# The role of tetraspanins in multinucleated giant cell formation

A thesis submitted in part fulfilment
of the requirements for the degree of
Doctor of Philosophy

by

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November, 2005

**DEDICATED TO MY BROTHER V. DEVARAJAN**

# SUMMARY

The tetraspanins are a superfamily of 4-pass membrane proteins with a wide distribution in multi-cellular organisms. Tetraspanins have been implicated in a number of basic cellular functions and recent findings showed that they are playing roles during pathological conditions such as cancer and viral infections e.g. HIV-1. Their functions appear linked to their ability to form interactions with other membrane proteins like integrins and it is suggested that they act as "molecular facilitators".

A recent report from Takeda and co-workers shed a light on the involvement of tetraspanins in mononuclear phagocyte fusion to form multinucleated giant cells (MGCs). The authors found that antibodies to CD9 and CD81 enhanced the fusion of Concanavalin A stimulated peripheral blood monocytes, and that monocytes/macrophages from CD9/CD81 double knockout mice showed an enhanced MGC formation (Takeda *et al*, *J. Cell. Biol.* **161**:945-956). This thesis describes further attempts to investigate the role of tetraspanins in MGC formation, primarily using recombinant proteins corresponding to their large extracellular (EC2) domains expressed as GST fusion proteins. Using a Concanavalin A induced monocyte fusion system, we confirmed that antibodies to CD9 enhanced MGC formation. However, in contrast to Takeda *et al* (2003), we found that various anti CD63 antibodies inhibited this function. Recombinant EC2CD9 and EC2CD63 inhibited MGC formation, whereas EC2CD81, EC2CD82, EC2CD151 and GST had no effect. The recombinant proteins did not appear to affect cell numbers and did not inhibit aggregation or normal monocyte adhesion, which are the pre-requisites for fusion. Flow cytometry analysis using EC2 tetraspanins labelled with fluorescent dyes indicated that EC2CD63 and EC2CD9 are binding specifically to the Concanavalin A stimulated monocytes.

In addition this thesis reports attempts to characterise a single chain Fv (scFv) antibody with putative specificity for CD63. This had been isolated using combinatorial library techniques from a patient with ocular melanoma by a previous worker. Studies indicate that although it binds to melanoma cells, the antibody is not specific to human CD63.

# ACKNOWLEDGEMENTS

# Abbreviations

| | |
|---|---|
| ADAMS | A disintegrin and a metalloproteinase |
| AGM | African green monkeys |
| aka | Also known as |
| AP | Adaptor Protein |
| APC | Antigen Presenting Cell |
| ARC | Australian Research Council |
| BCR | B-cell receptor |
| BFA | Brefeldin A |
| BITC | B-isothiocyanate |
| BMS | Biomedical Science |
| BSA | Bovine Serum Albumin |
| BSS | Balanced Salt Solution |
| CD | Cluster of Differentiation |
| cDNA | Complementary DNA |
| CDV | Canine distemper virus |
| CHAPS | 3-[(cholamidopropyl)-dimethylammonio]-1-propane sulphonate |
| $C_H1$ | Constant region (1) of heavy chain |
| CHO | Chinese Hampster Ovary |
| Con A | Concanavalin A |
| CR | Complement receptor |
| CSF | Cerebral spinal fluid |
| Da | Daltons |
| DC | Dendritic cells |
| DGM | Division of Genomic Medicine |
| DMEM | Dulbecco's Modified Eagles Medium |
| DMSO | Dimethyl Sulphoxide |
| DNA | Deoxyribonucleic acid |

| | |
|---|---|
| DNP-HSA | Dinitrophenol covalently linked to human serum albumin |
| dNTPs | Deoxyribonucleotide triphosphates |
| ds | Double-stranded |
| EC | Extracellular |
| EC2 | Extracellular region 2 |
| ECM | Extra-cellular matrix |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylene diamine tetra-acetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Endoplasmic Reticulum |
| EST | Expressed Sequence Tag |
| EtBr | Ethidium bromide |
| Fab/F(ab)$_2$ | Fragment antigen binding (univalent) /Fragment antigen binding (divalent) |
| FACS | Fluorescence Activated Cell Sorter |
| FAK | Focal Adhesion Kinase |
| FBGC | Foreign body giant cells |
| Fc | Fragment crystalline |
| FCS | Foetal Calf Serum |
| Fc□RI/Fc□RIβ | High affinity IgE receptor/ β chain |
| FcγRII/ FcγRIII | Low affinity IgG receptors |
| FITC | Fluorescein Isothiocyanate |
| FIV | Feline immunodeficiency virus |
| GFP | Green Fluorescent Protein |
| GPCR | G-protein coupled receptor |
| GPI | Glycosyl-phosphatidylinositol |
| GST | Glutathione S-transferase |
| hr | Hour |
| hrs | Hours |

| | |
|---|---|
| H chain | Heavy chain of antibody |
| HCV | Hepatitis C Virus |
| HDL | High-density Lipoproteins |
| HEPES | N-2-hydroxyethylpiperazine-N-2-ethane-sulphonate |
| HIV-1 | Human Immunodeficiency Virus-1 |
| HLA | Human leucocyte antigens |
| HMW-MAA | High molecular weight melanoma associated antigen |
| HPLC | High performance liquid chromatography |
| HRP | Horse radish peroxidase |
| HSC | Hepatic stellate cells |
| HTLV-1 | Human T-cell leukemica virus type-1 |
| HUVEC | Human Umbilical Vein Endothelial Cells |
| $IC_{50}$ | 50% inhibitory concentration |
| ICAM | Intercellular Adhesion Molecules |
| IFN-$\gamma$ | Interferon-gamma |
| Ig | Immunoglobulin |
| IGSF | Immunoglobulin superfamily |
| IL | Interleukin |
| INF | Interferon |
| IPTG | Isopropyl-$\beta$-D-galactosidase |
| ITIM | Immunoreceptor tyrosine based inhibitory motif |
| kb | Kilobase (s) |
| kDa | Kilo Dalton (s) |
| LB | Luria-Bertani |
| LBP | Lipopolysaccharide binding protein |
| L chain | Light chain of antibody |
| LEL | Large extracellular loop |
| LFA-1 | Leukocyte Function Antigen-1 |

| | |
|---|---|
| LGC | Langhans giant cells |
| LPS | Lipopolysaccharide |
| MAA | Melanoma associated antigen |
| mAb | Monoclonal antibody |
| MAP | Mitogen-Activated Protein |
| MDCK | Madin-Darby Canine Kidney |
| MDP | Muramyl-dipeptide |
| MFI | Mean Fluorescence Intensity |
| MFR | Mannosyl-fucosyl receptor |
| MGCs | Multinucleated giant cells |
| MHC | Major Histocompatibility Complex |
| MMP | Matrix metalloproteinases |
| MPS | Mononuclear phagocyte system |
| MQ | Milli-Q water |
| MT1-MMP | Membrane type-1 matrix metalloproteinases |
| $\mu g$ | Microgram |
| $\mu F$ | Micro- Farade |
| $\mu l$ | Microlitre |
| $\mu m$ | Micrometre |
| $\mu g.ml^{-1}$ | Microgram per ml |
| min | Minute(s) |
| NK | Natural Killer |
| NP40 | Nonidet P40 |
| NRK | Normal Rat Kidney |
| O.D. | Optical Density |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PEG | Polyethylene glycol. |
| PHA | Phytohemagglutinin-A |
| PI3-kinase | Phosphatidylinositol 3-kinase |

| | |
|---|---|
| PI4-kinase | Phosphatidylinositol 4-kinase |
| PMA | Phorbol mystirate acetate |
| PKC | Protein Kinase-C |
| ppm | Parts per million |
| PSG | Pregnancy specific glycoproteins |
| RBL | Rat Basophilic Leukaemia |
| RD | Rhabdomyosarcoma |
| Rhod BITC | Rhodamine B isothiocyanate |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| RPMI | Roswell Park Memorial Institute |
| RT | Room temperature |
| scFv | Single chain fragment variable |
| SDS-PAGE | Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis |
| SEL | Small extracellular loop |
| s/n | Supernatant |
| sq. in. | Square inches |
| Sulpho-NHS | Sulpho-N-hydroxysulfosuccinimide |
| SRB | Sulphorhodamine B |
| s.s. | Single-stranded |
| TAE | Tris acetate EDTA |
| *Taq* | *Thermus aquaticus* |
| TCA | Tri-chloroacetic acid |
| TCR | T-cell receptor |
| TEM | Tetraspanin enriched micro domain |
| Th | T-helper lymphocyte |
| TM | Transmembrane |
| TMB | Tetramethyl benzidine |
| TM4SF | Transmembrane 4 super family |
| TNF | Tumour necrosis factor |

| | |
|---|---|
| Tris | Tris (hydoxymethyl) methylamine |
| TRITC | Tetramethyl Rhodamine Isothiocyanate |
| Tween-20 | Polyoxyethylene sorbitol monolaurate |
| UL | Unlabelled |
| UV | Ultra Violet |
| V | Voltage |
| v | Volume |
| WPBs | Weibel-Palade Bodies |
| WT | Wild type |
| X-gal | 5-Bromo-4-chloro-3 indolyl-$\beta$-D-galactopyranoside |

| 1 letter code | 3 letter code | Amino acids |
| --- | --- | --- |
| A | Ala | Alanine |
| C | Cys | Cysteine |
| D | Asp | Aspartate |
| E | Glu | Glutamate |
| F | Phe | Phenylalanine |
| G | Gly | Glycine |
| H | His | Histidine |
| I | Ile | Isoleucine |
| K | Lys | Lysine |
| L | Leu | Leucine |
| M | Met | Methionine |
| N | Asn | Asparagine |
| P | Pro | Proline |
| Q | Gln | Glutamine |
| R | Arg | Arginine |
| S | Ser | Serine |
| T | Thr | Threonine |
| V | Val | Valine |
| W | Trp | Tryptophan |
| *X* | - | *Any residue* |
| Y | Tyr | Tyrosine |

**Table i**: One- and three-letter abbreviations for amino acids.

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# CHAPTER 1

# INTRODUCTION

Cell surface proteins are of crucial importance because they provide the cell with the means by which to sense, respond to and interact with the environment (Wright and Tomlinsons, 1994). A recently discovered protein family, which gained the attention of the research community, is the tetraspanin superfamily since they display numerous functions that indicate their physiological importance.

## 1.1. The tetraspanin superfamily of membrane glycoproteins

The tetraspanin superfamily is a group of genes that encode cell-surface proteins expressed in humans as well as primitive organisms (Tomlinson and Wright, 1996). The transmembrane proteins of the tetraspanin or transmembrane 4 (TM4SF) superfamily are assembled in multimeric complexes on the cell surface and the spatial orientation of tetraspanins within these complexes may affect signalling functions of the associated transmembrane proteins or receptors such as integrins. Tetraspanins first appeared in multicellular organisms and the family contains more than 30 members in mammals, 37 in *Drosophila* and 20 in *Caenorhabditis elegans* (Stipp *et al*, 2003). 33 tetraspanin genes have been identified in human (P.N.Monk, University of Sheffield, personal communication).

The existence of so many different tetraspanin molecules, as well as their expression in organisms as evolutionarily diverse as humans and schistomes, suggests a key role in biological systems (Wright and Tomlinons, 1994).

The diversity of expression of tetraspanin proteins is impressive and ranges from the developing nervous system, the vascular endothelium and most cells of the immune system. The involvement in function is equally diverse and includes adhesion, cell-cell contact and motility, antigen presentation, cell fusion, viral susceptibility and the control of cancer metastasis (reviewed in Wright and Tomlinson, 1994; Hemler *et al*, 1996; Maecker *et al*, 1997; Boucheix *et al*, 2001; Hemler, 2003; Martin *et al*, 2005).

The understanding of the molecular functions of tetraspanins may be a useful tool for the discovery of drug molecules for various life threatening diseases such as cancer, human immunodeficiency disorders etc.

Over the past few years extensive research has been carried out on the leukocyte tetraspanins such as CD9, CD37, CD53, CD63, CD81, CD82 and CD151 and the present study is focussed on functional characterisation of CD9 and CD63.

### 1.1.1. Molecular cloning and characterisation of tetraspanins

The melanoma associated membrane bound glycoprotein ME491 (later known as CD63) was the first tetraspanin cloned in mouse L-cell (Ltk⁻ cell) lines and characterised in 1988 (Hotta et al, 1988). In 1990 the tetraspanins such as CD9 (Boucheix et al, 1991), CO-029 (Szala et al, 1990), Sm23 (Wright et al, 1990), CD37 (Classon et al, 1990), CD81 [TAPA-1] (Oren et al, 1990) and CD53 (Angelisova et al, 1990) were cloned. Genes with homology to tetraspanins have also been identified in the nematode, *Caenorhabaditis elegans*, as part of its genome project (Stipp et al, 2003). Recently a tetraspanin encoding gene (PLS1) was discovered in the plant pathogenic fungus *Magnaporthe grisea*. The authors also found that the protein deduced from this gene defines a novel class of orthologous tetraspanin specific to fungi (Gourgues et al, 2002).

### 1.1.2. Structural features of tetraspanin proteins

Tetraspanins are 204-(SAS) to 355(oculospanin) amino acid surface proteins. They are characterised by four transmembrane domains (TM1-TM4) delimiting two extra cytoplasmic regions of unequal sizes, a small extracellular loop (EC1) containing 20-27 amino acids and large extracellular loop (EC2) containing 75-130 amino acids. The extracellular domains EC1 and EC2 are punctuated by a single intracellular loop flanked by a short intracellular amino terminus (typically 9-24 amino acids) and typically 4-40 amino acids in the carboxy terminus (Maecker et al, 1997; Berditchevski, 2001; reviewed by Hemler, 2003) (Fig.1.1.1). Apart from the conserved signature sequences (see below), the EC2 regions show the greatest sequence diversity between family members, and between species orthologs. These regions also contain crucial functional sites, and most tetraspanin-specific antibodies bind to the EC2 regions (reviewed in Stipp et al, 2003). The antibody epitope mapping and glycosylation pattern confirmed that the hydrophilic regions are indeed extracellular. In tetraspanin CD9, the small extracellular loop 1 (EC1) is thought to contain a glycosylation site (Boucheix et al, 1991) but in CD37, CD53, CD63 and CD82, glycosylation sites are found in extracellular loop 2 (EC2) region (Wright et al,

1994). Studies from our group indicate that the glycosylation site on CD9 may be on
EC2 (G.Moseley, PhD thesis, University of Sheffield, 2002).

**(A)**
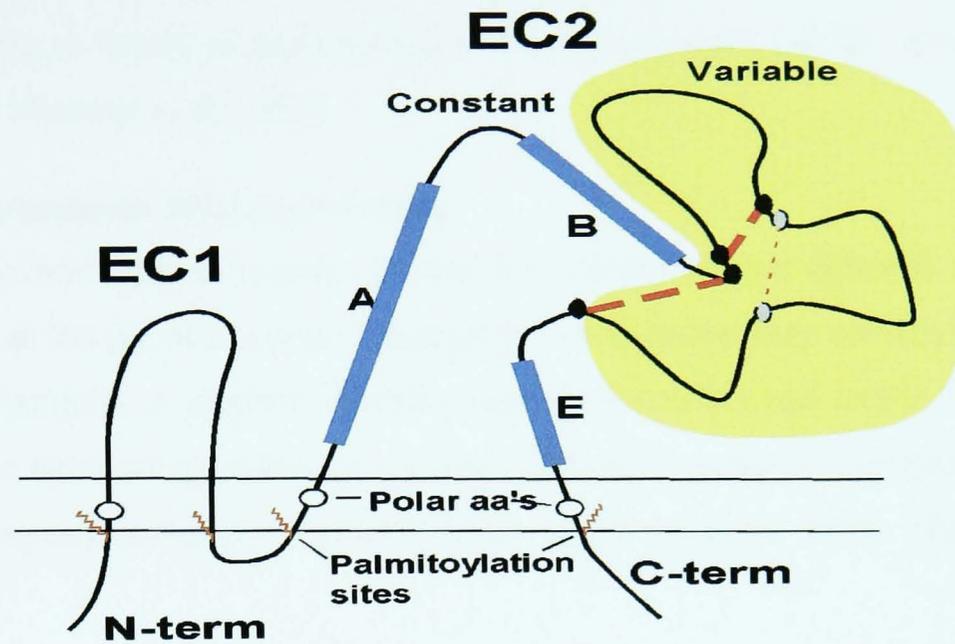


**(B)**
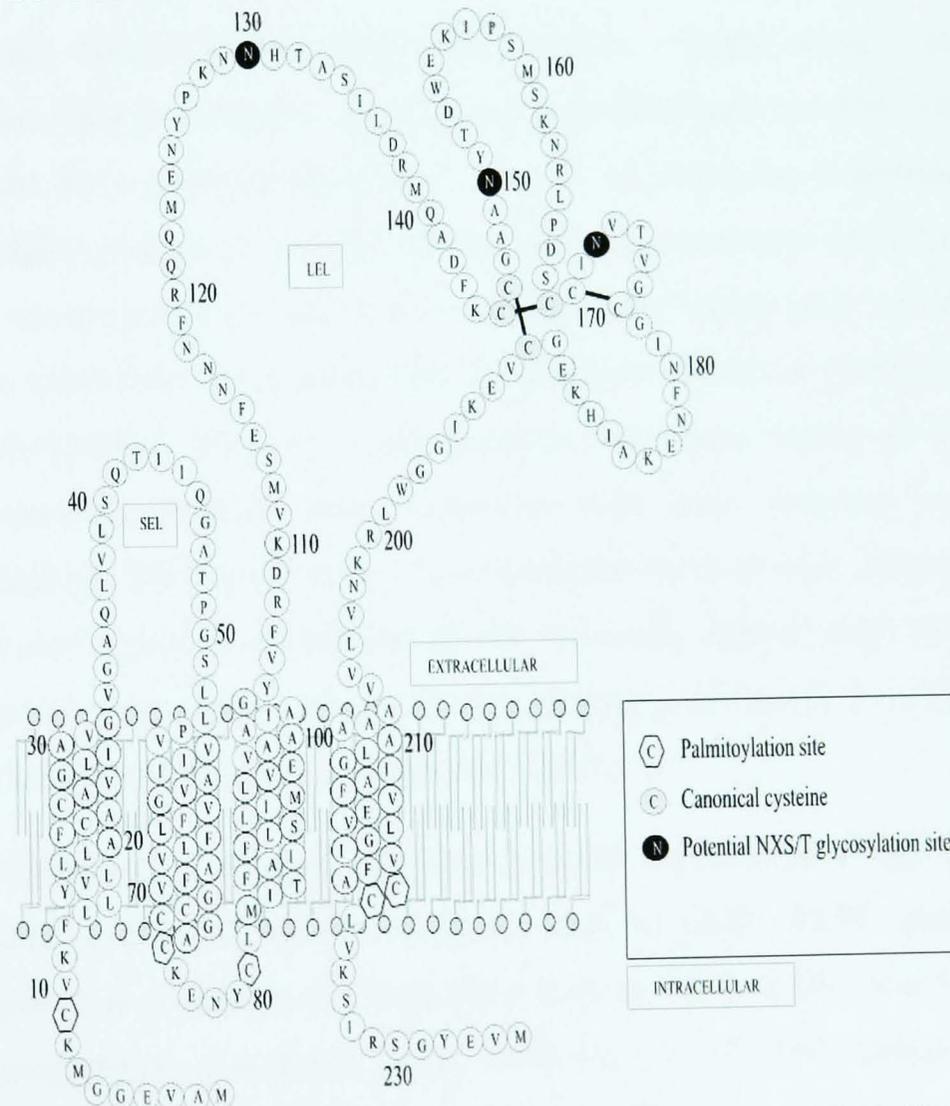


Fig.1.1.1. General structure of tetraspanin (A); structure of tetraspanin CD63 showing conserved amino acid residues (B). Fig.(A) adopted from Hemler,(2003); Fig.(B) courtesy of Dr.P.N.Monk, Dept. of Neurology, University of Sheffield. Where small and large extracellular loops are represented as EC1 or SEL and EC2 or LEL respectively.

The hydrophobic domains are highly conserved within the family and show no significant sequence similarity to other molecules with four transmembrane domains, such as CD20 and FcεR1β, the ligand–gated ion channels (acetyl choline receptor) or the connexins (a family of gap-junction membrane proteins) (Wright and Tomlinson *et al*, 1994; Maecker *et al*, 1997).

### 1.1.2.1. Tetraspanin defining residues

Although several types of proteins contain four transmembrane domains, they are not considered as the members of the tetraspanin family unless they contain many of the conserved residues. A number of highly conserved residues and motifs distinguishes tetraspanins from other proteins with four membrane-spanning domains, which are called tetraspanin-defining residues (Wright and Tomlinson, 1994; Maecker *et al*, 1997).

The transmembrane domain of the tetraspanins contains a larger number of polar amino acid residues than are found in 'single pass' transmembrane domains, although this is not unusual for multi-transmembrane proteins. In particular, transmembrane domain 1 has a highly conserved Asn (N) residue, and transmembrane domains 3 and 4 contain highly conserved Glu or Gln (E/Q) residues. These highly polar residues are of major interest, since their placement in the lipid bilayer would be predicted to be energetically unfavourable. However, such residues have been shown to mediate stable protein assembly through their interaction with polar residues on other transmembrane helices. The highly polar transmembrane residues may interact intra molecularly and play a part in the folding of the molecule; alternatively, they may form intermolecular bonds and be involved in the assembly of complexes with other transmembrane molecules (reviewed in Stipp *et al*, 2003).

The distinctive tetraspanin defining motifs present in the major extracellular domain contain four highly conserved cysteine residues such as CCG, PXSC and ECG. Binding of antibodies to a number of tetraspanins such as CD53, CD81 and Sm23 is sensitive to reducing agents (Tomlinson *et al*, 1993; Levy *et al*, 1991; Oligino *et al*, 1988). At least two of these cysteine residues are likely to participate in the disulphide bonding that is essential to the correct folding of this domain (Levy *et al*, 1991; Tomlinson *et al*, 1993). This has been confirmed for CD81, as described below.

Tetraspanins may contain up to 4 additional cysteines in the EC2 region that may be involved in disulphide bond formation (Seigneuret *et al*, 2001).

The cytoplasmic domains show little sequence similarity between different members of the superfamily, suggesting that these regions might have distinct functions (reviewed in Berditchevski, 2001), although some residues are conserved in most of the classical TM4SF molecules. These include: a Lys (K) residue in the N-terminal region; a Glu (E) residue between transmembrane domains 2 and 3; and an Ile (I) in the C-terminal region (Wright and Tomlinsons, 1994). The cytoplasmic domains are highly conserved with homologues in other species.
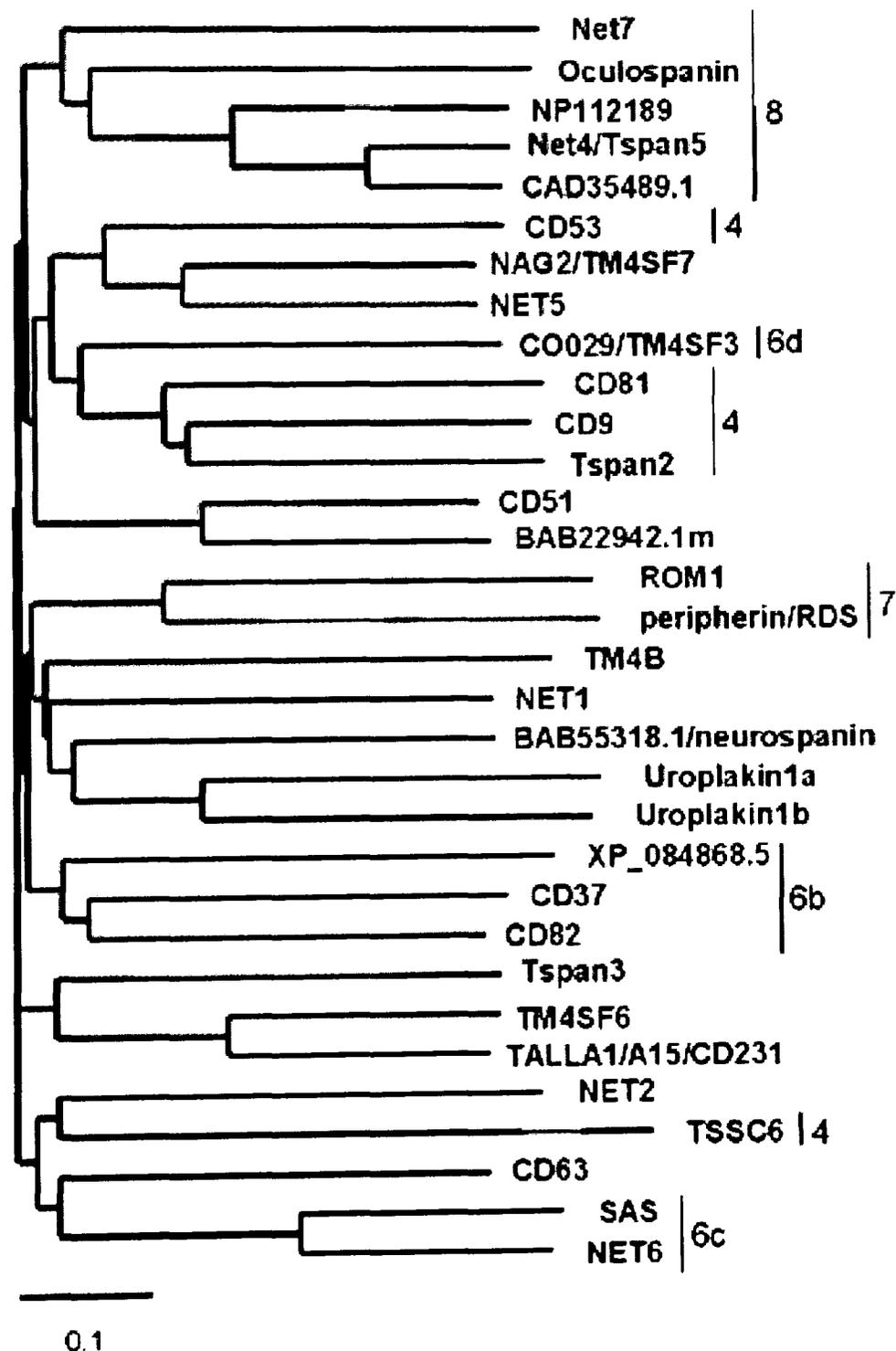
Fig.1.1.2. Evolutionary tree of the tetrasapanins. Image adopted from Hemler, (2003)

5

Peripherin and Rom-1 have long (79-81residues) carboxy-terminal cytoplasmic tails as compared to other family members (Maecker *et al*, 1997).

Distant members of the family have been reported that share the same transmembrane pattern and are related to each other and to the rest of the tetraspanins, based on amino acid homology, but lack the tetraspanins signature e.g. epithelial protein L6, which lacks the highly conserved N (TM1), E/Q (TM3), E/Q in TM4 and the CCG motif in EC2. These are no longer considered true tetraspanins (Wright *et al*, 2000; reviewed in Stipp *et al*, 2003). Figure.1.1.2 predicts the comparative relationship of the tetraspanins on the basis of amino acid homology.

### 1.1.2.2. Crystal structure of human CD81 large extra cellular domain

Kitadokoro and co workers have recently resolved the crystal structure of the large extracellular loop of human CD81 at 1.6 Å resolution using His-tag hCD81-EC2, a purified form of a recombinant protein (Kitadokoro *et al*, 2001) (Fig. 1.1.3).

The hCD81-EC2 is organised in a homodimeric structure, hosting 176 residues and 194 solvent molecules per asymmetric unit. Each subunit in the dimer is composed of five α helices, A, B, C, D and E (Fig.1.1.3). In addition to this a short helical segment covers residues Leu162-Ala 164. The α helices run anti parallel and are arranged in stalk and head sub domains. The helices A and E can be seen as the stalk of a mushroom-shaped molecule, whose head subdomain (~60 residues) is essentially composed of the last two turns of the A helix, the B, C and D helices and their intervening segments and the DE loop. The anti parallel pairing of the N and C terminal α helices allow the two termini of the expressed hCD81-LEL to fall spatially close to each other.

The sub unit fold of hCD81 is stabilised by a number of interactions. The stalk region is held together by hydrophobic contacts, along the A and E helices and by two salt bridges. The head subdomain is stabilised by two tetraspanin invariant disulphide bridges involving four cysteines. The disulfide bonds originate from two adjacent (156 and 157) cysteine residues and stretch approximately in opposite direction within the domain, connecting the C-terminal region of the B-helix (Cys156) to the first turn of the E helix (Cys190) and the BC segment (Cys 157) to the CD loop (Cys175).

The crystal structure of hCD81 contains two extended low polarity regions. The first one is a large hydrophobic cluster and has an area of 986 Å$^2$ and the second low polarity patch is located in a solvent-exposed region comprising the C and D helices. In the crystal, this region is virtually solvent inaccessible and the region is energetically unfavourable and therefore may be prone to mutation by natural selection (Kitadokoro *et al*, 2001).

Using the CD81 EC2 structure as a template, it is suggested the other tetraspanin EC2s are also composed of two subdomains (Seigneuret *et al*, 2001. The first sub domain is relatively conserved and corresponds to the two first helices (A and B) and the last helix (E) of the CD81 EC2 crystal structure. The second subdomain is much more variable between tetraspanins, with an overall structure determined by the conserved disulfide linking and a globally conserved chain topology including two specific residues, glycine and proline, which in each case are neighbour to a disulfide-linked cysteine and promote specific bends of the main chain (Seigneuret *et al*, 2001). The variable regions are thought to be involved in tetraspanin-specific protein-protein interactions whereas the constant region is speculated to be involved in dimerisation (reviewed in Stipp *et al*, 2003). A schematic diagram of this predicted structure is shown in figure 1.1.1.A.
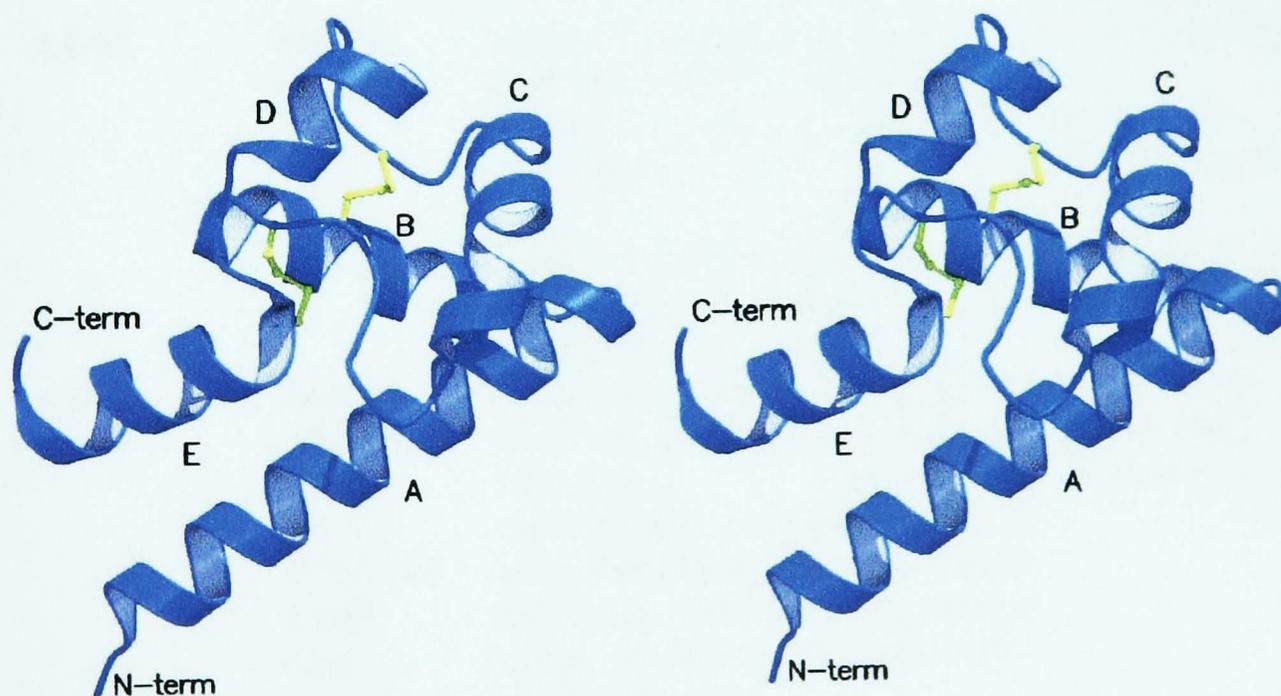


Fig.1.1.3. Crystal structure of large extra cellular domain of CD81

## 1.1.3. Expression pattern of tetraspanins

Some of the tetraspanins such as CD9, CD63, CD81, and CD82 show nearly ubiquitous tissue expression, whereas other tetraspanins such as CD53 and CD37 show a highly restricted pattern of expression. Some members of the tetraspanins appear to be highly expressed in the immune system. More recently it has been found that the tetraspanins are also expressed by the nervous system. The expression pattern of various tetraspanins are summarized in the table.1.1

| Tetraspanin | Other name (s) | Main cellular distribution | Reference |
|---|---|---|---|
| CD9 | MRP-1, DRAP 27 | Platelets, early B cells, activated and differentiating B cells, activated T cells, eosinophils and basophils, endothelial cells, brain and peripheral nerves, vascular smooth muscle, cardiac muscle and epithelial cells, acute myeloid and chronic lymphoid leukaemias, non lymphoid cells. | Rubinstein and de Hass, 1999<br><br>Wright and Tomlinson, 1994 |
| CD37 | MB-1 gp40-50 | Normal and neoplastic mature B cells, resting and activated T cells, neutrophils, granulocytes, monocytes and macrophages. | Schwartz-Albiez *et al*, 1998; Moldenhauer *et al*, 1999<br><br>Wright and Tomlinson, 1994 |
| CD53 | OX-44 | B and T cells, NK ells, Monocytes, macrophages, granulocytes, dendritic cells, osteoblasts, osteoclasts. | Conjeaud *et al*, 1997<br><br>Wright and Tomlinson, 1994 |
|  |  | Low levels of expression on keratinocytes and bladder epithelium. | Okochi *et al*, 1997<br>Okochi *et al*, 1999 |
| CD63 | ME491, NKI/C3, NGA, MLA1, PTLGP40, LIMP, Gp55, granulo-physin, Lamp3 | Lymphoid and non-lymphoid distribution including platelets, monocytes, neutrophils, macrophages, vascular endothelial cells, fibroblasts, glomeruli, sinus histocytes, smooth muscle, neural tissue, osteoclasts, dendritic cells and kupffer cells. | de Haas and Azorsa,1999￼DeHaas *et al*, 1997 |
|  |  | Largely associated with the glandular tissues, lysosomes of melanoma cell and Weibel Palade bodies of endothelial cells | Metzelaar *et al*, 1989￼Nishibori *et al*, 1992￼Smith *et al*, 1997￼Knol *et al*, 1991￼Hara *et al*, 1994 |

| | | | |
|---|---|---|---|
| CD81 | TAPA-1, M38 | Wide range of tissue expression including B and T cells, low expression on monocytes and granulocytes, thymocytes, follicular dendritic cells, eosinophils, monocytes and epithelial cell. | Wright and Tomlinson, 1994 Levy *et al*, 1998 Jones and Mason, 1997 |
| CD82 | C33, IA4, R2, 4F9,KAI1 | B and T cells, monocytes and granulocytes; platelets, NK cells. Various non haemopoietic cells | Lagudriere-Gesbert and Conjeaud, 1997 |
| CD151 | PETA-3, SFA-1 | Most of non lymphoid tissues, platelets, megakaryocytes, HTLV transformed T cells, epithelial cells, smooth and striated muscle and Schwann cells | Ashman *et al*, 1997 |
| L6 | | Lung, breast, colon, ovarian carcinomas, normal epithelial tissue | Marken *et al*, 1992 |
| A15 | TALLA-1 MXS1 CCG-B7 | Brain, skeletal muscle, spleen | Takgi *et al*, 1995 |
| CO-029 | | Colon carcinoma | Sazala *et al*, 1990 |
| Rom-1 | | Eye | Bascom *et al*, 1993 |
| UPIa, UPIb | | Bladder epithelium | Yu *et al*, 1994 |
| Lbm | | Expressed on the axons, terminal arbors, and growth cones of motorneurons of *Drosophila melanogaster* | Fradkin *et al*, 2002 |

Table.1.1. Tissue distribution of tetraspanins.

## 1.1.4. Molecular Associations of tetraspanins

### 1.1.4.1. Tetraspanin enriched microdomains and the tetraspanin web

The ability of tetraspanins to interact with each other, and other transmembrane proteins, has led to the hypothesis that tetraspanins predominantly exist in supramolecular complexes or tetraspanin enriched microdomains (TEMs) (Kurita-Taniguchi *et al*, 2002). The extended network of tetraspanin interactions is often referred to as the "tetraspanin web" (reviewed in Boucheix and Rubinstein, 2001). Not only do tetraspanins associate with each other, but also associate with Ig superfamily proteins, proteoglycans, complement regulatory proteins, integrins, growth factors, growth factor receptors, and signalling enzymes. As tetraspanins have no intrinsic enzymatic activity or signalling motifs, it has been suggested that tetraspanins act primarily as adaptor proteins, facilitating the interaction of associated molecules in tetraspanin signalling networks.

Of specific interest are recent reports evaluating tetraspanin complexes in relation to lipid microdomains ('rafts'). In lipid rafts, protein-protein interactions are facilitated by association with membrane regions enriched for cholesterol and glycosphingolipids (Simons and Ikonen, 1997). Tetraspanin complexes are thought to be autonomous units, existing both within lipid rafts and as non-raft domains (Claas *et al*, 2001; Kropshofer *et al*, 2002).

The assembly of the tetraspanin web/TEMS is based on multiple levels of interactions, which have been divided into three categories based on their susceptibility to different detergents (Boucheix and Rubinstein, 2001; Claas *et al*, 2001; Martin *et al*, 2005).

(a) The first level includes primary interactions between specific tetraspanins and other proteins. These interactions are direct and resist disruption by relatively hydrophobic detergents such as NP40 or Triton X-100. These interactions generally appear to be mediated by the EC2 regions of tetraspanins and can be captured by chemical cross-linking. Examples of primary interactions include that of CD151 with α3β1 integrin (Yauch *et al*, 2000), and CD9 and CD81 with the immunoglobulin gene superfamily (IGSF) members EWI-2 and EWI-F (Stipp *et al*, 2001; Charrin *et al*, 2001). Some homodimeric tetraspanin:tetraspanin interactions (e.g. CD81:CD81) are also considered to be primary interactions (Hemler, 2003).

(b) Second level interactions are indirect, more numerous, and much more sensitive to disruption by digitonin or Triton X-100. Soluble second level complexes are maintained in detergents such as Brij-96, Brij-97. In this type of complex, tetraspanins associate with each other, and thereby link together multiple primary complexes. Secondary interactions appear to involve the transmembrane and/or intracellular regions (Charrin *et al*, 2002). Level 2 associations are promoted or stabilized by tetraspanin palmitoylation (Yang *et al*, 2002).

(c) A third level of tetraspanin complex assembly occurs in the context of complexes stable only in mild detergents such as Brij-99 or and CHAPS ((3-[(3-Cholamidopropyl) dimethyl ammonio]-1 propane sulfonate)]. Tertiary interactions are large complexes containing multiple tetraspanins and associated molecules, and are hypothesised to result from coalescence of

higher order complexes. It has been shown that tertiary interactions can occur independently of lipid rafts or large detergent-insoluble fractions (Claas *et al*, 2001).

Palmitoylation may play a role in the incorporation of tetraspanins into TEMs. Charrin and co workers have found that all tetraspanins studied (CD9, CD37, CD53, CD63, CD81 and CD151) incorporated [$^3$H] palmitate. By site-directed mutagenesis, the authors found that CD9 was palmitoylated at any one of the four internal juxtamembrane cysteines. In addition to these the authors found that the palmitoylation of CD9 did not influence the partition in detergent-resistant membranes but contributed to the interaction with CD81 and CD53 (Charrin *et al*, 2002).
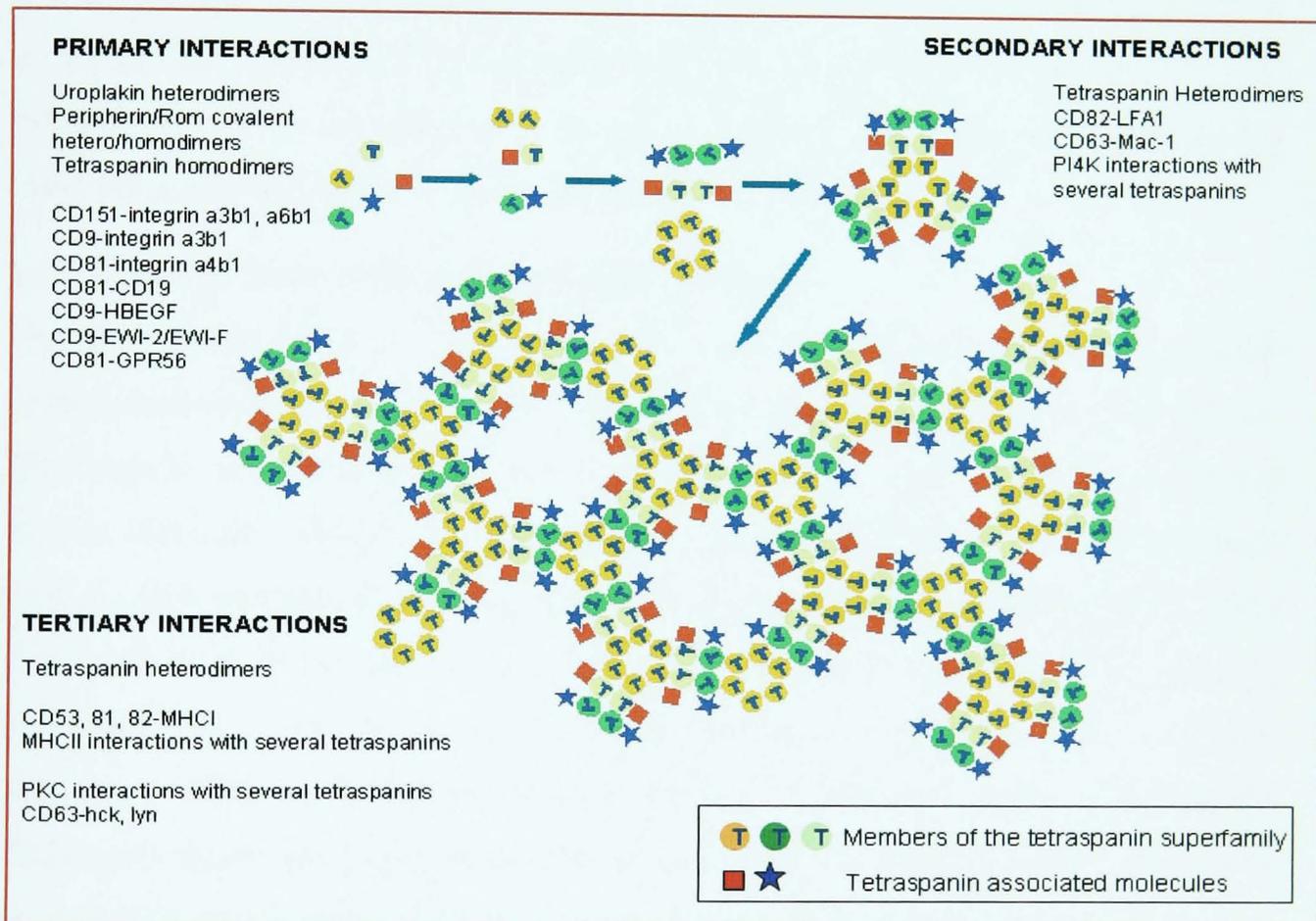


Fig. 1.1.4. Interactions of tetraspanins. Image courtesy of Martin *et al*, (2005).

TEMS may form extended structures of primary tetraspanin complexes indirectly connected in large networks (Figure.1.1.4). It is likely that TEMs are dynamic structures that can change in their composition or subcellular localisation. It is known, for example, that the location of tetraspanin complexes can change in response to stimulation. Thus CD63 is reported to associate with αIIbβ3 integrin-CD9 complexes on platelets after activation (Israels *et al*, 2001). In B cells, the interaction of the

CD81/CD21/CD19 coreceptor complex with lipid rafts has been shown to be modulated by palmitoylation of CD81 (Cherukuri *et al*, 2004).

In addition, soluble ligands have been identified for CD9. The pregnancy specific glycoproteins (PSGs) are a family of highly similar, placentally secreted proteins, originally isolated from the circulation of pregnant women. The human PSG induces IL-10, IL-6 and TGF$\beta_1$ expression in human monocytes. This suggests that PSGs, through the induction of these Th2 cytokines, may have a role in protecting the foetus from attack by the maternal immune system (Snyder *et al*, 2001). Recently it was found that CD9 acts as a receptor for the PSGs. Anti CD9 mAb inhibited the binding of PSG17 to CD9 on CD9 transfected cells and RAW 264.7 cells and PSG17 binding to macrophages from CD9 deficient mice was significantly reduced (Waterhouse *et al*, 2002). Very recently CD9 has also been shown to function as a receptor for the cytokine IL-16 as an alternative to CD4 receptor and mediates IL-16 related chemotaxis and activation of mast cells (Qi *et al*, 2005).

### 1.1.4.2. Association of tetraspanins with integrins

The involvement of various tetraspanins in adhesion and cell motility (and possibly other functions) seems to be related to their ability to form interactions with integrins. The integrins are heterodimeric, two-ways signalling receptors, which are composed of non- identical $\alpha$ and $\beta$ sub units, which recognize ligands through the extracellular domains and transmit intracellular signals through cytoplasmic tails (Liu *et al*, 1996; Kishimoto *et al*, 1999). Integrins are expressed on all nucleated cells, which regulate cell adhesion to extracellular matrix (ECM) proteins as well as intercellular cell-cell adhesive interactions. In humans there are at least 18 different integrin $\alpha$ chains and 8 different $\beta$ chains and they pair together noncovalently in specific patterns depending on the cell types in which they are expressed. The presence of particular $\alpha/\beta$ pairs on the cell surface confers the ability to bind the specific ECM proteins or counter receptors on the other cells. The activity of integrins is controlled by a process of "inside-out" (short cytoplasmic tail sequences of integrins alter ligand binding) signalling and "outside-in" (ligand binding triggers long-range alterations in the cytoplasmic domain interactions) signalling (reviewed in van der Flier, 2001). Changes in integrin affinity for ligands are the result of changes in integrin conformation. Certain cations (e.g. $Mg^{++}$) and antibodies may activate integrins by

stabilizing or inducing the active conformation. In addition, binding between integrins and ligands is increased by integrin clustering (van der Flier *et al*, 2001).

Beyond their role in regulating cell adhesion, it has become clear that integrins transduce signals inside the cell, which regulate the rearrangement of the actin cytoskeleton, cell movement, activation of specific cellular functions, gene transcription, cell proliferation and survival. These intracellular signals collaborate with signals transduced from growth factor receptors, cytokine receptors and other transmembrane receptors to regulate many anchorage-dependent cellular properties (reviewed in Berton *et al*, 1999). However, the short cytoplasmic regions of integrins have no intrinsic enzymatic activity. Instead, integrins interact with various cytoplasmic proteins including cytoskeletal proteins, potential signalling molecules and calcium binding proteins (reviewed in van der Flier, 2001). In addition they interact with transmembrane proteins including Ig superfamily members, growth-factor receptors and tetraspanins (reviewed in van der Flier, 2001; Berditichevski, 2001).

The association of leukocyte tetraspanins (including CD9, CD53, CD63, CD81, CD82 and CD151) with $\beta1$ integrins is well-documented (reviewed in Berditchevski, 2001). In addition, CD9, CD63 and CD151 are reported to associate with $\alpha II\beta3$, CD151 associates with $\alpha6\beta4$ and CD63 associates with $\alpha M\beta2$ on neutrophils and dendritic cells (Berditchevski, 2001; Mantegazza *et al*, 2004). Whilst some of these interactions are only preserved in relatively mild detergents (signifying secondary or tertiary interactions) the interactions between CD151 and $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$ and $\alpha7\beta1$ and between CD81 and $\alpha4\beta1$ integrins are classed as a primary interactions. The interaction between CD151 and $\alpha3\beta1$ has been extensively studied and shown to involve the EC2 of CD151 and the extracellular region of the $\alpha3$ subunit (Yauch *et al*, 2000).

Tetraspanins do not appear to alter integrin ligand binding, and do not modulate integrin-dependent static cell adhesion. Rather they are linked to processes such as cell morphology, spreading (reviewed in Berditchevski, 2001; Hemler, 2003) and adhesion strengthening (Lammerding *et al*, 2003). In addition, there are many reports that tetraspanins are involved in homotyic and heterotypic adhesion (Berditchevski, 2001) and in cell migration.

### 1.1.4.3. Interaction between tetraspanins, integrins and signalling molecules

Many studies suggest that integrin-associated tetraspanins are involved in adhesion-dependent signalling. In the Raji B cell line, ectopically expressed CD9 associated with α4β1 and α6β1 integrin and potentiates fibronectin and laminin-I dependent tyrosine phosphorylation of 130 kDa and 69 kDa proteins (Shaw et al, 1995). In addition, the expression of CD9 in fibrosarcoma cells specifically affects the de-phosphorylation rate of focal adhesion kinase (FAK) (Berditchevski and Odintsova 1999), and clustering of the α3β1-tetraspanin complexes in breast carcinoma cells stimulates the PI3-kinase-dependent signalling pathway (Sugiura and Berditchevski, 1999). The contribution of tetraspanins to adhesion-dependent signalling might be linked with their ability to recruit certain signalling enzymes into the integrin complexes (Hemler, 1998). Various tetraspanins are associated with phosphoinositol 4 –kinase (PtdIns 4-K) (Berditchevski et al, 1997; Yauch and Hemler, 2000; Berditchevski, 2001; Claas et al, 2001; Israels and McMillan-Ward, 2005) and protein kinase C isoforms (Zhang et al, 2001), and they may facilitate assembly of signalling complexes by tethering these enzymes to integrin heterodimers. At the plasma membrane, integrin-tetraspanin signalling complexes are partitioned into specific microdomains proximal to cholesterol-rich lipid rafts.

It has been suggested that tetraspanins can affect organization of the actin cytoskeleton. This may occur, for example, via the interaction of CD9 and CD81 with protein kinase C isoforms (Zhang et al, 2001; Berditchevski, 2001) or other signalling enzymes. A recent study shows that CD82, which act as a co-stimulator during T cell activation, may act as an adaptor protein, linking lipid rafts and the actin cytoskeleton in T cells during antigen presentation (Delaguillaumie et al, 2004). Previous studies had shown that CD82 activation involved Rho GTPases (Delaguillaumie et al, 2002). It has also been suggested that CD63 can modulate αIIbβ3-dependent cytoskeletal reorganization in adherent platelets via its interaction with PtdIns 4-K (Israels and McMillan-Ward, 2005).

It has recently been shown that CD9 and CD81 can associate with a G-protein coupled receptor (GPCR), GPR56. The findings indicate that these tetraspanins act as scaffolding proteins, mediating interactions between GPCR and their intracellular heterotrimeric G-protein subunits (Little et al, 2004).

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## 1.1.5. Functions of tetraspanins

As indicated previously, tetraspanins have been linked to many basic cell processes such as cell motility, cell differentiation, cell adhesion, cell signalling, cell fusion etc. (Wright and Tomlinson, 1994; Maecker *et al*, 1997; Boucheix *et al*, 2001; Hemler 2003; Martin *et al*, 2005). The functional properties of tetraspanins have been elucidated by different approaches. The main initial approach is the analysis of cellular effects of anti-tetraspanin antibodies, the second evaluates the effects of over expression by transfection and the third relates the phenotype of genetic defects caused either by gene knockout or by human genetic diseases (Boucheix *et al*, 2001). Their functions are undoubtedly linked to their ability to form association with one another and with other membrane proteins (e.g. integrins) as discussed in section 1.1.4. This has made it difficult to define the precise role of individual tetraspanins. Also studies using knockout animals have in most cases failed to show strong phenotypic defects, suggesting overlapping functions or functional redundancy amongst tetraspanins.

In considering the functions of tetraspanins, the main emphasis will be on examples relevant to those studies in this thesis i.e. CD9 and CD63.

### 1.1.5.1. Adhesion and motility

Cell adhesion and motility are complex processes involving extracellular matrix proteins, adhesion receptors, cytoskeletal components and signalling molecules. The role of tetraspanins in modulating adhesion/motility has been linked to processes such as wound-healing and cancer metastasis (Boucheix *et al*, 2001). The involvement of various tetraspanins in cell motility and adhesion seems to be related to their ability to form interactions with integrins (see section 1.1.4.2 and 1.1.4.3). A substantial fraction of tetraspanins co-localise with integrins in various intracellular vesicular compartments. It is proposed that tetraspanins can influence cell adhesion/migration by one of the following mechanism (1) modulation of integrin signalling (2) compartmentalisation of integrins on the cell surface or (3) direction of intracellular trafficking and recycling of integrins (reviewed in Berditchevski, 2001).

Although the role of tetraspanins in cell adhesion appears to relate to their association with integrin molecules, in the case of CD9, there is also evidence of a direct interaction with fibronectin. Cook and co workers (Cook *et al*, 1999) demonstrated a

role for CD9 in adhesion and spreading on the extracellular matrix fibronectin protein using a transfected Chinese Hamster Ovary (CHO) cell system. The transfection of CD9 in cells causes    morphological changes and a reduction in adhesion and spreading on fibronectin. The effects were reversed by ligation with anti CD9 antibody and with CD9 mutants lacking EC2 (Cook *et al*, 2002). In addition, direct binding of fibronectin to recombinant or platelet-derived CD9 has been shown (Longhurst *et al*, 2002).

Koyama *et al* (1990) showed that antibodies to a cell surface glycoprotein (later identified as CD63 (Koyama *et al*, 1998)) induced adhesion, spreading and morphological changes in myeloid cell lines HL-60, THP-1 and U937. In searching for integrin-associated proteins, Berditchevski *et al* (1995) identified an anti CD63 antibody that co-immunoprecipitated α3β1 and α6β1integrins from Brij 96 detergent lysates. CD63 co-localised with these integrins in cellular "footprints" suggesting a role in integrin adhesive functions. Binding of anti CD63 antibodies to neutrophils was reported to induce increased adhesion to human umbilical endothelial cells (HUVECs) and an association between CD63 and β2-integrin (CD11b/CD18) in neutrophils was reported (Skubitz *et al*, 1996). This association was only observed with CD63 that had been up-regulated to the cell surface after neutrophil activation (Skubitz *et al*, 2000). Tyrosine kinase activity was associated with CD63 in these cells. CD63 was also shown to be upregulated on activation of platelets and to associate with the αIIbβ3-CD9 complex on the plasma membrane (Israels *et al*, 2001). Antibodies to CD63 did not affect platelet adhesion, but inhibited platelet spreading (Israels and McMillan-Ward, 2005).

There are many reports that CD9 plays a role in regulating motility of cells. CD9 can induce motorgenic signals in association with β1 integrin and activation of protein tyrosine kinase. The association of CD9 with motility was demonstrated on the poorly motile B cell line Raji, which shows dramatically enhanced migration after ectopic expression of CD9. Enhanced levels of tyrosine-phosphorylation were observed in the transfected cells and migration was inhibited by the protein tyrosine kinase inhibitor herbimycin (Shaw *et al*, 1995).

CD9 appears to play a role in migration during wound-healing/angiogenesis. In an *in vitro* mechanical wound model, mimicking the angiogenic process, anti CD9

antibodies showed a dose dependent inhibition of endothelial cell migration during lesion repair, whereas anti β1antibodies affected both migration and proliferation. CD9 was shown to co-localise with β1 and β3 integrins on the endothelial cell plasma membrane (Klein-Soyer *et al*, 2000). CD9 and CD81 also inhibited keratinocyte motility in a wound healing migration assay, and accumulated with α3 and β1 integrins at intercellular junctions and in cellular footprints (Penas *et al*, 2000). Antibodies to CD9, CD63, CD81 and CD151 were shown to inhibit migration of hepatic stellate cells (HSC) that are involved in the progression of liver fibrosis, although cell adhesion was not affected (Mazzocca *et al*, 2002). Associations with β1 integrin were shown, although only that between CD9 and CD151 and β1 integrin were preserved in strong detergent (NP-40).

CD9 has also been implicated in the inhibition of cancer metastasis and is reported to inhibit motility of cancer cells *in vitro* (Boucheix *et al*, 2001). A co-operative effect between CD9 and the ganglioside GM3 was reported for the inhibition of motility in various human cancer lines (Ono *et al*, 2000). Kawakami and co workers found that the interaction of α3 and α5 integrins with CD9 is promoted by GM3 (Kawakami *et al*, 2002).

CD63 has also been reported to associate with tetraspanins such as CD9 and CD81 and β1 integrin on the surface of melanoma cell lines MV3 and MEL-FC as determined by co-immunoprecipitation using mild detergent (Radford *et al*, 1996). It was reported that transfection of human CD63 into a CD63 negative human melanoma cell line KM3 caused up regulated expression of β1 integrin. The motility of CD63 transfected KM3 cells was markedly suppressed but increased adhesion on β1 substrates was observed (Radford *et al*, 1997). However, the recent demonstration by our group that the KM3 cell line used in these studies is a rat cell line questions the validity of this work (Moseley *et al*, 2003). However, studies by Jang *et al*, (2003) also indicated that reduced CD63 expression during the malignant progression of human melanoma may promote metastasis. For the study the authors transfected CD63 cDNA into Mel Juso melanoma cells, which have endogenous CD63 expression. It was observed that decreased CD63 expression enhanced the invasiveness of the human melanoma cells.

Recently, antibodies to CD9, CD63, CD81 and CD82 were shown to enhance the chemokine-induced migration of dendritic cells (Mantegazza *et al*, 2004). It was shown that antibodies to CD63 and CD82 diminished the surface expression of CD29 (β1 integrin), CD11b (integrin αM), CD18 (β2 integrin) and α5 integrin. CD63 was shown to associate with CD11b and CD18 by co-immunoprecipitation in mild detergent (Mantegazza *et al*, 2004).

### 1.1.5.2. Role of tetraspanins in membrane trafficking

A number of the human leukocyte tetraspanins (CD151, CD63, A15 (CD231), CO-029, CD37, CD82 and Tspan-3) contain tyrosine-based sorting motifs at their C-terminus that might play a role in trafficking (Beridtchevski, 2001;Stipp *et al*, 2003). Tyrosine-based motifs (Y-X-X-ϕ, where ϕ is a hydrophobic residue) in the cytoplasmic domains of membrane proteins are recognized by clathrin adaptor complexes and play important roles in endocytosis and lysosomal targeting (Robinson and Bonifacino, 2001). These tetraspanins can be expressed in intracellular vesicles in addition to on the cell surface. The association of tetraspanins with a diverse array of other proteins could be used for the control of endocytosis and intracellular protein trafficking.

The tetraspanin CD63 resides in late endosomes, lysomes, secretary vesicles, and at the plasma membrane is rapidly internalised. CD63 contains a GYEVM motif at its C-terminus. The GY motif, which is unique amongst human tetraspanins, is a putative lysosomal targeting motif also found in many type 1 lysosomal glycoproteins e.g. Lamp-1 (Honing and Hunziker, 1995). Site-directed mutagenesis has shown that this region is crucial in determining the intracellular localisation and rapid plasma membrane internalization of CD63 (Rous *et al*, 2002; G. Mal, PhD thesis (2005), University of Sheffield).

### 1.1.5.3. Association of CD63 with membrane trafficking molecules

Duffield *et al*, 2003 reported that CD63 is present in tubulovesicular elements, the intracellular compartments that contain the H, K-ATPase (a heterodimeric ion pump) in unstimulated gastric parietal cells. The β-subunit of H, K-ATPase and CD63 complex colocalized in parietal cells. Expression of CD63 with β-subunit of H, K-ATPase induces the redistribution of the β-subunit from the cell surface to CD63+ intracellular compartments by enhanced endocytosis of H, K-ATPase β-subunit complexed with CD63. They found that the enhanced internalisation of β-subunit may

be due to the capacity of CD63 to interact with adaptor protein complexes 2 and 3, since the β-subunit did not internalise when co-expressed with CD63 containing a mutation at the C-terminal internalisation motif. From the study the authors concluded that the tetraspanin CD63 may play a role in the recyling of plasma membrane components to their appropriate intracellular compartment.

CD63 was also recently found to promote targeting and lysosomal proteolysis of membrane-type 1 matrix metalloproteinase (MT1-MMP) (Takino et al, 2003). Matrix Metallo Proteinases (MMP) are a family of $Zn^{2+}$ dependent enzymes anchored to plasma membrane, that are essential for extracellular matrix (ECM) turnover in normal and pathological conditions. MT1-MMP is believed to be a crucial for the invasion of malignant tumors. The authors found that the ectopic expression of CD63 accelerated degradation of MT1-MMP. In addition, MT1-MMP and CD63 were shown to form a complex through haemopexin-like domain of MT1-MMP and the N-terminal region of CD63. The enhanced internalisation was not observed with a CD63 mutant lacking the lysosomal targeting motif.

As discussed above, CD63 may also promote internalisation of integrins (Mantegazza et al, 2004). Thus, CD63 does appear to play a role in protein trafficking. Mantegazza et al, (2004) also suggested that CD63 may play a role in the internalisation of complex antigens, since internalisation of CD63 was observed during phagocytosis of yeast particles. An association between CD63 and the β-glycan receptor dectin-1 (which is involved in recognising particles such as yeast) was demonstrated by co-immunoprecipitation.

### 1.1.5.4. Tetraspanins in the immune response

There have been several recent reviews on the role of tetraspanins in the immune response (Tarrant et al, 2003; Wright et al, 2004; Levy and Shoham, 2005) and only certain aspects will be detailed here. Specific interactions of tetraspanins with a wide range of leukocyte receptors have been reported, including interactions with CD2, CD3, CD4, CD8, CD5, CD19, CD21, IgM, Fc receptors and MHC class I and class II (Tarrant et al, 2003; Wright et al, 2004; Levy and Shoham, 2005). As described previously, tetraspanins also associate with integrins and signalling enzymes expressed on leukocytes. Cross-linking tetraspanins at the cell surface can result in tyrosine phosphorylation, calcium fluxes and inositol phosphate turnover (Tarrant et

*al*, 2003). As mentioned previously, signalling is likely to be indirect via the presence of signalling molecules in TEMs.

### 1.1.5.4.1. Association of tetraspanins with MHC

The MHC molecules play an important role in the presentation of antigens to T-lymphocytes. Rubinstein *et al*, (1996), demonstrated that CD9, CD63, CD81 and CD82 associated with each other and with integrins and HLA–DR (MHCII) antigens on the cell surface. The authors demonstrated the interaction of tetraspanins with other proteins by over expression followed by immunoprecipitation studies. From the result it was observed that the different complexes (tetraspan/tetraspan/tetraspan, tetraspan/tetraspan/HLA-DR and tetraspan/integrin) are connected and that these complexes are organised in a cell type dependent manner. The authors suggested a possible function for the tetraspanins would be to play the role of surface organisers.

Lagaudriere-Gesbert *et al*, 1997, found that CD82 associated with HLA class I heavy chain using lysates prepared in mild detergent (CHAPS) and sequential immunoprecipitations and Western blot analysis. These association takes place at the cell surface and could interfere with the capacity of the MHC-I complex to protect targets from NK– mediated cytotoxicity.

MHC class II antigens have also been shown to be associated with CD9, CD53 and CD81 on the surface of dendritic cells (DCs) and CD9 and CD63 in cytoplasmic compartments (Engering and Pieters, 2001). Kropshofer *et al*, (2002) found that the MHC class-II molecules such as HLA-DR and HLA-DP are localised to membrane microdomains with tetraspanin proteins CD9, CD81 and CD82, the peptide editor HLA-DM and the co stimulator CD86. The deficiency of tetraspanin microdomains in antigen presenting cells results in a reduced capacity to activate $CD4^+$ T cells.

Engering *et al* (2003) reported that CD63, which associates with intracellulary localised MHC class-II molecules in immature dendritic cells, was modified post-translationally by poly N-acetyl lactosamine addition during maturation. This modification of CD63 was accompanied by a change in morphology of MHC class-II compartments from typical multivesicular organelles to structures containing densely packed lipid moieties. The authors postulated that post-translational modification of CD63 may be involved in the functional and morphological changes of MHC class II compartments that occur during dendritic cell maturation.

Recently the researchers focussed their attention towards the make-up of cytoplasmic MHC class II processing compartments (MIIC), a modified lysosomal compartments in which exogenous antigen is processed to peptides and loaded onto MHC class II molecules (Peters *et al*, 1991). Tetraspanins (CD37, CD53, CD63, CD81 and CD82) are located on a subset of MIIC internal vesicles, named exosomes, which are released extracellularly following fusion of the MIIC with the plasma membrane (Théry *et al*, 2002). The functions of tetraspanins in MIIC or exosomes is currently unknown but the exosomes are thought to present antigen, exchange membrane proteins with other cells or simply to dispose of redundant proteins (Théry *et al*, 2002). These vesicles might simply reflect an intermediate intracellular stage in the biosynthesis and assembly of tetraspanin–MHC complexes. Alternatively, tetraspanins could function in the endocytosis and/or trafficking of MHC class II molecules or indeed other molecules that they associate with, as discussed in 1.1.5.2.

### 1.1.5.4.2. Role of tetraspanis in allergy

Activation of basophils or mast cells enhances fusion of cytoplasmic granules with the plasma membrane and results in the release of inflammatory mediators, such as histamine. Expression of CD63 on the cell surface is strongly induced after activation of platelets, neutrophils and basophils, following mobilisation of the intracellular pool, for example on activation of human basophils with anti IgE (Knol *et al*, 1991). This has led to CD63 being used as a marker of platelet (Gutesohn *et al*, 1996) and basophil (Monneret *et al*, 2002) activation.

Mast cells and basophils express tetraspanins CD9 and CD63 in a constitutive manner (Valent and Bettelheim, 1992). Mast cells or basophils activated by IgE dependent stimuli or other agonists show significant changes in the expression of CD9 and CD63. Knol and co workers found that the activation of basophilic granulocytes with anti-IgE or with chemotactic peptide (formyl-methionyl-leucyl–phenyl alanine), increased the expression of CD63 up to 100 fold on the surface (Knol *et al*, 1991). Furuno *et al*, (1996) reported that surface expression of the rat homologue of CD63 (AD1) occurred simultaneously with the release of histamine corresponding to the degranulation in rat basophilic leukaemia (RBL2H3) cells. High levels of CD63 expression were observed in bone marrow mast cells from the adults with indolent systemic mast cell disease as compared to normal mast cells from healthy individuals (Escribano *et al*, 1998).

CD63 translocation has also been observed in activated eosinophils (Mahudi-Azer *et al*, 2002). In resting eosinophils, CD63 immunoreactivity was localized to the plasma and crystalloid granule membrane. In interferon-γ (IFN-γ)– or C5a/cytochalasin B stimulated cells, intracellular CD63 appears to shift to the cell periphery and plasma membrane, while stimulation with a cocktail of interleukin-3 (IL-3)/IL-5/granulocyte-macrophage colony-stimulating factor induced the appearance of discrete intracellular clusters of CD63 immunoreactivity (Mahudi-Azer *et al*, 2002).

There have been reports of an association between CD63 and the high affinity IgE receptor (FcεRI) on mast cells/basophils. An antibody directed against the rat homologue of CD63, AD1, was found to inhibit IgE mediated release from RBL2H3 cells. This suggested that CD63 and FcεRI were closely associated on the cell surface and that CD63 might be involved in the control of FcεRI function (Kitani *et al*, 1991). By contrast, mouse IgG1 anti human CD63 antibodies were shown to stimulate cell secretion from RBL2H3 cells transfected with human CD63 (Smith *et al*, 1995). Similar findings were observed for IgG1 anti CD9 antibodies on RBL2H3 cells transfected with CD9 (Higginbottom *et al*, 2000). Stimulation of RBL2H3 cells by the anti-tetraspanin antibodies was shown to be dependent on the interaction of the Fc region of the IgG1 antibodies with FcεRI. An association between human CD9 and rat FcεRI was suggested by their co-immunopreciptiation in mild detergent lysates of hCD9-transfected RBL2H3 cells (Higginbottom *et al*, 2000). Most recently, antibodies that inhibited FcεRI-mediated degranulation of rat RBL2H3 cells were shown to recognise CD63 (Kraft *et al*, 2005). Inhibition of degranulation was only observed for mast cells adherent to fibronectin and vitronectin ECM proteins. Furthermore, the anti-CD63 antibodies inhibited adhesion of mast cells to these ECM proteins. Inhibition of FεRI mediated degranulation therefore correlates with the effect of the antibodies on adhesion and is likely to relate to the known association of CD63 with β1 integrins. The same group also showed that an antibody directed to CD81 also inhibited mast cell degranulation (Fleming *et al*, 1997).

### 1.1.5.5. The role of tetraspanins in viral infection

There are many reports on the role of tetraspanins viral infectivity and life cycle (reviewed in Martin *et al*, 2005).

### 1.1.5.5.1. Hepatitis C virus and CD81

The tetraspanin CD81 is playing a role in the aetiopathogenesis of hepatitis 'C' virus (HCV), which infects 170 million people worldwide. The infection caused by HCV leads to hepatocellular cirrhosis and carcinoma and also immune disease related to lymphoid B cells (cryoglobulinaemia, lymphoproliferative disorders, and autoantibody production).

Flint and co workers have characterised the interaction of HCV envelope glycoprotein E2 and human CD81. The E2 glycoprotein of HCV binds specifically to tetraspanin CD81 (hCD81) transfected RBL2H3 and KM3 rat cell lines. Recombinant forms of hCD81 encoding both EC1 and EC2 or EC2 alone were expressed as -N or –C terminal fusion proteins were used to compete with cell surface expressed CD81 for binding of E2. From the result it was observed that GST-EC2 inhibited E2 binding to hCD81 expressed at the surface of rat or human cell equivalently. Furthermore they found that there was no binding of HCV-E2 in COS cells derived from African green monkey. The African green monkey CD81 differs from human CD81 by just 4 residues, all in the carboxyl terminal region of the large extracellular loop (EC2) implicating one or more of these residues as important for HCV E2 binding (Pileri *et al*, 1998; Flint *et al*, 1999). Higginbottom *et al*, 2000 defined the critical amino acid residues within the EC2 by mutating each of four positions in human CD81 to the corresponding African green monkeys (AGM) residues and expressing them as soluble GST-EC2 proteins in bacteria or as complete membrane protein in mammalian cells. Amino acid 186 of hCD81 was found to be critical for the interaction with HCV E2 and for binding of certain anti CD81 mAbs. Kitadokora *et al*, (2001) have demonstrated the residues reported to be involved in the interaction between hCD81 and HCV E2 glycoprotein reside in the smaller, solvent exposed apolar cluster comprising hcD81-LEL-C and D helices (see section 1.1.2.2).

Van Compernolle *et al*, (2003) have evaluated the possibility of using molecules designed to mimic CD81 helix D that may be able to competitively bind to HCV-E2 to prevent infection. The authors synthesised compounds that were structurally related by a common imidazole 4, 5-dicarboxamide (145DC) scaffold and presenting CD81 helix D amino acid side chains which acts as competitive inhibitors of HCV-E2 binding to human CD81. One compound almost completely blocked the HCV-E2 binding to CD81 in a dose dependent manner.

### 1.1.5.5.2. Canine distemper virus and CD9

CD9 is associated with the infection of cells with Canine Distemper Virus (CDV), a lymphotropic and neurotropic negative stranded RNA virus of morbilli virus genus. An anti-CD9 antibody inhibits the infection of cells with CDV (Loffler *et al*, 1997) although the virus is not directly associated with CD9, suggesting that CD9 is not the attachment receptor. Human CD9 transfected into NIH 3T3 or MDBK cells rendered these cells permissive for viral infection and raised virus production by a factor of 10 to 100. Anti CD9 antibodies reduced the number of infectious centres and the plaque size, possibly by steric hindrance of virus attachment or inhibition of fusion. These data indicate that CD9 is the component of a receptor complex for CDV, but it is also possible that CD9 acts via intracellular signalling, enhancing the expression or activity of a receptor molecule. CD9 may be necessary for the uptake of CDV by target cells, the formation of syncytia, and the production of progeny virus

### 1.1.5.5.3. HIV and CD63

Macrophages and CD4$^+$ T lymphocytes are the potential target cells for human immunodeficiency virus type-1 (HIV-1) infection. Recently von Lindern *et al*, (2003) identified CD63 as a new co-factor involved in the CCR5 but not CXCR4-mediated HIV-1 infection in macrophages. The role of CD63 in HIV-1 infection was demonstrated on macrophages, which were pre treated with anti CD63 mAb. Dose-dependent inhibition of new viral DNA formation for two separate R5 strains was observed, indicating that the block occurs early in the virus life cycle, prior to reverse transcription. Anti CD63 antibodies did not affect levels of CD4, CCR5 or β-cytokines. Von Lindern and co workers also assessed the possible role of CD63 in HIV-1 Env gp120-gp41 mediated cell-to-cell fusion. A quail fibrosarcoma line QT6, which expresses low levels of endogenous CD63, was used for the CD63 transfection experiments. Transfection of CD63 had no effect on HIV-1 Env mediated cell:cell fusion of these cells. Anti-CD63 antibodies also had no effect in a system where primary macrophages were induced to fuse with HIV-1 Env transfected 283T cells. The authors suggested that the anti CD63 antibodies might be affecting CD4-CCR5 complex formation (Von Lindern *et al*, 2003).

Most recently our research group in collaboration with Dr C. Chengmeyer (Aaron Diamond AIDS Research Center, New York) have found that recombinant GST-EC2 domain of human tetraspanins CD9, CD63, CD81 and CD151 potently inhibited

CCR5 (R5) and virions pesudotyped with CXCR4 (X4) tropic HIV-1 infection of macrophages at lower (nanomolar) concentrations. In this study we observed that the GST-EC2 tetraspanins did not decrease CD4 or co-receptor expression (Ho *et al*, manuscript submitted).

CD63 has been shown to accumulate preferentially in the viral envelope of HIV released from macrophages (Pelchen-Matthews *et al*, 2003). Since CD63 is associated with late endosomes, this suggested that most virus is released from late endosomes in these cells. It is not known if virus envelope-associated CD63 plays any role in virus transmission, although it may contribute to the ability of anti CD63 antibodies to inhibit HIV infection (von Lindern *et al*, 2003).

### 1.1.5.5.4. FIV and CD9

Feline immunodeficiency virus (FIV) infection appears to involve CD9. In cells expressing feline CD9, an anti CD9 antibody was found delay reverse transcriptase activity post-infection (deParseval *et al*, 2001). The antibody also reduced viral budding in infected cells and inhibited FIV spread in culture, suggesting an effect on viral assembly or release. FIV can infect CD9 -ve cells, but ectopic expression of CD9 enhances infectivity (Willett *et al*, 1994).

### 1.1.5.5.5. HTLV and CD82

In addition to these, as discussed in section 1.1.5.6 CD82 has been implicated in syncytium formation by human T-cell leukemia virus type 1 (HTLV-1) (Imai *et al*, 1992).

### 1.1.5.6.Tetraspanin and cell fusion

Tetraspanins CD9, CD81 and CD82 have also been implicated in cell fusion processes, including sperm-egg fusion, myoblast fusion and virus syncitium formation.

### 1.1.5.6.1. Sperm-Egg fusion

The integrin $\alpha6\beta1$ has previously been proposed to act as a sperm receptor for fertilin on the egg cell surface and antibodies to this integrin inhibit fertilization (Almeida *et al*, 1995). Since CD9 is known to associate with integrins (including $\alpha6\beta1$) its role in sperm:egg fusion was investigated (Chen *et al*, 1999). CD9 was found to be abundantly expressed and localised in the microvilli of eggs and densely concentrated on the sperm attachment site. The association of CD9 in the mouse sperm-egg fusion

process was demonstrated *in vitro* using anti CD9 mAb, which showed dose dependent inhibition of sperm-egg binding and fusion. The antibody disrupted binding of beads coated with native fertilin or a recombinant fertilin β disintegrin domain (ADAM 2).

The role of CD9 on sperm: egg fusion was conclusively demonstrated using CD9 knockout (CD9 $^{-/-}$) mice (Miyado *et al*, 2000; Le Naour *et al*, 2000; Kaji *et al*, 2000). CD9 deleted mice grew and developed normally without any obvious abnormalities. However, Miyado *et al* (2000) observed that the litter size of CD9 knockout female mice was less than 2% of that of the wild type. Sperm penetration through the zona pellucida and binding to the egg plasma membrane were normal, but oocytes from CD9 knockout mice showed severely inhibited sperm-egg fusion (Miyado *et al*, 2000; Kaji *et al*, 2000; Le Naour *et al*, 2000). Sperm injected directly into the cytoplasm of CD9 null oocytes yielded normal development, indicating that the role of CD9 is limited to initial sperm-oocyte fusion (Miyado *et al*, 2000). Kaji *et al* (2000) reported that intracellular calcium ($Ca^{2+}$) oscillations, which signal fertilization, was absent in almost all mutant (CD9 $^{-/-}$) eggs.

As described above, ealier studies indicated that α6β1 integrin was a receptor for sperm (Almeida *et al*, 1995), and since CD9 associates with integrins it was suggested that egg CD9 acts in association with the integrin during fusion (Chen *et al*, 1999). More recent studies indicate that α6β1 is not essential for sperm-egg binding and fusion (Miller *et al*, 2000). Eggs from mice lacking the α6 subunit showed no impairment of fertilization rate, fertilization index or sperm:egg binding relative to controls. Furthermore, inhibition of sperm fusion with α6 (-) eggs by anti CD9 antibody indicates that CD9 acts alone or in association with other proteins in gamete binding and fusion to eggs. The association of CD9 and CD81 with EWI-2, might be involved for the sperm-egg fusion (Stipp *et al*, 2001).

Normal fusion occurs after human or murine CD9 mRNA injection into the CD9 knock-out mice oocytes (Zhu *et al*, 2002; Kaji *et al*, 2002). The over expression of human CD9 and mouse CD81 on CD9 $^{-/-}$ eggs restored the fertilisation rate up to ~90 and ~50 % respectively, against the wild type eggs. This result suggests that CD81 has the potential to compensate for CD9 function in sperm-egg fusion (Kaji *et al*, 2002). Zhu and co workers reported that CD9 interacts through its EC2 domain with

an un identified egg surface protein, and recombinant CD9 EC2 inhibited sperm:egg fusion. In murine CD9, three residues SFQ (173-175) in variable subdomain of the EC2 are required for CD9s function in gamete fusion and mutating the SFQ residues to 173-175 SFQ to AAA abolished CD9 activity (Zhu *et al*, 2002).

Higginbottom *et al* (2003) further investigated sperm:egg fusion using recombinant tetrasapanin EC2 proteins. They showed that human CD9EC2 is as potent an inhibitor as mouse CD9 EC2 in mouse sperm-oocyte fusion assays and that CD9 EC2 can also inhibit sperm–oocyte binding. Using mutated human CD9 EC2s, the authors also demonstrated that the two disulphide bridges that define membership of the tetraspanin family are critical for structure and function of human CD9 EC2. Also mutation of residues in the EC2 variable subdomain encompassing those identified as essential in murine CD9 activity (Zhu *et al*, 2002) also affected the fusion inhibitory activity of human CD9 EC2.

### 1.1.5.6.2.Myoblast fusion

There is evidence that CD9 and CD81 are involved in muscle cell fusion. Tachibana and Hemler demonstrated that CD9 and CD81 are abundantly expressed on murine myoblast C2C12 cells and expression of CD9 is upregulated during the early phase of myogenic differentiation. The authors have also found that anti CD9 and CD81 antibodies delayed the fusion of C2C12 myoblast cells and RD rhabdomyosarcoma cells. Over- expression of CD9 promoted cell fusion in four independently transfected myoblast derived RD cell line (Tachibana and Hemler 1999). CD9 and CD81 therefore appear to accelerate muscle cell fusion and are involved in myotube maintenance (Tachibana and Hemler, 1999). CD9 on C212 cells associates with various β1 integrins, with α3β1 on α3-transfected RD cells (Tachibana and Hemler, 1999). Involvement of this tetraspanin in fusion may thus be indirect, relating to its control of integrin functions.

### 1.1.5.6.3. Role in viral syncytium formation

Earlier studies showed that antibodies to CD82 inhibited human T cell leukaemia virus type 1 (HTLV-1) but not HIV-1 induced syncitia formation (Fukudome *et al*, 1992; Imai *et al*, 1992). Co-immunoprecipitation experiments showed that HTLV-1 env glycoproteins associate with highly glycosylated forms of CD82 (Pique *et al*, 2000). The co expression of CD82 protein with HTLV-1 envelope glycoproteins resulted in marked inhibition of syncytium formation, whereas CD82 proteins had no

effect on syncytium formation induced by human immunodeficiency virus type 1 (HIV-1) envelop proteins. This effect was demonstrated using co transfection experiments in COS-1 cells (Pique *et al*, 2000).

#### 1.1.5.6.4.Role in cellular membrane fusion

As described in section 1.1.5.2 CD63 is associated with the membranes of intracellular vesicles and is upregulated on activation of several cell types. Studies using GFP-tagged CD63 show that on RBL2H3 cell activation, granules containing CD63 fuse with the plasma membrane within 2-3 min. (Amano *et al*, 2001). Also as described above, CD63 has been implicated in phagocytosis (Mantegazza *et al*, 2004). This has led some workers to speculate that CD63 may be involved in cellular membrane fusion (Mahudi-Azer *et al*, 2002), although there is no direct evidence for this.

#### 1.1.5.6.5.Tetraspanins in mononuclear phagocyte fusion

Recently a potential role for tetraspanins in the fusion of monocytes/macrophages to form multinucleated giant cells (MGCs) has been described. Takeda *et al* (2003) investigated the role of tetraspanins CD9 and CD81 in the Concanavalin A-induced fusion of mononuclear phagocytes (see section 1.2.4.3). They found that the expression of CD9 and CD81 and their complex formation with $\beta1$ and $\beta2$ integrins were up-regulated when blood monocytes were cultured under normal conditions but under fusogenic conditions in the presence of the lectin, the up regulation of CD9 and CD81 was inhibited and their complex formation with integrins was down regulated. In addition to this, the authors demonstrated that the anti CD9 and CD81 antibodies unexpectedly promoted the fusion of monocytes and alveolar macrophages. The effects did not appear to be due to effects of the antibodies on monocytes adhesion, aggregation or cytokine production. An anti CD63 antibody was reported to have no effect on fusion, although CD63 expression was upregulated under fusogenic conditions. Monocyte fusion was inhibited by a recombinant protein corresponding to the EC2 region of CD9. Alveolar macrophages and bone marrow cells of CD9 and CD81 null mice showed enhanced formation of multinucleated cells when stimulated *in vitro* or *in vivo* compared with those of wild type mice. In addition, CD9/CD81 double knockout mice showed spontaneous formation of multi-nucleated giant cells in the lung and enhanced osteoclastogenesis in the bone. From the results the authors suggest that the normal role of CD9 and CD81 is to co ordinately prevent the fusion

of mononuclear phagocytes (Takeda *et al*, 2003). As described above, this appears to be in contrast to their role in sperm:egg fusion.

CD9 has also been implicated in the formation of osteoclasts (Tanio *et al*, 1999). An anti CD9 antibody inhibited the formation of osteoclasts from haematopoietic stem cells on established stromal cell layers. This was due to effects of the antibody on CD9 expressed by the stromal cells, possibly blocking production of a factor required for osteoclastogenesis.

## 1.2. Multinucleated giant cells (MGCs)

Multinucleated Giant Cells (MGCs) are a characteristic histo-pathological feature of granulomatous inflammations (reviewed in Anderson, 2000; Fais *et al*, 1997) and their presence in tuberculosis granuloma was first described by Langhans (Langhans, 1868). Such MGCs, thought to be formed by fusion of cells of the monocytes/macrophage lineage, (Postlethwaite *et al*, 1982; Weinberg *et al*, 1984) were thereafter observed in a variety of infectious (mainly bacterial) and non-infectious granulomatous conditions (e.g. in response to foreign bodies such as implants, some cancers, and other disorders of unknown etiology such as Crohn's disease, sarcoidosis and primary biliary cirrhosis (reviewed in James, 2000; Okamoto *et al*, 2003; Weingberg *et al*, 1984)). Mostly the granulomatous inflammations are chronic, predominantly mononuclear host reactions to persisting, poorly degradable tissue irritants (Takashima *et al*, 1993). The release of lymphokines is likely to occur and the inflammatory conditions are known to lead to activation of macrophages (Abe *et al*, 1983).

In addition, the bone-resorbing osteoclasts, which are derived from monocytes precursors, may be considered giant cells (Anderson, 2000; Vignery, 2000). Some viruses, notably HIV-1, also induce the *in vitro* formation of syncitia (HIV-S) that share some of the characteristics of MGCs (Anderson, 2000; Fais *et al*, 1997). Since this thesis is concerned with MGCs derived from the mononuclear phagocyte lineage, a brief review of the properties of these cells will be given.
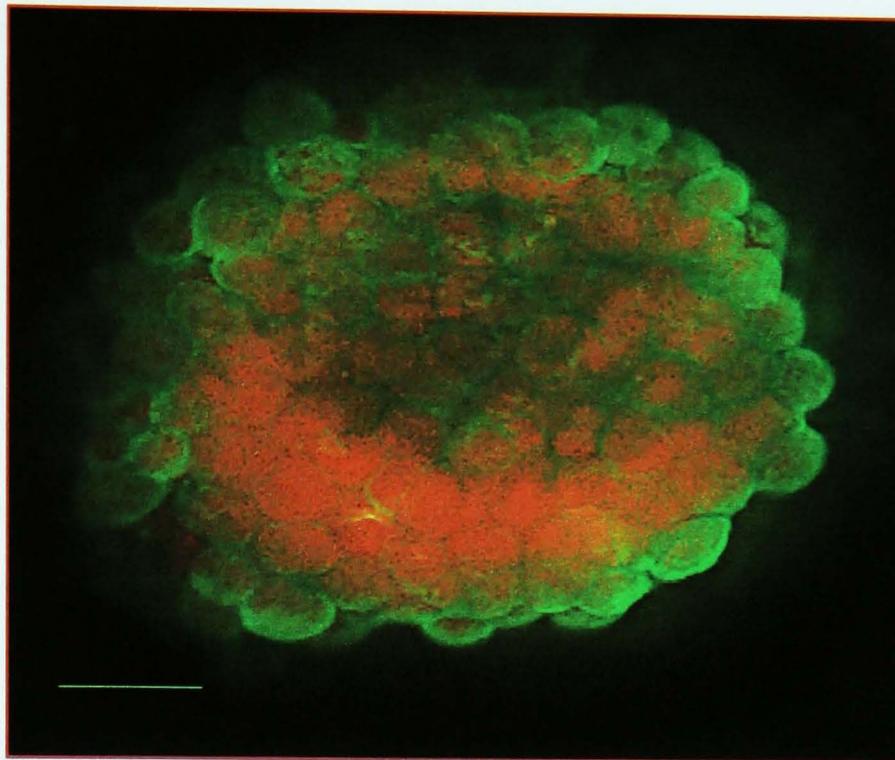
Fig.1.2.1. Multinucleate giant cells of monocytes (scale =20µm)

## 1.2.1. Mononuclear phagocytes

The immune system of vertebrates consists of organs and tissues composed of many different types of cells plus a variety of actively trafficking cells (Roitt *et al*, 1993). Among these cells the mononuclear phagocyte system (MPS) is a specialised tissue distributed throughout the body and composed of diverse differentiated cell types such as liver Kupffer cells, lung alveolar macrophages, serosal (peritoneal) macrophages, brain microglia, dendritic cells (DC), and osteoclasts, as well as granuloma-forming macrophages at sites of inflammation (Geissmann *et al*, 2003).


Fig.1.2.2. Monocyte represented by blue arrow

The mononuclear phagocytes develop in the bone marrow from dividing monoblasts and are released into the blood stream as non dividing cells, and enter tissues (Geissmann *et al*, 2003). They may continue to proliferate, mature, and become functionally activated in various tissues. Almost every organ of the body contains mononuclear phagocytes that possess unique characteristics for that location, while still sharing common features with analogous cells in other organs (reviewed in Wiktor-Jedrzejczak *et al*, 1996).

Morphologically, monocytes are characterised as mononuclear cells with bean/ horseshoe-shaped nuclei (Fig.1.2.2) and often contain faint azurophilic granules. The size of the monocyte is 10-18μm diameter, relatively larger than lymphocytes. Ultra structurally, the monocyte possesses ruffled membranes, a well-developed golgi complex and many intracytoplasmic lysosomes. These lysosomes contain peroxidase and several acid hydrolases, which are important in intracellular killing of microorganisms (Roitt *et al*, 1993). In addition the monocytes are characterised on the basis of expression of CD11b, CD11c, and CD14 in humans and lack of B, T, NK, and DC markers (Geissmann *et al*, 2003). Furthermore monocytes may be classified into subpopulations comprising $CD14^{++}/CD16^-$ and $CD14^{++}/CD16^+$ cells. $CD14^{++}/CD16^-$ cells are the major population and $CD14^{++}/CD16^+$ cells are regarded as proinflammatory monocytes (Okamoto *et al*, 2003). $CD14^{++}/CD16^+$ cells account for 10% of all blood monocytes and they are characterised by a low level expression of the CD14 molecule and a high level expression of the CD16 (FcγRIII) molecule (Ziegler-Heitbrock *et al*, 1993). The very important functions of mononuclear phagocytes are to remove particulate antigens by phagocytosis and to present antigen to lymphocytes. The mannosyl-fucosyl receptor (MFR) is a very important receptor for phagocytic activity of human monocytes/macrophages, which binds to sugars on the surface of microorganisms (Roitt *et al*, 1993). Monocytes/macrophages are highly expressing CD14 (Barclay *et al*, 1997), a receptor for lipopolysaccharide binding protein (LBP) normally present in serum and which coats gram-negative bacteria. In addition monocytes and macrophages constitutively express two distinct Fc receptors for IgG such as FcγRI (CD64) and FcγRII (CD32); a third FcγRIII (CD16) is predominantly expressed by macrophages (Barclay *et al*, 1997). These receptors have different functions, which include triggering extracellular killing, or opsonization and phagocytosis. In addition to these a range of other receptors are important in uptake

of microorganisms including the complement receptor, CR1 (C3b receptor, CD35) (Roitt *et al*, 1993). Molecules of monocytes/macrophages involved mainly in adhesion, include the β2 integrins (Roitt *et al*, 1993); complement receptor CR3 (C3bi receptor, Mac-1) are present especially on activated macrophages, together with the 'leucocyte function antigen' LFA-1 and p150,95 (Roitt *et al*, 1993). The integrins αL (CD11a), αM (CD11b) and αX (CD11c) form pairs with CD18 (β2 integrin) to form LFA-1, CR3 and p159,95, respectively (Barclay *et al*, 1997; Sanchez-Madrid *et al*, 1983). Both CD11b and CD11c are found in intra cytoplasmic vesicles (of macrophages) and are rapidly expressed following activation (Roitt *et al*, 1993). Class II MHC antigens are present on some of the monocytes/macrophages and are important in presentation of antigens to T cells (Roitt *et al*, 1993). None of these markers so far discussed are lineage specific, although FcγRI is a particularly useful marker (Roitt *et al*, 1993).

In addition to all of these molecules, monocytes and macrophages also have receptors for cytokines such as IL4, IFNγ and migration inhibition factor (Roitt *et al*, 1993). The functions of monocytes and macrophages can therefore be enhanced by T-cell derived cytokines through these receptors. Such activated monocytes/macrophages also generate cytokines including IFNs, IL-1 and TNF-α (Roitt *et al*, 1993). Complement components and prostaglandins are also produced (Roitt *et al*, 1993). Like neutrophils, monocytes contain peroxidase, which inactivates the peroxide ions that they generate while killing ingested microorganisms (Roitt *et al*, 1993).

Monocytes/macrophages can fuse with themselves to form MGCs in tissues during chronic inflammatory reactions or osteoclasts in bone (Vignery, 2005). In addition there is now evidence that macrophages may act as hemopoietic stem cells by fusing with non-proliferating somatic cells and tumor cells (Vignery, 2005; Ogle *et al*, 2005).

### 1.2.2. Types of multinucleated giant cell

On the basis of the degree of antigen-specific lymphocyte involvement in the developing lesion, granulomas are classified into immune-type and foreign body type, and MGCs are a feature of both (Fais *et al*, 1997). MGCs are generally classified into Langhans' giant cells or foreign body giant cells (FBGCs), based on their morphology. The Langhans' giant cells are characterised by a relatively small number

of nuclei, generally less than 20, arranged in a circular peripheral arrangement within the giant cell, whereas FBGCs generally consist of much larger number of nuclei, greater than 20, which are arranged in an irregular fashion throughout the giant cell (Anderson, 2000; Okamoto *et al*, 2003). Langhans' giant cells are commonly seen in immune granulomas with epithelioid-like macrophages and are associated with indigestible particles of organisms surrounded by a collar of mononuclear leukocytes that are principally lymphocytes. The lymphocytic collar surrounding the immune granuloma is indicative of T cell-mediated immunity to the inciting agent, and products of activated T lymphocytes are thought to initiate the fusion of monocytes and macrophages to form Langhans' cells (Anderson, 2000). FBGCs are most commonly found in foreign body granulomas, where the indigestible or poorly digestible material is surrounded or encapsulated within a thin (one or two cells in thickness) layer of macrophages and FBGCs. The lymphocyte collar seen in immune granulomas is not present in foreign body granulomas.
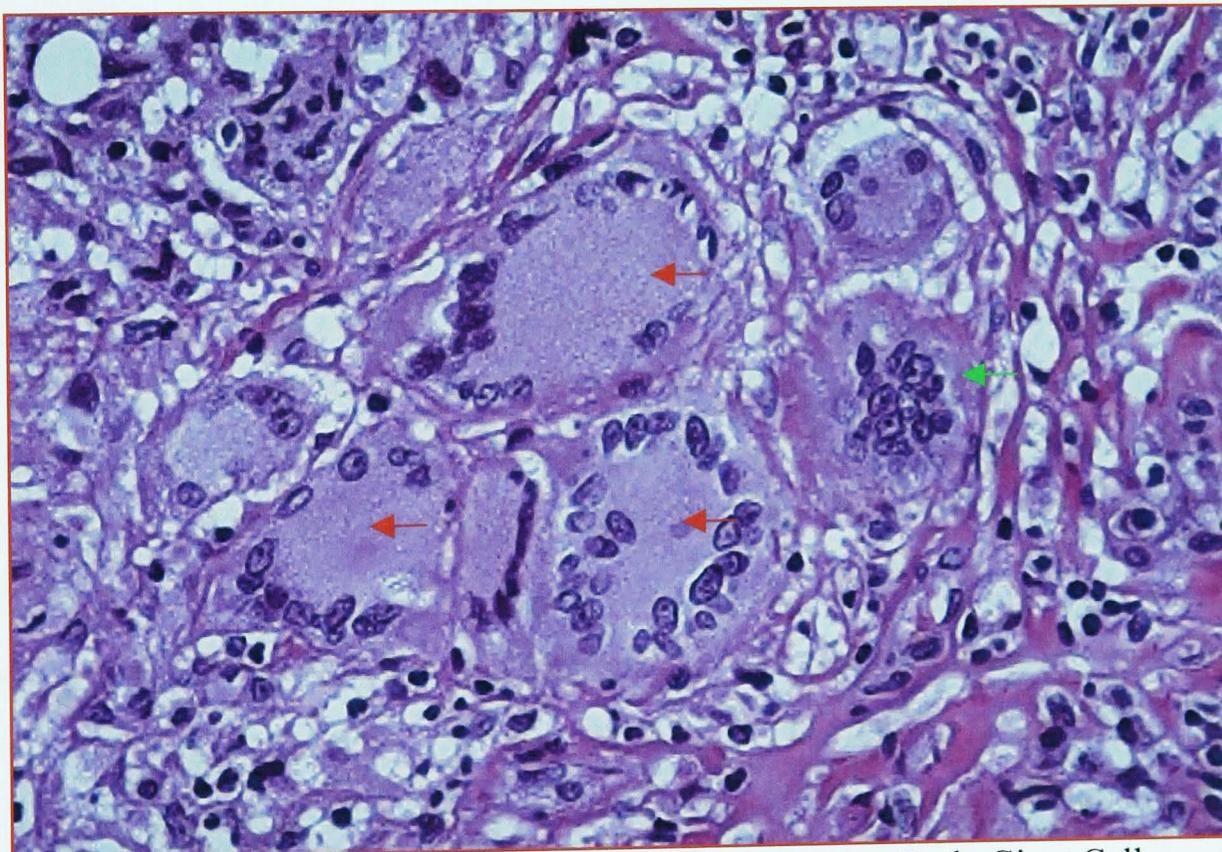


Fig.1.2.3. Langhans' Giant Cells (red arrows) and Foreign Body Giant Cells (green arrow) in tissue specimen.

The surface antigens of monocyte-macrophage lineage cells and adhesion molecules showed the same expression profiles between LGC and FBGC i.e. $CD1a^-$, $CD11a^{low+}$, $CD11b^+$, $CD11c^+$, $CD14^+$, $CD16^-$, $CD36^-$, $CD54^+$, $CD68^+$, $CD86^-$, $MAC387^-$ and 3A5 $^{cytoplasmic\ +}$. These immunohistochemical results suggest that both types of MGCs may be functionally as well as phenotypically similar cells with different distributions of

nuclei (Okamoto *et al*, 2003). However the *in vitro* MGC formation models show that LGCs and FBGCs are induced by different stimuli (Postlethwaite *et al*, 1982; Abe *et al*, 1983; Abe *et al*, 1986; McNally *et al*, 2003).

### 1.2.2.1. Langhans' giant cells

As described earlier, the Langhans' giant cells are seen in immune granulomas in the presence of indigestible particles of organisms such as the *M. tuberculosis*. They are also found in granulomatous disorders of unknown causes such as sarcoidosis and Crohns' disease (Anderson *et al*, 2000). As mentioned above, the association of lymphocytes with immune granulomas indicates a T cell mediated immune response to the infectious agent. Similarly the lesions of Crohn's disease consist of a diffuse infiltrate of lymphocytes and monocytes, together with epithelioid cells, MGCs and granulomas, which indicates that lymphocytes are very important for MGC formation here (Fais *et al*, 1997); spontaneous secretion of IFN-γ was observed in sarcoidosis and Crohn's disease (Fais *et al*, 1997). As described in section 1.2.4.1, various cytokines have been used to induce the formation of MGCs *in vitro*, to try to elucidate their role *in vivo*. Although some of the evidence is controversial, IFN-γ appears to be one of the pivotal factors promoting the formation of Langhans' cells. Antibodies to IFN-γ have been shown to inhibit the formation of giant cells *in vitro* (Most *et al*, 1990; Fais *et al*, 1994) and *in vivo* (Chensue *et al*, 1992). In addition the membrane bound molecules of mycobacterium in combination with cytokine containing supernatants directly induces the fusion of human monocytes *in vitro* to form MGCs. This suggests a direct effect of mycobacterium on the fusion process *in vivo* (Gasser and Möst, 1999).

Some studies *in vitro* indicate that monocytes are more able easily to form MGCs than macrophages. It is suggested that since monocytes are better at killing intracellular bacteria than macrophages, monocytes newly arrived at the site of infection may fuse with infected monocytes/macrophages to the benefit of the immune response (Most *et al*, 1997). It is reported that MGCs in sarcoidal lesions express monocyte (CD11a, CD14, 3A5) but not macrophage markers (CD16, MAC387) (Okamoto *et al*, 2003). This group also reported that MGCs are formed from the $CD14^{++}/CD16^{-}$, but not the $CD14^{+}/CD16^{+}$ subpopulation of monocytes *in vitro*.

However, the precise functions of the Langhans' cells are unknown and there are several ideas for the functional role of MGC in granulomas, including a disposal function for effete macrophages and specific functions in the phagocytosis of pathogens involved in granuloma formation (Möst *et al*, 1997). Byrd demonstrated that IFN-γ/IL-3 treatment reduced the growth of *Mycobacterium tuberculosis* in an *in vitro* infection assay (Byrd, 1998). This growth restriction was associated with the development of Langhans' giant cells from monocyte monolayers. Byrd observed dense growth of *M.tuberculosis* in the centre of the Langhans' cells and suggested that physical sequestration of the bacterium by giant cells might limit cell to cell spread. It has also been suggested that giant cells form because several phagocytes are necessary to ingest large aggregates of bacteria (Gasser and Most, 1999).

### 1.2.2.2. Foreign body giant cells

The most common foreign body granuloma is the suture granuloma, in which the macrophages and FBGCs are present at the surface of the sutures (Anderson, 2000). *In vivo* the FBGCs may remain at the implanted-tissue interface for the lifetime of the device, which in some cases may extend beyond 20 years (Anderson, 2000). FBGCs may be responsible for surface cracks, and degradation of biomaterial (Zhao *et al*, 1991; Nathan *et al*, 1987).

The research group of McNally and Anderson have extensively studied the biology of FBGCs *in vitro* (McNally and Anderson, 2003). The cell–derived factors responsible for the FBGCs formation were demonstrated by using recombinant human lymphokines with freshly isolated human monocytes. The authors reported that human interleukin-4 induced the formation of FBGCs from human monocyte derived macrophages, an effect that was optimised with either granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (Anderson, 2000). Interleukin-4 induced very large FBGCs with randomly arranged nuclei (~280) and extensive cytoplasmic spreading (Anderson *et al*, 2000). The morphology of FBGCs developed from human monocytes *in vitro* with IL-4 is indistinguishable from those that from on implanted biomaterials (McNally *et al*, 1995), which may be >1mm in diameter and occupy up to 25% of implant surface area (Zhao *et al*, 1992). IL-4 is produced by T-helper type2 (Th2) lymphocytes. Interestingly, IL-13, another Th2 cytokine, also induces FBGC formation *in vitro* (DeFife *et al*, 1997). IL-4 and IL-13 appear to act independently of one another (DeFife *et al*, 1997).

McNally and Anderson have also extensively investigated the role of substrate surface chemistry on monocytes adhesion and FBGC formation on biomaterials. In this process the adhesion of monocyte on implanted material is critical to biocompatible outcome because it initiates macrophage development and FBGC formation. The authors hypothesized that monocyte adhesion to biomaterial occurs via specific adhesion mechanisms i.e. interactions of cell surface receptors (e.g.integrins) with adsorbed proteins and that material-dependent protein adsorption determines the nature of adhesion molecules engaged. They have found that FBGC formation is significantly affected by the surface chemistry of the substrate to which the monocytes adhere, presumably reflecting differences in the composition and/or conformation of the proteins that are adsorbed (McNally *et al*, 1994; Anderson, 2000).

### 1.2.2.3. Osteoclasts

Bone is a complex, dynamic structure governed by the coupled actions of osteoclasts and osteoblasts (Bonewald *et al*, 2002). Most adult skeletal diseases (osteoporosis, periodontal disease, rheumatoid arthritis, multiple myeloma and metastatic cancers) are due to excess osteoclastic activity, leading to an imbalance in bone remodelling which favours resorption (Boyle *et al*, 2003). Osteoclasts are bone-degrading polykaryons, derived from monocyte-macrophage precursors at or near the bone surface (reviewed in Blair and Athanasou, 2004; Boyle, 2003). They are playing a regulatory role in balancing calcium homeostasis with skeletal modelling and repair (Blair *et al*, 2005). Recent studies have elucidated the factors involved in the differentiation of these cells and the biochemistry of bone resorption. A full description of these processes is outside the scope of this thesis. Briefly, however, osteoclasts are generated by the interaction between macrophage colony stimulating factor 'RANK' generated by monocytes/macrophages and TNF-family ligand 'RANKL' from mesenchymal cells in bone. The RANK and RANKL are considered as major determinants of osteoclast formation (Blair and Athanasou, 2005). In contrast, TNF-α, IL-6 and IL-11 have also been shown to promote osteoclast formation by RANKL-independent processes (reviewed in Blair and Athanasou, 2004; Blair *et al*, 2005). In addition the hormones, such as PTH/PTHrP, glucocorticoids and 1,25(OH)2D3, and humoral factors including TNF-α, IL-1, TGFs and prostaglandins also influence osteoclast formation (Blair and Athanasou, 2005).

Osteoclasts generally contain 10-20 nuclei per cell (Anderson, 2000). Whilst they are formed from precursors belonging to the monocytes/macrophage lineage, they appear distinct in terms of function and phenotype from Langhans' cells and FBGCs (Roodman, 1996; Anderson, 2000). Also, neither IFN-γ nor IL-4 have been identified as significant in their development.

### 1.2.2.4. HIV-mediated syncitium formation

Human immunodeficiency virus-1 infection of T lymphocytes and freshly isolated monocytes leads to the formation of giant cells also known as HIV-1 syncytia (HIV1-S) and MGCs respectively. The formation of HIV-1 infected $CD4^+$ T lymphocytes syncytia is highly dependent on interaction between intercellular adhesion molecule (ICAM-1) and LFA-1 (CD11a/CD18) (Fais et al, 1997; Anderson, 2000). In addition to $CD4^+$, the interaction between CD98 and CD147 is very essential in regulation of virus-induced cell fusion of monocytes; the cell fusion mediated by HIV-1 occurs between viral glycoprotein-gp160 and $CD4^+$ monocyte or lymphocytes. Mori and co-workers demonstrated the monocyte fusion in vitro using U937-2 cells ($CD4^+$ U937 cells) transfected with HIV-gp160 gene (Mori et al, 2004). Similarly the syncytia were generated in vitro by the infection of AIDS retrovirus with CD4 positive T cell lines JM (Jurkat) and VB (Lifson et al, 1986). The antibodies to CD4 specifically inhibited the fusion (Lifson et al, 1986). Steffy and co-workers showed that the HIV-2 fusion peptide gp41 is capable of inducing syncytium formation in vitro (Steffy et al, 1992). The morphology of giant cells produced by HIV-1 infection of normal human primary monocytes is similar to that observed with IFN-γ induced MGCs (Anderson, 2000). In contrast to these monocyte-derived macrophages did not produce multinucleated giant cells (Anderson, 2000).

### 1.2.3. Multinucleated giant cells in disease

### 1.2.3.1. Infections

The multinucleated giant cells containing granulomas are associated with infections caused by bacteria especially mycobacteria; parasites (e.g. schistosomiasis, leishmania); fungi (Aspergillus, Histoplasma, Coccidioides) (James, 2000) and viral infections such as HIV-1 and HIV-2 (Steffy et al, 1992; Alkhatib et al, 1996; Choe et al, 1998).

The HIV-1 induced $CD4^+$ T cell derived syncytia (HIV-S) have never been observed in vivo since they are cellular structures with high numbers of nuclei that inevitably

die shortly after infection. This is suggested as an explanation for the fact that HIV-S have not been identified in tissues of AIDS patients whereas the monocyte derived MGCs were observed *in vivo* in brain and lymph nodes of AIDS patients (Fais *et al*, 1997; Anderson, 2000); this may result from the homotypic fusion of HIV-1 infected blood monocytes recruited and activated in these tissues (Fais *et al*, 1997). Multinucleated giant cells produced by HIV-1 infection of monocytes/macrophages are bi-and trinucleated cells that remain viable for a considerable period of time (Fais *et al*, 1997).

### 1.2.3.2. Immune disorders

The multinucleated giant cells are common features of immune disorders such as Crohn's disease, sarcoidosis and primary biliary cirrhosis. It is speculated that these disorders may have an undiagnosed infection as a root cause (James, 2000; Okamoto *et al*, 2003). For example, sarcoidosis is a systemic granulomatous disease characterised by non-caseating granulomas (Okamoto *et al*, 2003). It is generally recognized that sarcoidosis is a disorder of T lymphocyte-mediated inflammatory response to the unknown antigenic stimuli (reviewed in Newman *et al*, 1997). In particular, mycobacterium and propionic bacteria have received much attention as the possible etiologic agents of sarcoidosis (Ishige *et al*, 1999; Eishi *et al*, 2002). Granulomas are also associated with some type III hypersensitivity reactions involving the lung e.g. farmers' lung. Granulomas also develop in response to some chemicals such as silica, (leading to silicosis), beryllium, zirconium and talc on inhalation (James *et al*, 2000). As described above, FBGC containing granulomas develop in response to surgical implants/devices and may shorten the life span of the implant.

### 1.2.3.3. Cancer

There is often a granulomatous component in malignant diseases. Sarcoid granulomas may be found in various tumors and in their draining lymph nodes, particularly those draining carcinoma of the lung, stomach, and uterus (James *et al*, 2000). Interestingly, the recent evidence suggests that tumor cells may fuse with themselves, as well as with monocyte/macrophages to create diversity, enhanced metastasis, chromosomal aberration and epigenetic regulation (Vignery, 2005).

## 1.2.4. Formation of MGCs *in vitro*

MGCs originate from fusion of monocytes or macrophages, but little is known about the mechanisms of the fusion process itself. Furthermore, it is not clear how monocyte fusion is induced *in vivo* and whether different mechanisms are involved in different pathological states.

### 1.2.4.1. Effect of stimulants used

There have been many attempts to generate MGCs *in vitro*, in an effort to understand how this process occurs and is controlled *in vivo* and in different pathological states. However, these have used different types of stimuli, different sources of monocytes/macrophages and different culture conditions. The list of factors that have been used to generate MGCs from human monocytes are given in table.1.2.1.

| Stimulants | Authors |
|---|---|
| SN of monocytes | Black *et al*, 1976 |
| Tetanus toxoid, PPD, NaIO$_4$ | Postlethwaite *et al*, 1982 |
| Con A | Kreipe *et al*, 1988, Takashima *et al*, 1993 Falzoni *et al*, 1995 |
| PMA | Hassan *et al*, 1989 Merrill *et al*, 1997 |
| IFN-γ | Hassan *et al*, 1989 Möst *et al*, 1990 Enelow *et al*, 1992 Fais *et al*, 1994 Falzoni *et al*, 1995 Byrd, 1998 |
| IL-3 | Enelow *et al*, 1992 Byrd, 1998 |
| IL-4 | Takashima *et al*, 1993 Kazazi *et al*, 1994 McNally *et al*, 1995 DeFife *et al*, 1997 Jenney *et al*, 1998 McNally *et al*, 2002 |
| Con A + IFN-γ | Falzoni *et al*, 1995 |
| PHA | Postlethwaite *et al*, 1982 Liu *et al*, 1996 |
| IL-4 + M-CSF | Akagawa *et al*, 1996 |
| Nippostrongylus brasiliensis | Seitzer *et al*, 1997 |
| SN of Con A stimulated mononuclear cells | Möst *et al*, 1997 |
| Heat-killed candida albicans | Heinemann *et al*, 1997 |
| IL-4 + GM-CSF | Dugast *et al*, 1997 |
| IL-13 | DeFife *et al*, 1997 Ikeda, *et al*, 1998 |
| IL-4 + M-CSF | Ikeda *et al*, 1998 |

| | |
|---|---|
| SN of HIV-transfected T cells + BCG | Gasser *et al*, 1999 |
| SN of Con A stimulated mononuclear cells ± MDP | Mizuno *et al*, 2001 |
| Vitamin E + α-tocopherol | McNally *et al*, 2003 |
| Vitamin D$_3$ | Abe *et al*, 1983 |

Table.1.2.1. Stimuli used for *in vitro* generation of MGCs from monocyte/macrophage. Where: SN=Supernatants; PHA=Phytohemagglutinin-A; Con A=Concanavalin A; PMA=Phorbol myristate acetate; MDP=Muramyl dipeptide.

In addition MGCs have been generated from various sources of animal macrophages and also from myeloid cell lines, as discussed below. Peripheral blood monocytes are generally considered as non-replicating cells and autoradiograpahy confirmed that fusion occurred between non-replicating monocytes, rather than by nuclear division (endomitosis) without cytoplasmic division (Postlethwaite *et al*, 1982; Weinberg *et al*, 1984).

Early studies showed that a factor derived from antigen or mitogen-stimulated T cells promoted the fusion of human monocytes to form MGCs *in vitro* (Postlethwaite *et al*, 1982). Subsequently, there have been various attempts to identify cytokines that may be involved in inducing fusion and many studies have indicated the importance of IFN-γ in MGC formation. Thus recombinant IFN-γ has been shown to induce fusion of human monocytes to form Langhans' type cells (Weinberg *et al*, 1984; Okamoto *et al*, 2003) and antibodies to IFN-γ inhibit fusion induced by cytokine-containing supernatants (Weinberg *et al*, 1984; Most *et al*, 1990; Fais *et al*, 1994). It has been reported that IL-3 enhances IFN-γ-induced fusion, whereas IL-4 had an inhibitory effect (Enelow *et al*, 1992); IL-4 has also been shown to inhibit Con A-induced monocyte fusion (Takashima *et al*, 1993). Interestingly, McNally and Anderson reported that Langhans' giant cells (LGC) were formed under the influence of IFN-γ whereas IL-4 induced the formation of FBGCs under otherwise identical culture conditions (McNally and Anderson, 1995). From these and other studies it is clear that the characteristics of MGCs are dependent on the stimulant used. DeFife and co-workers reported that the lymphokine interleukin IL-4 and the related lymphokine IL-13 are the only human macrophage fusion factors known to induce very large FBGCs (DeFife *et al*, 1997). Although IL-4 and IL-13 are strong inducers of monocyte/macrophage fusion, the maximum fusion was achieved by the combination of the two interleukins. The antibody studies showed that the fusion inducing effect of IL-13 is acting independently of IL-4 to promote foreign body giant cell formation but

IL-4 and IL-13 may trigger a common mechanism for macrophage fusion (DeFife *et al*, 1997). The addition of IL-3 or GM-CSF optimised the effect of IL-4 on the formation of FBGC (Okamoto *et al*, 2003; McNally and Anderson, 1995). In contrast to these, Takashima and co-workers found an inhibitory effect with IL-4 on Con A induced MGCs formation (Takashima *et al*, 1993). In general, TH1-type cytokines appear to promote Langhans' giant cell formation, whereas TH2-type cytokines appear to induce FBGCs (Anderson, 2000).

There are some reports that bacterial components influence the monocyte/macrophage fusion. Eishi, (1994) reported that muramyl dipeptide (MDP) from bacterial cell wall evokes epitheliod cell granulomas. Recently, Mizuno *et al*, (2001) found that the MDP significantly increased the Con A conditioned medium-induced fusion of $CD14^{++}/CD16^-$ peripheral blood monocytes. By contrast, Takashima and co-workers reported that bacterial lipopolysaccharide (LPS) inhibited Con A induced monocyte fusion (Takashima *et al*, 1993).

In their research on FBGC formation, McNally and Anderson showed that Vitamin E alone induced FBGC formation from monocytes to some extent and enhanced IL-4 induced fusion in their system (McNally and Anderson, 2003). They also showed that a component of vitamin E α-tocopherol was much more potent than vitamin E itself. α-tocopherol is reported to activate diacylglycerol kinase and an inhibitor of this enzyme (R59022) completely abrogated FBGC formation. The authors therefore suggest a role for diacylglycerol kinase in FBGC formation.

Other workers have investigated the effects of 1α, 25-dihydroxy vitamin D3, a known stimulator of osteoclast differentiation (Roodman, 1996) in MGC formation. Abe and co-workers have examined the effects of 1α, 25-dihydroxy vitamin D3 in the fusion of mouse alveolar macrophage. Conditioned medium from spleen cells co-cultured with 1α, 25-dihydroxy vitamin D3, stimulated fusion of alveolar macrophages and the vitamin alone also induced some cell fusion (Abe *et al*, 1983). Some effect of 1α, 25-dihydroxy vitamin D3 on MGC formation by human monocytes has also been reported (Enelow *et al*, 1992).

### 1.2.4.2. Effects of differentiation/activation

Attempts to look at the differentiation stage of cells that gives rise to MGCs have been made. In humans, it appears that MGCs are formed more easily from monocytes

than macrophages (Möst *et al*, 1997). Whilst freshly isolated monocytes gave high fusion rates when cultured with cytokine-containing supernatants, this decreased when the cells were induced to differentiate into macrophages by culturing them with human serum. Almost no MGC formation could be seen by day 8 of culture, and human serum had a negative effect on fusion. However, macrophages were still able to fuse with freshly isolated monocytes. Since the antimicrobial activity of monocytes declines during maturation in macrophages, the authors speculated that fusion with monocytes could benefit macrophages that take up certain bacteria or parasites in infectious granulomas. Fais and Pallone (1995) reported that virtually no MGC were formed from intestinal macrophages, in contrast to autologus peripheral monocytes. Interestingly, intestinal macrophages from Crohn's disease patients formed MGCs spontaneously; it suggests that MGCs in Crohn's disease might be recruited monocytes. However, the conditions required for the formation of MGCs *in vitro* may vary depending on the stimuli, and on the source and animal species of the mononuclear phagocytes used as starting material. Thus, Anderson and McNally have generated FBGC from human monocytes allowed to differentiate for 3 days, followed by the addition of IL-4 or IL-13 to induce fusion (McNally and Anderson, 2002). Also, Vignery and co-workers has generated MGCs using highly purified (over 99%) rat alveolar macrophages, which fuse spontaneously in culture to form MGCs containing large numbers of nuclei that express osteoclast markers. The relationship between these MGCs and classical bone-associated osteoclasts is unclear (Vignery, 2000). The fusion of rat alveolar macrophage initiates within few hrs after plating and is complete within 3 days in the absence of proliferation and differentiation factors such as M-CSF and RANKL, respectively, to induce their multinucleation. The researchers point out that this system differs from many others that use mixed populations of cells and may require growth factors M-CSF and RANKL to generate macrophages that are capable of fusing (Vignery, 2000).

The rate and extent of fusion thus also varies depending on the differentiation status of the starting population of cells and the stimulus used. Abe *et al* reported the differences in the time course of mouse macrophage fusion, depending on different treatments. The fusion rate induced by Con A-conditioned medium almost attained a plateau (~85% of cells fused) on day 2 and it increased further on day 3. However, fusion induced by IL-4 occurred as early as day 1 of culture but the maximal fusion

rate was only 30 to 40%. The 1α, 25-dihydroxy vitamin D3 induced the fusion of macrophages after 24 hrs, which then increased linearly until the maximal fusion rate (similar to that obtained with Con A-CM) was observed on day 3 (Abe *et al*, 1991). In humans, variations between donors in the efficiency of MGC formation from monocytes have been observed in response to the same stimuli (Möst, 1997; Spöt *et al*, 1995). In general, Langhans' type MGCs (induced by IFN-γ) start to appear by 24hrs in culture and fusion is maximal by 72-92hrs (Weingberg *et al*, 1984). FBGCs formed on day 2 from the bone marrow cells cultured with IL-4 and reached maximum fusion by day 5 (McInnes and Rennick, 1988); the interleukin 13 induced FBGCs formed within 3 days from the macrophage/monocytes (DeFife *et al*, 1997).

Myeloid cell lines have also been used to study fusion. For example, Weinberg and co-workers generated the polykaryons from the human promyelocytic leukaemia cell line HL-60 using IFN-γ. From these observation the authors are pointing out that small numbers of contaminating lymphocytes in blood monocytes will not affect the formation of polykaryons (Weinberg *et al*, 1984). Some investigators raised doubts about the relevance of MGCs generated from transformed cell lines (Anderson, 2000).

### 1.2.4.3. Concanavalin A induced formation of MGCs

In this thesis, a model system in which lectin Concanavalin A induces fusion of adherent human monocytes was used. Therefore it is pertinent to review what is known of this system.

Concanavalin A (Con A) is a member of a group of proteins called lectins, which is isolated from jack beans. Con A binds to a variety of eukaryotic cells through specific interactions with sugar residue or saccharide-containing cellular receptors and it has been widely used as a molecular probe in studies of cell membrane dynamics and cell division (Reeke *et al*, 1975). Con A is a well-known activator and mitogen for T lymphocytes (Ruscetti and Chervenick, 1975).

Initial studies used conditioned medium from Con A treated spleen cell cultures to generate MGCs from mouse alveolar macrophages (Abe *et al*, 1983). Subsequent studies indicated GM-CSF was a major fusion inducing factor in the Con A conditioned medium (Abe *et al*, 1992) but the relevance of these findings to Con A induced human monocytes fusion is unknown.

Most et al (1990) reported that conditioned medium from Con A stimulated peripheral blood mononuclear cells readily caused generation of MGCs from monocytes. This effect was completely abrogated by monoclonal antibody to IFN-γ. IFN-γ alone was less effective than the conditioned medium, but other cytokines (IL2, IL4 and TNF-α) considered to activate monocytes, neither induced the MGC nor enhanced the effect of IFN-γ. The authors reported that the formation of MGC could be almost entirely inhibited by mAb to α or β chain of LFA-1 and to a lesser extent by relatively high concentration of a mAb against ICAM-1, one of the ligands of LFA-1. In addition, mAb to LFA-1 very efficiently inhibited clustering of monocytes, which is a pre-requisite for the formation of MGC. This effect was not observed with mAb to IFN-γ.

Takashima et al (1993) reported that MGCs could be produced by incubation of highly purified human blood monocytes with Con A alone and that the effect was dose and time dependent. Efficient fusion was observed with $10\mu g.ml^{-1}$ Con A and MGC were observed by 24hrs, reaching a plateau by 72hrs. The MGC were predominantly of the Langhans' type on their morphological character basis. The authors reported that T cell-derived cytokines (IFN-γ, IL-2, IL-4, GM-CSF, TNF-α) did not induce MGC when added to the monocyte culture alone and an antibody to IFN-γ did not suppress Con A induced fusion. However, IFN-γ enhanced Con A induced MGC formation in a dose-dependent manner. By contrast, IL-4 suppressed MGC formation in a dose and time dependent manner and IL-4 antagonised the enhancing effect of IFN-γ. The suppressive effect on the formation of MGC was completely abrogated after treatment with anti IL-4 antibody. The involvement of monokines (IL-1α, IL-β, IL-6 and TNF-α) in Con A induced monocyte fusion was investigated by treatment with corresponding anti monokine antibodies. The anti TNF-α antibody suppressed Con A induced monocyte fusion in a dose dependent manner and a high dose of anti IL-6 antibody also had a partially suppressive effect. The authors argued that T cell-derived cytokines are not necessarily required to induce fusion and that monokines such as TNF-α, and the activation status of the monocytes may be important. The apparent discrepancy between these results and those reported by Möst and co-workers (1990) may be due to differences in experimental conditions. The recent report by Takeda and co-workers, and results described in this thesis confirm that incubation of human monocytes with Con A

alone can induce MGC formation (Takeda *et al*, 2003), but do not rule out effects of cytokines produced by contaminating lymphocytes.

## 1.2.5. Mechanism of mononuclear phagocyte fusion

As outlined above, there have been some difficulties in determining which signals (cytokines etc.) are required to stimulate MGC formation. This is in part due to the fact that different research groups have used different culture systems and different sources of monocytes/macrophages. Nevertheless, it is becoming apparent that the different types of mononuclear phagocyte-derived giant cells (Langhans' cells, FBGC and osteoclast) are formed in response to different signals. Prior to fusion itself, the cells must achieve a critical density, either by proliferating in situ, as may be the case for osteoclasts (Vignery, 2000) or accumulating from the bloodstream by chemotaxis. There must also be cell:cell contact and recognition (Vignery, 2000).

Recently, progress has been made in identifying cell surface molecules that may be involved in the monocyte/macrophage fusion process itself. However, the molecular basis of fusion is still be elucidated and it is unclear, as yet, whether a common mechanism will be shared by the different types of MGC. Prior to describing molecules that have been identified as of importance in mononuclear phagocyte fusion, it is useful to discuss what is known of other cell fusion mechanisms.

### 1.2.5.1. Membrane fusion: virus:cell and intracellular vesicle fusion

The fusion of viruses with cells, in particular influenza virus and human immunodeficiency virus (HIV), provided strong evidence that fusion is mediated both by viral proteins (ligand) and host cell surface molecules that function as viral receptors (reviewed in Vignery, 2005). The receptor-ligand interaction mediates the binding of viral membrane glycoproteins to a receptor on the target cells (Vignery, 2005; Ogle *et al*, 2005) e.g. the HIV viral membrane glycoproteins gp120 binds to its receptor CD4$^+$ in T lymphocytes and macrophages; similarly the hemagglutinin protein of influenza binds to its receptor sialic acid on epithelial cells (Vignery, 2005). The fusion proteins are integral membrane glycoproteins that exist as oligomers on the surface of each virion. Most viral fusion proteins contains a stretch of hydrophobic amino acids, known as a fusion peptide, which penetrates host cells, destabilising the lipid bilayer of the host cell. The fusion protein then undergoes a conformational change, forming a hairpin-like α-helical bundle, which acts like a spring to propel the viral membrane close enough to the cell membrane to trigger fusion (Fig.1.2.4)

(Vignery, 2005). Apart from these it has been reported that the chemokine family of G-protein coupled receptors, most notably CXCR4 and CCR5, are involved in HIV infection (Vignery, 2000). There are now at least 10 chemokine receptors identified as HIV co receptors (Vignery, 2000).
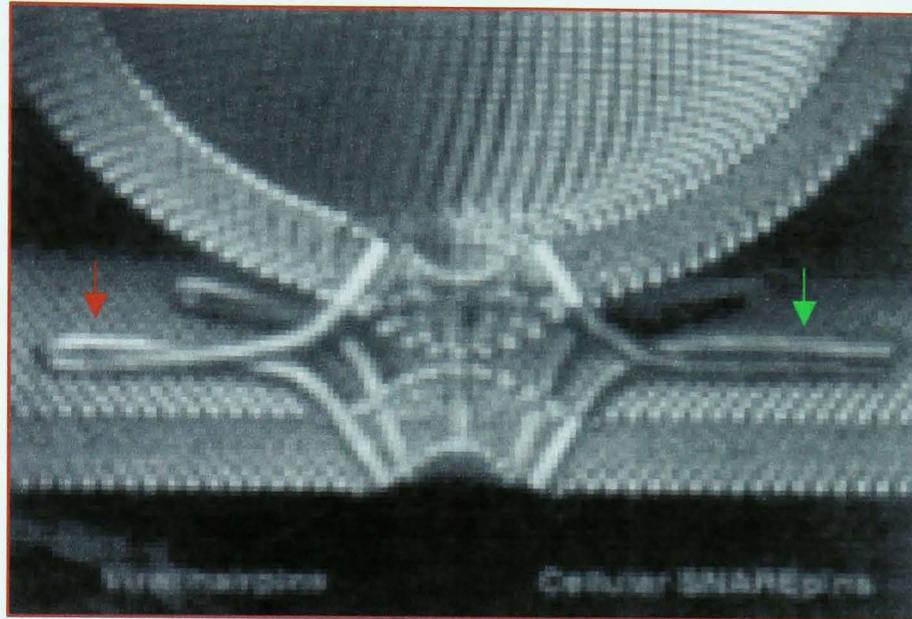


Fig.1.2.4. Hypothetical model of the synaptic fusion complex as it joins two membranes. In contrast to viral hairpins fusion protein (Red arrow), cellular SNARE pins (Green arrow) are formed from separate polypeptides that reside in different membranes before fusion. Cellular SNAREpins and viral hairpins suggest that they all employ a fundamentally similar mechanism to coalesce lipid bilayers (Image: Vignery, 2000).

Another type of fusion occurs between distinct membranes within a cell during intracellular trafficking between the endoplasmic reticulum and golgi apparatus; the fusion of an intracellular vesicle with its target membrane is mediated by a set of conserved proteins that are collectively called as SNAREs (soluble NSF attachment protein [SNAP] receptors) (Vignery, 2005). Many vesicle (v) – and target (t)-SNAREs have been characterized in yeast, plants, and animals, and shown to form a bundle of α-helices (SNAREpins) that bring opposing membranes close enough to fuse. The fusion process between intracellular membranes occurs in a manner analogous to that between viruses and cells (Vignery, 2000).

### 1.2.5.2 Cell: Cell fusion

A number of putative fusion proteins involved in cell:cell adhesion have recently been identified e.g. immunoglobulin superfamily proteins (reviewed in Chen and Olson, 2005). These proteins do not contain an obvious fusion peptide or α-helical spring, suggesting that cell:cell fusion may use different mechanisms from other types of

membrane fusion. Also, it is postulated that different types may have cell-type specific fusion mechanisms (Vignery, 2000).

Among the different types of cell fusions, fertilisation is perhaps the most well known form of cell-cell fusion. The membrane fusion between sperm and egg occurs after a series of pre-fusion events, including penetration of outer layer of the oocytes by sperm, secretion of enzymes by the lysosome-like acrosome in the sperm head, and penetration of the egg's inner layer, the zonapellucida, by the sperm. In addition proteins such as ADAM (A Disintegrin and Metallo protease) family transmembrane proteins on the sperm, such as fertilin α, fertilin β, and cyritestin, tetraspanin CD9 and integrins on egg surface have been postulated to mediate membrane fusion (Chen and Olson, 2005; Takahashi *et al*, 2001; Kaji *et al*, 2002, see section 1.1.5.6.1).
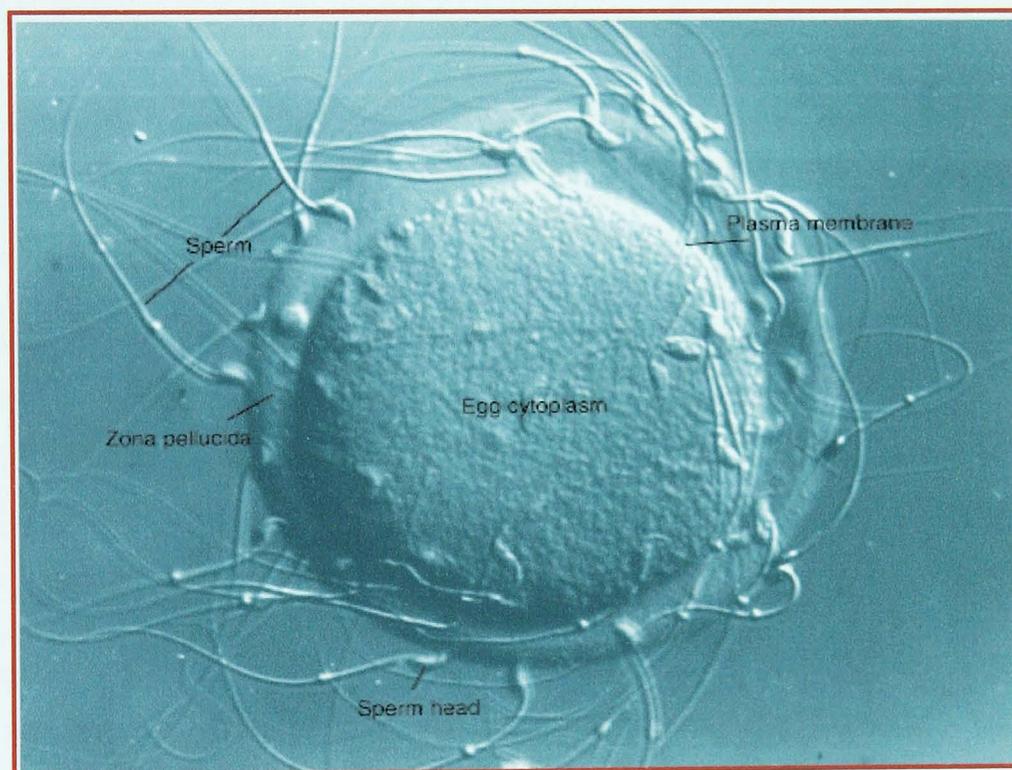


Fig.1.2.5. Sperm-egg fusion. Image adapted from Wassarman *et al*, (2001).

Similarly the fusion of mononucleated myoblasts to form multinucleated muscle fibers is an essential step in skeletal muscle differentiation. The embryo of Drosophilia contains two populations of myoblasts such as founder cells (seeds for future muscle fibers) and fusion competent cells, which undergo fusion by the activity of two classess of proteins such as Immunoglobulin (Ig) domain-containing transmembrane proteins Dumbfounded (Duf), Roughest (Rst), Sticks and stones (Sns) and Hibris (Hbs). These cell surface receptors are thought to mediate recognition and adhesion of the two types of muscle cells through direct interactions (Chen and Olson, 2005).

In addition the syncytium formation by fusion occurs during the development of placenta (Ogle *et al*, 2005), where for the mammalian placenta, trophoblasts fuse to form a syncytial layer of cells (syncytiotrophoblast) that functions as a barrier between maternal and fetal blood vessels. The proteins that are involved in this type of fusion are not known except syncytin (Chen and Olson, 2005). Syncytin is a single-pass transmembrane protein that can induce ecotopic cell-cell fusion in transfected cells and it is nearly identical to the envelope protein of the human endogenous retrovirus HERV-W. Syncytin functions as a class I fusion protein like hemagglutinin (HA). However, this mechanism of trophoblast fusion is unlikely to be universal, because syncytin is only present in primates and not in other mammals in which placental trophoblasts also undergo cell fusion to form the layer of syncytiotrophoblasts (Chen and Olson, 2005).

Cell fusion events are also involved in the regeneration of liver tissue and it was demonstrated that the bone marrow cells from mice fused with hepatocytes of mice (Ogle *et al*, 2005).

### 1.2.5.3. Cell surface molecules implicated in MGC formation

A number of putative fusion proteins have recently been identified in the plasma membrane of various types of fusing cells from different species, many of which belong to the immunoglobulin superfamily of proteins, which are discussed below (Vignery, 2003; Vignery, 2005).

**LFA-1 and ICAM-1**

Various groups have shown that antibodies to LFA-1 and ICAM-1 inhibit fusion (Most *et al*, 1990; Gasser and Most, 1999; Kurachi *et al*, 1993). Other integrins especially $\alpha V\beta 3$ have also implicated in osteoclast formation (Boissy *et al*, 1998). However, involvement of integrins and their ligands in fusion may be indirect since they are known to be involved in adhesion, spreading and aggregation, which are pre requisite for fusion. Thus Boissy and co-workers found that a cell permeable peptide that blocks $\alpha V\beta 3$ function inhibited spreading and fusion in an osteoclast like model system *in vitro*. In their studies on *in vitro* formation of FBGC McNally and Anderson reported that the initial monocyte adhesion is strongly mediated by $\beta 2$ integrins, whereas during the induction of macrophage fusion by IL-4, an additional dependence on $\beta 1$ integrins is acquired. This indicates that both $\beta 1$ and $\beta 2$ integrin play cooperative roles in cell adhesion during the formation of multinucleated giant cells,

but does not necessarily mean that they are involved in the fusion process (McNally and Anderson, 1994; McNally and Anderson, 2002).

**Cadherins**

E-Cadherin is a member of the homophilic calcium-dependent adhesion molecules family involved in cell:cell interactions. An antibody to E-Cadherin inhibited mouse osteoclast formation *in vitro*, but did not affect proliferation or adhesion (Mbalaviele *et al*, 1995).

**CD98 and integrin α3 (FRP-1 and FRP-2)**

Recently additional molecules, which regulates cell fusion have been identified and are designated as fusion regulatory protein-1 (FRP-1) and fusion regulatory protein-2 (FRP-2). FRP-1 and FRP-2 were identical to CD98 heavy chain and integrin α3, respectively (reviewed in Tsurudome and Ito, 2000). The FRP-1 system is involved in virus-mediated cell fusion and multinucleated giant cell formation of blood monocytes. Monoclonal antibody against human FRP-1 heavy chain induces polykaryocytes that have properties of osteoclasts (reviewed in Tsurudome and Ito, 2000). Similarly mAbs to FRP-1 and FRP-2 proteins induced MGC formation *in vitro* from monocytes (Tabata *et al*, 1994; Higuchi *et al*, 1998), and enhanced fusion of $CD4^+$ U937 cell line transfected with HIV gp160 gene or HeLa cells infected with Newcastle disease virus (Ohgimoto *et al*, 1995; Higuchi *et al*, 1998). CD98 is a type II integral membrane protein that associates covalently with several alternative light chains on the cell surface. It has been implicated in a range of functions including differentiation, oncogenic transformation, apoptosis and amino acid transport. It associates with integrins and has roles in regulating integrin signalling (Cho *et al*, 2003; Feral *et al*, 2005). It has been suggested that CD98 is a component of an egg surface "tetraspanin web" that includes CD9, CD81, integrins and integrin-associated proteins that is necessary for fusion (Takahashi *et al*, 2001).

**ADAM9**

The ADAMs (A Disintegrin And Metalloprotease) is a family of transmembrane proteins with putative roles in fertilisation and myoblast fusion. An anti ADAM9 antibody was found to block anti CD98 or RANKL mediated fusion of human monocytes, whereas it enhanced anti CD98 mediated adhesion. An inhibitor of ADAM9 metalloprotease activity also suppressed MGC formation. Interestingly ADAM9 contains a potential fusion peptide (Namba *et al*, 2001).

**Mannose receptor**

The mannose receptor is an endocytic/phagocytic receptor expressed by monocytes/macrophages. Inhibitors of mannose receptor activity (alpha-man-nan and synthetic neoglycoprotein conjugates) inhibited IL-4 induced FBGC formation (McNally *et al*, 1996). Inhibitors (swainsonine and castanospermine) of glycoprotein processing that interferes with the arrival of newly synthesized mannose receptors at the cell surface also attenuated macrophage fusion and the formation of giant cells. Mannose receptors were specifically up regulated by IL-4 in the culture system and were present and concentrated at macrophage fusion interfaces. These data suggest that the macrophage mannose receptor may be an essential participant in the mechanism of IL-4 induced macrophage fusion. IL-13, which also induces fusion, upregulates mannose receptor (DeFife *et al*, 1997). A role for this receptor has also been implied in a cell line (RAW264.7) model for osteoclast development (Morishima *et al*, 2003).

**P2Z/P2X$_7$ purinergic receptor**

Purinergic (pore-forming) receptors are emerging as one of the most interesting new families of plasma membrane receptors, which are also known as pore-forming receptor that binds ATP (Chiozzi *et al*, 1997; Falzoni *et al*, 1995). Falzoni and co-workers showed that human MGC formation induced by Con A was blockable with the P2X$_7$ blocker, oxidised ATP (Falzoni *et al*, 2000). Macrophage cell lines expressing high levels of P2X7 fuse spontaneously in culture, whereas those with low levels do not (Chiozzi *et al*, 1997). mAbs to these receptor block fusion (Falzoni *et al*, 2000), but fusion is enhanced by enzymes that destroy extracellular ATP. P2X7 receptor appears to be preferentially localised to sites of cell-to-cell contact in fusing macrophages (Falzoni *et al*, 2000).

**Macrophage fusion receptor (MFR)**

Components of the putative machinery that mediates the fusion of macrophages were identified initially by Vignery's research group using monoclonal antibodies that recognized cell surface determinants and altered fusion in tissue culture. The first protein identified in this way was macrophage fusion receptor (MFR) (Vignery, 2000). MFR is a type 1 transmembrane protein belonging to Ig gene superfamily with 3Ig (2C1,1V, Fig.1.2.6) loops, structurally closly resembling CD4. MFR (a.k.a CD172a, P84, SHPS-1, SIRPα and BIT) is expressed only by myeloid cells and

neurons (Vignery, 2000). MFR is strongly but transiently expressed in macrophages at the onset of fusion. mAbs to MFR and the recombinant extracellular domain of MFR block cell fusion; mAbs do not block aggregation, but a recombinant protein corresponding to extracellular domain did (Vignery, 2005). MFR contains 4 putative tyrosine phosphorylation sites belonging to immunoreceptor tyrosine based inhibitory motif (ITIM). An alternatively spliced form lacking two of the Ig domains is also produced transiently at the onset of fusion (Vignery, 2000). MFR binds to CD47 (Vignery, 2005).

**CD47**

CD47 is a ubiquitously expressed IgSF member with 5 transmembrane domains (Vignery, 2005). It shares the homology (28%) with vaccina virus protein A38L (which promotes calcium entry into cells, possibly by forming a pore). It also acts as a receptor for thrombospondin.
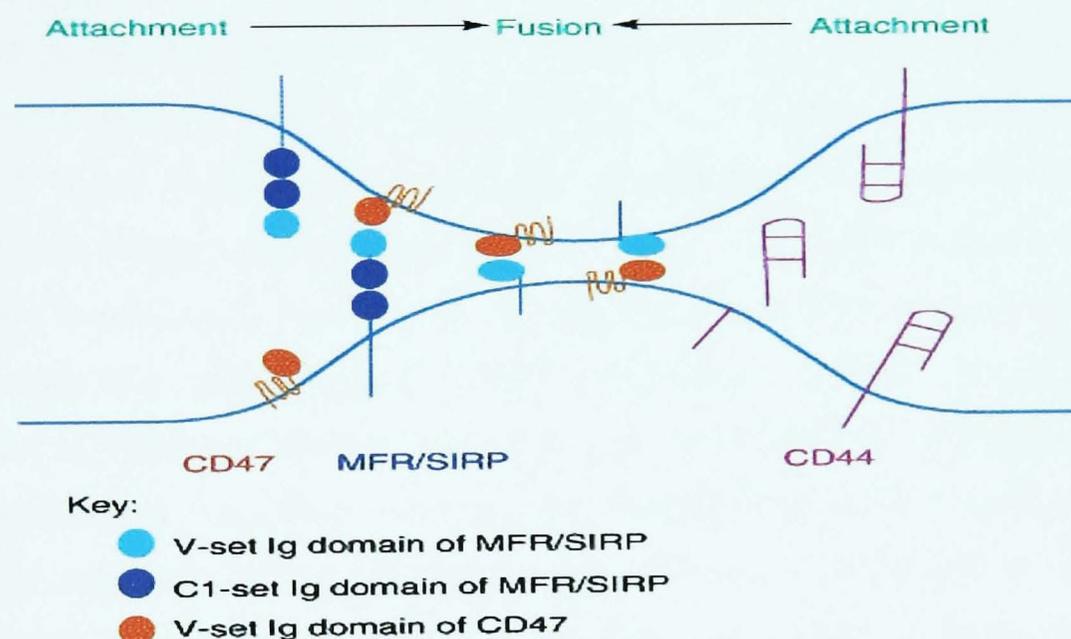


Fig.1.2.6. Hypothetical mechanism for homotypic fusion of macrophages: recognition of 'self'. Macrophage-macrophage adhesion is achieved by binding of the macrophage fusion receptor MFR to CD47, which allows cells to recognise each other as 'self'. The stepwise association of the long form of MFR and then the short form of MFR (MFR-s, lacking the C1 set Ig domains) with CD47 reduces the distance between the plasma membranes. This might be facilitated by the shedding of the extracellular domain of MFR. The distance between macrophage plasma membranes could be reduced to 5-10nm if MFR-s and CD47 bend upon binding. Meanwhile, the extracellular domain of CD44 might also be shed, further facilitating plasma membranes from apposing cells to get closer and fuse. Image courtesy of Vignery,A (2005).

CD47 contains one extracellular Ig variable domain (IgV), followed by five predicted transmembrane segments terminating in a cytoplasmic tail. As described above, the

MFR is expressed in two types, namely a long form and a short form, both with an extracellular N-terminal IgV domain. During the fusion CD47 and MFR appear to interact via the IgV domains (Vignery, 2005). Vignery speculates that the long form of MFR (with 2 IgC domains) first secures the attachment of cells, since their expression is more during fusion, and then switches to short form of MFR to bring the plasma membrane of cells closer to one another (Fig.1.2.6). However, CD47 KO mice develop normally (Vignery, 2000; Vignery, 2005).

**CD44**

CD44 is an integral membrane glycoprotein, a receptor for hyaluronan and membrane type 1 matrix metalloproteinase (MT1-MMP) (Vignery, 2005; Kajita et al, 2001). It is known to be involved in cell-cell and cell-substrate interactions and playing roles in cell migration, lymphopoiesis and lymphocyte homing (Vignery, 2000). This has also been implicated in monocyte/macrophage fusion. It is strongly and transiently induced at the onset of fusion (Vignery, 2005) and extracellular ligands of CD44 block the fusion and multinucleation. Recombinant extracellular domain in the form of GST fusion proteins blocks fusion by interacting with a putative cell-surface binding site (Vignery, 2000). Vignery and co-workers suggested that over expression of CD44 is required to override extracellular ligands and provide unoccupied CD44, which can then interact with a putative macrophage receptor. The extracellular domain of CD44 is cleaved from the cell membrane by MT1-MMP matrix metalloproteinase and sheds from the cell membrane in tumour cells promoting migration (presumably by allowing detachment from ECM (Kajita et al, 2001)). Vignery and co-workers suggest that this may also happen in fusing monocytes possibly allowing closer apposition of cells.

**DC-STAMP**

DC-STAMP was originally described as a molecule with 7 putative transmembrane domains expressed preferentially by (monocyte-derived) dendritic cells (Hartger et al, 2000). The molecule was later shown to be involved in osteoclast formation: its expression is rapidly induced on osteoclast progenitors by e.g. RANKL, and siRNA or antibody to DC-STAMP inhibited osteoclasts formation, whereas over expression enhanced RANKL-induced osteoclastogenesis (Kukita et al, 2004). More recently, studies in DC-STAMP knockout mice indicated a critical role for this molecule in both osteoclast and foreign body giant cell formation. Loss of osteoclast formation in

the knockout mice was restored by retroviral introduction of DC-STAMP (Yagi *et al*, 2005).

### 1.2.5.4. Multinucleated Giant Cell formation may involve phagocytosis

One of the important functions of macrophage is internalisation of apoptotic cells, pathogens and foreign bodies and their subsequent routing towards lysosomes for degradation. It has been proposed that MGC formation occurs via a process similar to phagocytosis or "cellocytosis" (Vignery, 2005). However, macrophages must use an alternative pathway for cellocytosis leading to fusion, because cellocytosed cells survive and become integrated into the new cell (Vignery, 2005). Apoptosis by macrophages is a multistep molecular event associated with sequential modification of plasma membranes, in addition to the high level expression of phosphoserine on the cell surface that is crucial for effective recognition and phagocytosis of target cells. The difference between cellocytosis of live cells and phagocytosis of apoptic cells might therefore involve specific cell-surface determinants, expressed by the internalised cell, such as CD47 (Vignery, 2005) (Fig.1.2.7). Most recently McNally and co-workers (2005) assessed the involvement of the endoplasmic reticulum and the mannose receptor for FBGC formation. In this study the authors found evidence for a phagocytic mechanism of macrophage fusion, including requirements for microtubules, V-type ATPase, and iPLA2. They also found that endoplasmic reticulum (ER) markers are present at fusion interfaces and on macrophage surfaces during FBGC formation, suggesting that the mechanism of macrophage fusion leading to multinucleated giant cell formation is supported by ER-mediated phagocytosis. Co-localisation of ER markers with Con A reactivity at fusion interfaces further raises the possibility that the ER presents ligands to mannose receptor (MR) during FBGC formation (McNally and Anderson, 2005). The involvement of mannose receptor (MR) in the macrophage-macrophage fusion the FBGC formation concurs with their earlier study (McNally and Anderson, 1996).

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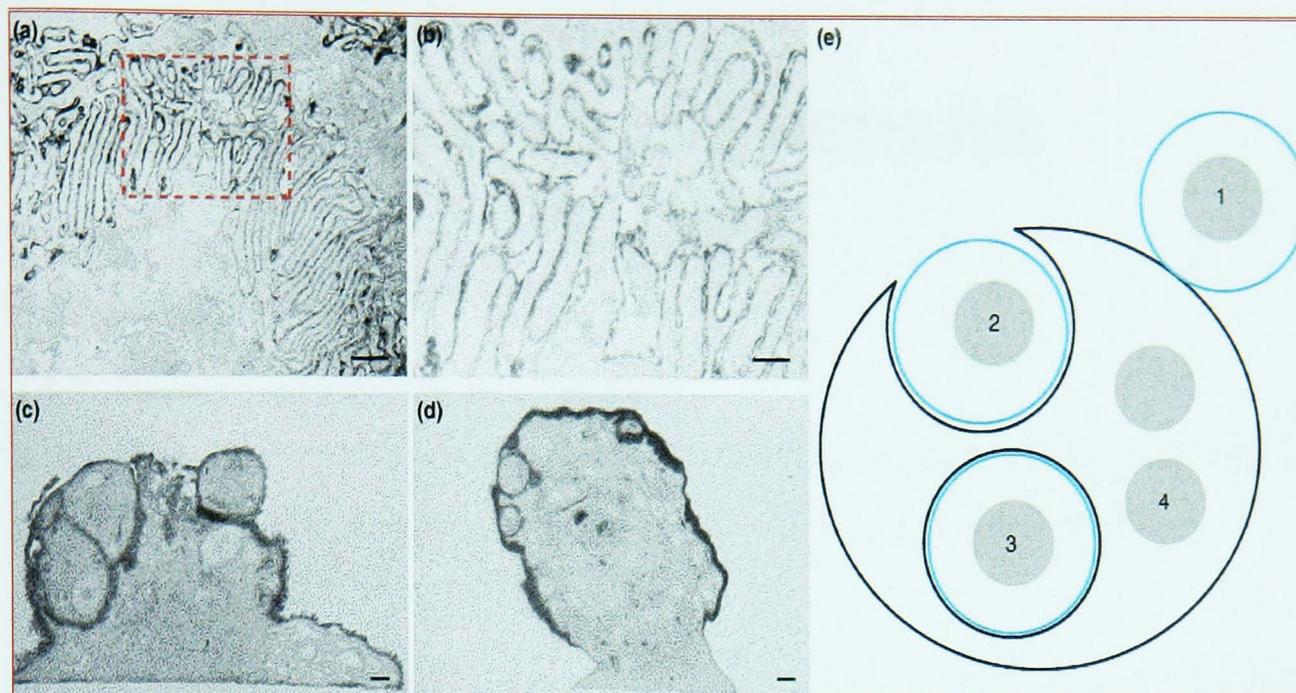
Fig.1.2.7. Macrophage 'Cellocytosis': (a) Extensive interdigitations of the plasma membrane between fusing macrophages; (b) Higher-magnification of the area enclosed by a red dotted line in (a); (c) Internalised, membrane-bound, but not fused, rat alveolar macrophages in tissue culture; (d) Multinucleate alveolar macrophages that contain a large number of nuclei; (e) Cellocytosis: a hypothetical model represents multinucleate cell expands. Image courtesy of Vignery, A (2005).

## 1.3. AIMS

The main aim of this project was to further investigate the role tetraspanins are playing in MGC formation, following the report of Takeda and co-workers (Takeda *et al*, 2003). Initially, a monocyte fusion assay was to be set up using Con A stimulation of normal human peripheral blood monocytes. The effects of different anti tetraspanin antibodies would be tested in this, to see if the findings reported by Takeda and colleagues could be reproduced and extended. The effects of a panel of tetraspanin GST-EC2 fusion proteins on Con A stimulated monocyte fusion was to be investigated. If the study elicited any interesting information, attempts would be made to elucidate the mechanism of action of the tetraspanins e.g. looking at their effects on adhesion, cell proliferation and aggregation. The binding of the tetraspanin EC2s to monocytes would also be assessed and attempts made to identify any interacting molecules.

Another initial aim of the project was to further characterise a human single chain Fv (scFv) antibody that was thought to bind CD63. This antibody had been isolated by Dr. Ann Marshall, previously a post-doctoral worker in the group. The antibody had been selected from a phage-display combinatorial library generated from patients with ocular melanoma. The background to this work is given in chapter 6.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. MATERIALS

### 2.1.1. General chemicals and reagents

Unless otherwise stated general chemicals and reagents were purchased from Sigma, BDH, Fisher Chemicals or Bio Rad and were of analytical grade or equivalent.

### 2.1.2. Water

Milli-Q water was prepared using millipore apparatus (PUR*I*TE NEPTUNE, Scientific Laboratory supplies Limited).

### 2.1.3. Sterilisation

Bacterial culture media, molecular biology buffers and glassware were sterilised by autoclaving at 121°C for 20min. at 15lb/sq.in. The media for tissue culture and BSS were filter sterilised using a 0.2μm micro culture capsule.

### 2.1.4. Media and Buffers

The media used for mammalian and bacterial cell culture and their method of preparation are summarised in section 2.1.4.1 and 2.1.4.2 respectively. Methods of preparation of commonly used buffers are given in section 2.1.4.3. Antibiotics used for the selection of mammalian and bacterial cells are given in section 2.1.4.3.10.

#### 2.1.4.1. Media for mammalian cell culture

The media used for the mammalian cell culture for the present research project are summarised in the table 2.1.

| Media | Preparation |
|---|---|
| DMEM (Dulbecco's Minimal Essential Medium) | 1 canister of powdered DMEM (Gibco, cat. no. 31600-075) was dissolved in 2L of MQ water with $1x10^6$units of benzyl penicillin (Sigma, cat. no. P3032) and $0.5x10^6$ units of streptomycin sulphate (Sigma, cat. no. S 0890). 50ml of 100x non-essential amino acids (Gibco, cat.no.11140-035), 1.46g of tissue culture grade solid glutamine (Gibco, cat.no.21051-024), 18.5g of tissue culture grade sodium bicarbonate (Sigma, cat. no. S5761) were added and sufficient MQ water was added to make 4.8L. The pH of the medium was adjusted between 7.2-7.4 before the final volume was adjusted to 5L. The medium was filter sterilised. |
| RPMI 1640 (Bicarbonate Buffered) | 1 canister of powdered RPMI 1640 (Gibco, cat. no. 3010755) was dissolved in 2L of MQ water with $1x10^6$ units of benzyl penicillin (Sigma, cat. no. P3032) and $0.5x10^6$ units of streptomycin sulphate (Sigma, cat. no. S0890). 10g of tissue culture grade sodium bicarbonate (Sigma, cat. no. S5761) were added. Sufficient MQ water was added to make 4.8L. The pH of the medium was adjusted to 7.2-7.4 before the final volume was adjusted to 5L. The medium was filter sterilised. |
| HEPES buffered RPMI 1640 | 1 canister of powdered RPMI 1640 with 25mM HEPES (Gibco, cat. no. 13018023) was dissolved in 2L of MQ water with $1x10^6$ units of benzyl penicillin (Sigma, cat. no. P3032), $0.5x10^6$units of streptomycin sulphate (Sigma, cat. no. S0890) and 4.25g of tissue culture grade sodium bicarbonate (Sigma, cat. no. S5761). Sufficient MQ water was added to make 4.8L. The pH of the medium was adjusted to 7.2-7.4 before the final volume was adjusted to 5L. The medium was filter sterilised. |

Table.2.1. Media used for mammalian cell culture.

**FCS**: Mycoplasma free FCS (Labtech. International, cat.no.4101500, batch no.1231 or Biowest Ltd, cat.no.SO4010S1810/50, batch no. SO4010S1810) was used for cell culture. For heat inactivation thawed FCS was placed in a water bath at 56°C for 30min. Different batches of FCS were tested for their ability to support growth of cells before use.

## 2.1.4.2. Media for bacterial culture

The media used for the bacterial cell culture are summarised in table 2.2.

| Media | Preparation |
|---|---|
| 2TY | 10g yeast extract (OXOID, cat. no. L21), 16g tryptone (OXOID, cat.no. LP0042), 5gm sodium chloride (BDH, cat. no.102415K) were dissolved in 980ml of MQ water using a magnetic stirrer. pH of the medium was adjusted to 7.0 with 10M sodium hydroxide solution and the final volume was adjusted to 1L with MQ water. The medium was sterilised by autoclaving. |
| 2TYE | 10g yeast extract (OXOID, cat. no. L21), 16g tryptone (OXOID, cat.no.LP0042), 5gm sodium chloride (BDH, cat.no.102415K) were dissolved in 980ml of MQ water using a magnetic stirrer and the pH of the medium was adjusted to 7.0 with 10M sodium hydroxide. The final volume was adjusted to 1L with MQ water. 15g bacto-agar (OXOID, cat.no. LP0011) was added. The medium was sterilised by autoclaving. |
| LB medium | 10g of tryptone (OXOID, cat. no. LP0042), 5g Nacl (BDH, cat. no. 102415K) and 5g yeast extract (OXOID, cat.no. L21) were dissolved in 980ml of MQ water using a magnetic stirrer and the pH of the medium was adjusted to 7.0 with 10M sodium hydroxide solutions. The final volume was adjusted to 1L. The medium was sterilised by autoclaving. |
| LB medium | 15g of bacto-agar added to 1L of LB medium. The medium was sterilised by autoclaving. The pH of the medium 7.0. |

Table.2. 2. Bacterial cell culture media used for the present study.

## 2.1.4.3. Buffers and reagents

### 2.1.4.3.1. General laboratory buffer solutions/reagents

The general laboratory buffers used for the present research project are summarised in table 2. 3.

| Buffer | Preparation |
|---|---|
| BSS (Balanced Salt Solution) | 43.36g sodium chloride (NaCl), 1.83g potassium chloride (KCl), 8.19g D-sorbitol, 3.0g dipotassium hydrogen phosphate ($K_2HPO_4.3H_2O$), 0.7g potassium dihydrogen phosphate ($KH_2PO_4$) and 12.09g HEPES. The ingredients were weighed accurately and dissolved in 4.8L of MQ water. The pH was adjusted between 7.2-7.4 with 1M sodium hydroxide solutions. The final volume was adjusted to 5L with MQ water. The solution was filter sterilised. Stored at 4°C in sterile bottles. |
| PBS (Phosphate Buffered Saline) 0.15M ($10x.L^{-1}$) PH 7.2 | 80g sodium chloride (Nacl), 2g potassium chloride (KCl), 11.5g di sodium hydrogen phosphate ($Na_2HPO_4$) and 2g potassium hydrogen phosphate ($KH_2PO_4$) were weighed and dissolved in 1L of distilled water. Usually 1:10 dilution in distilled water was used. |
| BBN (Wash Buffer for FACS) | 0.1% sodium azide and 0.2% BSA (tissue culture standard) dissolved in BSS. Stored at 4°C. |
| TAE ($50x,L^{-1}$) | 242g Tris base, 57.1ml glacial acetic acid, 100ml of 0.5M EDTA and water to 1L. |
| 2%Paraformaldeyde solution | 2g of paraformaldehyde was dissolved in 100ml of 1xPBS in a glass bottle by heating in a water bath for 1 hr. at 56°C. The solution was allowed to cool at RT and then stored at 4°C. |
| 20% PEG8000/ 2.5M Nacl | 100g PEG8000 was dissolved in 400ml of 2.5M sodium chloride solution by stirring with a magnetic stirrer. The volume was adjusted to 500ml with 2.5M NaCl. The solution was sterilised by autoclaving. Cooled to RT and then stored at 4°C. |

Table.2. 3. General laboratory buffers and reagents.

### 2.1.4.3.2. Reagent for freezing of mammalian cells

Freezing mixture is a combination of 90%FCS and 10% dimethyl sulphoxide (DMSO) prepared in advance and stored at 4°C in dark.

### 2.1.4.3.3. Reagents and solution for testing the viability of mammalian cells

The reagent and solution used for the testing the viability of mammalian cells were prepared as follows:

**(a) Erythrosine dye stock solution**: 0.4g erythrosine B salt, 0.81g sodium chloride, 0.06g potassium di-hydrogen orthophosphate 0.05g methyl-p-hydroxybenzoate. The ingredients were placed in 95ml of MQ water in a 250ml beaker, dissolved by heating to boiling in a heated stirrer. The pH was adjusted between 7.2-7.3 with 1M NaOH (about 8 drops). The final volume was adjusted to 100ml with MQ water. This solution is stable indefinitely at RT.

**(b) Trypan blue solution**: 1:4 ratio of trypan blue (Sigma,cat.no.T8154) in medium/1xPBS.

**(c) Wright stain modified**: A neat solution of Wright stain (Sigma,cat.no.WS-16) was used.

### 2.1.4.3.4. Reagents and solutions for ELISA

The reagents and solutions used for ELISA are summarised in table 2.4.

| Reagents | Preparation |
|---|---|
| Blocking buffer / dilution buffers | 5% milk powder and 0.01% tween 20 in 1xPBS. Mixed by stirring using a magnetic stirrer. |
| | 1% BSA, 2% dried milk powder in 1xPBS. Mixed by stirring using a magnetic stirrer. |
| | 0.2% gelatin in 1xPBS. Gelatin was dissolved by gentle heating in a microwave oven. |
| | 1% milk powder and 0.01% tween 20 in PBS. Mixed by stirring using a magnetic stirrer. |
| | 0.1% BSA and 1% dried milk powder in 1x PBS. Mixed by stirring using a magnetic stirrer. |
| Phosphate-Citrate buffer (0.05M) | 25.7ml of 0.2M dibasic sodium phosphate ($Na_2HPO_4$), 24.3ml of 1M citric acid were mixed together and the volume was adjusted to 50ml with MQ water. The pH of the solution was adjusted to 5 either using 0.2M dibasic sodium phosphate or 0.1M citric acid. |
| TMB substrate solution | A tablet (1mg) of 3,3', 5, 5' tetramethyl benzidine free base (Sigma, cat. no. T5525) was dissolved in a mixture of 1ml of DMSO + 9ml of 0.05M phosphate citrate |

| | buffer pH 5.0 with 4μl of 30% $H_2O_2$. A solution was achieved by vortex mixing. Prepared just before use. |
|---|---|
| 0.25% glutaraldehyde solution | 1ml of 25% glutaraldehyde solution mixed with 99ml of 1xPBS. Stored at 4°C. |
| 20mM carbonate /bicarbonate buffer (ELISA coating buffer) | 1.59g $Na_2CO_3$ and 2.93g $NaHCO_3$ were dissolved in 800ml of MQ water using a magnetic stirrer. The pH was adjusted to 9.6 using 1M HCl or 1M NaOH. The final volume was adjusted to 1L with MQ water. Stored at 4°C in clean glass bottles. |

Table.2.4. Reagents and solutions used for ELISA.

### 2.1.4.3.5. Reagents and solutions for FITC labelling of proteins

Conjugation buffer (0.5M carbonate-bicarbonate buffer) pH 9.5: 5.8ml of 5.3% $Na_2CO_3$ solution was added to 4.2ml of 4.2% NaHCO3 solution. The pH of the solution was adjusted to 9.5.

### 2.1.4.3.6. Reagents and solutions for SDS-PAGE gels

  (a) Precast gel used for SDS-PAGE: 12% Tris-HCl Ready Gel (Biorad)-10well, 30μl comb.

  (b) Protein standards for SDS-PAGE: Sigma marker $^{TM}$ high molecular weight range (6.5-205 kDa).

### 2.1.4.3.7. Reagent used for monocyte work

The monocytes were separated from the blood using Ficoll-Hypaque plus (Amersham Pharmacia Biotech, cat.no.17-1440-02). The monocytes were stimulated with Concanavalin A (Sigma, cat.no.C5275).

### 2.1.4.3.8. Reagents and solutions for SRB assay

  The reagents and solution used for the sulforhodamine B assay are summarised in table 2.5.

| Reagents | Preparation |
|---|---|
| 0.4%W/V SRB Solution | 40mg of Sulforhodamine B salt (Sigma, cat. no. S9012) in 10ml of 1% acetic acid solution. |
| 50% Trichloroacetic acid solution | 50g Trichloroacetic acid was dissolved in 80ml of MQ water. The final volume was adjusted to 100ml with MQ water. |
| Un buffered Tris base (10mM) pH 10.5 | 1.21g of tris base (Sigma, cat. no. T-1503) was dissolved in 95ml of MQ water and the pH was adjusted to 10.5 |

| | using a concentrated solution of tri base. |
|---|---|
| Poly L-lysine | $50\mu g.ml^{-1}$ solution was prepared in carbonate bicarbonate buffer. |

Table.2.5. Reagents and solutions used for SRB assay.

### 2.1.4.3.9. Reagents and solution for nuclear staining

**(a) Propidium iodide solution:** A stock solution of propidium iodide (Sigma,cat.no.P4170) @$1mg.ml^{-1}$ was prepared in 1xPBS and used at $1\mu g.ml^{-1}$.

**(b) Bisbenzimide (Hoechst dye):** A stock solution of bisbenzamide (Sigma,cat.no. B2261) @$1mg.ml^{-1}$ was prepared in MQ water and used at $1\mu g.ml^{-1}$.

### 2.1.4.3.10. Antibiotics used for mammalian cell culture and bacterial cell culture

The antibiotics used for the selection of mammalian and bacterial cells are summarised in table 2.6.

| Antibiotic | Stock Concentration $(mg.ml^{-1})$ | Working Concentration $(\mu g.ml^{-1})$ | Storage (°C) |
|---|---|---|---|
| Carbenicillin | 100 (in 50% ethanol) | 50/100 | -20 |
| Chloramphenicol | 25 (in 100% ethanol) | 25 | -20 |
| Kanamycin | 50 (in water) | 50 | 4 |
| Tetracycline | 10 (in water) | 10 | -20 |
| Geneticin (G418) | 20 (in culture medium) | 0.4 | 4 |
| Penicillin & streptomycin | 10,000 Units.$ml^{-1}$ | 100Units.$ml^{-1}$ | -20 |

Table.2.6. Antibiotics used for tissue culture and bacterial culture.

### 2.1.4.3.11. Reagents used for scFv phage propagation

(a) The expression of scFv phage was induced by isopropyl-β-D-Thiogalacto pyranoside (IPTG) (Sigma, cat. no. I6758).

(b) The helper phage used for the scFv probation is VCSM13 helper phage (Stratagene, cat. no. 200251).

### 2.1.4.3.12. Immunofluorescent mounting medium

The mounting medium used for the fluorescent slide preparation are: Immuno-Fluore mounting medium (ICN biomedical company, Ohio, US, cat. no. 62270); Citifluor mounting medium (Citifluor Ltd, London, cat. no. AFI+AF100); Mounting medium (The Binding Site Ltd, cat. no. CON195.2).

### 2.1.5. Primary antibodies and Isotype control

The primary antibodies used for the immunofluorescent studies, monocyte fusion assay and ELISA are summarised in table 2.7.

| Antibody | Target antigen | Species specificity | Label | Conc. used ($\mu g.ml^{-1}$) | Source | Cat. No | Isotype |
|---|---|---|---|---|---|---|---|
| Mouse anti human CD9 | CD9 | Human | FITC | 10 | Serotec | MCA 469 F | Mouse IgG1 |
| Mouse anti human CD9 (Alma-1) | CD9 | Human | None | 20 | Dr.F.Lanza, Strasbourg, France | NA | - |
| Mouse anti human CD9 602.29 | CD9 | Human | None | 20 | Prof.P.Andrews Dept.of BMS, Univ.of Sheffield | NA | Mouse IgG1 |
| Mouse anti human CD11b | CD11b | Human | FITC | 10 | Serotec | MCA 551F | Mouse IgG1 |
| Mouse anti human CD14 | CD14 | Human | FITC | 10 | Serotec | MCA2185F | Mouse IgG1 |
| Mouse anti human CD18 | CD18 | Human | FITC | 10 | Immunotec | 1568 | Mouse IgG1 |
| Rat anti human CD18 | CD18 | Human | FITC | 10 | Serotec | MCA503F | Mouse IgG1 |
| Mouse anti human CD29 | CD29 | Human | FITC | 10 | Serotec | MCA1949F | Mouse IgG1 |
| Mouse anti human CD37 | CD37 | Human | FITC | 10 | Serotec | MCA483F | Mouse IgG1 |
| Mouse anti human CD52 | CD52 | Human | FITC | 5 | Serotec | MCA 2188F | Mouse IgG1 |
| Mouse anti human CD63 (H5C6) | CD63 | Human | None | 15 | In house | None | Mouse IgG1 |
| Mouse anti human CD63 (H5C6) | CD63 | Human | FITC | 14 | In house | NA | Mouse IgG1 |
| Mouse anti CD63 (LP9) | CD63 | Human | FITC | 50 | In house | NA | Mouse IgG1 |
| Mouse anti CD63 (LP9) | CD63 | Human | None | 65 | In house | NA | Mouse IgG1 |
| Mouse anti CD63 (BEM1) | CD63 | Human | None | Neat S/N or 40 mg. $ml^{-1}$ | In house | NA | Mouse IgG2b |
| Mouse anti CD63 (16.1) | CD63 | Human | None | 20 | Dr.K.Skubitz, Dept.of Medicine, Univ. of Minnesota, U.S | NA | - |
| Mouse anti CD63 (16.5) | CD63 | Human | None | 20 | Dr.K.Skubitz, Dept.of Medicine, Univ. of Minnesota, U.S. | NA | - |

| Mouse anti human CD68 | CD68 | Human | None | 10 | Serotech | MCA759T | Mouse IgG1 |
|---|---|---|---|---|---|---|---|
| Mouse anti rat CD68 | CD68 | Human | FITC | 10 | Serotech | MCA341F | Mouse IgG1 |
| Mouse anti human CD81 | CD81 | Human | FITC | 10 | Serotech | MCA1847F | Mouse IgG1 |
| Mouse anti human CD82 | CD82 | Human | None | 10 | Serotech | MCA1311 | Mouse IgG1b |
| Mouse anti human CD151 | CD151 | Human | None | 10 | Prof.S.Levy,Stand ford Univ. Medical Centre, Standford, U.S. | NA | Mouse IgG1 |
| Goat Anti GST | GST | None | None | 5 | Amersham Pharmacia biotech | 27-4577-01 | Mouse IgG1 |

Table.2.7. Primary antibodies used for the fluorescent studies, monocyte fusion assay and ELISA, where NA =Not applicable; S/N=Supernate.

The isotype control used for the immunofluorescent studies are summarised in table 2.8.

| Isotype control | Target antigen | Species specificity | Type | Label | Conc. used ($\mu g.ml^{-1}$) | Source | Cat. No |
|---|---|---|---|---|---|---|---|
| JC-1 | Phosphonamidate hapten | N/A | IgG1 | None | 10 | In house (Muranova *et al*, 2004) | N/A |
| MOPC-21 (Mouse IgG1-FITC) | N/A | N/A | IgG1 | FITC | 10 | Sigma | F6397 |

Table.2.8. Isotype control used for the immunofluorescence assays, where NA=Not applicable.

## 2.1.6. Secondary antibodies/Immunodetection reagents

Secondary antibodies/ immuno detection reagents used for immunofluorescent studies, monocyte binding assays are summarised in table 2.9.

| Antibody/reagents | Species specificity | Label | Conc. used ($\mu g.ml^{-1}$) | Source | Cat. No | Isotype |
|---|---|---|---|---|---|---|
| Mouse anti GST | GST | Biotin | 10 | Serotec | MCA1352B | Mouse IgG1 |
| Mouse anti M13 | Phage-M13 | HRP | 1:5000 dilution | Amersham Pharmacia biotech | 27-9421-01 | Mouse IgG2a |
| Mouse anti M13, fd, F1 filamentous phages | Phage-M13 | FITC | 1 | Progen | 61497 | Mouse IgG2a |
| Mouse anti M13 bacteriophage | Phage M13 | Biotin | 1 | Serotec | MCA1858B | Mouse IgG2a |
| Mouse anti goat-sheep IgG | Goat IgG1 and IgG2 | HRP | 5 | Sigma | A9452 | N/A |

| Anti mouse IgG | Mouse IgG | FITC | 10 | Sigma | F6397 | N/A |
|---|---|---|---|---|---|---|
| Anti mouse IgG | Mouse IgG | HRP | 1:5000 | Sigma | A9174 | N/A |
| Anti mouse polyvalent immuno globulins | Mouse (IgG, IgA, or IgM). | FITC | 4 | Sigma | F1010 | N/A |
| Anti Goat IgG | Rabbit-Goat IgG | FITC | 5 | Sigma | F7367 | N/A |
| Anti Goat IgG | Goat IgG | HRP | 5 | Sigma | A5420 | N/A |
| Extravidin | - | FITC | 2.25 | Sigma | E2761 | N/A |
| Extravidin | - | HRP | 0.5 | Sigma | E2886 | N/A |

Table.2.9. Secondary antibodies/ immuno detection reagents

## 2.1.7. Bacterial strains and vectors

XL-1 Blue *E. coli* competent cells (*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F'*pro* AB *lac*I$^q$Z M15 *Tn*10 (Tet$^r$)]) were from Stratagene and were used for work involving human scFv clones.

Clones containing scFv genes in the phage display vector pAK 100 (Krebber *et al*, 1997) such as 4E and 5F were obtained from Dr. Ann Marshal (University of Sheffield). Clones containing pAK 100 vector alone (No insert) were used as controls. This vector was a kind gift from Professor Andreas Pluckthun, University of Zurich.

GST-EC2 fusion proteins cloned into the pGEX-KG GST fusion protein vector (Higginbottom *et al*, 2003) were expressed from BL21 RP Codon plus®-RIL *E.coli* (Stratagene).

The selection of bacterial strains were carried out using antibiotic stocks as given in section 2.1.4.3.10.

## 2.1.8. Cell lines

Various non-human and human cell lines were propagated and stocks established. The cell lines and their culture conditions are summarised in tables.2.10&2.11. Mammalian cell lines expressing human tetraspanins had been transfected with pEE6hCMV.neo vector (Celltech Ltd.) containing the human tetraspanin genes (Smith *et al*, 1995).

## 2.1.8.1. Non-human cell lines

| Cell line | Type | Source | Culture medium | Culture Condition |
|---|---|---|---|---|
| RBL2H3-hCD63 | Rat Basophilic Leukaemic cell line transfected with human CD63 | Dr.L.J.Partridge, Dept. of Mol.Biol. & Biotech., Univ. of Sheffield, U.K. | DMEM+10% FCS+G418 | 37°C in a humid 8%$CO_2$ |
| RBL2H3 | Rat Basophilic Leukaemic cell line-Untransfected | Dr.B.Helm, Dept. of Mol.Biol. & Biotech., Univ. of Sheffield, U.K. | DMEM+10% FCS | 37°C in a humid 8%$CO_2$ |
| KT-5 | Mouse cell line | Dr. P.N. Monk, Dept. of Neurol. Medical school, Univ. of Sheffield. U.K. | DMEM+10% FCS | 37°C in a humid 8%$CO_2$ |
| CD9/⁻⁻ and CD9 WT | Mouse macrophage | Dr.G. Dveksler Dept. of Pathol. Uniformed services Univ. of Health Sciences, Bethesda, MD, U.S. | DMEM+ 10% FCS | 37°C in a humid 5%$CO_2$ |

Table.2.10. Non-human cell lines used for the study and their culture conditions.

## 2.1.8.2. Human cell lines/cells

| Cell line | Type | Source | Culture medium | Culture Condition |
|---|---|---|---|---|
| Mewo | Melanoma cell line | Dr.J.Marshal, ICRF, London, UK. | DMEM+10% FCS | 37°C in a humid 8%$CO_2$ |
| Mel-17 | Melanoma cell line | Dr.J. Marshal, ICRF, London, UK. | DMEM+10% FCS | 37°C in a humid 8%$CO_2$ |
| U937 | Human monoblast leukaemic cell line | Dr.J. Gallagher, Dept. of human Met. Clin. Biochem. Univ. of Sheffield, UK. | Hepes-buffered RPMI 1640+10% FCS+100U.ml⁻¹ of penicillin and 100U.ml⁻¹ of streptomycin | 37°C in a humid 5%$CO_2$ |
| THP-1 | Human Monocytic leukaemic cell line | ECACC. | RPMI 1640+10% FCS +100U.ml⁻¹of penicillin and 100U.ml⁻¹ of streptomycin | 37°C in a humid 5%$CO_2$ |
| HL60 | Pro-myelocytic (Megakaryocytic cell line) | Dr.P.N.Monk, Dept. of Neurol. Medical school, Univ. of Sheffield, U.K. | RPMI 1640 +10% FCS +100U.ml⁻¹ of penicillin and 100U.ml⁻¹ of streptomycin | 37°C in a humid 5%$CO_2$ |

| K562 | Promyelocytic cell line | Dr.P.N.Monk, Dept. of Neurol. Medical school, Univ. of Sheffield. U.K | RPMI-1640 +10% FCS +100U.ml$^{-1}$of penicillin and 100U.ml$^{-1}$ of streptomycin | 37°C in a humid 5%$CO_2$ |
|---|---|---|---|---|
| DX3 | Melanoma cell line | Dr.J. Marshal, ICRF, London, UK. | DMEM+10% FCS | 37°C in a humid 8%$CO_2$ |
| VUP | Melanoma cell line | Dr.J. Marshal, ICRF, London, UK. | DMEM+10% FCS | 37°C in a humid 8%$CO_2$ |
| LoVo | Colorectal carcinoma cell line | ECACC. | DMEM+10% FCS | 37°C in a humid 5%$CO_2$ |
| IGROV1 | Ovarian carcinoma cell line | Dr.J.Bernard, INSERM. | RPMI+10% FCS | 37°C in a humid 5%$CO_2$ |

Table 2.11. Human cell lines used for the study and their culture conditions, where ECACC = European Collection of Animal Cell Culture.

## 2.1.9. Instrumentation

| Equipment | Supplier |
|---|---|
| Agarose gel documentation | Polaroid DS34 instant camera, linked to photodoc camera hood |
| Agarose gel electrophoresis | Hybaid HB1214 horizontal gel tank Jencons HU10 horizontal gel tank Consort E844 power supply Invitrogen X Cell Surelock T$^M$ electrophoretic cell |
| Thermal cyclers | MJ Research PTC 100, Techne Genius (Techne Limited) |
| Ultraviolet trans illuminator | Camlab UVP |
| Autoclaves | Express Portable Rodwell MP24 |
| Balances | Mettler PM460, AE100, AJ100 |
| Centrifuge | Beckman JA-25 MSE Mistral 3000 Sigma 3K15 |
| Microfuge | Sigma |
| Cold cabinet (4°C) | Sadia |
| Liquid nitrogen refrigerators and dewars | Taylor-Wharton, Jencons and Statebourne |
| Drying cabinet (55°C) | Belling |
| ELISA plate readers | Anthos HT2 linked to EPSON LX-850 printer |
| Water bath | Tempette Junior TE-8J |
| Freezers | Scandinova (-20°C), Sanyo Ultra Low (-70°C) |

| Heat block | Jenway 1200 |
|---|---|
| $CO_2$ incubators (tissue culture) | Herraeus, Forma Scientific water Jacketed incubator, SLS Galaxy R |
| Incubators | Gallenkamp (static), New Brunswick Scientific (Shaking) |
| Laminar flow cabinet | Medical Air Technology, Class II microbiological safety cabinet |
| Microwave oven | Hitachi MR 6400 |
| pH meter | Corning pH meter 220 |
| Pipettes (manual) | Gilson Pipetman P2, P10, P100, P200, P1000, P5000, Gilson 8 x 200µl multi channel |
| Pipette (automatic) | IBS, Jencons |
| Spectrophotometer | Perkin Elmer Lambda3 dual beam UV/Visible light spectrophotometer, Cecil Instruments CE272, WPA Light wave |
| SDS-PAGE gel electrophoresis | Biorad Protean 2 Vertical gel tank |
| Rocking table | Luckham R100 |
| Inverted light microscope | Nikon |
| Immunofluorescent microscope | Deltavision restoration microscope, Nikon Eclipse E400 microscope with DXM 1200 digital camera and Lucia image processing software for pc, Carl-Zeiss confocal microscope |
| Cytospin 2 | Shandon southern products |
| Turntable | Labinco |
| Vortex mixer | Rotamixer deluxe |
| Flow cytometer | CyAn ADP- Dako cytomation Beckmann-Coulter |
| Densitometer | Alpha Innotech corporation |

Table 2.12. General laboratory equipments and suppliers.

## 2.1.10. General laboratory consumables and plastic ware

| Consumable | Supplier |
|---|---|
| 0.2-0.4µm filters | PALL laboratories |
| 0.2µm, 0.4µm acrodisc | Sartorius |
| 0.5ml thin wall PCR tubes | Alpha laboratories |
| 0.6ml/1.5ml tubes | Eppendorf |
| Centrifuge tubes | Beckman, 30ml, 250ml, 500ml |
| Cling film | Cater Pak |
| Cocktail sticks | Plastico |
| Spectrophotometer cuvettes | Sarstedt |
| 6 well tissue culture plate | Corning |
| 96 well microtitre ELISA plate | Nunc, Maxisorp |
| 96 well microtitre (Tissue culture) plate | Corning Incorporated |
| 24 well tissue culture plate | Corning Incorporated |
| 96 well plate sealers | Corning Incorporated |

| | |
|---|---|
| Falcon tubes | Becton Dickinson & Co. |
| Gloves | Glovco, latex, powder free |
| 10ml, 25ml graduated pipettes | Becton Dickinson & Co. |
| 100mm bacterial culture plates | Bibby Sterilin |
| 100mm tissue culture dishes | Iwaki |
| 25cm tissue culture flask | Nunc |
| 175cm tissue culture flask | Nunc |
| Pipette tips | Gilson Diamon (non-sterile) |
| Dialysing tube (12-14KDa) | GeBAflex-tube Maxi (DO50) |
| Tissue paper | Kimberly Clark, Kimwipe |
| Lens cleaning tissues | Whatman International |
| Universal tubes and Bijous | Sterilin |
| Shandon cytocentrifuge filters | Shandon |
| Lab-Tek® II chamber slides | Nalge Nunc International, US |
| 22x64mm cover slip | BDH |
| 22x22mm cover slip | BDH |
| Ground edge microscope slide | BDH |
| Microscope slides | BDH |
| Hyper ladder I markers | Bioline |
| ID tapes | Star lab |
| 5ml disposable column | Qiagen |
| 10x10x 45 cuvettes | Sarstedt, Germany |
| Quartz cuvettes | Merck |
| Pipette tips 1000µl | Molecular bioproducts |
| Pipette tips 200µl, 0.1-10µl | Star lab |
| Parafilm | BDH |
| FACS tubes | Elkay lab products, U.K |
| Cryo vials | Nunc |
| Pasteur pipette 150mm &230mm | Mexcel |
| 0.2µm micro culture capsule | Gelman Laboratory |

Table 2.13. General laboratory consumables and plastic ware.

## 2.1.11. Software used

### 2.1.11.1. Flow cytometry

Flow cytometry data from the present research project was analysed using WINMDI 2.8 software (Joseph Trotter: http://facs.scripps.edu/software.html). The term mean fluorescence intensity (MFI) was used throughout the work as a measure of the intensity of staining of a population of cells.

### 2.1.11.2. Statistical analysis

Data of this research project was analysed using GraphPad Prism 4 software.

### 2.1.11.3. Deltavision restoration microscopy

The immunofluorescent images obtained using the Deltavision microscope were processed using Softrex- Deltavision software.

### 2.1.11.4. Extinction coefficient of protein

The protein concentration of rhodamine BITC/AlexaFluor 647® labelled GST proteins was estimated using Protparam Tool software [from the Swiss Protein Database, http://www.expasy.cg], which is based on the calculation of protein extinction coefficients from amino acid sequence data (Gilland Hippel (1989)).

# 2.2. METHODS

## 2.2.1. Tissue culture methods

Cell lines were cultured in standard tissue culture plastic plates or flasks in a humidified $CO_2$ incubator under conditions described in tables 2.10 and 2.11. Medium preparation is described in table 2.1.

### 2.2.1.1. Sub culturing of adherent cells

Once cells were confluent they were split in to 1:3 to 1:10 using trypsin/EDTA solution. To subculture the medium was removed, cells were washed with approximately 10ml of sterile BSS per 10cm petri dish and incubated with 5ml of 0.25% trypsin/EDTA solution (Sigma, cat. no. T4174) diluted 1/10 in BSS) at 37°C. After 5-10min. the cells in petri dish were dislodged by agitation using a sterile Pasteur pipette. Trypsin was neutralised by mixing with an equal volume of suitable medium containing 10% FCS (Tables.2.10&2.11). The cells were pelletted by centrifugation at 400g for 5min., re suspended in fresh medium and transferred at 15ml plate$^{-1}$.

### 2.2.1.2. Sub culturing of non-adherent cells

Non-adherent cells were sub cultured as necessary by diluting cells 1:3 to 1:10 with fresh medium. Non-adherent cells were routinely cultured in screw capped culture flasks.

### 2.2.1.3. Cell counting

A standard cell counting method using an improved Neubauer haemocytometer was used for cell counting.

### 2.2.1.4. Cryo preservation of cell lines

Cells were frozen in aliquots of 4-10x10$^6$. Cells with good viability (>90%) and in log phase were harvested by trypsinisation (adherent cells) or centrifugation

(suspension cells). After centrifugation, cells were resuspended in 1 ml cold FCS / 10% DMSO and transferred to chilled cryotubes (Nunc, cat. no. 368632). Vials were incubated in a biological freezing tray (Jencons) in the liquid nitrogen vapour phase to allow cooling at 1°C/2hrs. before lowering into liquid nitrogen for storage. The position and date of freezing were recorded in to an electronic logbook.

### 2.2.1.5. Resuscitation of cryo preserved cells

A vial from the liquid nitrogen was removed and agitated in a water bath at 37°C until nearly thawed. The contents were transferred quickly into a pre cooled universal tube on ice and washed by adding 10ml cold medium (no FCS) slowly. After centrifuging at 200g the cells were resuspended in appropriate medium containing 10% FCS.

### 2.2.1.6. Dye exclusion test for viability

The viability of the cells was assessed by exclusion of erythrosine dye or trypan blue solution (section 2.1.4.3.3). Stock erythrosine dye solution or trypan blue were mixed 1:10 or 1:4 with cell suspension, respectively and viability assessed after 4min. at RT.

### 2.2.2. Monitoring antigen expression by flow cytometry

### 2.2.2.1. Harvesting of cells for analysis of cell receptor/antigen analysis

For cell receptor/antigen analysis adherent cells were harvested non-enzymatically using cell dissociation solution (Sigma, cat.no.C5914). FACS analysis was performed with living cells; hence all manipulations were carried out on ice or at 4°C and in the presence of sodium azide (0.1%) to prevent capping, internalisation or shedding of antigen. Appropriate isotype controls (Table 2.8) were included in all assays.

### 2.2.2.2. Direct immunofluorescence assay

Cells were harvested, washed twice with cold wash buffer (BBN) (section 2.1.4.3.1) and re suspended to $0.5-1.0 \times 10^6$ cells.ml$^{-1}$ in BBN. 1ml aliquots were transferred into round bottomed 12x75mm plastic tubes ("FACS tubes"), and centrifuged at 400g for 5min and the cell pellet was incubated with 50µl of fluorescently-labelled antibody or GST proteins in BBN/PBS for 45-60min. on ice. Cells were washed 1x with 1ml of BBN and resuspended with 0.2ml of 2% cold paraformaldehyde solution prior to FACS analysis.

### 2.2.2.3. In-direct immunofluorescence assay

This was perfomed as in 2.2.2.2 except unlabelled antibodies/GST proteins were used for the first incubation. Cells were washed twice with 1ml aliquots of BBN then incubated with 50µl of fluorescently labelled secondary reagent (section 2.1.6) for

30min. on ice. The cells were then washed and resuspended with paraformaldehyde as described above.

## 2.2.3. Immunofluorescence in Lab-Tek® chamber slide

### 2.2.3.1. Direct method

The cells were harvested using trypsin:EDTA (section 2.2.1.1), resuspended in appropriate medium at $7x10^4$cell.ml$^{-1}$ and 0.5ml aliquots dispensed into the wells of an 8-chambered Lab-Tek® slide and cultured overnight. After washing with BSS, adherent cells were fixed and permeabilised with acetone (0.5ml. chamber$^{-1}$ for 5min. at RT) and washed twice with PBS. Acetone was removed by aspiration and slides washed briefly twice with 0.5ml of 1x PBS, with a third wash in a trough of PBS with stirring for 10min using a magnetic stirrer. PBS was removed by aspiration. The cells were incubated with 100μl of an appropriate dilution of FITC-labelled antibody or labelled GST protein in a humid atmosphere in the dark for 45-60min. The chambers were washed briefly twice with PBS and a third time in a trough of PBS with stirring for 8min. Where appropriate, cell nuclei were stained by incubating with 100μl of propidium iodide in PBS for 3min (section 2.1.4.3.9). The chambers were washed with 0.5ml of PBS and given a final wash in a trough of PBS with stirring for 15min as above. The PBS was removed by aspiration. All these manipulations were carried out on ice to avoid the drying of cells. The plastic chamber compartment was removed carefully from the slide according to the manufacturer's instruction. The cells were mounted using liquid mountant (section.2.1.4.3.12) at 12μl.chamber$^{-1}$, covered with 22x66mm cover slip and sealed using nail varnish. The slide was stored at 4°C until examination under fluorescence microscope. When viewing cells with the 100x objective, immersion oil (Cargille, cat. no. 16212 Type FF) was used.

### 2.2.3.2. Indirect method

This was performed as above but cells were incubated with 100μl of appropriate dilution of an unlabelled primary antibody (section 2.1.5)/scFv phage suspension. After washing, the cells were incubated with 100μl of an appropriate dilution of FITC labelled secondary antibody (section 2.1.6) for 30min. in dark. Washing, nuclear staining and mounting of the cells were as in section 2.2.3.1.

## 2.2.4. Immunofluorescence on cytocentrifuge slide

### 2.2.4.1. Direct method

Shandon cytocentrifuge chambers, filters and microscopic slides were assembled as per manufacturer's instructions and inserted in the cytocentrifuge.

Cells were harvested using cell dissociation solution (section 2.2.2.1) and re suspended to $1x10^5$ cells.ml$^{-1}$ with suitable culture medium. 100µl was added to each chamber and cells were deposited on the slide by centrifuging at 500rpm for 5min. The slides were air-dried and their position on the slides marked with a diamond marker. Cells were fixed and permeabilised in acetone (5min at RT) and the slides washed with PBS for 10min. One slide was removed at a time, the area around the cells wiped carefully with tissue paper and incubated with 50µl of an appropriate dilution of FITC labelled mAb (section 2.1.5) or labelled GST proteins and incubated for 30min. at RT in a damp container in the dark. After two brief washes in PBS, a third wash was done by immersing the slides in a trough of PBS for 10min. with stirring. One slide at a time was removed as above, and the nuclei of the cells were stained with propidium iodide/bisbenzamide in PBS/water respectively for 3min (section 2.2.3.1). The slides were washed briefly twice followed by a final wash in a trough of 1x PBS for 15min. using magnetic stirrer. Slides were mounted with 10µl mounting medium (section 2.1.4.3.12) using 22x22mm cover slips and sealed with nail varnish.

### 2.2.5. Titration of antibodies and antigens

#### 2.2.5.1. Titration of FITC conjugated anti CD63 antibody (H5C6) by FACS: Direct assay

RBL2H3-hCD63 cells were harvested non-enzymatatically and the assay was carried out as described in section. 2.2.2.2, The cell pellets were incubated with different concentrations of FITC conjugated anti CD63 antibody H5C6 in BBN, mixed by vortexing and incubated for 45-60min. on ice. The remaining steps are as in section 2.2.2.2.

#### 2.2.5.2. Titration of unlabelled anti CD63 antibody (H5C6) by FACS: Indirect assay

Unlabelled anti CD63 antibody (H5C6) was titrated against RBL2H3-hCD63 as described in section 2.2.2.3 using different concentrations in BBN. The FITC conjugated anti mouse polyvalent immunoglobulins (IgG, IgA & IgM) at 4µg.ml$^{-1}$ in

BBN was used as a secondary antibody. The remaining steps are same as in section 2.2.2.2.

### 2.2.5.3. Titration of Extravidin by FACS

Mouse KT-5 is a cell line, which is expressing high levels of CD81 was used for the titration of Extravidin-FITC. FACS analysis was carried out as described in section 2.2.2.3 using primary biotin-labelled mouse anti CD81 at $10\mu g.ml^{-1}$ and various dilutions of secondary Extravidin-FITC. The remaining steps are as in section 2.2.2.2.

### 2.2.5.4. Titration of GST-EC2CD63 against anti CD63 antibody by ELISA

A flat bottomed 96 well microtitre ELISA plate (Nunc, Maxisorb) was coated with $100\mu l$ of GST-EC2CD63 at $10\mu g.ml^{-1}$ in carbonate-bicarbonate buffer pH 9.6. overnight at 4°C. Excess GST-EC2CD63 was removed by flicking the plate and the plate was washed 1x with PBS. The wells were blocked with $300\mu l$ of Blotto (5% milk powder, 0.01% tween 20 in PBS) and incubating for 2hrs at RT. After removing blocking agent, the wells were incubated with $100\mu l$ of anti CD63 antibody H5C6 initially at 1:10 ($150\mu g.ml^{-1}$) and then with doubling dilutions in 1% milk powder, 0.01% tween 20 in PBS. The plate was incubated for 1hr at 37°C, washed 3x with PBS, and $100\mu l$ secondary antibody anti mouse IgG-HRP (whole molecule) at 1:1000 in dilution buffer was added. The plate was incubated for 1hr at 37°C, washed 2x in PBS followed by a final wash using MQ water to remove the trace salt from the plate. The binding of antibody was visualized by adding $50\mu l$ of TMB solution (2.1.4.3.4) for 20min. at 37°C. The enzyme-substrate reaction was quenched by the addition of $50\mu l$ of 2M $H_2SO_4$. The intensity of yellow colour developed by the reaction mixture was measured at 450nm using an ELISA plate reader.

### 2.2.5.5. Titration of EC2CD63 and GST in GST-EC2CD63 by ELISA

The correct folding and the optimal concentration of GST-EC2CD63 required for coating ELISA plates was determined by coating a plate with $100\mu l$ of GST-EC2CD63 initially at $10\mu g.ml^{-1}$, then doubling dilution in carbonate-bicarbonate buffer. After incubating and blocking as above, some rows were incubated with $100\mu l$ of goat anti GST antibody at $5\mu g.ml^{-1}$ and some rows with anti CD63 (H5C6) at $15\mu g.ml^{-1}$ in dilution buffer (1% milk powder, 0.01% tween 20 in PBS). After incubation and washing as above, H5C6 wells were incubated with goat anti mouse IgG-HRP at 1:5000 in dilution buffer, whereas anti GST antibody wells were

incubated with anti goat-sheep-HRP at $5\mu g.ml^{-1}$. The plates were developed as described above.

### 2.2.5.6 Titration of mouse anti GST-biotin against GST by ELISA

Plates were coated with GST-EC2CD63, as described above, then wells were incubated with 100µl of goat anti GST biotin antibody initially at $100\mu g.ml^{-1}$ and followed by doubling dilutions in 1% milk powder, 0.01% tween 20 in PBS. The plate was incubated and washed as above, then wells were incubated with a secondary Extravidin-HRP at $1\mu g.ml^{-1}$ in dilution buffer for 1hr at 37°C. The remaining steps are as in section 2.2.5.4.

### 2.2.5.7. Titration of mouse anti CD52-FITC antibody by FACS

Peripheral blood monocytes were harvested using cell dissociation fluid (section 2.2.2.1) and dilutions of anti CD52-FITC antibody in BBN were titrated by FACS as described in section 2.2.2.2.

### 2.2.6. Estimation of surface + intracellular antigen levels by flow cytometry

A fix and permeabilisation method was used for the estimation of surface and intracellular expression levels of antigens (CD63). Cells were permeabilised using a Caltag kit (cat. no. GAS-003), containing a permeabilising agent, which makes "pores" in the cell to allow antibodies to gain access. Half of the samples were fixed only (to assess surface staining) and half fixed and then permeabilised (to assess both surface and cytoplasmic staining). Cells were harvested and dispensed into FACS tubes as described above (2.2.2.2) and 50µl FITC labelled anti CD63 antibody or isotype control was added. Cells were incubated for 20min. on ice. 50µl reagent A (fixative) was added and the tubes incubated for 15min. at room temperature. Cells were washed 2x in B/B/N and 50µl of FITC-labelled anti CD63 antibody or isotype control and either 50µl reagent B (permeabilisation reagent) or 50µl B/B/N (for fixed only cells) was added. Cells were incubated at room temperature for 20min. in the dark, washed twice in B/B/N and fixed with 200µl of 1% paraformaldehyde solution before FACS analysis. Intracellular expression was calculated by subtraction of plasma membrane staining from total CD63 levels and expressed as a percentage of total CD63.

## 2.2.7. Antibody induced Internalisation assay

The antibody-induced internalisation of cell surface CD63 was estimated by FACS analysis. Levels of primary antibody remaining on the cell surface after incubating labelled cells at 37°C were measured. Cells were harvested non-enzymatically and resuspended at $10 \times 10^6$ cell.ml$^{-1}$ in internalisation buffer (BSS containing 0.2% BSA and 1mM CaCl$_2$). 50µl aliquots ($0.5 \times 10^6$ cells) were transferred to FACS tubes on ice. 50µl of primary monoclonal antibody at appropriate dilution in internalisation buffer was added and the tubes incubated for 30min. on ice. Cells were washed in ice-cold internalisation buffer to remove unbound antibody and the cell pellet was resuspended in 100µl of the same. Samples were placed in a water bath at 37°C, removed at timed intervals and quenched by addition of 2ml ice-cold B/B/N. Following centrifugation, remaining cell surface antibody was detected using 50µl of appropriate dilution of anti mouse IgG-FITC in B/B/N. Time zero (total) antibody binding was determined using samples that had been incubated with primary antibody on ice throughout. Appropriate primary isotype controls were also included. The percentage of antigen internalised after each time point ($\chi$ min.) was calculated as follows:

Antigen internalised (%)= 100 x MFI $_{(T=0)}$ - MFI $_{(T=\chi)}$/ MFI $_{(T=0)}$

## 2.2.8. Production of recombinant GST-EC2 tetraspanins

The Glutathione S-transferase (GST) gene fusion system is an integrated system for the expression of fusion proteins from *E.coli*. The recombinant GST-EC2 proteins of tetraspanins were produced from *E.coli* in our laboratory to assess their functional activity. In most cases, these proteins were produced and purified by Dr. Adrian Higginbottom or Francine Martin in our group, as described in Higginbottom *et al*, (2003). An overview of the method is given below.

### 2.2.8.1. Production of GST-EC2 fusion proteins

An aliquot of BL21 RP Codon plus®-RIL *E.coli* cells was defrosted. The bacterial cells were transformed with DNA encoding GST-EC2-tetraspanin @ 1µl of 0.1ng.µl$^{-1}$ for 20µl of bacterial suspension in a pre chilled eppendorf kept on ice. The contents of the eppendorf were mixed by gentle swirling and incubated for 30min. on ice and gently swirled every 2min. The DNA was transformed into *E.coli* by heat pulse for 25sec. at 42°C. (The transformation efficiency will decrease sharply if the duration of the heat pulse is <20sec. or >25sec.). After the transformation, the cells were

incubated on ice for 2min. and then inoculated into 500µl of pre warmed LB medium (section 2.1.4.2). After incubation for 1hr at 37°C with shaking at 220 RPM, 200µl of transformed bacterial cells was spread on an LB agar plate supplemented with carbenicillin to a final concentration of 50µg.ml$^{-1}$. The plate was incubated overnight at 37°C. A single colony from the plate was inoculated into 10ml of LB medium supplemented with 50µg.ml$^{-1}$ of carbenicillin. The cells were cultured overnight at 37°C in a shaker at @ 220RPM. 10ml of the overnight culture was inoculated into 400ml of fresh LB medium supplemented with 50µg.ml$^{-1}$ of carbenicillin and the cells were grown to log phase in a shaker @ 220RPM until the OD$_{600}$ of the culture reached between 0.2-0.4. The expression of GST protein was induced by supplementing 200µl of 0.5M IPTG (Sigma, cat.no.16758) to a final concentration of 0.5mM and the cells were grown for an additional 4hrs at 37°C in a shaker at @ 220RPM. The bacterial cells were separated from the culture supernatant by centrifugation at 4500g for 20min. and the supernatant was drained completely. The wet weight of the cell pellet was determined and frozen (-20°C) overnight before the protein was extracted using Bug buster.

### 2.2.8.2. Extraction and purification of GST-EC2 tetraspanins

The frozen cell pellet was thawed on ice, re suspended with Bug buster reagent (Novagen,cat.no.70584-3) @ 3ml.gm$^{-1}$ of wet weight of cell pellet by pipetting up and down and by gentle vortexing. This mixture was treated with protease inhibitors (Complete MINI$^{®}$, EDTA free protease inhibitor cocktail tablet (ROCHE, cat.no.10946900) to final concentration of 50µg.ml$^{-1}$ to prevent the degradation of expressed GST proteins by bacterial enzymes. The cell suspension was incubated on a shaking platform or rotating mixer at a slow setting for 1hr at RT. The insoluble cell debris was removed by centrifugation at 15000g for 20min. at 4°C. The supernatant was mixed with ~1.33ml of ethanol free glutathione beads in a universal tube, incubated at RT for 1hr/over night at 4°C. The contents were transferred into a disposable column and the flow-through was discarded. Then the column was washed 3x with ice cold PBS. The GST protein was eluted from the glutathione beads by transferring 0.5ml aliquots of 50mM glutathione in 10mM Tris-HCl pH 8.0 into the column. The protein concentration of the eluted fraction was estimated by Bradford assay (section 2.2.9.5) and fractions with similar protein content were pooled,

dialysed against PBS (~3x 10L dialysis/sample) to remove tris and glutathione. Samples were run on SDS gels and subjected to Coomassie staining to determine proteins concentration by densitometry using protein standards of known concentration for comparison. Western blotting was also carried out using conformation-sensitive antibodies to determine the position of correctly folded GST-EC2 tetraspanins.

## 2.2.9. Fluorescein conjugation of proteins

The FITC conjugation of proteins was carried out essentially as described by Johnson (Johnson, 1990). In this research project, anti CD63 antibody (H5C6) and GST-EC2 tetraspanins/GST were labelled with FITC for immunofluorescence experiments.

### 2.2.9.1. Fluorescein labelling of mouse anti CD63 antibody (H5C6)

30µg fluorescein isothiocyanate (FITC) (Sigma, cat. no. F7250/Sigma, cat. no. R 1755) per mg of antibody was dissolved in the appropriate amount of conjugation buffer (0.5M carbonate/bicarbonate, pH 9.5) in a volume equivalent to 10% of the protein. This was mixed by rotating gently on a blood mixer for 2hrs at room temperature in the dark. Conjugated protein was separated from unbound dye on a Sepharose G-25 column equilibrated with PBS. Sodium azide (0.1%) was added as preservative and the conjugated antibodies were stored in aliquots at -20°C. The molar ratio of FITC: protein was determined as described by The and Feltkamp (1970), using the formula: $2.87 \times O.D._{495}/ O.D._{280} - 0.35 \times O.D._{495}$. Briefly, this is derived by assuming $E_{280}$ for IgG = 1.4, $E_{495}$ for FITC = 200, m.wt. IgG = 160,000 and m.wt. FITC = 390, and takes into account that FITC absorbs at 280nm, with a factor of 0.35 of its absorbance at 495nm.

$$\text{Molecular FITC/protein ratio} = \frac{\text{Mol.wt.protein}}{\text{Mol.wt.FITC}} \times \frac{\text{Conc.FITC mg.ml}^{-1}}{\text{Conc.IgG mg.ml}^{-1}}$$

$$= 410 \times \frac{OD_{495}: 200}{(OD_{280} - 0.35 \times E_{495}): 1.4}$$

The FITC: protein ratios were typically between 1:1 and 5:1.

### 2.2.9.2. Fluorescein conjugation of GST-EC2 tetrasapanins

This was performed essentially as described above (section 2.2.9.1). GST-EC2CD63, GST-EC2CD9 and GST were labelled in the course of this project. For determining molar dye:protein ratios, molecular weights of 37,040, 35,730 and 26,000 were used

for GST-EC2CD63, GST-EC2CD9 and GST, respectively. The concentration of labelled protein was determined using the Bradford assay (2.2.9.5).

### 2.2.9.3. Rhodamine B-isothiocyanate labelling of GST-EC2 tetraspanins

The GST-EC2CD63 and GST proteins were also labelled with rhodamine B isothiocyanate (Sigma, cat. no. R1755) as described in section 2.2.9.1.

Since the fluorescent dye appeared to interfere with the Bradford assay, protein concentrations were measured spectrophotometrically using extinction coefficients derived using Protparam Tool software [from the Swiss Protein Database, http://www.expasy.cg], which is based on the calculation of protein extinction coefficients from amino acid sequence data (Gilland Hippel (1989)]. Extinction coefficients ($E_{280}$) of GST-EC2CD63 and GST were estimated as 1.474 and 1.516, respectively. Absorbances of GST proteins in 6M guanidium hydrochloride in 0.02M phosphate buffer pH 6.5 were measured at 280nm.

*2.2.9.3.1. Determination of degree of labelling (D.O.L)/ molar fluorochrome protein ratio of GST proteins labelled with rhodamine BITC*

$$D.O.L = \frac{Amax_{515} \times M.wt.of\ protein}{Conc.\ protein\ (mg.ml^{-1}) \times E_{dye}}$$

Where, $E_{dye} = 49,0000$; $Amax_{515}$ of free rhodamine BITC

* Formula for D.O.L supplied by Molecular probes.

### 2.2.9.4. AlexaFluor® 647 labelling of GST-EC2 tetraspanins

The labelling of GST-EC2CD63 and GST with Alexa Fluor®647 (Molecular probes, cat. no. A20006) was carried out as per the manufacturers instruction.

*2.2.9.4.1. Determination of protein concentration of labelled proteins*

The protein concentration of AlexaFluor® 647 labelled GST-EC2CD63 and GST was determined spectophotometrically as described above.

*2.2.9.4.2. Determination of degree of labelling (D.O.L)/ molar flurochrome: protein ratio of GST proteins labelled with AlexFluor®647*

$$D.O.L = \frac{Amax_{515} \times M.wt.of\ protein}{Conc.\ protein\ (mg.ml^{-1}) \times E_{dye}}$$

Where, $E_{dye} = 239,0000$; $Amax_{515}$ of free Alexa Fluor®647.

## 2.2.9.5. Estimation of proteins by Bradford assay

The Bradford protein assay was used to determine the protein concentration of flurochrome labelled GST-EC2 tetraspanins (Bradford (1976) and the information from:

http://www.chemistry.gatech.edu/class/peek/4581/techniques/bradford/bradord.html.) BSA standards (5,10,15,20,30,40 and 50µg.ml$^{-1}$) were prepared in PBS from the stock BSA solution. The test samples, blank and BSA standards for the protein estimation were prepared as given in table 2.14 using Bradford reagent kit (BioRad, cat.no.500-0001). The samples were incubated at RT for 10-30min., transferred into the disposable cuvettes and the absorbance of each sample measured at 595nm, a plot of absorbance of each BSA standard as a function of its theoretical concentration was obtained with the data and the best fit of the data to a straight line in the form of the equation 'y = mx + b' was prepared, where y = absorbance at 595nm and x = protein concentration. The protein concentration of the test samples were determined from the BSA standard graph by extrapolating their absorbance.

| Samples | Sample volume (µl) | Volume of water (µl) | Volume of Bradford reagent |
|---|---|---|---|
| Blank | 0 | 800 | 200 |
| BSA standard (5µg.ml$^{-1}$) | 10 | 790 | 200 |
| BSA standard (10µg.ml$^{-1}$) | 20 | 780 | 200 |
| BSA standard (15µg.ml$^{-1}$) | 30 | 770 | 200 |
| BSA standard (20µg.ml$^{-1}$) | 40 | 760 | 200 |
| BSA standard (20µg.ml$^{-1}$) | 50 | 750 | 200 |
| Test protein | 50 | 750 | 200 |

Table 2.14. Preparation of test samples, blank and standard samples for the Bradford protein assay.

## 2.2.10. Experiments using peripheral blood monocytes

### 2.2.10.1. Isolation of monocytes from blood

Peripheral blood mononuclear cells were derived from peripheral whole blood of healthy, anonymized human volunteers by Ficoll-Paque (Amersham Pharmacia Biotech., cat.no.17-1440-02) density centrifugation as described by Dockrell *et al*, (2001). The study was approved by the South Sheffield Research Ethics Committee (protocol number SSREC/02/299) and all donors gave informed consent to the donation of 250ml of blood. Blood was taken by Professor R.C. Read, Division of Genomic Medicine, University of Sheffield and preliminary preparation (Ficoll-Paque density centrifugation) was also carried out here, since some of the cells were to be used in other studies.

## 2.2.10.2. Purification of monocytes by adhesion on plastic

Monocytes were purified from the contaminating lymphocytes by adhesion to serum (FCS)/autologous plasma coated plastic tissue culture grade petri dishes by transferring 15ml of mononuclear cell suspension (2-5 x $10^6$ cell. $ml^{-1}$ in RPMI1640 - 10% FCS) into each plate. Cells were incubated at 37°C in a 5% $CO_2$ incubator over night. Alternatively the mononuclear cells were directly transferred into Lab-Tek® chambers slides for immunofluorescence microscopy. Non-adherent cells such as lymphocytes and platelets were separated from the over night cultured adherent monocytes by aspiration. The plates were washed thoroughly 3x with 10ml of pre warmed RPMI (without FCS) to ensure that all non-adherent cells were removed.

## 2.2.10.3. Monocyte cell fusion assay

### 2.2.10.3.1.Monocyte fusion assay in microtitre plate

Adhesion-purified monocytes were seeded at $2x10^5$ cells in 100μl of culture medium into a 96 well microtitre plate and cultured for 72hrs in the presence/absence of Con A at 10μg.$ml^{-1}$ as described by Takeda and co-workers. The supernatant was removed from the plate and the cells were stained with 100μl of Wright stain (section 2.1.4.3.3) for 20min. The stain was removed and the plate was air-dried. The plate was observed under the light microscope (Takeda *et al*, 2003).

### 2.2.10.3.2. Monocyte fusion assay in Lab-Tek® chamber slide

The mononuclear cells were seeded at $5x10^5$cells.chamber$^{-1}$ in 0.5ml RPMI-1640-10%FCS in an 8 chambered Lab-Tek® slide. After overnight culture, non-adherent cells were aspirated and the chambers washed 3x with 0.5ml pre-warmed RPMI-1640 (no FCS) then cultured in RPMI-1640-10%FCS in the presence/absence of Concanavalin A (Sigma, cat. no.17-1440-02) to a final concentration of 10μg.$ml^{-1}$ for 72hrs at 37°C. After the incubation period, the supernatant from the chambers of the slide was aspirated and each well washed with 0.5ml of 1xPBS. The cells were fixed and permeabilised with 500μl of acetone for 5min. The cells were then labelled with appropriate dilution of FITC conjugated antibody and nuclei counter-stained with propidium iodide as described in 2.2.3.1. The number of nuclei in fused cells and individual nuclei were counted in 6 randomly chosen fields using Nikon Eclipse E400 immunofluorescence microscope and the fusion index was calculated using a formula:

Fusion index = no. of nuclei in fused cells/ number of nuclei in total % (Abe, 1983; Weinberg *et al*, 1984; Takeda *et al*, 2003).

## 2.2.10.4. Functional effects of tetraspanins on monocyte fusion

### 2.2.10.4.1. Effects of anti tetraspanin antibodies/GST-EC2 tetraspanins on monocytes fusion

The mononuclear cells were seeded at $5 \times 10^5$ cells.chamber$^{-1}$ in a Lab-Tek® chamber slide. In the presence/absence of Con A ($10\mu g.ml^{-1}$) and anti tetraspanin antibodies or GST-EC2 tetraspanins or/GST ($20\mu g.ml^{-1}$) for 72hrs at 37°C. The slide was processed, stained with FITC labelled anti CD52 antibody and the fusion index was calculated as described in section 2.2.10.3.2.

### 2.2.10.4.2. Effect of short incubation of GST-EC2 tetraspanins on monocyte fusion

mononuclear cells were seeded into Lab-Tek® chamber slides as previously were incubated with GST-EC2 tetraspanins or GST ($20\mu g.ml^{-1}$) for 30min. at 37°C. The recombinant proteins were removed by 5x washing with pre warmed RPMI and the cells were cultured in RPMI+10%FCS in the presence of Con A ($10\mu g.ml^{-1}$) for 72hrs. The slide was processed and the fusion index was calculated as described in section 2.2.10.3 2.

## 2.2.10.5. Effect of GST-EC2 tetraspanins on functions related to monocyte fusion

### 2.2.10.5.1. Effect of GST-EC2 tetraspanins on monocyte adhesion: Lab-Tek® chamber slide assay

mononuclear cells were seeded into a Lab-Tek® chamber slides and incubated with GST-EC2 tetraspanins ($20\mu g.ml^{-1}$) for 72hrs at 37°C in the absence of Con A as previously described (2.2.10.4.1). The slide was processed and the cells were stained with anti CD52 antibody as described in section 2.2.10.3.2. The number of nuclei was counted in 6 randomly chosen fields using Nikon Eclipse E400 immunofluorescence microscope as above.

### 2.2.10.5.2. Sulforhodamine B (SRB) protein assay

The sulforhodamine B (SRB) assay is a rapid, sensitive and inexpensive method for measuring the cellular protein content of adherent cells and non-adherent cells in a 96 well microtitre plates (Skehan et al, 1990). In this research project the SRB assay was adopted to assess the effects of GST-EC2 tetraspanins on cell numbers and adhesion.

### 2.2.10.5.2.1. Titration of cell numbers by SRB cell stain assay

Adherent cells (RBL2H3 and monocytes) and non-adherent (THP-1 cells) were harvested and seeded into a 96 well microtitre plate (poly-L-lysine coated/uncoated) initially @ 500,000 cells.well$^{-1}$ in 200µl culture medium (sections 2.1.8.1&2.1.8.2)

and then with doubling dilutions of cell numbers in the following wells. These were incubated for 2hrs/4hrs at 37°C in a $CO_2$ incubator. (Some wells were incubated only with culture medium to measure the background optical density). The cells were fixed by layering 50µl of ice cold 50%TCA on the top of the growth medium to a final concentration of 10% TCA. The plate was left undisturbed for 5min. then incubated at 4°C for 1hr. The supernates was removed and wells were washed 5x with 200µl of MQ water and air-dried. Wells were stained with 100µl of 0.4%(w/v) sulforhodamine B sodium salt (Sigma, cat. no. S9012-5G) in 1% acetic acid for 30min. Unbound dye was removed with 1% acetic acid and the plate was air dried. Cell bound dye was solubilized with 100µl of 10mM unbuffered Tris base at pH 10.5 for 5min. using a gyratory shaker and the OD @ 570nm was measured using an ELISA plate reader.

### 2.2.10.5.2.2. Effect of GST-EC2 tetraspanins on monocyte adhesion: SRB assay

Adherent monocytes were harvested using trypsin: EDTA and seeded into a 96 well microtitre plate at $5x10^5$ cells.well$^{-1}$ in 200µl of medium. A duplicate plate was prepared with identical conditions. The plates were incubated with GST-EC2 tetraspanins to final concentration of 20µg.ml$^{-1}$ for 72hrs at 37°C in the absence or presence of Con A (10µg.ml$^{-1}$). After the incubation period one plate was fixed with TCA as described above, whilst the other was washed 3x with 200µl of BSS prior to fixation and the SRB assay was carried out.

### 2.2.10.5.2.3. Effect of GST-EC2/His$_6$/EC2 tetraspanins on the aggregation of monocytes

The effect of GST-EC2 or His$_6$-EC2 tetraspanins on monocyte aggregation was tested in 96 well microtitre plates, as described by Takeda et al (2003). Adherent monocytes were harvested using trypsin: EDTA or /cell dissociation solution (section 2.2.2.1). The monocytes were seeded into a 96 well microtitre plate at 200,000 cells.well$^{-1}$ in 100µl of medium and cultured for 12hrs in the presence of Con A (5µg.ml$^{-1}$) and GST-EC2 or His$_6$-EC2 tetraspanins (20µg.ml$^{-1}$). Control wells contained Con A alone or no Con A. The number of aggregates containing 4-10 and 10-20 cells were counted in 6 randomly chosen fields using an inverted light microscope.

### 2.2.10.5.2.4. Effect of GST-EC2 tetraspanins on THP-1 cell aggregation

THP-1 cells were seeded at 80,000 cells in 80µl of culture medium into a 96 well microtitre plate pre coated with 100µl of sterile poly L-lysine (50µg.ml$^{-1}$ in carbonate-bicarbonate buffer, incubated for 1hr at 37 C (poly L-lysine facilitates the adhesion of

non-adherent THP-1 cells). The cells were cultured for 24hrs in the presence/absence of Con A (5μg.ml$^{-1}$) and GST-EC2 tetraspanins or GST at 20, 10 and 2μg.ml$^{-1}$. Control wells contained Con A alone or no Con A. Aggregates containing 4-10; 10-20; 20-50; 50-100 and >100 cells were counted in 6 randomly chosen fields as above.

### 2.2.10.6. Binding GST-EC2 tetraspanins with monocytes

*2.2.10.6.1. Binding of GST-EC2 tetraspanins to monocytes: FACS-Indirect assay*

Adherent monocytes in 10cm culture plates were cultured in the absence or presence of Con A (10μg.ml$^{-1}$) 24hrs. Cells were harvested using cell dissociation solution and incubated with unlabelled GST-EC2 tetraspanins/GST (20μg.ml$^{-1}$, 1hr at 4°C) followed by goat anti GST antibody (10μg.ml$^{-1}$) then Extravidin-FITC (2.25μg.ml$^{-1}$) as described section 2.2.2.3.

*2.2.10.6.2. Binding of fluorochrome labelled GST-EC2 tetrasapanins to monocytes: FACS-Direct assay*

Monocytes were cultured as above (with/without Con A) for 2hrs. After harvesting, cells were incubated with FITC conjugated GST-EC2 proteins or FITC-GST (6μg.ml$^{-1}$ in RPMI-10%FCS) either at 4°C (to assess the surface binding) or at 37°C (to assess binding and uptake). The remaining conditions were as described for direct immunofluorescence (2.2.2.2.)

*2.2.10.6.3. Binding of GST-EC2 tetraspanins to monocytes: microscopy: Indirect assay*

Mononuclear cells were seeded into Lab-Tek® chamber slides as described previously (2.2.10.3.2) and cultured with/without Con A (10μg.ml$^{-1}$) in 500μl of medium for 2hrs at 37°C. Unlabelled GST-EC2 tetraspanins or GST (20μg.ml$^{-1}$ in BBN) were allowed to bind at 4°C for 1hr. The chambers were then washed 2x with PBS, fixed with acetone and processed as described for indirect immunofluorescence (2.2.3.2.). The primary antibody used was goat anti GST-biotin (10μg.ml$^{-1}$) followed by Extravidin-FITC (2.25μg.ml$^{-1}$).

*2.2.10.6.4. Binding of flurochrome labelled GST-EC2 tetrasapanins on monocytes: microscopy: Direct assay*

This was carried out as above (2.2.10.6.3) but cells were incubated with FITC/rhodamine BITC labelled GST-EC2 tetraspanins or GST (6μg.ml$^{-1}$) in RPMI+10%FCS either on ice or at 37°C to assess surface binding or binding and uptake, respectively . Slides were processed as described above (2.2.10.3.2).

*2.2.10.6.5. Binding of GST-EC2 fusion proteins on monocytes: ELISA*

Binding of GST-EC2 tetraspanins to monocytes in ELISA (whole cell ELISA) was essentially as described by Schober *et al* (2002). Adherent monocytes harvested using trypsin:EDTA were seeded into a 96 well microtitre plate at $3 \times 10^5$cells.well$^{-1}$ in 200µl of medium. After 12hrs in culture in the presence/absence of Con A (10µg.ml$^{-1}$), wells were washed with 200µl of 1xPBS, and cells fixed with 200µl of 0.25% glutaraldehyde.well$^{-1}$ (section 2.1.4.3.4) for 30min. at RT. Glutaraldehyde was removed and the plate was immersed in a trough of PBS. After removal of PBS, wells were blocked with 300µl of 2% milk power in PBS for 2hrs at 37°C. After removal of blocking buffer the wells were incubated with 50µl of GST-EC2 tetraspanins/GST (20µg.ml$^{-1}$) in dilution buffer (1% milk powder in PBS). After 1hr at 37°C the plate was washed 3x with PBS and 50µl of goat anti GST antibody (5µg.ml$^{-1}$) in dilution buffer added. After 1hr at 37°C, the plate was washed as before and incubated with 50µl of anti goat/sheep IgG HRP (5µg.ml$^{-1}$) in dilution buffer for 1hr at 37°C. The plate was washed, substrate added and colour developed as described in 2.2.5.4.

## 2.2.10.7. Binding of FITC labelled GST-EC2 tetraspanins to oocytes from mice

*2.2.10.7.1. Preparation of zona free oocytes from mice*

These experiments were carried out at ARC Centre of Excellence in Biotechnology and Reproductive Science, University of Newcastle, Australia under the guidance of Dr. Eileen McLaughlin.

Female Swiss mice (6-8 weeks old) were injected with 10 I.U. of Folligon to stimulate follicle development. 48hrs later a second injection of Chorulon was given to induce ovulation. 12hrs post-Chorulon injection, mice were $CO_2$ euthanaised. The ovaries and oviducts were excised and transferred into filter sterilised α-Minimum Essential Media (α-MEM) supplemented with 5% FCS, 1mM HEPES, 5U.ml$^{-1}$ penicillin G and 5µg.m$^{-1}$ streptomycin sulphate (all media components from Gibco). Using a dissection microscope (Carl-Zeiss) and fine tip forceps, oviduct tubules were carefully pinched allowing the release and collection of cumulus-oocyte complexes (CEOs). Oocytes were denuded (removal of surrounding cumulus cells) by incubating them with 1mg.ml$^{-1}$ hyalauronidase for 5min followed by 3x washes with 100µl of medium. Zona pellucidae of oocytes was removed by dissolving with a very brief (15-25sec) exposure to acidified tyrodes solution (pH 2.2). Oocytes were quickly washed

3x with 100µl media droplets and allowed to recover for 60min incubation at 37°C in 5% $CO_2$ incubator. After the recovery the oocytes were used for further experiments.

*2.2.10.7.2. Binding of FITC labelled GST-EC2 tetraspanins with mouse oocytes*

Approximately 12-15 oocytes were incubated in 100µl droplets of GST-EC2CD9-FITC, GST-EC2CD151-FITC and GST-FITC to a final concentration of 6µg.ml$^{-1}$ in media; the media alone was included as an additional control. After 1hr at 37°C in a 5% $CO_2$ incubator the oocytes were washed 3x with 100µl media droplets, mounted on slides with raised coverslips and observed under the Carl-Zeiss confocal microscope using 250x magnification.

## 2.2.11. Effect of Con A stimulation on the expression of tetraspanins and other leucocyte proteins by monocytes

The expression levels of cell surface membrane proteins by unstimulated/Con A stimulated monocytes or THP-1 cells were measured by FACS analysis as described in section 2.2.2.2. Adherent monocytes (section 2.2.10.2) or THP-1 cells in tissue culture plates were cultured without/with Con A (10µg.ml$^{-1}$) for 2hrs or 72hrs at 37°C. Cells were harvested and prepared for FACS analysis as described in 2.2.2.2 using appropriate concentrations of FITC labelled antibodies directed to CD9, CD11b, CD14, CD18, CD29, CD37, CD63, CD81, CD52, CD82, CD151 or FITC labelled mouse isotype control (section 2.1.5).

## 2.2.12. Characterisation of humanised recombinant scFv phage antibody
## 2.2.12.1. Colony insert PCR

Colony insert PCR is a rapid, direct technique employed on bacterial plaques or colonies to check the presence, size and orientation of DNA inserts by amplification with flanking primers circumventing all DNA preparation (Güssow *et al*, 1989).

Clones of XL1-Blue cells containing pAK100 vector with inserted scFv genes were streaked on TYE plates supplemented with 100µg.ml$^{-1}$carbenicillin and 1% glucose and incubated overnight at 37°C. A single colony from the plate was used to check for the presence of scFv encoding cDNA inserts. A clone known to contain insert encoding an anti alkaline phosphatase scFv was included as a positive control, and a clone containing pAK100 vector only was used as a negative control. The primers used for this reactions span the scFv insert and are an M13 primer LMB3 (5'-CAGGAAACAGCTATGAC-3') corresponding to vector sequence just before the insert site and a primer Fdseq1 (5'-GAATTTTCTGTATGAGG-3') corresponding to

sequence at the 3' end of the scFv insert. A single colony from the TYE plate mixed with 50μl of amplification reaction mix (5μl of 10x PCR buffer, 0.4μl of 25mM dNTP, 4μl of 25mM $Mg^{2+}$, 1μl of 20μM LMB3 primer, 1μl of Fdseq1 primer 20μM, 0.25μl of 5U. $μl^{-1}$ Taq polymerase) and the final volume was adjusted to 50μl with sufficient water. After a hot start (to break down bacterial cell membrane) at 94°C for 3min., 25 cycles of the following PCR conditions were used: 94°C-30s; 45°C-60s; 72°C-90s and a final extension at 72°C for 10min. Hold 4°C. Finally 8-10μl of the PCR product was analysed on a 1% agarose gel for the presence of insert (~800bp).

### 2.2.12.2. Preparation of scFv phage containing supernatant

On day 1 the 2TYE plates were streaked with clones of XL1 Blue containing vector with/without scFv insert and incubated at 37°C overnight. On day 2 a single colony was isolated and inoculated into 5ml of 2TY supplemented with 5μl of 25μg.$ml^{-1}$ chloramphenicol in a universal tube. The cell suspension was cultured over night at 37°C in a shaker at 250RPM. On day 3, a sufficient quantity bacterial culture was inoculated into a corresponding Falcon tube containing 50ml pre warmed NE medium (2TY+1%Glucose+25μg.$ml^{-1}$ chloramphenicol) to achieve $OD_{600}$ between 0.05-0.1. The cells were cultured at 37°C in 1L flask with shaking at 250 RPM until the $OD_{600}$ reached ~ 0.5. The $OD_{600}$ was recorded for the next 2hrs. The bacterial clone cultured in log phase was infected with 20-fold excess of VCSM13 helper phage (Stratagene) and protein expression was induced by adding 25μl of 1M IPTG (section 2.1.4.3.11).

The number of helper phage to be used for the infection of bacterial cells was calculated from the $OD_{600}$ of the culture. The $OD_{600}$ and their corresponding bacterial cell numbers are given below.

| $OD_{600}$ | Bugs.$ml^{-1}$ |
|:---:|:---:|
| 1 | $8.0 \times 10^8$ |
| 0.8 | $6.4 \times 10^8$ |
| 0.6 | $4.8 \times 10^8$ |
| 0.4 | $3.2 \times 10^8$ |
| 0.2 | $1.6 \times 10^8$ |

Table. 2.15. The $OD_{600}$ of bacterial culture and their corresponding cell numbers.

No. of phage particles to be used = No. of bugs.$ml^{-1}$ x vol. of culture x 20

The phage-infected bacterial cells were incubated for 30min at 37°C in a water bath (to facilitate the infection of helper phage). The culture was then supplemented with an additional 100ml LE medium (NE+50µl 1M IPTG). The cells were cultured in shaker for 2hrs at 26°C at 225rpm. The phage-infected bacteria were selected by the addition of 90µl of 50µg.ml⁻¹ kanamycin (final concentration of 30µg.ml⁻¹). The culture was grown over night at 26°C with shaking at 225rpm. On day 4 the cell suspension was transferred into a labelled Beckman centrifuge tube and centrifuged at 10,800g for 12min. at 4°C. (The centrifugation was repeated if the supernatant was not clear). The supernatant was decanted and transferred into another 250ml Beckman tube and mixed with 1/5 volume of cold PEG4000/Nacl (Sambrook *et al*, 2001) with respect to the volume of supernatant. After thorough mixing this was left on 1.5 to 3hrs, centrifuged at 15000g for 25min and the supernatant discarded. The tubes were inverted, left on a paper towel and the droplets of supernatant drained for 10min. The inner upper half of the Beckman centrifuge tubes was wiped with paper towel. The phage pellet was re suspended with 20ml of sterile MQ water + 4ml PEG4000/Nacl (1/5 Volume) in an Oakridge tube and incubated on ice for 30-45min. The tubes were centrifuged for 20min at 15000g, the supernatant discarded and the tube was inverted and dried for 10min at RT as before. Finally the phage particles were re suspended in 3ml of sterile 1x PBS and an aliquot of 1.5ml was transferred into eppendorfs and centrifuged for 1min. at 10,000g to remove the residual bacteria from the phage particles. This step was carried out twice or until the phage preparation was freed from bacterial contaminants. Clear supernatant was transferred into fresh eppendorfs and stored at 4°C with 0.1% sodium azide.

### 2.2.12.3. Quantification of scFv phage

Initial binding experiments were carried out using crude phage preparations, using phage-containing vector with no insert prepared under identical conditions as a negative control. With a view to standardising the number of phage particles in each binding experiments the quantification of phage preparation was carried out using (a) colony count (b) spectrophotometric method.

#### *2.2.12.3.1. Colony count method*

The number of infective phage particles present in the phage preparation can be estimated by colony counting method. However, this method does not give an estimate of number of phage that are able to bind, and falls off rapidly with time,

where as an antibody binding is stable for several weeks or even months (Steinberger *et al*, 2001).

10µl of stock XL1 Blue was inoculated into 10ml of prewarmed 2TY supplemented with 10µg.ml$^{-1}$ of tetracycline. The culture was grown over night at 37°C in a shaker at 225rpm. 20µl of overnight cultured XL1 Blue was sub cultured into another 10ml of prewarmed 2TY supplemented with 10µg.ml$^{-1}$ of tetracycline in falcon tube. The cells were grown in log phase at 37°C in a shaker at 225rpm until the OD$_{600}$ reached ~ 0.5. Dilutions ($10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}$ and $10^{-7}$) of phage were made in sterile 1xPBS. 1µl of diluted phage was mixed with 49µl of log phase XL1 Blue in sterile eppendorfs and incubated for 30min. at 37°C in a water bath. This mixture was spread into 2TYE plates supplemented with 1% glucose and 25µg.ml$^{-1}$ chloramphenicol. The plates were incubated overnight at 37°C. The number of colonies developed in the plate was counted and the number of infective phage particles present ml$^{-1}$ of phage preparation was estimated by using the formula: Number of colonies x dilution factor x 1000.

### 2.2.12.3.2. Spectrophotometric method

Quantification of phage was carried out by spectrophotometric method as described by Bonnycastle *et al* (2001). This method determines the number of phage particles, rather than just those that are infective. So as to get a concentration of approximately $10^{12}$ phage particles.ml$^{-1}$, the phage prep was diluted at 1:20 in 1xPBS and its OD$_{269}$ and OD$_{320}$ was measured using a spectrophotometer. The number of phage particles present in phage prep was determined using the formula:

$$\text{Phage particles.ml}^{-1} = \frac{(\text{Adjusted A}_{269}) \times (6 \times 10^{16})}{(\text{Number of nucleotides in the phage genome})}$$

Where, adjusted A$_{269}$ = measured A$_{269}$ − measured A$_{320}$; the number of nucleotides present in scFv phage genome is 7,307.

### 2.2.12.3.3. Titration of anti CD63 antibodies by whole cell ELISA

The melanoma cell lines Mewo or Mel-17 were harvested using 1:10 trypsin: EDTA solution (section 2.2.1.1). The cells were seeded into a 96 well microtitre plate at 1x10$^5$ cells.well$^{-1}$ in 100µl of medium and cultured overnight. The medium was removed and the cells fixed and blocked as described in section 2.2.10.6.5. The cells were incubated with 50µl of unlabelled primary antibodies such as H5C6, LP9 or BEM-1 and 50µl of secondary antibody anti mouse IgG-HRP (whole molecule) and developed as described in 2.2.5.4.

### 2.2.12.3.4. Binding of scFv phage to whole cells -three stage ELISA

Seeding and fixing of RBL2H3 cells, Mewo or Mel-17 in 96 well microtitre plate was as in 2.2.12.3.3. The cells were blocked with 300μl of 0.2% gelatin solution in 1xPBS for 1-2hrs in an incubator at 37°C, blocking agent removed and then wells incubated with 50μl scFv phage for 1hr at 37 C. After washing, anti M13 biotin-labelled antibody at 1μg.ml$^{-1}$ in dilution buffer was added and incubated for 1hr at 37°C. After washing, Extravidin-HRP at 0.5μg.ml$^{-1}$ in dilution buffer was added. Positive control wells were coated with 50μl of anti CD63 antibody (H5C6) at 5μg.ml$^{-1}$ and followed by anti mouse IgG-HRP (whole molecule) at 1:5000 in dilution buffer. After 1hr incubation at 37 C the colour was developed and the OD@450nm was measured.

### 2.2.12.3.5. Binding of scFv phage to whole cells-two stage ELISA

Seeding and fixing of RBL2H3 cells, Mewo or Mel-17 was as in section 2.2.12.3.3. The cells were blocked with 300μl of blocking buffer 2% milk powder in 1xPBS for 1-2hrs at 37°C. After removing blocking agent, cells were incubated with 50μl of scFv phage but the cells in +ve control wells were incubated with H5C6 at 14μg.ml$^{-1}$ in dilution buffer. The plate was incubated for 1hr at 37 C, washed as in section 2.2.10.6.5 and phage treated wells were coated with HRP labelled secondary anti M13 antibody at 1:5000 in dilution buffer. The positive wells were coated with 50μl of anti mouse IgG-HRP (whole molecule) at 1:5000 in dilution buffer. After incubation for 1hr at 37°C the plate was washed 3x with PBS+0.05% tween 20, 1x wash with PBS and a final wash with MQ water. After 1hr incubation at 37 C the colour was developed and the OD@450nm was measured.

### 2.2.12.3.6. Binding of scFv phage with GST-EC2 tetraspanins: ELISA

The binding of scFv phage on immobilized GST-EC2 tetraspanins was tested by ELISA as per the method described by Azorsa *et al* (1999) and Schober *et al* (2002). The coating of GST-EC2CD63 in 96 well microtitre plate, blocking and washings are same as in section 2.2.5.4. The wells were incubated with 50μl of scFv phage in 1% milk powder + 0.01% tween 20 in PBS. The positive wells were coated with anti CD63 antibody (H5C6) at 14μg.ml$^{-1}$ in dilution buffer. After 1hr incubation at 37°C, the plate was washed (section 2.2.5.4) and coated with a secondary antibody anti M13-HRP conjugate at 1:5000 in dilution buffer. The positive control wells were treated with anti mouse IgG-HRP in 1:5000 dilution buffer. After 1hr incubation at

37 C the colour was developed and OD@450nm was measured as described in section.2.2.5.4.

### 2.2.12.3.7. Inhibition ELISA

The coating of 96well microtitre plates with GST-EC2CD63, incubation, blocking were as in section 2.2.5.4. 50µl of scFv phage (at the dilution shown to give 50% maximal binding in the normal ELISA) was incubated with 100µl of serially diluted free GST-EC2CD63 in 1% milk powder and 0.01% tween 20 in PBS. The concentration of antigen (GST-EC2CD63) was initially at 0.5mg.ml$^{-1}$ then doubling dilutions in dilution buffer. The plate was incubated for 1hr at 37°C and the washing, coating with secondary anti M13-HRP antibody and developing, and measuring OD450nm are same as in section 2.2.5.4.

# CHAPTER 3

# EFFECTS OF TETRASPANINS EC2 REGIONS AND ANTI TETRASPANIN ANTIBODIES ON MONOCYTE FUSION

## 3.1. INTRODUCTION

As described in chapter 1, tetraspanin membrane proteins have been implicated in various cell fusion processes, such as sperm-egg fusion (Chen *et al*, 1999; Le Naour *et al*, 2000; Higginbottom *et al*, 2003); muscle cell fusion (Tachibana and Hemler, 1999) and the formation of viral syncytium (Pique *et al*, 2000). In this chapter, the role of tetraspanins in mononuclear phagocyte fusion was investigated. A recent report from Takeda and co-workers has suggested a role for tetraspanins proteins in the fusion of mononuclear phagocyte to form multinucleated giant cells (MGCs) (Takeda *et al*, 2003); Antibodies to CD9 and CD81 enhanced the formation of Con A induced monocyte fusion whereas no effect was observed with an anti CD63 antibody. Conversely, a GST fusion protein containing the large extracellular loop of CD9 (GST-EC2CD9) inhibited MGC formation. Stimulation of monocytes with Con A appeared to down-regulate the expression of CD9 and CD81, whereas the expression of CD63 was upregulated. The formation of integrin-CD81 and integrin-CD9 complexes was also down regulated. The authors also demonstrated that mononuclear phagocytes from CD9/CD81 double knockout mice showed a spontaneous MGC formation and concluded that tetraspanins CD9 and CD81 act as negative regulators of MGC formation (Takeda *et al*, 2003). This is in contrast to their role in sperm:egg fusion, where antibodies to CD9 and recombinant CD9EC2 regions inhibited the fusion (Takahashi *et al*, 2001).

### 3.1.1. Functional studies using anti tetraspanin antibodies

The functional properties of tetraspanins have been elucidated by various approaches. Among these, anti tetraspanin antibodies are important tools but they have number of disadvantages as they may recognise different epitopes and can act in multiple ways. The wide range of cellular responses stimulated by antibodies is thought to be due to perturbation not just of the specific tetraspanins, but also of the 'microdomains' in

which the tetraspanins are embedded. In most studies intact antibodies have been used, resulting in cross-linking of tetraspanins, with capping and internalisation likely consequences. Co-ligation of Fc receptors may also occur, causing FcR-related signalling events that are unrelated to normal tetraspanin functions (reviewed by Moseley, 2005). Higginbottom and co-workers, for example, showed the consequences of using whole molecule antibodies and their co-ligation with FcεRI using anti CD9 antibodies with CD9 transfected RBL cells (Higginbottom *et al*, 2000). Higginbottom and co-workers showed that the anti CD9 antibodies induced secretion by the transfected cells, but that this effect was dependent on the interaction of Fc region of the antibodies with the FcεRI naturally expressed by RBL2H3 cells (Higginbottom *et al*, 2000). Since monocytes express high levels of Fcγ receptors (Riemann *et al*, 2005) it may not be appropriate to use intact antibodies to study the functional properties of monocytes. However, to see if we could reproduce the findings described by Takeda and co-workers, studies with anti tetraspanin antibodies are described in this chapter. Alma-1 and 602.29 are mouse IgG1 anti CD9 antibodies (Azorsa *et al*, 1999; Andrews *et al*, 1981). H5C6 is a mouse IgG1 anti CD63 antibody (Azorsa *et al*, 1991). Fab fragments of this antibody were prepared by papain digestion and protein A-Sepharose purification and shown to be free of contaminating IgG, Fc or F(ab')$_2$ fragments by SDS-PAGE and western blotting (G. Mal, Ph.D thesis, University of Sheffield, 2005). LP9 is an IgM anti CD63 antibody, prepared in house (Smith *et al*, 1995). BEM-1 is an IgA anti-CD63 antibody prepared in house (McCullough *et al*, 1997).

### 3.1.2. Functional studies using EC2 fusion proteins

As an alternative to antibodies, the functional properties of tetraspanins are being studied by using recombinant tetraspanin large extracellular loop (EC2) domains, usually as fusion proteins with glutathione S-transferase (GST). It is likely that the tetraspanin EC2 domains form dimers or higher multimers (Kitadokoro *et al*, 2001), as proposed for full length tetraspanins (Hemler, 2003) and have a similar structure to native EC2 domains as demonstrated by the binding of conformation-dependent antibodies to both forms. The EC2 domains are critical to many, or perhaps all, tetraspanin specific activities described to date and probably act by clustering proteins at the cell surface into functional complexes (Boucheix and Rubinstein, 2001). Recombinant soluble EC2 domains appear to interfere with the formation of these

complexes, so inhibiting tetraspanin related cellular activities. Various studies have effectively used recombinant EC2 regions to assess the functional properties of tetraspanins. Thus GST-EC2CD9 was shown to inhibit sperm-oocyte fusion and the residues 173-175 (SFQ) in large extracellular loop were demonstrated to be essential to the sperm-egg fusion (Zhu *et al*, 2002). Higginbottom and co-workers also demonstrated that both recombinant mouse and human GST-EC2CD9 fusion protein were potent inhibitors of mouse sperm-egg fusion (Higginbottom *et al*, 2003). The role of the large extracellular loop of CD81 in its interaction with hepatitis C virus envelope glycoprotein E2 was demonstrated using recombinant GST-EC2CD81 and these proteins significantly inhibited E2 binding to hCD81 expressed at the surface of rat or human cells (Flint *et al*, 1999; Higginbottom *et al*, 2000; Petracca *et al*, 2000). As described in chapter 1 the role of CD9EC2 regions in the adhesion and pericellular fibronectin matrix assembly of CD9 transfected CHO cells was demonstrated using soluble GST-EC2CD9 protein (Cook *et al*, 1999). Most recently, in collaboration with Dr. Cecilia Cheng-Mayer (Aaron Diamond AIDS Research centre, New York) we have found that recombinant GST-EC2 tetraspanins such as CD63 and CD9 strongly inhibited the HIV-1 infection of human macrophages (unpublished work). Also, as mentioned above, Takeda and co-workers found that GST-EC2CD9 inhibited the Con A induced fusion of monocytes to form MGCs (Takeda *et al*, 2003). Recombinant EC2 regions have also been used for epitope mapping of monoclonal antibodies (Tomlinson *et al*, 1993) and for raising antibody against hEC2 domains of CD9, CD63, CD53, CD81, A15 or CO-029 (Azorsa *et al*, 1999). It appears that GST-EC2 regions of tetrapanins are recognised by conformation specific antibodies and reduced forms of GST-EC2 tetraspanins are non reactive (Azorsa *et al*, 1999; Hadlock *et al*, 2000 and our un published data). Recently, a $His_6$-tagged fusion protein of hEC2CD81 has been used to solve the crystal structure of the large extracellular loop of human CD81 (Kitadokoro *et al*, 2001).

### 3.1.3. Functional studies using monocytic leukaemic cell line and macrophages from CD9 WT and CD9$^{-/-}$ mice

The findings of Takeda and co-workers lead us to further investigate the role of tetraspanins in the fusion of monocytes. Most of the experiments described in this chapter relied on fresh human monocytes obtained from volunteers. However getting a good supply of fresh human monocytes is difficult and there may be considerable

variation between donors. Therefore attempts were also made to use the monocytic cell line THP-1 for the fusion assay. This cell line has been often used as a model system for functional studies of monocytes such as differentiation of monocytes into macrophages (Auwerx *et al*, 1991; Schwende *et al*, 1996; Kohro *et al*, 2004). Compared to other human monocytic cell lines (HL-60, U937, KG-1, or HEL), the differentiated THP-1 cells are believed to behave more like native monocyte-derived macrophages (Auwerx *et al*, 1991).

Similarly the role of CD9 on monocyte fusion was evaluated by using a macrophage cell line derived from CD9WT and CD9 knockout mice since these cells were successfully used to study the functional properties of human and murine pregnancy specific glycoproteins 17 (PSGs) (Ha *et al*, 2005).

### 3.1.4. Production and characterisation of GST-EC2 proteins

The production of the GST-EC2 proteins and their characterisation was described in previous publications from our group (Higginbottom *et al*, 2003) and is detailed in section 2.2.8. Correct folding of the proteins was determined by using conformation dependent antibodies, and is also indicated by their biological activity in other systems.

# 3.2. RESULTS

## 3.2.1. Monocyte cell fusion assay

### 3.2.1.1. Assay in microtitre plates

As mentioned in section 2.2.10.3.1, preliminary experiments with monocytes followed the procedures described by Takeda and co-workers (Takeda *et al*, 2003). The monocytes were isolated, cultured and stimulated in 96 well microtitre plates and subsequently stained with Wright stain. Although cell fusion was observed, staining of cells was not clear and the numbers of nuclei in fused cells were difficult to enumerate (Fig.3.2.1). Hence we tried an alternative approach, culturing the monocytes in Lab-Tek® chamber slides and staining cells using FITC labelled antibodies to achieve better cell morphology and allow more accurate counting.
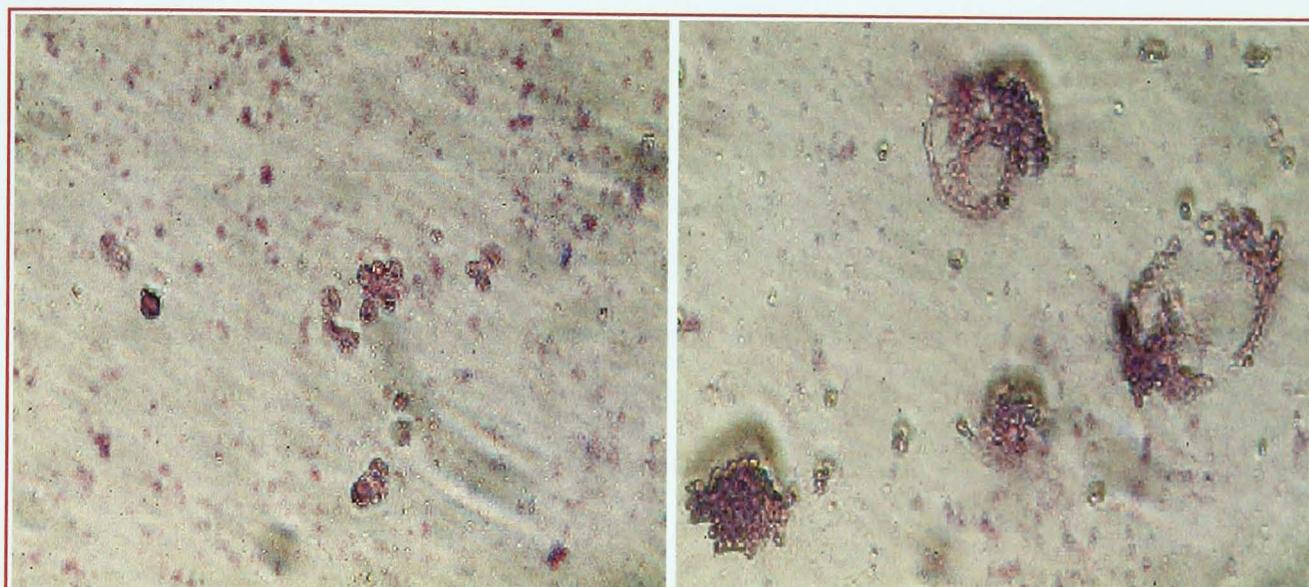


(A)                                                        (B)

Fig.3.2.1. Light microscopic images of monocytes stained with Wright stain. Monocytes at $2x10^5$ cells in 100µl of culture medium were plated into a 96 well microtitre plate and cultured for 72hrs in the presence/absence of Con A at 10µg.ml$^{-1}$: Monocytes alone (A); in the presence of Con A (B). The cells were stained as described in section 2.2.10.3.1 and the images were captured with a Nikon inverted microscope using 40x objective.


### 3.2.1.2. Assay in Lab-Tek® chamber slides

The monocytes were separated from the blood as mentioned in section. 2.2.10.1 and allowed to adhere to Lab-Tek® chamber slides. The purity of monocytes was assessed by staining with monocyte-specific markers such as FITC labelled anti CD14 or CD68 antibodies by immunofluorescence microscopy and the results indicated that ~ 80-90% of the slide-adherent cells were monocytes. The adherent cells were stimulated with Con A as described in section 2.2.10.3.2. After 72hrs, the cells were fixed, stained with a FITC-labelled primary antibody and the nuclei stained with

propidium iodide. Various FITC labelled antibodies (anti CD14, anti CD52, anti CD63 and CD68) were compared for their staining efficiency using fresh and thawed frozen stocks of monocytes (Audran *et al*, 1995) as described in section 2.2.10.3.2. These antibodies were chosen on the basis of their specificity and expression levels of corresponding antigens on the monocytes. CD14 is a glycosyl-phosphatidylinositol (GPI) linked glycoprotein predominantly expressed on the cell surface of monocytes (Barclay *et al*, 1997).

Surface expression of CD63 in monocytes is relatively low, but it has good intracellular expression (our unpublished observation). CD52 is an unusually short glycoprotein bearing GPI protein and leukocyte marker; its surface expression levels are very high on monocytes (Barclay *et al*, 1997). CD68 is a highly glycosylated lysosomal-associated membrane protein (lamp); its expression is less on the surface and more in the lysomes and endosomes of monocytes (Barclay *et al*, 1997).

From the staining studies with different antibodies it was observed that although freeze-thawed monocytes were stained well with FITC labelled anti CD63 (H5C6) and anti CD14 antibodies, their appearance on Con A stimulation was atypical of fresh monocytes. Thus, although they provided a convenient source of cells, frozen monocytes were not further used. Hence we used fresh monocytes for staining studies with FITC labelled anti CD52, anti CD63 and anti CD68 antibodies. Both anti CD52 and anti CD63 antibodies have given good staining with unstimulated and Con A stimulated monocytes; in contrast to these the anti CD68 antibody (monocyte marker) showed good staining with unstimulated monocytes but less staining with Con A stimulated monocytes. Even though the anti CD63 antibody is good for staining of unstimulated and Con A stimulated cells it was not suitable for the experiments involving recombinant GST-EC2CD63. Hence among all the antibodies tested the anti CD52 was selected as a suitable antibody for staining of unstimulated and Con A stimulated monocytes (Fig.3.2.2). To achieve optimum staining of monocytes with anti CD52-FITC antibody, the antibody was titrated by FACS analysis as described in section 2.2.5.7. The result showed that $5\mu g.ml^{-1}$ of antibody was the optimum concentration (Fig.3.2.3).

(A)

(B)

(C)

(D)

(E)

(F)
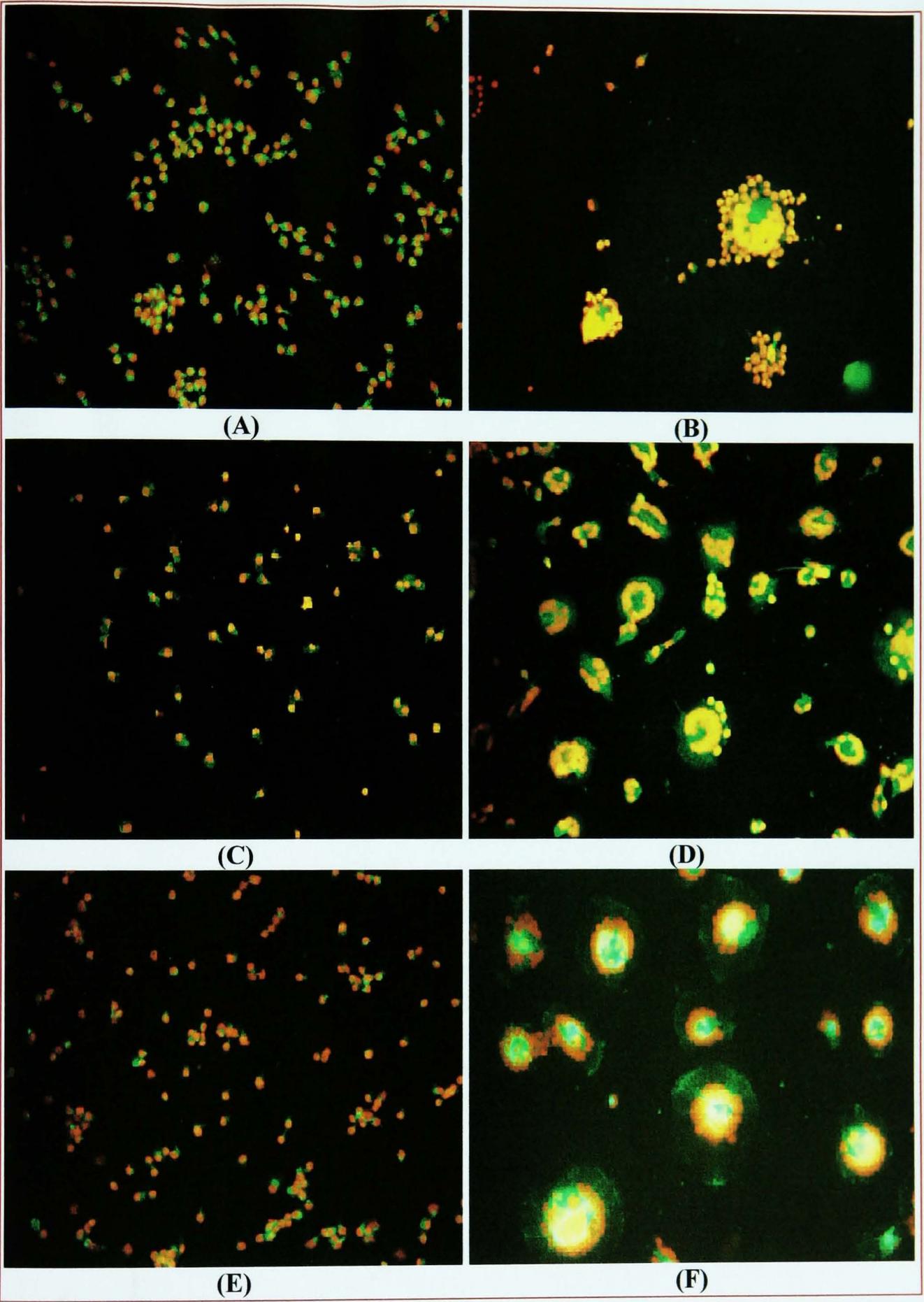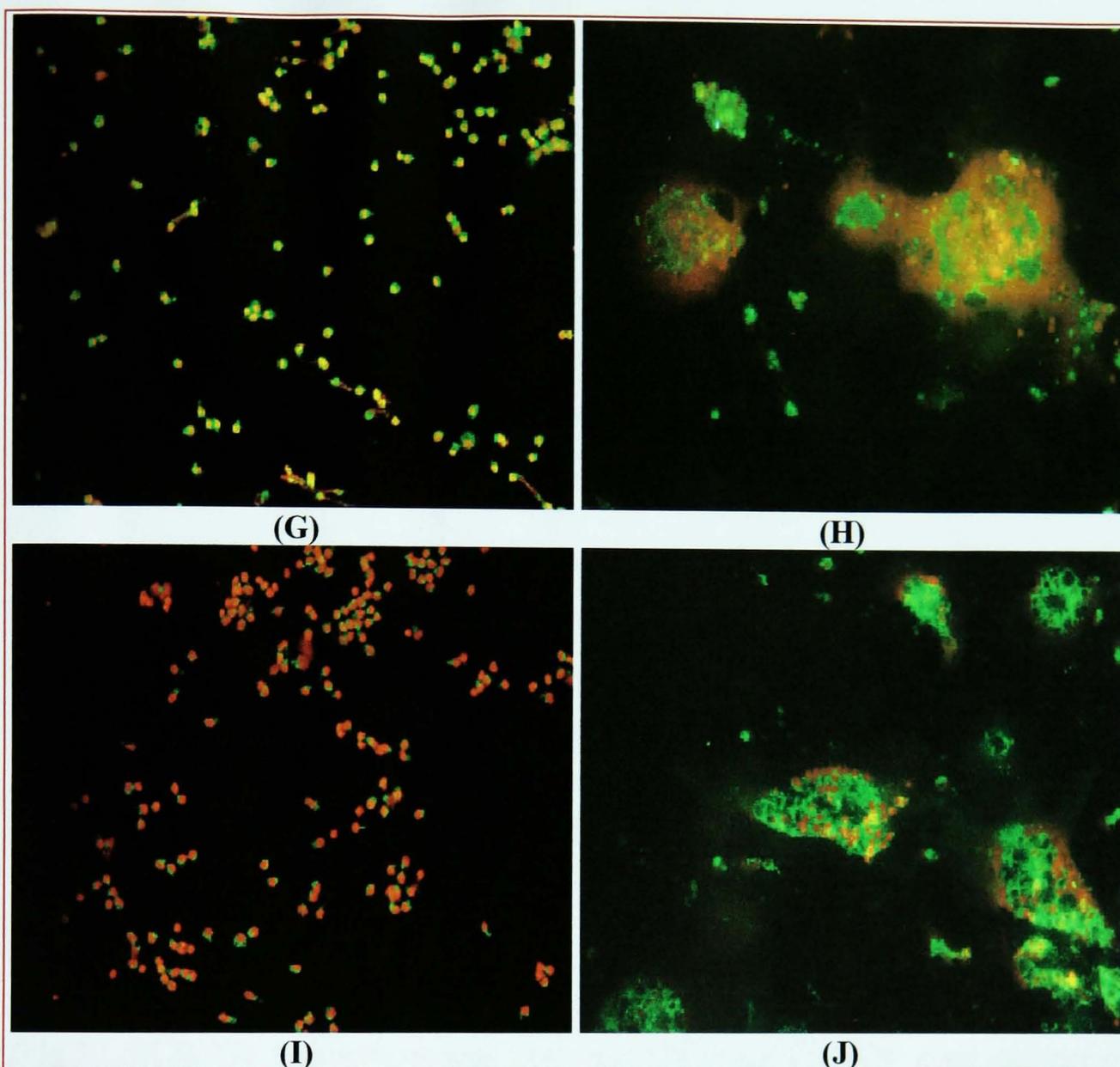
Fig.3.2.2. Immunofluorescence images of monocytes (fresh/freeze-thawed) cultured for 72hrs in Lab-Tek® chamber slides. The cells were fixed and stained with FITC-labelled monoclonal antibodies and propidium iodide as described in section 2.2.10.3.2. A, C, E, G, I-unstimulated; B, D, F, H, J- stimulated with Con A at $10\mu g.ml^{-1}$. The antibodies used were: anti CD68 (A) and (B); anti CD52 (C) and (D); anti CD63 (E) and (F); anti CD14 (G) and (H); anti CD63 (I) and (J). A-F corresponds to freshly prepared monocytes, (G)-(J) thawed, frozen monocytes. The images were captured with a Nikon Eclipse E400 immunofluorescence microscope using 20x objective.
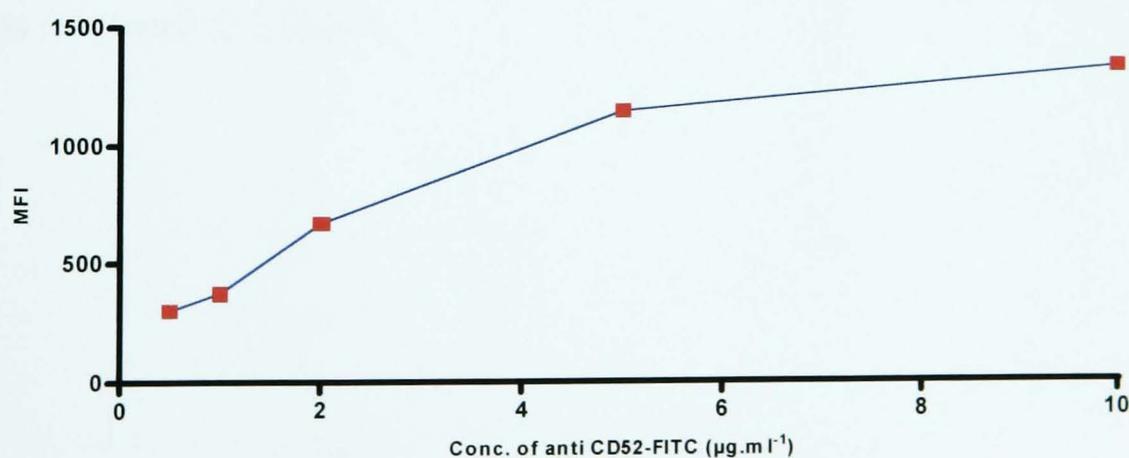


Fig.3.2.3. Titration of anti CD52-FITC antibody against monocytes. The FACS analysis was carried out as described in section 2.2.5.7.

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The stimulation of monocytes with Con A leads to fusion and produced mostly Langhans' type of MGCs with a circular peripheral arrangement of nuclei or horseshoe shaped arranged nuclei; occasionally we noticed some MGCs which resembled foreign body type MGC with scattered nuclei (Van der Rhee *et al*, 1979; Anderson, 2000; Mizuno *et al*, 2004). This varied between experiments and donors. The approximate numbers of nuclei in each fused cell were between 50-100 and this also varied between donors. Typical fusion indices (no. of nuclei in fused cells/ number of nuclei in total % (Abe, 1983; Takeda *et al*, 2003)) were 75-95%.

The Con A stimulated monocytes/MGCs appeared strongly adherent to the culture surface. By contrast the monocytes cultured under the same conditions in the absence of Con A did not fuse and they were less adherent i.e. they were more easily detached from the culture surface during slide processing.

## 3.2.2. Effects of anti tetraspanin antibodies on MGC formation

In preliminary experiments with antibodies to tetraspanins, it was apparent that some of these reagents greatly affected the size of the MGCs formed e.g. anti CD9 antibody Alma-1 induced the formation of very large MGCs containing hundreds of nuclei (Fig.3.2.5(C)). The fusion index term does not provide a particularly good measure of this effect, since the percentage of nuclei present in fused cells may remain high irrespective of the size of the MGCs (Fig.3.2.4(A)). Hence in the subsequent experiments with antibodies the fusion rate was estimated as a measure of number of nuclei in fused cells in randomly chosen fields. Based on the preliminary studies, the effects of anti CD9 antibodies (Alma-1 and 602.29) and anti CD63 antibodies (BEM-1, H5C6, 16.1, 16.5, LP9 and Fab fragments of H5C6) on Con A induced monocyte fusion was tested (2.2.10.3.2).

**(A)**



**(B)**
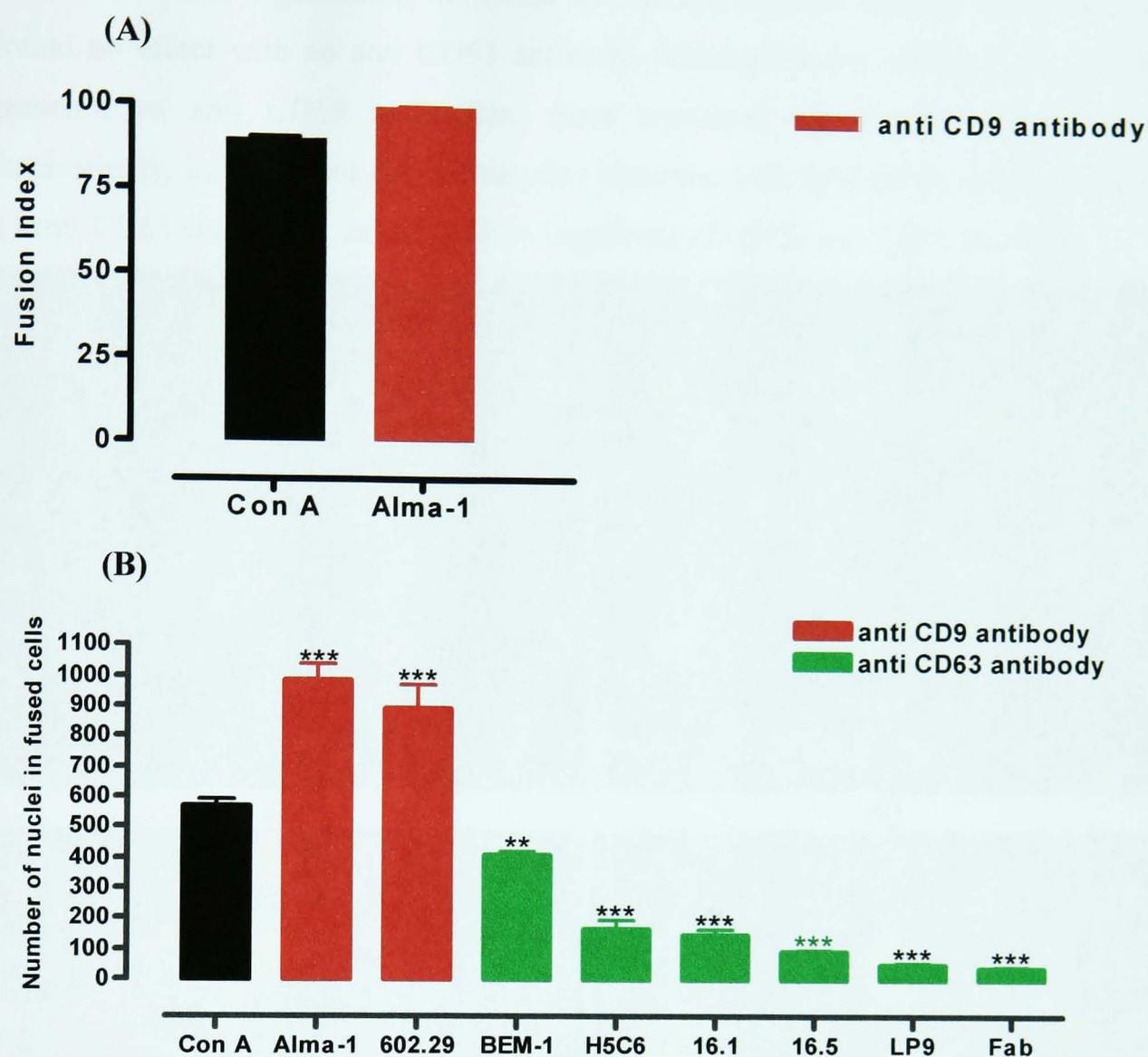


Fig. 3.2.4. Effects of anti CD9 and anti CD63 antibodies on Con A induced monocyte fusion. Monocytes in Lab-Tek® chamber slides were stimulated with Con A ($10\mu g.ml^{-1}$) and cultured for 72hrs in the presence or absence of antibodies ($20\mu g.ml^{-1}$ except for BEM-1, used at $40\mu g.ml^{-1}$) as mentioned in section 2.2.10.4.1. Fusion index (A); numbers of nuclei in multi nucleated giant cells (B) were calculated from 6 randomly chosen field after staining with anti CD52-FITC antibody as described in section 2.2.10.3.2. The data in Fig.3.2.4A is a means of 1 experiment. The data in Fig.3.2.4B represents means of 4 independent experiments in duplicate with Alma-1 and H5C6 and 2 independent experiments with other antibodies in duplicate carried out with monocytes of different blood donors. The level of significance between the Con A control and different treatments was tested by unpaired t test, where *** Significant at $P<0.0001$; ** Significant at $P<0.001$.

The effects of co-incubating the monocytes with the anti-tetraspanin antibodies are shown in Fig.3.2.4. The anti CD9 antibodies strongly enhanced the fusion of Con A stimulated monocytes and produced MGCs with large numbers of nuclei relative to Con A alone control. By contrast anti CD63 antibodies strongly inhibited the fusion. The effects of anti CD9 antibodies (Alma-1 and 602.29) are in agreement with the findings of Takeda and co-workers (Takeda *et al*, 2003). By contrast, all of the anti-

CD63 antibodies significantly inhibited the fusion whereas Takeda and colleagues found no effect with an anti CD63 antibody. Although some MGCs formed in the presence of anti CD63 antibodies, these contained significantly fewer nuclei. Interestingly, inhibition of fusion was also observed with IgM (LP9) and IgA (BEM-1) anti CD63 antibodies, as well as Fab fragments of H5C6 anti CD63 antibody.



(A)

(B)

(C)

(D)

(E)

(F)
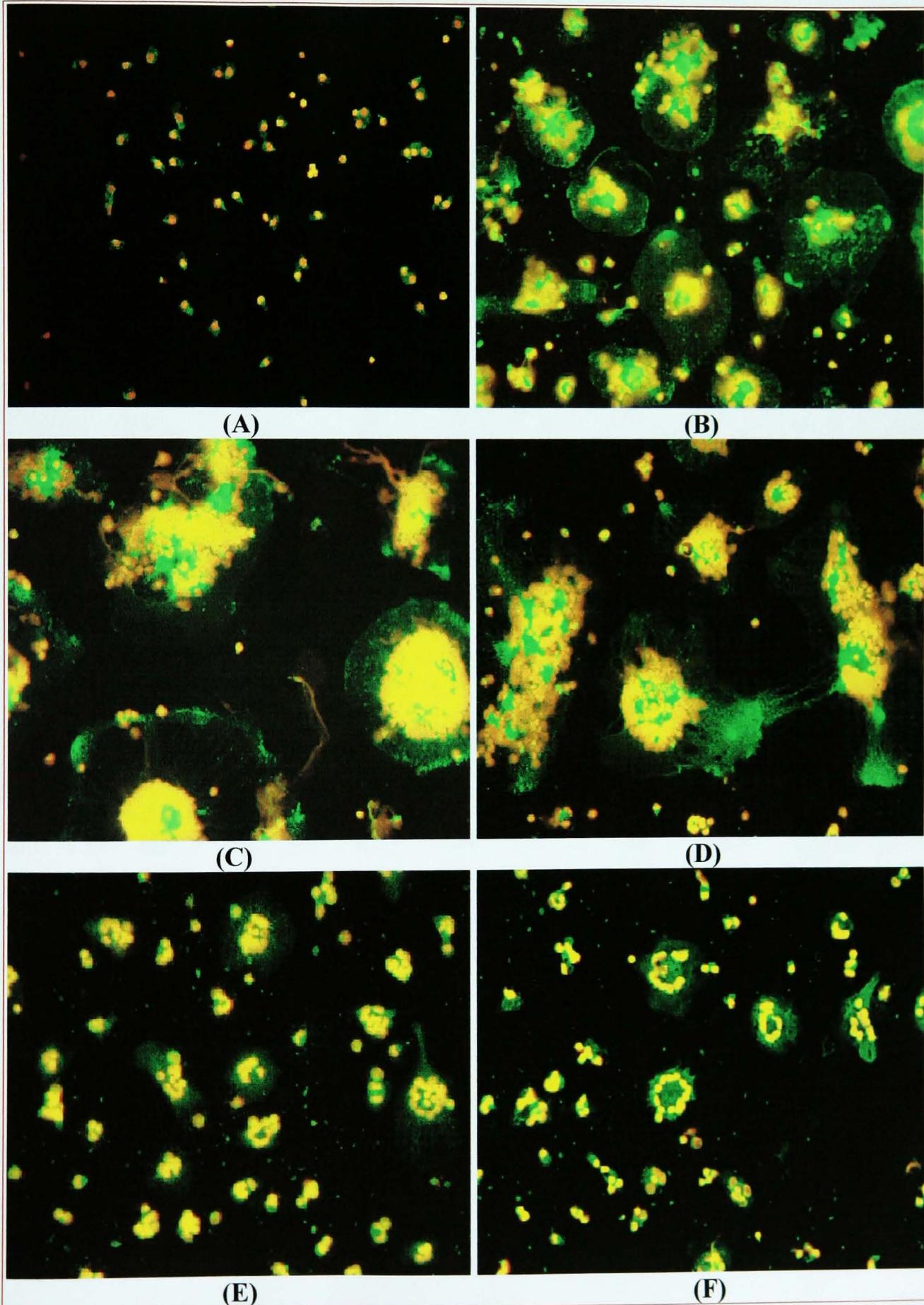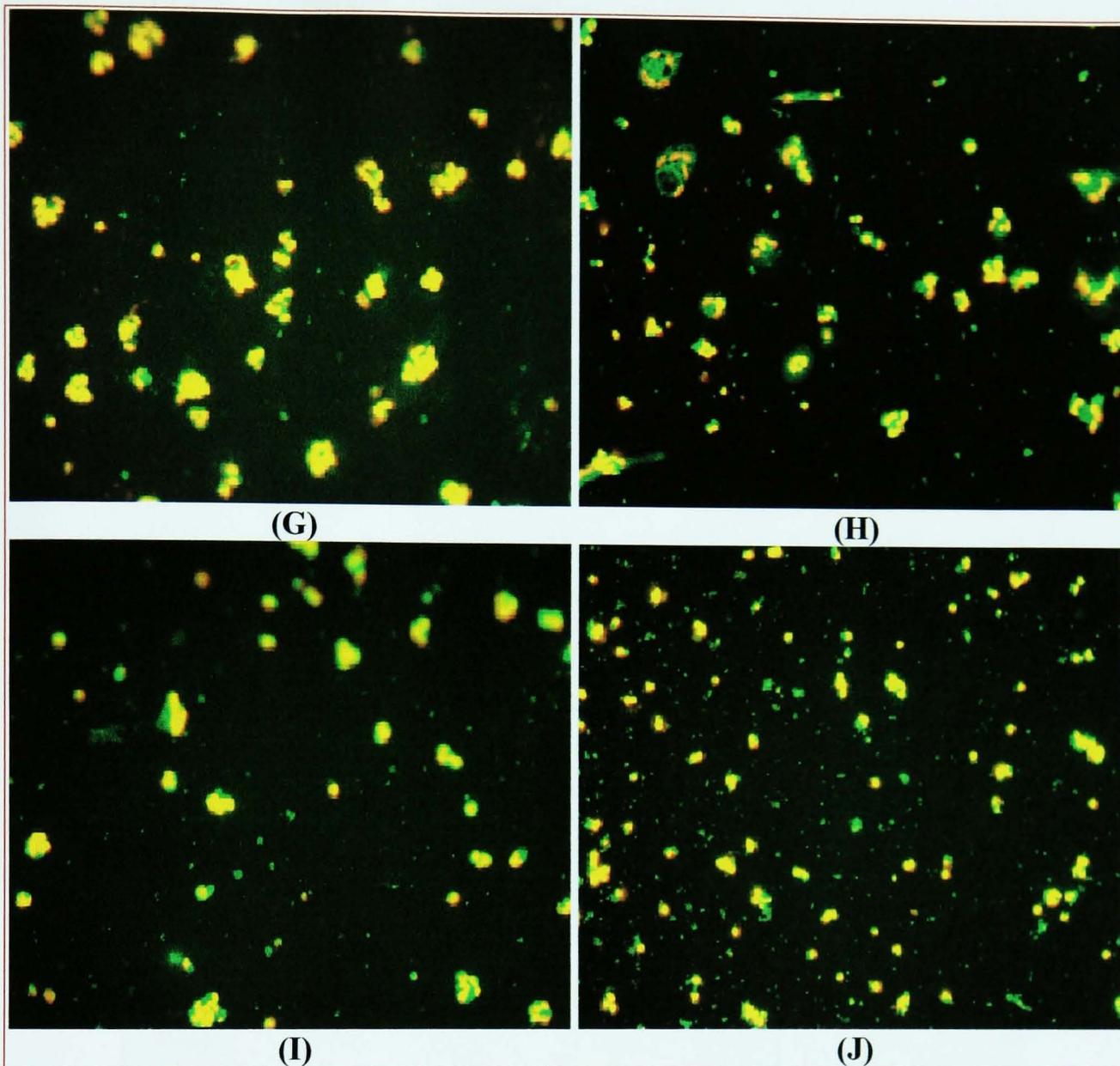
Fig.3.2.5. Effects of anti tetraspanin antibodies on Con A induced monocyte fusion. The conditions were as described in Fig.3.2.4. The monocytes incubated without Con A (A); Con A (B); Con A + anti CD9 antibody-Alma-1 (C); Con A + anti CD9 antibody-602.29 (D); Con A + anti CD63 antibody-BEM-1 (E); Con A + anti CD63 antibody-H5C6 (F); Con A + anti CD63 antibody-16.1 (G); Con A + anti CD63 antibody-16.5 (H); Con A + anti CD63 antibody-LP9 (I); Con A + Fab fragments of anti CD63-H5C6 (J). The images were captured with a Nikon Eclipse E400 immunofluorescence microscope using 20x objective.

### 3.2.3. Production and characterisation of GST-EC2 proteins

The expression, isolation and purification of GST-EC2 tetraspanins such as CD9 and CD63 were carried out as described in section 2.2.8. For functional assay described subsequently, production was carried out by Dr. Adrian Higginbottom or Francine Martin in the group. However, the results of a production carried out by myself are shown below. GST-EC2CD9 and GST-EC2CD63 were prepared in two batches of 400ml culture and the yield of GST-EC2CD9 was 21.6mg.L$^{-1}$; 12.9mg.L$^{-1}$ respectively, and of GST-EC2CD63 3.5mg.L$^{-1}$; 0.64mg.L$^{-1}$ respectively. The concentration of the proteins was determined by densitometry. The bands at 35KDa

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and 37KDa are corresponding to GST-EC2CD9 and GST-EC2CD63, respectively. The band at approximately 25KDa is free GST. Bands between 25-35KDa are presumed to be break down products of the GST-EC2 proteins. (The identity of these bands has been determined many times previously by Western blotting, but was not done in this instance). The bands below 25KDa and above 37KDa are the contaminants of the GST preparations and it may vary from preparations to preparation. For the functional studies described below, GST-EC2CD9 and GST-EC2CD63 yields were typically 15mg.L$^{-1}$ and 2-10mg.L$^{-1}$, respectively. The concentration of GST-EC2 varied from 20-60% of total protein.



Fig.3.2.6. Expression of GST-EC2CD9 and GST-EC2CD63 fusion proteins. The concentration of the proteins was determined by coomassie stained gel using densitometry. Lane 1: GST; Lane 2&3: GST-EC2CD9; Lane 4&5: GST-EC2CD63; Lane 6: BSA std (10mg); Lane 7: BSA std (5mg); Lane 8: BSA std (2.5mg) and Lane 9: BSA std (1mg). GST-EC2CD9 and GST-EC2CD63 were prepared in two batches. The bands corresponding to 25KDa is GST; the band at 35KDa (Red arrow) corresponding to GST-EC2CD9 and band at 37KDa (green arrow) GST-EC2CD63, were used for densitometry.

## 3.2.4. Effect of GST-EC2 tetraspanins at 20µg.ml$^{-1}$ on MGC formation

To further investigate the role of different tetraspanins on Con A induced mononuclear phagocyte fusion, the effects of a range of GST-EC2 tetraspanins proteins was tested. The GST-EC2 tetraspanins were co-incubated with monocytes and Con A for 72hrs in the fusion assay system as described above (Figs.3.2.4 and 3.2.5). Following staining, the fusion index (no. of nuclei in fused cells/ number of nuclei in total %) was determined.
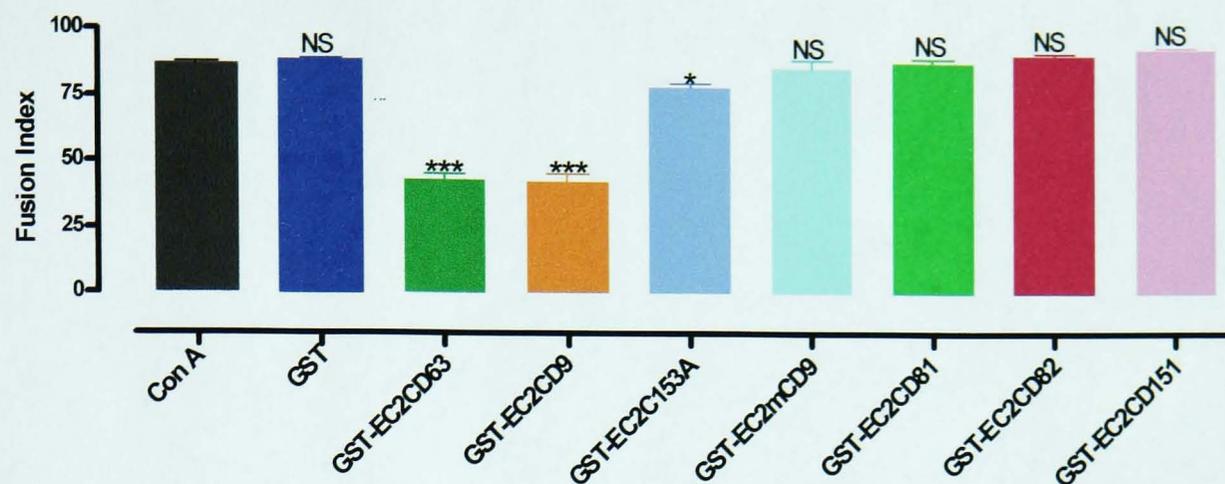
Fig.3.2.7. Effects of different GST-EC2 tetraspanins on Con A induced monocyte fusion. Monocytes in Lab-Tek® chamber slides were stimulated with Con A ($10\mu g.ml^{-1}$) for 72hrs in the presence or absence of GST-EC2 tetraspanins or GST control as described in section 2.2.10.4.1. The cells were stained with anti CD52-FITC and PI as described in section 2.2.10.3.2. The number of nuclei in fused and un-fused cells was counted in 6 randomly chosen fields. Fusion index (no. of nuclei in fused cells/ number of nuclei in total x100) was calculated. The results with Con A, GST, GST-EC2CD63 and GST-EC2C153A are the means of 17 experiments in duplicates carried out with monocytes from 12 blood donors; the results of GST-EC2CD9 are the means of 12 experiments with monocytes from 12 blood donors and the results of other GST-EC2 tetraspanins are the means of 3 experiments with monocytes from 3 different blood donors. The level of significance between the Con A control and different treatment was tested by unpaired t test, where *** Significant at $P<0.0001$; * Significant at $P<0.01$; NS=Non significant.

From the results it was observed that GST-EC2CD9 and GST-EC2CD63 showed a strong and a highly significant ($P<0.001$) inhibitory effect on Con A induced monocyte fusion when compared with Con A alone. The inhibitory effect observed with GST-EC2CD9 is in agreement with Takeda and co-workers (Takeda *et al*, 2003). GST proteins alone had no effect on monocyte fusion. In addition GST-EC2CD9 mutant C153A (a mutation of one the cysteines thought to be involved in EC2 disulphide bond formation, which is not recognised by conformation-dependent antibodies) showed a mild inhibitory effect ($P<0.01$). The other GST-EC2 tetraspanins CD81, CD82, CD151 and mouse CD9 did not show any effect on Con A induced monocyte fusion.

(A)

(B)

(C)
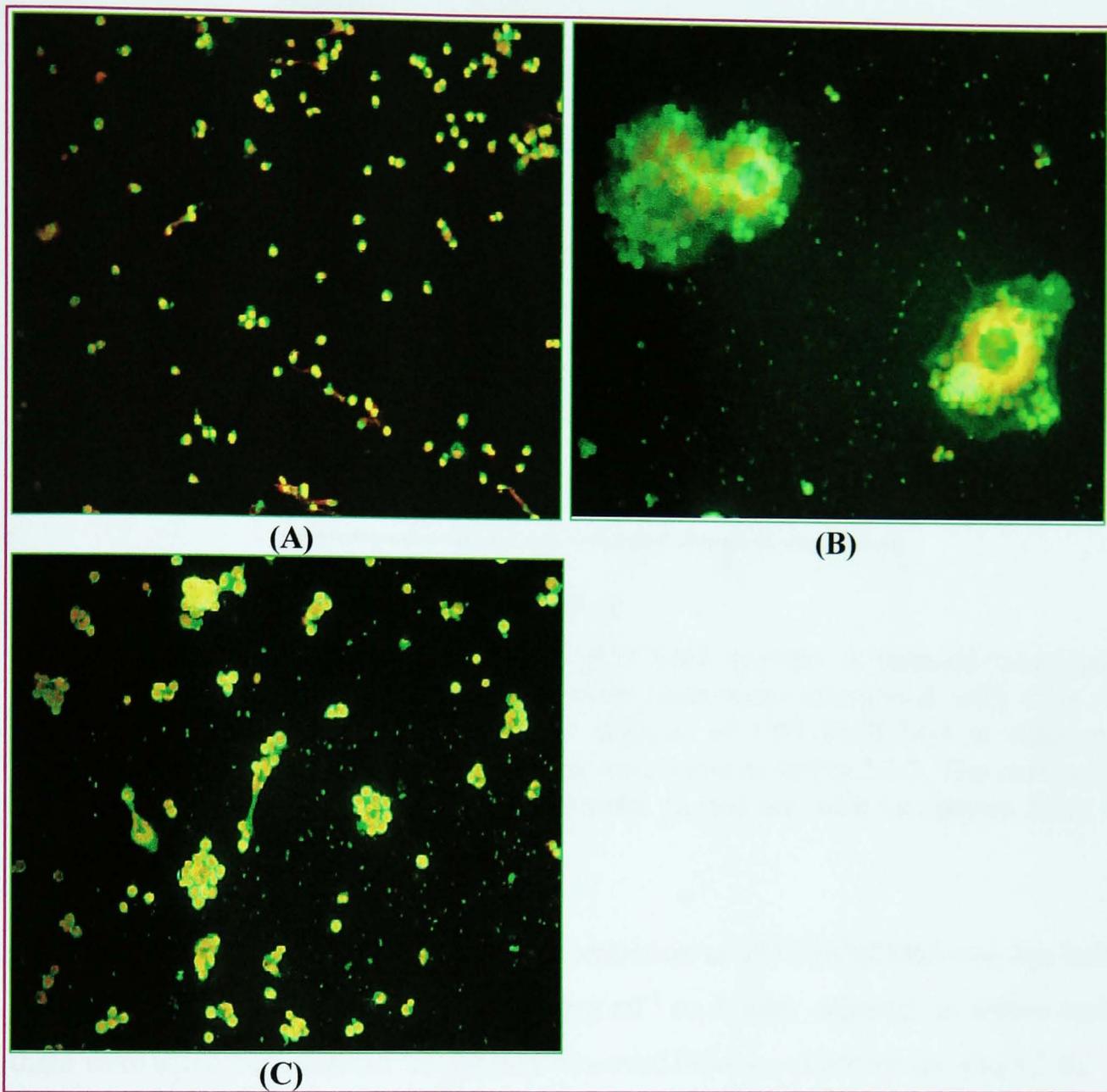
Fig.3.2.8. Effect of recombinant GST-EC2CD63 at $20\mu g.ml^{-1}$ $(0.6\mu M)$ on Con A induced monocyte fusion. The conditions were similar as described in Fig.3.2.4. The images were captured with a Nikon Eclipse E400 immunofluorescence microscope using 20x objective. Monocytes alone (A); Con A (B); Con A+ GST-EC2CD63 (C).

## 3.2.5. Dose/response effect of GST-EC2CD63

Since a role for CD63 in MGC formation had not previously been observed, some further experiments concentrated on GST-EC2CD63. Since an inhibitory effect was observed with GST-EC2CD63 at $20\mu g.ml^{-1}$ (equivalent to $0.57\mu M$) on Con A induced monocyte fusion, the dose/response effects of GST-EC2CD63 were examined.
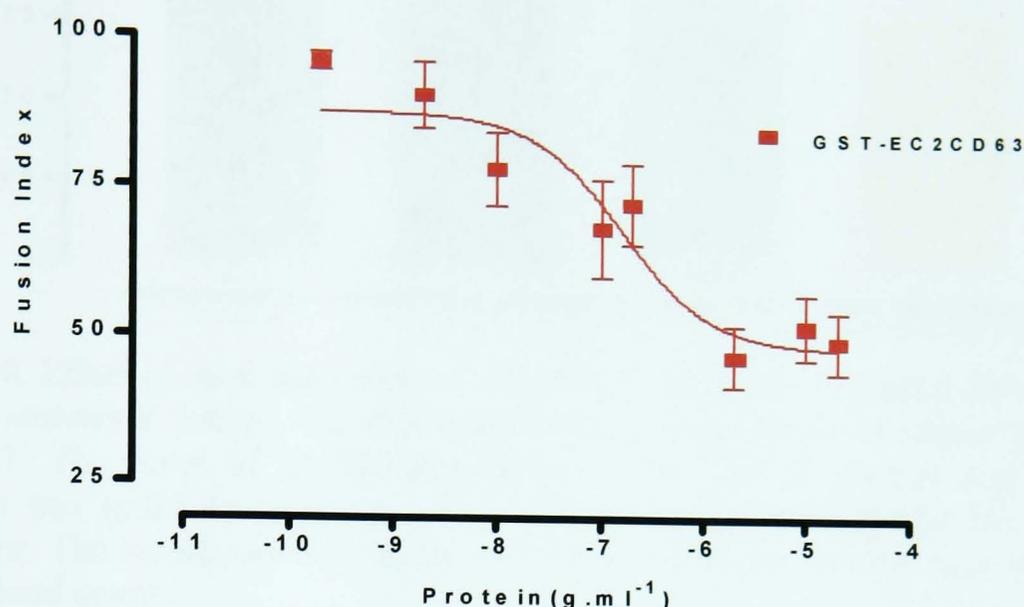
Fig.3.2.9. Dose response effects of GST-EC2CD63 on Con A induced monocyte fusion. The monocytes in Lab-Tek® chamber slide were stimulated with Con A ($10\mu g.ml^{-1}$) for 72hrs in the presence or absence of GST-EC2CD63 at different concentrations and the remaining conditions were same as in Fig.3.2.7. The results of GST-EC2CD63 are the means of 6 experiments carried out with monocytes from 5 blood donors.

Analysis of the results shows that the concentration of GST-EC2CD63 that has half the maximal inhibitory effect ($IC_{50}$) is 160ng $ml^{-1}$ or 4.3nM, although as shown later there were some variations in the potency observed between experiments (Fig.3.2.9).

### 3.2.6. Effect of short incubation of GST-EC2CD63 and GST-EC2CD9 on MGC formation

Since the GST-EC2CD63 and GST-EC2CD9 strongly inhibited the fusion of Con A stimulated monocytes at $20\mu g.ml^{-1}$, the effect of a short incubation of GST-EC2 tetraspanins on monocyte fusion was tested as described in section 2.2.10.4.2. In this study the monocytes were incubated with GST-EC2 tetraspanins for 30min. at 37°C. After washing to remove the proteins, cells were cultured in presence of Con A for 72hrs. From the data it was observed that there was still some slight inhibition of the fusion process, suggesting that the tetraspanins does not need to be present throughout the incubation period of 72hrs.
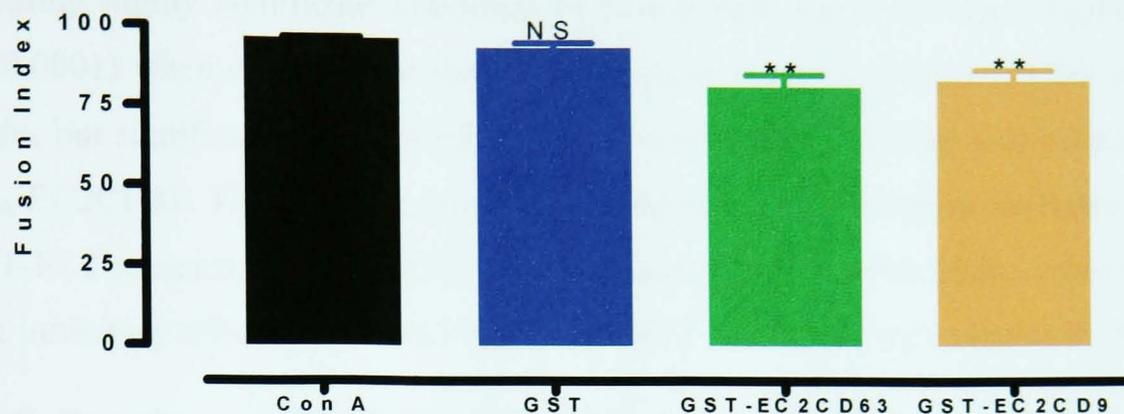
Fig.3.2.10. Effect of short incubation of GST-EC2CD63 and GST-EC2CD9 on Con A induced monocyte fusion. The experiment was carried out as described in section 2.2.10.4.2. The level of significance between the Con A control and different treatment was tested by unpaired t test, where ** Significant at $P<0.05$; NS=Non significant. The results are the means of 2 experiments carried out with monocytes from 2 blood donors.

### 3.2.7. Effect of His$_6$-tagged tetraspanins at 20µg.ml$^{-1}$ on MGC formation

Although GST alone had no effect on MGC formation, we wished to investigate the effects of tetraspanin EC2s where this fusion protein was absent. Highly purified His$_6$-tagged EC2 tetraspanins (CD9, CD63 and CD81) were obtained from our collaborators Christopher Liu and Richard Hynes (Massachusetts Institute of Technology, U.S) and tested for their effects on Con A induced monocyte fusion. In this study the above His$_6$-EC2 tetraspanins were tested at 20µg.ml$^{-1}$ against Con A stimulated monocytes. In addition the effect of GST-EC2CD63 was also tested in parallel with His$_6$-EC2CD63 for comparison.
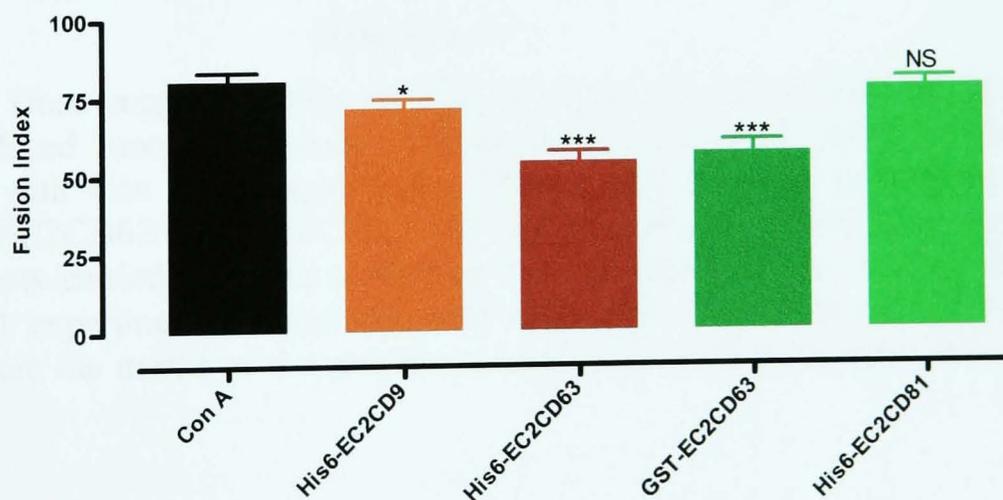


Fig.3.2.11. Effects of His$_6$-EC2 tetraspanins on Con A induced monocyte fusion. Monocytes in Lab-Tek® chamber slides were stimulated with Con A (10µg.ml$^{-1}$) for 72hrs in the presence or absence of His$_6$-EC2/GST-EC2 tetraspanins at 20µg.ml$^{-1}$ as described in section 2.2.10.4.1. The results are the means of 4 experiments carried out with monocytes from 2 blood donors. The level of significance between the Con A control and treatment group was tested by unpaired t test, where *** Significant at $P<0.0001$; * Significant at $P<0.01$; NS=Non significant at $P<0.05$.

A strong highly significant inhibition in fusion was observed with $His_6$-EC2CD63 (P<0.0001) when compared to the Con A control, where as $His_6$-EC2CD9 showed a slight, but significant (P<0.01) inhibition. No inhibition in fusion was observed with $His_6$-EC2CD81. These results are in general agreement with results observed with the GST-EC2 proteins, although GST-EC2CD9 appeared to inhibit fusion more strongly. The inhibitory effect of $His_6$-EC2CD63 and GST-EC2CD63 were similar at $20\mu g.ml^{-1}$

### 3.2.8. Dose/response of $His_6$-EC2CD63 and EC2CD63

The dose/response effect of $His_6$-EC2CD63 and EC2CD63 (generated by thrombin cleavage of GST-EC2CD63) on monocyte fusion was examined as described in section 2.2.10.4.1. The dose/response effect of GST-EC2CD63 was examined in parallel for comparison.
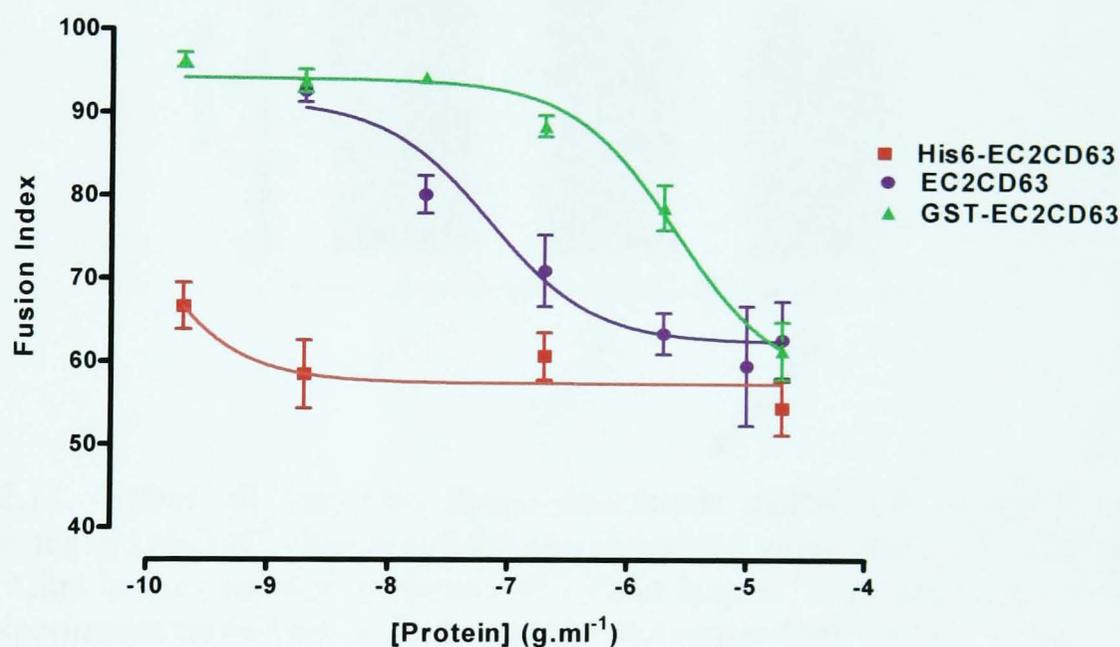


Fig.3.2.12. Dose response effect of $His_6$-EC2CD63/EC2CD63/GST-EC2CD63 on Con A induced monocyte fusion. Monocytes in Lab-Tek® chamber slide were stimulated with Con A ($10\mu g.ml^{-1}$) for 72hrs in the presence or absence of $His$-EC2CD63/EC2CD63/GST-EC2CD63. The results of $His_6$-EC2CD63 are the means of 5 experiments carried out with monocytes from 3 blood donors; EC2CD63 are the means of 4 experiments carried out with monocytes from 3 blood donors; GST-EC2CD63 are the means of 4 experiments carried out with monocytes from 3 blood donors.

All preparations of recombinant CD63 inhibited MGC formation. The EC2CD63 showed an $IC_{50}$ value of $69ng.ml^{-1}$ or 5.8nM. The latter value is similar to that observed overall with the GST-EC2CD63 (section 3.2.5). However, in this set of experiments, the GST-EC2CD63 was less potent ($IC_{50}$ of $2.5\mu g.ml^{-1}$ or 68nM). Possible reasons for these types of variations are discussed in Discussion 3.3. The

His$_6$-EC2CD63 gave very potent inhibition, that was not titrated out in these experiments. This was unexpected, and ideally this titration would have been repeated.

### 3.2.9. Effect of bacterial lipopolysaccharide (LPS) on monocyte fusion

Since recombinant proteins were used for these studies, there were concerns that LPS present as a contaminant in GST-EC2 tetraspanin preparations (produced in *E.coli*) might inhibit the fusion of monocytes (Takashima *et al*, 1993). Therefore, we investigated the effects of bacterial cell wall protein LPS at 20, 10 and 2µg.ml$^{-1}$ on unstimulated or Con A stimulated monocytes as described in section 2.2.10.4 .1.
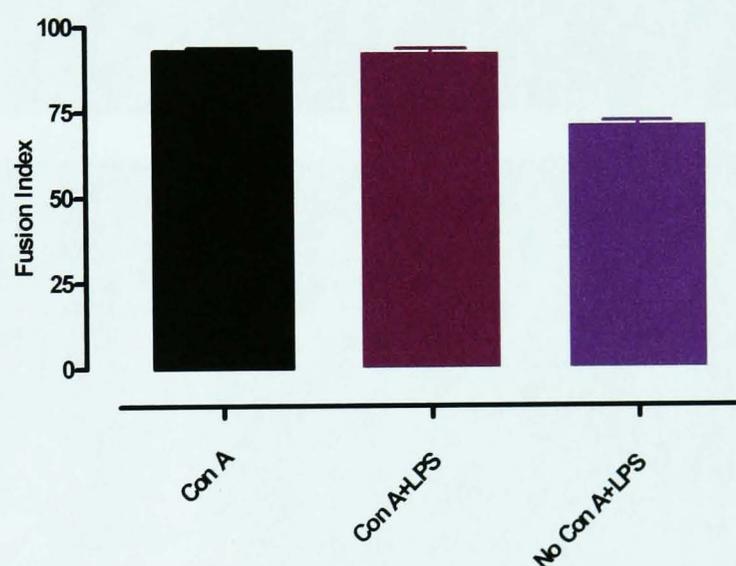


Fig.3.2.13. Effect of bacterial lipopolysaccharide (LPS) on monocyte fusion. Monocytes in Lab-Tek® chamber slide were stimulated with/without Con A (10µg.ml$^{-1}$) for 72hrs in the presence or absence of LPS at 2µg.ml$^{-1}$. The results are the means of 2 experiments carried out in duplicate with monocytes from 1 blood donor.

From this study it was observed that LPS did not show any inhibitory effect on the fusion of monocytes. By contrast, LPS alone at 2µg.ml$^{-1}$ induced the fusion of monocyte in the absence of Con A. The fusion index shown by LPS (2µg.ml$^{-1}$) in presence of Con A was 92.89%, very close to the fusion index of Con A control (92.99%). LPS alone at 2µg.ml$^{-1}$ induced fusion in the absence of Con A and its fusion index was 71.69%. The size of the MGCs produced by LPS in the absence of Con A was smaller (4 -10 nuclei) than the MGCs produced in presence of Con A. In addition LPS produced Langhans type of MGCs with Con A stimulated monocytes (~ >50 nuclei); this effect is similar to the effect reported with muramyl dipeptide (Mizuno *et al*, 2001). Interestingly LPS promoted the adhesion of monocytes to the culture surface in the absence of Con A. The size of MGCs produced by LPS in

presence of Con A was very large when compared to the MGCs of Con A stimulated monocytes alone. Thus it appears that LPS may actually enhance Con A induced fusion.
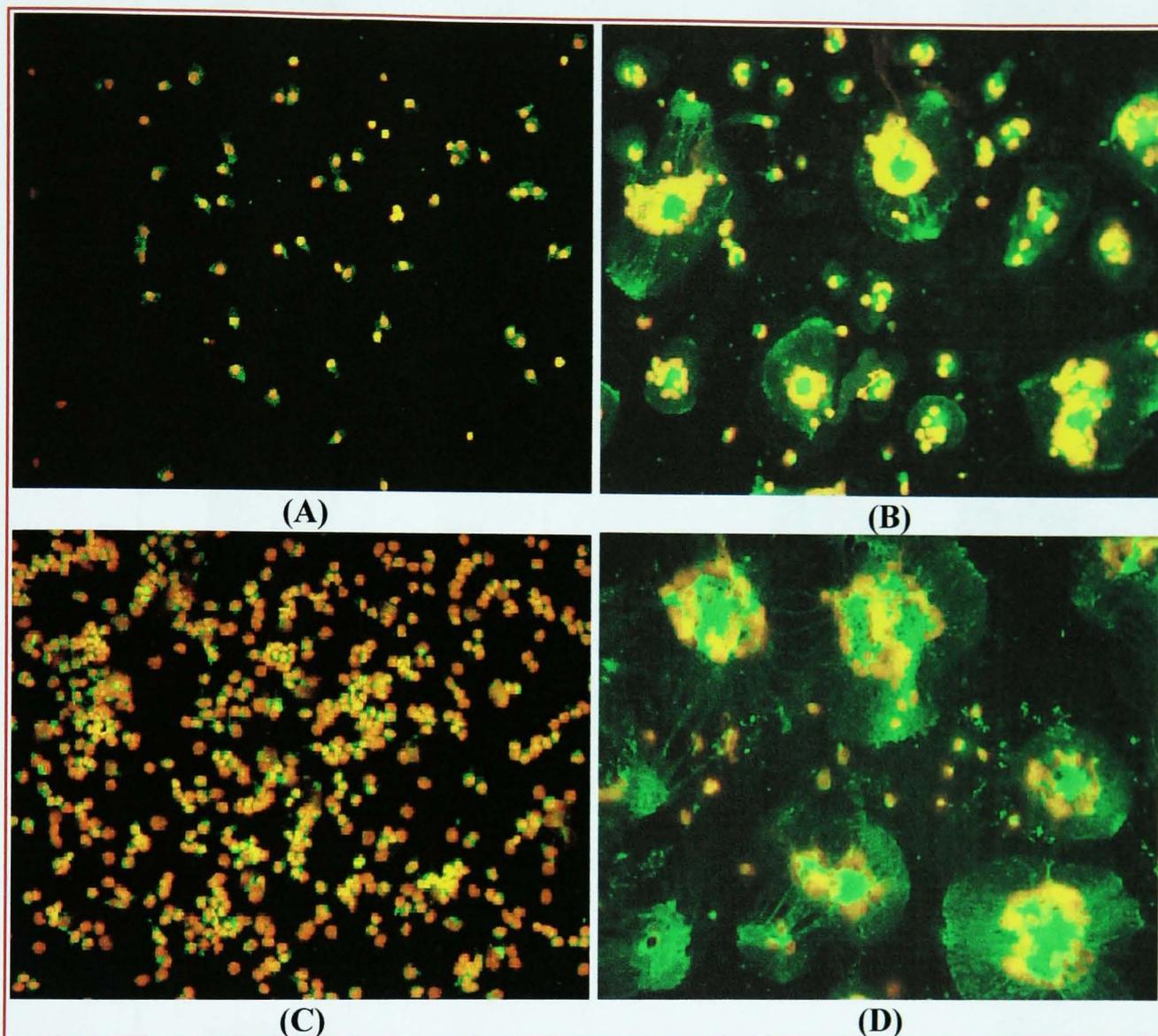


Fig. 3.2.14. Effect of LPS on unstimulated and Con A stimulated monocytes. The conditions were as described in Fig.3.2.4. Monocytes alone (A); Con A ($10\mu g.ml^{-1}$) (B); No Con A+LPS at $2\mu g.ml^{-1}$(C); Con A+ LPS at $2\mu g.ml^{-1}$. (D). The images were captured with a Nikon Eclipse E400 immunofluorescence microscope using 20x objective.

### 3.2.10. Studies on the rate of MGC formation

The rate at which the multinucleated giant cells (MGCs) are forming in response to Con A stimulation was examined at different time points (24hrs, 48hrs and 72hrs). These study showed that although optimum (i.e. largest size) MGC formation was observed after 72hrs (in agreement with previous reports (Chiozzi *et al*, 1997)), smaller MGCs were observed after 24hrs. The effects of GST-EC2 tetraspanin on the formation of MGC at these stages were examined. As shown in Fig.3.2.15 inhibition of MGC formation by GST-EC2CD63 and GST-EC2CD9 was observed from 24hrs onwards. Slight inhibition by GST-EC2CD9C153A was observed only after 72hrs.

110
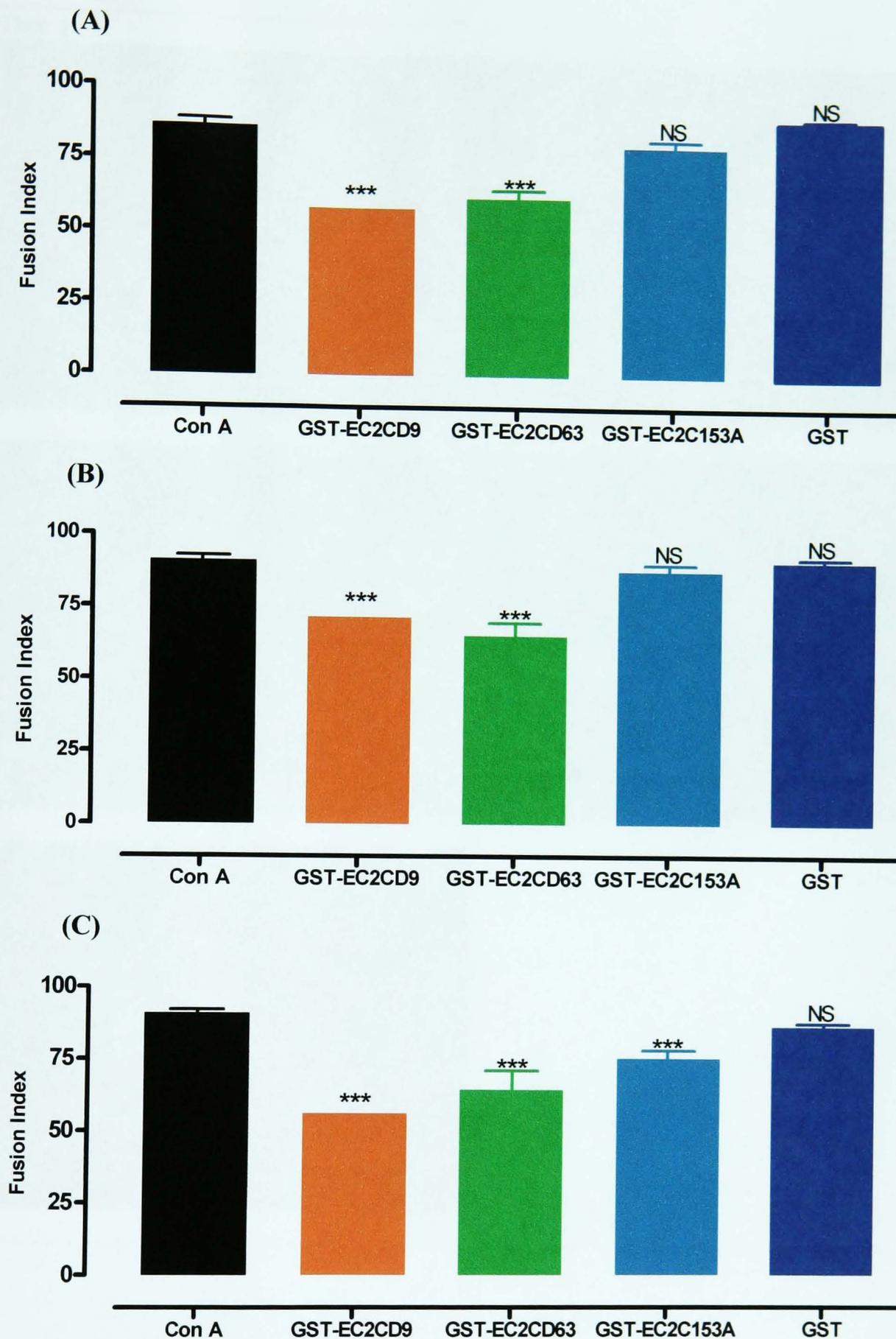
Fig.3.2.15. Effect of GST-EC2 tetraspanins on rate of MGCs formation. The experiment was carried out as described in section 2.2.10.4.1 in Lab-Tek® chamber slide using 20µg.ml⁻¹ concentration of GST-EC2 tetraspanins; fusion index was calculated on day 1, day 2 and day 3. The results are the means of 2 experiments carried out with monocytes from 2 different blood donors. The level of significance between Con A and different treatments were tested by unpaired t test, where *** Significant at P<0.0001; ** Significant at P<0.001; NS=Non significant at P<0.05.

**Day 1**



(A)

(B)

(C)

(D)

(E)

**Day 2**



(A)

(B)

(C)

(D)

(E)
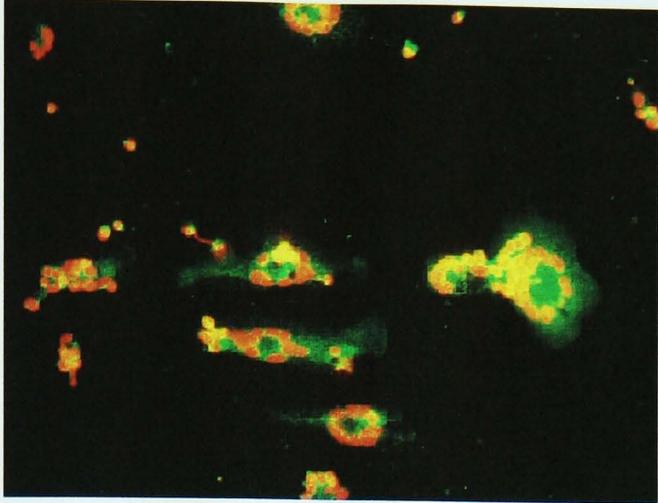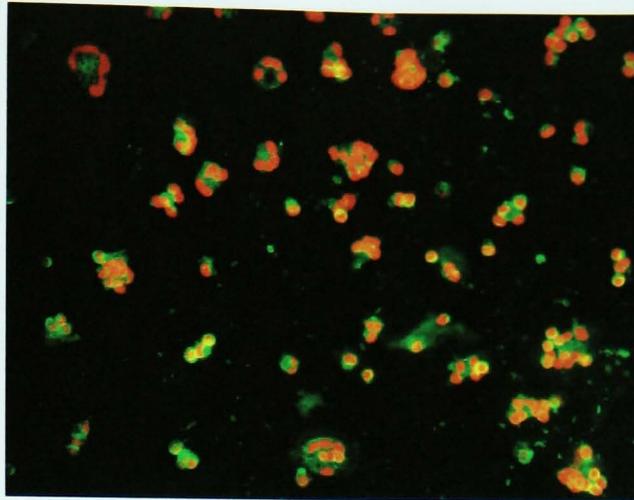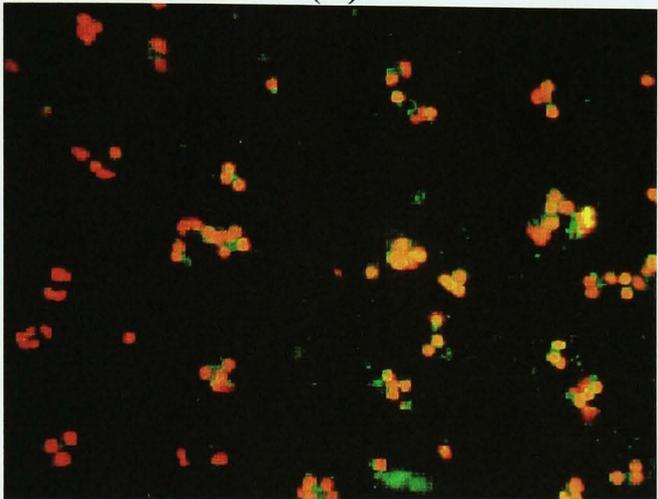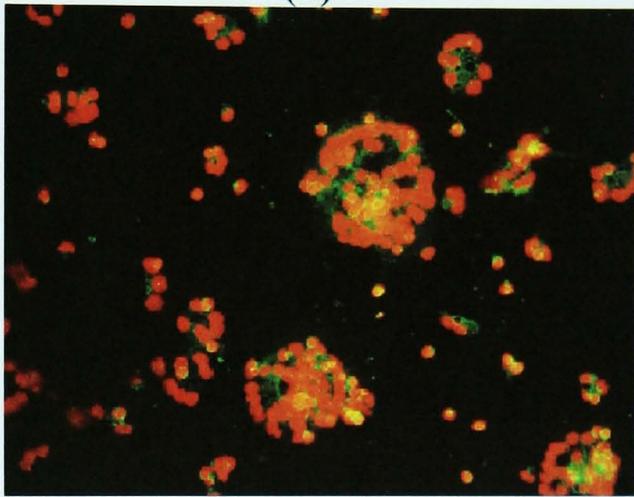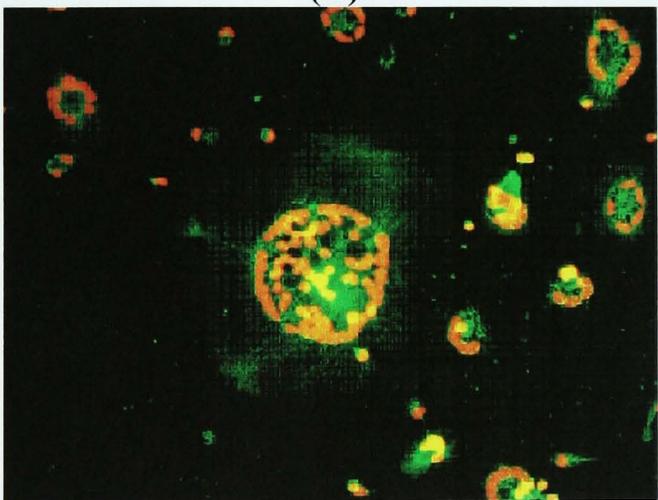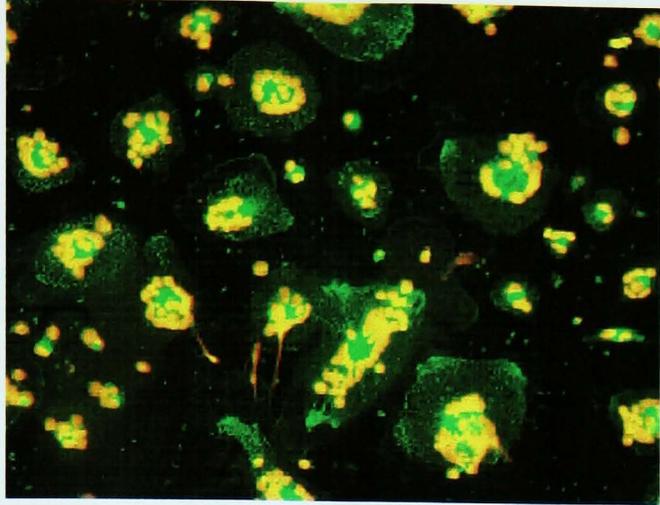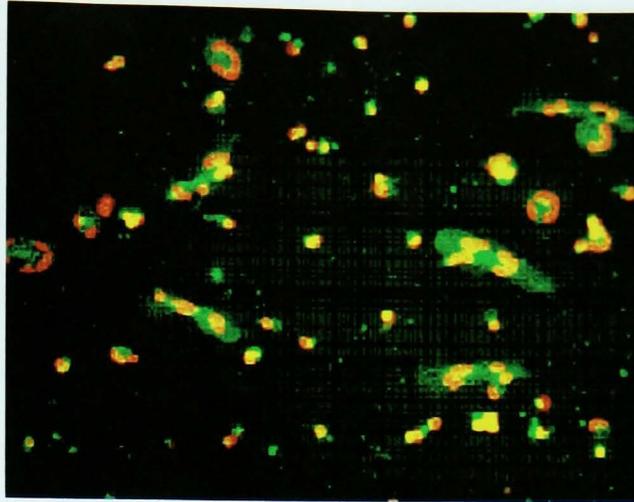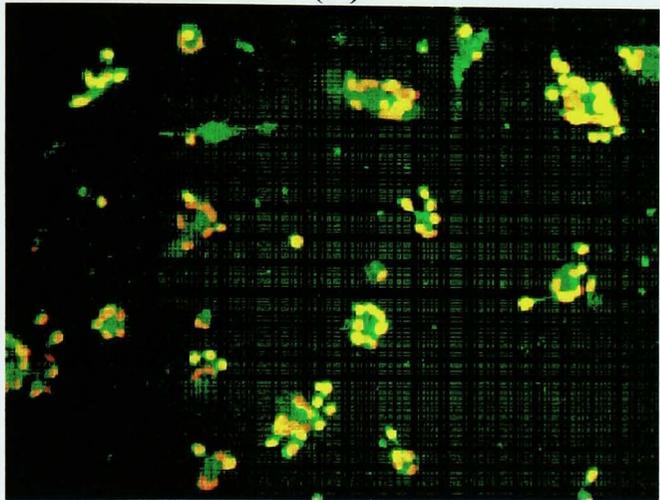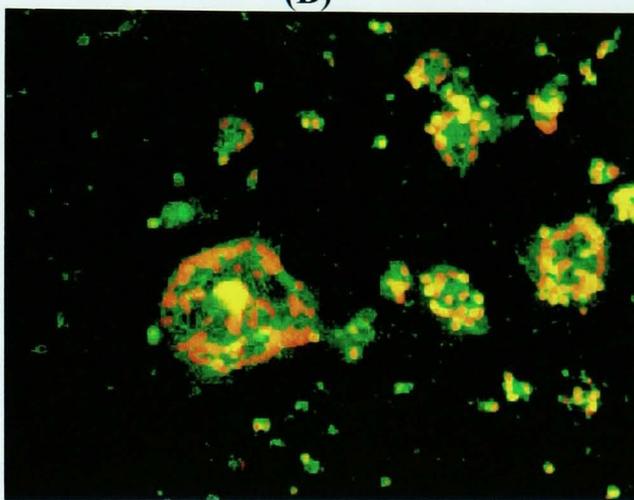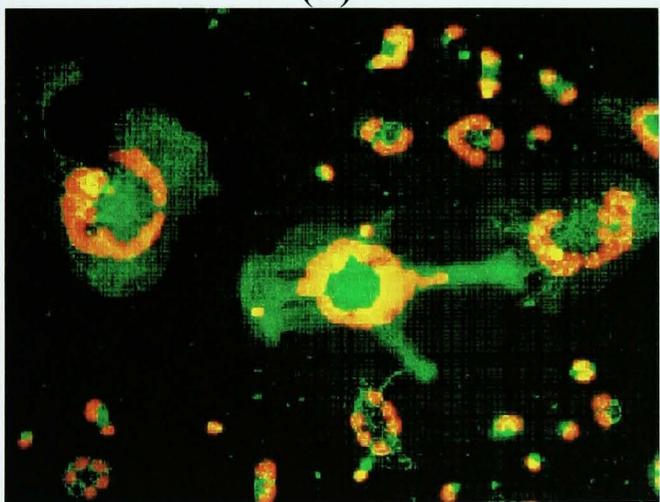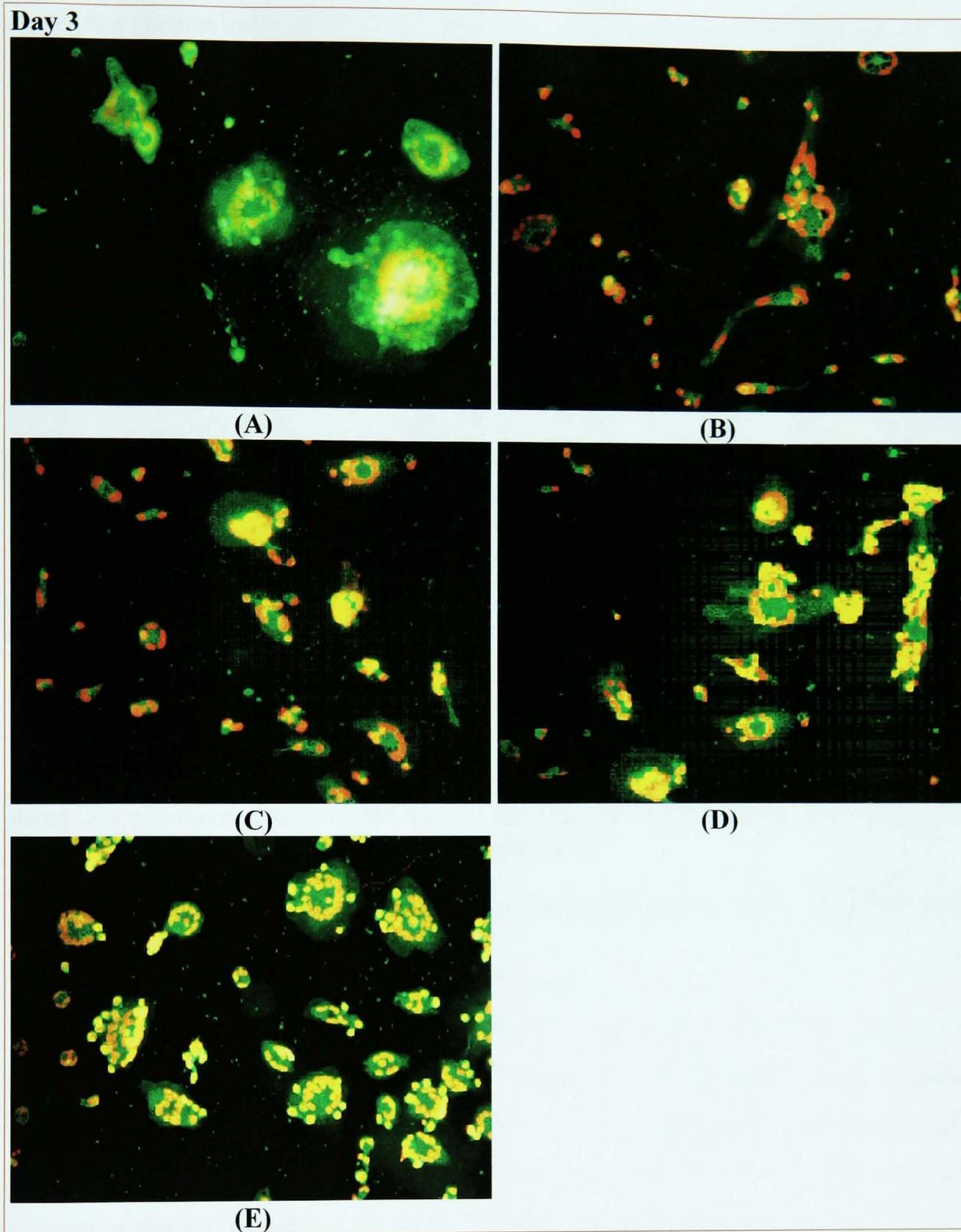
**Day 3**



Fig. 3.2.16. Effect of tetraspanins on rate of MGCs formation. The experiment was carried out as described in section 2.2.10.4.1 in Lab-Tek® chamber slide consecutively for three days: day 1, day 2 and day 3. The cells were stained with anti CD52-FITC antibody and nuclei stained with propidium iodide as described in section 2.2.10.3.2. The images were captured with Nikon Eclipse E400 immunofluorescence microscope using 20x objective. Con A alone (A); Con A+ GST-EC2CD9 (B); Con A+ GST-EC2CD63 (C); Con A+ GST-EC2C153A (D); GST (E).

Similarly the rate at which the multinucleated giant cells (MGCs) are forming in response to Con A stimulation was examined after 2hrs. 2hrs of Con A stimulation induced aggregation and there was evidence of monocyte fusion within these

aggregates (fusion index of 30-40%). The fused cells neither resembled Langhans' or FBGC types of MGCs. This suggests that some fusion occurs within a short period of time in response to Con A but these do not yet correspond in morphology to MGCs (Fig.3.2.17).



**(A)**             **(B)**
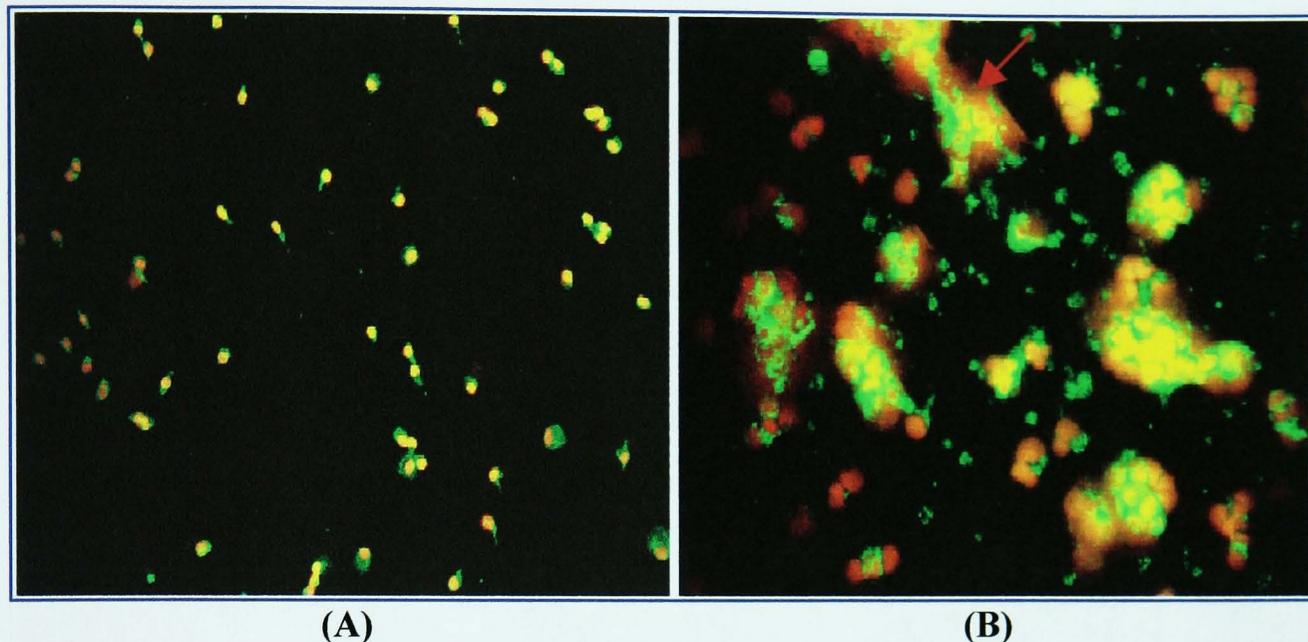
Fig.3.2.17. Effect of 2hrs Con A treatment on monocytes. The experiment was carried out as described in section 2.2.10.3.2 in Lab-Tek® chamber slide. The cells were stained with anti CD52-FITC antibody and nuclei stained with propidium iodide. The images were captured with a Nikon Eclipse E400 immunofluorescence microscope using 20x objective. Unstimulated monocytes (A); 2hrs Con A stimulated monocytes (B). An arrow represents the fusing/fused monocytes.

### 3.2.11. Studies with THP-1 cells and macrophages from CD9WT and CD9$^{-/-}$ mice

### 3.2.11.1. Stimulation of THP-1 cells with Con A

An attempt was made to stimulate the monocytic leukaemic cell line THP-1 with Con A to fuse in Lab-Tek® chamber slides pre-coated with/without poly L-lysine (50$\mu$g.ml$^{-1}$) (poly L-lysine allows THP-1 cells, which normally grow in suspension, to become adherent). THP-1 cells were incubated with Con A at different final concentrations and monitored for up to 72hrs as described in section 2.2.10.3.2. At all concentrations, Con A induced aggregation of THP-1 cells, but not fusion.

### 3.2.11.2. Stimulation of THP-1 cells with Con A conditioned medium

Previous experiments showed that Con A was not capable of inducing the fusion of THP-1 cells hence an another attempt was made to fuse THP-1 cells with Con A conditioned medium (a supernatant from monocytes and lymphocytes cultured in the presence of Con A for 2hrs or 72hrs), since Con A conditioned medium is known to be capable of inducing the fusion of monocytes (Postlethwaite *et al*, 1982; Weinberg *et al*, 1984; Most *et al*, 1990). In this assay the monocytes cultured for 24hrs, 48hrs

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and 72hrs in presence of Con A conditioned medium by replacing the whole of the culture medium. The results showed that Con A conditioned medium did not induce the fusion of THP-1 cells.

### 3.2.11.3. Studies with macrophages from CD9WT and CD9$^{-/-}$ mice

Attempts to induce the fusion of macrophages derived from CD9$^{-/-}$ (knockout) and CD9WT mouse were made (The macrophage cell lines were kindly provided by Gabriela Dveksler, Dept. Pathol. Uniformed Services University of Health Sciences, Bethesda, MD, US) (Ha *et al*, 2005). Cells were cultured in Lab-Tek$^{®}$ chamber slides with Con A at a final concentration of $10\mu g.ml^{-1}$ as described in 2.2.10.3.2 for 2hrs, 24hrs, 48hrs and 72hrs. Concentrations of cells were varied from 1-$5x10^{5}$cells.chamber$^{-1}$. However no fusion of macrophages from CD9WT and CD9KO mouse was observed. Con A caused some aggregation of macrophages of CD9$^{-/-}$ mouse but not with the cells from CD9WT mouse (Fig.3.2.18).
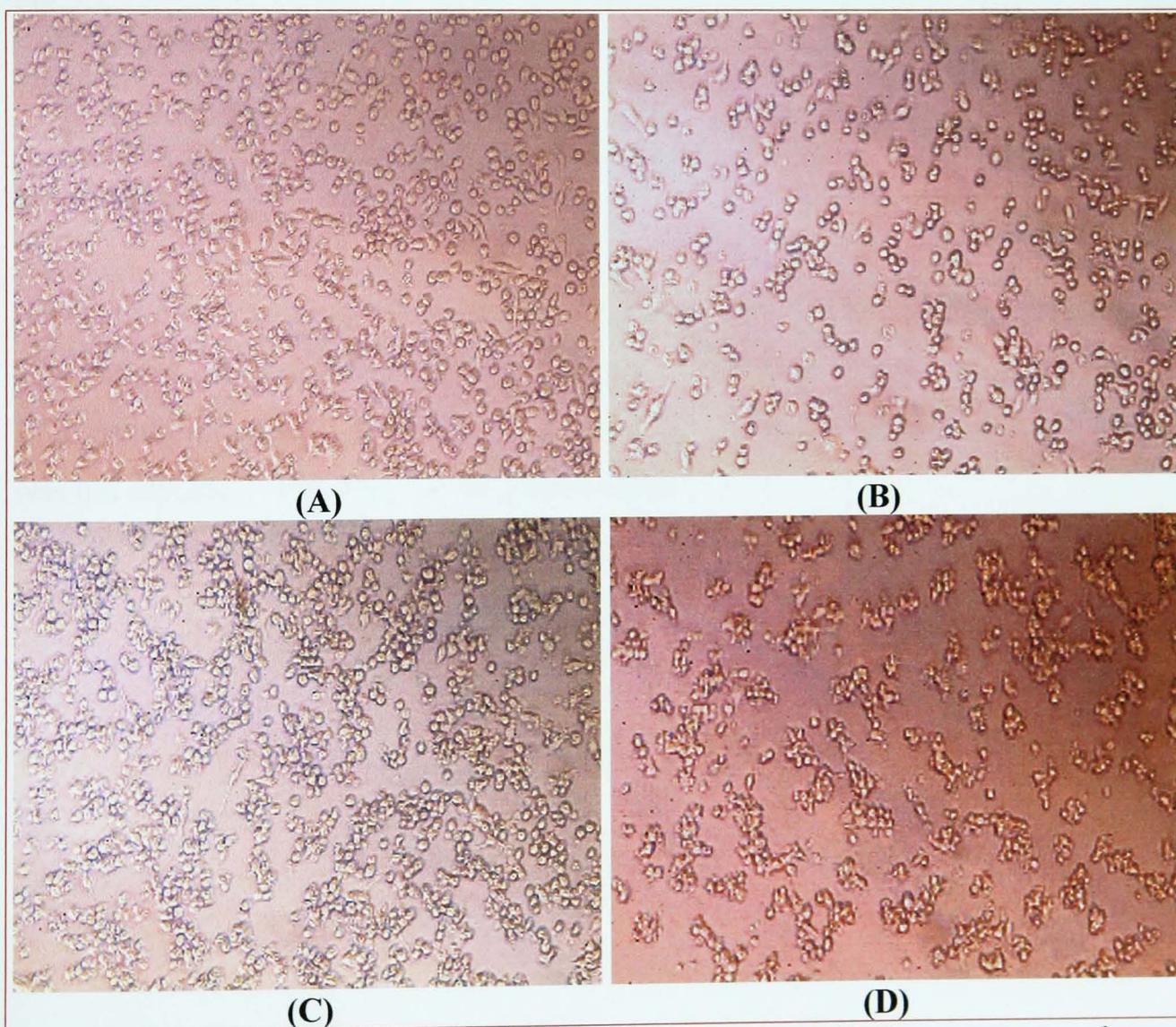


**(A)** **(B)** **(C)** **(D)**

Fig.3.2.18. Effect of Con A stimulation on CD9WT and CD9KO mouse macrophages. $2x10^{5}$ cells were seeded in to a Lab-Tek$^{®}$ chamber slides, stimulated with Con A ($10\mu g.ml^{-1}$) for 2hrs. CD9WT + No Con A (A); CD9WT + Con A (B); CD9KO + No Con A (C); CD9KO + Con A (D). The light microscopic images were captured with Nikon inverted microscope using 20x objective.

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Preliminary microscopic observation indicated that Con A might be preferentially promoting division of CD9$^{-/-}$ macrophages. Therefore the effect of Con A on proliferation of macrophages from CD9WT and CD9$^{-/-}$ mouse was assessed by SRB assay (2.2.10.5.2.1). No significant difference in cell numbers between the no Con A/Con A treated CD9WT and CD9$^{-/-}$ cells was observed.
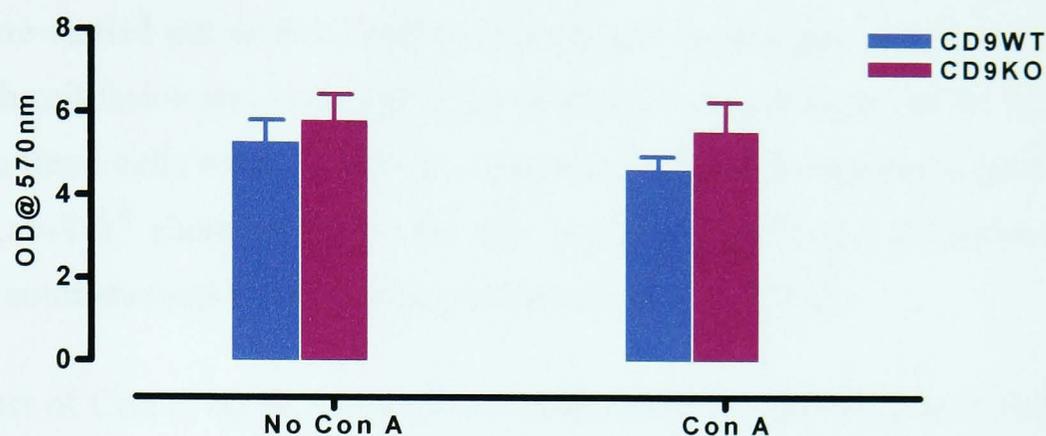


Fig.3.2.19. Effect of Con A on cell proliferation of macrophages from CD9$^{-/-}$ and CD9WT mouse by SRB assay. Cells were plated at 9 x 10$^4$ cells.well$^{-1}$ in 96 well microtitre plate, with/without Con A (final concentration of 10µg.ml$^{-1}$) for 24hrs at 37°C. The cells were fixed with TCA and SRB assay was carried out as described in section 2.2.10.5.2.1. The results are the means of 1 experiment carried out in quadruplicate.

An attempt was made to induce fusion of CD9KO and CD9WT mouse macrophages with various concentrations of TNF-α (instead of Con A) for 24hrs, 48hrs and 72hrs at 37°C as per the method described in section 2.2.10.3.2. The study showed that TNF-α was not capable of inducing the monocyte fusion.

# 3.3. DISCUSSION

## 3.3.1. Monocyte cell fusion assay

The data presented in this chapter details the role of anti tetraspanin antibodies and GST fusion proteins of EC2 tetraspanins on monocyte fusion. Initially monocyte isolation, culture, stimulation in 96 well microtitre plates and staining with Wright stain were carried out as described by Takeda and co-workers (Takeda *et al*, 2003). Although cell fusion was observed, staining of cells was not clear and the numbers of nuclei in fused cells were difficult to enumerate. Thereafter experiments were carried out in Lab-Tek® chamber slides; the cell membrane and nuclei stained with FITC labelled antibody (anti-CD52) and propidium iodide respectively.

The effect of Con A on monocytes was tested using this system. Con A induced the monocytes to become MGCs, which were mainly Langhans' type (with a circular peripheral arrangement of nuclei or horseshoe shaped arranged nuclei) although some foreign body type were also seen (Van der Rhee *et al*, 1979; McNally and Anderson *et al*, 1995; Mizuno *et al*, 2004). The variations in the type of MGCs formed might be due to variability between the experiments such as different batches of Con A or more likely, the use of monocytes from different donors. As described in chapter 1, Langhans' type and foreign type MGCs are thought to be mainly induced by Th1 and Th2 type cytokines, respectively (Anderson, 2000). Contaminating lymphocytes were certainly present and it might be expected that they would produced cytokines in response to Con A stimulation, and the type produced might influence the type of MGCs formed. It has been reported that there were highly significant inter-individual variations in the production of different cytokines (IL-1$\beta$, IL-2, TNF-$\alpha$, IL-10, INF-$\gamma$ ) when the mononuclear cells were stimulated with Con A or LPS. This suggested that the level of production of this cytokine is characteristic of an individual (Yaqoob *et al*, 1999). Similarly Whitney and co-workers found a significant gender bias in expression of IFN-GR2, the $\beta$ chain of the INF-$\gamma$ receptor complex in peripheral blood mononuclear cells, which has been implicated as a potentially important factor in determining the number of functional receptor complexes that transduce INF-$\gamma$ signals (Whitney *et al*, 2003).

In addition it was noted that Con A-induced MGCs strongly adhere to the culture surface compared with unstimulated monocytes. McNally and Anderson reported

increased expression of β1 integrins during the formation of foreign body type MGCs *in vitro*, and showed that whilst β2 integrins were important in initial adhesion of monocytes, both β1 and β2 integrins were involved in adhesion of MGCs (McNally and Anderson 2002).

As discussed in chapter 1 (1.2.4.) different researchers have suggested different mechanisms involved in Con A induced MGC formation. Con A conditioned medium i.e. supernatant of Con A stimulated peripheral blood mononuclear cells readily caused generation of MGCs (Abe *et al*, 1986; Most *et al*, 1990) and this effect was completely abrogated by monoclonal antibody to IFN-γ (Most *et al*, 1990). Purified recombinant IFN-γ from *E.coli* also produced MGCs from monocyte (Weingberg *et al*, (1984). In contrast, it was reported that MGCs could be formed by incubating highly purified monocytes with Con A alone (Takashima *et al*, 1993). In this system it was claimed that INF-γ produced by contaminating lymphocytes was not responsible for Con A induced MGC formation, since formation was not inhibited by anti- INF-γ antibodies (Takashima *et al*, 1993). Instead the authors found that TNF-α plays a central role in MGC formation, since antibodies to this cytokine caused inhibition. This was not addressed in our study, but the rate of formation and type of MGCs (mainly Langhans, containing high numbers of nuclei) were consistent with that described by Takashima and co-workers.

### 3.3.2.Effects of monoclonal antibodies

In the first instance the effect of anti CD9 and anti CD63 antibodies was tested on MGC formation. The anti CD9 antibodies Alma-1 (Azorsa *et al*, 1999) and 602.29 (Andrews *et al*, 1981) strongly enhanced the fusion of Con A stimulated monocytes and produced larger MGCs with many nuclei compared with the Con A control. By contrast, anti CD63 antibodies H5C6 (IgG) (Azorsa *et al*, 1991), 16.1, 16.5 (Skubitz *et al*, 1996), LP9 (IgM) (Smith *et al*, 1995), BEM-1 (IgA) (McCullough *et al*, 1997) and the Fab fragment of H5C6 inhibited MGC formation and produced MGCs with few nuclei. It is very interesting that the IgG, IgM and IgA anti tetraspanin antibodies showed similar inhibitory effects and the results suggest that the inhibitory effect of the anti CD63 antibody is not mediated through Fc gamma receptors. In addition, the finding that highly purified Fab fragments inhibit MGC formation demonstrates that inhibition is independent of antibody cross-linking. The Fab fragments of H5C6

appeared to show a better inhibition of fusion than the whole molecules IgG; however, the molar concentration of Fab antibody (0.4µM) is 3x higher than the whole IgG molecule (0.14µM) in these experiments. The exact mechanism in which the anti tetraspanin antibodies are affecting MGC formation is presently unknown. The results that we observed with the anti CD9 antibodies are in agreement with Takeda and co-workers (Takeda *et al*, 2003). However our studies showed that anti CD63 antibodies strongly inhibited the Con A induced MGC formation whereas they found no effect with their anti CD63 antibody. The difference in results may be due the fact that different anti-CD63 antibodies were used, that may differ potency or in the epitopes recognised. As discussed in chapter 1 (1.1.5.2.) cell surface CD63 is rapidly internalised on antibody cross-linking (Audran *et al*, 1995; Barrio *et al*, 1998). The rate of internalisation is little reduced even when monovalent Fab fragments are used (Mantegazza *et al*, 2004; G. Mal , PhD Thesis, University of Sheffield, 2005). Inhibition of MGC formation by anti-CD63 antibodies might therefore be due to internalisation of CD63 or a molecule that it interacts with.

### 3.3.3. Effects of EC2 tetraspanins

Based on the observations with anti tetraspanin antibodies, the effects of GST-EC2 tetraspanins (CD9, CD63, CD81, CD82 and CD151) on MGC formation was tested. In addition, murine CD9 EC2 and a CD9 EC2 mutant (C153A) were tested. Cysteine 153 is likely to be involved in formation of the subloop structure of CD9-EC2 (Kitadokoro *et al*, 2001) and this mutant is not recognised by conformation-dependent antibodies. The GST-EC2CD9 and GST-EC2CD63 showed a strong and highly significant inhibitory effect at 20µg.ml$^{-1}$ (0.57µM and 0.55µM respectively). No effect was observed with GST-EC2CD81, GST-EC2CD82, GST-EC2CD151 and mouse GST-EC2CD9. There is therefore some evidence of sequence specificity for CD9, since mouse CD9 shows 77% sequence identity with human in the EC2. A slight inhibitory effect was observed with the GST-EC2CD9 mutant C153A, indicating that regions outside of the variable subloop region of CD9 EC2 might be involved. GST-EC2CD9 mutant C153A also showed some inhibitory effect on the infection of macrophages by HIV-1 infection (Ho *et al*, unpublished observation), indicating that it may have some biological activity.

Takeda and co-workers also showed that GST-EC2CD9 human inhibited MGC formation, whereas GST-EC2CD9 mouse had no effect. However, they showed that anti CD81 antibodies, like anti CD9 antibodies, enhanced fusion. If, as they argued, both CD9 and CD81 were acting as negative regulators of fusion, it might be expected that GST-EC2CD81 would also inhibit. This was not the case in our studies. The GST-EC2CD81 used here was correctly folded, as determined using conformation specific antibodies. It has also been shown to have biological activity in other assays such as inhibition of HCV protein E2 binding (Higginbottom et al, 2000) and inhibition of HIV-1 infection of human monocytes (Ho et al, 2005 manuscript submitted). Our results are therefore not consistent with a negative regulatory role for CD81 in MGC formation. The enhancement of fusion observed by Takeda et al, with anti CD81 antibodies may have been due to indirect effects e.g. due to the association of CD81 with CD9 in TEMs (Takeda et al, 2003).

The effect of a short incubation with GST-EC2CD63 and GST-EC2CD9 on Con A stimulated monocyte fusion was tested. Monocytes were pre incubated with the GST-EC2 tetraspanins for 30 min. at 37°C, then the cells were washed to remove excess GST-EC2 before being cultured for 72hrs in presence of Con A. The results showed slight inhibition of MGC formation, suggesting that the tetraspanins does not need to be present throughout the incubation period. If time had permitted, it would have been useful to repeat this, removing the GST-EC2s after various time points.

Although GST alone had no effect on MGC formation, it was possible that the GST component of GST-EC2CD63 and GST-EC2CD9 might affect their biological activity. To investigate this, MGC formation assays using highly purified $His_6$ tagged EC2CD63, CD9, CD81 and GST-EC2CD63 at $20\mu g.ml^{-1}$ was carried out. In addition CD63 EC2 generated from GST-EC2CD63 by thrombin cleavage (by Francine Martin of the group) was tested. $His_6$ protein samples were obtained from collaborators Christopher Liu and Richard Hynes (Massachusetts Institute of Technology, U.S). These had been purified and analysed by reverse-phase HPLC connected to an LCQ electrospray mass spectrometer (Finnigan-MAT, San Jose, CA), which allows purification of correct conformers. The folding of the proteins was also tested by using conformation specific antibodies. $His_6$-EC2CD63 showed a highly significant (P<0.0001) inhibitory effect, which is comparable with GST-EC2CD63 but $His_6$-

EC2CD9 showed little (P<0.01) inhibition; no effect was observed with $His_6$-EC2CD81. The reduced effect with $His_6$-EC2CD9 may indicate that GST enhances the activity of EC2CD9.

Dose/response effects were investigated for GST-EC2CD63, EC2CD63 (generated by thrombin cleavage from GST-EC2CD63) and $His_6$-EC2CD63. GST-EC2CD63 and EC2CD63 gave similar levels of inhibition overall, with $IC_{50}$ values in the nanomolar range. This indicates a very potent effect on MGC formation. Interestingly, the GST-EC2CD63 has been also shown to inhibit HIV infection of macrophages at the nanomolar concentrations (P. Monk, Academic Unit of Neurology, University of Sheffield, personal communication and see chapter 5). However, the dose/response curves did highlight some variations between sets of experiments. This may be partly due to donor variation, and some individuals may be more sensitive to inhibition of MGC formation by tetraspanin EC2s. However, it is likely that there may also be variations between different batches of the same recombinant tetraspanin. Hence although correctly folded protein was checked for using conformation-sensitive antibodies, the proportion of correctly folded protein may have varied between preparations. Also some free GST was present in the preparations and the amount of this might vary between batches. As found above, free GST had no effect on MGC formation, but it might be affecting the activity of GST-EC2s. The $His_6$-EC2CD63 did not titrate out well in these experiments and it would be useful to repeat this if more $His_6$-EC2CD63 was available.

It is also interesting that complete inhibition of MGC formation was not observed, even at the higher concentrations of GST-EC2s. However, MGCs at these higher concentrations tended to be much smaller and contain fewer numbers of nuclei as seen in microscopy images (e.g. Figures 3.2.8. and 3.2.16). In retrospect, it may have also been useful here to compare the number of nuclei present in MGCs, rather than using the conventional fusion index term.

### 3.3.4. Effects of LPS in monocyte fusion

It has been reported that bacterial proteins can affect MGC formation. Muramyl dipeptide (MDP) from bacterial cell walls evokes epitheliod cell granulomas (Eishi. Y 1994) and MDP has significantly increased the MGC formation when used with conditioned medium from Concanavalin A stimulated peripheral blood monocytes

(Mizuno *et al*, 2001). In contrast Takashima and co-workers reported that in spite of the ability to activate monocytes to produce TNF-α, lipopolysacharide (LPS) inhibited MGC formation (Takashima *et al*, 1993). Since GST-EC2 tetraspanins were produced from the *E.coli,* we were concerned that LPS could be a possible contaminant present in some GST-EC2 preparations. Therefore, the effect of LPS on monocyte fusion was tested, since CD14 of monocyte acts as a receptor for LPS and induces the synthesis of TNF-α (Barclay *et al*, 1997). High concentration of LPS in the presence of Con A did not inhibit the fusion; by contrast, LPS treated cells showed a higher fusion index than untreated cells. The treatment of monocytes with LPS produced Langhans type of MGCs in presence of Con A and this effect is similar to muramyl dipeptide (Mizuno *et al*, 2001); LPS alone in the absence of Con A produced much smaller MGCs. Interestingly, LPS appeared to promote the adhesion of monocytes to the culture surface. This might be due its interaction with CD14, promoting the adhesive activity of CD11b/CD18, since these types of interaction have been observed with neutrophils (Barclay *et al*, 1997). It is concluded that the inhibitory effect shown by GST-EC2CD63 and GST-EC2CD9 on Con A induced MGC formation is not due to LPS contaminants of GST of proteins.

### 3.3.5.Rate of MGC formation

The rate of MGC formation was examined. As reported by others, although MGC formation was maximal at 72hrs, MGCs could be observed within 24hrs (Takashima *et al*, 1993; Takeda *et al*, 2003). Inhibition of MGC formation by GST-EC2CD63 and GST-EC2CD9 was observed form 24hrs onwards, showing that the proteins are active before this time.

The effect of Con A stimulation on MGCs formation was examined after 2hrs of Con A stimulation. The result showed that 2hrs. Con A stimulation induced monocyte aggregation and cell fusion, but the fused cells neither resembled Langhans' nor FBGC types of MGCs. It may be that the formation of MGCs with typical morphology takes longer, or that more time is needed for the concentration of cytokines involved in cell fusion to increase.

### 3.3.6. Studies with monocyte cell lines

Since it was difficult always to get a good supply of fresh human monocytes from volunteers, some attempts were made to see if MGC formation could be induced from

cell lines. The THP-1 cell line was chosen, since it is thought to have the most mature phenotype of commonly available monocyte cell lines (Auwerx *et al*, 1991). Con A alone induced aggregation of THP-1 cells, but no fusion. Fusion was also not induced with Con A conditioned medium, which is presumed to contain cytokines that induce fusion (Takashima *et al*, 1993; Most *et al*, 1990; Mizuno *et al*, 2000). It may be that THP-1 cells lack components of the fusion mechanism (e.g. cell surface fusion receptors), or require different stimulants to blood monocytes.

An attempt was made to induce fusion in a macrophage cell lines derived from CD9$^{-/-}$ (knockout) and CD9WT mice. These cell lines were used successfully by Dr.Dveksler to study the functional properties of pregnancy specific glycoproteins (PSGs) (Ha *et al*, 2005). No fusion of macrophages from CD9 WT and CD9$^{-/-}$ mouse was observed with Con A, but some aggregation of CD9$^{-/-}$ cells but not CD9 WT was observed. Since TNF-α is reported to promote MGC formation (Takashima *et al*, 1993), the effects of this cytokine were also investigated, but no fusion was observed. Takeda *et al*, (2003) reported enhanced fusion *in vitro* with macrophages from CD9$^{-/-}$ mice. However, they used freshly isolated alveolar and bone marrow macrophages and stimulated them with 1α25(OH)$_2$D$_3$ and splenocyte conditioned medium. If different agents had been tested it may have been possible to induce fusion of the CD9$^{-/-}$ macrophage cell line *in vitro*.

Overall, our results with anti CD9 antibodies enhancing MGC formation of Con A stimulated monocytes and EC2CD9 inhibiting are in agreement with Takeda and co-workers. They are consistent with their hypothesis that CD9 normally plays a negative role in monocyte fusion (Takeda *et al*, 2003). In contrast, we found an inhibitory effect with anti CD63 antibodies and EC2CD63 on Con A stimulated monocytes. This suggests a positive role for CD63 in MGC formation. The inhibition of MGC formation seen with the EC2 regions indicates that they may interact directly with a type of receptor that is involved in controlling fusion. However it is also possible that the recombinant EC2 proteins may be affecting processes in MGC formation that precede fusion such as cell adhesion, aggregation etc. Investigations into this form the basis of experiments described in the next chapter.

# CHAPTER 4

# EFFECTS OF EC2 TETRASPANINS ON FUNCTIONS RELATED TO MONOCYTE FUSION

## 4.1. INTRODUCTION

This chapter explores the possible involvement of tetraspanins in functions related to fusion. The results described in the previous chapter indicated that tetraspanin CD63 and CD9 play a role in MGC formation. At this stage, however, it was not clear whether the tetraspanins were involved in the monocyte fusion mechanism, or events that precede this. In order for MGC formation to occur, monocytes must be adherent, and able to come together to reach a "critical density" (cell aggregation) (Vignery, 2000). The experiments described in this chapter therefore attempted to investigate whether the GST-EC2 tetraspanins might be affecting these functions.

### 4.1.1. Cell adhesion

Cell adhesion is an important step involved in monocyte mediated MGCs formation (McNally *et al*, 1995; McNally *et al*, 2002; Brodbeck *et al*, 2002). Adhesion of monocytes to glass/plastic surfaces *in vitro* is believed to be due to interactions of monocytes receptors with proteins, such as ECM components, that have been adsorbed to the surfaces (McNally and Anderson, 1994; Jenney and Anderson, 1999). Adhesion to ECM proteins is mediated predominantly by integrins. For monocytes, the $\beta 1$ (CD29) integrin family are known to be involved in attachment to ECM proteins: $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (collagens and laminins), $\alpha 4\beta 1$ (fibronectin), $\alpha 5\beta 1$ (fibronectin) and $\alpha 6\beta 1$ (laminins) (de Fougerolles and Koteliansky, 2002). The last three integrins are well expressed by monocytes, whereas $\alpha 1\beta 1$ is induced on activation (de Fougerolles and Koteliansky, 2002). Monocytes also express $\beta 2$ integrins, $\alpha M\beta 2$ (CD11b/CD18) and $\alpha X\beta 2$ (CD11c/CD18) that are reported to interact with a wide range of ligands including C3bi and fibrinogen (Barclay *et al*, 1997) that may adsorb to the surfaces from serum (McNally and Anderson, 2002). Boissy and co-workers reported that *αvβ3* (CD51 and CD61) is a key integrin expressed by monocytes, which is mediating adhesion of multinucleated osteoclasts during bone resorption and stimulation (Boissy *et al*, 1998).

CD9 is reported to associate with all of the β1 integrins described above and associations of CD63 with α3β1, α4β1 and α6β1 have been observed (see chapter 1, 1.1.4.2; reviewed in Berditchevski, 2001; Tarrant *et al*, 2003). There is evidence that CD63 associates with β2 integrins on leukocytes (Skubitz *et al*, 1996; Mantegazza *et al*, 2004). Whilst tetraspanins are generally not thought to be involved in static cell adhesion mediated by integrins, there is evidence that they affect cell spreading, morphology and integrin-dependent migration (Hemler, 2003). The involvement of tetraspanins in adhesion-dependent signalling might be linked with their ability to recruit certain signalling enzymes into the integrin complexes (see chapter 1, 1.1.4.3; Berditchevski, 2001).

McNally and Anderson have investigated monocyte membrane proteins that mediate adhesion during the formation of FBGC *in vitro*. In their system, adherent monocytes are allowed to differentiate in culture for 3 days, and then FBGC formation is induced by addition of IL-4 (McNally and Anderson, 2002). Using specific antibodies, they demonstrated that initially adhesion of monocytes to culture surfaces (within 1hr of plating) was specifically inhibited by anti β2 integrin antibodies, but not by anti β1 integrin antibodies. However, macrophage/FBGC adhesion following IL-4 treatment was reduced by both anti β2 and anti β1 antibodies. Whilst anti β2 antibodies also appeared to inhibit FBGC formation, this was not the case for anti β2 antibodies. The authors concluded that whilst initial monocyte adhesion was strongly dependent on β2 integrins, the β1 and β2 integrins play a co-operative role in cell adhesion during macrophage fusion and FBGC formation (McNally and Anderson, 2002). Consistent with this, they observed expression of β2 integrins on both initially adherent monocytes and macrophage/FBGCs, whereas β1 staining was not observed initially but increased during macrophage differentiation and fusion. In fusing macrophages, β1 integrins were observed throughout the cytoplasm. In multinucleated FBGCs, β1 integrins were observed to be most concentrated in central cytoplasmic areas (McNally *et al*, 2002).

It is not known whether β1 and β2 integrins play similar roles in the adhesion and formation of Langhans' giant cells. However, Takeda and co-workers found that antibodies to β1 and β2 integrins inhibited the adhesion of monocytes induced with Con A. Anti β2 antibodies were also shown to inhibit MGC formation of monocytes cultured with IFN-γ containing mononuclear cell supernatants (Most *et al*, 1990;

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Gasser and Most, 1999). However, this may have been due to inhibition of cell aggregation, since anti ICAM1 antibodies were also inhibitory to some extent (Most *et al*, 1990).

### 4.1.2. Cell proliferation/cytotoxicity

In order to fuse, monocytes must also reach a "critical cell density" (Vignery, 2003). Since monocytes are not thought to undergo proliferation *in vitro* (Weinberg *et al*, 1984) this is likely to be achieved by migration of the plated cells to form aggregates. However, in the Con A induction system used here, it is likely that contaminating T lymphocytes will divide in response to the mitogen (Baral *et al*, 2004; Rashid *et al*, 2005) and that this may be critical in generating sufficient cytokines for MGC formation. The possibility that the recombinant proteins were having cytotoxic effect was also considered. Hence the effect of GST-EC2 tetraspanin on monocyte adhesion and cell numbers or cytotoxic effects was measured by SRB (Sulforhodamine B) assay. The SRB assay is a rapid, highly sensitive and inexpensive method of measuring the cell numbers in 96 well microtitre plate as a function of protein content of cells using a spectrophotometer by measuring the OD@570nm. The relationship of cell protein content is linear with cell number over a broad range of cell densities (Skehan *et al*, 1990). The proliferation/cytotoxicity assay was not carried out using pure populations of monocytes since the monocyte preparations always contained contaminating lymphocytes, hence the Con A may affect the cell numbers of lymphocytes.

### 4.1.3. Cell migration and aggregation

There are numerous reports that tetraspanins are involved in the motility of various cells types (see chapter 1, 1.1.5.1; reviewed by Berditchevsky, 2001). Most recently Mantegazza and co workers reported that mAb directed to CD9, CD63, CD81 and CD82 increased the chemokine-induced migration of immature dendritic cells (Mantegazza *et al*, 2004). Similarly, there is a great deal of evidence that tetraspanins play a role in homotypic or heterotypic cell:cell interactions (Berditchevski, 2001; Yanez-Mo *et al*, 2001). Whilst Takeda and co-workers reported that their anti tetraspanin antibodies had no inhibitory effect on monocyte aggregation in response to Con A, it was possible that the recombinant EC2 regions might affect cell:cell interactions. Masumoto and co-workers reported that antibody to CD9 significantly induced the aggregation of eosinophils (Matsumoto *et al*, 1999); anti CD9 antibodies

are also involved in aggregation and activation of platelets through interaction with Fc receptors (Worthington *et al*, 1990).

### 4.1.4. Membrane expression of tetraspanins

In the present chapter we analysed the involvement of tetraspanins and integrins on monocyte fusion by comparing their expression levels during normal and fusogenic conditions. Takeda and co-workers found that under the normal culture conditions the expression of CD9 and CD81 were up regulated, reached a peak at ~2day, and were sustained until 3 day after incubation. CD63 also appeared to be gradually up regulated. The up regulation of CD9 and CD81 was inhibited when the monocytes were cultured in the presence of Con A; in contrast to these, the up regulation of CD63 was enhanced in the presence of Con A. Takeda and co-workers further reported that during normal culture conditions, the complexes of CD9 and CD81 with integrins $\beta_1$ and $\beta_2$ were also up-regulated but during the fusogenic conditions in presence of Con A the tetraspanin-integrin complexes were down regulated (Takeda *et al*, 2003). The overall expression of $\beta_1$ and $\beta_2$ was reported to be little affected. By contrast, as mentioned previously, McNally and Anderson reported that there was little expression of $\beta_1$ integrin initially in their system, but that expression increased in culture/fusogenic conditions (McNally and Anderson, 2002). Takeda and co-workers used immunoprecipitation and immunoblotting techniques to analyse the effect of Con A on the expression pattern of tetraspanins CD9, CD63 and CD81 and its association with integrins in monocytes. Thus they were looking at total cellular expression. We used FACS analysis to try to assess the cell surface expression levels of tetraspanins and integrins.

# 4.2. RESULTS

## 4.2.1. Effect of GST-EC2 tetraspanins on monocyte adhesion: Lab-Tek® chamber slide assay

To investigate possible effects of the GST-EC2 tetraspanins on adhesion, initially their effects on adhesion of unstimulated monocytes were assessed by incubating with monocytes in Lab-Tek® chamber slides for 72hrs as described in 2.2.10.5.1.
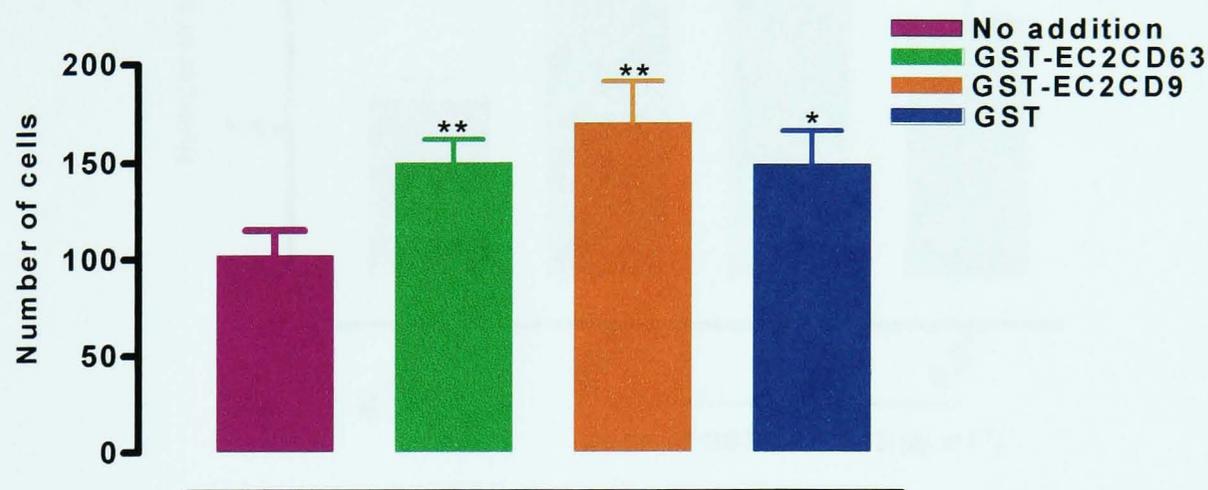


Fig.4.2.1. The effect of GST-EC2 tetraspanins on the adhesion of unstimulated monocytes cultured at $5 \times 10^5$ cells.chamber$^{-1}$ in a Lab-Tek® chamber slide with/without GST-EC2 tetraspanins/GST at $20\mu g.ml^{-1}$ for 72hrs (section 2.2.10.5.1). The slide was processed and stained with FITC labelled anti CD52 antibody as described in section 2.2.10.3.2. The numbers of cells were counted in 6 randomly chosen fields. The results are the means of 2 experiments carried out in duplicate with monocytes from 2 blood donors. The level of significance between the monocyte alone and different treatments was tested by unpaired t test, where ** Significant at $P<0.005$; * Significant at $P<0.05$. Unpaired t test showed no significant enhancement of adhesion by GST-EC2CD63 and GST-EC2CD9 when compared to GST control.

The results showed that both GST-EC2CD9 and GST-EC2CD63 did not inhibit the adhesion of monocytes. Interestingly these recombinant proteins significantly ($P<0.005$) enhanced the adhesion of monocytes compared to monocytes alone or monocytes incubated with GST ($P<0.05$). The effect of GST-EC2 tetraspanins on adhesion of Con A stimulated monocytes was not examined, since Con A induces the formation of MGCs, which are strongly adherent on Lab-Tek® chamber slides relative to normal monocytes, as described in chapter 3. Since the GST-EC2CD63 and GST-EC2CD9 inhibit the formation of the strongly adherent MGCs, the results would be biased.

We further investigated whether lower concentrations of tetraspanins affect the adhesion of monocytes by examining the dose/response effect of GST-EC2CD63 on adhesion of unstimulated monocytes.
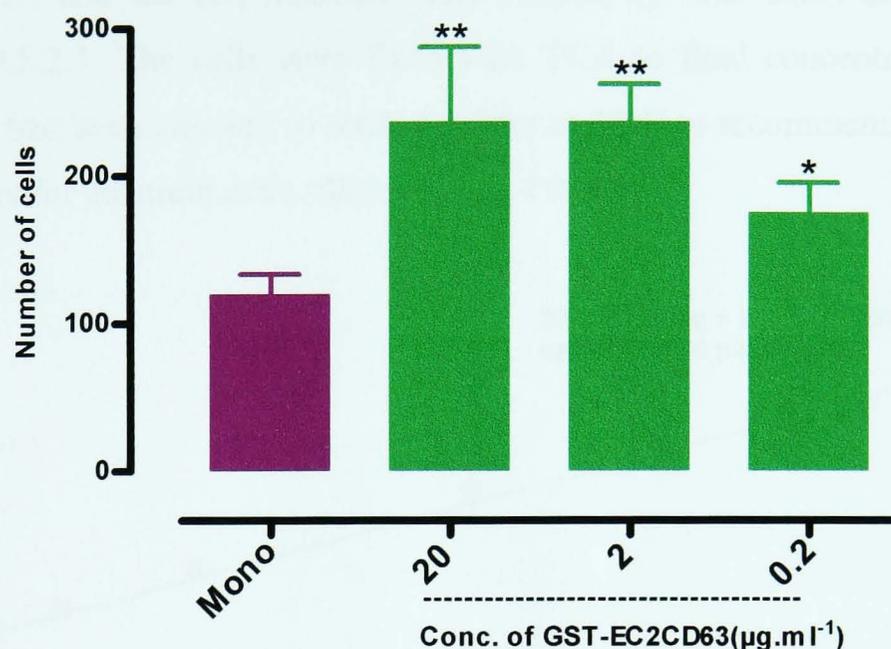


Fig.4.2.2. The dose response effect of GST-EC2 tetraspanins on the adhesion of unstimulated monocytes cultured at $5 \times 10^5$ cells.chamber$^{-1}$ in a Lab-Tek® chamber slide with/without different concentrations of GST-EC2CD63/GST ($20\mu g.ml^{-1}$) for 72 hrs (section 2.2.10.5.1), the slide was processed and stained with FITC labelled anti CD52 antibody as mentioned in section 2.2.10.3.2. The numbers of cells were counted in 6 randomly chosen fields. The results are the means of 2 experiments carried out in duplicate with monocytes from 2 blood donors. The level of significance between the monocyte alone and different treatments was tested by unpaired t test, where ** Significant at $P<0.005$; * Significant at $P<0.05$.

The results of the dose/response study with GST-EC2CD63 showed that down to $2\mu g.ml^{-1}$ GST-EC2CD63, there was still some enhancement of adhesion of unstimulated monocytes.

## 4.2.2. Titration of cell number by SRB cell stain assay

Estimating cell numbers by counting microscopically was laborious. Therefore we decided to use an alternative quantitative method, the SRB assay (section 2.2.10.5.2.1), to investigate the effect of GST-EC2 tetraspanins on overall cell numbers and monocyte adhesion. This method estimates cell number as a function of the total protein present in the wells of a microtitre plate (Skehan *et al*, 1990). Before using the SRB assay with monocytes, trial experiments were carried out with an adherent cell line (RBL2H3) and a non-adherent cell line (THP-1) to optimise experimental conditions and to check that the relationship between cell numbers and

optical density was linear. Adherent and non-adherent cells were used since we were unsure how strongly adherent the 'monocyte' population (which is likely to contain contaminating lymphocytes) would be in culture. The RBL2H3 cells were harvested (section 2.2.1.1) and the cell numbers were titrated by SRB assay as described in section 2.2.10.5.2.1. The cells were fixed with TCA to final concentration of 10% after the cells had been allowed to settle for 2hrs at 37°C as recommended by Skehan and co-workers for adherent cells (Skehan *et al*, 1990).
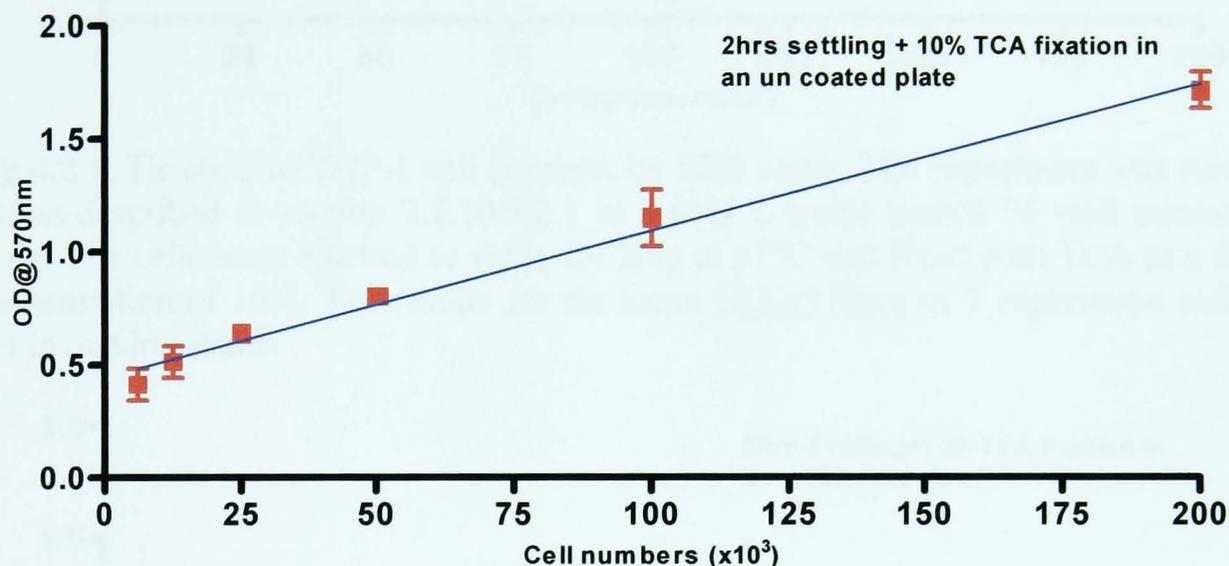


Fig.4.2.3. Titration of RBL2H3 cell numbers by SRB assay. The experiment was carried out as described in section 2.2.10.5.2.1 in an uncoated 96 well microtitre plate. The cells were allowed to settle for 2hrs at 37°C and fixed with TCA to a final concentration of 10%. The results are the mean OD@570nm of 1 experiment carried out in quadruplicate.
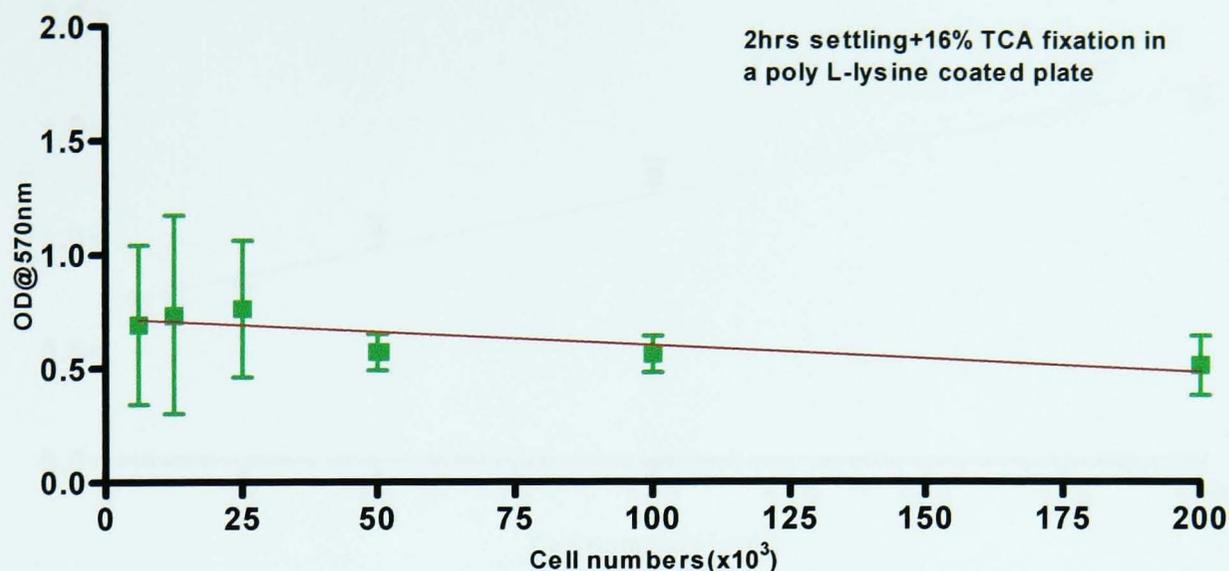
Fig.4.2.4. Titration of THP-1 cell numbers by SRB assay. The experiment was carried out as described in section 2.2.10.5.2.1 in a poly L-lysine coated 96 well microtitre plate. The cells were allowed to settle for 2hrs at 37°C and fixed with TCA to a final concentration of 16%. The results are the mean OD@570nm of 1 experiment carried out in quadruplicate.
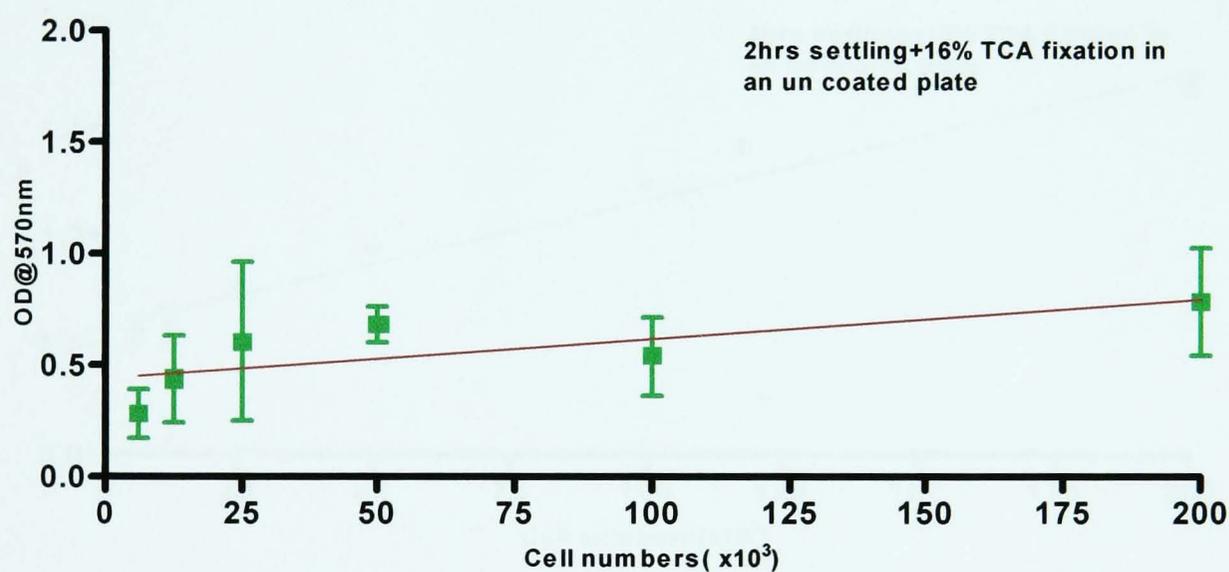


Fig.4.2.5. Titration of THP-1 cell numbers by SRB assay. The experiment was carried out as described in section 2.2.10.5.2.1 in an uncoated 96 well microtitre plate. The cells were allowed to settle for 2hrs at 37°C and fixed with TCA to a final concentration of 16%. The results are the mean OD@570nm of 1 experiment carried out in quadruplicate.
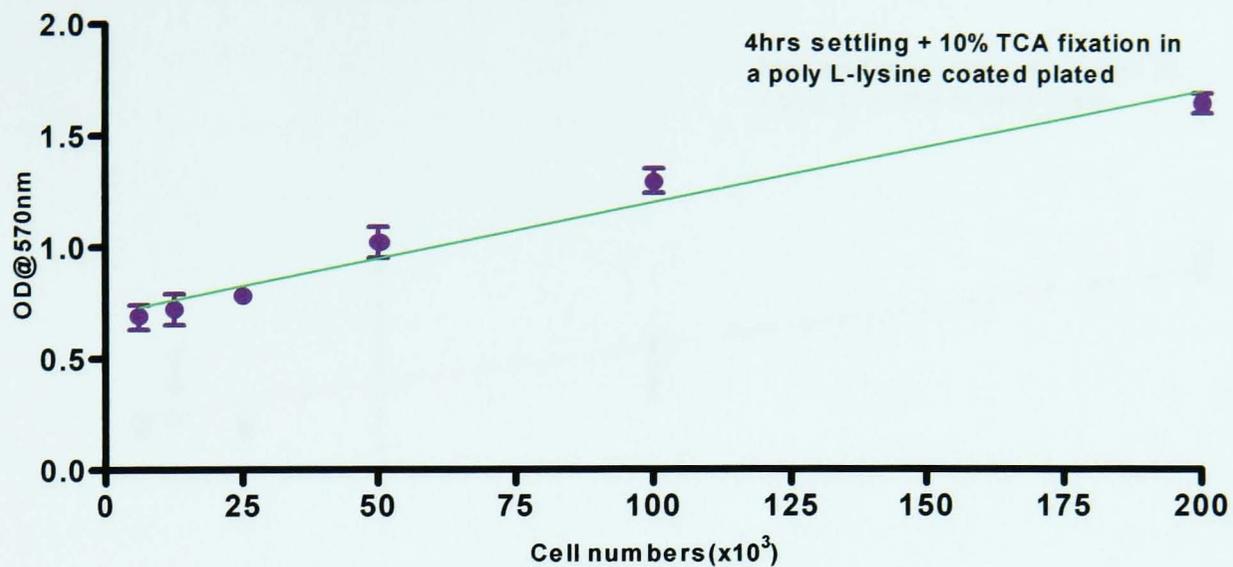
Fig.4.2.6. Titration of THP-1 cell numbers by SRB assay. The experiment was carried out as described in section 2.2.10.5.2.1 in a poly L-lysine coated 96 well microtitre plate. The cells were allowed to settle for 4hrs at 37°C and fixed with TCA to a final concentration of 10%. The results are the mean OD@570nm of 1 experiment carried out in quadruplicate.
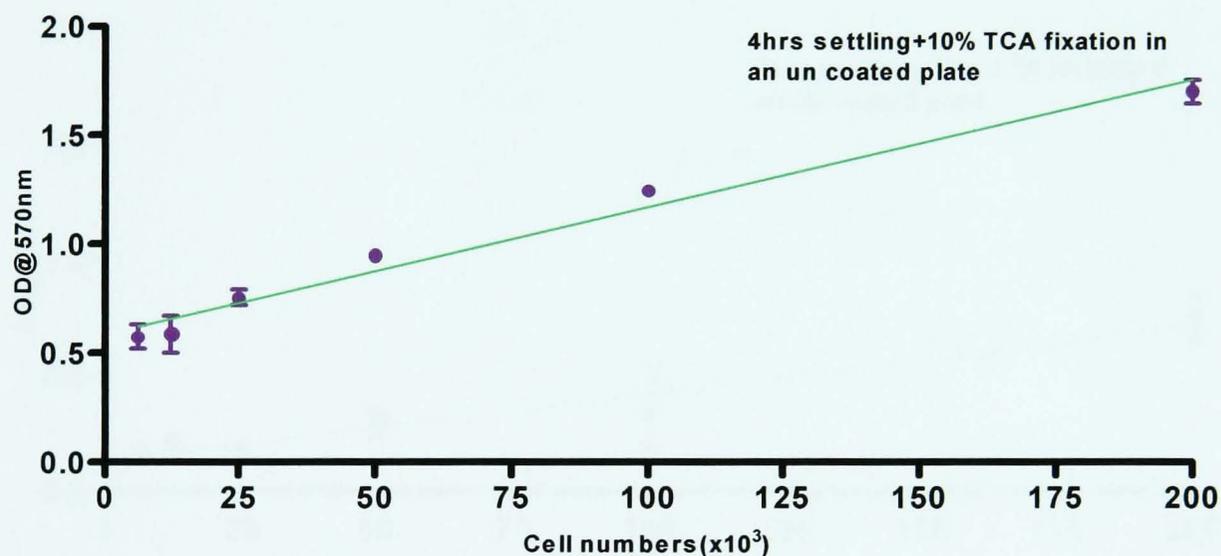


Fig.4.2.7. Titration of THP-1 cell numbers by SRB assay. The experiment was carried out as described in section 2.2.10.5.2.1 in an uncoated 96 well microtitre plate. The cells were allowed to settle for 4hrs at 37°C and fixed with TCA to a final concentration of 10%. The results are the mean OD@570nm of 1 experiment carried out in quadruplicate.
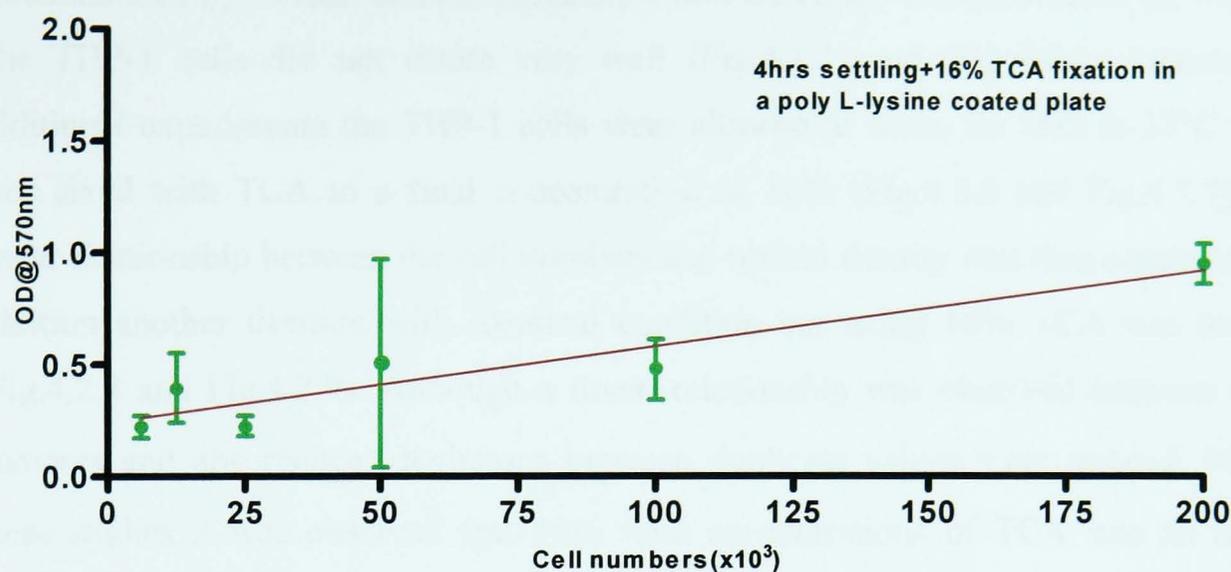
Fig.4.2.8. Titration of THP-1 cell numbers by SRB assay. The experiment was carried out as described in section 2.2.10.5.2.1 in a poly L-lysine coated 96 well microtitre plate. The cells were allowed to settle for 4hrs at 37°C and fixed with TCA to a final concentration of 16%. The results are the mean OD@570nm of 1 experiment carried out in quadruplicate.
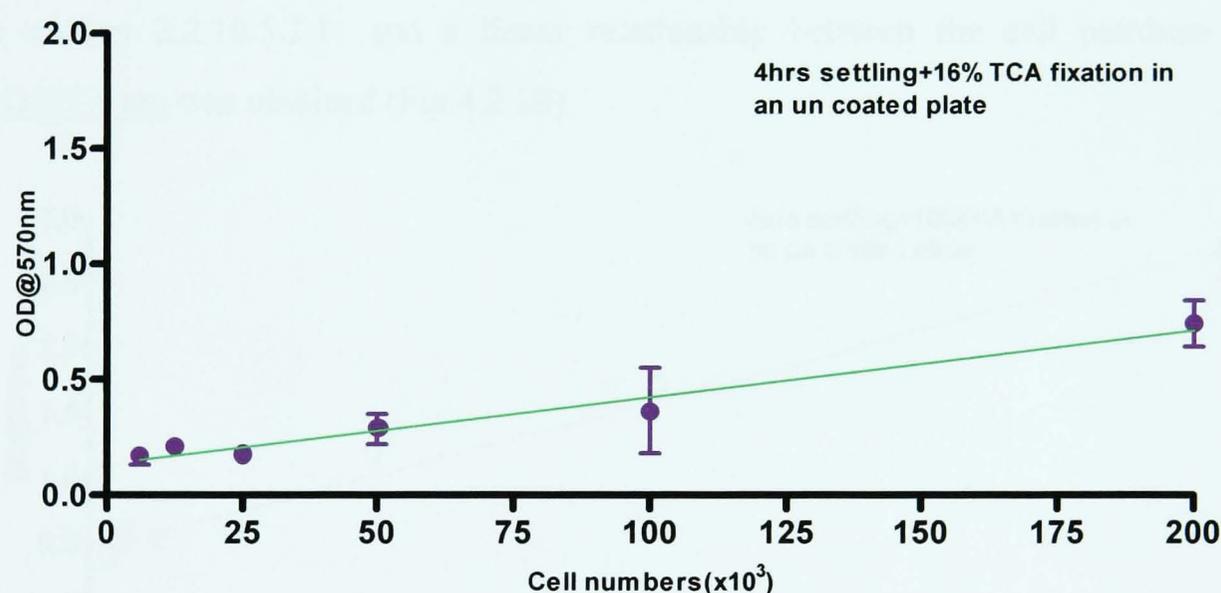


Fig.4.2.9. Titration of THP-1 cell numbers by SRB assay. The experiment was carried out as described in section 2.2.10.5.2.1 in an uncoated 96 well microtitre plate. The cells were allowed to settle for 4hrs at 37°C and fixed with TCA to a final concentration of 16%. The results are the mean OD@570nm of 1 experiment carried out in quadruplicate.

The results of SRB assay with RBL2H3 (Fig.4.2.3) cells showed a linear relationship between cell numbers and optical density. Hence an attempt was made to titrate the cell numbers of non-adherent THP-1 cells as described in section 2.2.10.5.2.1. either in poly L-lysine coated or uncoated 96 well microtitre plates. After 2hrs of incubation at 37°C the cells were fixed with TCA to a final concentration of 16% as

recommended by Skehan and co-workers for non-adherent cells (Skehan *et al*, 1990). The THP-1 cells did not titrate very well (Fig.4.2.4) and (Fig.4.2.5). Hence, in additional experiments the THP-1 cells were allowed to settle for 4hrs at 37°C and then fixed with TCA to a final concentration of 10% (Fig.4.2.6 and Fig.4.2.7). A linear relationship between the cell numbers and optical density was then obtained. In addition another titration with identical condition but using 16% TCA was tested (Fig.4.2.8 and Fig.4.2.9). Although a linear relationship was observed between cell numbers and absorbance, deviations between duplicate values were noticed. From these studies it was observed that 10% final concentrations of TCA was an ideal concentration for fixing the THP-1 cells. These results showed that the SRB assay could be used as a quantitative measure of cell number, for both adherent and non-adherent cells.

Based on these observations the cell numbers of monocytes were titrated as described in section 2.2.10.5.2.1. and a linear relationship between the cell numbers and OD@570nm was obtained (Fig.4.2.10).
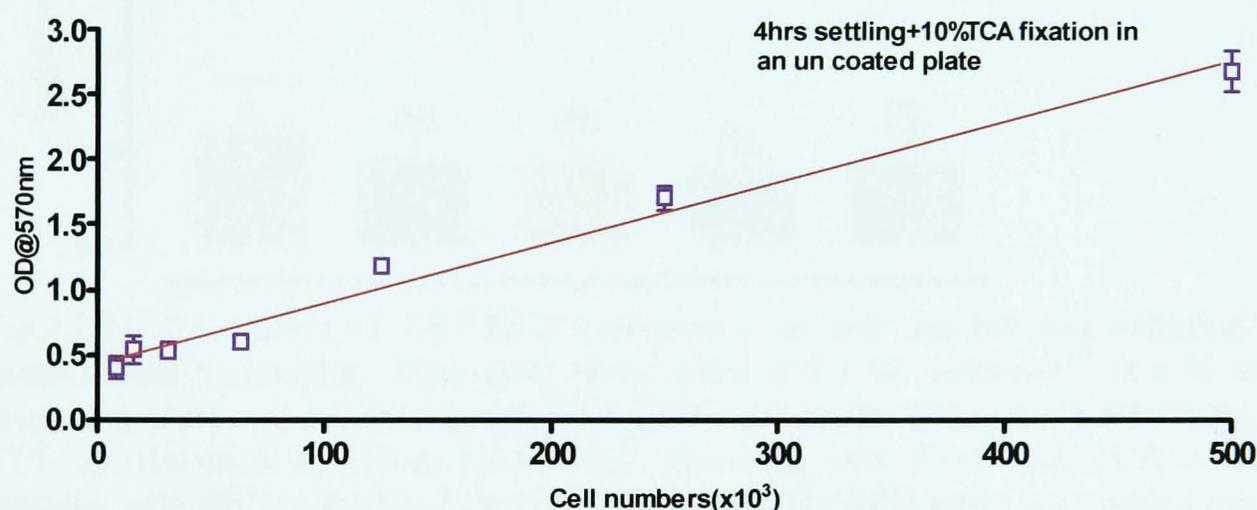


Fig.4.2.10. Titration of monocyte cell numbers by SRB assay. The experiment was carried out as described in section 2.2.10.5.2.1. The non-adherent cells from the overnight-cultured peripheral blood monocytes were removed by three washings with pre warmed RPMI and the adherent monocytes were harvested using 0.25% trypsin: EDTA solution as described in section 2.2.1.1. The cells were plated into an uncoated 96 well microtitre plate, allowed to settle for 4hrs at 37°C and fixed with TCA to a final concentration of 10%. The results are the mean OD@570nm of 1 experiment carried out in quadruplicate.

### 4.2.3. Effect of GST-EC2 tetraspanins on monocyte adhesion: SRB assay

The SRB assay was used to investigate the effect of GST-EC2 tetraspanins on overall cell numbers and monocyte adhesion. In these experiment monocytes were cultured in

96 well microtitre plates as described in 2.2.10.5.2.2 for 72hrs. After the incubation period one plate was fixed with TCA without washing and the other plate was washed 3x with BSS to remove any non-adherent cells prior to fixation (Fig.4.2.11). The SRB assay was then performed.

**(A)**



**(B)**
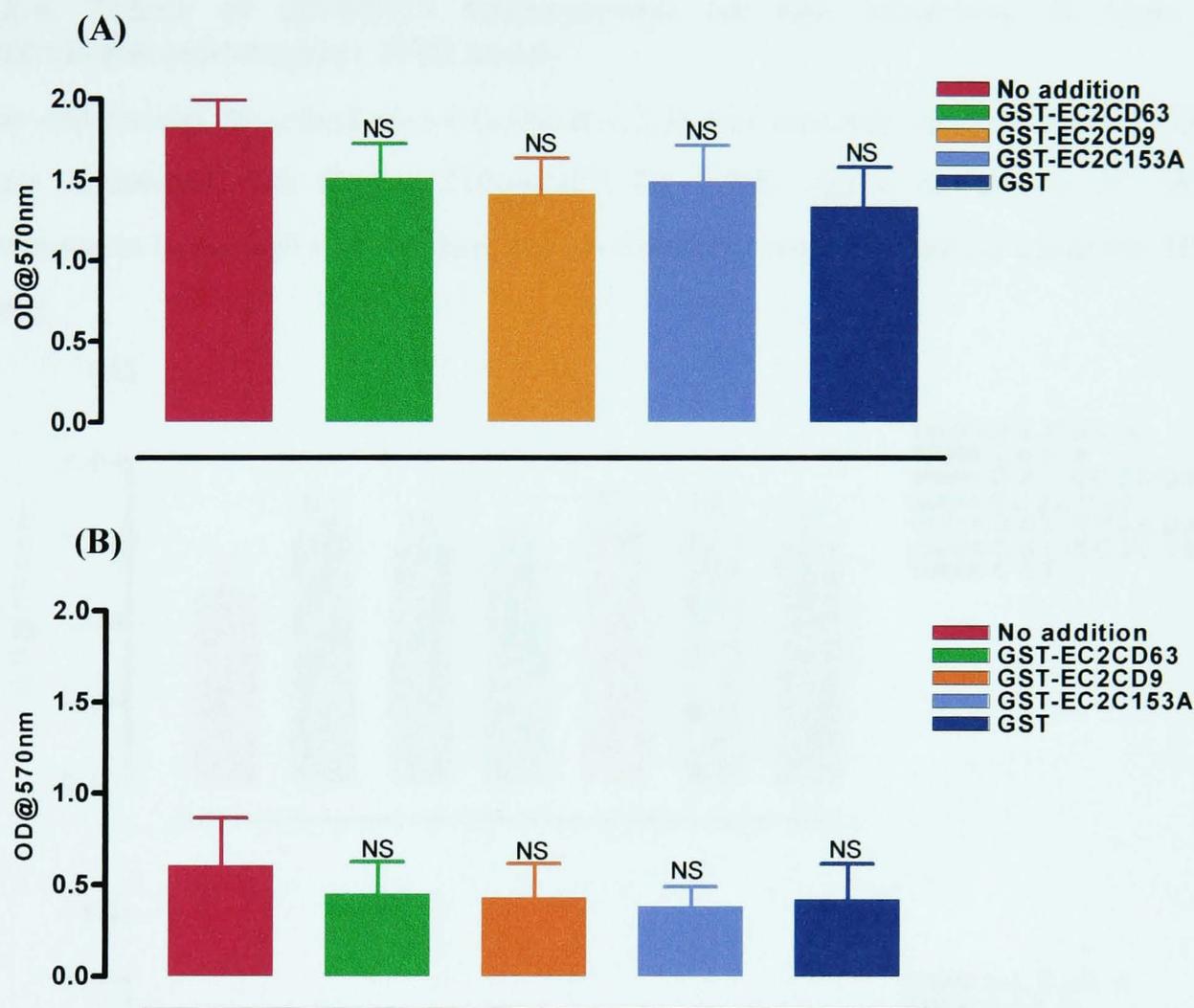


Fig.4.2.11. The effect of GST-EC2 tetraspanins on cell number and adhesion of unstimulated monocytes. Monocytes were plated at $5 \times 10^5$ cells.well$^{-1}$ in a 96 well microtitre plate and incubated with GST-EC2 tetraspanins ($20\mu g.ml^{-1}$) for 72 hrs at 37°C as described in section 2.2.10.5.2.2. The cells were fixed with TCA without washing with BSS (A); after 3x washing with BSS (B). SRB assay was carried out as described in section 2.2.10.5.2.2. The results are the mean absorbance of 3 experiments carried out in quadruplicate with monocytes from 3 different blood donors. The level of significance between the no addition control and different treatment was tested by unpaired t test, where: NS=Non significant at $P<0.05$.

The results show that GST-EC2CD63, GST-EC2CD9, GST-EC2C153A and GST did not have any significant effect on the adhesion of unstimulated monocytes. In addition, the GST-EC2 tetraspanins did not show any significant effect on monocyte cell numbers as is evident for the plate where cells were not removed by washing. This indicates that the recombinant proteins are not affecting monocyte viability or proliferation. There was around 3x reduction in overall cell numbers for the washed

plate compared to the unwashed plate, reflecting the loss of cells during washing. This indicates that a large proportion of the cells are not strongly adherent. The EC2 tetraspanins did not induce significant further loss of cells when compared to monocytes alone, but in this assay they did not enhance adhesion.

## 4.2.4. Effect of GST/EC2 tetraspanins on cell numbers of Con A stimulated monocytes: SRB assay

The experiment described above (section 4.2.3) was repeated using monocytes that were stimulated with Con A ($10\mu g.ml^{-1}$) for 72hrs. Again the effects of GST-tetraspanins on overall cell number, and on adhesion, were determined using the SRB assay.

**(A)**



**(B)**
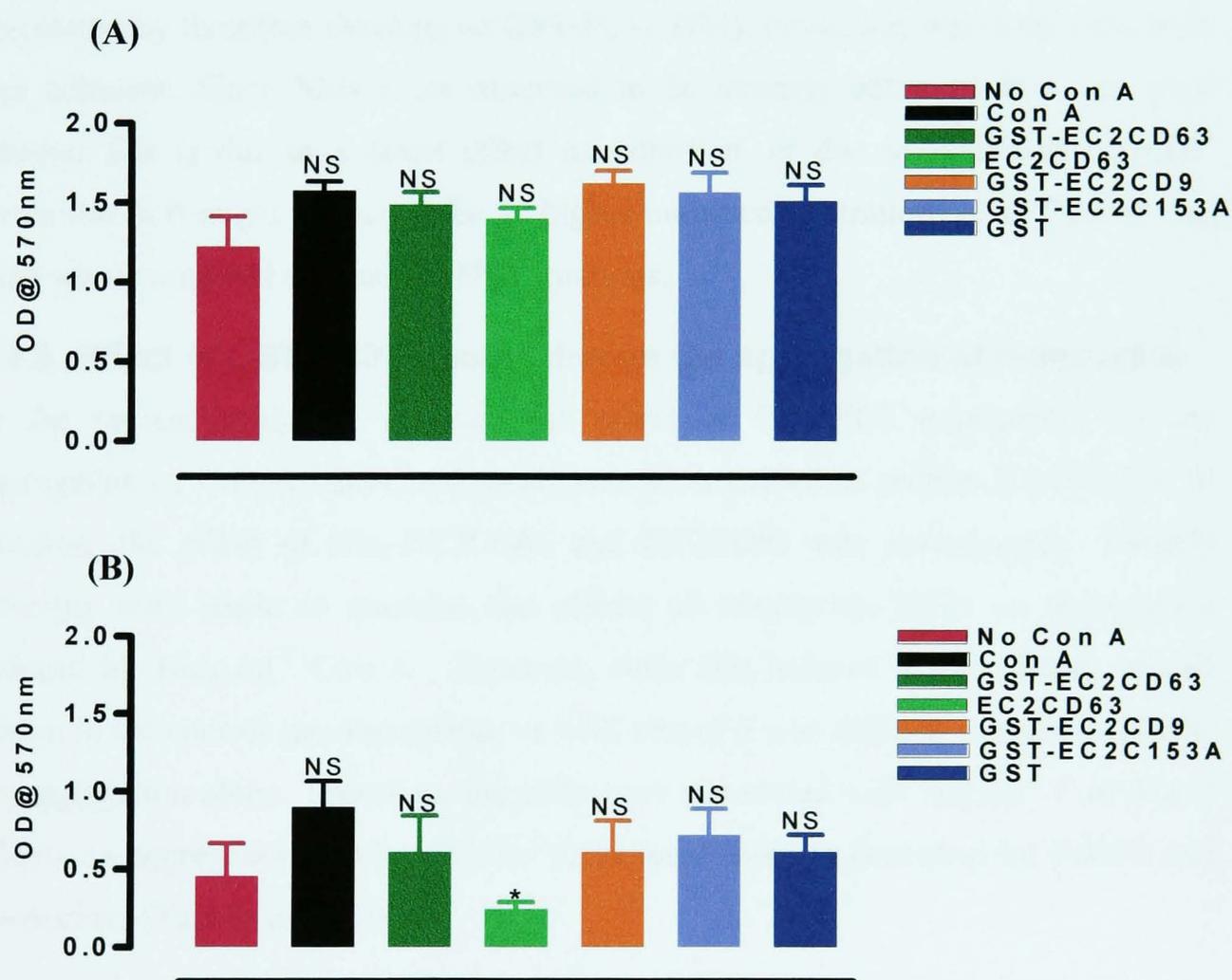


Fig. 4.2.12. Effect of GST-EC2/EC2 tetraspanins on cell number of Con A stimulated monocytes. The monocytes were stimulated with Con A ($10\mu g.ml^{-1}$) and cultured in presence/absence of GST-EC2/EC2 tetraspanins ($20\mu g.ml^{-1}$) for 72hrs at 37°C as described in section 2.2.10.5.2.2. The cells were fixed with TCA without washing (A); after 3x washing with BSS (B). The SRB assay was carried out as described in section 2.2.10.5.2.2. The results are the mean absorbance of 3 experiments carried out in quadruplicate with monocytes from 3 different blood donors. The level of significance between no Con A and Con A; Con A and different treatments was tested by unpaired t test, where * Significant at $P<0.05$; NS= Non significant at $P<0.05$.

From the results of the SRB assay where the plate was not washed, Con A does not seem to induce proliferation of monocytes significantly, although a slight increase in cell number was seen for all Con A stimulated cells. In addition, co-incubating these cells with tetraspanins does not significantly affect overall cell numbers i.e. the tetraspanins do not show any cytotoxic or anti-proliferative effect on Con A stimulated monocytes. From the results of the SRB assay where the plate was washed, Con A appeared to induce a slight increase in adhesion of monocytes but this was not significant. In addition, the GST-EC2 tetraspanins neither significantly increased nor decreased the adhesion of Con A stimulated monocytes. However, after washing, there were significantly fewer cells in wells that had been incubated with EC2CD63 (generated by thrombin cleavage of GST-EC2CD63), indicating that these cells were less adherent. Since MGCs are observed to be strongly adherent, it is not clear whether this is due to a direct effect on adhesion, or due to inhibition of MGC formation or it might be due to the 3x higher molar concentrations of EC2CD63 was used when compared to other GSTEC2 proteins.

## 4.2.5. Effect of GST-EC2 tetraspanins on the aggregation of monocytes

In the present study we assessed the effect of GST-EC2 tetraspanins on the aggregation of Con A stimulated monocytes as described in section 2.2.10.5.2.3. In addition, the effect of $His_6$-EC2CD63 and EC2CD63 was investigated. Initially attempts were made to examine the effects of tetraspanin EC2s on aggregation induced by $10\mu g.ml^{-1}$ Con A. However, since this induced a high degree of cell fusion in the control (no tetraspanin, or GST alone) it was difficult to monitor effects on aggregation alone. Therefore, the cells were stimulated with $5\mu g.ml^{-1}$ Con A and effects on aggregation monitored after 12hrs incubation, as described by Takeda and co-workers (Takeda *et al*, 2003).

The initial aggregation assays were carried out with monocytes harvested by using trypsin:EDTA. The number of aggregates with <4 cells were counted as described (Takeda *et al*, 2003), and it appeared that the tetraspanins caused some inhibition of aggregation (Fig.4.2.13). However, microscopic observation showed that the results were misleading: GST-EC2 tetraspanins in the presence of Con A ($5\mu g.ml^{-1}$) produced larger aggregates of <20 monocytes, which lead to an overall reduction in the number of small aggregates (between 4-10 monocytes) in the chosen field during the counting than Con A control. In addition, monocytes without any treatment also

showed some degree of aggregation. Therefore, this experiment was repeated with monocytes that had been harvested non-enzymatically and aggregates of 4-10 and 10-20 cells were recorded separately (Fig.4.2.14).
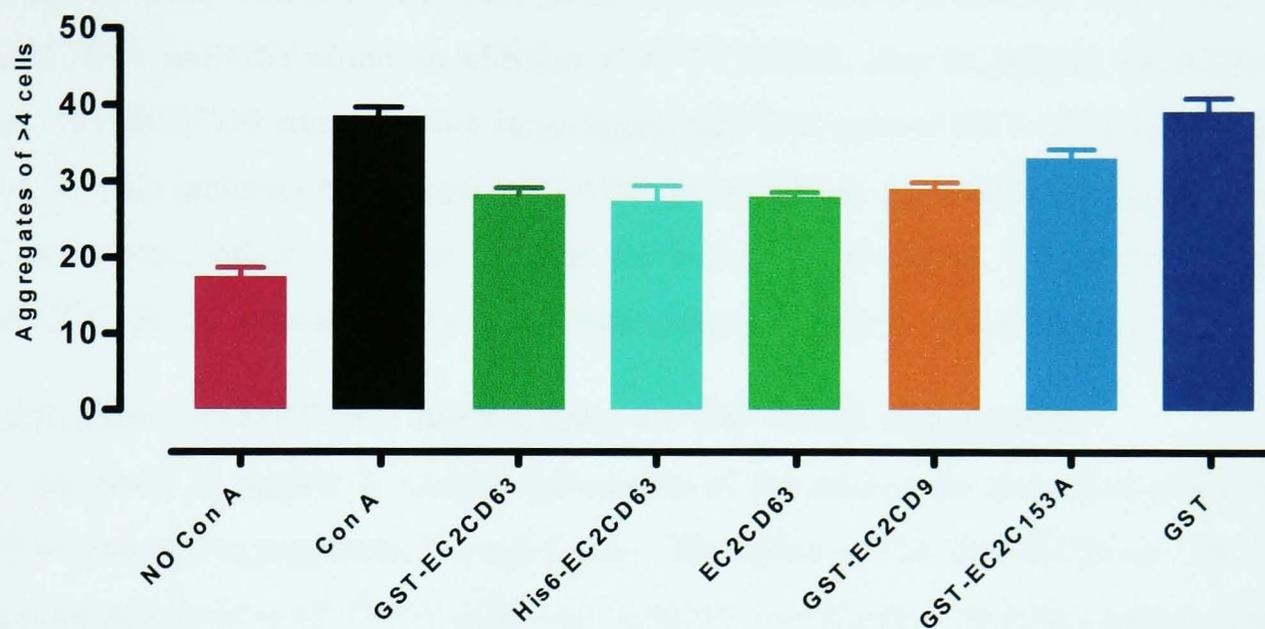


Fig.4.2.13. Effect of GST-EC2/EC2/His$_6$-EC2 tetraspanins on the aggregation of monocytes. The monocytes were harvested using trypsin: EDTA, plated at $2 \times 10^5$ cells.well$^{-1}$ in a 96 well micro titre plate and the assay was carried out as described in section 2.2.10.5.2.3. The number of aggregates with <4 monocytes were counted from 6 randomly chosen fields. The results are the mean value of 3 experiments carried out in quadruplicate using monocytes from 3 different blood donors.
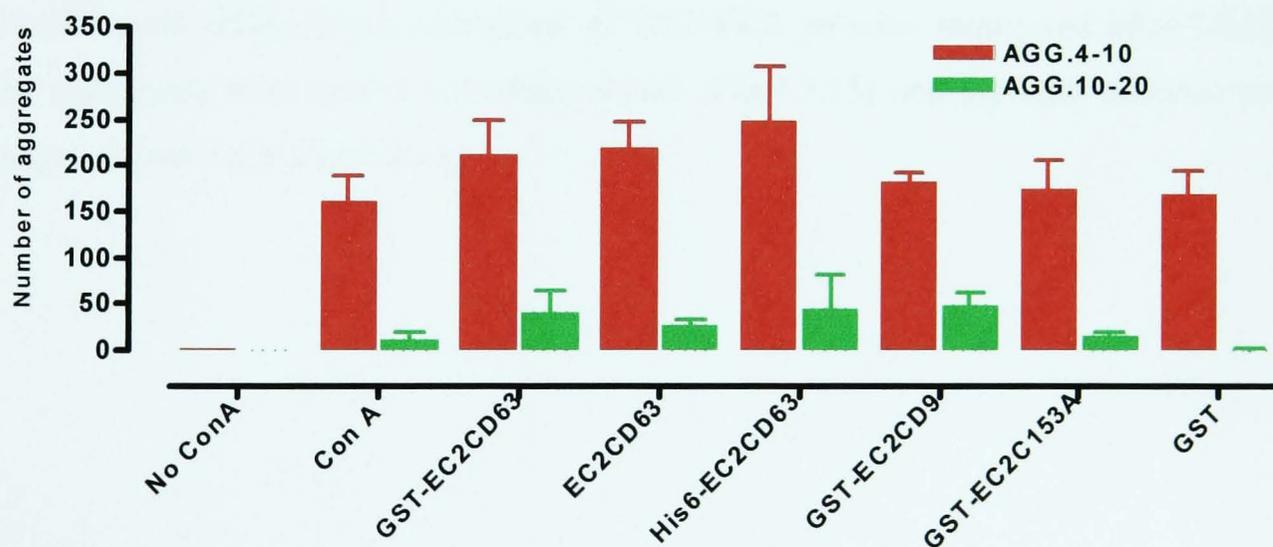


Fig.4.2.14. Effect of GST-EC2/EC2/His$_6$-EC2 tetraspanins on the aggregation of monocytes. The monocytes were harvested using cell dissociation solution, plated at $2 \times 10^5$ cells.well$^{-1}$ in a 96 well microtitre plate and the assay was carried out as described in section 2.2.10.5.2.3. The number of aggregates between 4-10 and 10-20 monocytes was counted from 6 randomly chosen fields. The results are the mean value of 1 experiment carried out in triplicate.

The results of this aggregation assay indicate that GST-EC2/EC2/His$_6$-EC2CD63 and GST-EC2CD9 increased the aggregation of Con A stimulated monocytes. In this experiment, there were slightly increased numbers of small aggregates with GST-EC2CD63, His$_6$-EC2CD63 and EC2CD63 treatments relative to GST-EC2CD9, GST-EC2C153A and GST alone. In addition, GST-EC2CD63, His$_6$-EC2CD63, EC2CD63 and GST-EC2CD9 showed more large aggregates than control GST-EC2C153A and Con A. This indicates that tetraspanins CD9 and CD63 do not inhibit the aggregation of monocytes, which is prerequisite for the fusion of monocytes. By contrast, CD9 and CD63 EC2s appear to show some enhancement of Con A induced aggregation.

## 4.2.6. Effect of GST-EC2 tetraspanin on THP-1 cell aggregation

As described in chapter 3, Con A stimulation of the monocytic leukaemic cell line THP-1 induced aggregation, but not fusion. The effect of the GST-EC2s on Con A induced aggregation of THP-1 cells was therefore investigated. In initial aggregation assays cells were incubated with 10μg.ml$^{-1}$ Con A and GST-EC2 tetraspanins (20μg.ml$^{-1}$) for 72hrs and then the number of aggregates with more than 4 cells were recorded. No clear difference was observed between the GST-EC2 tetraspanin treated cells and the controls since at 10μg.ml$^{-1}$ Con A, huge aggregates of THP-1 cells formed in all groups. A reduced concentration of Con A (5μg.ml$^{-1}$) was then used and the effects of different concentrations of GST-EC2 proteins monitored after 24hrs. The aggregates were scored according to size (Fig.4.2.15) and the light microscopic images are shown in Fig.4.2.16.
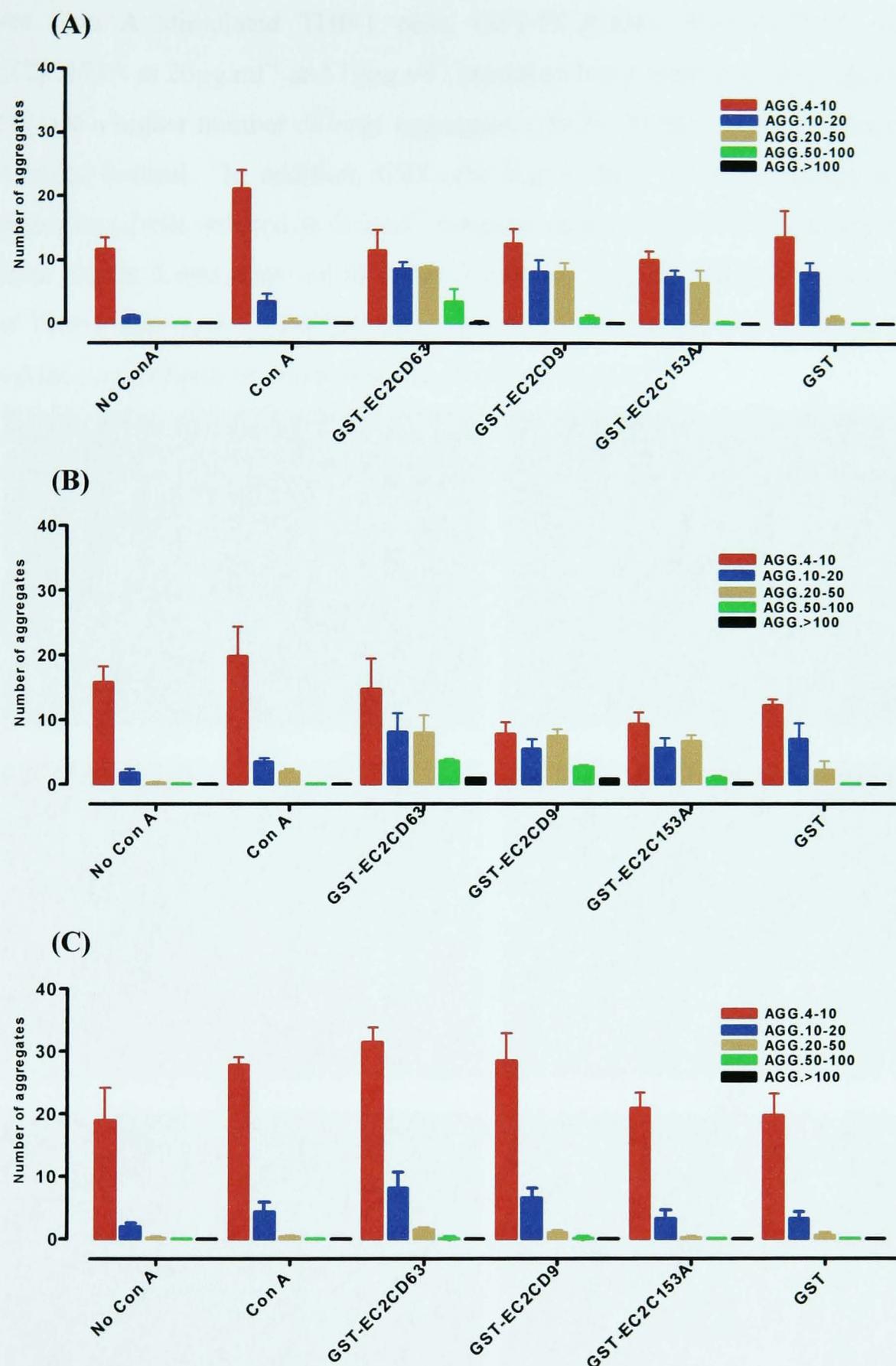
Fig.4.2.15. Effect of GST-EC2 tetraspanins on the aggregation of THP-1 cells. The cells were plated at 80,000 cells.well$^{-1}$ in a 96 well microtitre plate, incubated with Con A (5µg.ml$^{-1}$) and GST-EC2 tetraspanins/GST at 20µg.ml$^{-1}$ (A); 10µg.ml$^{-1}$ (B); 2µg.ml$^{-1}$ (C) for 24hrs at 37°C as described in section 2.2.10.5.2.4. The numbers of aggregates in different categories were counted from 6 randomly chosen fields. The results are the mean value of 1 experiment carried out in quadruplicate.

For Con A stimulated THP-1 cells, GST-EC2CD63, GST-EC2CD9 and GST-EC2C153A at 20µg.ml$^{-1}$ and 10µg.ml$^{-1}$ produced low numbers of small aggregates (4-10) and a higher number of large aggregates (10-20; 20-50 and 50-100) than the Con A alone control. In addition, GST also has a slight effect on aggregation. The aggregation was reduced at 2µg.ml$^{-1}$ concentrations of GST-EC2 tetraspanins. From these results it was observed that Con A alone at 5µg.ml$^{-1}$ induces some aggregation of THP-1 cells and GST-EC2CD63, GST-EC2CD9 and GST-EC2C153A appear to enhance aggregation at concentrations down to 10µg.ml$^{-1}$.
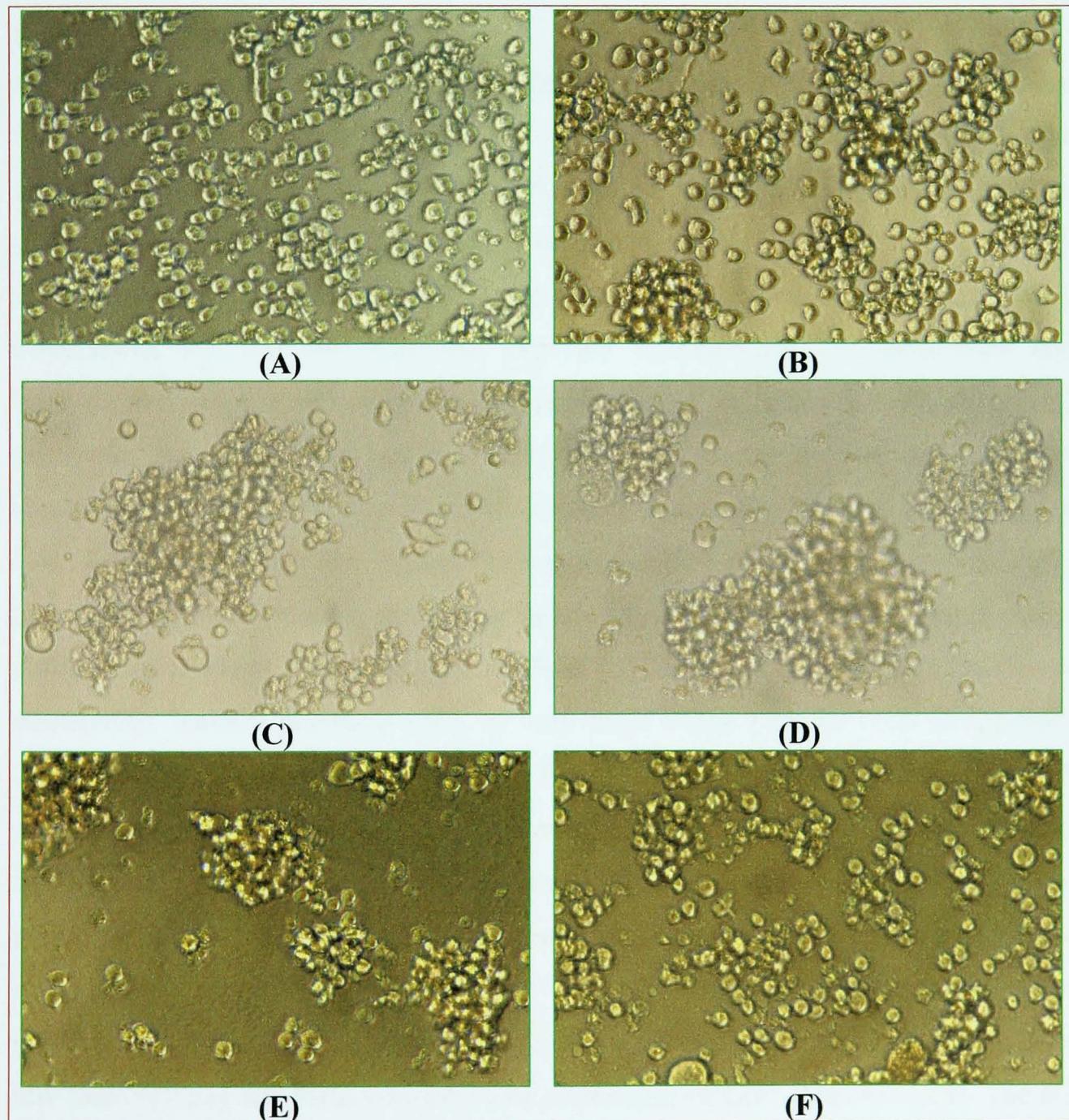


Fig. 4.2.16. Effect of GST-EC2 tetraspanins on the aggregation of THP-1 cells. The assay was carried out with GST proteins at 20µg.ml$^{-1}$ as described in section 2.2.10.5.2.4 in the presence/absence of Con A (5µg.ml$^{-1}$). No addition (A); Con A (B); GST-EC2CD63 (C); GST-EC2CD9 (D); GST-EC2C153A (E); GST (F). The light microscope images were captured with Nikon inverted microscope using 20x objective.

## 4.2.7. Effect of Con A stimulation on the expression of tetraspanins and other leucocyte proteins by monocytes

To try to further elucidate the role of tetraspanins in monocyte fusion, their expression levels on monocytes with and without Con A stimulation were examined by FACS analysis. The expression of integrins CD18 (β2 subunit), CD29 (β1 subunit), CD11b (CR3 α subunit) was also examined, along with the monocyte marker CD14 and the lymphocyte/monocyte marker CD52. The expression study was carried out by FACS analysis as discussed in section 2.2.11 using appropriate FITC labelled antibodies.

### (a) 2hrs Con A stimulation

During the FACS analysis it was noticed that there were four distinct cell populations in our preparation of unstimulated monocytes and five distinct cell populations with the Con A stimulated cells (Fig.4.2.17A and B), gateable on the basis of their granularity and size. The populations were identified using the anti CD14 and anti CD52 antibodies, and the well-known scatter profiles of monocytes and lymphocytes. Populations R1 and R4 have the expected characteristics of monocytes and lymphocytes, respectively. However the population of cells labelled R2 also stains positively for CD14, but has a slightly different scatter profile and expresses significantly more CD52 than R1. This population seems to increase in number on Con A stimulation. The R5 population is negative for CD14, but positive for CD52 and presumably represents another lymphocyte subpopulation since lymphocytes express low levels of CD14 and high levels of CD52 (Barclay *et al*, 1997). On Con A stimulation, another population R3, of very large CD14 positive cells is apparent. These presumably represent fusing cells. The results of FACS analysis concurs with evidence from microscopy that 2hrs of Con A stimulation has induced the monocytes to start fusing (Fig.3.2.17). The histogram represents only the MFI of CD14 +ve populations. (The lymphocytes (R4) expressed very low levels of tetraspanins; R5 had some CD9 (MFI=32.37) and CD81 (MFI=13.81), and this did not change on Con A stimulation). The level of expression of CD63 on normal monocytes was surprisingly low (MFI=17.21) and there was moderate expression of CD9 (MFI=127.83). The R3 gate contains very few cells in the unstimulated population, which presumably represent larger than average monocytes. Their overall expression profile is very similar to the R1 cells, but the cells are more brightly stained, presumably because of their increased size.

On 2hrs Con A stimulation, most markers expressed on surface of R1 and R2 cells increased slightly, except CD18, CD11b and CD14, which decrease. All markers increase on R3 cells, but this may simply be increased staining due to increased cell size (although relative to other markers, CD18 and CD29 appear to increase more on Con A stimulated cells). Similarly the effect of 72hrs of Con A stimulation on the expression levels of CD9, CD63, CD81, CD82, CD11b, CD14, CD18, CD29 and CD52 on monocytes was tested by FACS as described in section 2.2.11.

**(b) 72hrs Con A stimulation**

The surface expression of CD9, CD81, CD14, CD18, CD29 and CD52 in R1 cells are considerably increased during normal culture at 37°C, whereas CD63, CD11b and CD82 show little increase. The pattern of expression is similar on cells included in the R3 gate, but higher, presumably due to the larger size of the cells. Thus these larger cells do not represent a separate population. Interestingly, after 72hrs of culture in the presence of Con A, all markers with the exception of CD63, CD52 and CD82 (which is low in any case), appear to be down-regulated on R1 cells. The R3 cells exhibit a similar profile, although CD29 expression is high, comparable to unstimulated cells. The R3 population is relatively low in numbers in the 72hrs Con A stimulated cells, compared with 2hrs stimulation. This may be due to loss of very large MGCs, which are difficult to harvest and may not pass through the FACS machine (Fig.4.2.18.).
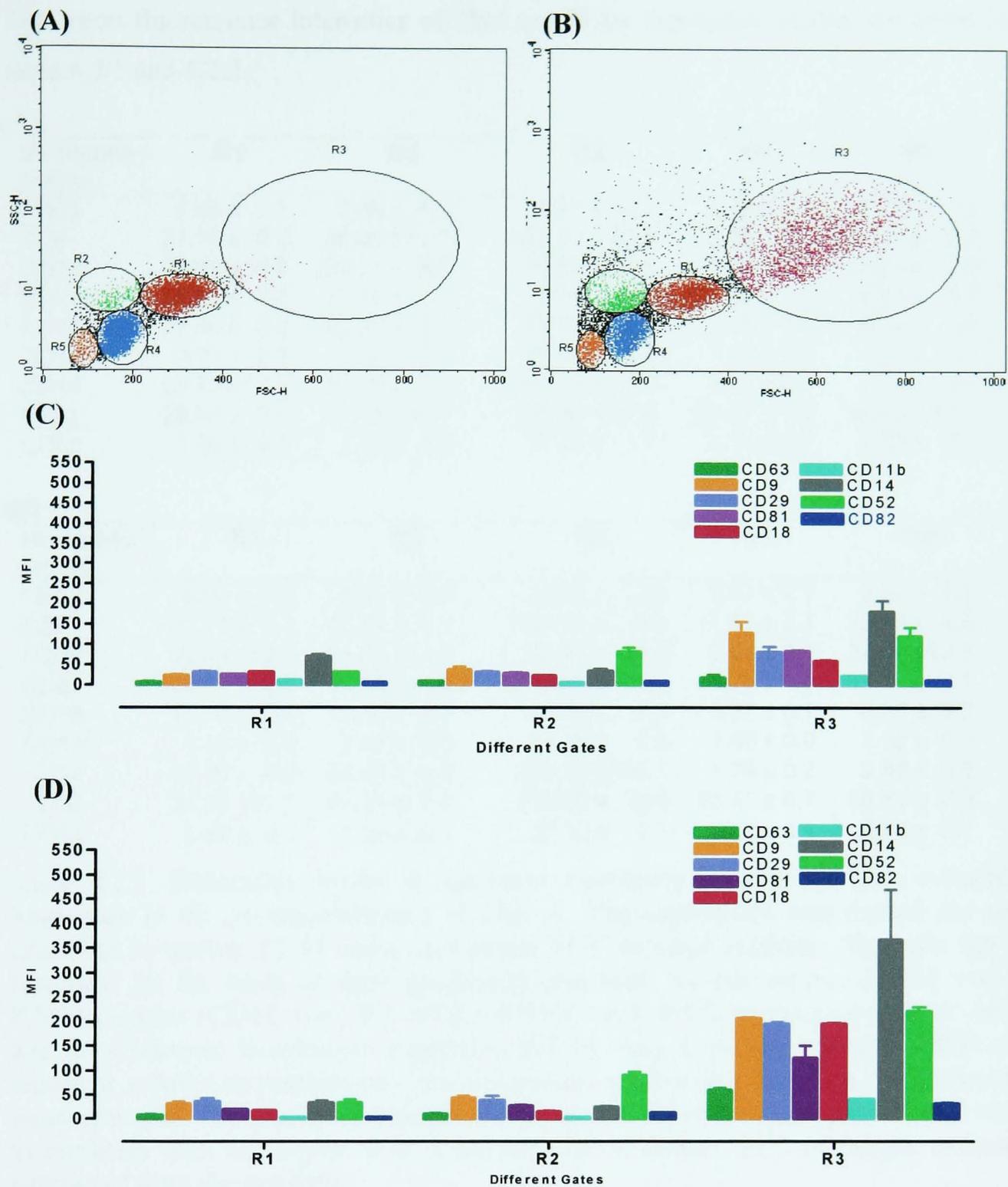
Fig.4.2.17. Expression levels of leucocyte membrane proteins on 2hrs cultured leucocytes in the presence/absence of Con A. The experiment was carried out as described in section 2.2.11 using appropriate FITC labelled antibody. The cells were separated on the basis of their granularity and size: R1=Monocytes (CD14 +ve); R2=Monocytes (CD14 +ve); R3=MGCs (CD14 +ve); R4=Lymphocytes (CD14 –ve) and R5=Unknown lymphocyte population (CD14–ve). Scatter profiles of unstimulated (A); 2hrs Con A stimulated (B) leucocytes; expression levels of leucocyte membrane proteins on 2hrs cultured monocytes (C); 2hrs Con A stimulated monocytes (D). The results of C and D are the mean values of 2 experiments carried out in duplicate with monocytes from 2 different blood donors. MFI of isotype control subtracted from the test MFI.

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The mean fluorescence intensities of 2hrs and 72hrs expression studies are given in table 4.2.1 and 4.2.2.

**(A)**

| Membrane protein | R1 | R2 | R3 | R4 | R5 |
|---|---|---|---|---|---|
| CD63 | 4.95 ± 2.7 | 6.69 ± 4.2 | 17.21 ± 12.3 | 1.16 ± 0.1 | 2.72 ± 1.3 |
| CD9 | 21.74 ± 6.2 | 36.26 ±14.3 | 127.83 ± 53.8 | 3.48 ± 1.3 | 32.37 ± 18.9 |
| CD29 | 29.81 ± 6.8 | 29.27 ± 6.6 | 79.63 ± 30.9 | 7.97 ± 0.9 | 40.85 ± 37.5 |
| CD81 | 23.05 ± 1.8 | 27.69 ± 2.1 | 83.61 ± 7.2 | 11.82 ± 1.3 | 13.81 ± 4.7 |
| CD18 | 29.45 ± 2.0 | 22.05 ± 3.3 | 58.80 ± 6.6 | 7.36 ± 0.5 | 6.34 ± 1.6 |
| CD11b | 9.13 ± 0.3 | 5.63 ± 0.1 | 21.09 ± 1.0 | 1.35 ± 0.0 | 1.32 ± 0.1 |
| CD14 | 68.70 ±11.7 | 33.88 ± 9.5 | 182.19 ± 51.3 | 1.18 ± 0.1 | 2.54 ± 0.8 |
| CD52 | 28.68 ± 3.8 | 81.15 ±18.1 | 118.99 ± 37.6 | 19.89 ± 1.0 | 58.80 ± 11.1 |
| CD82 | 3.96 ± 0.1 | 7.74 ± 1.9 | 11.02 ± 1.7 | 1.19 ± 0.1 | 4.58 ± 0.3 |

**(B)**

| Membrane protein | R1 | R2 | R3 | R4 | R5 |
|---|---|---|---|---|---|
| CD63 | 8.09 ± 5.1 | 9.87 ± 6.9 | 58.22 ± 13.0 | 2.67 ± 1.1 | 3.62 ± 2.2 |
| CD9 | 32.17 ± 7.4 | 42.98 ± 7.2 | 204.83 ± 5.3 | 12.63 ± 2.8 | 33.16 ± 6.2 |
| CD29 | 36.60 ±12.5 | 38.38 ±19.3 | 193.77 ± 11.2 | 9.97 ± 3.4 | 24.04 ±13.8 |
| CD81 | 18.61 ± 2.0 | 25.96 ± 4.4 | 123.33 ± 51.0 | 7.75 ± 1.0 | 11.42 ± 3.1 |
| CD18 | 17.94 ± 0.4 | 16.40 ± 1.4 | 195.16 ± 6.9 | 6.37 ± 0.1 | 6.38 ± 0.3 |
| CD11b | 4.13 ± 0.0 | 3.43 ± 0.0 | 41.38 ± 0.8 | 1.56 ± 0.0 | 1.32 ± 0.0 |
| CD14 | 33.75 ± 9.9 | 24.44 ± 5.9 | 366.23 ±204.7 | 1.78 ± 0.2 | 2.80 ± 0.5 |
| CD52 | 34.82 ±11.1 | 90.36 ± 8.8 | 219.68 ± 15.9 | 23.31 ± 0.7 | 56.88 ± 3.9 |
| CD82 | 3.46 ± 0.1 | 11.65 ± 0.1 | 32.30 ± 2.1 | 1.63 ± 0.1 | 4.74 ± 0.1 |

Table 4.2.1. Expression levels of leucocyte membrane proteins on 2hrs cultured leucocytes in the presence/absence of Con A. The experiment was carried out as described in section 2.2.11 using appropriate FITC labelled antibody. The cells were separated on the basis of their granularity and size: R1=Monocytes (CD14 +ve); R2=Monocytes (CD14 +ve); R3=MGCs (CD14 +ve); R4=Lymphocytes (CD14 –ve) and R5=Unknown lymphocyte population (CD14 –ve). Expression levels (MFI) of leucocyte membrane proteins on 2 hrs cultured monocytes (A); 2hrs Con A stimulated monocytes (B). The results of A and B are the mean MFI of 2 experiments carried out in duplicate with monocytes from 2 different blood donors. MFI of isotype control subtracted from the test MFI.

Fig.4.2.18. Expression levels of leucocyte membrane proteins on 72hrs cultured monocytes in the presence/absence of Con A. The experiment was carried out as described in section 2.2.11 using appropriate FITC labelled antibody. The cells were separated on the basis of their granularity and size: R1=Monocytes (CD14 +ve); R2=Monocytes (CD14 +ve); R3=MGCs (CD14 +ve); R4=Lymphocytes (CD14 –ve) and R5=Unknown lymphocyte population (CD14 –ve). Scatter profiles of 72hrs cultured unstimulated (A); 72hrs Con A stimulated (B) leucocytes; Expression levels of leucocyte membrane proteins on 72hrs cultured monocytes (C); 72hrs Con A stimulated monocytes (D). The results of C and D are the mean values of 2 experiments carried out in duplicate with monocytes from 2 different blood donors. MFI of isotype control subtracted from the test MFI.

**(A)**

| Membrane protein | R1 | R2 | R3 | R4 | R5 |
|---|---|---|---|---|---|
| CD63 | 8.11 ± 3.9 | 14.45 ± 9.1 | 14.83 ± 6.1 | 1.14 ± 0.1 | 3.33 ± 1.6 |
| CD9 | 206.09 ± 61.2 | 92.92 ± 6.9 | 461.71 ± 123.8 | 4.76 ± 0.5 | 28.69 ± 4.7 |
| CD29 | 73.33 ± 10.9 | 70.13 ± 36.6 | 103.08 ± 25.4 | 11.27 ± 1.8 | 26.45 ±19.2 |
| CD81 | 151.99 ± 50.3 | 59.33 ± 13.4 | 252.83 ± 49.7 | 16.47 ± 0.9 | 16.52 ± 1.5 |
| CD18 | 49.03 ± 0.3 | 19.74 ± 0.1 | 68.83 ± 4.2 | 3.73 ± 0.0 | 5.69 ± 0.3 |
| CD11b | 12.55 ± 0.0 | 4.29 ± 0.3 | 19.14 ± 0.8 | 1.16 ± 0.0 | 1.35 ± 0.1 |
| CD14 | 206.01 ± 32.2 | 24.42 ± 4.7 | 305.86 ± 97.4 | 1.16 ± 0.1 | 2.96 ± 0.3 |
| CD52 | 57.40 ± 2.7 | 179.37± 21.4 | 144.66 ± 11.7 | 17.64 ± 3.7 | 51.31 ± 5.5 |
| CD82 | 5.94 ± 0.5 | 18.98 ± 0.5 | 14.30 ± 0.3 | 1.26 ± 0.1 | 5.43 ± 0.6 |

**(B)**

| Membrane protein | R1 | R2 | R3 | R4 | R5 |
|---|---|---|---|---|---|
| CD63 | 12.87 ± 6.6 | 26.76 ± 18.4 | 51.75 ± 30.5 | 2.24 ± 0.8 | 6.04 ± 3.9 |
| CD9 | 38.07 ± 3.2 | 67.00 ± 2.5 | 95.51 ± 30.3 | 9.38 ± 1.4 | 46.84 ± 1.6 |
| CD29 | 36.57 ± 4.4 | 71.26 ± 15.7 | 108.79 ± 15.5 | 8.27 ± 1.2 | 40.02 ±17.7 |
| CD81 | 27.62 ± 0.6 | 28.06 ± 1.6 | 85.66 ± 9.8 | 6.25 ± 0.2 | 8.94 ± 3.3 |
| CD18 | 11.00 ± 0.4 | 10.44 ± 0.2 | 36.37 ± 1.3 | 2.02 ± 0.1 | 3.21 ± 0.3 |
| CD11b | 2.33 ± 0.1 | 2.73 ± 0.1 | 8.31 ± 0.1 | 1.13 ± 0.0 | 1.17 ± 0.0 |
| CD14 | 5.22 ± 0.5 | 5.75 ± 1.0 | 15.81 ± 1.5 | 1.26 ± 0.1 | 1.83 ± 0.4 |
| CD52 | 54.42 ± 1.6 | 88.86 ± 4.9 | 155.93 ± 51.5 | 13.87 ± 3.8 | 37.79 ±12.6 |
| CD82 | 5.78 ± 0.2 | 8.58 ± 0.1 | 18.00 ± 1.0 | 1.45 ± 0.2 | 2.76 ± 0.3 |

Table 4.2.2. Expression levels of leucocyte membrane proteins on 72hrs cultured leucocytes in the presence/absence of Con A. The experiment was carried out as described in section 2.2.11 using appropriate FITC labelled antibody. The cells were separated on the basis of their granularity and size: R1=Monocytes (CD14 +ve); R2=Monocytes (CD14 +ve); R3=MGCs (CD14 +ve); R4=Lymphocytes (CD14 –ve) and R5=Unknown lymphocyte population (CD14–ve). Expression levels (MFI) of leucocyte membrane proteins on 72hrs cultured monocytes (A); 72hrs Con A stimulated monocytes (B). The results of A and B are the mean MFI of 2 experiments carried out in duplicate with monocytes from 2 different blood donors. MFI of isotype control subtracted from the test MFI.

## 4.2.8. Comparison of tetraspanin expression on THP-1 cells and monocytes

As described in section 3.2.11.1 Con A did not induce the fusion of THP-1 cells, although it did promote aggregation of these cells. Hence the expression levels of tetraspanins on unstimulated/Con A stimulated non-fusogenic THP-1 cells and fusogenic monocytes were compared in identical experimental conditions by FACS analysis (Fig.4.2.19 and 4.2.20).
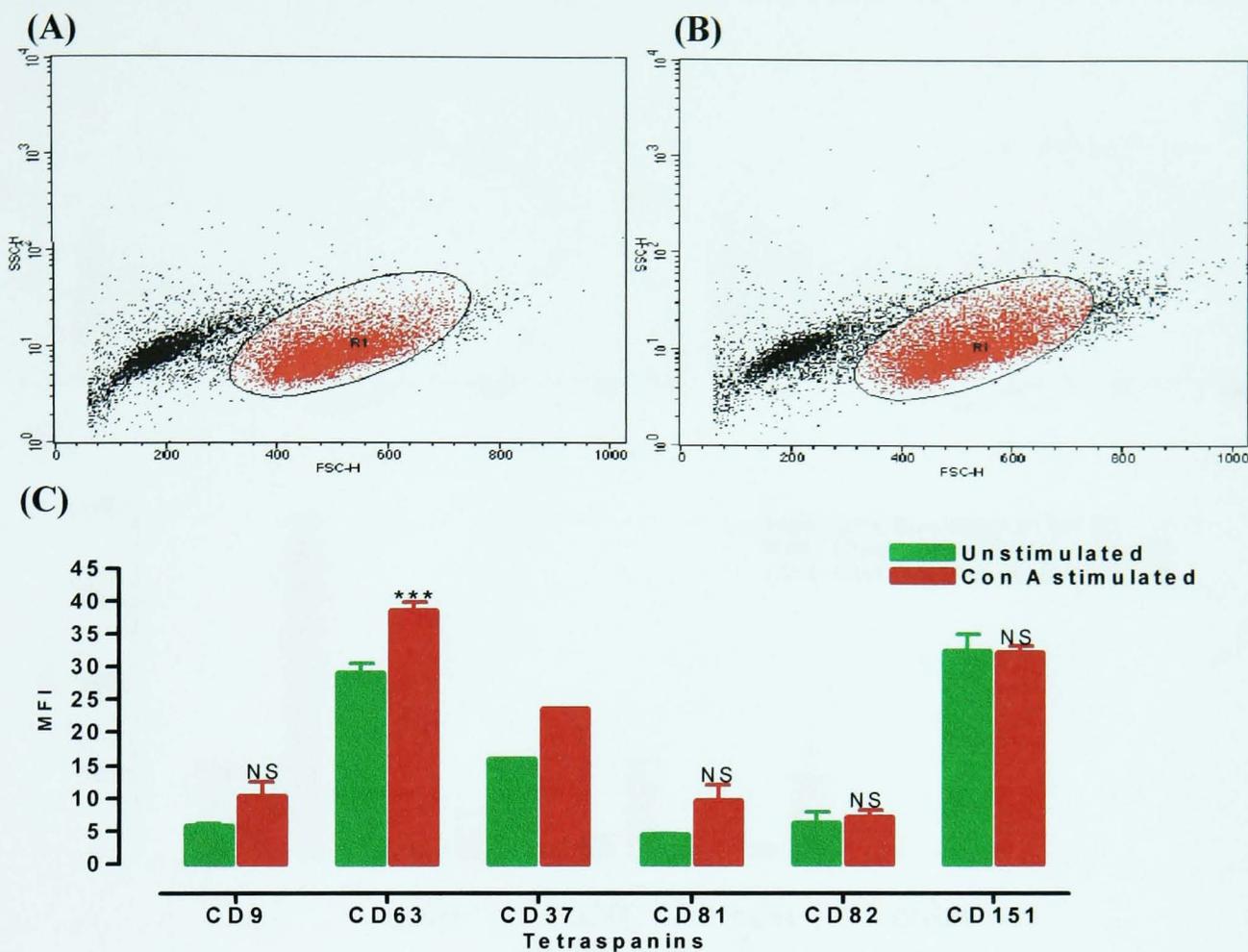
Fig.4.2.19. Expression levels of tetraspanins on 2hrs cultured THP-1 cells in the presence/absence of Con A $(10\mu g.ml^{-1})$. The experiment was carried out as described in section 2.2.11 using appropriate FITC labelled antibody. Scatter profiles of 2hrs cultured unstimulated (A); 2hrs Con A stimulated (B) THP-1 cells. Expression levels of tetraspanin proteins on 2hrs unstimulated/Con A stimulated THP-1 cells (C). The results of CD9, CD63, CD81 and CD151 are the mean values of 2 experiments carried out in triplicate; the result of CD37 was from a single experiment. The level of significance was tested by unpaired t test by comparing the MFI of unstimulated cells with Con A stimulated cells in R1, where ***Significant at $P<0.05$; NS=Non significant at $P<0.05$.

Unstimulated THP-1 cells showed higher expression levels of CD63 (MFI=27.06) and CD151 (MFI=35.08) when compared to other tetraspanins i.e. the expression of CD63 and CD151 were around 5 times more than the expression of CD9. However, after 2 hrs of Con A stimulation little increased expression levels of CD9, CD63, CD37 and CD81 were observed, whilst there was no change in expression CD82 and CD151. However, the increased expression was only significant for CD63.
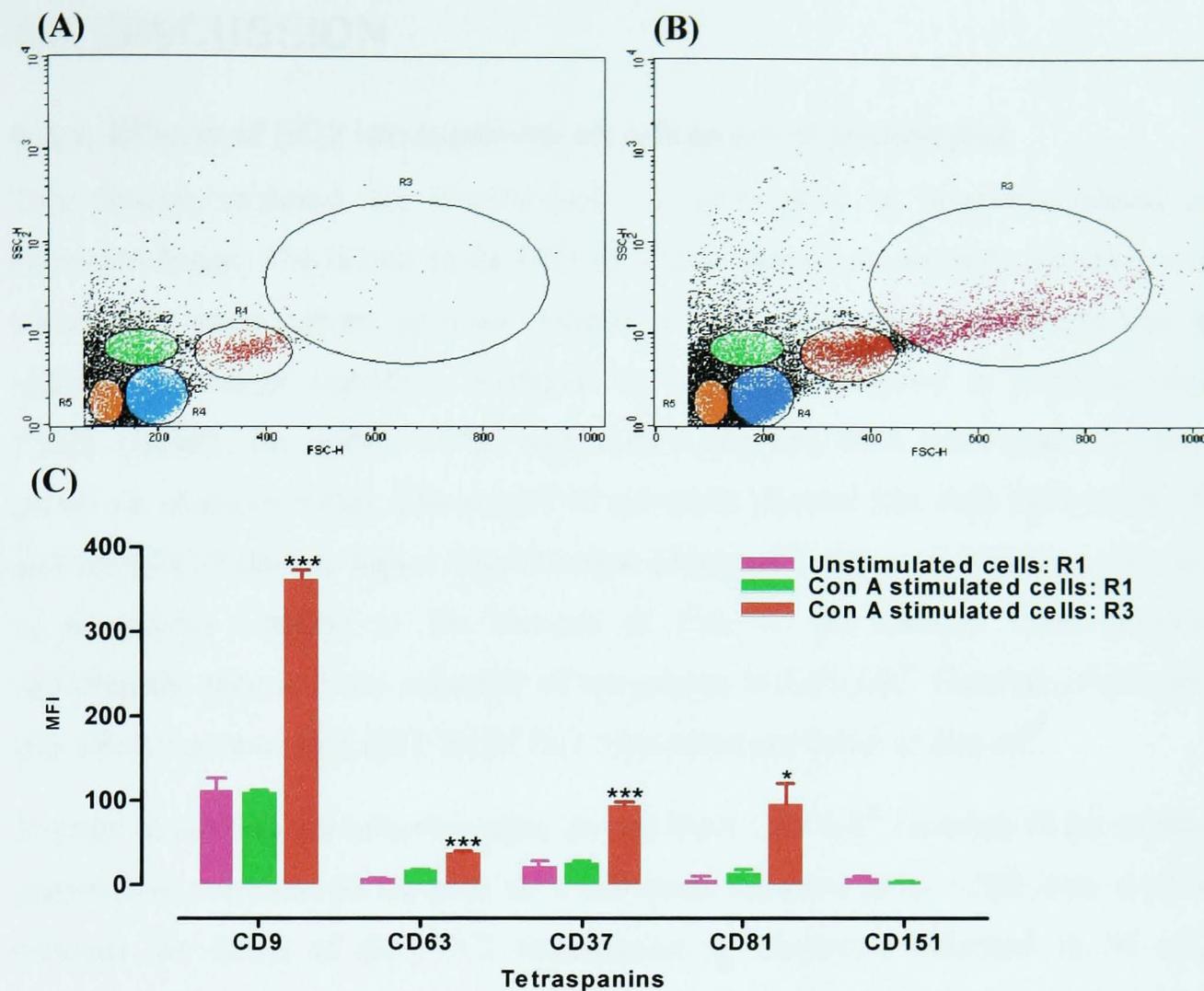
Fig.4.2.20. Expression levels of different tetraspanins on unstimulated/Con A stimulated monocytes. The experiment was carried out as described in section 2.2.11 using appropriate FITC labelled antibody. R1=Monocytes (CD14+ve); R2=Monocytes (CD14 +ve); R3=MGCs (CD14 +ve); R4=Lymphocytes (CD14 –ve) and R5=Unknown lymphocyte population (CD14–ve). The results are the mean values of 1 experiment carried out in triplicate. The level of significance was tested by unpaired t test by comparing the MFI of unstimulated cells in R1 and Con A stimulated cells in R3, where *** Significant at $P<0.0001$; * Significant at $P<0.05$.

Compared with THP-1 cells unstimulated monocytes examined at the same time under identical conditions express much higher levels of CD9 (MFI=111.10). CD63, CD37, CD81, and CD151, however, are more weakly expressed by monocytes in R1 and no expression of CD82 was observed. The monocytes population (R1) of Con A stimulated cells did not show much change in expression values of these tetraspanins. In contrast to these increased expression levels of CD9 (MFI=363.00), CD63 (MFI=37.8), CD37 (MFI=92.69) and CD81 (MFI=96.4) with Con A stimulated cells in R3 gated cells were seen and these results are in agreement with the observations made in section 4.2.7. No expression of CD82, CD151 was observed (Fig.4.2.20). The higher fluorescence with Con A stimulated cells in R3 may simply be increased staining due to increased cell size.

# 4.3. DISCUSSION

## 4.3.1. Effects of EC2 tetraspanins on adhesion of monocytes

This chapter explored the involvement of tetraspanins in functions related to monocyte fusion. The effects of the GST-EC2 tetraspanins on monocyte adhesion was tested since many reports provide evidence for the involvement of tetraspanins in adhesion-dependent signalling mediated by integrins (reviewed in Berditchevski, 2001). Initially, the effects of the recombinant proteins were investigated on cells grown on chamber slides. The results of our study showed that both GST-EC2CD9 and GST-EC2CD63 at higher concentration ($20\mu g.ml^{-1}$) did not inhibit the adhesion of monocytes cultured in the absence of Con A. Interestingly these proteins significantly enhanced the adhesion of monocytes in Lab-Tek® chamber slides, and this effect was seen with GST-EC2CD63 concentrations down to $2\mu g.ml^{-1}$.

In order to confirm the adhesion assay results from Lab-Tek® chamber slides another quantitative colorimetric method, the SRB assay (Skehan *et al*, 1990) was used to estimate the effect of GST-EC2 tetraspanins on monocyte adhesion in 96 well microtitre plates as a function of cell numbers. Preliminary experiments confirmed that this assay gave a linear relationship between OD and cell number. The results of the adhesion assay by the SRB method indicated that GST-EC2 tetraspanins did not have any significant inhibitory effect on adhesion of monocytes cultured in the absence of Con A. However, there was no apparent enhancement of adhesion by GST-EC2CD9 or GST-EC2CD63 in this assay. The differences between the results on chamber slides and microtitre plates may have been due to the differences in surface chemistry of surfaces the monocytes adhere to. Extensive studies by Anderson and co-workers indicate that surface chemistry affects the adhesion of monocytes and fusing/fused macrophages, presumably due to difference in the type and conformation of proteins that are adsorbed (Jenney *et al*, 1998; McNally and Anderson, 2002).

In addition the SRB assay was also used to examine the effect of GST/EC2-tetraspanins on Con A stimulated monocytes. It is apparent that Con A stimulation slightly increased the adhesion of monocytes, possibly through increased expression/activation of integrins. None of the GST-EC2 proteins significantly inhibited adhesion of Con A induced monocytes at concentration that gave strong inhibition of MGC formation. However, reduced cell numbers were observed for Con

A induced monocytes incubated with EC2CD63 alone (generated from GST-EC2CD63 by thrombin cleavage). Thus, part of the effect of this protein on MGC formation might be due to an effect on cell adhesion. However, we had observed in chamber slides that MGCs were strongly adherent, and less likely to be removed during slide processing than single cells (chapter 3, 3.2.1.2). The apparent effect of cleaved CD63EC2 on adhesion might alternatively be resulting from potent inhibition of MGC formation by this preparation. Also, at the same protein concentration ($20\mu g.ml^{-1}$), 3x higher molar concentrations of EC2CD63 was used for this assay when compared to GST-EC2CD63.

In contrast to our studies, Takeda and co-workers tested the effects of antibodies to tetraspanins CD9 and CD81 on adhesion of monocytes in presence of Con A ($10\mu g.ml^{-1}$) using MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay. They reported that antibodies to CD9 and CD81 did not have any effect on adhesion but anti $\beta 1$ and anti $\beta 2$ integrin antibodies significantly inhibited monocyte adherence to the tissue-culture treated surfaces, consistent with the known adhesive functions of these integrins. From these observations we conclude that GST-EC2CD63 and GST-EC2CD9 do not inhibit MGC formation due to inhibition of adhesion.

## 4.3.2. Effects of EC2 tetraspanins on cell numbers

The effect of GST-EC2 tetraspanins on overall cell number was also assessed by SRB assay. These experiments were designed to investigate any possible anti-proliferative or cytotoxic effects of the recombinant proteins, and looked at cell numbers prior to vigorous washing to remove loosely adherent cells. Neither GST-EC2 tetraspanins nor Con A or a combination of both induced significant proliferation or showed any cytotoxic effect on monocytes since overall cell numbers were similar between treatments. Although Con A is a T cell mitogen (Baral *et al*, 2004; Rashid *et al*, 2005) it did not appear to induce the proliferation of monocytes. This effect was supported by our additional studies using macrophages from CD9KO and CD9WT mice (section 3.2.11.3). We therefore conclude that the effects of GST-EC2CD9 and GST-EC2CD63 on MGC formation are not due to anti-proliferative or cytotoxic activity.

### 4.3.3. Effects of EC2 tetraspanins on aggregation of monocytes and THP-1 cells

The aggregation of monocytes is prerequisite for the fusion process (Vignery *et al*, 2000; Takeda *et al*, 2003). Hence we analysed the effects of various recombinant EC2 tetraspanins on the aggregation of monocytes. As detailed in 4.2.5, there were some initial problems with setting up and scoring this assay. After these problems were resolved, the results indicated that there was no inhibition of Con A induced aggregation by the recombinant proteins. By contrast, there was an indication that CD9 and CD63EC2s enhanced aggregation. Takeda and co-workers reported that anti CD9 and CD81 antibodies did not have any effect on aggregation of Con A stimulated monocytes (Takeda *et al*, 2003), whereas anti β2 integrins were inhibitory. By contrast, other workers have shown that anti CD9 antibodies activate and induce the aggregation of platelets (Wright *et al*, 1994) and eosinophils (Matsumoto *et al*, 1999).

In addition the effect of different concentrations of GST-EC2 tetraspanins on the aggregation of THP-1 cells in presence of Con A (5µg.ml$^{-1}$) was tested. Again no inhibition of aggregation was observed. By contrast, as the concentration of GST-EC2CD63 and GST-EC2CD9 increased the aggregation of THP-1 cells also increased. The results of the aggregation assay with THP-1 cells concur with results from the monocyte aggregation assay. We conclude that the effect of the recombinant CD9 and CD63EC2 proteins on MGC formation is unlikely to be due to inhibition of aggregation, or of the movement of cells coming together to form aggregates.

### 4.3.4. Expression levels of leucocyte membrane proteins

Attempts were made to investigate the possible involvement of tetraspanins and other leucocyte membrane proteins in fusion of monocytes by comparing their expression levels on the surface of unstimulated and Con A stimulated (2hrs and 72hrs) monocytes by FACS analysis. It has been reported that the expression levels of tetraspanins and integrins were modulated under fusogenic conditions (McNally and Anderson, 2002; Takeda *et al*, 2003). From the scatter profile of FACS analysis it was observed that there were four distinct cell populations of leucocytes in our preparation of unstimulated cells and five distinct cell populations with Con A stimulated cells. These populations were gated on the basis of their granularity and size and were identified using anti CD14 and anti CD52 antibodies. The populations R1 and R2 in unstimulated cell preparations are monocytes since both stain with anti

CD14 antibody. However, the R2 cells have a slightly different scatter profile and are smaller than R1 cells. They express less CD14 and more CD52 than R1 cells. They might possibly correspond to the pro-inflammatory (CD14$^+$ CD16$^+$) subpopulation of monocytes (Ziegler-Heitbrock *et al*, 1993; Geissmann *et al*, 2003). Staining with anti CD16 antibody would be needed to try to verify this. On 2hrs Con A stimulation, a population of large CD14 $^+$ ve cells (R3) was observed, that presumably correspond to the fusing monocytes observed microscopically (section 3.2.10). For the R1 cells, CD9, CD63, CD81, CD29 and CD52 showed a slight increase on Con A stimulation, whereas CD14 and integrin CD18 and CD11b staining decreased by approximately 50%. All markers increased on the R3 population of cells; however this may be largely due to increased cell size. CD63 staining, however, which was low on unstimulated R1 cells, does appear to have increased more relative to other markers. Because increased cell size results in increased staining by FACS, it is difficult to relate the data unequivocally to increased expression of the different markers. In retrospect, immunofluorescence microscopy may have given a better indication of changes in expression. Alternatively, immunoblotting of cell lysate samples containing equivalent amounts of protein could have been used.

Similarly the expression pattern of all of the above membrane proteins was assessed using monocytes cultured for 72hrs in presence/absence of Con A, The expression of CD9, CD81, CD14, CD18, CD29 and CD52 were increased during normal culture condition at 37°C for 72hrs. The increase in the expression of the tetraspanins CD9 and CD81 is particularly marked after 72hrs in culture. This is in agreement with the findings of Takeda and co-workers, who used immunoblotting to look at changes in protein expression. However, for cells treated with Con A for the same period of time, decreased expression levels of CD9, CD81, CD14, CD29 and CD18 proteins were observed for R1 cells, whereas CD63 increased and levels of CD52 and CD82 were similar to untreated cells. The population gated as R3 on 2hrs Con A induced cells was much less evident after 72hrs, possibly due to the formation of very large MGCs that were difficult to harvest or did not pass through the FACS machine. The cells gated as R3 showed a similar overall staining profile to the R1 cells. The decreased expression of CD9 and CD81, and increased expression of CD63 on Con A treatment is again agreement with the findings of Takeda and co-workers.

Interestingly, Wang *et al* have investigated the role of inflammation and macrophage activation in the regulation of CD9 expression (Wang *et al*, 2002). It was found that the expression of CD9 on primary cultures of murine peritoneal macrophages was down regulated by IFN-γ. This down regulation was concentration dependent and maximal by 48hrs. The mechanism by which IFN-γ decreases CD9 expression appears to be through the stat-1 signalling pathways, since stat-1 knockout mice did not demonstrate any reduction in CD9 expression by IFN-γ treatment. It is possible that cytokines such as IFN-γ produced on Con A stimulation may lead to the down-regulation of CD9 observed here and by Takeda *et al* (2003).

Although THP-1 cells are non-fusogenic in response to Con A stimulation (section 3.2.11.1) they undergo aggregation. Hence FACS analysis was carried out to assess the differences in expression pattern of tetraspanins in THP-1 cells and monocytes with and without 2hrs Con A stimulation. The THP-1 cells weakly express the tetraspanins and on stimulation with Con A there is little change in expression. By contrast the surface expression of tetraspanins in monocytes is higher than THP-1 cells and expression increases on treatment with Con A. The low expression of tetraspanins of THP-1 cells may in part explain why they are unable to form MGCs.

The overall observation from this chapter is that recombinant tetraspanins EC2s did not alter the functions related to monocytes fusion such as cell adhesion, aggregation and cell numbers. From these we could conclude that the inhibitory effect shown by GST-EC2/EC2/His$_6$-EC2CD9 and CD63 on Con A induced monocyte fusion could be due to direct effect on fusogenic receptors of monocytes or alterations in the organisation of essential microdomains for the fusion process. To address these issues we were motivated to carry out studies described in the next chapter to assess whether these tetraspanins exert their effects by direct binding to monocytes.

# CHAPTER 5

# INVESTIGATION OF EC2 TETRASPANIN BINDING TO MONOCYTES

## 5.1. INTRODUCTION

As described in chapter 3, the GST fusion proteins EC2CD63 and EC2CD9 showed a profound inhibitory effect on the fusion of Con A stimulated monocytes. This did not appear related directly to inhibition of adhesion or aggregation. To investigate their role further, attempts were made to determine if the recombinant proteins were binding specifically to monocytes. Initially attempts were made to investigate binding using indirect techniques, using antibodies directed against the GST protein. Due to non-specific binding of antibodies clear binding was not detected. The GST proteins were therefore labelled directly with fluorescent dyes, and their binding to monocytes investigated.

Recently FITC, AlexaFluor 647® and rhodamine BITC labelled GST-EC2CD63 and GST proteins have been sent to our collaborator Dr. Cecilia Cheng-Mayer (Aaron Diamond AIDS Research Centre, New York, US) for their investigations on the role of tetraspanins in HIV-1 infection of human macrophages (Ho *et al*, manuscript submitted). They had previously found that recombinant GST-EC2 domain of human tetraspanins CD9, CD63, CD81 and CD151 potently inhibited CCR5 (R5) HIV-1 infection of macrophages with $IC_{50}$ values of 0.11-1.2nM, in contrast to a weak inhibition by GST alone or by mouse CD9EC2. In addition these proteins also inhibited macrophage infection by virions pesudotyped with CXCR4 (X4) tropic HIV-1 or VSV-G glycoproteins at higher concentrations and showed a partial inhibitory effect on the infection of PBMC by R5 and VSV-G was but not X4 pseudotyped viruses. The tetraspanins did not decrease CD4 or co-receptor expression. The study using the labelled proteins also showed the specific uptake of tetraspanin proteins by virus-infected cells. There were some instances of HIV-1 co-localising with CD63. The binding of the flurochrome labelled proteins in their experimental system was assessed by FACS analysis and by fluorescent microscopy

and some of the results are presented in section 5.2.2.5. In addition we tested the binding efficiency of FITC labelled GST-EC2CD9, GST-EC2CD151 and GST on mouse oocytes in collaboration with Dr. Eileen McLaughlin, ARC Centre of Excellence in Biotechnology and Reproductive Science, University of Newcastle, Australia and preliminary results are shown in section 5.2.2.6. It is known that CD9 plays an important role in sperm-egg fusion, but the involvement of CD151 is unknown.

# 5.2. RESULTS

## 5.2.1. Binding of GST-EC2 tetraspanins to monocytes: Indirect assays

### 5.2.1.1. Indirect immunofluorescence on unstimulated monocytes

Attempts were made to assess the binding of GST-EC2 tetraspanins to monocytes using indirect immunofluorescence. In the initial attempts, fresh/matured (120hrs cultured) unstimulated monocytes were incubated with GST-EC2CD63, GST-EC2CD9, or GST (10 and 20μg.ml$^{-1}$) followed by goat anti GST and rabbit anti goat IgG-FITC in Lab-Tek® chamber slides as described in section 2.2.10.6.3. No difference in fluorescence between monocytes incubated with recombinant proteins or secondary and tertiary reagent alone was observed. Despite careful titration of the antibodies, and attempts to block non-specific binding by pre-incubating monocytes with rabbit serum no differential binding of EC2 proteins above background could be discerned microscopically (data not shown). Similarly labelled monocytes were analysed by FACS. No difference in fluorescence intensities (MFIs) was observed between controls (cells incubated with B/B/N) and test samples (cells incubated with GST-fusion proteins), confirming observations made by microscopy.

Since high backgrounds were observed using these antibodies, a different combination of secondary and tertiary reagent was used: mouse monoclonal anti GST antibody labelled with biotin followed by Extravidin-FITC. It was assumed that a monoclonal antibody would show greater specificity, and mouse IgG1 antibodies show little binding to human monocytes Fc receptors (Partridge *et al*, 1986; Woof *et al*, 1986). Following titration of the reagents, they were used to assess binding of GST-recombinant proteins to monocytes cultured in Lab-Tek® chamber slides as described previously. No difference in staining pattern was observed between the tests and negative control (cells incubated with BBN instead of recombinant protein). The Extravidin-FITC showed strong non-specific binding with monocytes even at low concentrations (~2μg.ml$^{-1}$). It is likely that the Extravidin-FITC binds to intracellular biotin molecules of monocytes when the cells are fixed and permeabilised with acetone. To avoid this problem binding experiments were carried out by fixing the cells with 2% paraformaldehyde rather than using acetone as a fixative. However, no differential binding of tetraspanins EC2s was clearly observed.

## 5.2.1.2. Indirect immunofluorescence on Con A stimulated monocytes

We surmised that tetraspanin EC2s might only bind to Con A stimulated monocytes. Also, since inhibition of MGC formation by the tetraspanin EC2s occurred within 24 hrs (section 3.2.10), we reasoned that any binding would occur fairly early after Con A stimulation. To investigate this, we carried out further binding assays by FACS analysis using Con A stimulated (24hrs) monocytes. The indirect binding assay was carried out (section 2.2.10.6.1) with GST-EC2CD9, GST-EC2CD63, GST-EC2C153A and GST and using mouse anti GST biotin at $1\mu g.ml^{-1}$ and Extravidin-FITC at $2.25\mu g.ml^{-1}$ as secondary and tertiary reagents, respectively. During the FACS analysis we observed three different cell populations, which were R1 (monocytes); R3 (MGCs) and R4 (lymphocytes). (In this experiment, the R2 and R5 populations of cells described in section 4.2.7. and 5.2.2.3 were not evident).
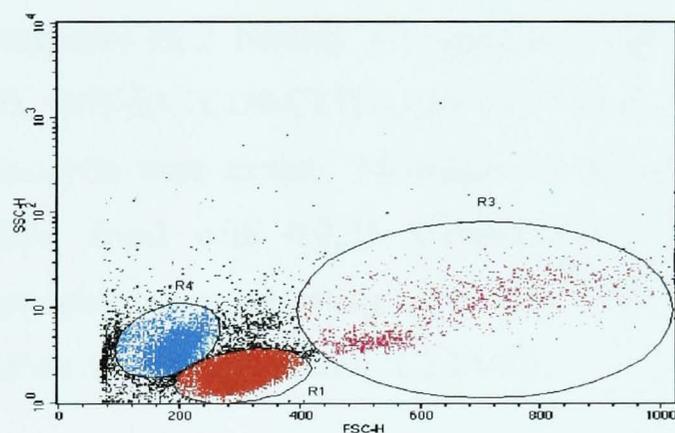


Fig.5.2.1.Scatter profile of flow cytometry analysis with Con A stimulated monocytes. The cells were separated on the basis of their granularity and size: R1=Monocytes (CD14 +ve); R3=MGCs (CD14 +ve); R4=Lymphocytes (CD14 –ve).
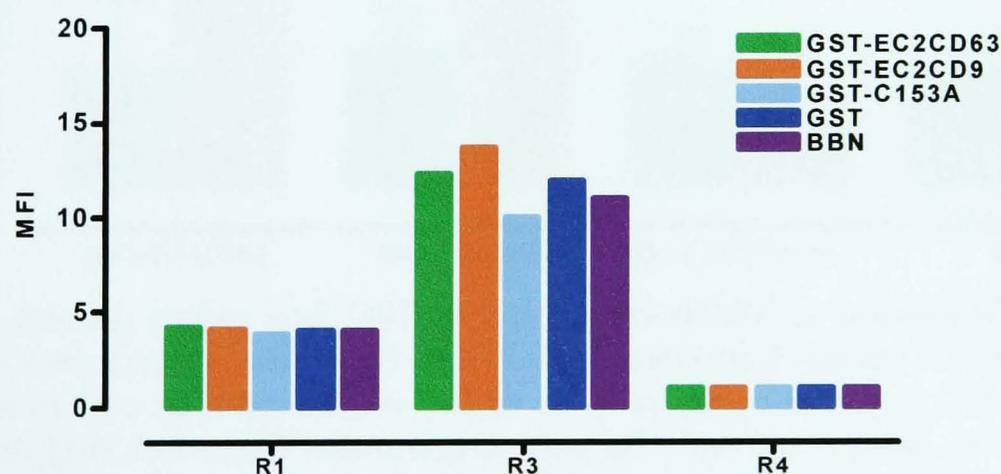


Fig.5.2.2. Binding of GST-EC2 tetraspanins/GST to Con A stimulated monocytes. The monocytes were stimulated with Con A $(10\mu g.ml^{-1})$ for 24hrs at 37°C. The indirect binding assay was carried out as described in section 2.2.10.6.1 using mouse anti GST biotin antibody at $10\mu g.ml^{-1}$ and Extravidin-FITC at $2.5\mu g.ml^{-1}$. The results are the means of 1 experiment carried out in duplicate. Where R1=Monocytes (CD14 +ve); R3=MGCs (CD14 +ve); R4=Lymphocytes (CD14 –ve).

It appeared that the cell population R3 corresponding to MGCs showed very slightly higher binding values with GST-EC2CD9 than controls GST-EC2CD9-C153A, GST and BBN. No such difference was observed from cells in populations R1 and R4, which correspond to monocytes and lymphocytes respectively. Unstimulated monocytes tested under identical condition did not show binding (data not shown). This suggested that Con A stimulation may affect the binding of GST-EC2 tetraspanins. However, it was difficult to determine if differential binding of GST-EC2 tetraspanins was occurring when similar experiments were carried out on monocytes cultured in chamber slides (data not shown).

### 5.2.1.3. Whole cell ELISA on monocytes

Whole cell ELISA (section 2.2.10.6.5) was used as an alternative method to investigate tetraspanin EC2 binding to monocytes. The binding of GST-EC2CD9, GST-EC2CD63, GST-EC2CD9-C153A and GST alone on unstimulated and Con A stimulated monocytes were tested. Monocytes were cultured in 96 well microtitre plates for 12hrs, fixed with 0.25% glutaraldehyde and the binding of GST-recombinant proteins assessed using goat anti GST antibody and mouse anti goat/sheep HRP as described in section 2.2.10.6.5.
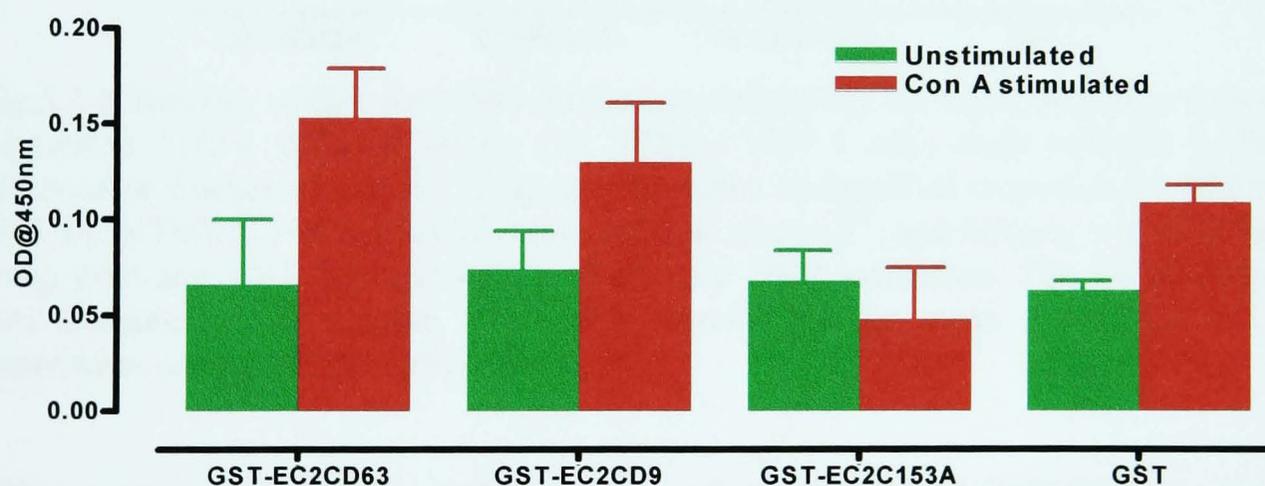


Fig.5.2.3. Binding studies with GST-EC2 tetraspanins/GST on unstimulated /Con A stimulated monocytes by whole cell ELISA. Monocytes were cultured in the presence or absence of Con A ($10\mu g.ml^{-1}$) for 12hrs as described in section 2.2.10.6.5. GST-EC2CD63/CD9/C153A/GST was tested at $20\mu g.ml^{-1}$, and binding was detected using goat anti GST and mouse anti goat/sheep HRP antibodies. The OD@450nm was measured using a plate reader. The results are the mean absorbance of 1 experiment carried out in duplicate.

GST-EC2CD63 showed a slightly higher binding value with Con A stimulated monocytes as compared to GST-EC2CD9, GST-EC2C153A and GST control. No differences in binding values were observed between the test and control with unstimulated monocytes. However, a problem with this assay system was that during processing, monocytes detached from the 96 well microtitre plates, giving rise to variable results when attempts were made to repeat this.

### 5.2.1.4. Whole cell ELISA on THP-1 cells

As an alternative to using peripheral blood monocytes, attempts were made to investigate binding of GST-EC2 tetraspanins to the monocytic leukaemic cell line THP-1. These cells were stimulated to adhere by culturing them in 96 well microtitre plates pre coated with poly-L-lysine at $50\mu g.ml^{-1}$.
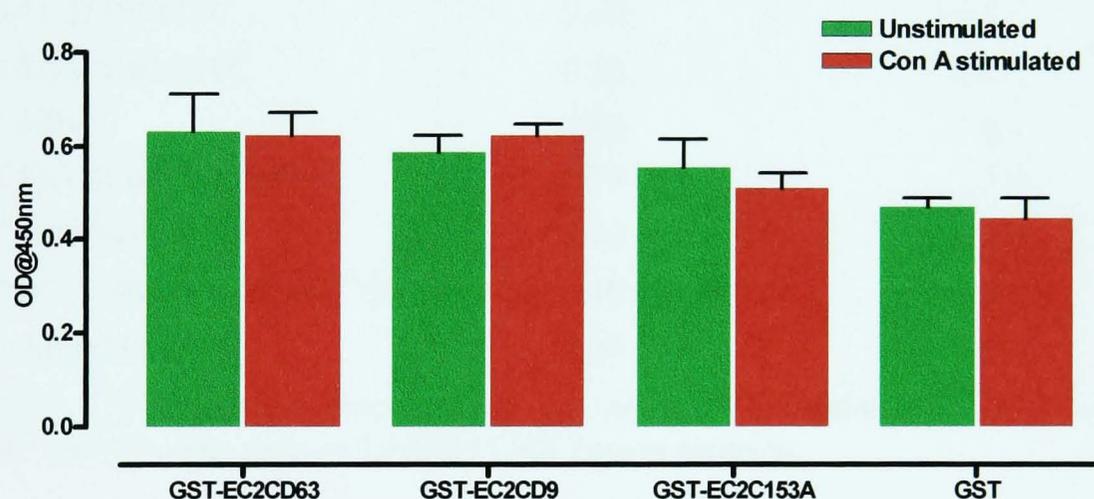


Fig.5.2.4. Binding studies with GST-EC2 tetraspanins/GST on unstimulated or Con A stimulated THP-1 cells by whole cell ELISA. THP-1 cells were cultured in the presence or absence of Con A ($10\mu g.ml^{-1}$) for 12hrs as described in section 2.2.10.6.5. GST-EC2CD63/CD9/C153A/GST was tested at $20\mu g.ml^{-1}$, and binding was detected using goat anti GST and mouse anti goat/sheep HRP antibodies. The OD@450nm was measured using a plate reader. The results are the mean absorbance of 1 experiment carried out in triplicate.

The results showed slightly higher binding values with GST-EC2CD63 and GST-EC2CD9 on both unstimulated and Con A stimulated THP-1 cells relative to GST alone (Fig.5.2.4). However, since the THP-1 cells could not be induced to undergo MGC formation (Section 3.2.11.1), the relevance of this low level of binding to monocyte fusion is unclear.

## 5.2.2. Binding of GST-EC2 tetraspanins to monocytes: Direct assays

### 5.2.2.1. Labelling GST-EC2CD9, GST-EC2CD63 and GST with fluorescent dyes

After the problems encountered in trying to use indirect binding assays to investigate the binding, direct labelling of these proteins with fluorescent dye (FITC) was attempted as described in section 2.2.9.2. The labelling appeared successful and the protein concentrations of the labelled proteins (estimated by Bradford assay as described in section 2.2.9.5) and the estimates of the molar FITC:protein ratio is shown in table.5.2.1.

| Labelled Protein | Conc. (mg.ml$^{-1}$) | Molar flurochrome: Protein ratio |
|---|---|---|
| GST-EC2CD9-FITC | 0.40 | 4.2:1 |
| GST-EC2CD63-FITC | 0.36 | 4.8:1 |
| GST-FITC | 0.40 | 4.8:1 |
| GST-EC2CD63-rhod BITC | 1.08 | 1.5:1 |
| GST-rhod BITC | 1.70 | 1.4:1 |
| GST-EC2CD63-AlexaFluor® 647 | 1.40 | 3.5:1 |
| GST-AlexaFluor® 647 | 1.60 | 1.8:1 |

Table.5.2.1. The proteins concentration and molar FITC/rhodamine-BITC/ Alexa Fluor® 647: Protein ratio of labelled GST fusion proteins.

The possible consequences of FITC labelling of GST-EC2CD9 and GST-EC2CD63 on proper folding was assessed by testing the biological activity of the FITC labelled proteins at 20µg.ml$^{-1}$ concentrations on Con A induced monocyte fusion assay in Lab-Tek® chamber slides as described in section 2.2.10.4.1. After 72hrs of incubation at 37°C light microscope images were captured.
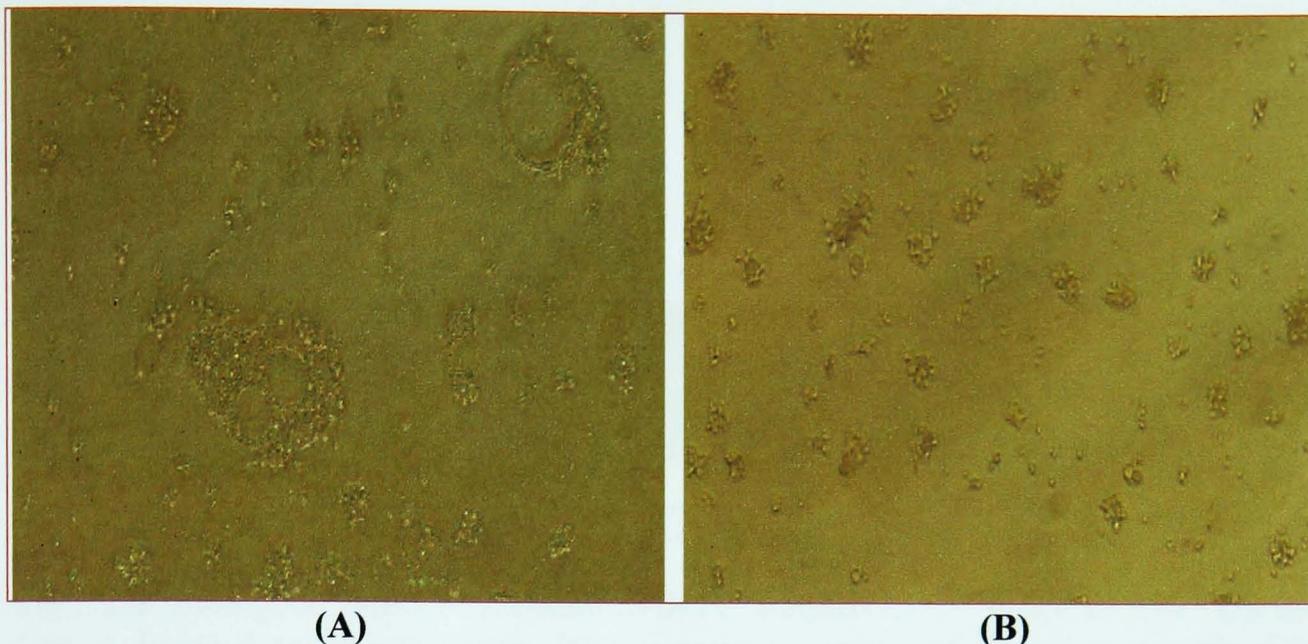
**(A)**            **(B)**

Fig.5.2.5. Biological activity of FITC labelled GST-EC2CD63 on Con A stimulated monocytes. The experiment was carried out as described in section 2.2.10.4.1 with 20μg.ml$^{-1}$ GST-EC2CD63-FITC. The light microscope images were captured with Nikon inverted light microscope using 20x objective. Con A stimulated monocytes (A); Con A stimulated monocytes with GST-EC2CD63-FITC (B).

The FITC labelled GST-EC2CD63 showed strong inhibition of Con A induced MGC formation compared to Con A control (Fig.5.2.5). This finding indicates that the FITC labelling conditions do not affect the biological activity of the proteins. Similarly the GST-EC2CD63 and GST were labelled with rhodamine B isothiocyanate (rhodamine-BITC) and with AlexaFluor 647® as described in section 2.2.9.3 and 2.2.9.4 and the biological activity of the labelled proteins was tested (section 2.2.10.4.1). Labelling appeared successful and the estimates of protein concentration were determined using Protparam Tool software [from the Swiss Protein Database, http://www.expasy.cg, which is based on the calculation of protein extinction coefficients from amino acid sequence data (Gilland Hippel (1989)] as described in section 2.2.9.3.1 (since the dyes interfere with the Bradford assay). The protein concentration and molar dye:protein ratios are given in table.5.2.1. The labelled proteins were biologically active (Fig.5.2.6&Fig.5.2.7).
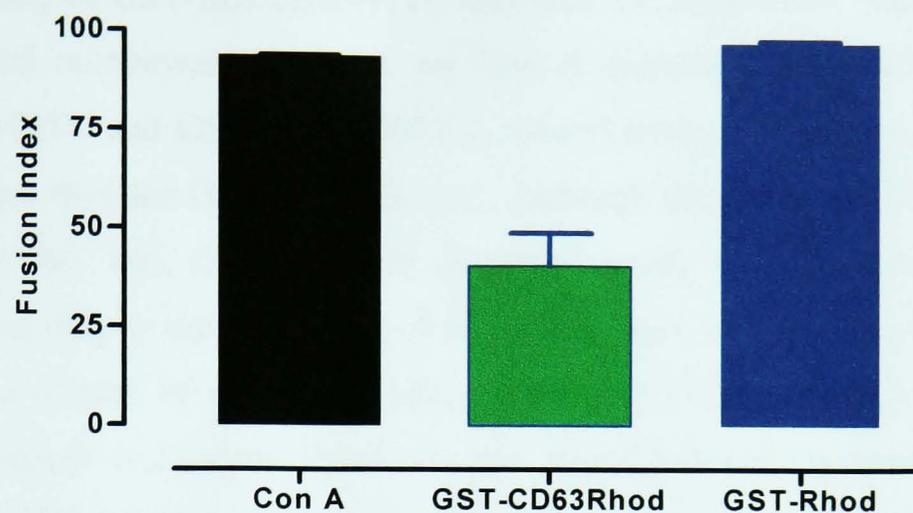
Fig.5.2.6. Biological effect of rhodamine-BITC labelled GST-EC2CD63 and GST on Con A induced monocyte fusion. The experiment was carried out as described in section 2.2.10.4.1 with/without rhodamine labelled GST proteins at $20\mu g.ml^{-1}$. The cells were stained with FITC labelled anti CD52 antibody and the nuclei stained with propidium iodide. The results are the mean values of 1 experiment carried out in triplicate.
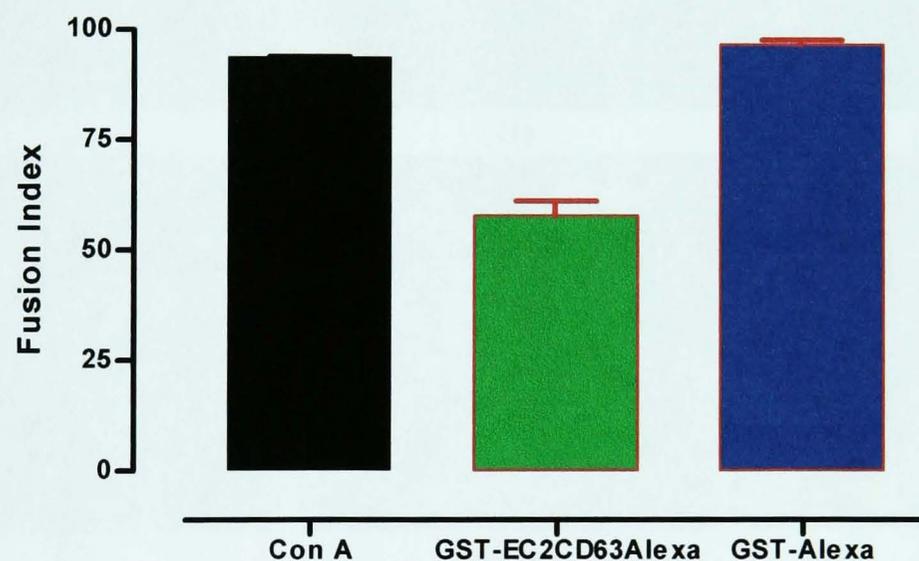


Fig.5.2.7. Biological effect of AlexaFluor 647® labelled GST-EC2CD63 and GST on Con A induced monocyte fusion. The experiment was carried out as described in section 2.2.10.4.1 with/without AlexaFluor 647® labelled GST proteins at $20\mu g.ml^{-1}$. The cells were counter stained with FITC labelled anti CD52 antibody and the nuclei stained with propidium iodide. The results are the mean values of 1 experiment carried out in triplicate.

## 5.2.2.2. Binding of labelled GST-EC2 tetraspanins to monocytes: microscopy

Binding assays with FITC labelled GST-EC2CD63, GST-EC2CD9 and GST were carried out with monocytes cultured for 2hrs in the presence/absence of Con A in Lab-Tek® chamber slides as discussed in section 2.2.10.6.4. The FITC labelled proteins were used at $6\mu g.ml^{-1}$ and $3\mu g.ml^{-1}$ since at $20\mu g.ml^{-1}$ the fluorescence observed was too bright.

Little binding of GST-EC2CD63-FITC and GST-EC2CD9-FITC was observed with unstimulated monocytes. However, on Con A stimulated cells, binding of GST-EC2CD63-FITC and GST-EC2CD9-FITC was observed. The fluorescence intensity was stronger than for GST-FITC binding. Although the staining with FITC labelled GST-EC2CD63 and GST-EC2CD9 appeared good, a bright fluorescence was concentrated only in certain regions of stimulated/fused cells. It was thought that this might be a cluster of receptors/binding motifs for GST-EC2CD63/GST-EC2CD9, which emerged and accumulated on the stimulated cell surface after Con A stimulation (Fig.5.2.8).
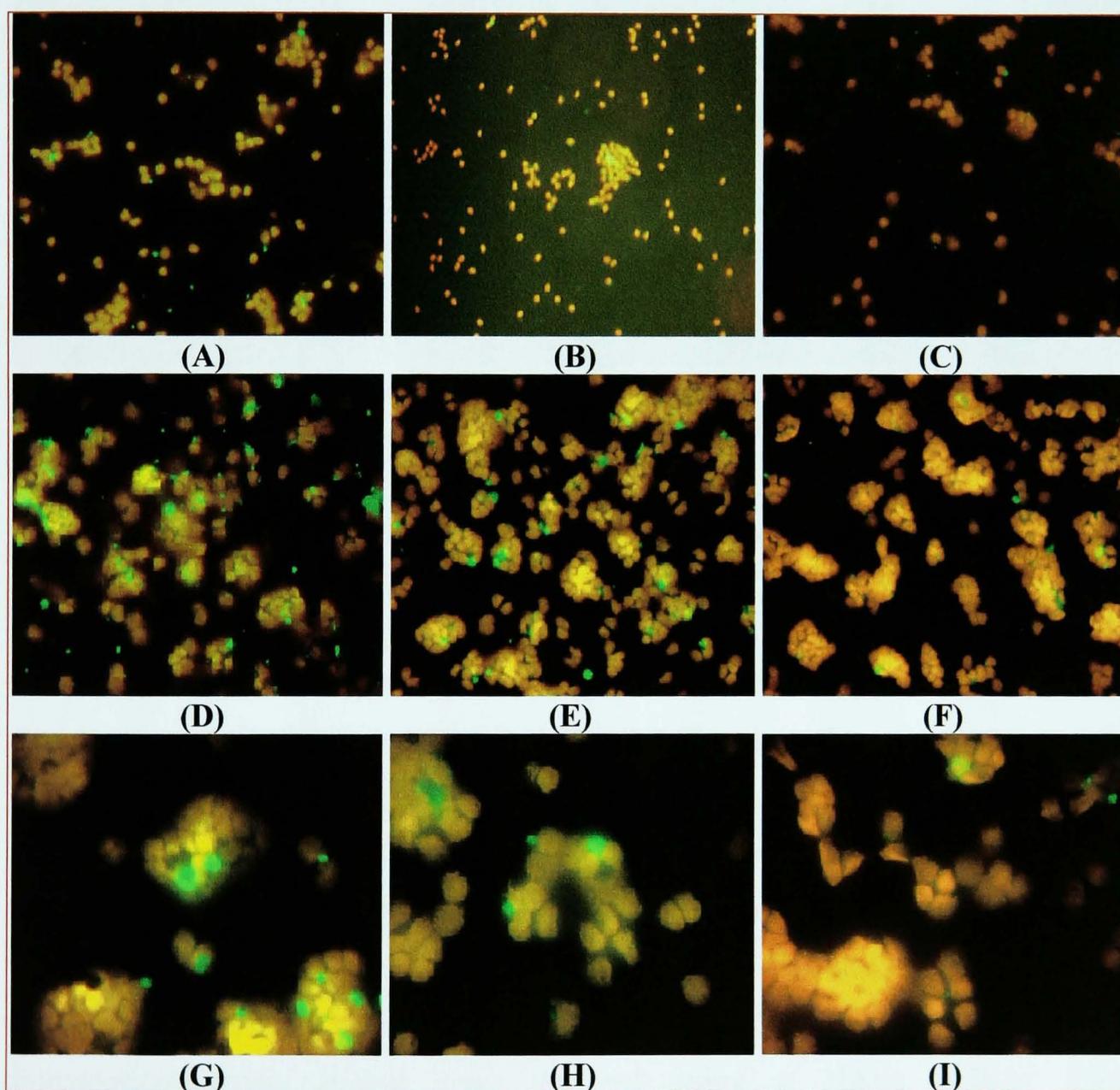


Fig.5.2.8. Binding of FITC labelled GST proteins to monocytes in Lab-Tek® chamber slides. The experiment was carried out as described in section 2.2.10.6.4 using live cells at 4°C with FITC labelled GST-EC2CD63/CD9/GST at 6μg.ml$^{-1}$. After fixation the nuclei of the cells stained with propidium iodide. Unstimulated monocytes (A-C); Con A stimulated monocytes (D-I). Treatments: GST-EC2CD63-FITC (A, D&G); GST-EC2CD9-FITC (B, E&H); GST-FITC (C, F&I). The images were captured with Nikon Eclipse E400 immunofluorescence microscope using 20x objective (A-F); 40x objective (G-I).

The binding of FITC labelled GST-EC2CD63 on 2hrs Con A stimulated monocytes was also assessed with Deltavision restoration microscopy using Lab-Tek® chamber slide preparation. The nuclei of stained cells were counter stained with propidium iodide or bisbenzimide. The quality of the images was disappointing, but GST-EC2CD63-FITC again showed a strong localised binding to some of the stimulated cells (Fig.5.2.9).



(A)                                            (B)
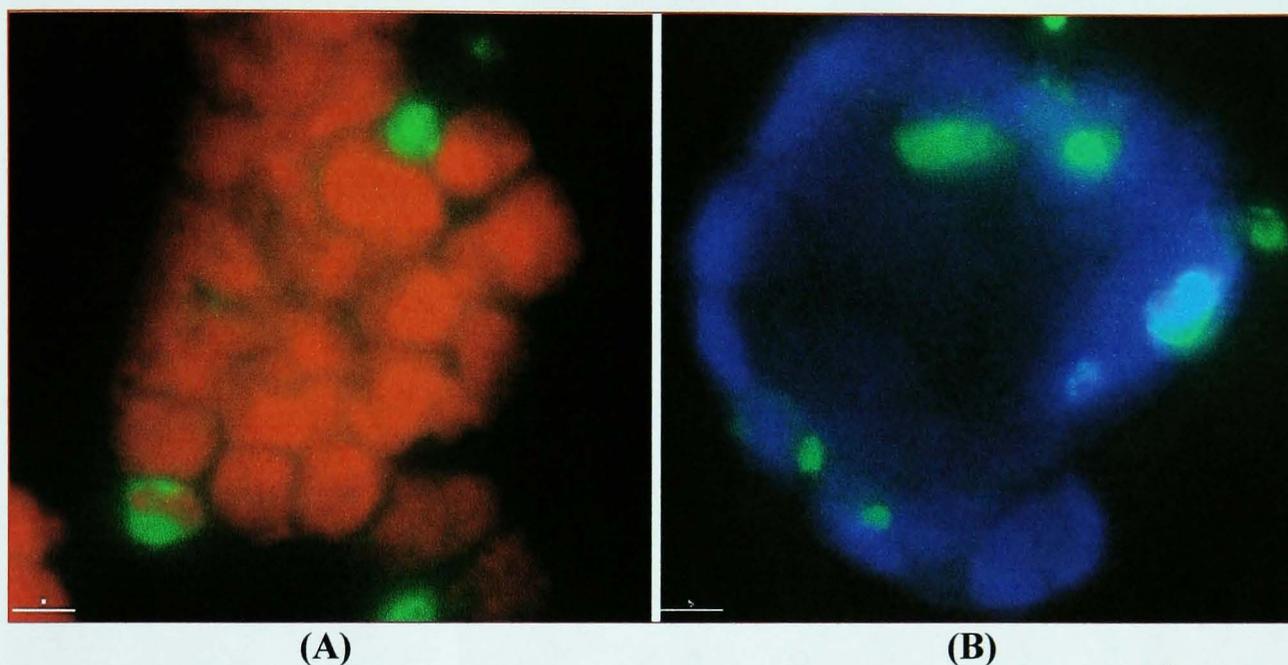
Fig.5.2.9. Binding of FITC labelled GST proteins to monocytes in Lab-Tek® chamber slides. The experiment was carried out as described in section 2.2.10.6.4 with FITC labelled GST-EC2CD63 at $6\mu g.ml^{-1}$. The nuclei of the cells stained either with propidium iodide or bisbenzimide (Hoechst dye) as described in section 2.2.10.3.2, where nuclear staining with propidium iodide (A): bisbenzimide (B). The images were captured with Deltavision restoration microscope using 100x objective.

The binding of FITC labelled GST-EC2CD9/CD63 and GST on Con A stimulated monocytes was also assessed using cytospin slide preparation. In this experiment the monocytes were stimulated with Con A to a final concentration of $10\mu g.ml^{-1}$ for 2hrs (section 2.2.11), the cells were harvested (section 2.2.2.1) and the cytospin slides were prepared as mentioned in section 2.2.4.1 by incubating the fixed and permeabilised cell smear with FITC labelled GST-EC2CD9/CD63 and GST control. The immunofluorescence images were captured using a Nikon Eclipse E400 immunofluorescence microscope at 100x objective.

(A)  (B)

(C)  (D)
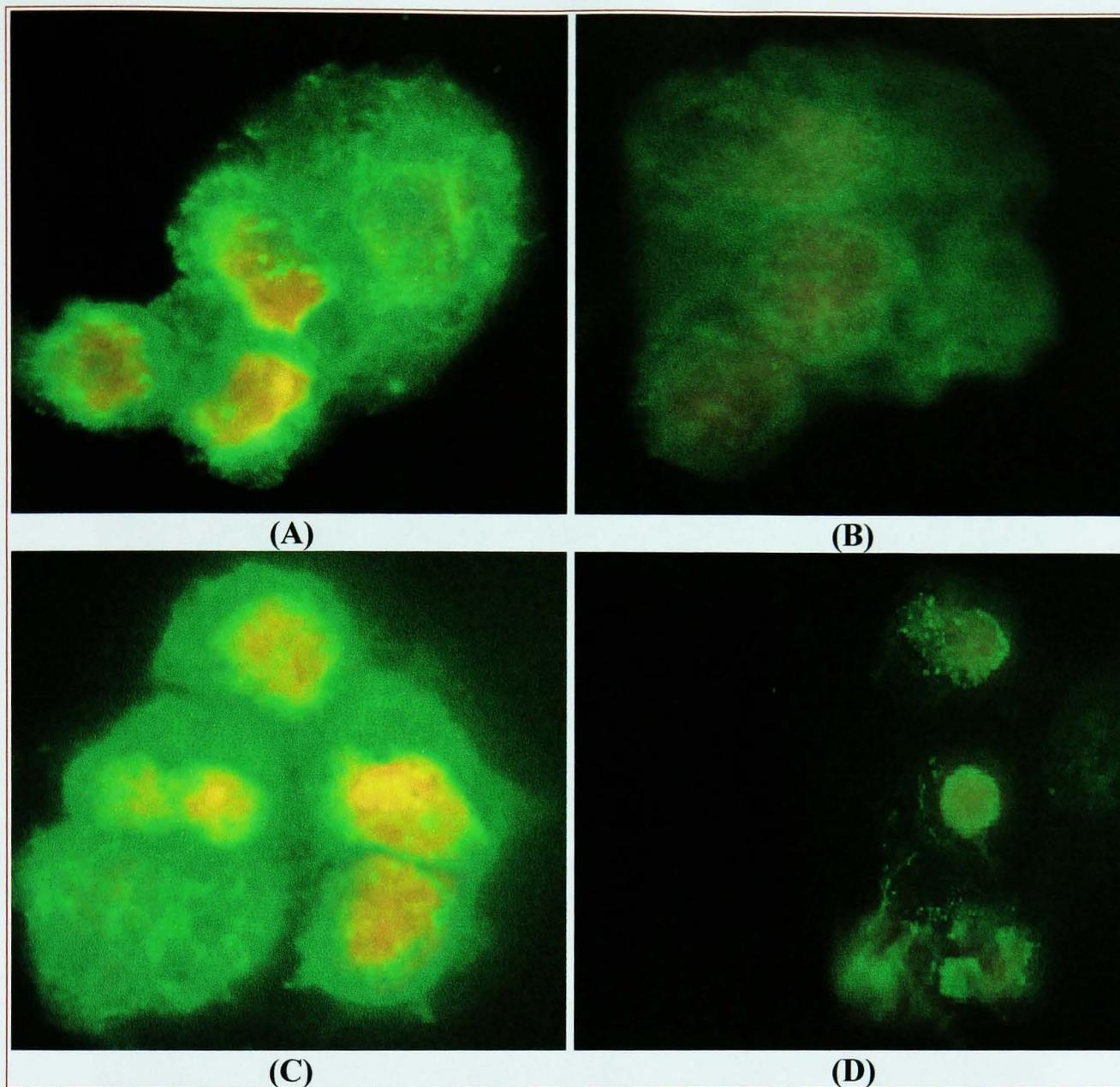
Fig.5.2.10. Binding of FITC labelled GST-EC2 tetraspanins/GST control on 2hrs Con A stimulated monocytes by direct cytospin slide preparation. The cells were fixed in slides with acetone as described in section 2.2.4.1 and incubated with 50μl of FITC labelled GST-EC2CD63/GST at 6μg.ml$^{-1}$ for 1hr at 4°C. The nuclei of cells were stained with propidium iodide and the slide was processed as mentioned in section 2.2.4.1. The different treatments are: Con A stimulated monocytes stained with GST-EC2CD63-FITC (A)&(C); GST-FITC (B)&(D). The immunofluorescence images were captured with Nikon Eclipse E400 immunofluorescence microscope using 100x objective.

Stronger binding was observed with FITC labelled GST-EC2CD63 than GST on Con A stimulated monocytes (Fig.5.2.10). In this case, all cells appeared to be stained but it was not really possible to determine from these images what cell structures are stained.

A final attempt was made to use Deltavision restoration microscopy to investigate the binding of rhodamine BITC labelled GST-EC2CD63 and GST proteins on 2hrs Con A stimulated monocytes in Lab-Tek® chamber slides. The cells were counter stained

with anti CD52-FITC antibody as described in section 2.2.10.3.2. The Deltavision restoration microscopy images were captured using the 100x objective.

**(A)**
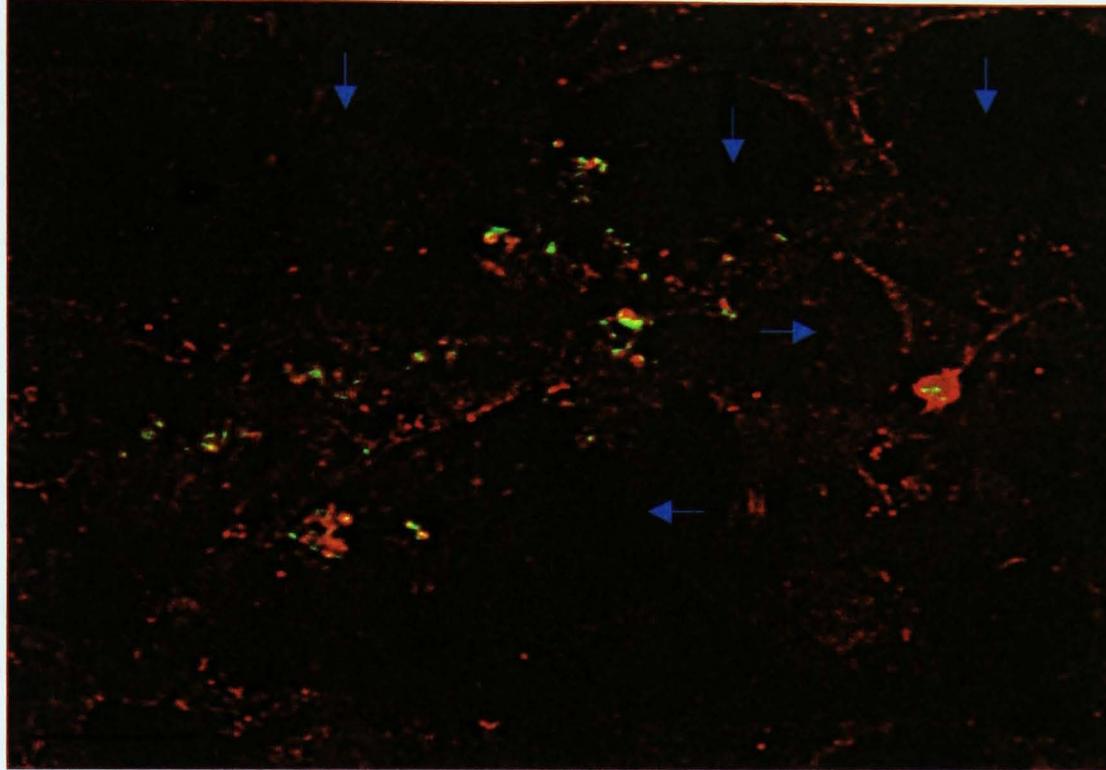


**(B)**
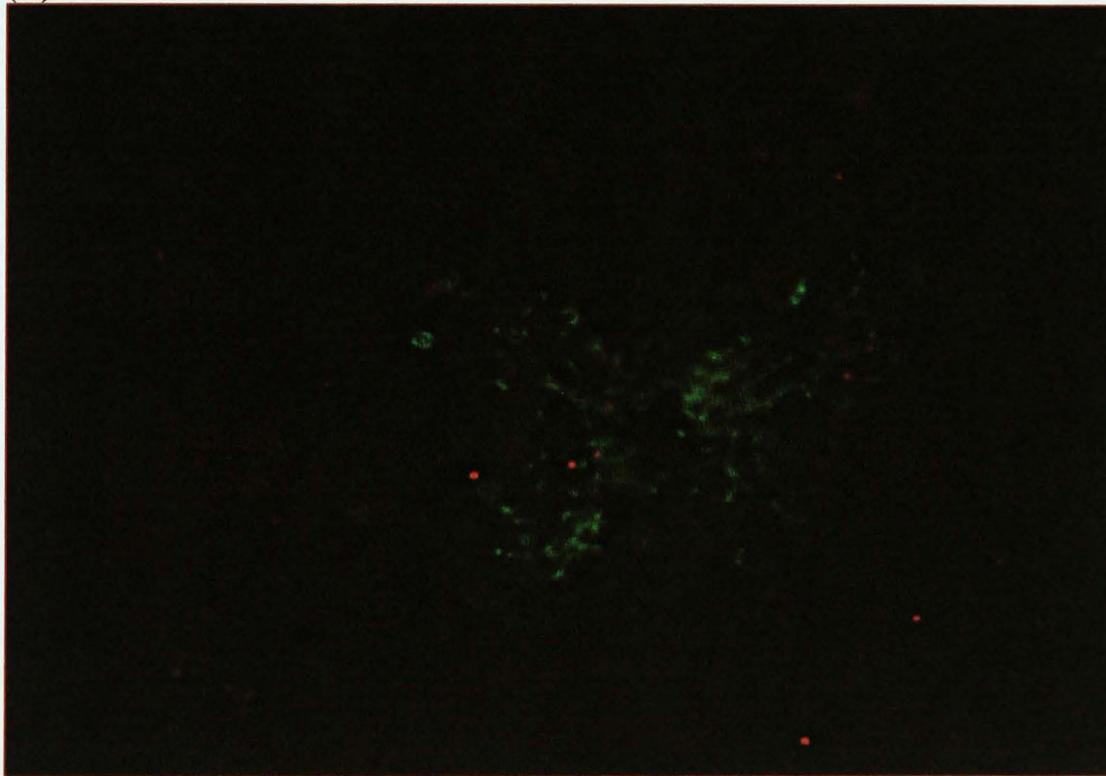


Fig.5.2.11. Binding of rhodamine BITC labelled GST proteins on Con A stimulated monocytes. The experiment was carried out as described in section 2.2.10.6.4 with rhodamine BITC labelled GST-EC2CD63/GST at $6\mu g.ml^{-1}$. The cells were counter stained with FITC labelled anti CD52 antibody as described in section 2.2.10.3.2. The images were captured with Deltavision restoration microscope using 100x objective. Where Con A stimulated monocytes with GST-EC2CD63-rhodamine BITC (A); GST-rhodamine BITC (B). The blue arrows represent the nuclei of fusing/fused monocytes.

The GST-EC2CD63 rhodamine BITC showed some preferential binding with 2hrs Con A stimulated monocytes relative to GST-rhodamine BITC (Fig.5.2.11). Again, all cells here appeared to give some staining. There appeared to be granular staining with GST-EC2CD63 around the nucleus of Con A stimulated cells. Unfortunately, the anti CD52 counter stain did not give bright staining here. We were unable to repeat this experiment to obtain clear images of the cellular structure due to problems with Departmental Deltavision microscopy facility during the later stages of the project.

### 5.2.2.3. Binding of labelled GST-EC2 tetraspanins to monocytes: FACS analysis

To assess binding of GST-EC2s quantitatively flow cytometry (FACS) analysis was used (section 2.2.10.6.2). Monocytes were cultured in the presence or absence of Con A for 2hrs prior to harvesting and analysis of binding. As described in section 4.2.7, scatter profiles of unstimulated and Con A stimulated monocytes (Fig.5.2.12) indicated that there were four distinct cell populations for unstimulated cells and five distinct cell populations with Con A stimulated cells.



(A)                                    (B)
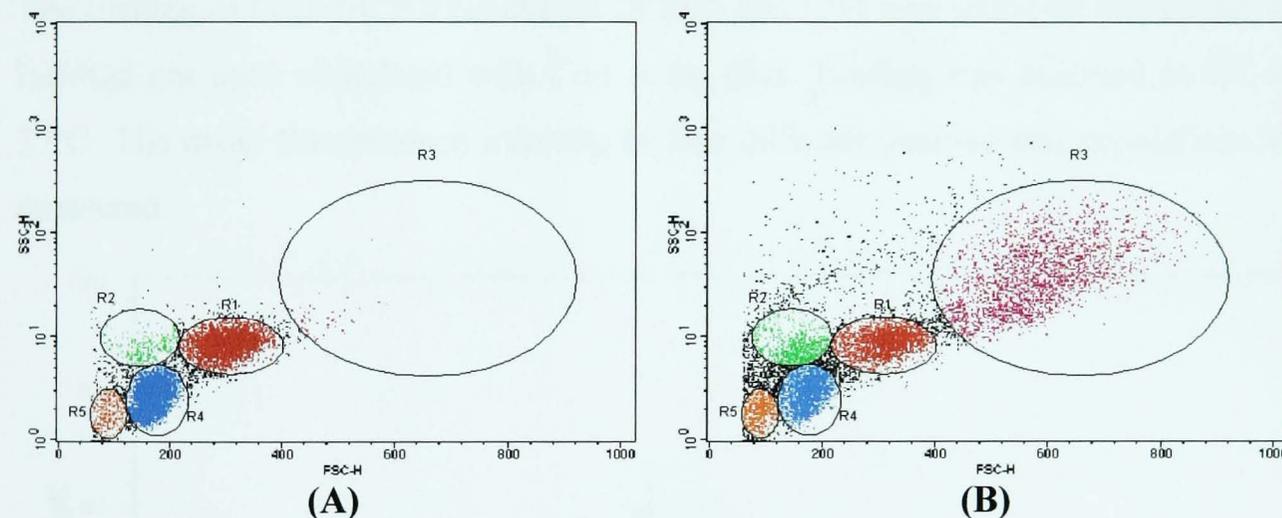
Fig.5.2.12. Scatter profiles of flow cytometry analysis with unstimulated and Con A stimulated monocytes. The cells were separated on the basis of their granularity and size: R1=Monocytes (CD14 +ve); R2=Monocytes (CD14 +ve); R3=MGCs (CD14 +ve); R4=Lymphocytes (CD14–ve) and R5=Unknown lymphocyte population (CD14 –ve). Unstimulated (A); 2hrs Con A stimulated (B) leucocytes.

As described in chapter 4, based on their scatter profiles and staining with the monocyte marker (anti CD14) and lymphocyte/monocyte marker (anti CD52) the cells and their corresponding regions are: R1=monocytes; R2=monocytes; R3=MGCs; R4=lymphocytes and R5=unknown lymphocyte population. The population R5 is CD14 negative and CD52 positive hence it is not a monocyte population but it could be another lymphocyte population. In addition Con A stimulation appears to induce increased cell numbers of the R5 population when

compared to unstimulated cells in the same gate. The percentages of cells gated in the different regions are given table 5.2.2.

| Leucocytes | Total numbers of cells in different populations (%) | | | | |
|---|---|---|---|---|---|
| | R1 | R2 | R3 | R4 | R5 |
| Unstimulated | 47.06 | 3.16 | 0.49 | 47.42 | 1.82 |
| Con A stimulated | 25.43 | 10.00 | 27.45 | 30.50 | 6.57 |

Table.5.2.2. Total numbers of cells in different populations (%) gated during the FACS analysis of leucocytes. R1=Monocytes (CD14 +ve); R2=Monocytes (CD14 +ve); R3=MGCs (CD14 +ve); R4=Lymphocytes (CD14–ve) and R5=Unknown population (CD14 –ve).

From these studies it is clear that changes in monocyte morphology occur upon stimulation with Con A and this result reconfirms the observation that made in section 4.2.7. Our study mainly focussed on the binding of FITC labelled GST-EC2 tetraspanins on large multinucleated giant cells of monocytes (MGCs) apart from other cell populations.

The binding of GST-EC2CD9/GST-EC2CD63 and GST was tested on monocytes that had/had not been stimulated with Con A for 2hrs. Binding was assessed at 4°C and 37°C. The mean fluorescence intensity of five different positive cell populations was measured.
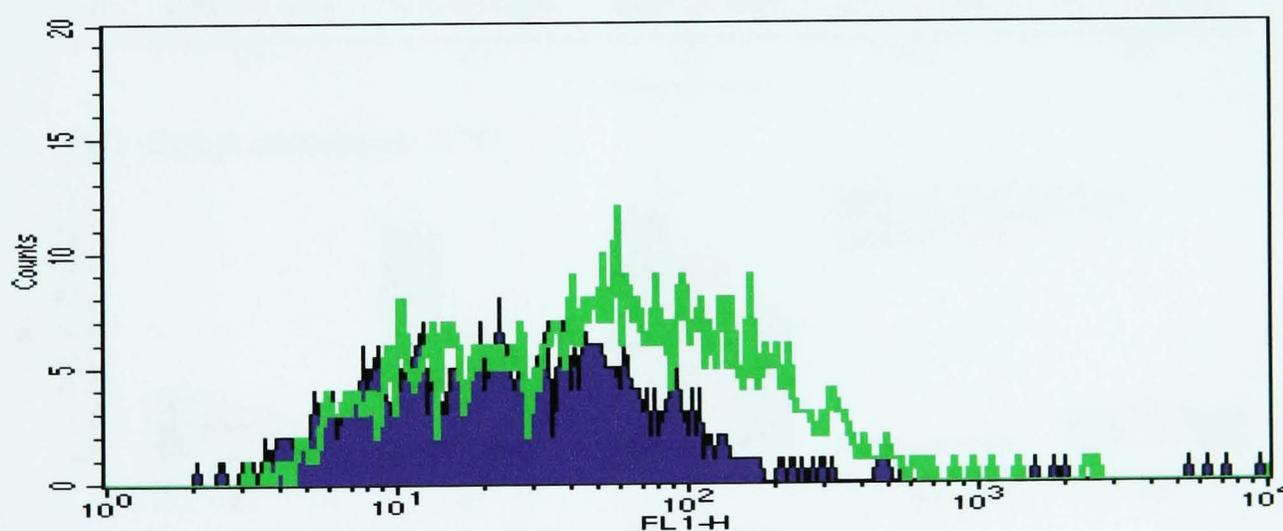


Fig.5.2.13. Binding of FITC labelled GST-EC2CD63 and GST control on 2hrs Con A stimulated monocytes. The plot shows the number of gated cells (counts) as a function of their fluorescence intensity (FL1-H) in R3 (MGCs), where GST-EC2CD63-FITC binding shift (Green); GST-FITC binding shift (Blue).

**(A) Unstimulated: 4°C**

**(B) Con A stimulated: 4°C**

**(C) Unstimulated: 37°C**

**(D) Con A stimulated: 37°C**
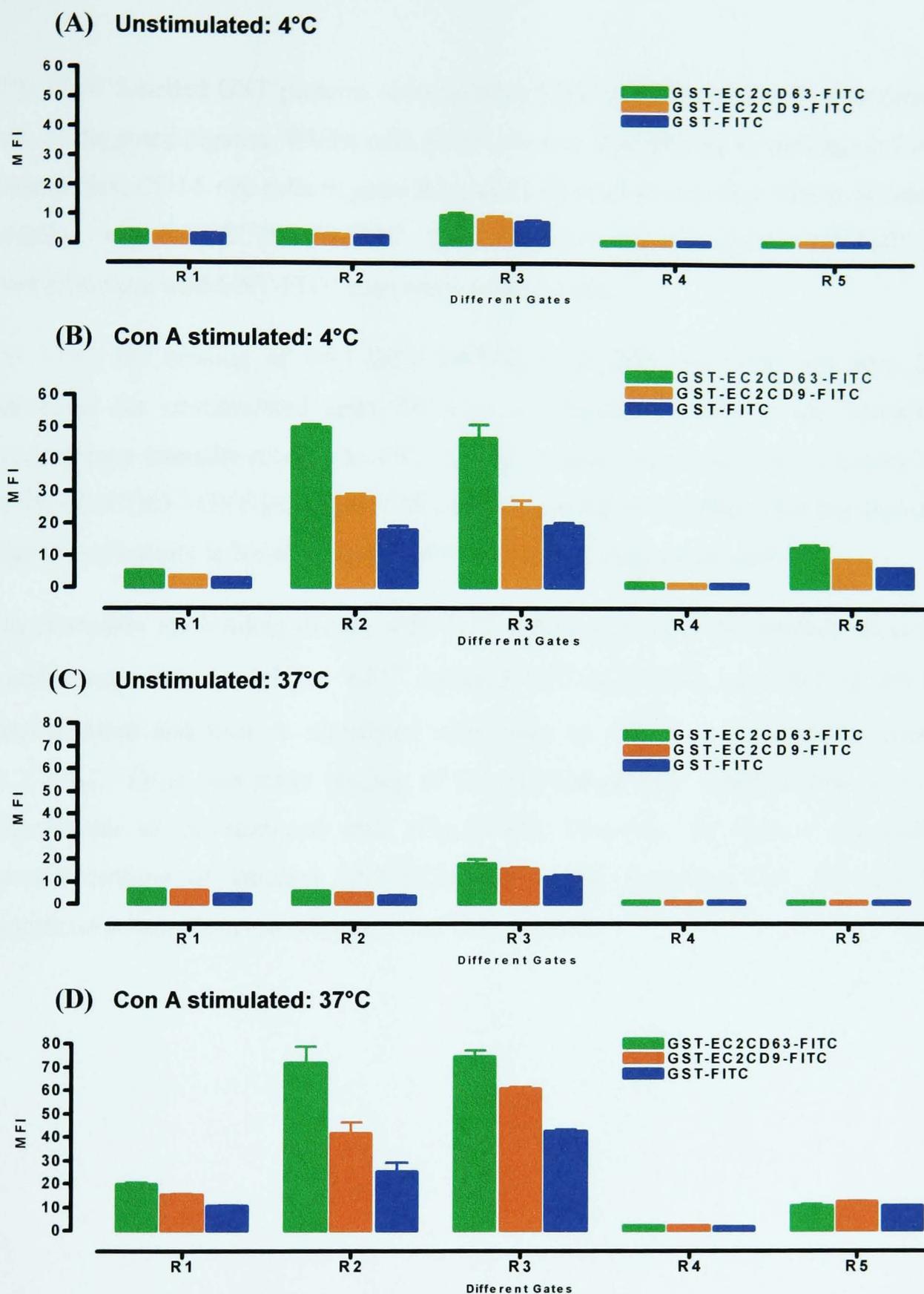
Fig.5.2.14. Binding of FITC labelled GST-EC2CD63, GST-EC2CD9 and GST to cells cultured in the presence/absence of Con A (10µg.ml$^{-1}$ for 2hrs). Binding of recombinant GST-EC2 tetraspanins (6µg.ml$^{-1}$) and GST (12µg.ml$^{-1}$) was assessed at 4°C or 37°C as described in section 2.2.10.6.2 by FACS. Where R1=Monocytes (CD14+ve); R2=Monocytes (CD14+ve); R3=MGCs (CD14+ve); R4=Lymphocytes (CD14–ve) and R5=Unknown lymphocyte population (CD14–ve). Unstimulated (A&C); 2hrs Con A stimulated (B&D) leucocytes. Binding study at 4°C (A&B); 37°C (C&D).

The FITC labelled GST proteins showed little binding to unstimulated monocytes in any of the gated regions. Whilst cells in R1 showed little change in binding on Con A stimulation, CD14 +ve cells in gates R3 and R2 showed around five fold more intense staining with GST-EC2CD63-FITC, three fold more with GST-EC2CD9-FITC and two fold more with GST-FITC than unstimulated cells.

At 37°C, the binding of GST-EC2CD9/GST-EC2CD63 and GST was very little increased for unstimulated cells. On Con A stimulation, there is an increase in fluorescence intensity relative to 4°C, but the overall pattern observed is similar (i.e. GST-EC2CD63 >GST-EC2CD9>GST) for R2 and R3 monocytes. This indicates that there is selectivity in binding/uptake of the GST-EC2 tetraspanin proteins.

To reconfirm the binding results with FITC labelled proteins the binding assay was carried out with AlexaFluor 647® labelled GST-EC2CD63 and GST at 4°C on unstimulated and Con A stimulated monocytes by FACS as described in section 2.2.10.6.2. There was some binding of the AlexaFluor 647® labelled proteins to all populations of unstimulated cells (Fig.5.2.15). However, on Con A stimulation, greater binding of labelled GST-EC2CD63 to the monocyte (R1, R2 and R3) populations was observed relative to the GST control.

**(A)**



**(B)**
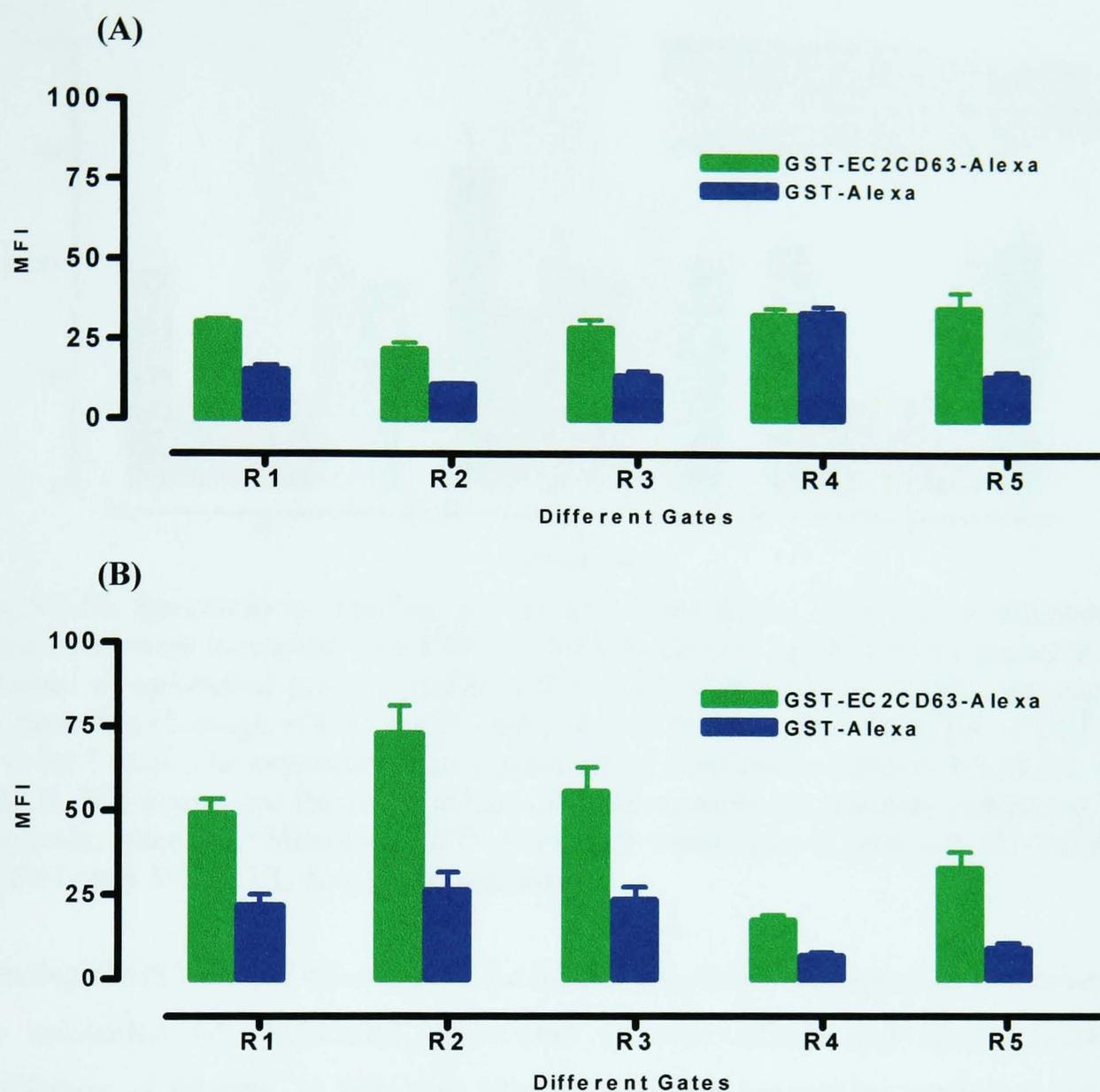


Fig.5.2.15. Binding of AlexaFluor 647® labelled GST-EC2CD63 and GST to cells cultured in the presence/absence of Con A (10µg.ml$^{-1}$ for 2hrs). Binding of recombinant proteins (6µg.ml$^{-1}$) was assessed at 4°C as described in section 2.2.10.6.2 by FACS. Where R1=Monocytes (CD14+ve); R2=Monocytes (CD14+ve); R3=MGCs (CD14 +ve); R4=Lymphocytes (CD14–ve) and R5=Unknown lymphocyte population (CD14–ve). Unstimulated (A); 2hrs Con A stimulated (B) leucocytes. The results are the mean value of 1 experiment carried out in triplicate (A); 2 independent experiment in triplicate (B).

To determine if this binding was specific, attempts were made to block the binding of GST-EC2CD63-Alexa to Con A stimulated cells with excess unlabelled recombinant proteins.
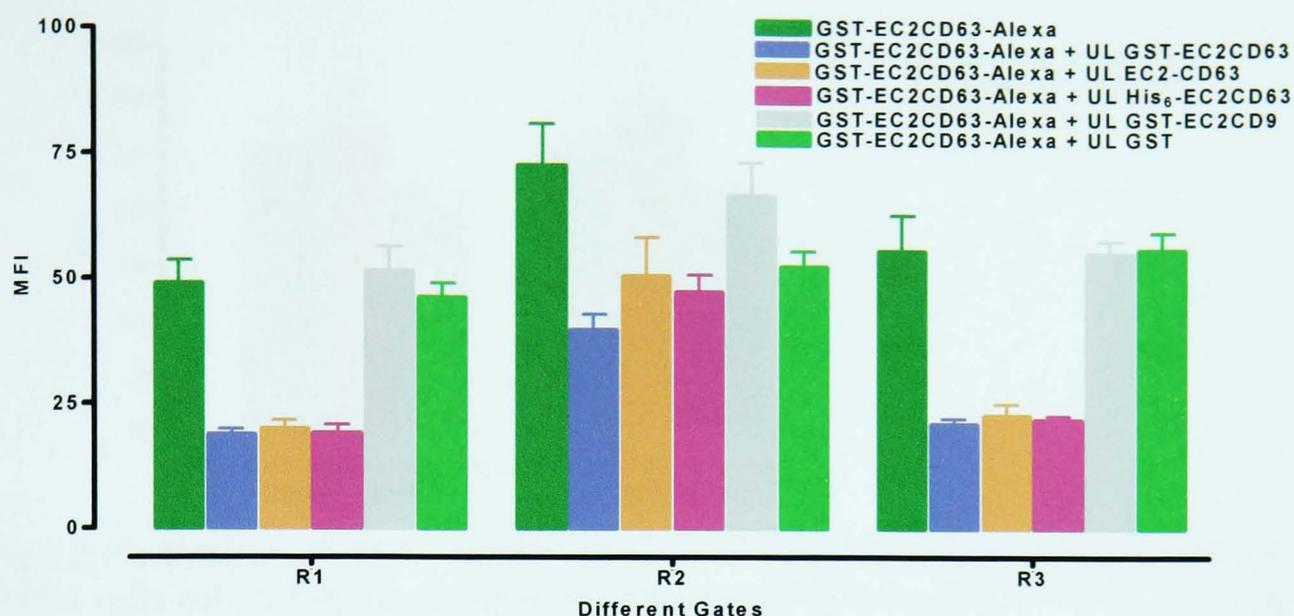
Fig.5.2.16. Specificity of binding of GST-EC2CD63-Alexa. 2hrs Con A stimulated monocytes were incubated with GST-EC2CD63-Alexa (6μg.ml$^{-1}$) in the presence or absence of unlabelled proteins (100μg.ml$^{-1}$): GST-EC2CD63, EC2CD63 (generated by thrombin cleavage of GST-EC2CD63), His$_6$-EC2CD63, GST-EC2CD9 or GST at 4°C. by FACS. The experiment was carried out as described in section. 2.2.10.6.2 by FACS. The results are the mean values of 2 independent experiments carried out in triplicate, where R1=Monocytes (CD14+ve); R2=Monocytes (CD14+ve); R3=MGCs (CD14 +ve). Where UL denotes unlabelled.

Binding of GST-EC2CD63-Alexa to the R3 sub population was specifically inhibited by unlabelled GST-EC2CD63, EC2CD63 (cleaved CD63) and His$_6$-EC2CD63 (inhibition of 69.09%, 61.90% and 70% respectively) but not by unlabelled GST-EC2CD9 or GST. Binding of GST-EC2CD63 to R1 monocytes also appeared specific, since a similar inhibition pattern was observed. The specificity of binding to the R2 population is less clear, since unlabelled GST gave some inhibition. Overall, however, the results indicate that GST-EC2CD63 can bind to Con A stimulated monocytes specifically (Fig.5.2.16).

### 5.2.2.4. Binding of fluorescent labelled GST-EC2 tetraspanins to THP-1 cells: FACS analysis

The binding of FITC labelled GST-EC2CD9, GST-EC2CD63 and GST was tested on unstimulated and Con A stimulated THP-1 cells at 4°C by FACS as described in section 2.2.10.6.2. The results are given in Fig.5.2.17.
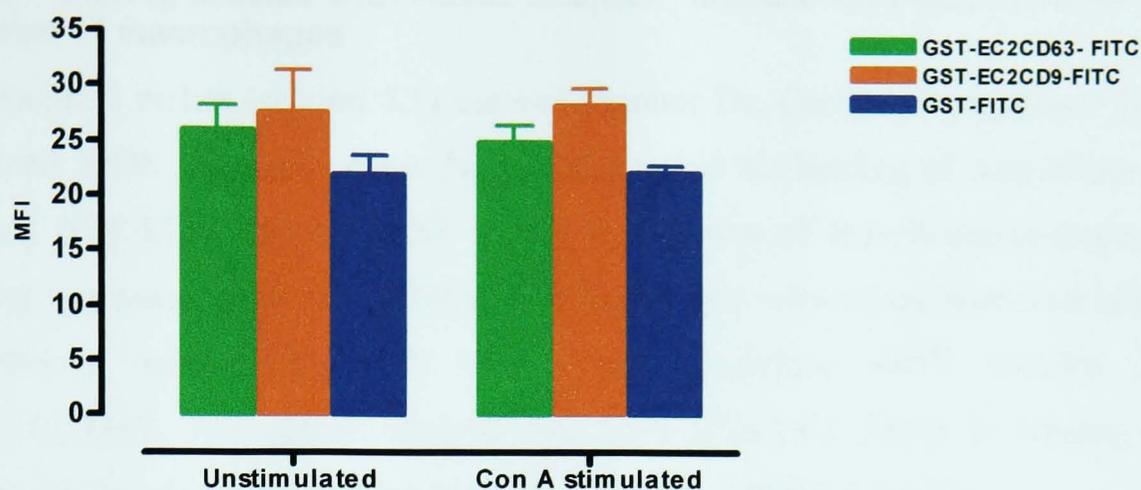
Fig.5.2.17. Binding of FITC labelled GST-EC2CD63, GST-EC2CD9 and GST on THP-1 cells cultured in the presence/absence of Con A ($10\mu g.ml^{-1}$ for 2hrs). Binding of recombinant proteins ($6\mu g.ml^{-1}$) was assessed at 4°C as described in section 2.2.10.6.2 by FACS. The results are the mean values of 1 experiment carried out in triplicate.

No difference in binding between unstimulated and Con A stimulated cells was observed. It indicates that GST-EC2CD63 and GST-EC2CD9 may only bind to cells capable of fusing.

Similarly the binding of AlexaFluor 647® labelled GST-EC2CD63 and GST was tested on unstimulated and Con A stimulated THP-1 cells at 4°C by FACS as described in section 2.2.10.6.2 (Fig.5.2.18).
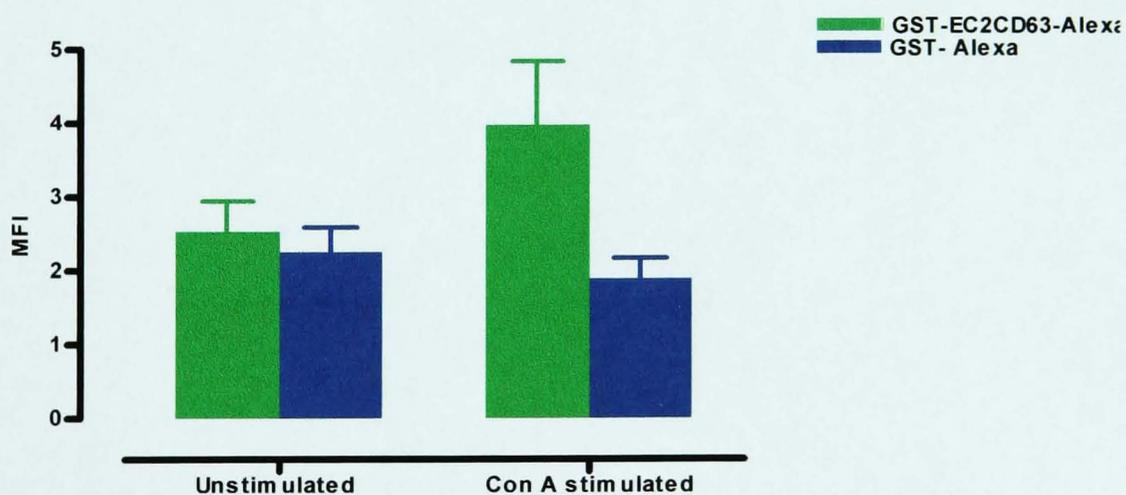


Fig.5.2.18. Binding of AlexaFluor 647® labelled GST-EC2CD63 and GST on THP-1 cells cultured in the presence/absence of Con A ($10\mu g.ml^{-1}$ for 2hrs). Binding of recombinant proteins ($6\mu g.ml^{-1}$) was assessed at 4°C as described in section 2.2.10.6.2 by FACS. The results are the mean values of 1 experiment carried out in triplicate.

A slight increase in GST-EC2CD63-Alexa binding was observed on Con A stimulation. However, the mean fluorescence intensities observed with GST-EC2CD63-Alexa on unstimulated and Con A stimulated THP-1 cells were very low (~15 times less than the values observed with monocytes).

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### 5.2.2.5. Binding studies with AlexaFluor 647® labelled GST-EC2CD63 on HIV-1 infection of macrophages

As described earlier (section 5.1) our collaborator Dr. Cecilia Cheng-Mayer (Aaron Diamond AIDS Research centre, New York) tested the binding of AlexaFluor 647® labelled GST-EC2CD63 and GST in HIV-1 infection of human macrophages. The binding was assessed by using Deltavision restoration microscopy with dual labelling experiments using Vpr-eGFP virus and AlexaFluor 647® labelled GST-EC2CD63/GST. The results showed that GST-EC2CD63 Alexa is binding more strongly to the macrophages than GST-Alexa (Fig.5.2.19&Fig.5.2.20).



(A)　　　　　　　　　　　　　　　(B)

(C)　　　　　　　　　　　　　　　(D)
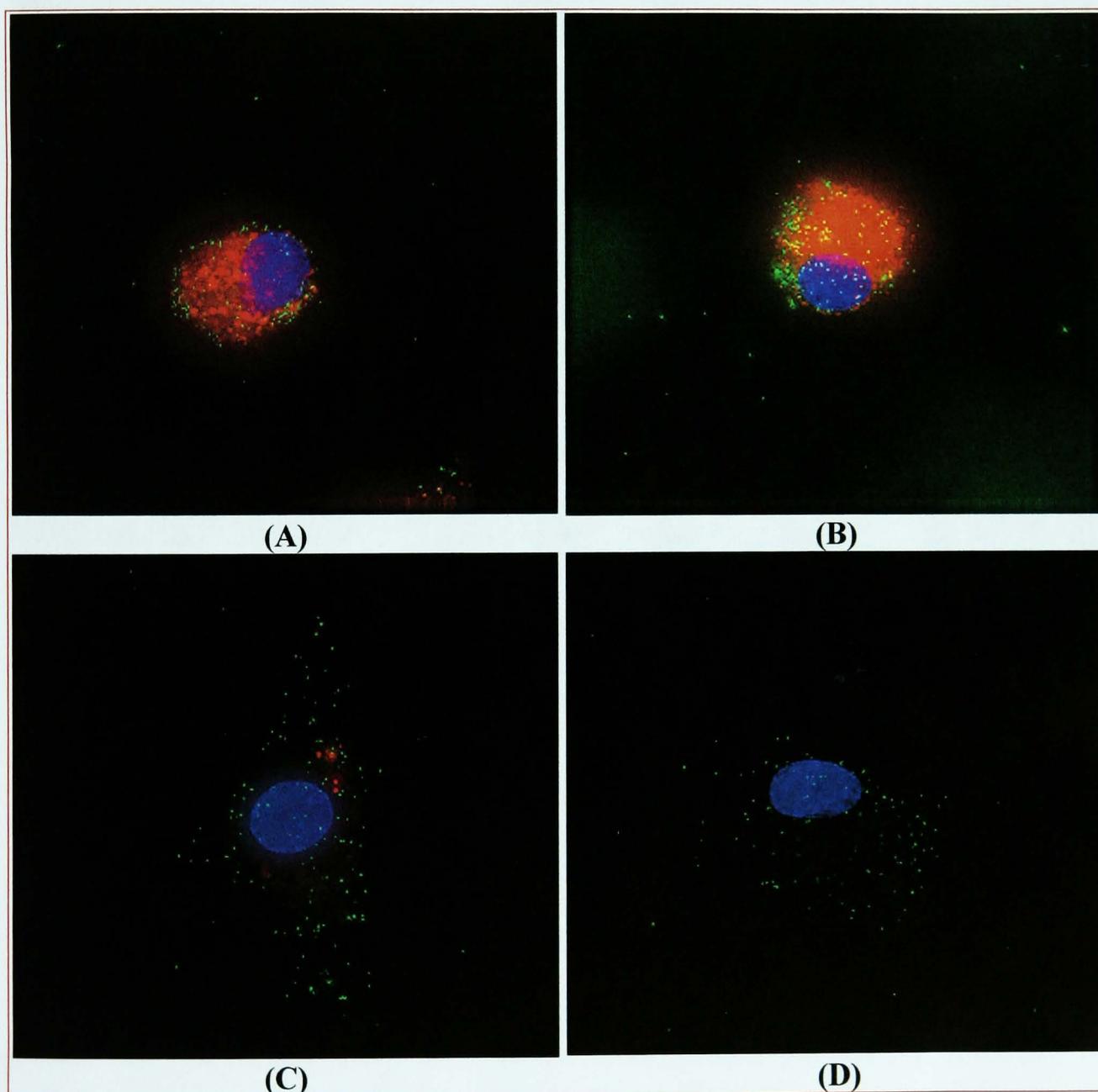
Fig.5.2.19. Binding of GST-EC2CD63AlexaFluor 647® with HIV-1 infection of peripheral blood macrophages. The cells were pre incubated with 0.11µM GST-EC2CD63/GST labelled with AlexaFluor 647® (red) for 1hr before the addition of Vpr-eGFP virus (green). After fixation and permeabilization, cell membranes were stained with Con A-rhodamine (blue). Treatments: GST-EC2CD63 Alexa (A&B); GST-Alexa (C&D). The images were captured with Deltavision restoration microscope.
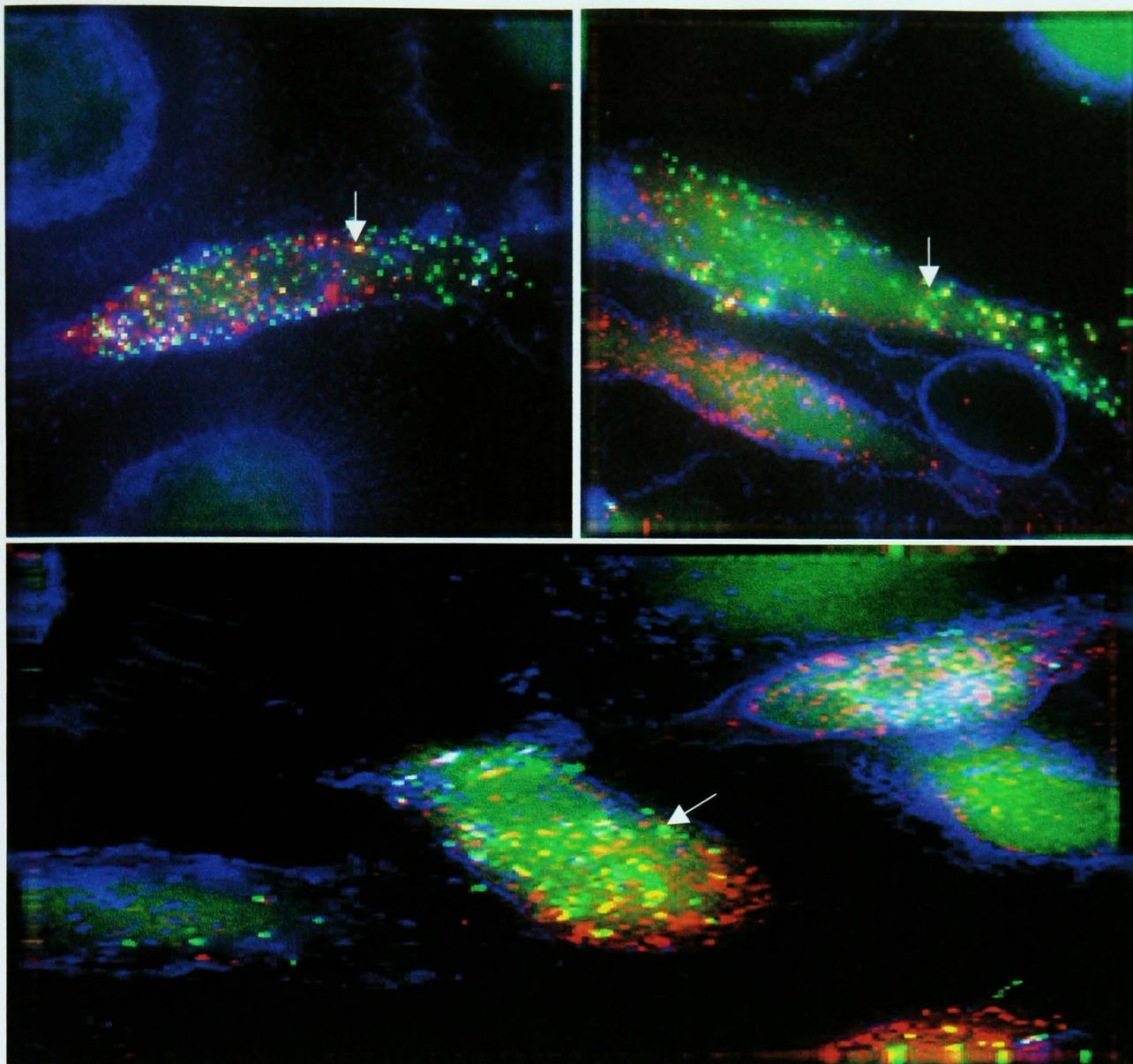
Fig.5.2.20. Binding of GST-EC2CD63 AlexaFluor 647® with HIV-1 infected peripheral blood macrophages/monoyctes. The cells were pre incubated with 0.11μM GST-EC2CD63 labelled with AlexaFluor 647 (red) for 1hr before the addition of Vpr-eGFP virus (green). After fixation and permeabilization, cell membranes were stained with Con A-rhodamine (blue). Three representative images from Deltavision restoration microscopy are shown, with co-localisation of virus and CD63EC2 indicated by arrows.

## 5.2.2.6. Binding of FITC-labelled GST-EC2 tetraspanins with mouse oocytes

The FITC, AlexaFluor 647® and rhodamine BITC labelled GST-EC2CD63 and GST-EC2CD9 showed binding reactivity in our experiments on monocyte fusion and in studies by our collaborator Dr. Cecilia Cheng-Mayer on HIV-1 infection of macrophages. We further evaluated the binding of FITC labelled GST-EC2CD9 and GST-EC2CD151 on mouse oocytes at our collaborator Dr. Eileen McLaughlin's laboratory at University of Newcastle, Australia. The experiment was carried out as described in section. 2.2.10.7.
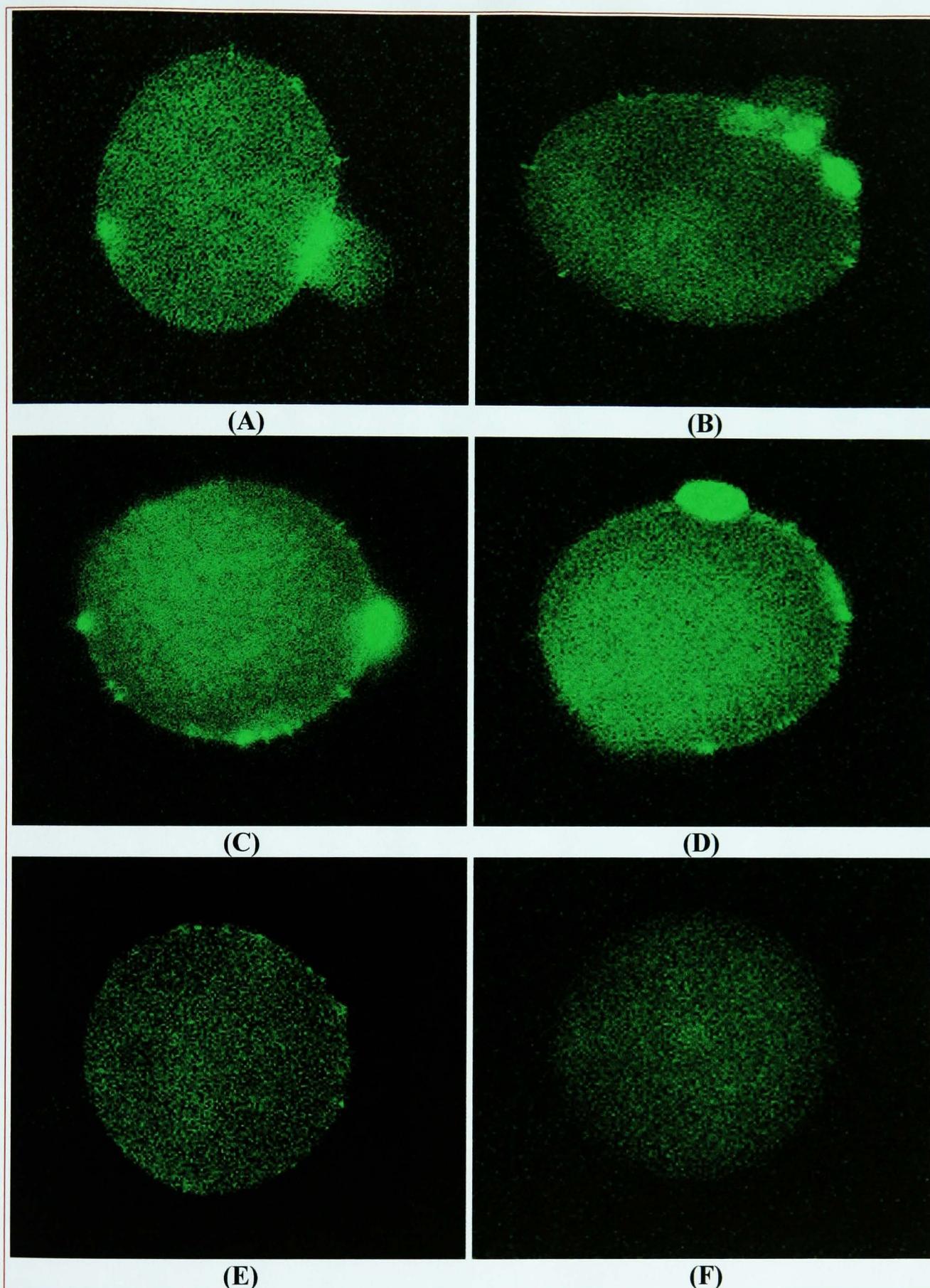
Fig.5.2.21.Binding of FITC labelled GST-EC2 tetraspanins to oocytes. The experiment was carried out as described in section 2.2.10.7 with FITC labelled GST-EC2CD9/CD151/GST at $6\mu g.ml^{-1}$. The images were captured using Nikon Eclipse E400 immunofluorescence microscope. GST-EC2CD9-FITC (A&B); GST-EC2CD151-FITC (C&D); GST-FITC (E); Media control (F). The images were captured with Carl-Zeiss confocal microscope using 250x objective.

The results showed that the GST-EC2CD9-FITC and GST-EC2CD151-FITC are binding to mouse oocytes more strongly than GST-FITC or the media control. The binding is prominent at the microvilli region of the oocytes. This indicates that the binding of GST-EC2-FITC labelled proteins is specific, although it would be necessary to show inhibition of binding by unlabelled proteins to confirm this.

## 5.3. DISCUSSION

The experiments described in chapter 4 indicated that the effects of CD63 and CD9 EC2s on MGC formation were not directly related to inhibitory effects on cell adhesion, aggregation or cell numbers. To further investigate their possible mechanism of actions, attempts were made to determine whether they were binding, or being specifically taken up, by monocytes. It was not possible to unequivocally demonstrate binding of GST-EC2 tetraspanins using indirect techniques due to non-specific binding of secondary reagents and also the binding was not very strong. Labelling of GST-EC2CD9 and GST-EC2CD63 with fluorescent dye (FITC/rhodamine BITC/AlexaFluor 647®) was therefore attempted. The conjugations were successful and yielded proteins that were still biologically active (i.e. inhibited MGC formation). Microscopic analysis with FITC labelled proteins showed some preferential binding of GST-EC2CD63 and GST-EC2CD9 to Con A stimulated cells, although GST alone was also binding to some extent. Interestingly, in some preparations it appeared that there was bright labelling of only certain Con A stimulated cells. As mentioned in chapter 1, studies using knockout mice have very recently shown that the seven-transmembrane spanning molecule DC-STAMP is crucial to the cell-cell fusion giving rise to osteoclasts and FBGCs (Yagi *et al*, 2005). It was shown that osteoclast precursors from the DC-STAMP knockout mice could still undergo fusion with precursors from wild type mice. This suggests that expression of DC-STAMP only by a "founder" cell that initiates fusion is necessary. This is consistent with one macrophage taking the lead in "cellocytosing" another (Vignery, 2005a; Vignery, 2005b). Vignery suggests that once fusion of two cells has been initiated, that cell becomes the "leader" or "master" fuser and can fuse with other monocyte or multinucleated cells (Vignery, 2005b). Possibly labelled tetraspanin EC2s might be staining such "master" cells. However, other microscopy data was not consistent with this. Unfortunately, due to problems with Departmental Deltavision microscopy facility during these studies, it was difficult to obtain clear images of the cellular structures that the GST-EC2 proteins were labelling. Had time permitted these experiments would have been repeated and extended e.g. using subcellular markers. It would also have been interesting to look at different time points after Con A stimulation.

To try to verify if specific binding was occurring and to obtain more quantitative data, binding of the labelled GST-EC2 proteins was analysed by flow cytometry. FACS analysis showed increased binding of GST-EC2CD63 and GST-EC2CD9 on Con A stimulated monocytes relative to the GST control. FITC labelled GST-EC2CD63 and GST-EC2CD9 showed good binding at 4°C, which indicates specific binding, not just uptake. Increased staining was observed at 37°C, which presumably reflects some uptake, but the preferential staining of GST-EC2CD63 and GST-EC2CD9 relative to GST was still apparent, indicating specificity. GST-EC2CD63 labelled with AlexaFluor 647® also showed preferential binding to Con A stimulated monocytes, and this conjugate appeared more stable than FITC-labelled protein (FITC appeared to dissociate from the proteins on storage leading to reduced specific binding and higher backgrounds). Since GST also shows some binding to Con A stimulated monocytes the specificity of GST-EC2CD63 binding was assessed using excess unlabelled protein to try to block binding of labelled protein. Unlabelled GST-EC2CD63, EC2CD63 (generated by thrombin cleavage from the GST fusion protein) and $His_6$-EC2CD63 inhibited the binding of GST-EC2CD63-Alexa to Con A stimulated monocytes. However, no inhibition of binding to the R1 and R3 populations of monocytes was seen with unlabelled GST or GST-EC2CD9, indicating that the binding was CD63-EC2 specific.

It is not yet clear how the binding of the tetraspanin EC2 regions to Con A stimulated monocytes relates to their inhibition of MGC formation. Both indicate a direct interaction of EC2CD63 with a monocyte membrane protein. It is possible that the proteins are binding to an as yet unidentified receptor and it will be of great interest to try to identify the molecule/s that these proteins are interacting with. The receptor could be a known tetraspanin-associated molecule, such as an integrins, or may be a newly emerging fusion receptor. If time had permitted, we might have carried out immuno-precipitation and cross-linking studies to identify the binding motif of EC2CD63 and associated molecular partners respectively. To date, no direct interaction of CD63 with other proteins has been reported, although many secondary or tertiary interactions, probably within TEMs, are known (Berditchevski, 2001; Hemler, 2003). Such interactions are assumed to be weak or indirect. However, it is possible that the recombinant EC2s, particularly if present as dimers or higher multimers (Kitadokoro et al, 2001) could interact with TEM components with

sufficient affinity to give detectable binding. Hence EC2 might bind to TEM-associated proteins such as integrins and disrupt interactions required for fusion. It is also conceivable that the EC2s might participate in homotypic or heterotypic interactions with tetraspanins expressed on the monocyte membranes.

The binding of GST-EC2CD9-FITC with Con A stimulated monocyte does not appear to be as strong as EC2CD63. Also, since unlabelled GST-EC2CD9 does not inhibit GST-EC2CD63 binding to monocytes, it appears that the proteins may bind to distinct sites. Hence EC2CD9 effects on monocyte fusion might be different from GST-EC2CD63. Direct interactions of CD9 with transmembrane immunoglobulin gene superfamily members named EWI-F and EWI-2 (also called PGLR, CD9P-1, and FPRP) are known to occur (chapter 1, 1.1.4.1; Hemler, 2003). Whether these proteins are involved in fusion is not yet known. As discussed above, it is also possible that CD9EC2 could be interacting with components of TEMs and modifying their activity.

Little or no binding of labelled GST proteins to THP-1 cells was observed, even on Con A stimulation. This is further evidence that binding of GST-EC2CD63 or CD9 is specific to stimulated monocytes.

Although some tetraspanins have been shown to participate in primary interactions via their EC2 regions (reviewed in Martin et al, 2005; Hemler, 2003) there have been few demonstrations of direct binding of tetraspanin EC2 proteins to cells. Wong and co-workers have demonstrated the specific binding of GST-EC2CD9 and GST-EC2CD81 on hamster oocytes by indirect immunofluorescence assay (Wong et al, 2001). Since the surface expression of CD9 and CD81 are high on oocytes it is possible that GST-EC2CD9 and GST-EC2CD81 could form dimers or higher multimers with naturally expressed CD9 or CD81. Otherwise there may be direct binding to unidentified receptors or CD9 and CD81 associated proteins such as integrins, which may affect tetraspanin mediated cell signalling essential for the sperm-egg fusion.

In addition we sent FITC, Alexa-Fluor 647® and rhodamine BITC conjugated GST-EC2CD63 and GST to our collaborator Dr. Cecilia Cheng-Mayer, New York, U.S. to test the binding of GST-EC2CD63 on macrophages in their experimental system using HIV-1 virus. They found that GST-EC2CD63 is specifically binding/taken up

by macrophages and prevents the infection of HIV-1 virus with macrophages (Ho *et al*, manuscript submitted).

We tested the binding efficiency of FITC conjugated GST-EC2CD9 and GST-EC2CD151 on mouse oocytes at ARC Centre of Excellence in Biotechnology and Reproductive Science, University of Newcastle, Australia. The tetraspanin proteins showed stronger binding to the oocytes than GST control, indicating that the binding of GST-EC2 proteins are specific. Already it has been reported that CD9 is essential for the sperm egg-fusion (Miyado *et al*, 2000; Le Naour *et al*, 2000; Kaji *et al*, 2000). At this stage the collaborators are unable to conclude the role of CD151 on sperm-egg fusion based on the binding results.

# CHAPTER 6

# PRELIMINARY CHARACTERISATION OF A HUMAN scFv WITH PUTATIVE SPECIFICITY FOR CD63

## 6.1. INTRODUCTION

This chapter describes attempts to define the specificity of a putative anti CD63 human scFv antibody. The antibody was selected from a phage display library generated from peripheral B-lymphocytes of patients with ocular melanoma. Library generation and selection of the antibody was carried out by a previous worker in the laboratory, Dr. Ann Marshall.

Melanoma is a very serious form of skin cancer that begins in melanocytes and occurs more often in people with fair skin that burns or freckles easily (http://www.melanoma.com). The incidence of malignant melanoma has risen over the last 30 years and is now increasing at a faster rate than any other cancer in the US (Chung et al, 2004; Smith et al, 2001). Although early stage of the disease can be treated successfully with surgery, once melanoma has spread to regional lymph nodes or distant sites, the prognosis is much worse (e.g. survival is predicted to be less than 12 months for patients with stage IV disease (Smith et al, 2001)). Among the different types of melanoma, uveal melanoma is the most common intra ocular tumour in adults. Current treatment, such as radiation and enucleation can control primary tumour growth (Muller et al, 2005), but neither is able to control metastatic tumour spread i.e. melanoma appears to be relatively resistant to conventional chemotherapy and radiotherapy, leading to a search for alternative treatments. Since there is evidence for an immune response to melanoma, there has been a great deal of interest in exploring immunotherapeutic approaches (Smith et al, 2001; Komenaka et al, 2004). Whilst these have mostly been based on using melanoma antigens as T cell vaccines, there has also been interest in using antibody–based strategies (Lutzky et al, 2002; Altomonte and Maio, 2002).

Traditionally, tumor-associated antigens have been identified by hybridomas derived from mice immunised with tumor cells. Several types of immunologic probes have

been used to characterize the antigenic profile of human melanoma cells. Among them, mouse mAbs have been the most effective in identifying a number of human melanoma associated Ags (MAAs) with distinct immunochemical and functional properties (Reisfeld *et al*, 1987; Herlyn *et al*, 1988; Graf *et al*, 1989). Attempts have also been made to identify human melanoma associated antigens (MAAs) using both sera from patients with malignant melanoma and human mAb. Human mAb have been successful only to a limited extent primarily because of the methodological difficulties in constructing stable human mAb secreting hybridomas and cell lines (Irie *et al*, 1982; Houghton *et al*, 1983). More recently, antibody phage display has been used for the production of antibodies (Burton 1991; Winter *et al*, 1994). The main advantage of this technique is that human antibodies can be produced much easier than is possible with human hybridomas. These can then be developed for therapeutic use without the need to humanize mouse mAb. Furthermore, by changing the selection conditions, the antigen binding properties of antibody phage can be influenced, e.g. by selection for high affinity antibodies or specificity for a pre-determined epitope (Meulemans *et al*, 1994; Hawkins *et al*, 1992). The random pairing of V domains has the potential to generate artificial antibody specificities and could overcome the problem of the immune dominance of some antigens and epitopes in mice that limit the spectrum of specificities obtained with hybridomas.

Antibody phage display techniques have been used both to characterise melanoma immunogens (Desai *et al*, 1998; Li *et al*, 2001) and to try to develop reagents for passive immunotherapy (Kupsch *et al*, 1999; Wang *et al*, 1999). Noronha *et al*, 1998 have isolated a human single chain fragment (scFv) by panning a synthetic phage antibody library with human melanoma cell lines such as S5 and SK-MEL28. The authors demonstrated that the scFv fragment reacts specifically with melanoma associated high molecular weight antigen 'HMW-MAA', which appears to be immunodominant *in vitro* and *in vivo*. Kupsch *et al*, (1999) have constructed a human single chain scFv phage library using peripheral blood mononuclear cells of donor with increased auto- antibody titre to melanoma. The positive selection of library was carried out with human melanoma cells lines such as DX3 and HMB2 and the negative selection on human peripheral blood mononuclear cells. In this study the authors isolated two scFv clones that appeared largely specific to tumour cells although the antigen was not identified.

Originally CD63 was described as melanoma antigen, ME491, whose expression decreases as the disease progresses (Atkinson *et al*, 1985; Hotta *et al*, 1988; Kondoh *et al*, 1993) but normal melanocytes express little CD63 (Sikora *et al*, 1987; Hotta *et al*, 1988). Antibodies to CD63 have been identified in the sera of melanoma patients (Smith *et al*, 1997; Li *et al*, 2003) and it has been suggested that decreased expression of CD63 in metastatic melanoma is consistent with immune escape (Smith *et al*, 1997) and enhanced invasiveness of human melanoma cells (Jang *et al*, 2003). In contrast to these, some reports state that CD63 is also expressed by metastatic melanoma (Barrio *et al*, 1998). Evidence of an immune response to this protein has lead to the suggestion that CD63 might be a suitable vaccine candidate for malignant melanoma: When given in combination with interleukin 2, vaccination with recombinant hCD63 was found to inhibit tumour growth in hCD63 transgenic mice (Li *et al*, 2003).

In addition, CD63 has been suggested as a target for therapeutic antibodies in the treatment of cancers, since the molecule is rapidly internalised from the cell surface (e.g. macrophages, some tumours) and would therefore deliver drugs effectively (Barrio *et al*, 1998; Audran *et al*, 1995). Antibodies to CD63 could target drugs directly to cancer cells that express the protein on the cell surface (e.g. melanoma cells) (Barrio *et al*, 1998). Alternatively, antibodies could target drugs (e.g. cytokines) to tumour-infiltrating macrophages that express CD63, causing their activation (Audran *et al*, 1995).

This chapter describes the preliminary characterisation of a putative human scFv anti CD63 antibody isolated from a phage display library generated from peripheral blood lymphocytes of patients with ocular melanoma. Such an antibody may have potential in the treatment of melanoma and other type of cancers.

In this project we wished to compare the reactivity of human scFv generated against CD63 with existing whole molecule mouse anti CD63 antibodies. Initially, mouse mAb were tested with RBL2H3 cell line transfected with hCD63 gene (also known as RBL-hCD63); melanoma cell lines Mewo, Mel-17, VUP and DX3; myeloid cell lines THP-1, HL60 and K562; colorectal carcinoma cell line Lovo and ovarian carcinoma cell line IGR-OV. Except for RBL2H3-hCD63, these cells are naturally expressing different levels of CD63. RBL2H3hCD63 were used as a positive control since these

cell line has been widely used in the laboratory to study the structure/function of ectopically expressed human tetraspanin proteins (Smith *et al*, 1995; Higginbottom *et al*, 2000). The rat cell line RBL2H3 is not naturally expressing human CD63 but it expresses rat CD63 (Kitani *et al*, 1991), which is 70% homologous to human CD63. Most mouse monoclonal antibodies that have been raised against human CD63 are specific to the human protein.

As discussed previously, CD63 is predominantly intracellular in most cells and internalises rapidly from the plasma membrane, due to the presence of a lysosomal targeting sequence (GYEM) at its C-terminus (Rous *et al*, 2002). This has led to suggestions that CD63 could be used to target drugs. It was therefore of interest to compare surface:intracellular distribution and internalisation of CD63 on the different cell lines.

### 6.1.1. scFv phage library

In previous work carried out in the laboratory by Dr. Ann Marshall, antibody phage display libraries were generated from the peripheral blood B-lymphocytes of patients with ocular melanoma. Genes corresponding to $V_H$ (IgG1) and $V_L$ ($\kappa$ and $\lambda$) were amplified from patient mRNA using specific primers (Sblattero and Bradbury, 1997) and cloned in to the pAK100 vector (Krebber *et al*, 1997) (Fig.6.1.1). To try to select for antibodies to CD63, libraries from 5 individual patients were pooled to give a library with approximately $10^7$ members. The pooled library was subjected to 4 rounds of panning against RBL2H3 cells transfected with human CD63 (RBL2H3-hCD63), with 2 rounds of negative panning against untransfected RBL2H3 cells. Following the final round of panning, 50 randomly chosen clones were analysed by FACS analysis. 9 clones appeared to show differential reactivity with RBL2H3-hCD63 compared to untransfected cells. scFv-encoding DNA from 4 of the clones was sequenced and found to be identical (Fig.6.1.2). Analysis of the sequence using V-BASE shows that the heavy chain belongs to the $V_H4$ subgroup (locus 4-34) and corresponds to the germline sequence. The light chain belongs to the V$\kappa$2 subgroup (locus A3) and shows 2 amino acid mutations from germline (highlighted in red in Fig.6.1.2).

**Recombinant human antibody library construction**

mRNA from human peripheral blood lymphocytes/splenic lymphocytes

RT − PCR : 5′ − − − − 3′

Light chain cDNA

Heavy chain cDNA

VL gene primers

VH gene primers

Sfi1          Sfi1

Light chain / Linker sequence / Heavy chain

Lac I  Lac P/O  Pel B          myc  gene III

ColE1

pAK 100 phagemid display vector

F 1 ori

CAM resistance

Complete phage particle displaying phagemid encoded scFv fragment

Fig. 6.1.1. Schematic representation of the strategy for cloning heavy and light chain genes to create a combinatorial library for scFv antibody. RNA from the lymphocytes ($5 \times 10^7$) of uveal melanoma patient was reverse transcribed to cDNA. Immunoglobulins heavy chain and light chain V regions were amplified by PCR. The heavy and light chains were randomly combined and expressed on the surface of phage as scFv fragments. The clones with desired specificity were selected by binding to antigen.

```
1                              CDR1           40
DIVMTQSPLS LPVTPGEPAS ISCRSSQSLL HSNGYNYLDW

41                 CDR2                        80
YLQKPGLPPQ LLIYLGSIRA GVPDRFSGSG SGTDFTLKIR

81              CDR3
RVEAEDVGVY YCMQAIQSPS TFGPRDTTGD AVLT
```

**HEAVY CHAIN V REGION**

```
1                              CDR1           40
QVQLQQWGAG LLKPSETLSL TCAVYGGSFS GYYWSWIRQP

41              CDR2                           80
PGKGLEWIGE INHSGSTNYN PSLKSRVTIS VDTSKNQFSL

81              CDR3                          121
KLSSVTAADT AVYYCARGRG LIWKQQLVRT GMDVWGQGT
```

Fig.6.1.2. Amino acid sequences (single-letter code) of the variable regions of the scFv light chain (VL) and heavy chain (VH). The CDRs are underlined and in bold. Residues that differ from germline sequence are shown in red. The numbering of the residues is according to Kabat (Kabat *et al*, 1991). The Kabat antibody sequence database was accessed as described by Martin (Martin, 1996). Variable region genes were assigned using the MRC Centre for Protein Engineering VBASE (http://vbase.mrc-cpe.cam.ac.uk).

Prior to studies on the scFv antibody, the expression of CD63 on a variety of human cell lines was examined using conventional mouse monoclonal antibodies. In these studies RBL2H3 cells transfected with the human CD63 gene were used as a positive control since this cell line has been widely used in the laboratory to study the structure/function of ectopically expressed human tetraspanin proteins (Smith *et al*, 1995; Higginbottom *et al*, 2000). Although the RBL2H3 cells contain endogenous rat CD63 (Kitani *et al*, 1991), most mouse monoclonal antibodies that have been raised against CD63 are specific to the human protein.

Previous work by Dr. Ann Marshall had demonstrated very poor soluble expression of the scFv clones selected from the melanoma antibody phage display library. The studies on their specificity here were therefore carried out using scFv expressed on the surface of phage. Two of the identical scFv-expressing clones (4E and 5F) were studied, along with a control comprising vector with no insert.

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# 6.2. RESULTS

## 6.2.1. Comparison of total, cell surface and intracellular expression of CD63 on human cell lines

The surface and intracellular expression of CD63 in different cell lines were assessed using fixed and permeabilised cell as described in section 2.2.6 by FACS. There was considerable variation in the total amount of CD63 expressed by the cell lines. Generally, as expected, melanoma cell lines (Mewo, Mel-17, DX3 and VUP) expressed high levels. There was moderate expression by the myeloid cell lines (U937, THP-1 and K562) although expression by HL60 was low. CD63 was also expressed by colorectal carcinoma cell line LOVO and Ovarian carcinoma cell line IGR-OV.
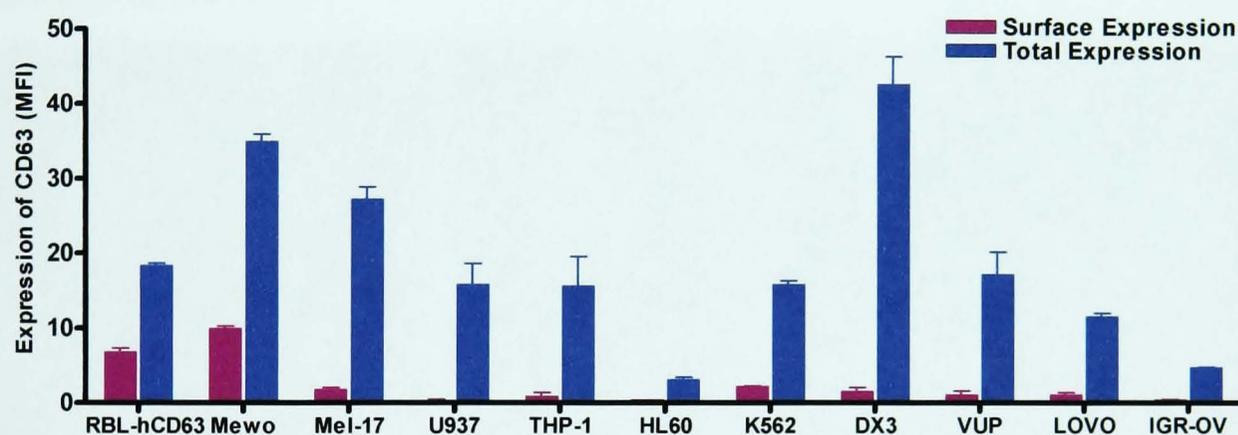


Fig.6.2.1. Surface and intracellular expression levels of CD63 in different cell lines. The expression levels of CD63 was measured by FACS as described in section 2.2.6 using anti CD63 antibody H5C6-FITC at $14\mu g.ml^{-1}$. The CD63 expression levels are expressed as MFI. The results are the mean values of 2 experiments in triplicate.
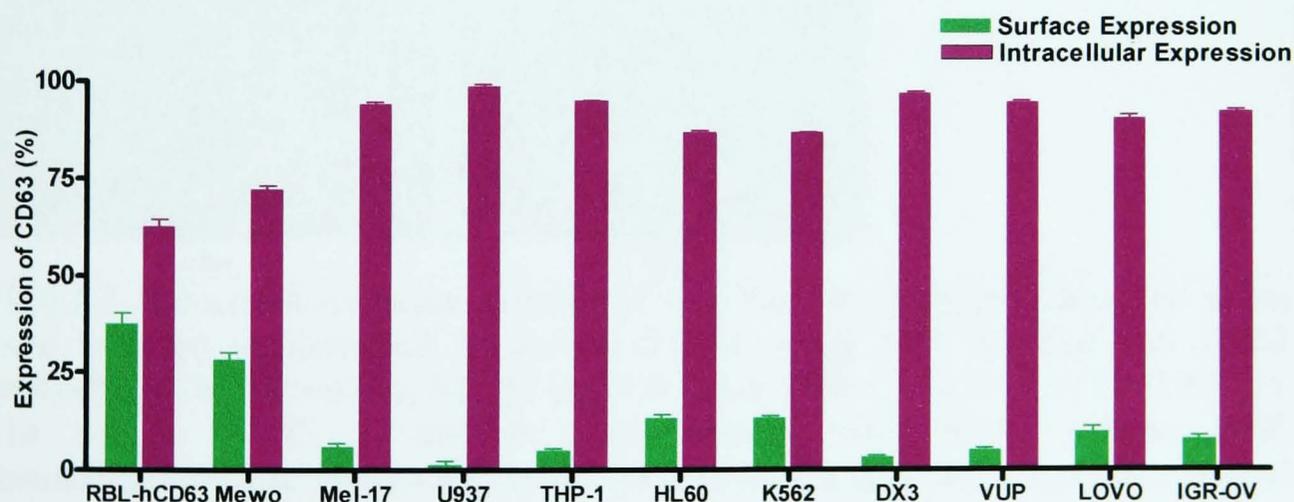


Fig.6.2.2. Surface and intracellular expression levels of CD63 in different cell lines. The expression levels of CD63 was measured by FACS as described in section 2.2.6 using anti CD63 antibody H5C6-FITC at $14\mu g.ml^{-1}$. The CD63 expression levels are expressed as %. The results are the mean values of 2 experiments in triplicate.

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As expected, expression of CD63 was predominantly intracellular. Interestingly, the melanoma cell line Mewo expresses high levels of CD63 on the surface, almost as high as the hCD63 transfected RBL2H3 cell line (28.27% and 37.33% respectively). The monocytic leukaemic cell lines HL60 and K562 also express some amounts of CD63 on their surface i.e. 13.33% and 13.45% respectively. In contrast the surface expression of CD63 was very low (4%) with monocytic leukaemic cell line THP-1 and melanoma cell lines, Mel-17, DX3 and VUP. The results are given in Fig.6.2.2.

### 6.2.1.1. Immunofluorescent microscopy with cell lines expressing CD63

The structural features of various CD63 expressing cell lines such as Mewo, Mel-17, U937, THP-1 and RBL2H3-hCD63 were studied by immunofluorescence microscopy using cytospin slide preparation as described in section 2.2.4.1 with anti CD63 antibody H5C6-FITC.
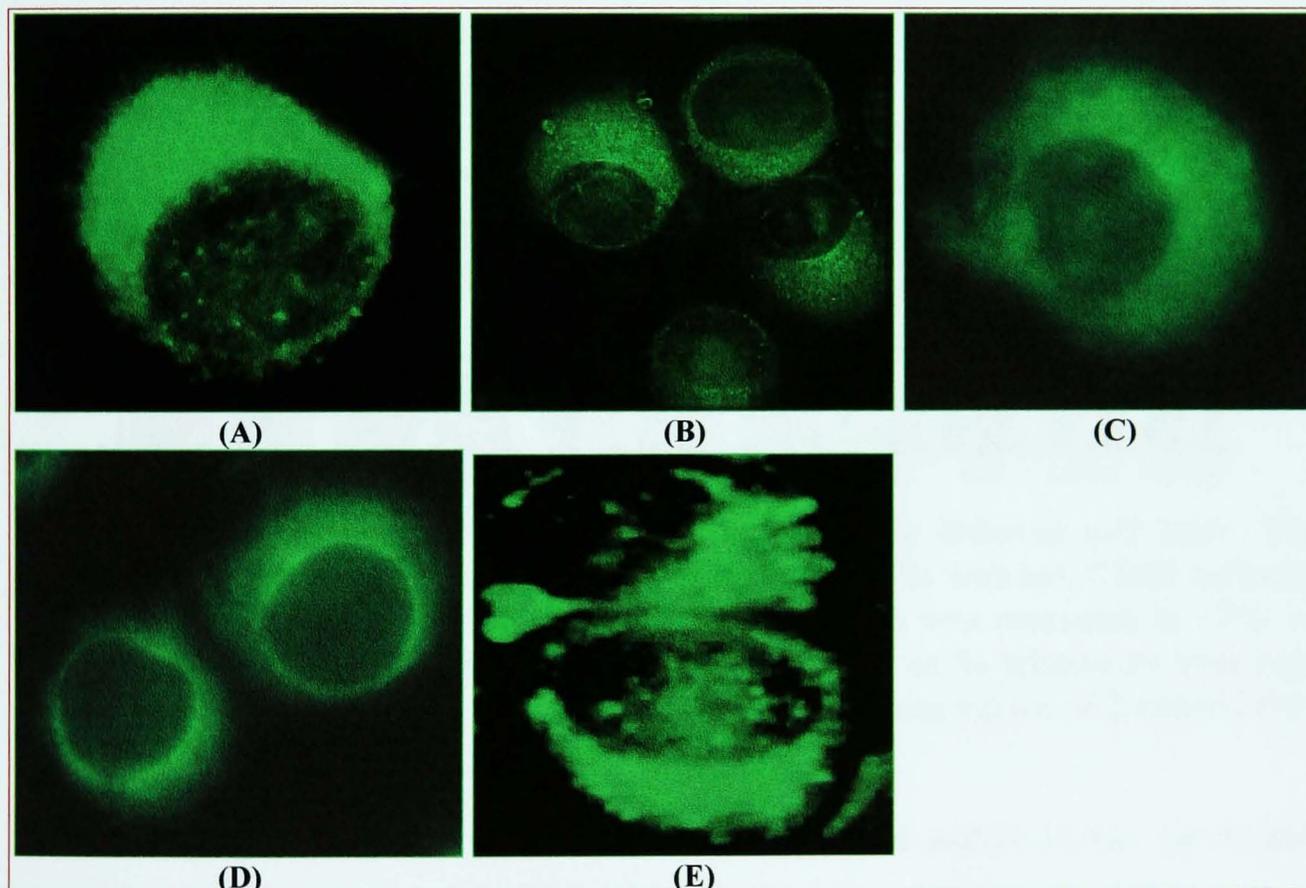


Fig.6.2.3. Immunofluorescence staining of cell lines in cytospin slides. The slides were prepared as described in section 2.2.4.1 using FITC labelled anti CD63 antibody-H5C6. Mewo (A); Mel-17 (B); U937(C); THP-1 (D); RBL2H3-hCD63 (E). The images A, C, D and E were captured with Nikon Eclipse E400 immunofluorescence microscope using 100x objective and the image (B) captured with Deltavision restoration microscope using 40x objective.

Strong surface staining was observed with Mewo and RBL2H3-hCD63 cells since these cells are expressing more CD63 on their surface and a moderate staining with Mel-17. Relatively less surface staining was observed with U937 and THP-1 cells in

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agreement with the FACS data. All cell lines showed granular cytoplasmic staining (Fig.6.2.3).

## 6.2.2. Comparison of antibody induced internalisation of CD63 on human cell lines

Previous work from our laboratory has shown that human CD63 transfected into RBL2H3 cells is rapidly internalised in response to antibody ligation at 37°C (G.Mal, Ph.D thesis, University of Sheffield, 2005). In the present study we investigated the internalisation of CD63 in cells that naturally express this proteins. To induce internalisation, cells that had been pre-incubated with unlabelled anti CD63 antibody at 4°C were transferred to 37°C. Samples were removed at timed intervals and the amount of remaining cell surface bound anti CD63 antibody determined as described in section 2.2.7.
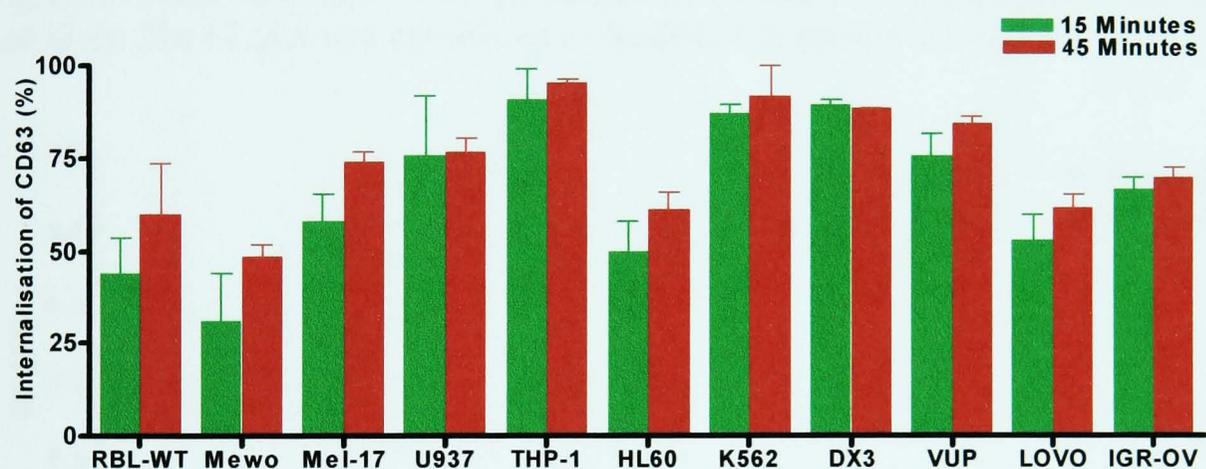


Fig.6.2.4. Antibody induced internalisation of hCD63 in different cell lines. The internalisation was measured by FACS by labelling the cells with anti CD63 antibody -H5C6 at 15µg.ml$^{-1}$ at 4°C, then antibody internalisation was measured at 37°C as described in section 2.2.7. Internalisation was expressed as % relative to time zero (cells incubated throughout at 4°C). The results are the mean values of 2 experiments in triplicate.

Rapid internalisation of cell surface CD63 was observed within 15min. on all cell lines, with little further internalisation occurring after 45min. Thus, rapid internalisation appears to be general feature of this molecule. There were, however, some differences between cell lines in the extent of internalisation e.g. most of the cell surface CD63 was internalised after 45min on THP-1 and K562 cells, whereas only ~50% was internalised on Mewo cells (Fig.6.2.4).

## 6.2.3. Reactivity of mouse anti CD63 antibodies in whole cell ELISA

The binding reactivity of mouse monoclonal antibodies to CD63 such as H5C6 (IgG1), LP9 (IgM) and BEM-1 (IgA) was tested against Mewo and Mel-17 cell lines by whole cell ELISA as described in section 2.2.12.3.3.
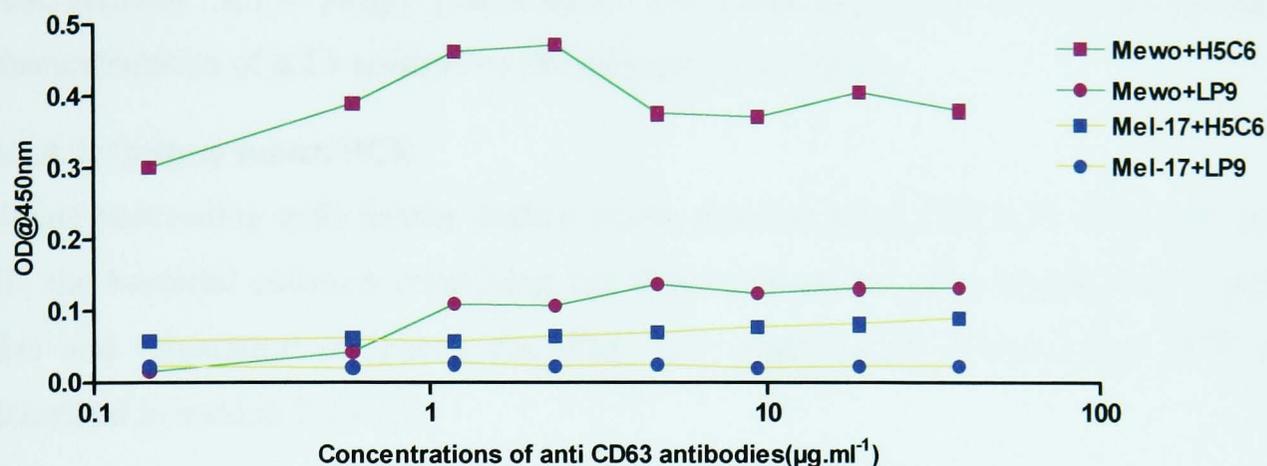


Fig.6.2.5. Titration of anti CD63 antibodies-H5C6 and LP9 against Mewo and Mel-17 cell lines. The ELISA was carried out as described in section 2.2.12.3.3.
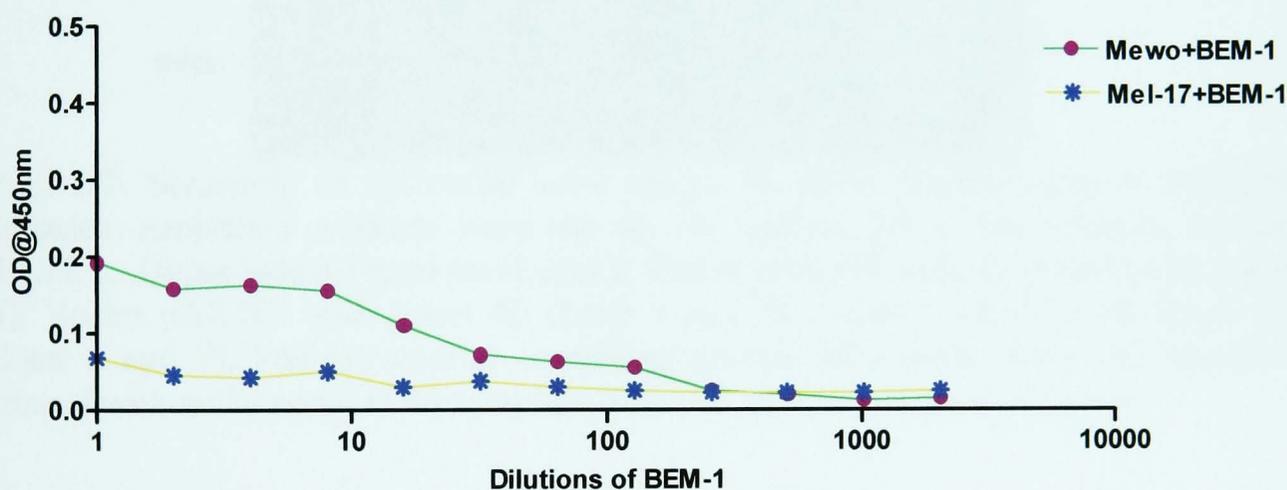


Fig.6.2.6. Titration of anti CD63 antibody-BEM-1 (supernatant) against Mewo and Mel-17 cell lines. The ELISA was carried out as described in section 2.2.12.3.3.

H5C6 and LP9 showed good and moderate binding reactivity respectively with Mewo cells in ELISA even at $15\mu g.ml^{-1}$ concentrations since the surface expression of CD63 is higher in Mewo cells. In contrast, both H5C6 and LP9 showed little binding with Mel-17 cells (Fig.6.2.5). In addition the mouse hybridoma supernatant BEM-1 was also showed a moderately good binding reactivity with Mewo but not Mel-17 (Fig.6.2.6).

## 6.2.4. Preparation of scFv phage

For the studies on the putative anti CD63 scFv antibody, phage were prepared from 2 clones, 4E and 5F, which had been shown by sequence analysis to contain identical

scFv genes. As a negative control colonies containing the pAK100 vector with no inserted scFv encoding genes were used. All studies were carried out using scFv expressed on the surface of phage (scFv phage), since extensive previous attempts to express the scFv in soluble form were unsuccessful (Dr. Ann Marshall, unpublished observations). scFv phage preparations are frequently used in the preliminary characterisation of scFv antibodies (Steinberger *et al*, 2001).

### 6.2.4.1. Colony insert PCR

Before proceeding with further studies on the putative anti CD63 scFv clones 4E and 5F, the bacterial colonies containing the phagemid vector were assessed for correct size and orientation of inserts encoding scFv fragment by colony direct PCR as described in section 2.2.12.1.



Fig.6.2.7. Screening of individual scFv clones by direct amplification of bacterial colonies. Amplified products were run on 1% agarose gel as described in section 2.2.12.1. Hyper ladder I markers (Lane1); Vector pAK100 without insert (Lane 2 and 3); Vector pAK100 with insert 4E (Lane 4 and 5); Vector pAK100 with insert 5F (Lane 6 and 7); Positive control containing known scFv clone (Lane 8); Negative control with no bacterial DNA template (Lane 9), where bp-denote base pair.

From the agarose gel it was observed that there were strong 800bp bands corresponding to the scFv insert in clones 4E, 5F and positive control clones. No band was developed with the colony from the no insert (vector only) control. The bands below 100bp might be due to unused primers in the reactions. Hence these results confirmed that the 4E and 5F bacterial colonies contained vector with correctly sized and correctly orientated scFv insert DNA (Fig.6.2.7).

### 6.2.4.2. Preparation of scFv phage containing supernatents

In the initial studies, crude phage-containing supernatants obtained after overnight infection of phagemid containing bacteria (3ml cultures) with helper phage, were used. However to improve phage numbers, supernates from larger volume (150ml) cultures were used and subjected to PEG precipitation as described in section 2.2.12.2 (Sambrook *et al*, 2001). This resulted in a higher yield of phage numbers in each

phage preparations. The phage-containing vector with no insert prepared under identical conditions was used as a negative control.

### 6.2.4.3. Quantification of Phage

#### 6.2.4.3.1. Colony count method

The initial studies were carried out with crude phage preparations, which are similar in principle to using crude hybridoma supernates or antisera. Hence to standardise the number of phage particles to be used in each experiment, such as ELISA and fluorescence microscopy, attempts made to quantify the phage particles present in each preparation either by colony count (section 2.2.12.3.1) or spectrophotometric method (section 2.2.12.3.2). The colony counting method estimates the number of infective phage particles present in the phage preparation. However, this method does not give an estimate of the number of phage that are able to bind, since the number of infective phage falls off rapidly with time, whereas antibody binding is stable for several weeks or even months (Steinberger *et al*, 2001). The estimates of phage numbers are given in table 6.2.1.

#### 6.2.4.3.2. Spectrophotometric method

Alternatively a spectrophotometric method (section 2.2.12.3.2) was used to measure the total phage content of an scFv phage preparation, although this method has the limitation that it will measure only the total number of phage particles as a function of protein content. This method will not give any indication about the antigen specific antibody fragment. From this study the number of phage particles per ml of phage preparations are given in table 6.2.1.

| Phage | Colony count method (Phage particles. ml$^{-1}$) | Spectrophotometric method (Phage particles. ml$^{-1}$) |
|---|---|---|
| No insert | $3.74 \times 10^{12}$ | $3.77 \times 10^{13}$ |
| 4E | $2.25 \times 10^{13}$ | $2.87 \times 10^{14}$ |
| 5F | $5.60 \times 10^{12}$ | $1.48 \times 10^{14}$ |

Table.6.2.1. Estimates of number of phage particles ml$^{-1}$ of PEG concentrated phage preparation by colony count method and spectrophotometric method. The experiments were carried out as described in sections 2.2.12.3.1 & 2.2.12.3.2. The values are from a single experiment.

Using these phage preparations various experiments such as whole cell ELISA, titration against GST fusion proteins of hCD63EC2 region and inhibition ELISA etc. were carried out.

## 6.2.5. Assessment of scFv-phage specificity by whole cell ELISA

### 6.2.5.1. Three stages ELISA

In the initial stages of this work, a very limited range of anti-M13 phage reagents was commercially available. Hence the initial studies were therefore carried out using a "3-stage" whole cell ELISA, consisting of phage supernatants followed by anti-M13 phage antibody labelled with biotin, followed by HRP-labelled Extravidin. Results obtained under standard conditions are shown for RBL2H3 cells (hCD63 transfected or untransfected) and melanoma cell lines Mewo and Mel-17.

**(A)**



**(B)**
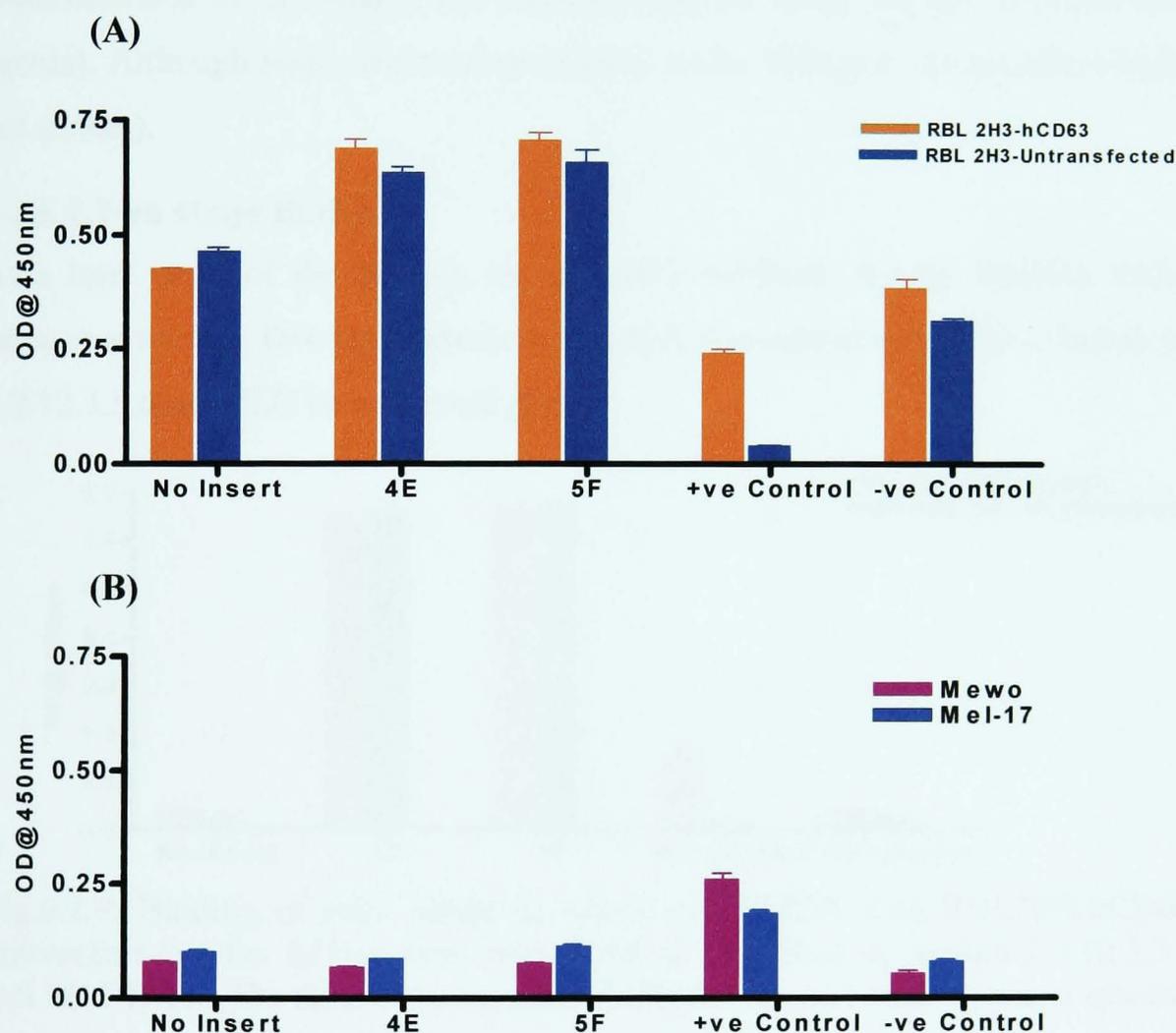


Fig.6.2.8. Binding in whole cell ELISA using scFv phage with RBL2H3/Mewo/Mel-17 cells. ELISA was carried out as described in section 2.2.12.3.4 using anti M13-biotin antibody at $1\mu g.ml^{-1}$ and Extravidin-HRP at $0.5\mu g.ml^{-1}$. The cells in positive control were labelled with mouse anti CD63 antibody-H5C6 and followed by anti mouse IgG-HRP; the cells in negative control were labelled only with anti M13 HRP and Extravidin-HRP, where the binding studies with RBL2H3-hCD63 transfected and untransfected cells (A); Mewo and Mel-17 (B).

Surprisingly, the 4E and 5F clones appeared to react with untransfected RBL2H3 cells. However, although slightly higher binding values were observed with phage 4E and 5F, the no insert phage control also gave high binding. The negative control (cells

were labelled only with anti M13 HRP and Extravidin-HRP in the absence of phage) also showed a high backgrounds colour, indicating some non-specific binding of the secondary or tertiary reagent to these cells (Fig.6.2.8A). The binding of same phage preparations was tested with Mewo and Mel-17 cells. Almost no difference in binding was observed between the test phage (4E&5F) and controls (no insert and –ve control) (Fig.6.2.8B).

Extensive attempts were made to optimise the assay (e.g. using different concentrations of secondary and tertiary reagents, using various different blocking agents). Although some improvements were made, backgrounds remained high (data not shown).

### 6.2.5.2.Two stage ELISA

At a later stage of the project, an anti M13 antibody directly labelled with HRP became available. Two layer whole cell ELISA was carried out as described in section 2.2.12.3.5 using PEG concentrated phage.
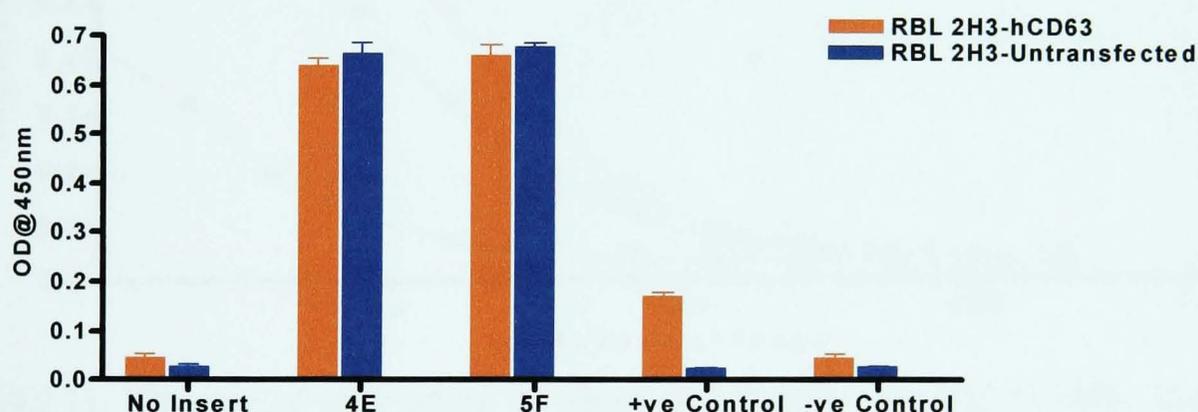


Fig.6.2.9. Binding of scFv phage in whole cell ELISA with RBL2H3-hCD63 and untransfected cells. ELISA was carried out as described in section 2.2.12.3.5 using anti M13-HRP. The results are the mean OD@450nm of 1 experiment in triplicate.

In the two layers ELISA very low levels of background colour was measured (Fig. 6.2.9). In addition the phage 4E and 5F showed a significant higher binding value with RBL2H3-hCD63 and untransfected cells than no insert control. The binding of phage to untransfected cells was unexpected. Based on this interesting result the same phage preparation was titrated against Mewo and Mel-17 cell lines under identical experimental conditions as described in section 2.2.12.3.5.

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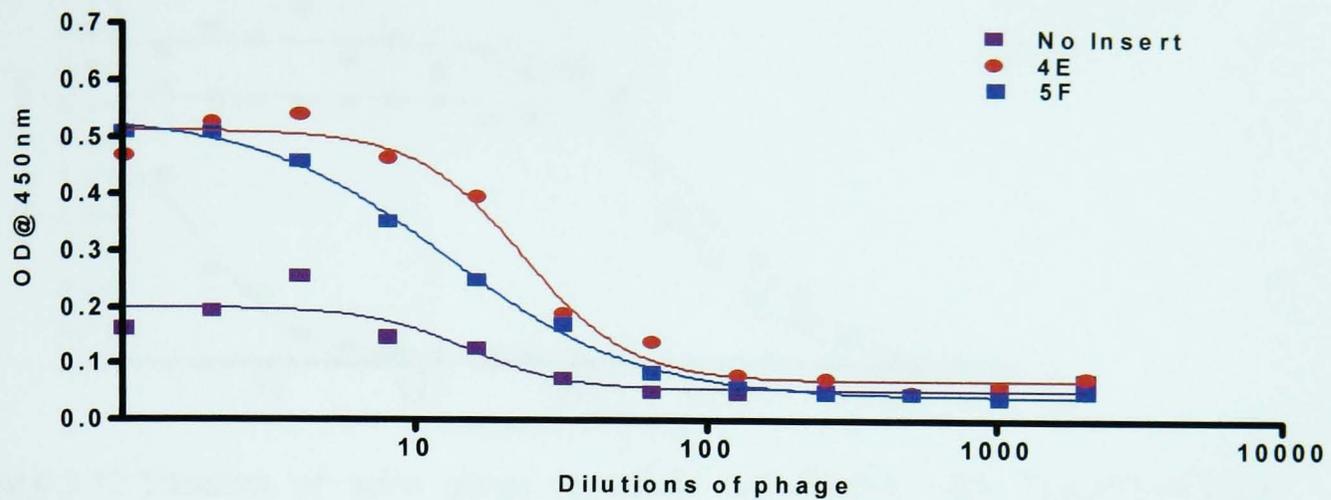
Fig.6.2.10. Titration of scFv phage in whole cell ELISA with Mewo cells. ELISA was carried out as described in section 2.2.12.3.5 using anti M13-HRP antibody. The results are the mean OD@450nm of 1 experiment in triplicate.
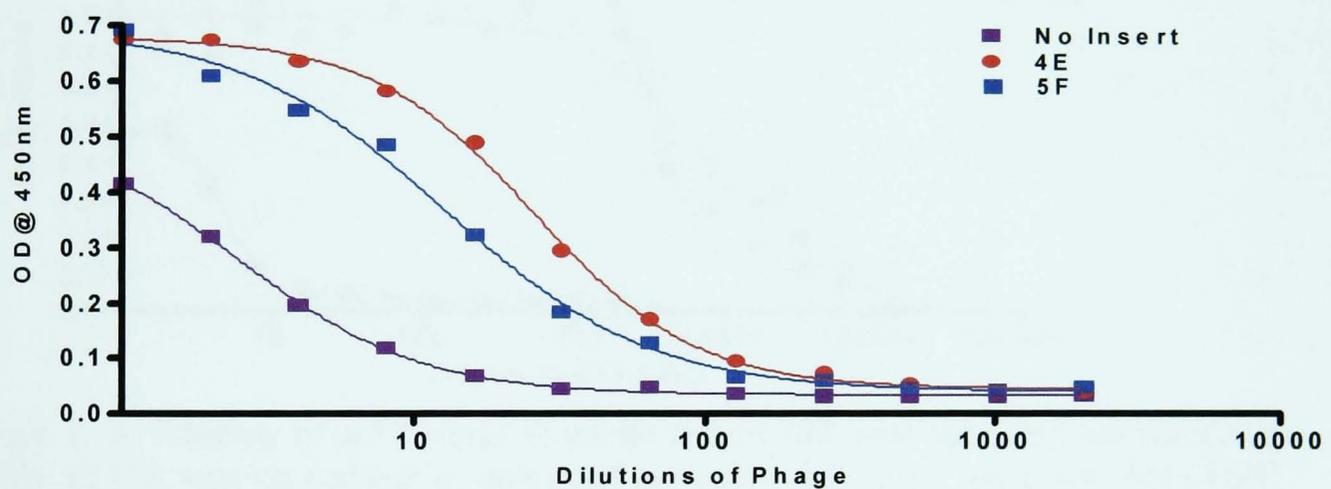


Fig.6.2.11. Titration of scFv phage in whole cell ELISA with Mel-17 cells. ELISA was carried out as described in section 2.2.12.3.5 using anti M13-HRP antibody. The results are the mean OD@450nm of 1 experiment in triplicate.

The results showed that the binding of phage 4E and 5F with Mewo and Mel-17 was ~ two folds higher than no insert control (Fig.6.2.10&Fig.6.2.11).
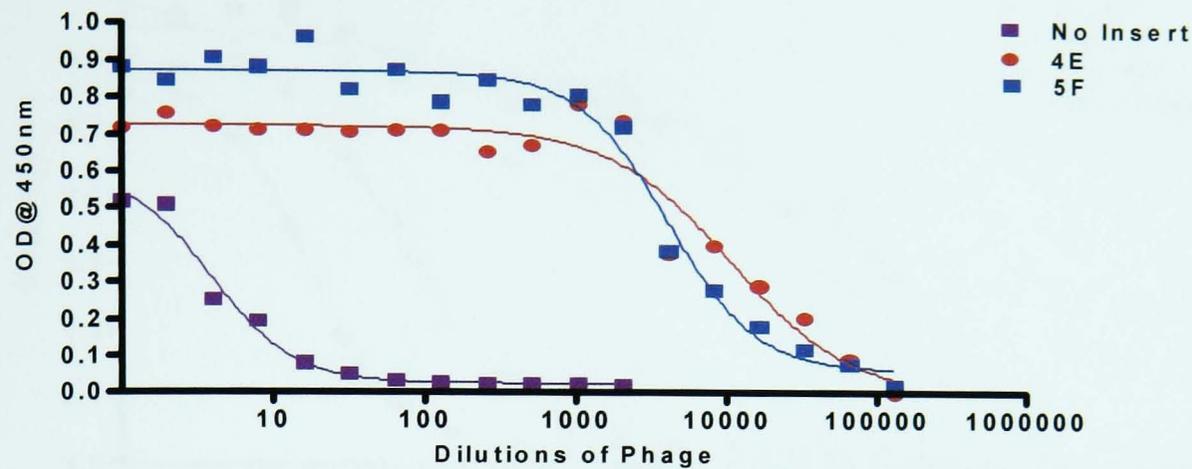
Fig.6.2.12.Titration of scFv phage in whole cell ELISA with RBL2H3-hCD63. ELISA was carried out as described in section 2.2.12.3.5 using anti M13-HRP antibody. The results are the mean OD@450nm of 1 experiment in triplicate.
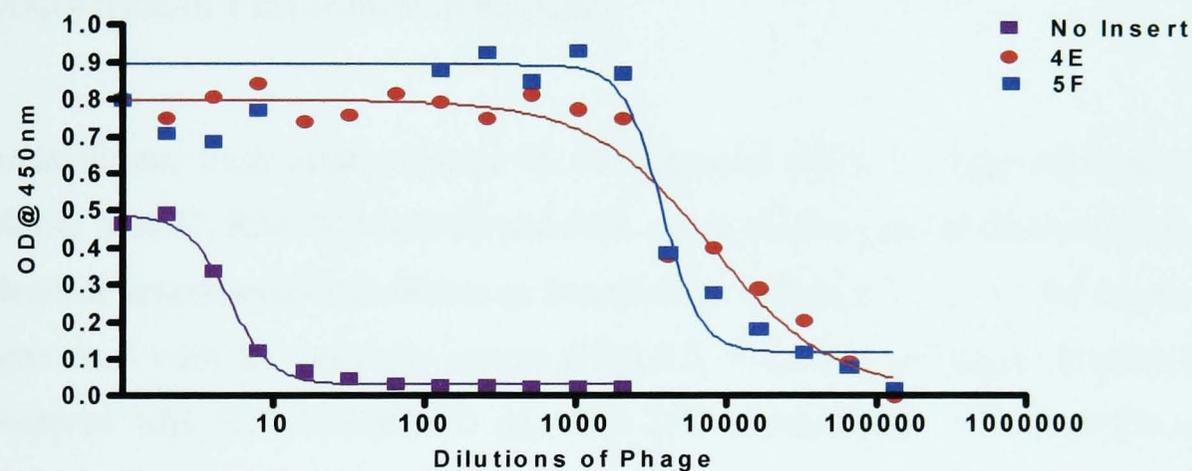


Fig.6.2.13. Titration of scFv phage in whole cell ELISA with RBL2H3-untransfected cells. ELISA was carried out as described in section 2.2.12.3.5 using anti M13-HRP antibody. The results are the mean OD@450nm of 1 experiment in triplicate.

The binding reactivity of phage no insert, 4E and 5F were also tested with RBL2H3-hCD63 and RBL2H3 untransfected cells. The 4E and 5F showed higher binding values than no insert control with RBL2H3-hCD63 and RBL-untransfected cells (Fig. 6.2.12&Fig.6.2.13). The results indicate that at least in the whole cell ELISA assay, the scFv phage are recognising an antigen that is expressed at high levels on transfected and untransfected RBL2H3 cells.
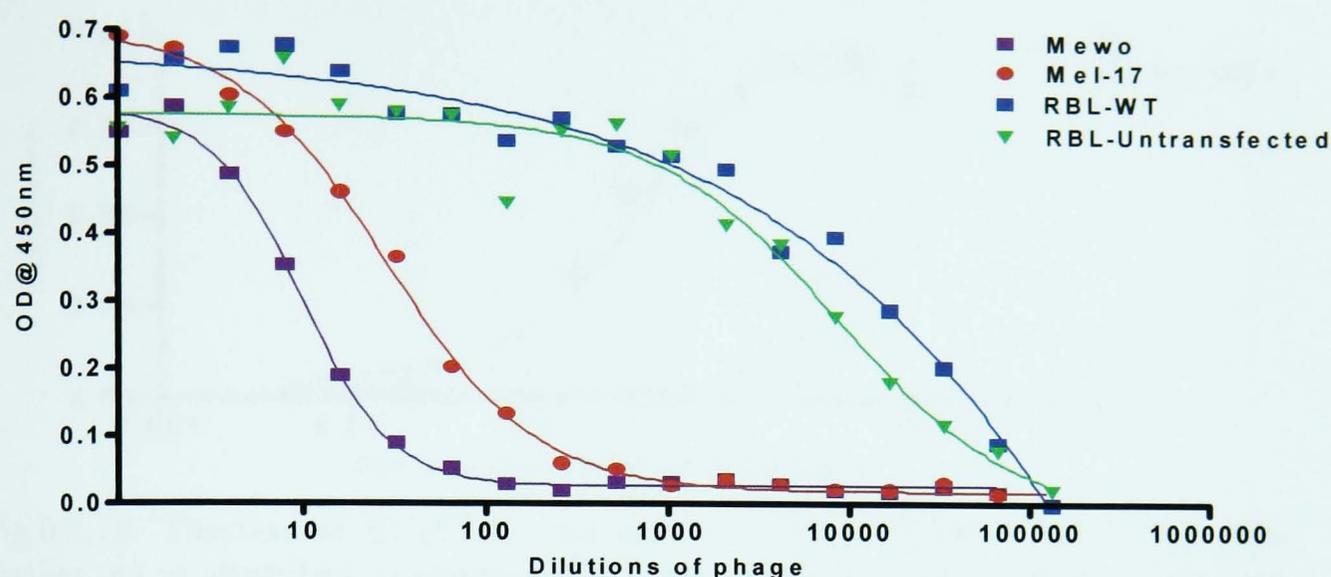
Fig.6.2.14. Titration of scFv phage 4E in whole cell ELISA with RBL2H3-hCD63, RBL2H3-untransfected cells, Mewo and Mel-17. ELISA was carried out as described in section 2.2.12.3.5 using anti M13-HRP antibody. The results are the mean OD@450nm of 1 experiment in triplicate.

An additional fresh batch of phage 4E was prepared and its binding was tested against Mewo, Mel-17, RBL2H3-hCD63 and RBL untransfected cells at the same time under identical experimental conditions as described in section 2.2.12.3.5. The results are in agreement with the previous results (Figs.6.2.10-6.2.13) i.e. higher binding values observed with RBL2H3-hCD63 and RBL2H3 untransfected cells than Mewo and Mel-17 (Fig.6.2.14). This indicates that scFv are reacting with an antigen that is highly expressed by the RBL2H3 cells.

## 6.2.6. Binding of scFv phage to tetraspanins EC2 proteins

### 6.2.6.1. Titration of GST and EC2CD63

The binding reactivity of the phage with GST-EC2CD63 prepared as described in section 2.2.8.1 was also tested. The preliminary experiments with GST-EC2CD63 were carried out to optimise the concentration of GST-EC2CD63 to be coated on the ELISA plate. Different concentrations of immobilised GST-EC2CD63 were titrated against anti CD63 antibody-H5C6 and anti GST antibody as described in section 2.2.5.5.
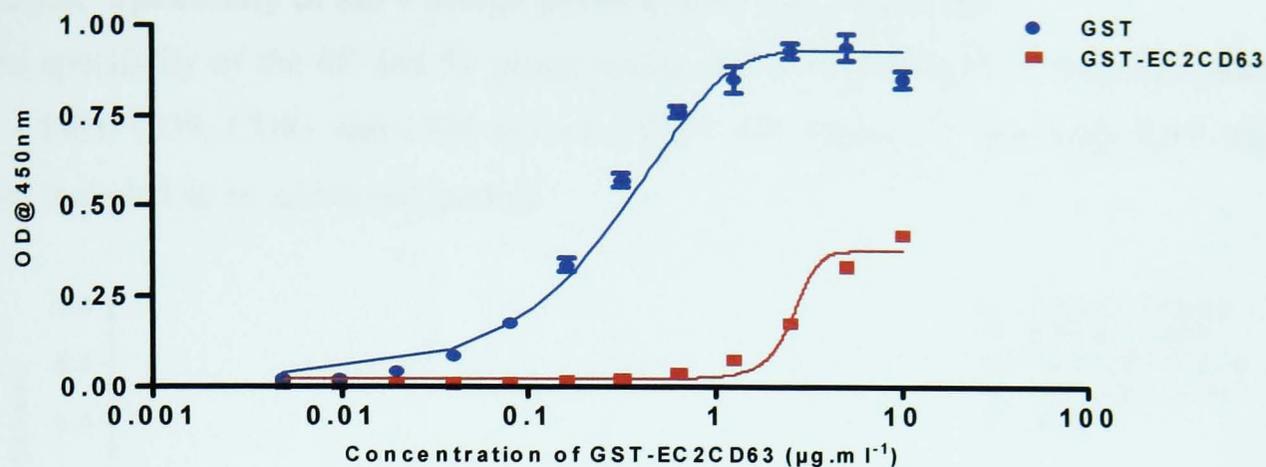
Fig.6.2.15. Titration of EC2CD63 and GST in GST-EC2CD63. The ELISA was carried out as described in section 2.2.5.5. EC2CD63 was estimated by coating the plate with anti CD63 antibody H5C6 (15μg.ml⁻¹) and followed by Goat anti mouse IgG-HRP (1:1000). The concentration of GST was estimated by coating the plate with anti goat GST antibody (5μg.ml⁻¹) and followed by mouse anti Goat-Sheep HRP (5μg.ml⁻¹). The results are the mean OD@450nm of 2 experiments in duplicate.

From the result it was observed that the GST-EC2CD63 at 10μg.ml⁻¹ was an optimum concentration for coating the ELISA plate for the binding studies (Fig.6.2.15).

## 6.2.6.2. Binding reactivity of scFv phage with GST-EC2CD63

The binding reactivity of phage no insert, 4E and 5F were tested against immobilised GST-EC2CD63 as described in section 2.2.12.3.6.
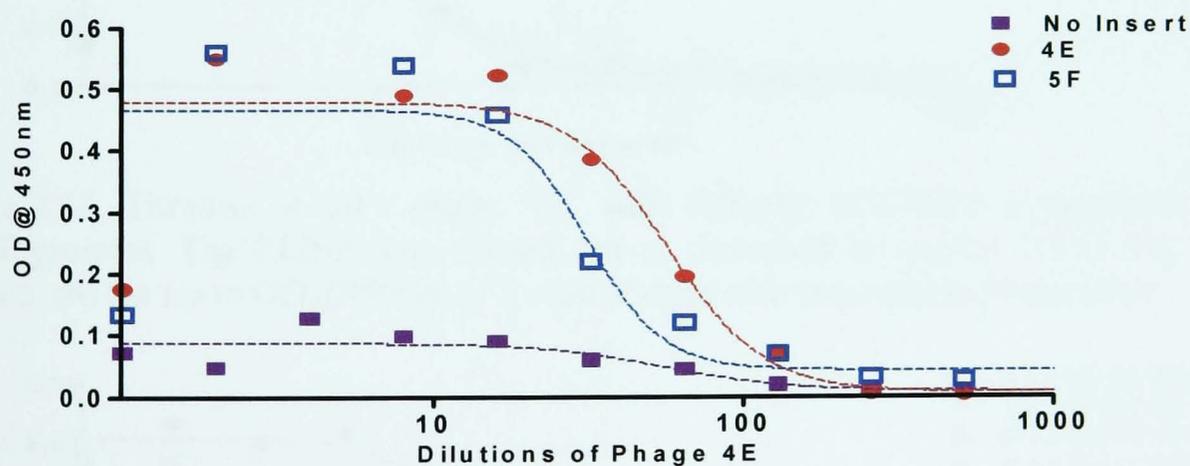


Fig.6.2.16. Titration of scFv phage: No insert, 4E and 5F with GST-EC2CD63. The ELISA was carried out as described in section 2.2.12.3.6. The results are the mean OD@450nm of 2 experiments in duplicate.

The phage 4E and 5F showed higher binding with GST-EC2CD63 than no insert phage i.e. OD values of 4E and 5F were ~four folds higher than no insert control (Fig. 6.2.16).

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### 6.2.6.3. Specificity of scFv phage binding with GST-EC2CD63

The specificity of the 4E and 5F phage against different GST-EC2 tetraspanins such as CD63, CD9, CD81 and CD9 mutant C153A was tested. In this study GST was also included as an additional control.
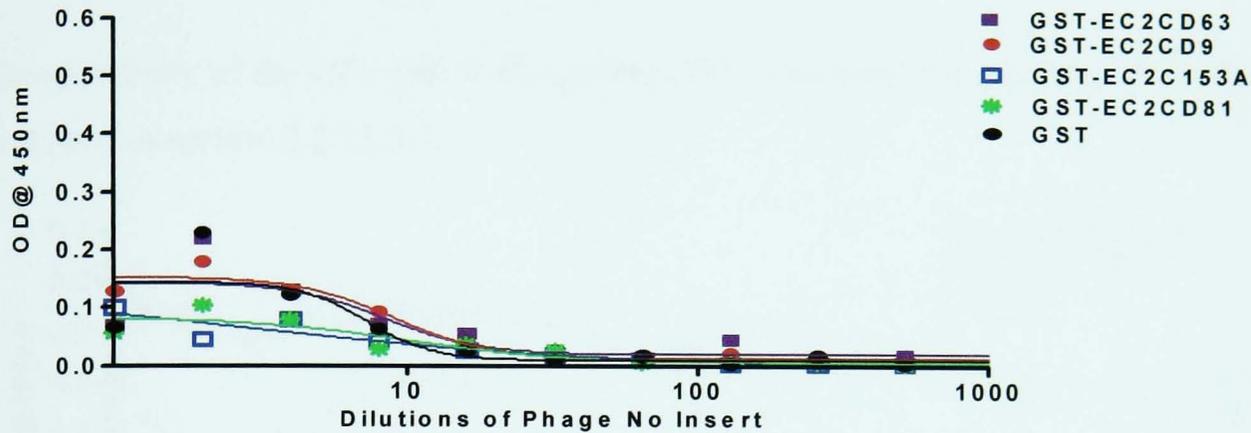


Fig.6.2.17. Titration of phage 'no insert' with different GST-EC2 tetraspanins and GST proteins. The ELISA was carried out as described in section 2.2.12.3.6. The results are the mean OD@450nm of 2 experiments with same phage preparation.
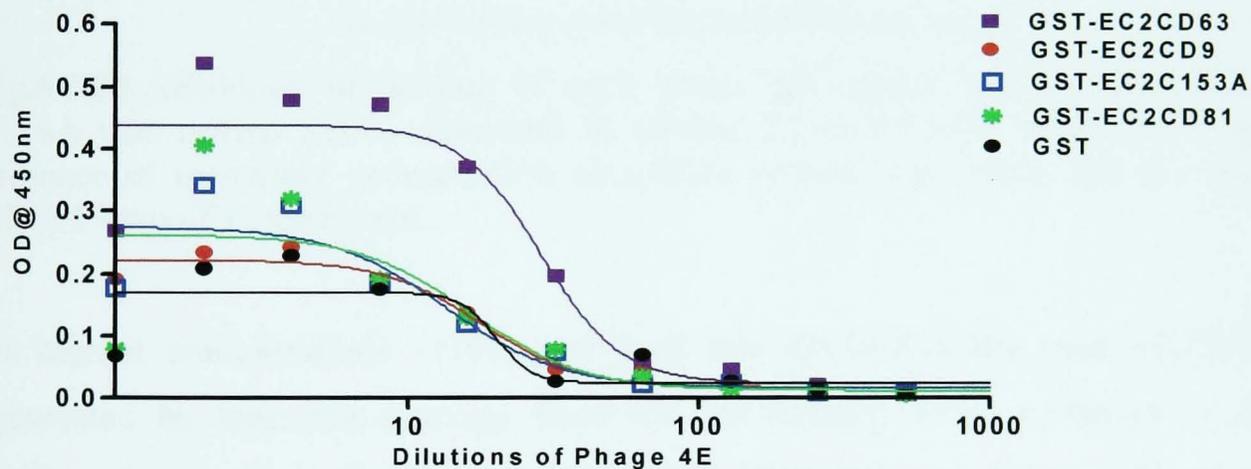


Fig.6.2.18. Titration of scFv phage '4E' with different GST-EC2 tetraspanins and GST proteins. The ELISA was carried out as described in section 2.2.12.3.6. The results are the mean OD@450nm of 2 experiments with same phage preparation.
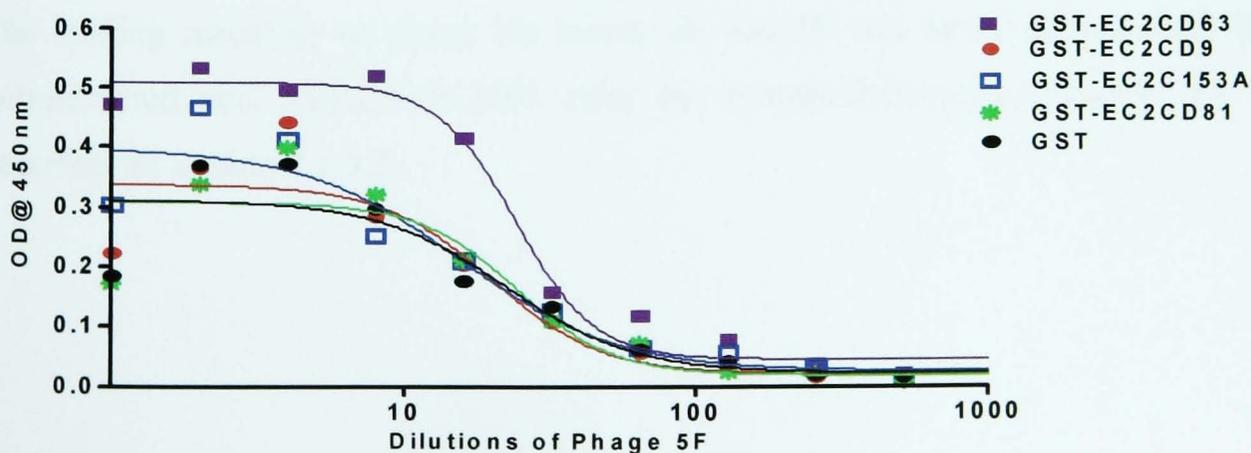


Fig.6.2.19. Titration of scFv phage '5F' with different GST-EC2 tetraspanins and GST proteins. The ELISA was carried out as described in section 2.2.12.3.6. The results are the mean OD@450nm of 2 experiments with same phage preparation.

The no insert phage showed little difference in binding between the GST-EC2CD63 and other EC2 tetraspanins or GST control (Fig.6.2.17). In contrast 4E and 5F showed higher binding values with GST-EC2CD63 than the other GST-EC2 tetraspanins or GST. However, some binding to the other recombinant proteins was observed.

The specificity of the scFv phage 4E against CD63 was tested by inhibition ELISA as described in section 2.2.12.3.7.
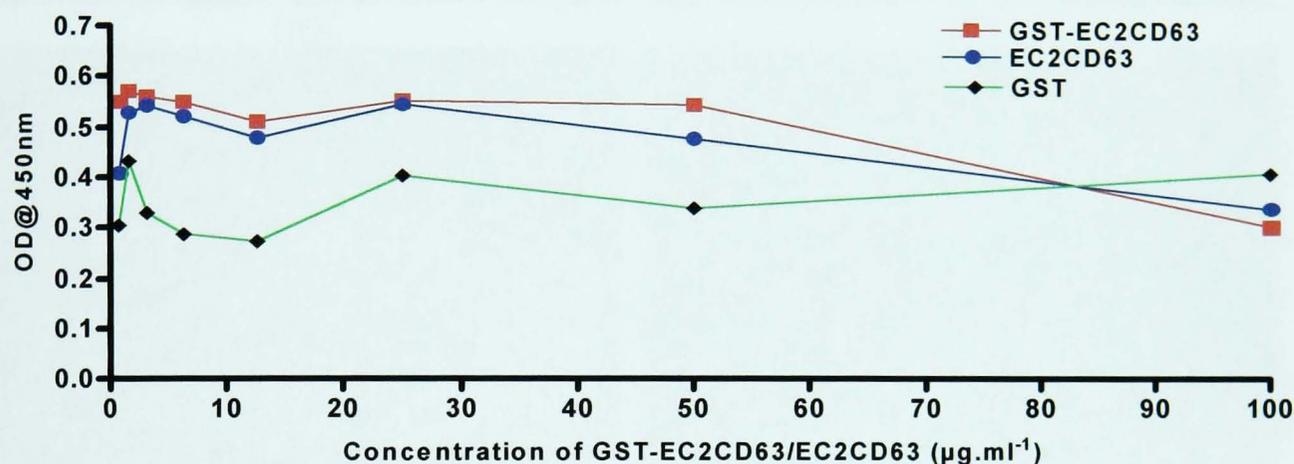


Fig.6.2.20. Inhibition of binding of scFv phage 4E against GST-EC2CD63. The ELISA was carried out as described in section 2.2.12.3.7 with phage 4E, in the presence of increasing concentration of soluble protein. The results are the mean OD@450nm of 1 experiment.

At highest concentrations (100µg.ml$^{-1}$) of free GST-EC2CD63 and EC2CD63 (generated by thrombin cleavage from GST-EC2CD63) some inhibition of the binding of scFv phage 4E with immobilised GST-EC2CD63 was observed. However, this experiment would have to be repeated to see if the result is significant.

### 6.2.6.4.Immunofluorescence microscopy with scFv phage

The binding reactivity of phage No insert, 4E and 5F was tested against RBL2H3 untransfected and RBL2H3-hCD63 cells by immunofluorescence microscopy as described in section 2.2.3.2.
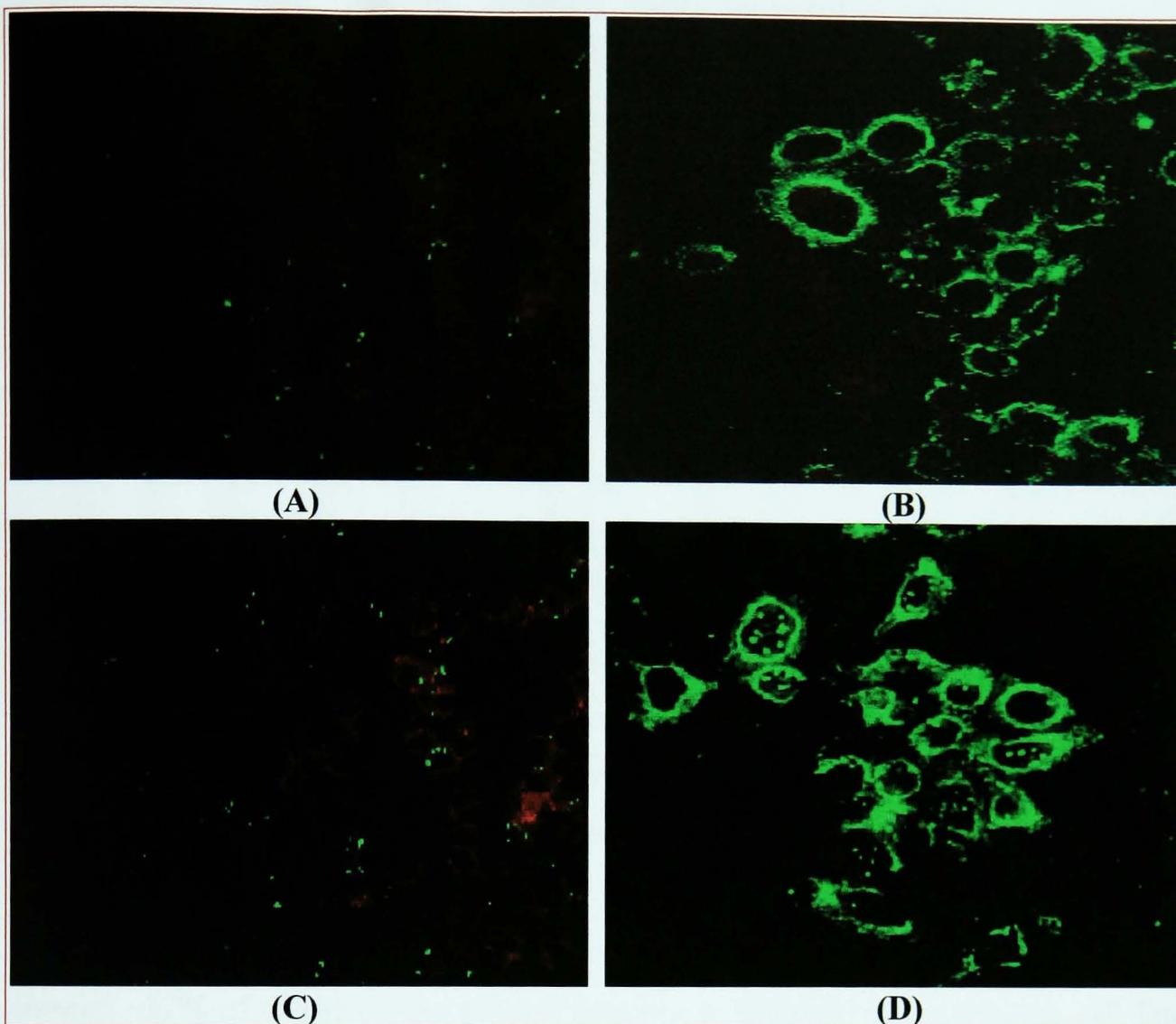
Fig.6.2.21. Immunofluorescence images of scFv phage binding with RBL2H3-hCD63 and RBL2H3 untransfected cells in Lab-Tek® chamber slide. The assay was performed as described in section 2.2.3.2 using anti M13-FITC monoclonal antibody. Where RBL2H3 untransfected cells (A&B); RBL2H3-hCD63 cells (C&D). The phage used: No insert (A&C); 4E (B&D). The images were captured with Nikon Eclipse E400 immunofluorescence microscope using 20x objective.

The results of fluorescence microscopy are in agreement with the results from whole cell ELISA i.e. the phage 4E showed strong binding with RBL untransfected and RBL 2H3-hCD63 cells and almost no binding was observed with no insert phage (Fig. 6.2.21).

# 6.3.DISCUSSION

The combinatorial library technology is a novel innovation for generating recombinant proteins. This technique has been exploited to produce human antibodies to tumour antigens. This chapter describes attempts to further characterise a human scFv phage antibody cloned from a patient with ocular melanoma with putative specificity for human CD63. This chapter also compares the reactivity of human scFv antibody and mouse (whole molecule) anti CD63 antibodies.

Initially, the expression levels of total, cell surface and intracellular expression of CD63 were compared in different human tumour cell lines by FACS analysis using the mouse monoclonal antibody, H5C6. There was variation in total amount of CD63 expressed, with melanoma cell lines usually showing highest levels. All cell lines showed a higher intracellular expression compared with surface expression. This was expected since CD63 has a lysosomal/internalisation motif (GYEVM) at its C-terminus (Rous *et al*, 2002). However, some cell lines showed higher levels of cell surface CD63. As expected, the "control" RBL2H3-hCD63 cell line (transfected with the human CD63 gene and selected by FACS sorting for high surface expression) showed ~37% of CD63 on the plasma membrane. Notably, the melanoma cell line Mewo expressed ~28% of CD63 on the surface, whereas the other melanoma cell lines had ≤ 6% as surface CD63. Immunofluorescence microscopic examination of CD63 staining was performed for some of the tumour cell lines. These were consistent with results obtained from FACS analysis.

The internalisation of surface CD63 at 37°C in response to antibody cross-linking was compared for the different tumour cell lines. Rapid internalisation of cell surface CD63 has previously been observed for macrophages (Audran *et al*, 1995), breast cancer cell lines (Barrio *et al*, 1998), dendritic cells (Mantegazza *et al*, 2004) and hCD63 transfected RBL2H3 cells (G. Mal, PhD thesis, University of Sheffield, 2005). This property may make CD63 useful as an antigen for drug targeting (Audran *et al*, 1995; Barrio *et al*, 1998). All cell lines showed rapid internalisation of CD63, with a high amount of CD63 internalising within 15min. However, whilst some cell lines showed internalisation of most cell surface CD63 by 45min (e.g. ~ 80% for THP-1 and K562 cells) others showed less (e.g. less than 50% for Mewo cells). Whilst further work is needed to reveal the significance of this, it suggests that whilst some cell surface CD63 is free to rapidly internalise on these cells, a proportion is not.

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On the basis of FACS results, the Mewo and Mel-17 melanoma cell lines, expressing high and low amounts of surface CD63 respectively, were selected for initial testing of scFv-phage, along with hCD63 transfected and untransfected RBL2H3 cells. Although the scFv phage had been initially selected by FACS analysis, subsequently problems using phage in FACS were encountered, such as high non-specific binding. Since whole cell ELISA assays had been used successfully by other workers in the group to analyse scFv phage to cell surface antigens, this assay was employed. Mouse antibodies to CD63 were titrated against Mewo and Mel-17 initially. All antibodies (H5C6, IgG; LP9, IgM; BEM-1, IgA hybridoma supernate) gave reactivity with Mewo cells but not Mel-17. This may reflect the level of CD63 on the surface of the two cell lines. Lack of reactivity with Mel-17 may indicate that this assay is less sensitive than FACS, or that epitope(s) recognised by the mouse anti-CD63 are sensitive to fixation.

Two clones, expressing identical scFv genes, were used to generate scFv phage, along with negative (no scFv insert) control. After optimization these were tested in whole cell ELISA. Both clones showed good reactivity with RBL2H3-hCD63, but surprisingly also with untransfected RBL2H3 cells. They also reacted with human Mewo and Mel-17, showing similar reactivity but less than with the rat cell line. The clones were then tested against recombinant tetraspanins EC2s in conventional ELISA. Although some higher reactivity with GST-EC2CD63 was seen, there was also reactivity with the other recombinant proteins. An attempt was made to inhibit binding of the scFv-phage to plate-bound GST-EC2CD63 with free GST-EC2CD63. Some inhibition was seen, but only at high concentrations of free protein, and this should be repeated.

From these results it seems clear that the scFv phage is not specific for human CD63 and the original observations may have been mistaken. It is possible that there is reactivity with rat CD63, and some cross-reactivity with human CD63, which shows 70% identity (Kitani et al, 1991). If there had been time, the scFv phage would have been tested against other cell lines from other animal species transfected with human CD63 (e.g.Chinese Hamster Ovary cells). The scFv phage could also have been tested in Western blotting with cell lysates to determine the molecular weight of the antigen. It is possible that the scFv is not very specific and low affinity. The sequence is very similar to germline antibody sequence, as found in low affinity IgM antibodies.

# CHAPTER 7

# GENERAL DISCUSSION

Tetraspanins appear to play roles in a number of crucial cellular functions including activation, motility and fusion. Recent reports state that these proteins are also involved in various pathological conditions such as cancer and viral infections. Recently Takeda and co-workers reported the involvement of tetraspanins in multinucleated giant cell formation from monocytes (Takeda *et al*, 2003). Hence in the work described in chapters 3-5 of this thesis we further investigated the roles of tetraspanins in mononuclear phagocyte fusion using a panel of different recombinant EC2 tetraspanins.

Previously Takeda and co workers reported that anti CD9 and CD81 antibodies enhanced the fusion of Con A stimulated peripheral blood monocytes, whereas no effect was observed with anti CD63 antibodies (Takeda *et al*, 2003). In contrast GST-EC2CD9 inhibited MGCs formation. As described in chapter 3, we successfully established a Con A-induced fusion system using fresh human peripheral blood monocytes. In agreement with the studies described above, we found that anti CD9 antibodies enhanced MGC formation. However, a panel of different anti CD63 antibodies, including IgG, IgA and IgM isotypes and Fab fragments inhibited MGC formation. GST-EC2CD9 and GST-EC2CD63 inhibited MGC formation whereas GST alone, and GST-EC2CD81, GST-EC2CD82 and GST-EC2CD151 had no effect. CD63-EC2 alone (without the GST fusion protein) also inhibited MGC formation. GST-EC2CD63 and GST-EC2CD9 inhibited the fusion within 24hrs and produced very small MGCs, whereas in their absence the size and number of nuclei in MGCs were increased as the incubation period increased (section 3.2.10).

From the dose/response studies, overall it appeared that GST-EC2CD63 and EC2CD63 inhibited MGC formation with $IC_{50}$ values in the nanomolar range (section 3.2.5. and 3.2.8). Interestingly, nanomolar concentrations of GST-EC2CD63 completely inhibited CCR5-tropic HIV infection of macrophages (Ho *et al*, manuscript submitted). In these cases, however, GST-EC2CD9, GST-EC2CD81 and GST-CD151 were also inhibitory and GST alone had effects at micromolar concentrations.

As discussed in chapter 3, there were some variations in the activities of different preparations of the GST-EC2 tetraspanins. Although all preparations of EC2CD63 and EC2CD9 inhibited MGC formation at high concentrations, these varied in potency. These proteins were expressed in a bacterial system, and although correct folding was checked using conformation-specific antibodies, the level of correctly folded protein could vary. Also, in the case of GST-EC2 proteins, there was variable contamination with GST. Although GST had no effect on MGC formation, it might be preferable to use pure EC2 proteins. Alternatively, improved purification techniques, such as reverse-phase HPLC, allowing isolation of correct conformers could be used. The yields of GST-EC2CD63 were also generally lower than of GST-EC2CD9. This may be due to the larger size of CD63EC2 and that it has two more cysteine residues that are thought to give an extra disulphide bond (Seigneuret *et al*, 2001, see Fig 1.1.1.B). CD63 is also heavily glycosylated *in vivo*. More recently, the better yields of GST-EC2 tetraspanins have been obtained by expressing constructs in *E.coli* strains such as Origami, that facilitate disulphide bond formation (Francine Martin, University of Sheffield, personal communication). Attempts have been made to express tetraspanin EC2s in mammalian cells, but the yields were very poor (Dr L. Partridge, personal communication).

A problem with the fusion assay with human monocytes was variability between donors. The fusion index observed on Con A stimulation was typically 75-95% but poorer rates were observed with some individuals, which could not be used for experiments. Variability in fusion index may be due to blood donor variability since the expression of certain cytokines varies between individuals (Yaqoob *et al*, 1999). Some individuals may also be more sensitive to inhibition by EC2 tetraspanins than others. Attempts to induce fusion in cell lines were not successful here, but it might be possible to induce fusion in cell lines using different cytokines. Another source of variability in the fusion index between the different experiments may be due to Con A. Since Con A requires optimum pH of 7 (Becker *et al*, 1975) for its activity, if the media used for the experiments is slightly acidic/alkali it may affect the function of Con A.

It was important to confirm whether the inhibitory effect CD63 and CD9 EC2s on MGC formation is due to altering molecular mechanisms involved in the fusion process or these proteins are affecting the functions which are related to monocyte

fusion such as adhesion and aggregation. It was also possible that the recombinant proteins might be having cytotoxic or anti-proliferative effects; however, as described in chapter 4, the tetraspanin EC2s did not affect cell numbers. Whilst Takeda *et al.*, and colleagues found that anti-β1 and anti-β2 integrins inhibited monocytes adhesion and aggregation, respectively, their anti CD9 and anti CD81 antibodies had no significant effects. This indicated that the inhibition of MGC formation by the anti-tetraspanin antibodies was not related to involvement in these integrin-mediated functions. The EC2CD9 and EC2CD63 proteins did not inhibit aggregation or initial adhesion of monocytes in our system. However, incubation with CD63 EC2 (with GST removed by cleavage) resulted in reduced numbers of adherent cells, which had been stimulated with Con A. As discussed in chapter 4, this may be because the EC2 inhibits formation of MGCs, which we had observed to be strongly adherent relative to cultured monocytes/macrophages. However, it is possible that EC2CD63 might interfere with adhesion strengthening or spreading, which may be associated with MGC formation (McNally and Anderson, 2002).

Chapter 5 describes attempts to investigate the binding/uptake of GST-EC2 proteins by monocytes. Although further microscopy studies are needed, FACS analysis indicate that GST-EC2CD9 and GST-EC2CD63 are able to bind specifically to Con A stimulated monocytes. As mentioned in chapter 5, it would be of great interest to carry out chemical cross-linking and "pull down" experiments to try to determine what proteins the tetraspanin EC2s are interacting with.

Overall, our results indicate roles for tetraspanins CD9 and CD63 in MGC formation. Since inhibitory effects were observed with recombinant EC2 regions, and these proteins showed some binding to monocytes, we can conclude that these regions of the CD9 and CD63 molecules are involved. The effects are tetraspanin-specific, since no inhibition was observed with recombinant EC2s of CD81, CD82 or CD151. In addition no inhibition was observed with murine CD9 EC2, which shows 77% sequence identity in this region.

Our results for CD9, with anti CD9 antibodies enhancing MGC formation whereas CD9 EC2 inhibits, confirm the observations made by Takeda and co-workers (Takeda *et al*, 2003). Whilst they also found enhancement of MGC formation with anti CD81 antibodies, the lack of an effect with CD81 EC2s suggests a less direct role for this

tetraspanin, perhaps in modulating the activity of CD9. The authors also reported that the expression levels of CD9 and CD81 were upregulated during the normal culture condition for ~2days but under the fusogenic conditions Con A down regulated the expression of CD9 and CD81, whereas the expression of CD63 was up regulated. In addition to this, alveolar and bone-marrow derived macrophages from CD9$^{-/-}$ and CD81$^{-/-}$ mice showed enhanced MGC formation compared to macrophages from control mice both *in vitro* and *in vivo* (in response to a *Propioibacterium acnes*-induced lung inflammation). Finally they showed that mononuclear phagocytes from CD9/CD81 double knock out mice showed enhanced fusion and spontaneous MGCs formation in the lung and enhanced osteoclastogenesis in the bone. This led them to suggest that CD9 and CD81 normally act to negatively regulate MGC formation and our findings are consistent with this hypothesis, at least for CD9. Hence when CD9 is "masked" by antibody or its expression down-regulated, a suppressive effect is removed and MGC formation occurs. When exogenous CD9 EC2 is added, it blocks a component/s required to generate MGCs and the process is inhibited. The effects of CD9 EC2 suggest a direct interaction of this protein in *cis* or in *trans* with molecules on the monocyte surface and binding of CD9 EC2 to Con A stimulated monocytes is consistent with this.

CD9 might associate with other molecules that have been implicated in monocyte fusion, such as CD44 and CD47 (chapter 1, 1.2. reviewed in Vignery, 2000 and Vignery, 2005a), CD98 (Tabata *et al*, 1994) or ADAMS (Namba *et al*, 2001). Although no direct interactions between these proteins have been reported, there is evidence that they may occur in *trans* within the context of TEMs. Hence there are reports that CD9 associates with CD44 (Jones *et al*, 1996; Toyo-oka *et al*, 1999; Yashiro-Ohtari *et al*, 1998) and with CD47 (Longhurst *et al*, 1999). An association between CD9 and ADAMS proteins was reported in sperm:egg fusion (Zhu and Evans, 2002). There is evidence that CD9 on the egg cell surface interacts in *trans* with ADAMS on sperm cells (Takahashi *et al*, 2001). CD98 also associates with integrins and has been implicated in sperm:egg fusion. It has been suggested that CD98 may be a component of an egg surface "tetraspanin web" that includes tetraspanins CD9 and CD81, integrins and integrin-associated proteins that is necessary for fusion (Takahashi *et al*, 2001). A "tetraspanin web", including CD9, CD81, CD98, integrins and ADAMS proteins has been suggested to be involved in

testicular development (Tres and Kierszenbaum, 2005). The interaction of tetraspanins with other molecules recently implicated in monocyte fusion, such as the purinergic receptor (Falzoni *et al*, 1995; Chiozzi *et al*, 1997; Falzoni *et al*, 2000) or DC-STAMP (Yagi *et al*, 2005), has not to our knowledge been investigated. It is also possible that the interaction of CD9 with the EWI proteins is in some way involved in MGC formation. It would be interesting to examine the expression of some of these molecules under fusogenic conditions (i.e. on induction of monocytes with Con A).

Very recently, CD9 has also been shown to function as a receptor for the cytokine IL-16 on mast cells (Qi *et al*, 2005). IL16 also acts as a chemokine and activator of monocytes (Cruikshank *et al*, 1987). Although no role for IL-16 has been ascribed to IL-16 in MGC formation, it is conceivable that this T-cell derived cytokine might be involved in regulating the process.

The suppressive role of CD9 in monocyte/macrophage fusion is in contrast to studies suggesting a positive involvement of this tetraspanin in other fusion processes. Hence, as described in chapter 1, anti CD9 antibodies (Chen *et al*, 1999; Miyado *et al*, 2000; Takahashi *et al*, 2001) and recombinant CD9 EC2s (Zhu *et al*, 2002; Higginbottom *et al*, 2003) inhibit sperm:egg fusion, whilst CD9 null mice show loss of fertility due to a defect in oocyte fusion (Miyado *et al*, 2000; Le Naour *et al*, 2000; Kaji *et al*, 2000). Anti CD9 antibodies are also reported to inhibit virus-induced fusion (Loffler *et al*, 1997) and inhibit or delay muscle cell fusion (Tachibana and Hemler, 1999). This suggests that these fusion processes differ from MGC formation, or that CD9 has different functions depending on the cells where it is expressed.

It is worth noting that the negative regulatory role proposed for CD9 in MGC formation is partly based on the observation that anti CD9 antibodies enhance MGC formation. There are possibilities that these antibodies might cause cell activation, interact with Fcγ receptors, or cross-link proteins associated with CD9 in the tetraspanin web. It would be interesting to examine the effects of Fab fragments of anti CD9 antibodies on monocyte fusion.

Previously Takeda *et al* (2003) reported that substantially higher numbers of MGCs were observed in the air space of CD9 and CD81 null mice challenged with *Propionibacterium acnes* than wild type mice, suggesting that CD9 and CD81 co-ordinately prevent the fusion of mononuclear phagocytes (Takeda *et al*, 2003). In

contrast the same research group very recently (Yamane *et al*, 2005) reported that CD9 may be needed to promote the formation of certain types of granuloma (Yamane *et al*, 2005). In an *in vivo* model of hepatic granuloma formation using injected *Propionibacterium acnes*, compared to normal litter mates CD9-knockout mice showed dissemination of bacteria and an 80% reduction in number and size of granulomas. Production of granuloma-inducing cytokines, TNF-alpha and IFN-gamma, was delayed and chemotactic activity for macrophages was suppressed in the liver of mutant mice. In wild type mice CD9 expression was upregulated on hepatocytes when challenged with *P. acnes,* although the monocytes/macrophages recruited into granulomas were reported to stain weakly for CD9 (Yamane *et al*, 2005).

The inhibitory effects of several anti CD63 antibodies and CD63 EC2s strongly suggests a positive role for this tetraspanin in MGC formation. Takeda and co-workers noted that CD63 expression was increased under fusogenic conditions, but saw no inhibition with an anti CD63 antibody (Takeda *et al*, 2003). This lack of an effect may be due to differences in the antibodies, or slight differences in assay systems used. Unlike CD9, to date, there are no reports of a role for CD63 in cell:cell fusion, although the translocation of CD63 from granules to the plasma membrane in neutrophils and eosinophils has lead to speculation that it could be involved in intracellular membrane fusion (Mahmudi-Azer *et al*, 2002). The molecule has well-reported roles in membrane trafficking (Ryu *et al,* 2000; Kobayashi *et al*, 2000; Rous *et al*, 2002). CD63 has a lysosomal targeting/internalisation motif (GYXX$\phi$, where $\phi$ is a hydrophobic residue (Robinson and Bonifacino, 2001)) at its C-terminus. This part of the molecules has been shown to be important in the CD63-mediated co-internalisation of $\beta$-subunit of the gastric H, K–ATPase ion pump (Duffield *et al*, 2003) and a membrane-type 1 matrix metalloproteinase (Takino *et al*, 2003). Hence, one of its functions appears to be as an "adaptor" protein, inducing rapid internalisation (and thus moderating the functions) of molecules that it associates with. Cell surface CD63 is rapidly internalised on antibody cross-linking (Audran *et al*, 1995; Barrio *et al*, 1998), and even when monovalent Fab fragments are used (Mantegazza *et al*, 2004; our unpublished observations). Inhibition of MGC formation by anti CD63 antibodies might therefore be due to internalisation of a required cell surface component, either CD63 or a molecule that it interacts with. The effects of

CD63 EC2s suggest a direct interaction with a molecule involved in MGC formation. The binding of CD63 EC2 to Con A stimulated monocytes also suggests a direct interaction with a monocytes membrane protein. The identity of such a protein is a matter of speculation; it could be a molecule already implicated in monocyte fusion, or an unknown protein. To date, no direct interactions of CD63 with other cell surface proteins have been reported, although many secondary and tertiary interactions have been demonstrated by co-immunoprecipitation in mild detergents (Hemler, 2003). Such interactions occur in *cis*, probably within the context of TEMs, and are assumed to be weak or indirect. However, as mentioned in chapter 5.3, it is possible that the recombinant EC2s, which might be present as multimers (Kitadokoro *et al*, 2001), could bind to components of the TEMS.

As discussed in chapter 1 (1.1.5.) tetraspanins are known to interact with integrins and to regulate integrin-dependent functions (Berditchevski, 2001; Hemler, 2003). There are reports that CD9 and CD63 associate with $\beta1$ integrins in various cell types (reviewed in Berditchevsky, 2001) and with $\beta1$ integrins and $\beta2$ integrins in monocytes (Takeda *et al*, 2003). These interactions have been demonstrated by co-immunoprecipitation in mild detergents, indicating that they probably occur within TEMs (Hemler, 2003). Antibodies to $\beta1$ and $\beta2$ integrins have been shown to inhibit MGC formation (Tabata *et al*, 1994; Most *et al*, 1990; Gasser and Most, 1999; Takeda *et al*, 2003), although these antibodies may inhibit monocyte adhesion (McNally and Anderson, 2002) and/or aggregation (Tabata *et al*, 1994; Takeda *et al*, 2003) rather than fusion itself. Complex formation between CD9 and integrins is reported down-regulated under fusogenic conditions (Takeda *et al*, 2003). The implications of this for monocyte fusion are unclear, but may relate to the known role of tetraspanins in adhesion dependent signalling mediated by integrins (Berditchevski, 2001).

Interestingly, antibody-induced internalisation of CD63 has been shown to lead to reduced cell surface expression of CD29 ($\beta1$ integrin subunit), CD11b ($\alpha M$ integrin subunit), CD18 ($\beta2$ integrin subunit) and $\alpha5$ intregrin in dendritic cells with concomitant increased migration (Mantegazza *et al*, 2004). CD63 is also implicated in integrin-mediated adhesion-dependent signalling and associations with phosphatidylinositol 4-kinase (PI 4-kinase) (Hemler, 1998; Yauch and Hemler, 2000) and the Src tyrosine kinases Lyn and Hck (Skubitz *et al*, 1996) have been observed.

As suggested above, there is a possibility that CD63 might be involved integrin-mediated adhesion strengthening or spreading of MGCs. A recent report showed that anti CD63 antibodies inhibited FcεRI-dependent degranulation of adherent, but not nonadherent, mast cells (Kraft *et al*, 2005). The anti CD63 antibodies also inhibited mast cell adhesion to fibronectin and vitronectin. Anti CD63 antibodies were shown to impair signalling via the Gab2-PI3 kinase pathway, which is known to be essential for both β1-dependent adhesion and degranulation. Hence, antibodies to CD63 or EC2CD63 might affect adhesion-dependent signalling in monocytes undergoing MGC formation.

As discussed in chapter 1 (1.2.5.4.), it has been suggested that MGC formation occurs through a process akin to phagocytosis. Several groups have observed that phagocytosis of whole cells appears to occur during MGC formation (Gasser and Most, 1999 and references therein; Vignery, 2005a) leading to a model termed "cellocytosis" (Vignery, 2005a). According to this model, after the binding of one macrophage to another, one cell takes the lead and phagocytoses the other. McNally and Anderson also very recently demonstrated that fusion to form multinucleated giant cells (FBGCs) exhibits features of ER-mediated phagocytosis (McNally and Anderson, 2005). CD63 has also been implicated in phagocytosis. For example, it has been shown that CD63 is selectively internalised from the plasma membrane during phagocytosis of yeast particles by immature dendritic cells (Mantegazza *et al*, 2004). It is possible that CD63 might also be involved in "cellocytosis" giving rise to multi-nucleated giant cells. It would be interesting to investigate the effects of the tetraspanin EC2s on phagocytosis.

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