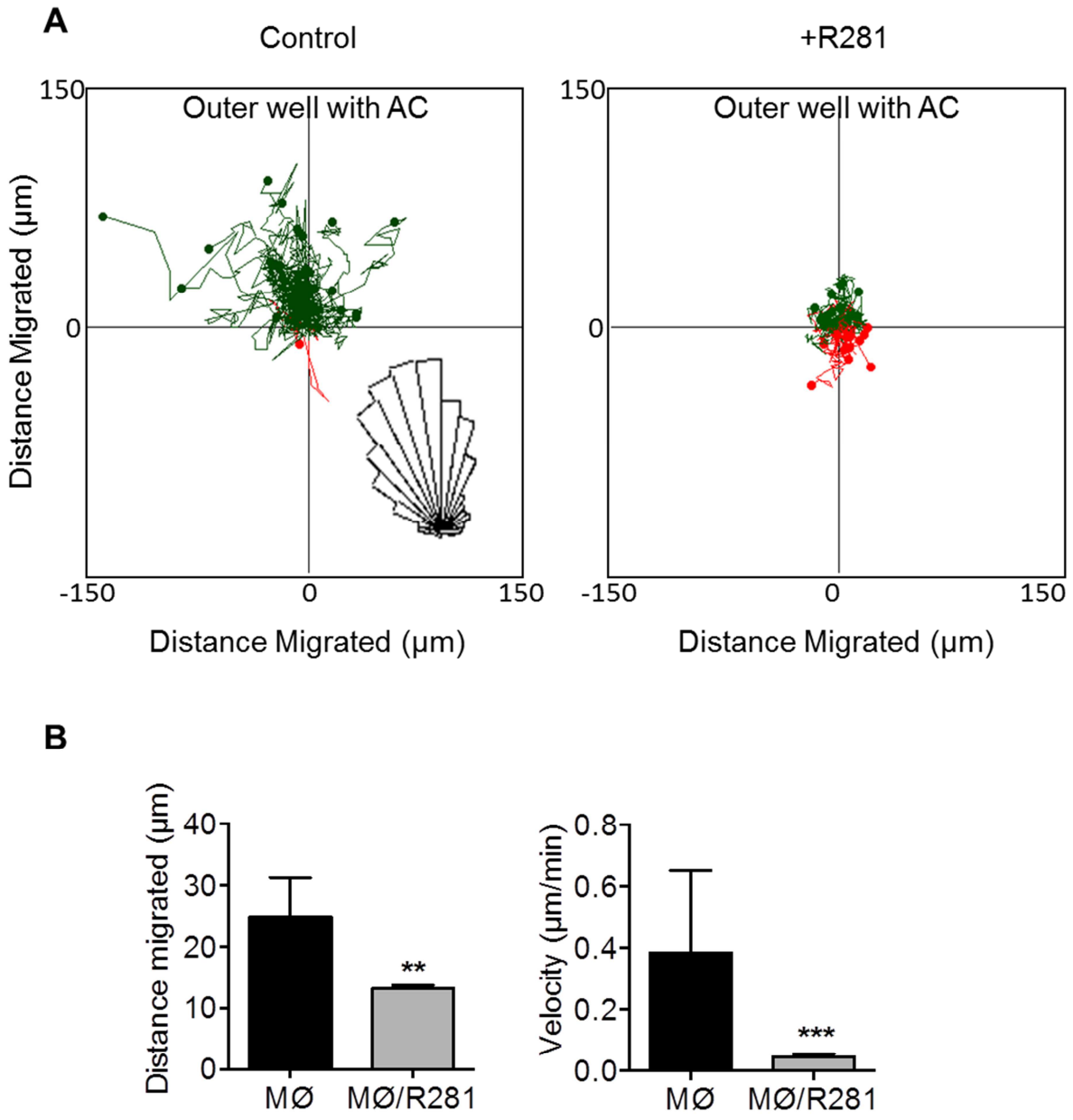


FIGURE 31. TG2 inhibitor R281 affects MØ migration towards AC. ***A,*** The effect of TG2 inhibitor R281 on MØ chemoattraction towards AC was tested using the Dunn chemotaxis chamber coupled with time-lapse imaging at 37^{o}C for 20h. Cell migration was tracked using ImageJ software and tracks were analysed using Chemotaxis and Migration toll 2.0, IBIDI, and were plotted as distance migrated/µm. All MØ are mapped to the cross hairs at the start of the assay and their final destination is plotted by the closed circle with the line showing the path taken. In each case, the AC (attractant) is positioned at the top of the plots. Untreated MØ show migration towards the AC (green lines), whilst inhibitor treated MØ show no direction (green and red lines). Altered directionality is shown in the rose diagram. ***B***, represents the Euclidean distance migrated by the MØ, and ***C,*** velocity at which the cells migrated both in presence and absence of TG2 inhibitor. Data shown is mean ±SEM for n≥3 independent experiments. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (** P<0.01; *** P<0.001).

A dramatic loss of MØ migration towards AC is seen in MØ treated with TG2 inhibitor R281 (Figure 31), with loss in MØ directionality as shown by rose diagram (Figure 31A). Euclidean distance migrated (Figure 31B) and velocity of migration (Figure 31C) had altered significantly with TG2 inhibitor treatment. Taken together, it is evident that MØ TG2 is needed for directional migration to, bind to and engulf AC.

4.10 Discussion

Transglutaminase 2 (TG2) is a ubiquitously-expressed allosterically-regulated enzyme with well-documented enzymatic and non-enzymatic function. It is a multifunctional enzyme with transglutaminase, GTPase/ATPase, deamination, protein disulphide isomerase, and protein kinase functions. While being ubiquitously expressed, it is well known for its involvement in many cellular functions including adhesion, migration, growth, differentiation, survival and apoptosis and at the same time involved in various pathological conditions e.g. inflammation and autoimmunity. Most importantly, with respect to present study, it is known to be expressed in monocyte/macrophages. A series of in vivo mouse model studies to date have showed that TG2 plays a prominent role in monocyte extravasation to sites of inflammation, whose expression upregulated during monocyte differentiation to macrophage (Murtaugh et al., 1983) and involved primarily in macrophage phagocytosis (Szondy et al., 2003). Simultaneously, macrophage infiltration to sites of infection will generate TGF-β1 contributing to the degree of inflammation, which in turn may increases TG2 expression via its response element in TGM2 gene promoter (Ritter and Davies, 1998). Here, for the first time, under *in vitro* conditions, using different cell-permeable and impermeable TG2 inhibitors and knocking down TG2, the role of TG2 in human macrophages using the human monocytic leukaemia cell line, THP-1 while simultaneously working on the human blood monocyte-derived macrophages was studied.

Though TG2 is predominantly a cytoplasmic protein, a protein with non-ER/Golgi-dependent externalization, it is known to be externalised at the cell surface by an ill-defined mechanism (Griffin et al., 2002, Collighan and Griffin, 2009). Several possible externalisation agents such as β-integrins (Akimov and Belkin, 2001, Mangala et al., 2007), fibronectin and syndecan-4 bound heparan sulphates (Scarpellini et al., 2009) and recycling endosomes (Zemskov et al., 2011) were detailed. It had been proposed that TG2 is expressed by macrophages and it is well demonstrated that it is needed for efficient phagocytic portal formation (Toth et al., 2009). Moreover, TG2-/- mice studies revealed its importance in phagocytosis, where loss of TG2 resulted in impaired apoptotic cell clearance *in vivo* (Szondy et al., 2003, Falasca et al., 2005). However, to date, no detailed study defining the molecular mechanism by which TG2 mediates AC clearance was available and there is no evidence about during which stage of clearance process (recognition, tethering, signalling and

engulfment) does TG2 play a prominent role. Moreover, TG2 involvement in AC clearance in human system has never been identified.

Following the TG2 expression studies from chapter 3 of this thesis, where a detectable level of whole cell TG2 expression (Figure 12) and cell surface expression via biotinylation of cell surface proteins (Figure 14A) is revealed by western blot analysis in the most adherent MØ types THP-1/PMA, THP-1/DS and HMDMØ. Similarly, cell surface expression of TG2 in THP-1/DS is clearly revealed with indirect immunofluorescence microscopy (Figure 14B) while no detectable TG2 expression is revealed in Mutu cells (used as a source of apoptotic model). Here, this chapter was focussed on understanding the role of TG2 in human macrophage function using different TG2 inhibitors targeting both cell surface and intracellular TG2. Similarly analyses were carried out by knocking down TG2 using small interfering RNAs specifically targeting human TG2.

Several TG2 inhibitors have been used to date to inhibit TG2 and to understand its role in different pathologies. In this study, cell permeable TG2 irreversible inhibitors R283 and Z-DON, cell impermeable TG2 irreversible inhibitors R281 and R294 along with amine competitive inhibitor monodansylcadaverine (MDC) were used depending on the specific study. All the inhibitors were designed to target the active cysteine residue in the catalytic triad of the TG2 core domain. Upon treating THP-1/MØ with either cell-permeable or cell-impermeable TG2 inhibitors, a profound degree in inhibition in interaction between MØ-AC was seen in THP-1/PMA and THP-1/DS, in a dose dependent manner, while no such significant reduction in interaction is seen with THP-1/VD3 (Figure 16). This is consistent with the low degree of differentiation seen in THP-1/VD3 which continue to be monocyte-like and TG2 expression studies by western blotting analysis showed THP-1/VD3 expressed no detectable TG2 (Figure 12 & Figure 14A) suggesting that type of stimulus and degree of differentiation may contribute to TG2 expression in THP-1/MØ and that there is no specific target in/on THP-1/VD3 to be specifically targeted by TG2 inhibitors. However, observed dose dependency could be either because of small levels of TG2 not easily detected by western blotting, or could be an effect on other TG family members in the cells. Though THP-1/VD3 expressed no TG2 they are well established as THP-1/MØ either in terms of phagocytic function or cytokine production (Devitt et al., 1998). Moreover, most encouragingly, HMDMØ responded in the same manner to TG2 inhibitors as did THP-1/PMA or THP-1/DS, suggesting THP-1/MØ are a true replica of HMDMØ in terms of TG2

expression and phagocyte function. THP-1/MØ showed a similar interaction efficiency to resin beads, yeast or *E.coli* which is inhibited in presence of TG2 inhibitors, suggesting that TG2 is needed and plays a common role in phagocytosis of AC and other immune stimulatory particles. It is not clear however if TG2 mediates clearance of these different particles in the same way.

TG2 is well acknowledged in apoptosis as a cross-linking protein, facilitating packing of intra cellular contents and resolving inflammation (Smethurst and Griffin, 1996). Similarly, upregulation of TG2 expression during monocyte to macrophage differentiation is well documented (Murtaugh et al., 1983). Whilst the presence of TG2 in MØ has been noted (Hodrea et al., 2010) its role in dealing with AC is not clear. TG2 expression studies by western blotting and immunofluorescence staining of cell surface TG2 revealed that TG2 is present at the MØ cell surface while no detectable TG2 expression is noted in viable or apoptotic Mutu. Moreover, MØ alone treated with TG2 inhibitors but not AC alone treated resulted in a significant reduction in MØ-AC interaction implying that TG2 inhibitor affected MØ-TG2 and is critical in facilitating AC clearance. However, slight reduction in MØ-AC interaction is also seen when AC alone treated with TG2 inhibitors, even though no TG2 expression is seen in AC (Figure 15) and this may be the TG2 inhibitor treatment effect on AC cells continues to effect MØ-AC interaction as no toxicity effect is evident upon TG2 inhibitor treatment (Figure 18). Taken together these data suggest, for the first time, that cell surface TG2 on the human MØ is likely to be crucial for promoting AC clearance.

To further confirm the importance of TG2 in MØ function, AC clearance studies were done upon knocking down TG2 expression in MØ using human TG2 specific siRNA. In line with the noted TG2 inhibitor effect, reduced expression of TG2 resulted in reduced MØ-AC interaction. Transient transfection of THP-1 with siRNA before stimulating them to differentiate with PMA or DS resulted in no knockdown in TG2 expression. However transfecting THP-1 cells following 24h of differentiation resulted in a robust and consistent knockdown in TG2 expression. It may be that without stimulation the siRNA have no target as TG2 isn't expressed in THP-1 cells (Figure 12). Moreover, as seen from figure 13, TG2 is expressed 6-12h following differentiation in THP-1 cells. However, fully differentiated THP-1/DS (48h differentiated) resisted siRNA transfection suggesting that THP-1 cells differentiation to 'complete' MØ-like cells may inhibit efficient transfection or MØ being 'hard core' phagocytic cells, may be simply taking up and destroying siRNA.

TG2 is known to have a great role in MØ function and as evident from TG2 expression studies and effect of TG2 inhibitors and TG2 knockdown studies on MØ-AC interaction, MØ cell surface TG2 is active for AC clearance. All the four site-directed TG2 inhibitors used to check TG2 involvement in MØ- AC interaction (Figure. 16) are irreversible inhibitors and can both block the transamidase enzyme activity and alter TG2 conformation (Siegel and Khosla, 2007). Such changes to TG2 conformation affect binding of TG2 to its high affinity ligands such as heparan sulphates (Wang et al., 2010). To address the involvement of TG2 activity and/or conformation in MØ-AC interactions, monodansylcadaverine (MDC: blocks TG2 crosslinking function without any irreversible change in TG2 conformation [(Siegel and Khosla, 2007)]) was used. MDC reduced MØ-AC interaction to the same degree as other inhibitors used suggesting alterations to TG2 activity rather than conformation was responsible for altered AC clearance. Given that the activity of cell surface TG2 is important in AC clearance, we next sought to confirm the TG2 activity in live cells. Cell surface TG2 activity was measured via biotin-cadaverine incorporation into fibronectin (Scarpellini et al., 2009, Wang and Griffin, 2012). Interestingly, a loss in cell surface TG2 activity is seen upon treatment with TG2 inhibitors in THP-1/MØ. HMDMØ showed a similar pattern of cell surface TG2 activity. Additionally, TG2 knockdown resulted in loss of cell surface TG2 activity consistent with reduced TG2 expression. However, 'add back' of purified exogenous active TG2 to the inhibitor treated MØ resulted in partial compensation in TG2 activity suggesting that active TG2 on MØ cell surface is needed for mediating MØ-AC interaction. Furthermore, a TG2 activity blocking Ab that may only bind cell surface exposed TG2 inhibited MØ-AC interaction in a dose dependent manner. Taken together these data demonstrate an important role for TG2 cross-linking activity at the surface of MØ in mediating AC removal.

TG2 is well-documented for its role in MØ differentiation, adhesion and spreading in co-operation with integrins, migration and phagocytosis (Akimov and Belkin, 2001, Szondy et al., 2003). Involvement of TG2 in MØ growth and differentiation, adhesion and phagocytosis is studied by knocking down TG2 or by functional loss of TG2 using TG2 inhibitors. Treating adherent THP-1/MØ with TG2 inhibitors for 1h for MØ-AC interaction studies showed no effect on MØ adhesion. However, treating THP-1 cells with TG2 inhibitors before differentiation, or treating them while stimulating them to differentiate to MØ-like with PMA or PMA/VD3 resulted in loss of cell adhesion and spreading. As MØ migration to dying cells

is an important event for in vivo phagocytic removal of AC and as MØ has been shown to migrate towards AC along a gradient of AC-derived microparticles (Torr et al., 2012) we focused on determining the role of TG2 in MØ migration to AC. THP-1/DS cells were assessed for their migration towards AC microparticles in the presence or absence of the TG2 inhibitor R281 using a Dunn chemotaxis chamber (Hawkley DCC100) in conjugation with time-lapse microscopy. Macrophage migration and chemoattraction towards apoptotic Mutu is dramatically reduced (Figure 31A), with loss in MØ directionality as shown by rose diagram. Euclidian distance migrated and velocity of migration had altered significantly with TG2 inhibitor treatment suggesting the involvement of TG2 for MØ directional migration to, bind to and engulf AC.

It is evident from this chapter that:

1. TG2 mediates human MØ binding to AC
2. TG2 mediates human MØ interaction with AC
3. TG2 mediates human MØ directional migration to AC
4. MØ cell surface TG2 rather than intracellular TG2 is important for MØ-AC interaction
5. Activity of the enzyme rather than conformation is important for mediating MØ-AC interaction

Taken together, these suggest looking for partners at the MØ cell surface that may work with TG2 to get it to the surface and act as a substrate for TG2 to mediate AC clearance. This now forms the focus for next chapter.

Chapter 5

TG2 associates with syndecan-4 on MØ cell surface

Chapter 5

Result 3: TG2 associates with Syndecan-4 on MØ cell surface

5. 1 Introduction

TG2 is predominantly an intracellular protein, membrane-associated as well as in cytosolic form (Upchurch et al., 1991, Aeschlimann et al., 1995, Verderio et al., 1998, Griffin et al., 2002, Collighan and Griffin, 2009). Though predominantly being a cytosolic protein that lacks the hydrophobic leader sequence, TG2 is a protein with classical non-ER/Golgi-dependent externalization. Once externalised TG2 is found on the cell surface and in association with the extracellular matrix (Aeschlimann et al., 1995, Balklava et al., 2002, Collighan and Griffin, 2009) where it is known to promote cell-matrix interactions via cross-linking fibronectin and collagen (Aeschlimann and Thomazy, 2000, Chau et al., 2005). TG2 is also reported to mediate non-enzymatic functions in the matrix as an integrin co-receptor to fibronectin (Akimov et al., 2000, Telci et al., 2008) leading to cell adhesion and integrin clustering and integrin-mediated signalling (Janiak et al., 2006). More interestingly, MØ cell surface TG2 is known to mediate the formation of β3 integrin and MFG-E8 complexes for promoting AC engulfment (Toth et al., 2009) where MFG-E8 is already established to link to phosphatidylserine on AC. Loss of TG2, MFG-E8 or both, impairs AC clearance by macrophages leading to autoimmunity (Szondy et al., 2003, Hanayama et al., 2006).

Whilst, cellular stress is a key trigger for TG2 externalization (Lorand and Graham, 2003, Ientile et al., 2007), the mechanism of how TG2 is targeted to the cell surface is still ambiguous. Fibronectin and integrins are the two main binding partners of TG2 and have been highlighted for their possible role in TG2 externalization (Akimov and Belkin, 2001, Telci et al., 2008). Recently, Scarpellini et al. (2009) have shown that TG2 has a very high affinity for heparan sulphates by using heparin/heparan sulphate (HS) solid binding assays and by surface plasmon resonance. Heparan sulphates are covalently bound to the core protein of cell–surface proteoglycans including syndecans.

Syndecans are family of four membered transmembrane cell surface proteoglycans bearing heparan sulphate (HS) glycosaminoglycan (GAG) (Bernfield et al., 1999) that are virtually expressed in all cell types (Bernfield et al., 1992). They are well-known to be involved in multiple cellular processes e.g. cell adhesion, proliferation, differentiation, migration and signal transduction (Tumova et al., 2000). Scarpellini et al. (2009) also proposed that

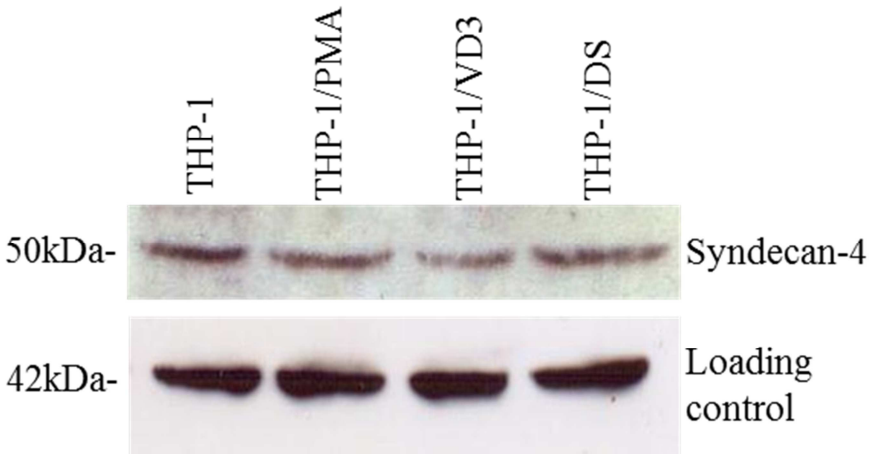
syndecan-4 mediates membrane trafficking of TG2 whilst demonstrating that fibroblasts deprived of syndecan-4 failed to externalize TG2 effectively. They also showed that the extracellular cross-linking activity of TG2 is dependent upon heparan sulphate proteoglycans (HSPG). Another possible means for TG2 externalization is through recycling of endosomes, as TG2 is known to associate with various cytoplasmic early and late, recycling endosomes and lysosomes (Zemskov et al., 2011).

Irrespective of the method of externalization, TG2 is found on the human MØ cell surface and loss of TG2 activity in presence of TG2 inhibitors or loss of cell surface TG2 expression by knocking down TG2 resulted in reduced MØ-AC interaction suggesting that TG2 activity at MØ cell surface plays a prominent role in mediating MØ interaction with AC (Figure 16 & 24). In this context, focus was laid on possible TG2 partners at the MØ cell surface and as HSPG syndecan-4 is a known binding partner for TG2, its role in exhibiting TG2 and in its possible function in TG2 mediated clearance of AC was studied in this chapter.

5.2 Syndecan-4 is expressed in THP-1/MØ and associates with TG2

TG2 has a strong binding affinity for heparan sulphates (HS) of the cell surface receptor syndecan-4 which is important in the translocation of TG2 to the cell surface and extracellular matrix (Scarpellini et al., 2009, Wang and Griffin, 2012). Changes in the expression of HSPGs are known to mediate MØ activation (Laskin et al., 1991, Edwards et al., 1995). Syndecan-4, a heparin sulphate proteoglycan; (HSPG) acts as a receptor for TG2 via its HS chains and can mediate an RGD-independent cell adhesion mechanism via a TG2-FN complex (Wang et al., 2010). Syndecan-4 is known to be expressed in both monocyte and MØ (Yeaman and Rapraeger, 1993). In this context, we first analysed the expression profile of syndecan-4 in our panel of THP-1/MØ by western blotting and immunofluorescence microscopy (Figure 32).

A



B

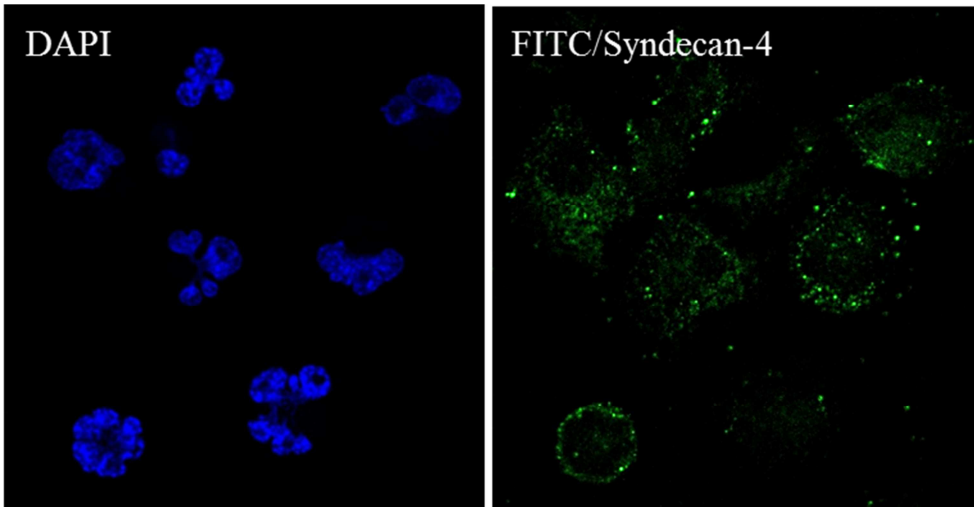


Figure 32. Syndecan-4 is expressed by THP-1 and THP-1/MØ. *A*, Whole cell lysates of undifferentiated THP-1(THP-1) or those stimulated to differentiate with VD3 (THP-1/VD3); PMA (THP-1/PMA) or both (THP-1/DS) were quantified and 50µg equivalent of cell lysates were analysed by western blotting. Membranes were probed using monoclonal anti-syndecan-4 antibody 5G9 and mAb binding was revealed by HRP-conjugated anti-mouse secondary antibody. Stripped membranes were re-probed with anti-β-actin antibody to ensure equal loading. ***B***, Highly adherent THP-1/DS which were also known to express TG2 were immunostained with monoclonal anti-syndecan-4 antibody 5G9 and mAb binding was revealed by FITC-conjugated anti-mouse secondary antibody. Specimens were fixed following antibody staining and imaged using confocal for cell surface TG2 expression.

Though TG2 expression is specific to PMA and PMA/VD3 stimulated THP-1 cells (Figure 12), syndecan-4 expression is seen in THP-1 cells as well as in TG2 expressing and non-expressing THP-1/MØ (Figure 32). Immunostained specimens of THP-1/DS that showed strong TG2 expression also revealed a clear punctate staining for syndecan-4 (Figure 32B). As TG2 is known to have a strong binding affinity for heparan sulphate (HS)/heparin and which is studied to mediate the translocation of TG2 to the cell surface and extracellular matrix (Scarpellini et al., 2009, Wang and Griffin, 2012), possible interaction between TG2 and syndecan-4 was studied via co-immunoprecipitation analysis. Syndecan-4 immuno-complexes, generated as a result of incubating pre-cleared whole cell lysates of THP-1/DS with monoclonal anti-syndecan-4 antibody were precipitated using protein A-sepharose beads and TG2 antigen in the syndecan-4 immuno complexes was resolved by PAGE and western blotting by probing the membranes using anti-TG2 antibody (TG100; Figure 33).

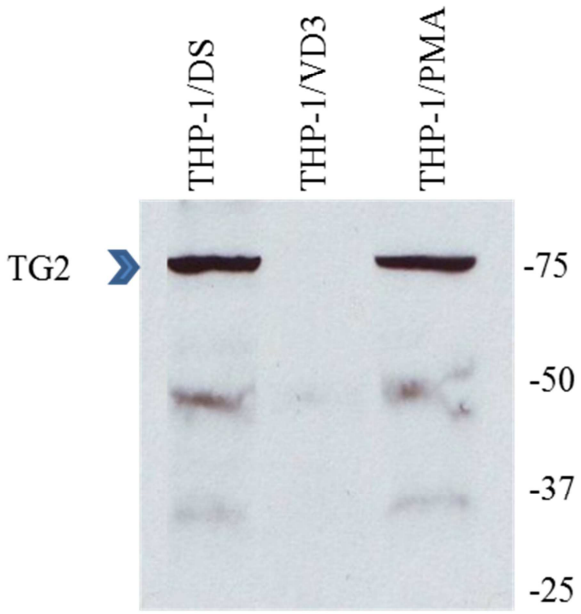


Figure 33. TG2 interacts with syndecan-4. ***A***, 150µg of protein equivalent THP-1/MØ cell lysates pre-cleared with protein A-Sepharose beads slurry was incubated with mouse monoclonal syndecan-4 antibody 5G9 to form syndecan-4 immuno-complex. So formed immuno-complexes were precipitated with protein A-Sepahrose beads, extracted into Laemmli buffer and were resolved by western blotting. Membranes were immunoblotted using anti-TG2 antibody, TG100 to detect TG2 antigen in the complex. TG2 antigen in syndecan-4 immuno-precipitates were pointed by arrow heads. Blot shown is the representative of 4 independent repeats.

As seen from figure 33, TG2 co-precipitates with syndecan-4 on THP-1/MØ. Interestingly, TG2 antigen is revealed in syndecan-4 immuno complexes from THP-1/PMA and THP-1/DS which were known to express TG2 but not in THP-1/VD3 which is consistent with lack of TG2 expression in THP-1/VD3 (Figure 12) standing as a known positive control. However, lack of a standard isotype control IP is the limitation of this experiment.

5.3 Loss of syndecan-4 in THP-1/DS reduces cell surface TG2 expression

Having reported that HSPGs (e.g. syndecan-4) acts as a receptor for TG2 via their HS chains (Scarpellini et al., 2009, Wang et al., 2012), the effect of syndecan-4 knockdown on TG2 and MØ-AC interaction was evaluated.

Four different human SDC4 specific siRNAs (Qiagen) were tested for their ability to reduce SDC4 expression following transfection into cells using the Hi-Perfect transfection reagent. Transfecting THP-1 cells with siRNA for 48h and then stimulating the transfected THP-1 cells to differentiate to MØ-like cells for a further 48h resulted in loss of cell adherence and spreading in THP-1/DS. As HSPGs are known to be involve in cellular processes including cell adhesion, migration and signal transduction (Tumova et al., 2000), loss of syndecan-4 may have also resulted in loss of cell adhesion. Moreover, as cell surface TG2, found in association with syndecan-4 is also known for its role in mediating cell adhesion and migration, loss of syndecan-4 may have also resulted in loss of cell surface TG2 finally resulting in loss of THP-1/MØ adhesion. Relying on earlier experience with efforts to knockdown TG2 where proper knockdown was seen by transfecting THP-1 cells after their differentiation was induced. Similar methodology was followed for syndecan-4 knockdown work. Following 24h differentiation with PMA and VD3, THP-1/DS were transfected with one of the four different siRNA targeting human syndecan-4 using HiPerfect transfecting reagent as per the manufacturer's instructions for a further 48hrs (taking the overall differentiation time to 72h). Non-specific (NS) siRNA was used as a control. Following washing, whole cell lysates were analysed by western blotting to assess the level of syndecan-4 expression.

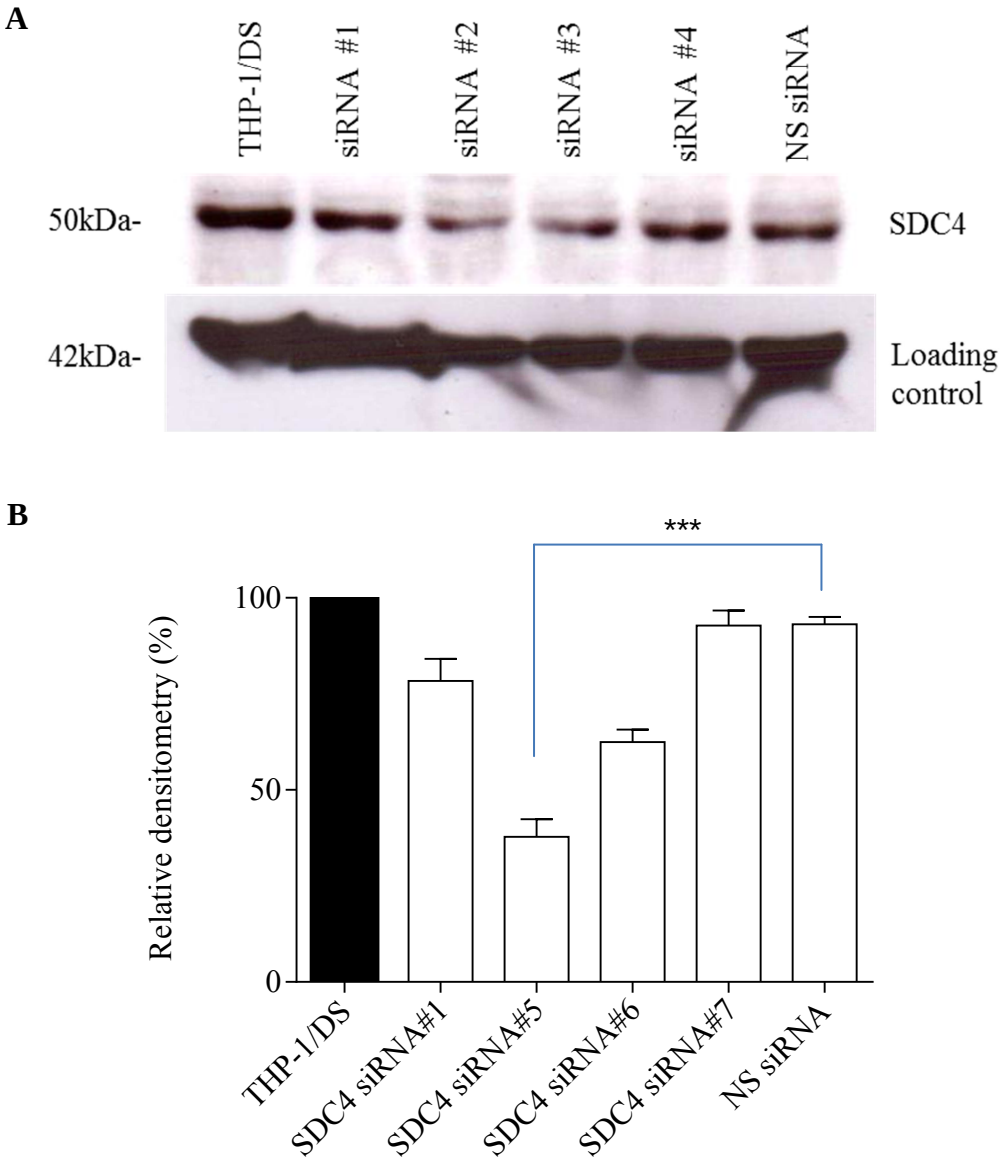


Figure 34. THP-1/DS syndecan-4 knockdown. A, THP-1 cells double stimulated to differentiate to MØ-like cells for 24h, were transfected with four different siRNA targetting human syndecan-4 for a further 48h using HiPerfect transfecting reagent. Whole cell lysates of syndecan-4 siRNA transfected THP-1/DS were analysed by western blotting for syndecan-4 expression. THP-1/DS transfected with non-specific (NS) siRNA was used as a control. Stripped membranes were re-probed with anti-β-actin antibody to ensure equal loading. **B,** Densitometric analysis of syndecan-4 expression knockdown in THP-1/DS using ImageJ. Densitometry values relative to the loading control were calculated and are shown as a % of DS. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** $P<0.001$). Data shown is a representative of three independent experiments. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** $P<0.001$).

As seen from figure 34, syndecan-4 siRNA#2 showed significant knockdown in syndecan-4 expression in comparison to NS siRNA and loading control. Subsequent work in this thesis

with syndecan-4 knockdown used this construct, SDC4 siRNA#2 for knockdown of syndecan-4. Given that TG2 is bound to syndecan-4, it is possible that, loss of syndecan-4 may result in loss of TG2 as well. With this idea in mind, TG2 cell surface expression was analysed, via biotinylation of cell surface proteins, on THP-1/DS cells following syndecan-4 knockdown (Figure 35A). Similarly, whole cell TG2 expression in syndecan-4 knockdown THP-1/DS (Figure 35A) and syndecan-4 expression in TG2 knockdown THP-1/DS were also analysed (Figure 35B).

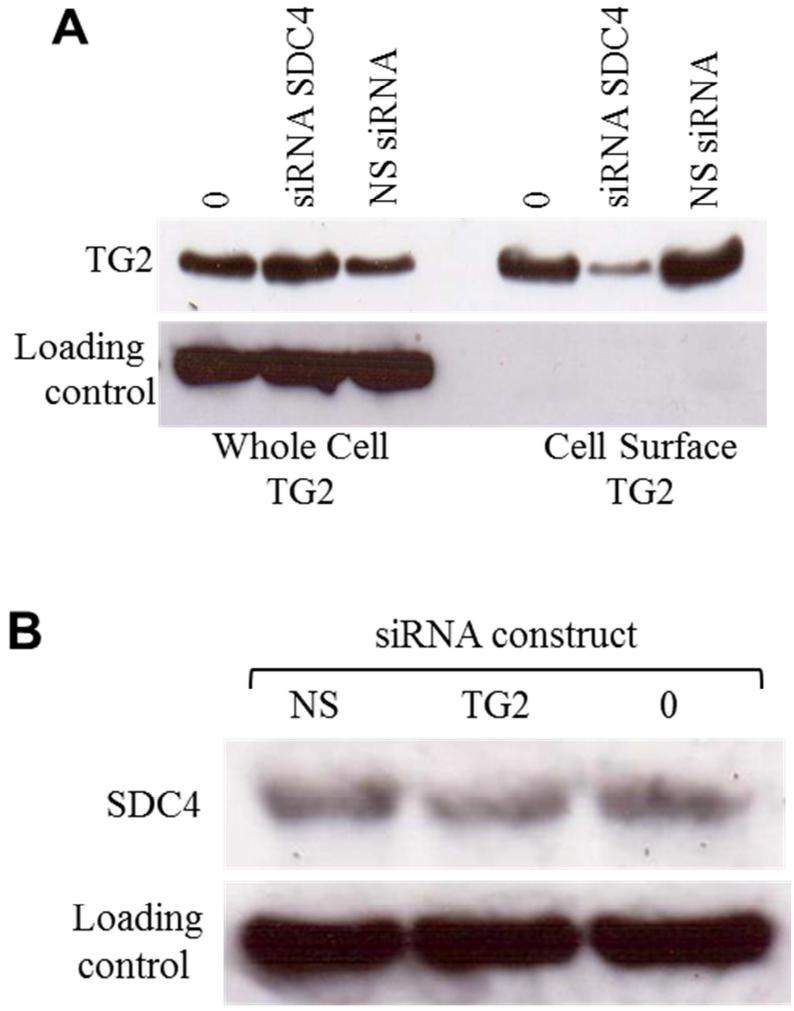


Figure 35. Loss of Syndecan-4 results in loss of cell surface TG2. ***A***, Expression of whole cell TG2 and cell surface TG2 (upon on biotinylation of cell surface proteins) in SDC4 knockdown THP-1/DS was analysed by western blotting. NS siRNA transfected THP-1/DS and non-transfected THP-1/DS were used as control. ***B***, Syndecan-4 expression in TG2 knockdown THP-1/DS was analysed by western blotting. NS siRNA transfected THP-1/DS and non-transfected THP-1/DS were used as control. Stripped membranes were re-probed with anti-β-actin antibody to ensure equal loading. Data shown is the representative of four independent experiments.

As seen from figure 34, syndecan-4 siRNA#5 showed significant knock down in syndecan-4 expression in comparison to NS siRNA and this knockdown is robust as seen from all four repeats. Similarly, loss of syndecan-4 in THP-1/DS resulted in the loss of cell surface TG2 expression (figure 35A). However, no change in whole cell TG2 expression is seen with syndecan-4 knockdown suggesting that cell surface TG2 is anchored to syndecan-4 and loss of which resulted in loss of cell surface TG2 expression. Interestingly, no loss of syndecan-4 expression is seen upon knocking down TG2 in THP-1/DS (Figure 35B) strengthening the idea that TG2 is held anchored to syndecan-4 on MØ cell surface.

5.4 Loss of syndecan-4 in THP-1/DS reduces cell surface TG2 activity and MØ-AC interaction

Our results from chapter 4 suggest that cell surface TG2 activity is important in AC clearance by MØ. Loss of TG2 activity as a consequence of TG2 inhibitors or as a result of TG2 knockdown resulted in reduced MØ interaction with AC (Figure 16 & 24A). In line with these studies and the results of figure 35, cell surface TG2 activity following syndecan-4 knockdown was analysed in THP-1/DS cells (Figure 36).

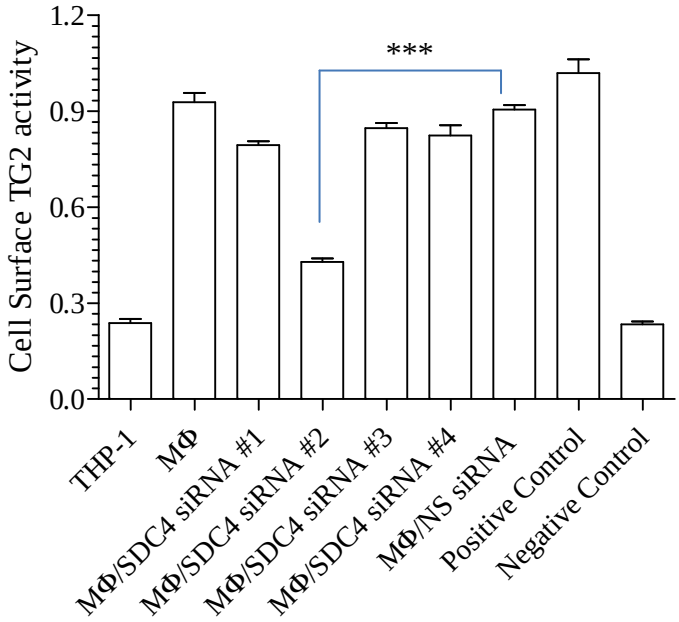


Figure 36. Cell surface TG2 activity is reduced following syndecan-4 knockdown in THP-1/DS cells. Cell surface TG2 activity was tested via biotin cadaverine incorporation into fibronectin. 24h differentiated THP-1/DS transfected with four different syndecan-4 siRNA were seeded on fibronectin coated plates and incubated in presence of biotin-X-cadaverine. Fibronectin incorporated TG2 is conjugated with Extr-Avidin peroxidase and colour development following treatment with 3,3',5,5'-Tetramethyl benzidine in DMSO representing cell surface TG2 activity is expressed as mean absorbance at 450nm. NS siRNA transfected THP-1/DS was used as control. Guinea pig liver TG in serum free medium containing biotin-X-cadaverine and 10mM EDTA are used as known positive and negative controls. Data shown is mean ±SEM for n≥3 independent experiments. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** $P<0.001$).

As seen from figure 36, a significant loss in cell surface TG2 activity as a result of syndecan-4 knockdown is evident which is consistent with loss of cell surface TG2 expression (Figure. 35A). As TG2 activity on the MØ cell surface is found to be crucial for mediating MØ-AC interaction, the ability of THP-1/DS to interact with AC following knockdown of syndecan-4 was analysed. Non-transfected and NS siRNA transfected THP-1/DS were used as controls. Simultaneously, syndecan-4 siRNA or NS siRNA transfected and non-transfected THP-1/DS were treated with R281 to check any further reduction in MØ-AC interaction (Figure 37).

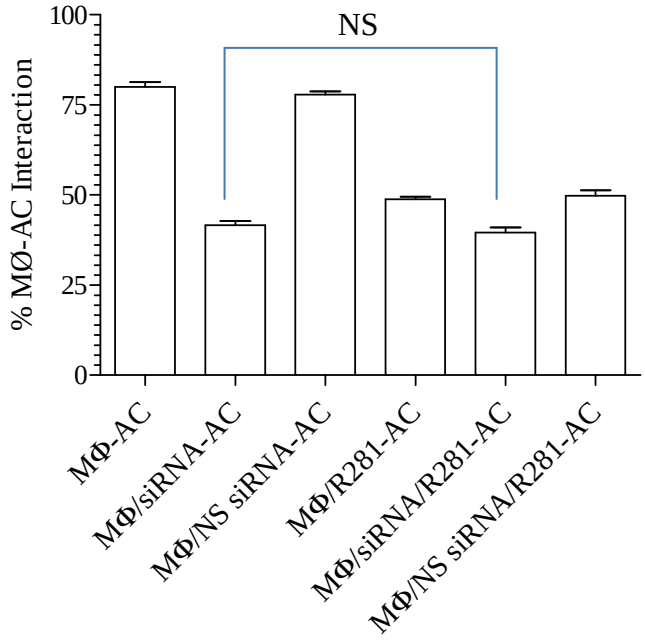


Figure 37. Syndecan-4 knockdown in THP-1/DS cells impairs MØ-AC interaction. Syndecan-4 siRNA transfected, NS siRNA transfected and non-transfected THP-1/DS cells treated with or without cell surface TG2 inhibitor R281 were co-cultured with apoptotic Mutu for 1h at 37^{o}C. Unbound AC were washed off and MØ were fixed with 1% w/v formaldehyde, stained with Diff quik II and scored as a percentage of MØ interacting with AC using light microscopy. Atleast 200 MØ were scored in each replicate well. Data shown is mean ±SEM for n≥3 independent experiments. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (NS: Non-significant).

As seen from figure 35, loss of syndecan-4 resulted in loss of MØ cell surface TG2 activity which was shown to affect MØ-AC interaction (Figure 26). Similarly, loss of syndecan-4 even resulted in reduced MØ-AC interaction (Figure 37) implying that loss of syndecan-4 resulted in loss of cell surface TG2 activity leading to a significant reduction in MØ interaction with AC (Figure 37), on par with inhibiting effect shown by cell impermeable TG2 inhibitors R281. However, when syndecan-4 knockdown THP-1/DS treated with R281, no further reduction in MØ-AC interaction is seen suggesting that either knocking down syndecan-4 leading to the loss of cell surface TG2 or inhibiting cell surface TG2 activity has the same inhibiting effect on MØ-AC interaction.

5.5 Heparan sulphate side chains of syndecan-4 are the receptors of cell surface TG2

HS chains on the core syndecan-4 are complex polysaccharides consisting of alternating *N*-acetylated or *N*-sulphated glucosamine units and uronic acids with highly negatively charged

binding sites for protein ligands (Bishop et al. 2007, Lortat-Jacob et al. 2012). They are known to immobilize and regulate the turnover of ligands that act at the cell surface (Bernfield et al., 1999). With TG2 being a well-known binding partner for syndecan-4 via its HS chains with a unique binding site within the family of transglutaminases (Lortat-Jacob et al., 2012), we further looked to identify, whether loss of HS will have a direct impact on mediating MØ-AC interaction. Upon treating THP-1/DS with heparinase II, an enzyme that depolymerizes heparan sulphate glycosaminoglycans through a beta-elimination mechanism (Shaya *et al.*, 2010), cell surface TG2 activity was measured by incorporation of biotinylated cadaverine into fibronectin, as described in experimental procedures (Figure 38).

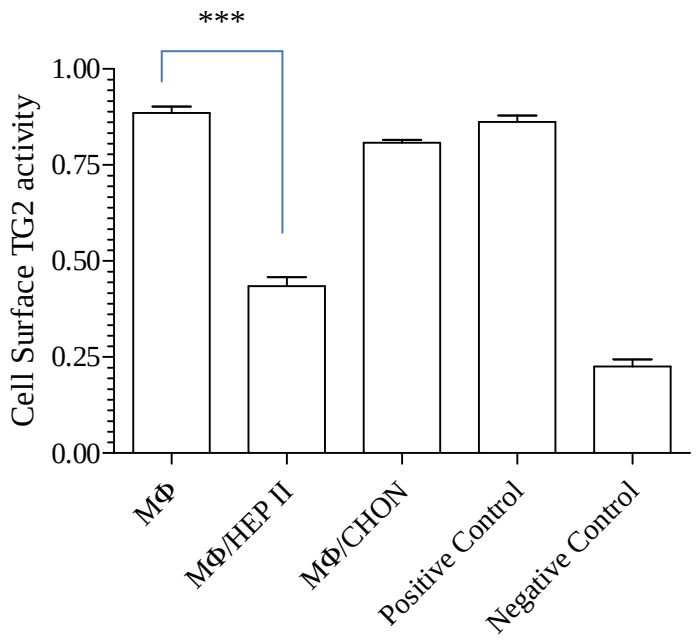


Figure 38. Loss of HS chains of syndecan-4 reduce cell surface TG2 activity. Cell surface TG2 activity was tested via biotin cadaverin incorporation into fibronectin. THP-1/DS treated with heparinase II (HEP II) to digest HS side chains of syndecan-4 were seeded on fibronectin coated plates and incubated in presence of biotin-X-cadaverine. Fibronectin incorporated TG2 is conjugated with extravidine peroxide and colour development following treatment with 3,3',5,5'-Tetramethyl benzidine in DMSO representing cell surface TG2 activity is expressed as mean absorbance at 450nm. THP-1/DS treated with chondroitinase (CHON) to digest chondroitin sulphate is used a control for heparinase II digestion, while guinea pig liver TG in serum free medium containing biotin-X-cadaverine and 10mM EDTA are used as known positive and negative controls. Data shown are mean ±SEM for n≥3 independent experiments. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** P<0.001).

Loss of HS upon digestion with heparinase II resulted in loss of cell surface TG2 activity while no significant change is seen with chondroitinase treatment (Figure 38). With loss in cell surface TG2 activity upon loss of HS, effect of HS loss on MØ-AC interaction was analysed (Figure 39).

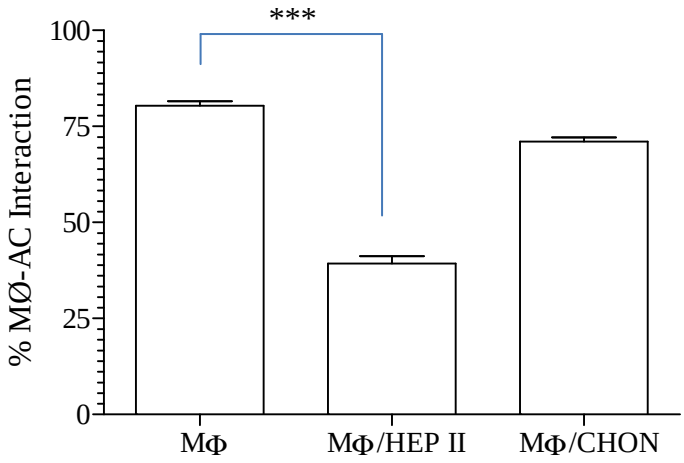


Figure 39. Loss of HS reduces MØ-AC mediated by loss of TG2. THP-1/DS treated with heparinase II enzyme to digest HS chains of syndecan-4 were washed twice before co-culturing with AC for 1h at 37^{o}C. Unbound AC were washed, **MØ** were fixed with 1% w/v formaldehyde, stained with Diff quik II and scored as a percentage of macrophages interacting with AC using light microscopy. THP-1/DS treated with chondroitinase enzyme and untreated were used as controls. Data shown is mean ±SEM for n≥3 independent experiments. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (*** P<0.001). (Heparinase II: HEP II and Chondroitinase: CHON)

Removal of HS through digestion with heparinase II results in reduced MØ interaction with AC (Figure 39). This result is in parallel with reduced AC removal following loss of cell surface TG2 activity through treatment with TG2 inhibitors (Figure 27); knockdown of TG2 (Figure 28) or knockdown of syndecan-4 (Figure 36). Treatment with chondroitinase as a control showed no changes either in TG2 activity (Figure. 38) or in MØ-AC interaction (Figure. 39) identifying HS as prominent receptors for TG2 at the human MØ cell surface.

5.6 Syndecan-4 bound TG2 via HS is crucial in mediating MØ interaction with AC

To further strengthen our understanding of TG2-syndecan-4 binding via HS on the MØ cell surface, the P1 peptide (^{200}NPKFLKNAGRDCSRRSS^{216}), which competes for the heparin binding domain within the TG2 molecule was used. Wang *et al*. (2012) demonstrated that P1

peptide showed specific binding towards syndecan-4 and was able to block the interaction between TG2 and syndecan-4. To check the effect of P1 peptide on MØ-AC interaction, THP-1/DS treated with varying concentrations of P1 peptide and P1s were co-culture with apoptotic B-cells for 1h at 37^{o}C.

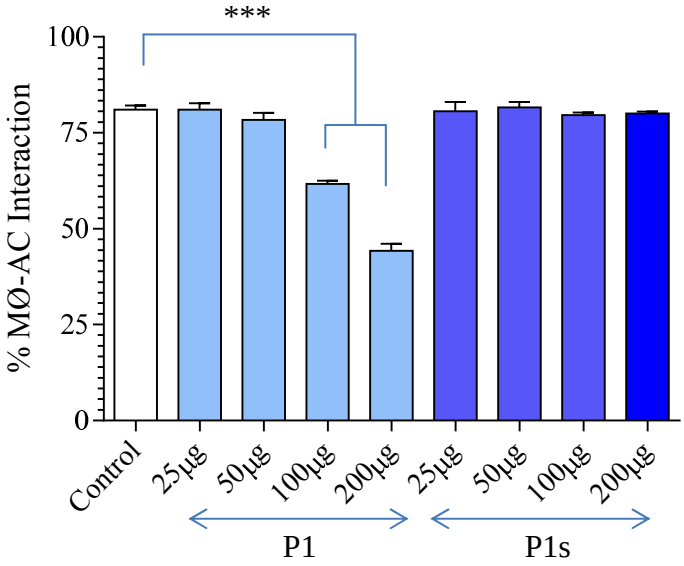


Figure 40. TG2 heparan binding domain competing P1 peptide results in a dose dependent reduction in MØ-AC interaction. THP-1/DS treated with varying concentrations of P1 peptide were co-culture with apoptotic B-cells for 1h at 37^{o}C. Unbound AC were washed, macrophages were fixed with 1% w/v formaldehyde, stained with Diff quik II and scored as a percentage of MØ interacting with AC using light microscopy. P1s peptide used at similar concentrations was used as control. Data shown is mean ±SEM for n≥3 independent experiments. Statistical analysis was conducted using ANOVA followed by Dunnett's post-test (*** P<0.001).

As seen from figure 40, P1 peptide (but not a scrambled control peptide) reduces MØ-AC interaction in a dose-dependent manner. A significant reduction in MØ-AC interaction is seen with P1 peptide at 100-200µg/ml in TG2 expressing THP-1/DS which could be through the loss of TG2-HS binding leading to the loss of active TG2 from the MØ cell surface. No such reducing effect is seen with the scrambled analogue P1s (FNRADLKPRCGSSNKSR) suggesting that the importance of HS bound TG22 is crucial in mediating MØ interaction with AC.

To further confirm the involvement of TG2 activity in MØ-AC interaction, THP-1/DS treated with P1 peptide were treated with active TG2 exogenously (Figure 41).

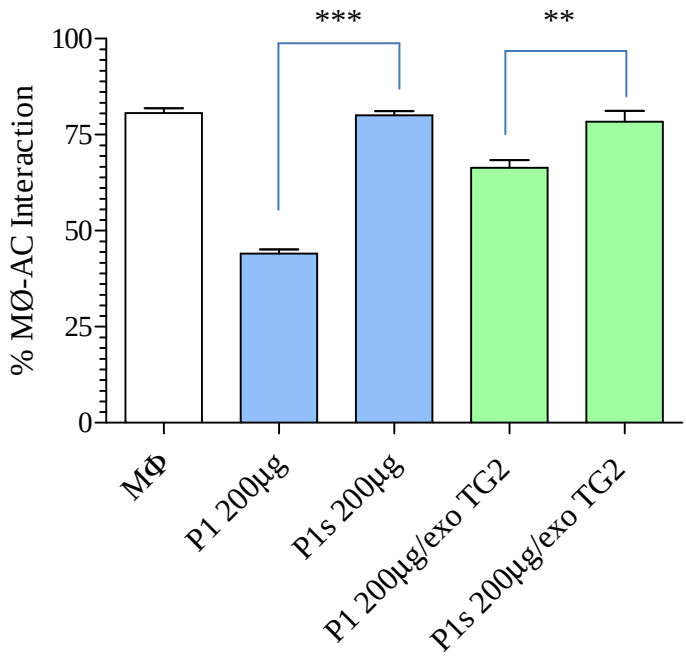


Figure 41. Exogenous TG2 partially compensates the loss of TG2 activity. THP-1/DS treated with P1 peptide were further treated with active TG2 exogenously and co-cultured with AC for 1h at 37^{o}C. Unbound AC were washed, macrophages were fixed with 1% w/v formaldehyde, stained with Diff quik II and scored as a percentage of macrophages interacting with AC using light microscopy. THP-1/DS/P1s treated with TG2 exogenously is used as control. Data shown is mean ±SEM for n≥3 independent experiments. Statistical analysis was conducted using ANOVA followed by Bonferroni post-test (** P<0.01; *** P<0.001).

THP-1/DS treated with P1 peptide (P1; which competes the heparin-binding domain within TG2 for binding to HS) but not a scrambled P1 peptide (sP1) show reduced interaction with AC. However, upon addition of active TG2 exogenously the loss of MØ-AC interaction was found to be rescued outcompeting the blocking effect of P1, replacing TG2 at the cell surface (Figure 41), and it fits with the rescue seen in the knockdown experiments too suggesting that the importance of syndecan-4 bound cell surface TG2 via HS is crucial in mediating MØ interaction with AC.

5.7 Discussion

TG2 is a multi-functional, ubiquitous protein better known for its cross-linking enzymatic reaction in a Ca^{2+} dependent manner. TG2 is predominantly a cytoplasmic protein whose activity was reported to be regulated by Ca^{2+} and GTP/GDP levels while its cross linking activity will be enhanced in presence of high levels Ca^{2+} (Verderio et al., 2004). Through its non-ER/Golgi-dependent externalization, the presence of this enzyme in the extracellular environment is evident, an ideal location with high Ca^{2+} and low GTP concentrations for its transaminase activity (Verderio et al., 1998). Once externalized, it either remains tightly bound to the cell surface or is released to the extracellular matrix and mediates several enzymatic and non-enzymatic functions in collaboration with its extracellular binding proteins like fibronectin, collagen and integrins (Akimov et al., 2000, Balklava et al., 2002, Telci et al., 2008). Not all the externalised TG2 is active enzymatically and most of it is found to remain inactive in spite of prevailing favourable conditions of high Ca^{2+}/GTP in the extracellular environment (Siegel et al., 2008). Whilst cellular stress is the key trigger for TG2 externalisation (Nicholas et al., 2003, Lorand and Graham, 2003, Ientile et al., 2007), even its extracellular activation is purely dependent on stress. It is the demand for activation like cell proliferation/differentiation in response to tissue injury/repair or by TGF-β in response to oxidative stress will trigger from its latent phase to a catalytically active.

Several theories to date have been put forward as a means of TG2 externalization. Fibronectin and integrins are the two main binding partners of TG2 and have been highlighted for their possible role in TG2 externalization (Akimov et al., 2000, Telci et al., 2008). Having known for its affinity to heparin (Gambetti et al., 2005), a highly sulphated analogue of heparin sulphate side chains on the syndecans, Scarpellini *et al.* (2009) proposed heparan sulphate proteoglycan (HSPG) as the key receptors for cell surface TG2 and their possible involvement in TG2 externalization. Binding of TG2 to heparin is also known to protect TG2 from thermal unfolding and proteolytic degradation (Gambetti et al., 2005). In line with the affinity studies of TG2 to HS (Scarpellini et al., 2009), coupled with our observations in terms of TG2 surface localisation and its involvement in mediating MØ-AC interaction, possible involvement of syndecan-4 and its anchored HS side chains in TG2 mediated MØ phagocytosis was studied in this chapter.

Syndecan-4 is a well-established member of the syndecan family of transmembrane type heparan sulphate proteoglycans (Bernfield et al., 1999). Syndecan-4 was shown to be

expressed by mouse peritoneal macrophages and murine monocyte-macrophage cell lines (Yeaman and Rapraeger, 1993, Boyanovsky et al., 2009). Changes in the expression of HSPGs are even known to mediate MØ activation (Laskin et al., 1991, Edwards et al., 1995). With an established affinity of TG2 for HS, the importance of syndecan-4 in TG2 externalisation and its expression in mouse macrophages, syndecan-4 expression profile in THP-1/MØ model system was analysed. Through the use of western blotting and immunofluorescence staining, a clear expression of syndecan-4 in THP-1/DS model system that also shows strong TG2 expression was revealed. Though TG2 expression is quite specific to PMA or double stimulated THP-1 cells, syndecan-4 is expressed by THP-1 as well as by all three THP-1/MØ models (Table 4).

Table 4. TG2 and syndecan-4 expression profile in THP-1 cells and THP-1/MØ

	TG2 Expression	Syndecan-4 Expression
THP-1	NO	YES
THP-1/PMA	YES	YES
THP-1/VD3	NO	YES
THP-1/DS	YES	YES

More interestingly, co-immunoprecipitation analysis revealed the possible interaction between cell surface TG2 and syndecan-4 in TG2 expressing THP-1/MØ confirming that cell surface TG2 is in syndecan-4-bound form. In support of the above statement, loss of syndecan-4 as a result of siRNA knockdown resulted in the loss of cell surface TG2 (which is evaluated by cell surface protein biotinylation). However no such loss in syndecan-4 expression is seen upon knocking down TG2 clearly suggesting that cell surface externalised TG2 is in syndecan-4 anchored form either directly or indirectly. As TG2 is known to have strong binding affinity to HS of syndecan-4, treating THP-1/DS with heparinase II for digesting HS side chains of the core syndecan-4 molecule resulted in loss of cell surface TG2 expression. Data presented here indicates that the loss of syndecan-4 or loss of HS results in loss of cell surface TG2 and its associated activity. Thus the loss of TG2 from the cell surface is the likely explanation for syndecan-4 knockdown effects have to reduce interaction between MØ and AC. This is strongly supported by the ability of exogenous TG2 to rescue MØ-AC interaction inhibited by the loss of syndecan-4. This is in line with the outcome from

chapter 3, suggesting that MØ cell surface TG2 activity is a key regulator for MØ interaction with AC.

Understanding TG2 activity in mediating MØ interaction with AC was further strengthened by using TG2 heparin-binding domain mimicking peptide 'P1'. This peptide from TG2 lacks TG2 activity but competes with TG2 for binding to HS. Use of P1 peptide at a concentration of 200µg/ml showed a significant reduction in MØ-AC interaction and this effect was dose-dependent. This effect noted in THP-1/DS expressing TG2 upon treatment with P1 peptide at a high concentration may possibly replace cell surface TG2 overtime, leading to suppression of MØ-AC interaction through loss of TG2 activity. Interestingly, upon addition of active TG2 exogenously, the loss of MØ-AC interaction as a result of P1 peptide was found to be rescued, suggesting that the importance of syndecan-4 bound TG2 via HS is crucial in mediating MØ interaction with AC. Quantifying the cell surface TG2 activity on THP-1/MØ following P1 peptide treatment would have provided a convenient explanation for the TG2 activity need on the MØ cell surface for mediating MØ-AC interaction. Further addition of exogenous TG2 coupled with TG2 inhibitor treatment to the P1 peptide treated THP-1/MØ could confirm whether there the requirement of TG2 presence or TG2 activity for the rescue effect seen with exogenous TG2.

Taken together with the inhibitory effect of the P1 peptide, which competes with the heparin binding domain within the TG2 molecule, these data support the notion that cell surface TG2, in association with HS of syndecan-4 mediates MØ-AC interaction. Knockdown of syndecan-4 expression and inhibition of TG2 activity with TG2 inhibitors showed no further reduction in AC clearance suggesting TG2 exerts most, if not all of its cell surface effects on AC clearance through its association with syndecan-4. However, there could be a possibility that cell surface legends other than HS chains on syndecan-4 may be responsible for TG2 association and thus mediating MØ clearance of AC.

Chapter 6

TG2 interacts with CD44 at the macrophage cell surface: A possible role for TG2 in mediating CD44 cross-linking

Chapter 6

Result 4: TG2 interacts with CD44 at the macrophage cell surface: A possible role for TG2 in mediating CD44 cross-linking.

6.1 Introduction

The transmembrane glycoprotein CD44 is an HSPG and an established receptor for hyaluronan that is known to play a critical role in immune cells modulating proliferation and differentiation, cell adhesion, migration, and inflammation (Fadok et al., 1998, Pure and Cuff, 2001, Ponta et al., 2003). CD44 is encoded by a single, highly-conserved gene and is located on chromosome 2 in mice and chromosome 11 in humans (Naor et al., 1997). The CD44 gene is composed of 20 exons of which exons 1-5 and 16-18 are constant, whereas exons 6-15 and 19-20 are variants (Screaton et al., 1992). CD44S ('standard') or CD44H expressed by haematopoietic cells represent the basic form of CD44 with a molecular mass of 40kDa (Jalkanen et al., 1986). However, by undergoing complex alternative mRNA splicing and posttranslational glycosylation of CD44S (Levesque and Haynes, 1999), results with a molecular mass of 80-100kDa on SDS-PAGE. Similarly, expression of variant isoforms in parallel with glycosylation results in the generation of multiple isoforms of different molecular sizes (80-230kDa) especially by different carcinomas (Gunthert et al., 1991). Primary blood monocytes are known to express CD44S, but increased expression of variant isoforms along with CD44S is evident as monocytes differentiate to macrophages, in vitro (Mackay et al., 1994). Similarly, expression of various CD44 isoforms by macrophages is known to be increased at sites of chronic inflammation (Levesque and Haynes, 1996).

Structurally, CD44 comprises a 248 amino acid extracellular domain that includes binding site for hyaluronan, other CD44 ligands including collagen, laminin and fibronectin (Ishii et al., 1993, Jalkanen and Jalkanen, 1992) and sites for *N*- and *O*-linked glycosylation and chondroitin sulphate binding. Its transmembrane domain comprises of a 23 amino acid and its intracellular domain comprising 70 amino acids with 70-80% homology between species which mediates CD44 interaction with intracellular binding partners (Stamenkovic et al., 1989). Though, lacking intrinsic kinase activity, the cytoplasmic tail of CD44 interacts with a variety of signalling mediators. It also contains binding sites for the actin-cytoskeleton adaptor proteins ankyrin and members of ERM (exrin/radixin/moesin) family for actin cytoskeleton reorganization for mediating cell adhesion and motility.

As a well-known cell-cell and cell-matrix interactions mediator, CD44 is associated with several inflammatory processes (Pure and Cuff, 2001). Its roles in recruiting peripheral blood monocytes to the sites of inflammation (Guazzone et al., 2005) and lymphocyte homing during inflammation (Stoop et al., 2002) are well documented. Moreover, CD44 is also known to be a ligand for E-selectin which, in cooperation with P-selectin, facilitates neutrophil extravasation into inflamed sites (Katayama et al., 2005). Phagocytosis is a hallmark of MØ and substantial evidence is available establishing CD44 as a prominent phagocytic regulator (Hart et al., 1997, Teder et al., 2002, Leemans et al., 2003, Vachon et al., 2006, Hart et al., 2012). Hart et al., (2012) recognised the multifunctional cell surface receptor, CD44 as one of the key regulators in defining MØ capacity of AC clearance confirming that, when cross-linked by CD44 monoclonal antibody (mAb), augments AC clearance. A well-defined role for CD44 in AC binding, ingestion and clearance is evident as mice deficient in CD44 showed a moderately decreased clearance in AC following bleomycin-induced lung injury with a 13-fold increase in uncleared AC compared to wildtype mice (Teder et al., 2002). Compelling evidence is now available establishing CD44 as a phagocytic receptor to AC (Vachon et al., 2006) while CD44-/- mice are characterised by impaired apoptotic neutrophil clearance, accumulation of low molecular weight hyaluronan fragments with proinflammatory functions, and impaired activation of TGF-β1 following lung injury. Similarly, a well characterised effect of MØ CD44 cross-linking in association with augmented AC clearance is provided by Hart et al. (2012).

Interestingly, a monoclonal antibody (6B9) that was reported to recognize cell surface TG2 (Mohan et al., 2003) was confirmed to be an antibody recognizing CD44 (Stamnaes et al., 2008) leading to the possibility that CD44 and TG2 interact. Given (a) that CD44 (an HSPG) cross-linking is required on MØ to augment AC clearance, (b) the established link between TG2-CD44 (via mAb 6B9), (c) that TG2 binds HS and that TG2 exhibits strong cross-linking activity, the possibility that TG2 may interact with CD44 to promote AC clearance was investigated, initially focussing on the expression profile of CD44 in our THP-1/MØ and primary HMDMØ cell systems.

6.2 CD44 is expressed in macrophage.

To assess CD44 expression in our macrophage system, whole cell lysates of THP-1 and THP-1/MØ, primary HMDMØ, viable and apoptotic Mutu were analysed by western blotting for CD44 expression (Figure 42).

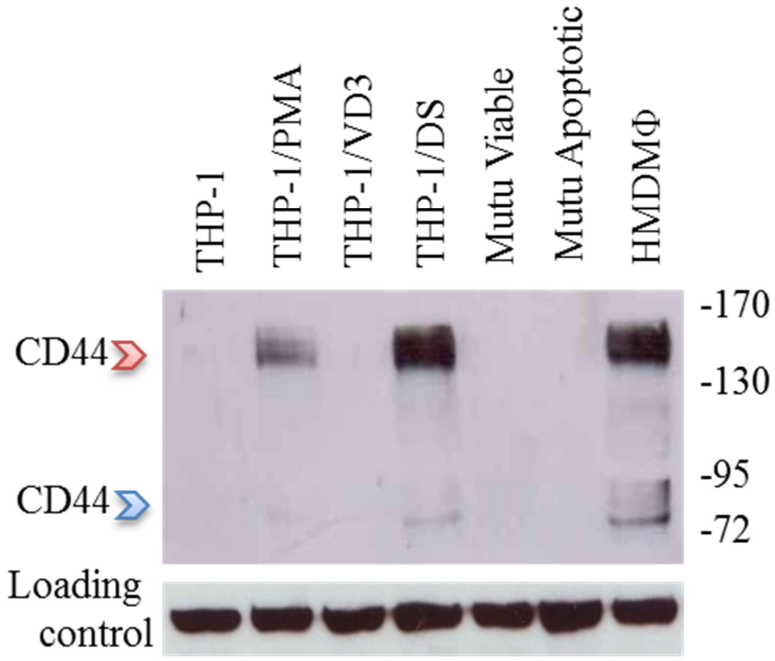


Figure 42. CD44 expression profile in THP-1/MØ, HMDMØ and apoptotic cells. 50µg equivalent whole cell lysates of THP-1, THP-1/MØ, HMDMØ, viable and apoptotic Mutu were analysed by western blotting using a 5% gel. Membranes were probed using monoclonal anti-CD44 antibody and mAb binding was revealed by HRP-conjugated anti-mouse secondary antibody. Stripped membranes were re-probed with anti-β-actin antibody to ensure equal loading. Data shown are the representative of three independent repeats.

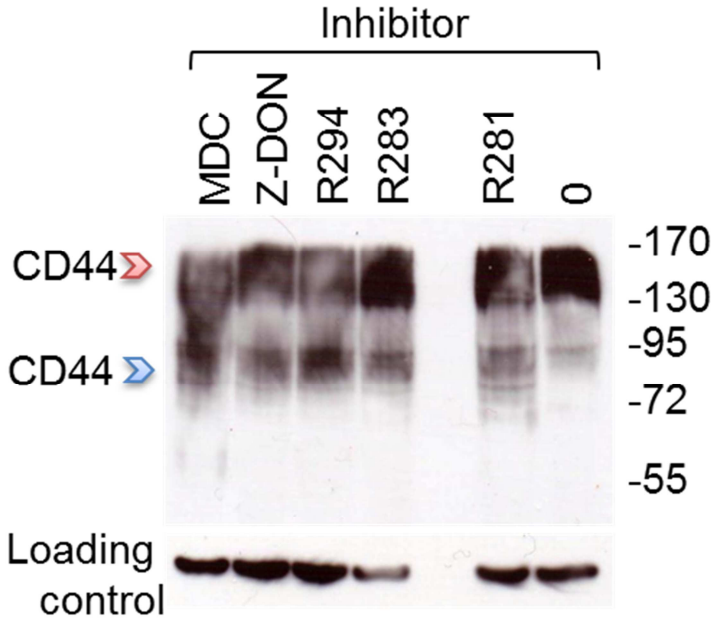
As evident from figure 42, western blot analysis of CD44 in THP-1/PMA, THP-1/DS and HMDMØ reveals detectable CD44 expression that paralleled TG2 expression (Figure. 10, 14). Interestingly, two distinct molecular weight bands for CD44 were expressed one equivalent to ~130-160kDa and the other equivalent to ~80-90kDa. Red and blue arrowheads indicate high and low molecular weight bands for CD44 (Figure 42). CD44 is known to contribute a range of isoforms due to extensive *N*- and *O*-glycosylation or alternative splicing resulting in expression of varied isoforms ranging from 80 to 200 kDa (Screaton et al., 1992, Skelton et al., 1998). Human monocytes are known to express CD44S but following

differentiation express high molecular weight isoforms along with low molecular weight isoforms suggesting that CD44v expression is associated with monocyte differentiation to tissue macrophages (Levesque and Haynes, 1996). More interestingly, high molecular weight CD44 isoform expression is consistent with TG2 expression in THP-1/MØ though the low molecular weight isoform expression is not seen in undifferentiated THP-1. The expression of TG2 and high molecular weight CD44 with THP-1 differentiation to MØ-like cells suggests that the high molecular weight CD44 could be a result of TG2 bound isoform.

6.3 TG2 cross link CD44 on MØ cell surface

Hart *et al*. (2012) have proposed that cross-linking of CD44 by mAb augments AC clearance. While TG2 is well known for its cross-linking function, there could be a possibility that the high molecular weight CD44 (as evident from figure 42) could be a TG2 cross-linked product of CD44 that may be generated through TG2 activity. If this hypothesis were true, treatment with TG2 inhibitors would result in a reduction or loss of the high molecular weight CD44 band, as TG2 cross-linking were inhibited. To address the possibility that the high molecular weight CD44 is a TG2 cross-linked form [cross-linked to itself (CD44-CD44) or other partner molecules, possibly including TG2], CD44 expression in TG2 inhibitor treated cells was examined (Figure 43).

A



B

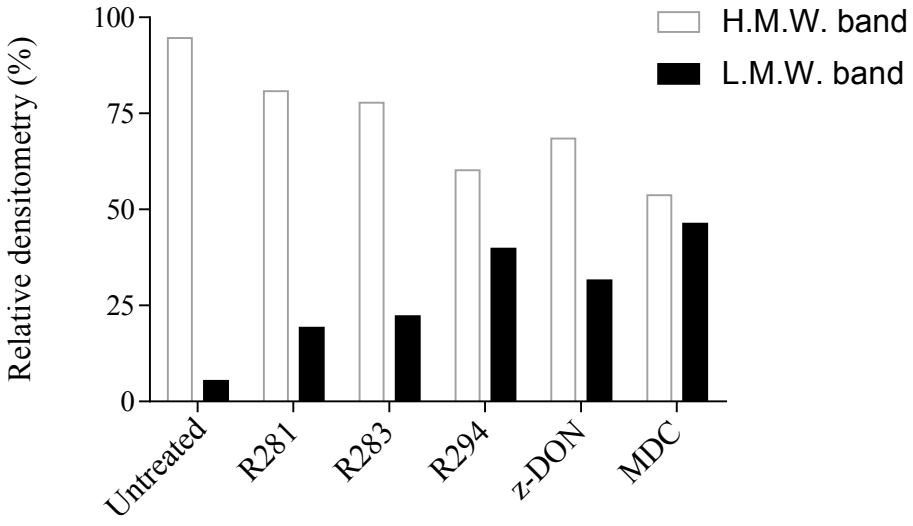


Figure 43. TG2 cross-link CD44. A. 50µg equivalent whole cell lysates of THP-1/DS ±TG2 inhibitors were analysed for CD44 expression by western blotting. Membranes were probed using monoclonal anti-CD44 antibody and mAb binding was revealed by HRP-conjugated anti-mouse secondary antibody. Stripped membranes were re-probed with anti-β-actin antibody to ensure equal loading. **B**. Densitometric analysis of the high and low molecular weight bands of CD44 bands obtained with different TG2 inhibitor treatments, using ImageJ. Densitometry values relative to the loading control were calculated and are shown as a % of untreated control. Blot shown is the representative of three different independent experiments, while the densitometry shown is the representative of the blot shown.

In agreement with the hypothesis, inhibition of TG2 was associated with a significant loss in the high molecular CD44 (~150-160kDa, red arrow head) coupled with an increase in the intensity of low molecular weight CD44 (~80-90kDa, blue arrow head) consistent with the notion that the high molecular weight CD44 species arises from TG2 activity (Figure 42). As MØ CD44 cross-linking is known to associate with augmented AC clearance (Hart et al., 2012), this may suggest a possible mechanism by which TG2 exerts its effect on promoting MØ clearance of AC adding an extra bit of weight to earlier established data in a way that TG2 may be the possible CD44 cross-linker favouring CD44 mediated MØ clearance of AC.

To look specifically into possible interactions between TG2 and CD44, whole cell lysates of both THP-1/DS and HMDMØ were subjected to co-immunoprecipitation analysis. TG2 immuno-complexes generated as a result of incubating pre-cleared whole cell lysates of THP-1/DS and HMDMØ with monoclonal TG2 antibody (TG100) were precipitated using protein A-Sepharose beads. Following western blotting, CD44 antigen in the TG2 immuno complexes was resolved by probing the membranes using an anti-CD44antibody (Figure 44).

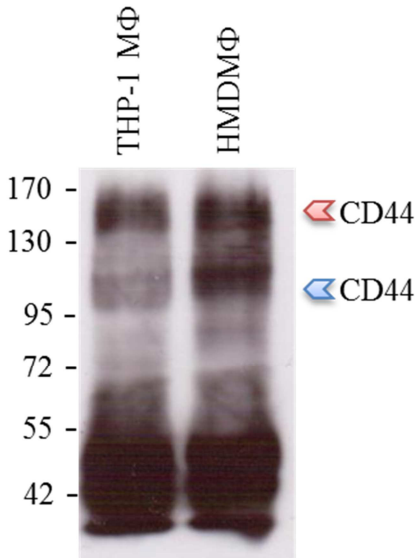


Figure 44. TG2 co-precipitates with CD44. 150µg of protein equivalent THP-1/MØ and HMDMØ whole cell lysates precleared with protein A-Sepharose beads slurry were incubated with TG2 antibody to form TG2 immuno-complex. So formed immuno-complexes were precipitated with protein A-Sepharose beads, extracted into laemmli buffer and were resolved by SDS-PAGE using 8% gels and western blotting. Membranes were immunoblotted using anti-CD44 antibody, to detect CD44 antigen in the TG2 immuno-complex. CD44 in TG2 immuno-complexes from THP-1/MØ and PMDM were pointed with the arrow heads. Molecular weight standards shown in kDa.

Precipitation of TG2 resulted in the co-precipitation of CD44 (Figure 44) suggesting a possible interaction between these proteins. Interestingly, in parallel with CD44 western analysis, both high and low molecular weight bands for CD44 were detected in co-immunoprecipitation. However, the low molecular weight band (~95-110kDa; Figure 44) detected were not identical to the molecular weight of the band (~85-95kDa; Figure 42) seen in western blots suggesting that CD44 bands above 85-95kDa may be the consequence of either partial or complete posttranslational modification. As CD44-TG2 interaction is evident, the possibility that TG2 inhibitors may reduce the interaction was tested. MØ treated with and without TG2 inhibitors were analysed by co-immunoprecipitation (Figure 45).

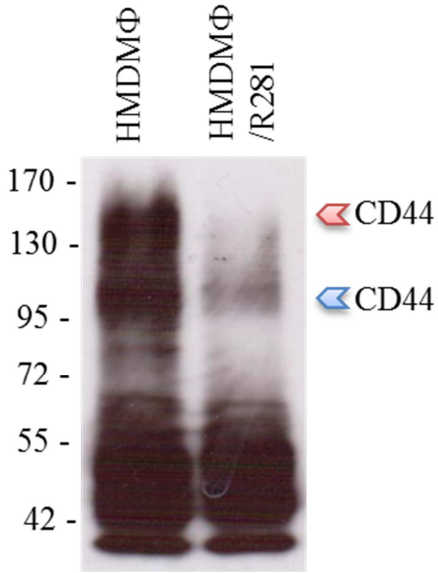


Figure 45. TG2 inhibitors blocked TG2-CD44 interaction. 150µg of protein equivalent whole cell lysates of HMDMØ treated with and without TG2 inhibitor were pre-cleared with protein A-Sepharose beads slurry and incubated with TG2 antibody to form TG2 immuno-complex. So formed immuno-complexes were precipitated with protein A-Sepharose beads, extracted into laemmli buffer and were resolved by SDS-PAGE using 8% gels and western blotting. Membranes were immunoblotted using anti-CD44 antibody, to detect CD44 antigen in the TG2 immuno-complex. CD44 in TG2 immuno-complexes HMDMØ were pointed with the arrow heads. Molecular weight standards shown in kDa.

Notably, as evident from co-immunoprecipitation analysis, TG2-CD44 interaction was significantly reduced following treatment with TG2 inhibitor R281 as was the expression of the high molecular weight CD44(~150kDa, Red arrow head) (Figure 45). This suggests that by inhibiting TG2 cross-linking activity, there is a great reduction in the upper band.

However, the precipitation of the lower band would suggest that TG2 and CD44 are still capable of interacting, mostly likely through TG2-HS interactions.

To further strengthen our preliminary observations, CD44-TG2 cross-linking by TG2 was analysed by co-immunoprecipitation in TG2 knockdown or SDC4 knockdown THP-1/DS, while observing the presence of CD44 in the anti-TG2 (TG100) immunoprecipitates (Figure 46).

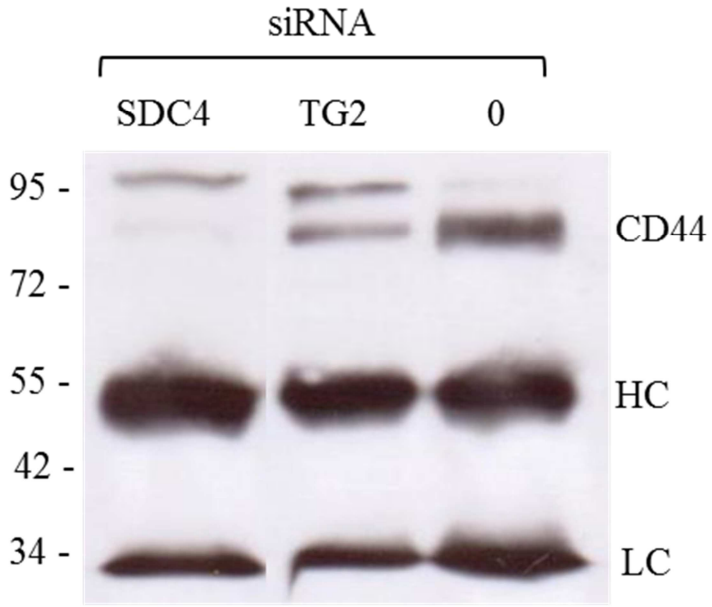


Figure 46. Loss of TG2 or syndecan-4 results in loss of CD44 antigen in TG2 immunoprecipitates. THP-1/DS were transfected with TG2 siRNA or syndecan-4 siRNA using HiPerfect transfecting reagent. 150µg of protein equivalent cell lysates of TG2 or syndecan-4 knockdown along with non-transfected THP-1/DS were pre-cleared with protein A-Sepharose beads slurry and incubated with TG2 antibody (TG100). So formed immuno-complexes were precipitated with protein A-Sepahrose beads, extracted into laemmli buffer and were resolved by SDS-PAGE using 8% gels and western blotting. Membranes were immunoblotted using anti-CD44 antibody, to detect CD44 antigen in the anti-TG2 immuno-complex. Antibody heavy chain (HC) and light chain (LC) were marked. Molecular weight standards shown in kDa.

Loss of TG2 or syndecan-4 (which reduces cell surface TG2 expression and activity; Figure 35A & 36) resulted in the loss of the low molecular weight form of CD44 (Figure 46) suggesting that TG2 interacts with CD44. However, CD44 interaction with syndecan-4, a known binding partner for TG2 on MØ cell surface cannot be ruled-out. To address the

possible interaction between syndecan-4 and CD44, syndecan-4-CD44 interaction was analysed by co-immunoprecipitation in TG2 knockdown THP-1/DS (Figure 47).

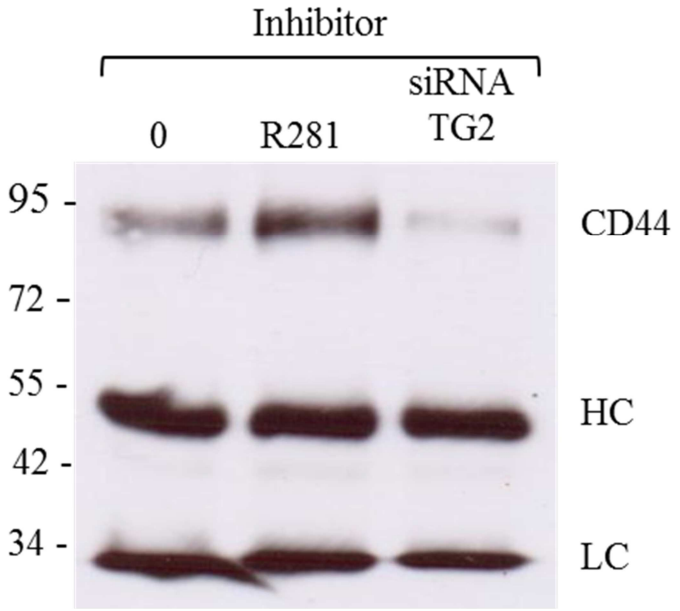


Figure 47. Loss of TG2 results in loss of CD44 antigen in syndecan-4 immunoprecipitates. THP-1/DS were transfected with TG2 siRNA using HiPerfect transfecting reagent. 150µg of protein equivalent cell lysates of TG2 knockdown along with non-transfected THP-1/DS and R281 treated THP-1/DS were pre-cleared with protein A-Sepharose beads slurry and incubated with syndecan-4 antibody. So formed immuno-complexes were precipitated with protein A-Sepahrose beads, extracted into laemmli buffer and were resolved by SDS-PAGE using 8% gels and western blotting. Membranes were immunoblotted using anti-CD44 antibody, to detect CD44 antigen in the anti-syndecan-4 immuno-complex. Antibody heavy chain (HC) and light chain (LC) were marked. Molecular weight standards shown in kDa.

Interestingly, loss of TG2 results in loss of CD44 in syndecan-4 immuno-complex suggesting that CD44 interacts with TG2 and not with syndecan-4. Taken together, it is quite evident that TG2 interacts with CD44, on the MØ cell surface and this crosslinking can be a possible TG2 mediated molecular mechanism in MØ clearance of AC.

6.4 Discussion

Interaction of cell surface adhesion molecules either with components of extracellular matrix or with neighbouring cells is known to influence various cell behaviours including adhesion, migration, proliferation and signalling (Fadok et al., 1998, Pure and Cuff, 2001, Ponta et al., 2003). Cell surface CD44 is one such cell adhesion receptor known for its interaction with its principal ligand hyaluronic acid (HA), a polymer with repeating disaccharide units of N-acetyl-o-glucosamine and D-glucuronic acid (Underhill, 1992, Knudson, 1998). CD44 is also well known for its active participation in a number of interactions with ECM components like fibronectin, collagen, laminin, osteopontin, and matrix metalloproteinases (MMPs) (Weber et al., 1996, Cichy and Pure, 2003, Ponta et al., 2003).

CD44 is known to be expressed in several cell types, including erythrocytes, epithelial cells, endothelial cells, fibroblasts and smooth muscle cells, glial cells, leukocytes and a variety of tumour cells (Xu et al., 2002, Lesley et al., 1993, Alam et al., 2004, Cao et al., 2006, Rajarajan et al., 2008, Vigetti et al., 2008, Shinoe et al., 2010). CD44 is even found to be expressed in embryo (Wheatley et al., 1993). CD44 plays a critical role in immune cells modulating proliferation and differentiation, cell adhesion, migration, and inflammation (Fadok et al., 1998, Pure and Cuff, 2001, Ponta et al., 2003). In cooperation with HA, CD44 is known to regulate directed migration in neutrophils (Alstergren et al., 2004) and through being an E-selectin ligand in co-operation with P-selectin facilitate controlled neutrophil rolling and extravasation to inflamed sites (Katayama et al., 2005). Moreover, it is known for its requirement for peripheral blood monocyte recruitment to sites of inflammation (Guazzone et al., 2005) with reduced numbers of MØ in atherosclerotic lesions from CD44/ApoE double-deficient mice (Cuff et al., 2001). A well-defined role for CD44 in AC binding, ingestion and clearance is evident as mice deficient in CD44 show a moderately decreased clearance of AC following bleomycin-induced lung injury with a 13-fold increase in uncleared AC compared to wildtype mice (Teder et al., 2002). Hart *et al.* (2012) recognised the multifunctional cell surface receptor CD44 as one of the key regulators in defining the MØ capacity of AC clearance confirming that, when cross-linked by monoclonal antibody, augments AC clearance.

Results from chapters 4 and 5 reveal a functional role for MØ cell surface TG2 with a clear role for MØ cell surface TG2 activity in mediating MØ-AC interaction. As CD44 (an HSPG)

cross-linking is required on MØ to augment AC clearance, and that TG2 exhibits strong cross-linking activity, it is a possibility that TG2 may interact with CD44 to promote AC clearance. Work presented here shows CD44 is expressed in TG2-expressing MØ (THP-derived and HMDMØ) suggesting that TG2 might exert its effect through CD44. Interestingly, two distinct molecular weight CD44 bands equivalent to ~130-160kDa and ~80-90kDa were detected (which may be due to heterogeneous glycosylation). CD44 exists in variant isoform as a result of alternative splicing or variable glycosylation (Screaton et al., 1992, Skelton et al., 1998, Levesque and Haynes, 1999, Ponta et al., 2003) and the observed CD44 bands were in line with earlier findings (Jalkanen et al., 1986, Stamenkovic et al., 1989). CD44s ('standard') is a single chain type1 transmembrane protein containing 341 amino acids is the most prevalent form with a molecular mass of 40kDa. However, extensive post-translation glycosylation results in CD44s of 80kDa (Underhill, 1992, Mackay et al., 1994). In terms of TG2, the high molecular weight CD44 was assumed to be a possible TG2 cross linked isoform. As, MØ-AC interaction studies with TG2 inhibitors revealed their ability to inhibit the cross-linking activity of TG2, treatment with TG2 inhibitors should result in loss of high molecular weight CD44 band as a result of loss of TG2 activity. In agreement with the hypothesis, inhibition of TG2 with a cell-impermeable inhibitor resulted in a significant loss in the high molecular CD44 band detected by western blotting. This was associated with an increase in the relative intensity of the low molecular weight CD44, suggesting a possible interaction between TG2 and CD44. As MØ CD44 cross-linking is known to mediate augmented AC clearance (Hart et al., 2012), loss of assumed TG2-cross-linked CD44 band as a result of treatment with TG2 inhibitor implies that TG2 may be a possible CD44 cross-linker favouring CD44 mediated MØ clearance of AC.

In agreement with the western blotting analysis, co-immunoprecipitation studies revealed an interaction between TG2 and CD44, with the expression of both high and low molecular weight bands of CD44. However, it is not clear whether the high molecular weight band is a CD44-CD44 cross-linked form or CD44 cross-linked to other partner molecules, including TG2. Future work looking at the presence of TG2 in high molecular weight band of CD44 by mass spectrometry analysis could suggest the presence of TG2 and CD44 together in the high molecular band. If TG2 is interacting with CD44 via HS, there might be a possibility that the TG2 will not be the final component that is covalently linked to CD44 as it gets dissociated in the western blot analysis, unless the TG2-CD44 association is SDS resistant. (Iismaa et al

2009). Similarly, if TG2 is not seen in the high molecular weight band of CD44, it can be presumed that the upper band may be a CD44 containing complex that was generated by TG2 activity and TG2 is not a final component of the CD44 complex. Here, cross reactivity of the anti-TG2 antibody used with CD44 (Mohan et al., 2003) can be ruled out as TG100 cannot recognise CD44 (Hodrea et al., 2010). Co-immunoprecipitation studies of TG2 revealed complete loss of the high molecular weight CD44 band upon treatment with TG2 inhibitors. This suggests TG2 activity at the cell surface is responsible for the formation of the high molecular mass CD44 band through possible cross-linking of CD44. Furthermore, loss of TG2 or syndecan-4 (which reduces cell surface TG2) resulted in reduced co-precipitation of the low molecular weight CD44 band. This confirms that TG2 interacts with CD44 via heparin binding sites possibly providing a physiological equivalent of monoclonal antibody cross-linking which contributed for augmented AC clearance by MØ (Hart et al., 2012). Moreover, loss of TG2 resulted in loss of CD44 in syndecan-4 immuno-complex suggesting that TG2 interacts with CD44 and not with syndecan-4. Taken together, these data would support the hypothesis that TG2 exerts its effect on augmenting MØ clearance of AC, at least in part, through CD44 cross-linking while binding MFG-E8, a protein known to bridge another binding partner for TG2, β3 integrin to AC and mediate its uptake via signalling the recruitment of CrkII-DOCK180-Rac1 complex and thus activating GTPase Rac1 (Hanayama et al., 2002, Akakura et al., 2004, Toth et al., 2009) (Figure. 48). Although data presented here suggest that TG2 mediated CD44 cross-linking augments AC clearance by MØ, further work is required to assess the details of this interaction and cross-linking e.g. to assess CD44 is homo-dimerised or linked to other partners possibly including TG2. Similarly, loss of CD44 cross-linking as a result of loss of TG2 activity or TG2 expression resulted in reduced MØ clearance of AC, further work needs to look into the effect of MØ clearance of AC due to loss of CD44 expression by knocking down CD44 expression in MØ.

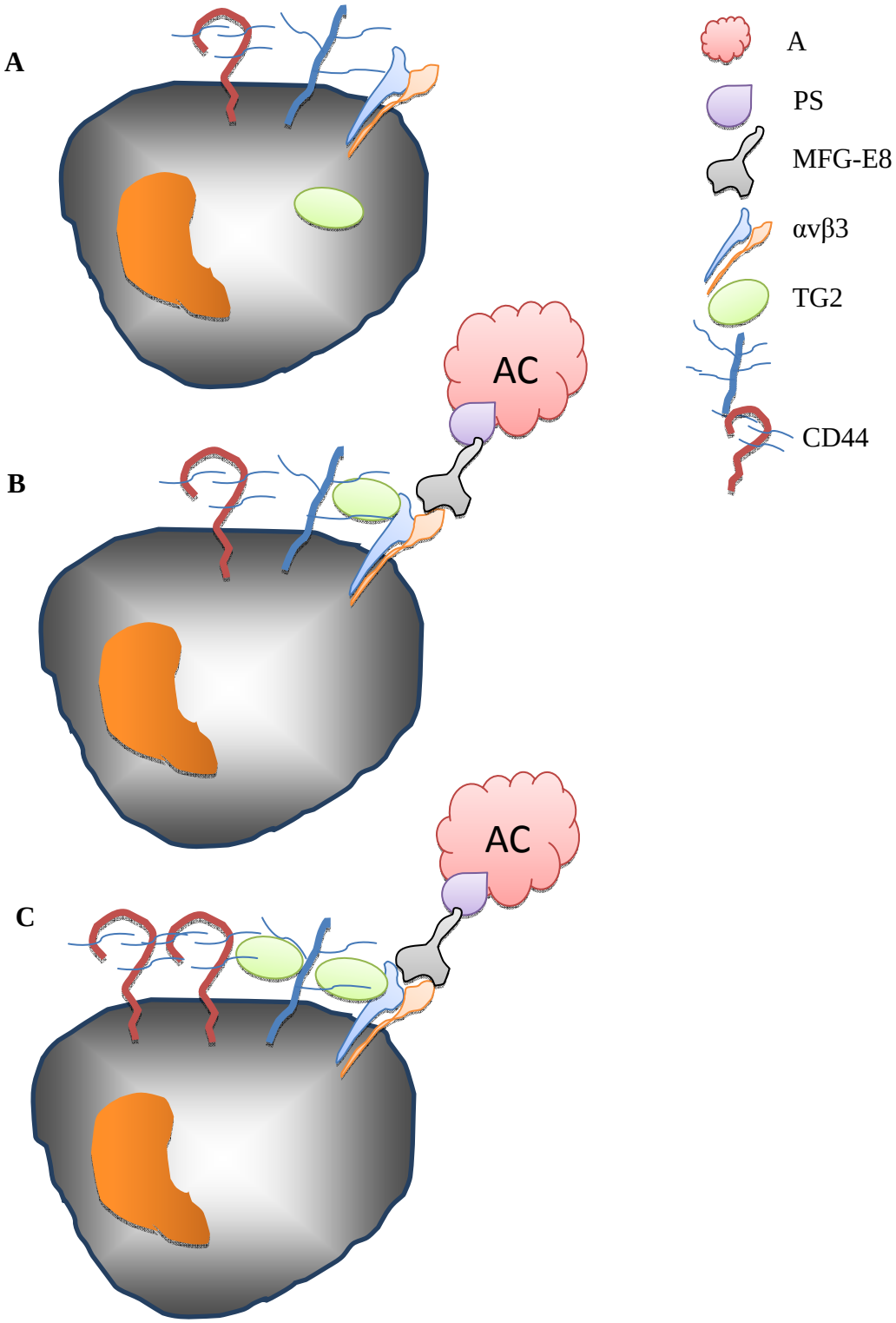


Figure 48. CD44-TG2 cross talk augments macrophage clearance of apoptotic cells. ***A.*** Cytoplasmic TG2 gets externalized and ***B***, binds to HSPGs on the MØ cell surface and interacts with MFG-E8 via integrin β3, which specifically binds AC by recognizing phosphatidylserine (PS), the best characterized 'eat-me' signal expressed on the surface of AC and mediate its uptake while ***C,*** simultaneously cross-linking surface glycoprotein CD44 in the process of augmenting MØ clearance of AC.

CD44 has been implicated in cell adhesion to a variety of matrix components and may be associated with cytoskeletal proteins and intracellular signalling pathways (Lesley et al., 1993). CD44 is widely known for mediating leukocyte migration to sites of inflammation (DeGrendele et al., 1997), a process managed by cytoskeletal rearrangements and downstream signalling events. Earlier studies also emphasised CD44's involvement in cytoskeletal-dependent phagocytosis of heat-killed *Staphylococcus aureus* by polymorphonuclear cells (Moffat et al., 1996), *Mycobacterium tuberculosis* by murine MØ (Leemans et al., 2003) and apoptotic polymorphonuclear leukocytes (PMNs) by human MØ (Hart et al., 1997). MØ-CD44 ligation mediated augmented AC intake is evident both *in vitro* by treating mice MØ with CD44 mAb, prior to co-culture with apoptotic neutrophils (Hart et al., 1997, Hart et al., 2012) and *in vivo* by injecting PMN labelled with 5-chloromethylfluorescein diacetate (CMFDA) following injecting CD44 mAb into the peritoneal cavity of wild type or $CD44^{-/-}$ mice and estimating the proportion of MØ which phagocytised apoptotic PMN within 7 min by flow cytometry (Teder et al., 2002, Hart et al., 2012). Studies by Cuff *et al*. (2001) demonstrated CD44 promotes MØ recruitment to atherosclerotic lesions (sites of extensive cell death), while MØ from $CD44^{-/-}$ mice showed reduced migration into areas of inflammation (Stoop et al., 2002) and a defective clearance of AC (Teder et al., 2002). Cross-linking CD44 may signal within the cells as the cytoplasmic domain of CD44 has been reported to interact with the cytoskeletal and intracellular transduction molecules (Isacke, 1994). With reference to MØ migration studies from chapter 4 (Figure. 31), loss of CD44 cross-linking due to loss of TG2 activity as a result of TG2 inhibitors could be the possible explanation for reduced migration and directionality towards chemoattractants. Moreover, altered cytoskeletal dynamics following CD44 cross-linking may alleviate MØ interaction with AC as members of ezrin/radixin/moesin (ERM) family (Tsukita et al., 1994) and *src* family kinases (Ilangumaran et al., 1998) are established as molecular linkers of CD44 and actin-based cytoskelton. As with professional MØ, several semi-professional, so-called 'amateur' phagocytes including fibroblasts, endothelial, epithelial and mesenchymal cells (Gregory and Devitt, 2004, Devitt and Marshall, 2011) are known to express TG2 (Olsen et al., 2011, Oh et al., 2013) and CD44 (Rajarajan et al., 2008, Vivers et al., 2002). Given that CD44 is expressed in 'amateur' phagocytes and so is TG2, a similar AC clearance mechanism by amateur' phagocytes can be anticipated. However, in terms of activity of Rho family GTPases which has been linked to control of AC phagocytosis (Leverrier and Ridley, 2001, Hart et al., 2012) have shown a specific induction

of Rac2 activation in mouse MØ following CD44 mAb treatment, which is not expressed by non-professional phagocytes such as fibroblasts. This may provide a clue that CD44 cross-linking may not augment AC clearance in amateur phagocytes as with in professional MØ.

TG2 mediated CD44 cross-linking leading to altered cytoskeletal dynamics may result in reduced MØ migration favouring stabilised interaction of MØ with AC. Taken together the above results possibly identify a previously unrecognized role for TG2 in AC clearance by MØ suggesting that TG2 and CD44 may work together to mediate MØ migration to and clearance of AC.

Chapter 7

Discussion & Future Work

Chapter 7

7.1 Discussion & Future work

Prompt and efficient removal of apoptotic cells is needed to prevent undesired immune responses to apoptotic cell-derived potential self-antigens, which may lead to chronic inflammatory and autoimmune diseases (Peng and Elkon, 2011, Miyanishi et al., 2012, Nagata et al., 2010, Munoz et al., 2010). Substantial evidence has been generated over the years in revealing the molecular mechanisms surrounding phagocytic clearance of apoptotic cells. Removal of apoptotic cells by professional phagocytes involves an array of phagocyte receptors which directly or indirectly, via many soluble intermediate bridging molecules facilitate recognition of apoptotic cell-associated ligands and their uptake (Savill et al., 2002). Deficiency of molecules involved in the clearance of apoptotic cells such as MFG-E8 or C1q can result in activation of the immune system and can lead to systemic lupus erythematosus (SLE)-type chronic autoimmune diseases (Pickering and Walport, 2000, Yamaguchi et al., 2010). The MFG-E8$^{-/-}$ mice develop splenomegaly and suffer from glomerulonephritis as a result of autoantibody production (Hanayama et al., 2006). An understanding of the molecular mechanisms involved in the clearance of apoptotic cells may provide substantial evidence associated with autoimmunity and potential therapies. As evident from TG2 knockout studies, TG2 is reported to play a prominent role in the clearance of apoptotic cell clearance, a process known to be immunosuppressive and anti-inflammatory (Szondy et al., 2003).

TG2 is a multi-functional extracellular protein (Belkin, 2011, Wang and Griffin, 2012) known to accumulate in cells undergoing apoptosis, under both *in vivo* and *in vitro* conditions (Fesus, 1993, Szondy et al., 2003). It is also known to correlate with apoptosis induction. However, studies have reported that TG2-mediated cross-linking of intracellular proteins is not essential to the apoptotic process (Griffin et al., 2002). Other studies demonstrate that TG2 knockout mice do not display a morphological phenotype associated with dysregulated apoptosis (De Laurenzi and Melino, 2001) suggesting that other TG enzymes (Grenard et al., 2001) could be compensating for the loss of TG2 or that TG2 is not so important. Moreover, Deasey *et al.* (2013) have shown that the compensation shown by other TG members is tissue specific and includes transcriptional and functional compensation. The extent of redundancy within the TG family with respect to apoptotic cell clearance is not known. There is still

extensive evidence indicating that TG2 is up-regulated in cells that are more prone to, or destined to undergo, programmed cell death (Piacentini et al., 2002). From the apoptotic cell point of view, death might trigger TG2 expression. This may not be needed for AC clearance but may be a cause or an effect of the apoptotic process. However, increased expression of TG2 occurs in the process of monocyte differentiation to MØ (Akimov and Belkin, 2001) and it is thought to contribute to formation of the phagocytic portal in association with the phagocytic receptor, $\alpha_V\beta_3$ and its bridging molecule, MFG-E8, which specifically binds phosphatidylserine (PS) on the surface of AC, in the process of apoptotic cell engulfment by MØ (Toth et al., 2009). TG2 also mediates cross-talk between MØ and AC via activation of TGFβ1, a well-known immunoregulatory cytokine, which is specifically released by macrophages ingesting AC (Fadok et al., 1998) by the recognition of PS on the AC (Fadok et al., 1992) while TGFβ1 is required for in vivo induction of TG2 in both MØ and AC (Szondy et al., 2003). Defective cross-talk between MØ and AC in TG2 null mice highlighted the role of TG2 in apoptotic cell clearance by MØ. However, the functional role played by TG2, the underlying molecular mechanisms involved and possible TG2 partners that may be involved in the process of MØ clearance of AC are still not fully understood. The current study act to address the role of cell surface TG2 in the human MØ in the process of AC clearance.

This study demonstrates that irreversible TG2 inhibitors (including both cell permeable and impermeable inhibitors) cause a significant reduction in human MØ interaction with AC. The effect of TG2 inhibitors on MØ-AC interaction is seen with both THP-1/MØ and primary HMDMØ. Treatment of MØ alone was sufficient to inhibit AC clearance suggesting human MØ TG2 is key to AC clearance as no TG2 is expressed by apoptotic mutu. Additionally, the ability of cell-impermeable TG2 inhibitors also suggests that cell surface TG2 on the human MØ is key. Given the effect of inhibitors on the MØ and taking the inhibitory effect shown by the cell impermeable TG2 inhibitor R281 into consideration, it can be suggested that TG2 on the human MØ cell surface is crucial in contributing to the uptake of AC by MØ. This is confirmed by the comparable inhibition shown by the extracellular acting inactivating TG2 antibody D11D12. Furthermore, TG2 inhibitors have been shown here for the first time to inhibit MØ tethering to AC through undertaking the MØ-AC assay at 20^oC, a temperature non-permissive for phagocytosis (Devitt et al., 2004). Thus TG2 plays a role in both MØ binding to and subsequent engulfment of AC. In parallel with this TG2 inhibitor approach, a significant reduction in MØ-AC interaction is seen with knock-down of

TG2 using small interfering RNA, again highlighting the need for TG2 in MØ clearance of AC. Importantly, treatment of 'TG2 knock-down MØ' with TG2 (including cell-impermeable) inhibitors together showed no further (i.e. additive) reduction in AC clearance. This suggests TG2 activity exerts most, if not all of its effects on AC clearance at the cell surface of human MØ. Interestingly, the TG2 inhibitor effect is also seen with primary HMDMØ thus confirming THP-1/MØ as an excellent MØ model system for TG2 mediated AC clearance studies.

A dose dependent inhibition of AC clearance by TG2 inhibitors was shown with testing at three different concentration (500, 50 and 5µM), irrespective of the inhibitor used. Inhibition of AC clearance may also be explained by toxic effects by TG2 inhibitors on MØ. However, toxicity studies (XTT assay) showed no sign of altered cellular metabolic activity in TG2 inhibitor treated MØ compared with untreated. Similarly, microscopic observations revealed no sign of alterations in morphology, attachment and spreading with one hour TG2 inhibitor treatment (the inhibitor treatment period for MØ-AC interaction studies). This would suggest that the effects of TG2 inhibition on AC clearance are not the result of reagent toxicity. However, prolonged (2 days) incubation of THP-1/MØ with TG2 inhibitors, especially during the process of THP-1 differentiation to MØ-like cells induced signs of altered cell adhesion as evident from suspension cell counts. This altered MØ adhesion could be the result of non-specificity of the TG2 inhibitors or their role in affecting cell adhesion via TG2 inhibition, as TG2 is well acknowledged as a cell adhesion molecule (Akimov and Belkin, 2001, Verderio et al., 2003, Mangala et al., 2007). As demonstrated by Akimov and Belkin, (2001) and Fok *et al*. (2006), cell surface TG2 was found to be co-localized with β1- and β3-integrins in podosomes (specialized adhesive structures) of adherent macrophages. Knock-down of TG2 via siRNA or treatment with the functional blocking antibody, D11D12 led to significant decrease in cell adhesion and migration of monocytes (Akimov and Belkin, 2001, Fok et al., 2006). In the current study, prolonged MØ treatment with TG2 inhibitors resulted in loss of cell adhesion, though no effect on cell viability is evident from DAPI or acridine orange staining and assessment of nuclear morphology. Treatment with TG2 inhibitors also resulted in reduced MØ migration towards AC and is in line with earlier studies by Balajthy *et al.* (2006) who showed impaired migration by neutrophils in $TG2^{-/-}$ mice. This highlights the importance of TG2 in the migration of human macrophages for the first time. However, effect of TG2 inhibitors on human MØ migration towards AC may or may not be specific and

future work needed to assess the effectiveness of TG2 inhibitors in inhibiting human MØ migration towards other chemo attractants.

Having confirmed whole cell and cell surface TG2 expression in THP-1/MØ (PMA and DS stimulated but not VD3) and HMDMØ, and having seen the effect of TG2 inhibition and TG2-knockdown using siRNA on MØ-AC interaction, the functional role of TG2 in the context of MØ-AC interaction was addressed. Given that the TG2 inhibitors used may affect TG2 function either by inhibiting TG2 activity directly (competitive inhibition) or by changing its conformation (an indirect effect on activity), the effect of the TG2 competitive primary amine substrate, monodansylcadaverine (MDC) was used. MDC blocks TG2 crosslinking with no change in enzyme conformation (Siegel and Khosla, 2007). Inhibition of AC clearance by MDC suggests that TG2 activity is crucial for effective MØ-AC interaction. In parallel, altered cell surface TG2 activity measured via biotin-X-cadaverine incorporation into fibronectin following TG2 inhibitor treatment, TG2 knockdown and the effect of the TG2 activity blocking antibody provides evidence in support of TG2 activity in mediating MØ-AC interaction. Moreover, the use of 'P1 peptide' (a peptide that mimics the heparin binding domain of TG2) resulted in a significant reduction in MØ-AC interaction in comparison to its scrambled analogue. More interestingly, the compensation effect seen with the active TG2 added exogenously suggested that the activity of TG2 is crucial in mediating MØ interaction with AC. Similarly, adding back active TG2 even compensated for the loss of MØ-AC interaction by both TG2 inhibition and TG2 knockdown. Taken together, with no detectable TG2 expression in B cells (viable or apoptotic), it can be concluded that the cell surface TG2 activity in human MØ plays a crucial role in efficient clearance of AC by MØ.

Following this, the current study focussed on identification of possible TG2-binding partners at the MØ cell surface. Though TG2 is predominantly a cytosolic protein that lacks a hydrophobic leader sequence for ER-mediated externalisation, TG2 is exported to the cell surface and associates with extracellular matrix binding proteins including fibronectin, collagen and integrins (Akimov et al., 2000, Aeschlimann and Thomazy, 2000, Balklava et al., 2002, Chau et al., 2005, Telci et al., 2008), Fibronectin is a well-established binding partner for TG2 in the extracellular matrix (Hang et al., 2005). Similarly, TG2 is known to have a very strong affinity for heparin (Gambetti et al., 2005) and interacts with heparan sulphate proteoglycans including syndecan-4 (Scarpellini et al., 2009, Wang et al., 2012).

Thus a possible syndecan-4 mediated TG2 interaction in THP-1/MØ model system was analysed.

Syndecan-4 is expressed in THP-1 cells and in both TG2 expressing (THP-1/PMA and THP-1/DS) and non-expressing THP-1/MØ (THP-1/VD3) MØ models. Through the use of western blotting and immunofluorescence staining of non-permeable macrophages, a clear expression of syndecan-4 in THP-1/DS model system that also shows strong TG2 expression was revealed. Due to time constrains, this study addressed syndecan-4 expression in only THP-1/ MØ and not in primary HMDMØ. Interestingly, as evident from co-immunoprecipitation studies, TG2 was found to interact with syndecan-4 and is consistent with earlier studies (Scarpellini et al., 2009, Lortat-Jacob et al., 2012, Wang et al., 2012). Loss of syndecan-4 by siRNA resulted in significant loss of cell surface TG2 expression thus supporting earlier studies by Scarpellini *et al.* (2009) and Wang *et al.* (2012) who proposed HSPG as mediators for TG2 cell-surface trafficking. Similarly, loss of either syndecan-4 or digestion of cell surface HS chains, upon treatment with heparinase II, resulted in a significant reduction in MØ cell surface TG2 activity and MØ interaction with AC supporting the notion that HSPGs are the cell surface adhesion receptors for externalised TG2 which is known to be in heterocomplex with fibronectin in the extracellular environment (Scarpellini et al., 2009, Telci et al., 2008, Verderio and Scarpellini, 2010). Using a TG2 heparin binding domain mimic peptide 'P1' (Wang et al., 2012) which competes with TG2 for binding to syndecan-4 data here showed a significant reduction in MØ-AC interaction in dose-dependent manner, further strengthening the need for TG2 binding to cell surface syndecan-4. Interestingly, addition of active TG2 exogenously, rescued the loss of MØ-AC interaction seen as a result of treatment with P1 peptide, again suggesting the importance of TG2 activity via syndecan-4 as crucial in mediating MØ interaction with AC.

Syndecan-4 is a signalling molecule, implicated in integrin co-signalling induced by interaction with extracellular TG2 or free TG2 in the extra cellular space (Telci et al., 2008, Verderio et al., 2009). Clustering of syndecan-4 has been shown to initiate a signalling cascade that results in PIP_2-dependent PKCα and Rac1 activation in focal adhesion formation and directional migration acting via the syndecan-4 cytoplasmic domain (Woods et al., 2000) (Lim et al., 2003, Keum et al., 2004, Tkachenko et al., 2005, Tkachenko et al., 2006, Bass et al., 2007). PKCα is also known to serve as a major target for PMA (Kikkawa et al., 1989)

stimulation which results in the expression of TG2 in THP-1/MØ. Moreover, once externalized, available TG2-activating concentrations of Ca^{2+} and subsequent structural changes brought about by HS binding may favour the transition from a closed inactive conformation of TG2 towards an open active form and may thus contribute to extracellular TG2 activity.

Interaction of cell surface adhesion molecules either with components of the extracellular matrix or with neighbouring cells are known to influence cell behaviour including phagocytosis. Cell surface TG2 is known to interact with both integrins and HSPGs (Scarpellini et al., 2009, Wang et al., 2012) thus influencing their synergistic signalling and cell adhesion. HSPGs may also bind TG2 on adjacent cells in the process of cell-cell interaction and signalling. Similarly, TG2 may also bind CD44 (a HSPG), a principal surface receptor for the extracellular matrix molecule hyaluronan and a well-known cell-cell and cell-matrix interaction mediator. CD44 is known for recruiting peripheral blood monocytes to sites of inflammation (Guazzone et al., 2005) and in lymphocyte homing during inflammation (Stoop et al., 2002). Several studies have provided substantial evidence establishing CD44 as a prominent phagocytic regulator (Vivers et al., 2002, Vachon et al., 2006, Hart et al., 2012). CD44 is also implicated in apoptotic cell clearance (Teder et al., 2002) where co-signalling between CD44 and other receptors (e.g. phosphatidylserine receptors, CD36 scavenger receptors, vitronectin receptors ($\alpha_v\beta_3$) and calreticulin (Fadok et al., 1998) (Fadok and Henson, 2003, Gardai et al., 2003)) expressed by phagocytes is evident. Mice deficient in CD44 show defective clearance in AC following bleomycin-induced lung injury suggesting a role for CD44 in AC binding, ingestion and clearance (Teder et al., 2002). CD44 is also recognised as a key regulator in controlling MØ capacity to clear AC as CD44 cross-linking with bivalent monoclonal antibodies profoundly augments phagocytosis of apoptotic neutrophils, an effect which was found to be independent of its principal ligand, hyaluronan (Hart et al., 1997). Given this role of CD44 crosslinking in augmenting AC clearance and the established role of TG2 in cell surface heparin binding and protein cross-linking, the possibility of an interaction between TG2-CD44 was hypothesised. Such an interaction may be mediated by mAb 6B9 which in reality binds to CD44 rather than cell-surface TG2 (Stamnaes et al., 2008) but augments phagocytosis, focus was laid on understanding any functional link between CD44 and TG2 in the context of MØ-AC interaction.

TG2 expressing MØ (THP-1/PMA, THP-1/DS and HMDMØ) express detectable CD44 with two distinct bands, a light band equivalent to ~80-90kDa and an intense larger band equivalent to ~130-160kDa. CD44 exists in various isoforms as a result of alternative splicing or variable glycosylation (Screaton et al., 1992, Ponta et al., 2003). CD44s ('standard') is found to be the most prevalent form of CD44 with molecular mass of 40kDa which with extensive post-translation glycosylation results in CD44s of 80-100kDa (Underhill, 1992, Mackay et al., 1994). Thus the 80-90kDa band noted in MØ in the current study corresponds to this. The higher molecular mass band (130-160kDa) detected by anti-CD44 western blotting, was considered as a possible TG2 cross-linked isoform. Surprisingly, upon treatment with TG2 inhibitor R281, a shift in the intensity of high molecular weight CD44 band (~150-160kDa) to low molecular weight (~80-90kDa) CD44 band was evident suggesting that CD44 cross-linked by TG2 may contribute to the strong, high molecular weight CD44 band. Camp et al. (1991) showed that in resident macrophages, there is a detergent insoluble CD44 fraction, but in elicited macrophages all of the CD44 was detergent soluble. As CD44 (an HSPG) cross-linking has been shown to augment AC clearance (Hart et al., 2012), it is possible that TG2 may interact with CD44 mediated via syndecan-4 binding to promote its crosslinking and AC clearance. In parallel with western blot analysis, CD44 is detectable in the immunoprecipitates of TG2 suggesting interaction between the TG2 and CD44. Co-immunoprecipitation of TG2-CD44 with cell lysates treated with TG2 inhibitor completely abolished the high molecular weight band suggesting dissociation of TG2-CD44 cross-linking upon TG2 inhibitor treatment.

MØ-AC interaction studies in the presence of TG2 inhibitors reveal their ability to inhibit TG2 activity thus contributing to loss of AC interaction. Treatment with TG2 inhibitors should have resulted in loss of the putative TG2 cross-linked high molecular weight CD44 band as a result of loss of TG2 activity. In agreement with this hypothesis, inhibition of TG2 with a cell-impermeable inhibitor R281 resulted in a significant loss of the high molecular CD44 band and increase in the relative intensity of the low molecular weight CD44 suggesting a possible functional interaction between TG2 and the high-molecular weight CD44. As MØ CD44 cross-linking is known to mediate augmented AC clearance loss of assumed TG2-cross-linked CD44 band as a result of treatment with TG2 inhibitor implies that TG2 may be the possible CD44 cross-linker favouring CD44 mediated MØ clearance of AC *in vivo*.

As cell surface TG2 is suggested to be in a syndecan-4 bound form via HS, loss of either TG2 or syndecan-4 resulted in complete loss of TG2-CD44 interaction suggesting that CD44 is cross linked by TG2 via a mechanism requiring binding to cell surface syndecan-4, providing a physiological equivalent of monoclonal antibody cross-linking which contributes to augmented AC clearance by MØ. Moreover, loss of TG2 resulted in the loss of CD44-syndecan-4 interaction, as evident from co-immunoprecipitation studies, suggesting that TG2-CD44 cross-linking is independent of syndecan-4. Taken together, these data support the hypothesis that TG2 exerts an effect on MØ clearance of AC, at least in part, through TG2-CD44 cross-linking. Although the preliminary data presented here suggest that TG2-mediated CD44 cross-linking augments AC clearance by MØ, additional work is required to assess the details of this interaction and cross-linking e.g. to assess if CD44 is homo-dimerised or linked to other partners possibly including TG2 by mass-spectrometry analysis. Similarly, loss of CD44 cross-linking as a result of loss of TG2 activity or TG2 expression results in reduced MØ clearance of AC, further work is needed to detail the effect of MØ clearance of AC due to loss of CD44 expression by knocking down CD44 expression in MØ. Similarly, re-confirming the above using either TG2-/- or CD44-/- *in vivo* systems will provide a more complete understanding of TG2-CD44 interaction leading to augmented macrophage clearance of apoptotic cells. This also provides a platform to further understand and assess whether TG2 is functional for AC clearance on cells that are CD44-/-.

MØ-CD44 augmented AC intake following its crosslinking is evident both *in vitro* and *in vivo* (Hart et al., 1997, Hart et al., 2012) conditions. Earlier studies by Hart *et al*., (1997) ruled out the possibility of antibody-mediated bridging via FcγR while emphasising the requirement for CD44 cross-linking for augmented phagocytosis. Similarly, studies by Hart *et al*., (2012) ruled out serum opsonin-dependent, glucocorticoid-induced Mer/protein S pathways mediated (McColl et al., 2009), antibody-induced CD44 shedding leading to reduced/loss of CD44 expression in CD44 mediated AC clearance augmentation. Earlier studies emphasised CD44 involvement in cytoskeleton-dependent phagocytosis of heat killed *Staphylococcus aureus* by polymorphonuclear cells (Moffat et al., 1996), *Mycobacterium tuberculosis* by murine macrophages (Leemans et al., 2003) and apoptotic PMNs by human macrophages (Hart et al., 1997). Similarly, both ‘inside-out’ and ‘outside-in’ signalling processes that render cytoskeletal alterations lead to macrophage migration and phagocyte portal formation in facilitating AC engulfment. CD44 is known to activate Rap1 protein, a

small Ras-like GTPase or Ras-related protein-1 which binds the α-subunit of integrins via its effector RAPL (Katagiri et al., 2004). Similarly, it even mediates the binding of talin to the β-chains of integrins via Rap1-GTP-interacting adaptor molecule (RIAM). Binding of talin to the cytoplasmic tail of β3-subunit promotes integrin activation (Lee et al., 2009). Simultaneously, 'outside-in' signalling, in response to MØ tethering to AC via SDC4/TG2/MFG-E8/$\alpha_V\beta_3$ could be responsible for complex stimulating formation of the phagocytic cup via actin cytoskeletal remodelling and recruitment of AC.

Altogether, it suggests that CD44 regulates Rap-1-mediated integrin activation through effects on the actin cytoskeleton (Wang et al., 2006), work by Vachon *et al.* (2007) also suggested that CD44 induces 'inside-out' signalling through the GTPase Rap-1 to mediate phagocytosis in murine macrophages. On the other hand, an observed increase in phosphorylation of paxillin (the cytoskeletal protein involved in actin-membrane attachment at focal adhesions and vinculin binding, actin localisation, organisation of podosome-rich regions and activation of Rho family GTPases (Rac2) following CD44 treatment in MØ) predicted possible alterations in cytoskeletal dynamics following CD44 ligation which may have profound effects on MØ migration and capacity for AC clearance. In support of the above observations, Cuff *et al*. (2001) demonstrated CD44 promotes MØ recruitment to atherosclerotic lesions, a site of prominent cell death, while MØ from $CD44^{-/-}$ mice show delayed migration into areas of inflammation (Stoop et al., 2002) and a defective clearance of AC (Teder et al., 2002). Similarly, MØ chemotaxis studies reveal loss of TG2 activity, as a result of TG2 inhibitors led to delayed and non-directional migration suggesting TG2 has a direct impact on CD44 mediated clearance, emphasising the need of TG2 activity in the overall process of AC clearance by MØ.

CD44 is known to interact with members of the ERM family of cytoskeletal proteins (that includes ezrin, radisin and moesin) which function as cross-linkers between plasma membrane and the actin-based cytoskeleton (Legg et al., 2002). In resting cells, CD44 is constitutively phosphorylated at Ser325. However, protein kinase C (PKC) activation results in dephosphorylation of Ser325 and phosphorylation of Ser291 of the cytoplasmic domain of CD44 leading to disassociation of CD44-ezrin complex and controlled directional migration (Legg et al., 2002). On the other hand, activation of PKC following PMA treatment alters CD44 binding to its native ligand HA and acts as a substrate for PKC phosphorylation. Kim

et al. have shown CD44 mediated cell motility via activating PKCα signalling involving cytoskeletal rearrangements (Kim et al., 2008). This is coincidental with PMA-mediated PKCα activation leading to differentiation of THP-1 and increased TG2 expression or syndecan-4 cytoplasmic domain mediated PKCα activation via binding phosphatidylinositol 4, 5-bisphosphate (PIP2), following binding of cell surface TG2 and regulators of TG2 to syndecan-4 which can also regulate TG2 activity (Scarpellini et al., 2009, Oh et al., 1998). This suggests that TG2 activation and CD44 function are inter-related and possibly mutually-regulated with respect to macrophage clearance of apoptotic cells.

Taken together, it may be suggested that cell surface TG2 has a novel role in human MØ clearance of AC and the current study indicates that HSPG-bound TG2 at the MØ cell surface may interact with MØ-CD44, promoting its cross-linking, which in turn regulates MØ migration to and, binding of AC and resulting in subsequent clearance (Figure. 49).

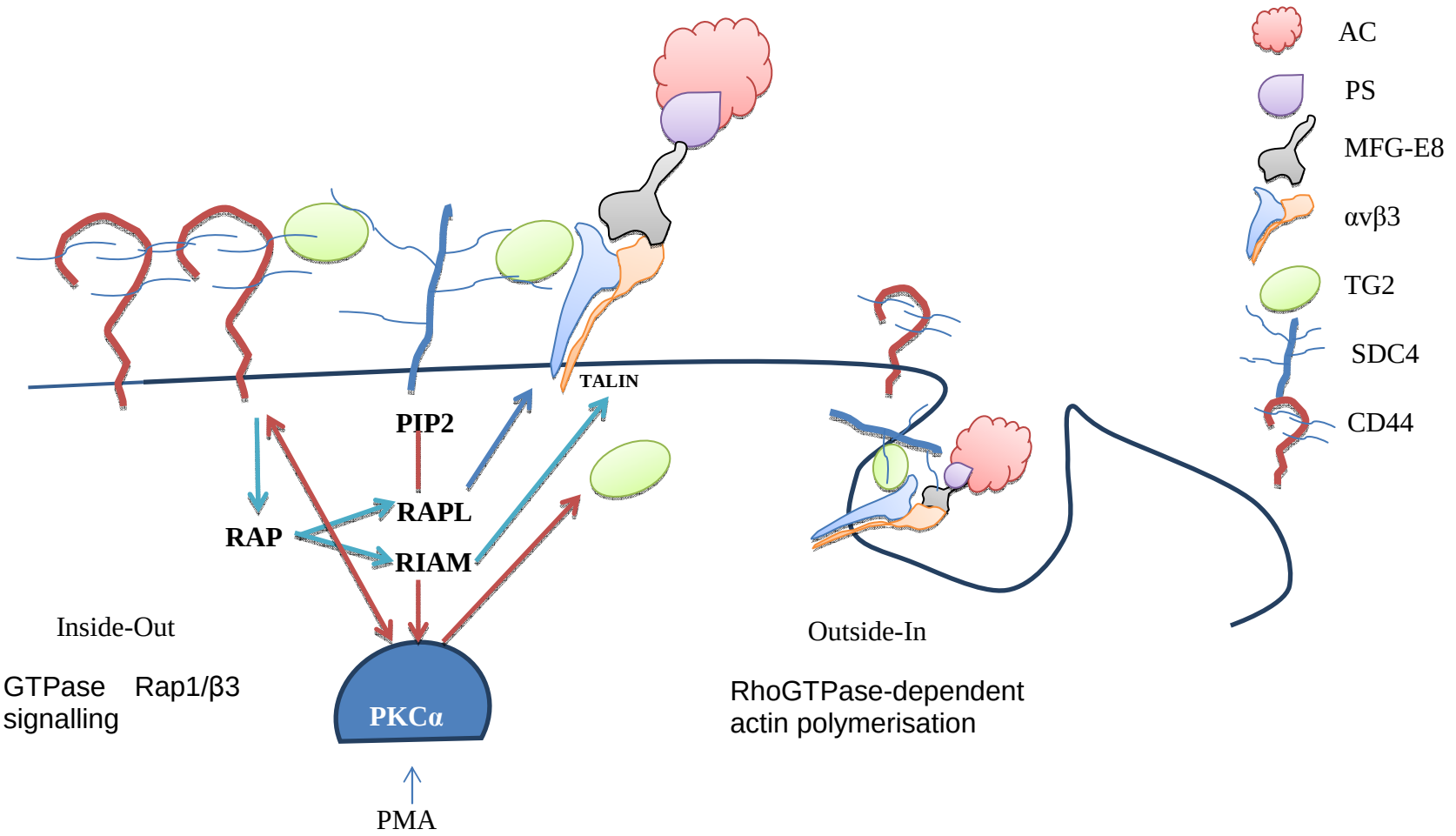


Figure 49. Possible mechanism by which TG2 may mediate macrophage clearance of apoptotic cells. HSPG bound macrophage cell surface TG2 mediates AC binding via the bridging molecule MFG-E8 linking PS on AC and macrophage vitronectin receptor. On the other hand, interaction with CD44 can mediate augmented macrophage clearance of AC. Inside-out signalling stimulated with CD44 cross-linking, leading to activation of integrin via RAP1 and PKCα and outside-in signalling following ligand binding leading to integrin clustering and cytoskeletal changes to form the phagocyte portal results in recruitment of apoptotic body within the cell via a RhoGTPase-dependent actin polymerisation manner.

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