Plasma Membrane-derived Vesicles: their role in the terminal differentiation of monocytes and in inhibiting the phagocytosis of apoptotic cells

Presented by

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PUBLICATIONS

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ABBREVIATIONS

ABCA	ATP-binding cassette (ABC) transporter
ACs	Apoptotic cells
AML	Acute myelocytic leukaemia
APL	Acute promyelocytic leukaemia
ATRA	all-*trans*-retinoic acid
BAK	Bcl-2 homologous antagonist/killer
BANK	B-cell scaffold protein with ankyrin repeats
Bcl-2	B-cell lymphoma 2
BLK	B lymphocyte kinase
CD	Cluster of differentiation
CDKN	cyclin-dependent kinase inhibitor
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-O, O'-bis (2-aminoethyl)-N, N, N', N'-tetraacetic acid
FACS	Fluorescence Activated Cell Sorting
FDC	Follicular dendritic cells
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GM-CSF	Granulocyte- monocyte colony-stimulating factor
HDACL	Histone deacetylase
ICAM	Inter-Cellular Adhesion Molecule
IL	Interleukin
IRF	Interferon regulatory factor
ITGAM	Integrin alpha M
JAKS	Janus kinase ("Just another kinase")
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MiAC	Mitochondrial induced apoptosis channel
M-CSF	Monocyte colony-stimulating factor
MΦ	Macrophage
NBT	Nitroblue Tetrazolium
NCOR	Nueclear co-repressor
NHS	Normal Human Serum
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PECAM	Platelet endothelium adhesion molecule
PMA	Phorbol 12-myristate 13-acetate
PM	Promyelocytic leukaemia
PMVs	Plasma Membrane-derived Vesicles
PS	Phosphatidylserine
PTPN22	Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)
RARα	Retinoic acid receptor alpha
ROCK	Rhoassociated kinase
SMACs	Second mitochondria-derived activator of caspases
SSC	Side scatter
TBM	Signal Transducers and Activators of Transcription
STAT	Tingible body macrophages
TNFSF	Tumor necrosis factor superfamily
VCAM	Vascular cell adhesion molecule
VDAC	Voltage-dependent anion channels

ABSTRACT

Plasma Membrane-derived Vesicles (PMVs) are membrane-coated vesicles of diameter 0.1 to 1 μm, carrying various proteins inherent in their parental cells. PMVs are released when cells undergo activation/apoptosis via blebbing and shedding and have a function in intercellular communication. Exposition of phosphotidylserine (PS) on the outer membrane leaflet that mark them as a biologically distinct entity could explain a role for PMVs in phagocytosis and thrombosis.

There is currently a large amount of variation between researchers with regards to characterisation of PMVs as a result of less or no standardization in the pre and post analytical steps employed in PMV work. Efforts are being made to combat these issues, but still, proper standardization of analytical procedures is yet to be achieved. The purpose of this work is to be able to properly analyse, categorise and characterise PMVs to help lay the groundwork for PMV testing in clinical settings.

We have examined the role of PMVs, in the differentiation of HL-60 promonocytes. The myeloid differentiating agents ATRA, PMA and histamine, which inhibit promonocyte proliferation, induced an intracellular Ca^{2+}-mediated PMV (as opposed to exosome) release from HL-60 promonocytes. These PMVs caused HL-60 cells to enter G0/G1 cell cycle arrest and induce terminal monocyte-to-macrophage differentiation through TGF-β1 mediation.

Patients with inflammatory diseases such as SLE show increased PMV levels in the plasma, accompanied by persistent apoptosis and defective clearance, which correlates with the disease activity. Although still unclear, impaired clearance of apoptotic cells (ACs) may represent a mechanism for the development or enhancement of SLE. We found that PMVs dose dependently inhibit the phagocytosis of ACs by competing for the PS receptor on macrophages via which ACs are phagocytosed. PMVs can therefore modulate phagocytosis of ACs and may play a role in the aetiology of autoimmune diseases, in particular SLE.

CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

This introduction aims to outline the current understanding of the biosynthesis of Plasma Membrane-derived Vesicles (PMVs). The current protocols for isolation and separation from exosomes are discussed, as is the need for standardization of such methods. This thesis will investigate the involvement of PMVs in monocyte development in the bone marrow with respect to APL, acute promyelocytic leukaemia so this introduction will explain the etiology and current therapy for the disease As I have found PMVs to inhibit phagocytosis the introduction will then describe the role of tissue macrophages and outline the mechanism of phagocytosis as a precursor to describing SLE, systemic lupus erythematosus, the autoimmune disease in which monocyte-derived macrophages are unable to phagocytose apoptotic cells.

1.1 Microparticles/Plasma membrane vesicles (PMVs)

Microparticles or Plasma membrane-derived vesicles (PMVs) are a heterogeneous group of small, membrane-coated vesicles with a diameter of 0.1 to 1μm (Abid-Hussein MN *et al* 2003, Aupeix K *et al* 1997). PMVs are phospholipid rich particles containing certain membrane receptors as well as other proteins inherent in their parental cells (Baj-Krzyworzeka M *et al* 2002). PMVs are released from the cellular membrane when various types of cells undergo activation or apoptosis and the presence of a cell-specific antigen or combination of antigens allows identification of their cellular origin (Barry OP *et al* 1999). Various names have been used to describe vesicles released by cells including microparticles, particles, microvesicles, vesicles, and ectosomes probably because the mechanism involved in their release was not always studied in detail (Barry OP *et al* 1999, Barry OP *et al* 1998).

The presence of basal levels of PMVs is common in healthy individuals and an increase in their release although a controlled event, is a hallmark of cellular alteration. Therefore pharmacological modulation of circulating PMV concentrations could become a major therapeutic target in the future (Berckmans RJ *et al* 2001; Boulanger CM *et al* 2001). The field of PMVs has gained interest over the past ten years, and is constantly gaining momentum as more workers are exposed to the subject (Bird DA *et al* 1999). Various publications have been released over the years describing the molecular and functional characteristics of PMVs. These suggest the importance of PMVs as a key role player in various cell processes rather than just inert bi-products of cellular activation (Boulanger CM *et al* 2001; Bird DA *et al* 1999).

1.2 Historical Background of PMVs

It has been known since the 1940's that human plasma and serum contained a subcellular factor that facilitates fibrin formation (Chargaff E *et al* 1946; O'Brien JR 1955). Using electron microscopic techniques, Wolf in 1967 demonstrated that activated platelets shed membrane fragments (subcellular factor) and was able to show that this subcellular factor consisted of small vesicles which was originally described as "platelet dust" now "PMVs" (Wolf P 1967). These PMVs showed procoagulant activity comparable to that of intact platelets (Wolf P 1967) and they were associated with phospholipid-related procoagulant activity known as platelet factor 3 (PF3). The procoagulant activity of these PMVs was designated as platelet factor 3 (PF3) (Hardisty RM *et al* 1966). Subsequently, it was shown *in vitro* that platelet-derived PMVs were formed during the attachment of platelets to the vascular wall (Warren BA 1972).

In recent years, the interest for PMVs has increased substantially, not only because of their procoagulant properties but also because of the role they are thought to play in inflammation processes and their ability to directly affect endothelial functions (Freyssinet JM *et al* 1999; Horstman LL, Ahn YS 1999) which was demonstrated for the first time in patients with Idiopathic Thrombocytopaenic Purpura (ITP) (Nieuwland R *et al* 2002). They are also indicated in a number of autoimmune diseases as well as malaria infestation whereby the number of PMVs in the plasma of the affected individual is known to increase. The most abundant of circulating PMVs in blood is derived from platelets (Janowska-Wieczorek *et al* 2001) although PMVs in the periphery can also arise from erythrocytes, granulocytes, monocytes, lymphocytes and endothelial cells, which usually circulate at lower numbers (Diamant M *et al* 2002).

1.3 Structural Components of PMVs

PMVs are released from the membranes of cells and therefore contain cell surface or membrane proteins and cytoplasmic components of their parent cells (Bevers EM *et al* 1982). This implies that PMVs derived from monocytes and platelets possess different components as they contain some antigenic markers derived from their cell of origin (Fadok VA *et al* 2001). This is why markers on the PMVs membrane are generally used to identify which cell line they were produced from as different cell lines express different markers and proteins on their surfaces (Lodish, H *et al* 2004;Piccin A *et al* 2007). The type of stimulus that releases the PMVs also determines their constituents, therefore PMVs generated by cell activation processes or apoptosis contain different cell markers even if they are produced by the same cell type (Diaz C *et al* 1996).

In general PMVs carry proteins characteristic of their parental cell. PMVs shed by polymorphonuclear leukocytes (PMN) express selectins, intergrins, complement regulators, HLA-1 and other markers of nuetrophils whereas, monocyte derived microparticles express tissue factor and P-selectin glycoprotein ligand-1 (PSGL-1) as well as CD14 (lipopolysacharide receptor). Lymphocytic PMVs express CD4 and CD8 with erythrocytes staining for glycophorine A (Rauch U *et al* 2000; Takaoka A *et al* 2003). Platelet microparticles express platelet endothelium adhesion molecule (PECAM-1; CD31), CD62p (P-selectin), glycoproteins IIb–IIIa, P-selectin / CD42a, CD41, (GpIb) and CD63 (Alimonti JB *et al* 2003). Endothelial PMVs express CD31, CD34, CD54, CD51, CD146 (S endo 1), CD105 (endoglin), 62E (E-selectin) (Butikofer P. *et al* 1989). PMVs can also express a different set of surface markers than the precursor cells as has been observed with erythrocyte PMVs, but the rules for the incorporation of different proteins into PMVs are not known (Hamilton, K K *et al* 1990; Alimonti, JB *et al* 2003).

1.3.1 PMVs are surrounded by a phospholipid bilayer

Cells from which PMVs originate are surrounded by a plasma membrane consisting of a phospholipids bilayer, containing phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyeline (SM) (Bevers EM *et al* 1982; Connor J *et al* 1992). In unstimulated or resting cells, the various phospholipid species are distributed asymmetrically in the bilayer (Huber J *et al* 2002). However, this asymmetrical phospholipid distribution is usually disturbed or altered during PMVs formation (Zwall RF *et al* 1997) resulting in the exposure of negatively charged phospholipids such as PS and PE on the PMV surface (Diamant M *et al* 2002; Huber J e*t al* 2002). The PS surface exposure most likely plays a role in some of the *in vivo* effects of PMVs since PS efficiently binds coagulation factors and their receptors on cellular surfaces (Zhou Z *et al* 2007).

1.3.2 PMVs also contain actin

Actin is a globular, ubiquitous, roughly 42kDa protein. It is a monomeric subunit of microfilaments and one of the three major components of the cytoskeleton of a eukaryotic cell present in concentrations of over 100μM (Abid-Hussein MN *et al* 2003; Berckmans RJ *et al* 2001). There are at least six isoforms of actin in mammals coded for by separate genes and these are further subdivided into three classes (alpha, beta and gamma) according to their isoelectric point. Alpha actin is found in muscle whereas beta and gamma isoforms are prominent in non-muscle cells (Cotran *et al* 1998). Individual subunits of actin are known as globular actin (G-actin), which assembles in F-actin.

Actin participates in many important cellular functions, including muscle contraction, cell motility, production of pseudopodia in phagocytic cells, cell division and cytokinesis, vesicle and organelle movement, cell signalling, the establishment and maintenance of cell junctions, and cell shape (Cotran *et al* 1998; Fox JE *et al* 1990). Actin exists mainly as a fibrous polymer, and is associated with protrusions of the plasma membrane followed by PMVs formation (Werlen G *et al* 2003). It is also cleaved under apoptotic conditions by caspase-3.

1.4 Formation of PMVs

Before we look at how PMVs are produced, it is important for us to understand the normal constitution of the cell membrane and how it is maintained in a normal physiologically active cell (Boulanger CM *et al* 2001). As referred to earlier, the phospholipid bilayer membrane of cells is composed of highly specific phospholipids that are distributed in a precise manner. A form of asymmetry is constantly maintained in a dynamic manner in that phosphatidylcholine (PC) and sphingomyelin (SM) are located on the external membrane whereas

phosphatidylserine (PS) and phosphatidylethanolamine (PE) are positioned on the inner membrane (Piccin A *et al* 2007).

This asymmetry is actively maintained by various enzymes such as the aminophospholipid-translocase or floppase (Iwata M *et al* 1996), scramblase, calpain and gelsolin (present only in platelets) (Piccin A *et al* 2007). These enzymes maintain a dynamic asymmetric steady state and allow membrane phospholipids to move to the outside of the cell membrane while at the same time, the aminophospholipids are redirected to the inner side of the cell membrane (Dejean LM *et al* 2006; Piccin A *et al* 2007). The conservation of this asymmetry is essential for cell survival and physiology, and is achieved by a combination of transmembrane enzymes (Fig 1a-1d) that are also implicated in PMV production (Fox JE *et al* 1990; Jimenez JJ *et al* 2003).

1.4.1 Gelsolin

Gelsolin is an enzyme that is specific to platelets only (Piccin A *et al* 2007) and functions by removing proteins that cap the ends of actin filaments of the platelet cytoskeleton. This in turn allows actin to reorganize and hence causes platelet activation and contraction together with PMV generation. The enzyme is activated during increased levels of cytosolic calcium (Martinez MC *et al* 2005).

1.4.2 Aminophospholipid translocase

This is an ATP dependant enzyme that is involved in the constant transportation of PS and PE from the outer layer of the cell membrane to the inner layer. One molecule of ATP is required for the transfer of each PS molecule. The enzyme is inhibited by increased levels of cytosolic calcium (Baj-Krzyworzeka M *et al* 2002).

1.4.3 Floppase

This is another ATP dependent enzyme that is thought to work in conjunction with aminophospholipid translocase. Its main action is to transport lipids from the inner to the outer leaflet; however its full function is not currently well understood (Piccin A *et al* 2007).

1.4.4 Lipid Scramblase

The function of this enzyme is to allow the movement of phospholipids across the cell membrane. The enzyme is activated during increased levels of intracellular calcium, which simultaneously inhibits Aminophospholipid translocase (Connor J *et al* 1992; Ziegler U & Groscurth P 2004). This therefore causes a loss of membrane asymmetry and a stable expression of PS is maintained on the outer membrane, which is a sign of microparticle production (Horstman LL, Ahn YS 1999; Piccin A *et al* 2007). This enzyme is thought to play an important role in platelet coagulant activity and microparticle production. Patients who have Scott syndrome (have a defect in lipid scramblase enzyme) have shown a defect in platelet coagulation activity as well as a reduction in microparticles. This is due to reduced PS expression on the outer membrane hence reduced production of PMVs proving the importance of the enzyme in PMV formation (Piccin A *et al* 2007).

1.4.5 Calpain

This is an enzyme that belongs to the cysteine proteinase of the papainase family. Calpain is activated during increased levels of cytosolic calcium and when activated is associated with various functions that aid in PMV production such as cleaving of cytoskeletal filaments, facilitating PMV shedding, and activating apoptosis via various pathways (Huber J *et al* 2002; Piccin A *et al* 2007).

Fig.1 Schematic presentation of modes of emission of PMVs

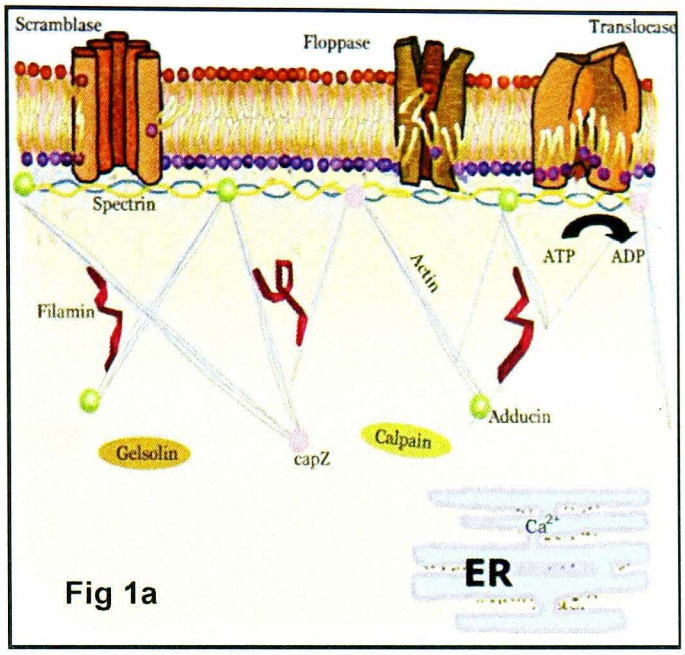


Fig 1a

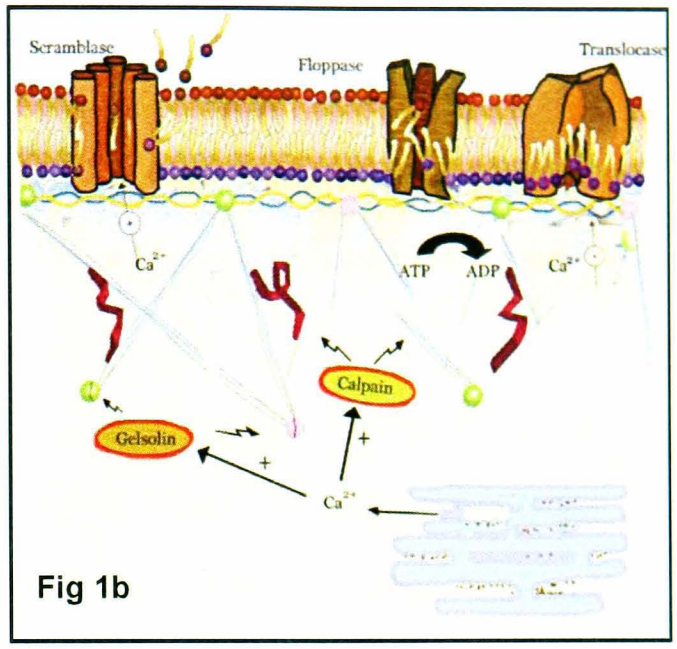


Fig 1b

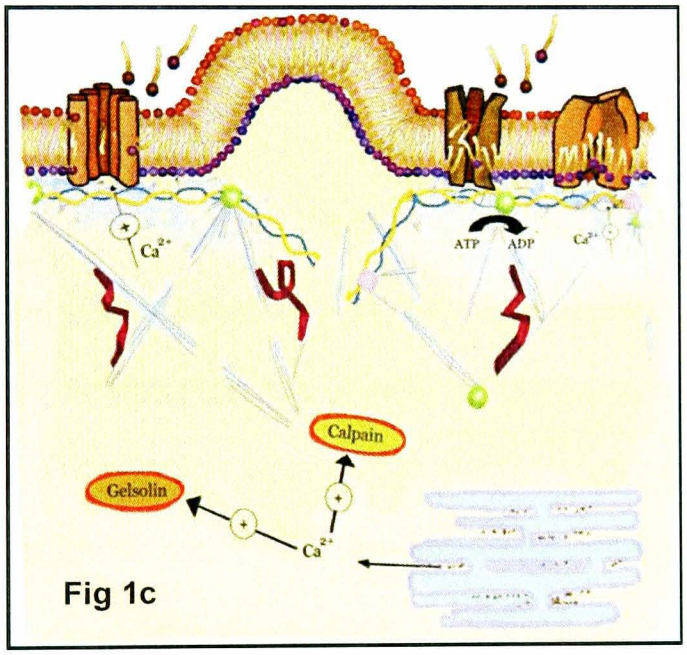


Fig 1c

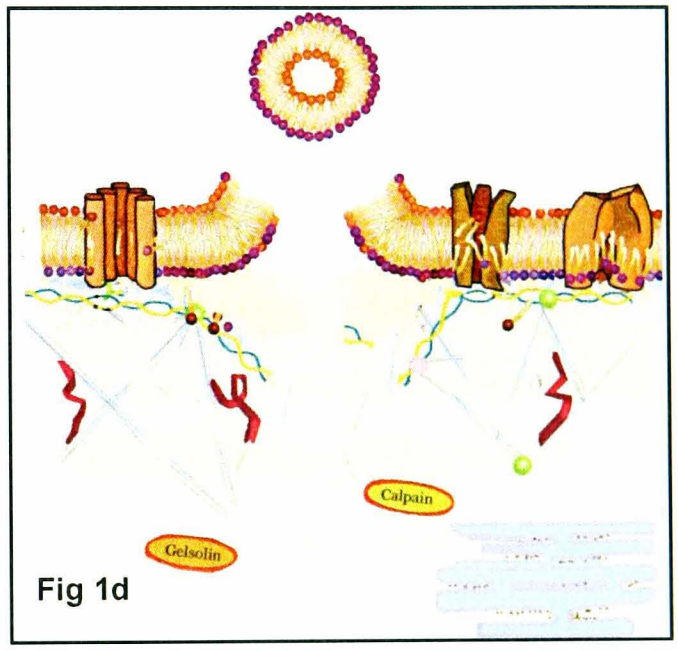


Fig 1d

Fig 1a: *Representation of the resting cell*: Scramblase is inactive while Translocase and Floppase is active maintaining membrane asymmetry (Piccin A *et al* 2007).

Fig 1b: *Cell Activation*: Scramblase, Calpain and Gelsolin are activated due to calcium release from ER. Calpain cleaves long actin filaments. Gelsolin cleaves actin-capping proteins. Translocase is inactivated. Membrane asymmetry is compromised (Piccin A *et al* 2007).

Fig 1c: *Cytoskeletal Disruption*: protein anchorage to the cytoskeleton is disrupted allowing membrane budding (Piccin A *et al* 2007).

Fig 1d: *Microparticle (PMV) formation*: increased phosphatidylserine is exposed on the microparticle external surface (Piccin A *et al* 2007).

1.4.6 Mechanism of PMV formation

The intracellular mechanisms underlying the stimulus leading to the vesiculation process and actual release of PMVs are as yet not fully understood (Barry OP et al 1999; Diamant M *et al* 2002). While all subcellular structures may have physiological activities, they likely vary depending upon the array of molecules present (Abid-Hussein MN *et al* 2003; Barry OP *et al* 1998). Experiments have shown that PMV release from cells occurs with both activation and cell death either through apoptosis or necrosis (Dejean LM *et al* 2006; Eguchi K. 2001). PMVs are released from the cell surface membrane following cellular activation or death by either chemical stimuli such as cytokines, endotoxins or a physical stimulus such as shear stress (Cohen JJ *et al* 1992; Connor J *et al* 1992).

On cell activation there is a rise in cytosolic calcium [ca^{2+}] concentration possibly through the entry of extracellular ca^{2+} via ca^{2+} channels in the plasma membrane which are normally closed at physiologic or resting membrane potential, but are activated (*i.e.*, opened) at depolarized membrane potentials (Berckmans RJ *et al* 2002; Bevers EM *et al* 1982). This consequently leads to the activation of enzymes such as calpain, gelsolin, scramblase as well as protein kinases. Simultaneously, enzymes such as translocase (Fig 1b), and phosphatases are inhibited, therefore resulting in cytoskeletal reorganisation, loss of membrane asymmetry, membrane blebbing and hence PMV formation and release (Cohen JJ *et al* 1992; Connor J *et al* 1992). PMVs have also been shown to be released during apoptosis induced *in vitro* by growth factor deprivation or by complement deposition (Hamilton KK *et al* 1990; Jimenez JJ *et al* 2003). This membrane modification can cause PS exposure and bleb formation, leading to the extrusion of PMVs which incorporates surface proteins and other contents of the originating cell (Martinez MC *et al* 2005).

During programmed cell death (apoptosis), the release of microparticles is associated with membrane blebbing, which is a characteristic feature of apoptosis. Blebbing involves a dynamic redistribution of cellular contents, perhaps related to volume stress that occurs as cells die and the activation of Rho associated kinase 1 (ROCK-1) in this process is critical (Distler JH *et al* 2005). ROCK 1 is one of the kinases that have been identified to play a role in membrane blebbing and is thought to be key in the formation of PMVs (Connor J *et al* 1992; Distler JH *et al* 2005). The ROCK kinases are activated by GTP bound Rho and are important mediators of cytoskeletal modifications such as myosin light chain phosphorylation and actin myosin coupling to the plasma membrane (Distler JH *et al* 2005). Experiments carried out with mouse fibroblasts and human epithelial breast cancer cells showed a decrease in PMV formation on application of a ROCK 1 activity inhibitor (Y27632) (Cotran *et al* 1998; Fox JE *et al* 1990) hence suggesting that they indeed play a role in PMV formation in apoptosis. The caspase inhibitor z-VAD-fmk also blocked the cleavage of ROCK-1 and subsequently membrane blebbing and PMV release thereby suggesting a role of caspases (involved in apoptosis) as well in the activation of ROCK-1 (Fox JE *et al* 1990; Goeddel DV *et al*).

Although ROCK-1, an effector of Rho GTPases, is essential for apoptotic membrane blebbing, not all cells bleb, and even when they do, the blebbing process can differ during the stages of apoptosis (Berckmans RJ *et al* 2001; Berckmans RJ *et al* 2002). PMV release appears to occur late in the death process and may occur concurrently with cell fragmentation and the formation of apoptotic bodies. Apoptotic bodies represent shrunken and collapsed cells with nuclear fragmentation (Boettner DR *et al* 2008). As these considerations suggest, the mechanisms for PMV formation likely differ in the settings of cell activation and apoptosis.

These differences could lead to variations in PMV size as well as macromolecular composition, both surface and internal (Chargaff E *et al* 1946; Connor J *et al* 1992). As an example of these differences, the expression of cell membrane molecules on endothelial PMVs varies depending upon whether their formation results from cell activation or apoptosis (Jimenez JJ *et al* 2003). What is not yet known is whether these structural differences influence function.

1.5 Physiological and exogenous stimuli inducing PMV release

Though ectocytosis is a general biological property of most eukaryotic cells, cells release PMVs in response to a variety of stimuli. PMV formation *in vitro* occurs whenever a stimulus is applied which then induces either cell activation or apoptosis (Diamant M *et al* 2002). These stimuli include chemical stimuli, such as cytokines, thrombin and endotoxin, or physical stimuli, such as shear stress or hypoxia (Van Wijk MJ *et al* 2002). Complement membrane attack complex C5b-9, in the presence or absence of antibodies, Ca^{2+} ionophore A23187, phorbol esters, epinephrine, adenosine diphosphate, and microbial peptides such as formyl-methionyl-leucyl-phenylalanine (fMLP) are potent stimuli for PMV release as well (Abid-Hussein MN *et al* 2003; Aupeix K *et al* 1997). In addition to many biological stimuli, shear stress is an important mechanical factor inducing PMV release in platelets and many cell types. It also should be noted that many stimuli can be additive or even synergistic (Boettner DR *et al* 2008; Butikofer P *et al* 1989).

1.6 Concept of PS-Positive PMV Release

The concept of plasma membrane blebbing leading to the shedding of PMVs is based on a transverse migration of anionic phospholipids such as PS from the inner layer to the outer layer of the plasma membrane (Schroit AJ *et al* 1984; Zwaal RF *et al* 1997). The composition and the distribution of cell membrane phospholipids are

highly specific but cellular activation or death processes cause an imbalance in the symmetry of the plasma membrane phospholipid distribution as mentioned earlier which results in the exposure of PS on the outer cell surface of the PMVs through which they can be identified. PS has become a dominant marker for identifying PMVs and other proteins (Connor J *et al* 1992)

1.7 Detection of PMVs

Though several methods for analysis of PMVs have been reported, Flow-cytometry and ELISA are the most widely adopted (Jy W *et al* 2004). It is, however, difficult to make direct comparison of results because several groups have used different approaches to basic procedures such as centrifugation, re-suspension and washing of the PMV pellet (Jy W *et al* 2004; Piccin A *et al* 2007). Furthermore, the cell lineage markers adopted are broadly different. Flow cytometry is the method of choice for analysing and studying of plasma PMVs (Hay S *et al* 2002; Jy W *et al* 2004). The samples to be tested are obtained from platelet-free plasma after centrifugation although PMVs can be detected in blood samples or fractions thereof as well as in other body fluids such as synovial fluid (Berckmans RJ *et al* 2001; Horstman LL *et al* 1999).

Using labelled antibodies against cell-specific antigens and/or activation markers and annexin V, a protein that binds specifically to negatively charged phospholipids in the presence of calcium ions, PMV fractions or subpopulations can be quantified and concurrently their cellular origin as well as their 'activation status' established (Jy W *et al* 2004). Of each event detected by the flow cytometer, the size (forward scatter-FSC) and density or granularity (sidescatter-SSC) are determined electronically, as well as the fluorescence in various channels (Aupeix K *et al* 1997). Fluorescence reflects the amount of antibody bound and therefore is an estimate for

the amount of antigen exposed on the membrane surface (Miyazaki *et al* 1996). Also, ELISA and electron microscopy technique can be used for PMVs detection and especially for distinguishing PMVs and exosomes. One of the most frequently used ELISAs to quantify cell-derived PMVs employs a plate coated with annexin V (Aupeix K *et al* 1997; Miyazaki *et al* 1996; Nieuwland R *et al* 2002).

1.8 Exosomes, Endosomes and PMVs are distinct

Exosomes were described in the early 1980s as 5'-nucleotidase activity-containing vesicles and to range in size from 30 to 90 nm (Fig. 2). In contrast to PMVs, exosomes are preformed membrane vesicles, which are stored in cellular compartments named multivesicular bodies (MVB), and secreted when the MVBs fuse with the cell membrane (Bettina T. *et al* 2007). Many eukaryotic cells release vesicles like exosomes spontaneously or under appropriate stimulation. They were shown to be involved in the removal of the transferrin receptor from maturing reticulocytes by 'invagination' blebbing. These blebs (called endosomes) pinch off very small (30–90nm) 'intraluminal' vesicles (Bettina T *et al* 2007; Denzer K *et al* 2000). These endosomes containing 'intraluminal vesicles' are stored in the MVB and once the 'intraluminal' vesicles become secreted, i.e. after membrane fusion of MVB and the surrounding plasma membrane, they are called exosomes (Denzer K *et al* 2000).

Endosomes are membrane-bound vesicles, formed via a complex family of processes collectively known as endocytosis, and found in the cytoplasm of virtually every animal cell. The basic mechanism of endocytosis is the reverse of what occurs during exocytosis or cellular secretion. It involves the invagination of a cell's plasma membrane to surround macromolecules or other matter diffusing through the extracellular fluid (Hess C *et al* 1999). The encircled foreign material is then brought

into the cell, and following a pinching-off of the membrane (termed budding), is released into the cytoplasm in a sac-like vesicle (Abid-Hussein MN *et al* 2003).

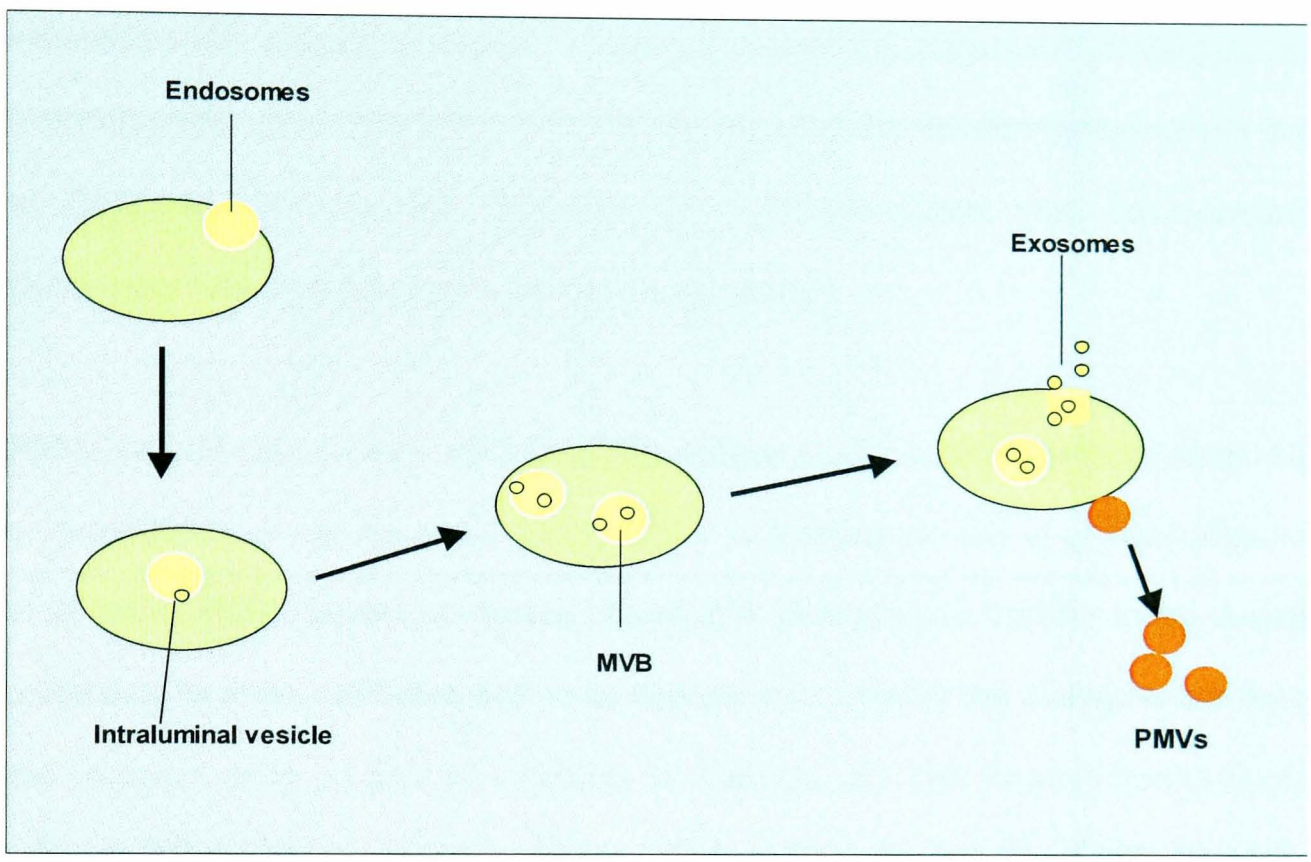


Fig.2 Schematic presentation of modes of emission of PMVs vs. exosomes. This figure shows the formation of an endosome by invagination. By inward blebbing of the endosomal membrane, intraluminal vesicles are formed. Endosomes containing intraluminal vesicles are called multivesicular bodies (MVBs). Cells release the contents of their MVB when the membrane of the MVB fuses with the plasma membrane and in contrast to exosomes, PMVs are formed by major structural rearrangements of the cytoskeleton and are 'budded' off from the outer cell membrane.

1.9 Functions of PMVs

The most frequently described characteristic of both *in vitro* and *in vivo* released PMVs is their procoagulant activities (Berckmans RJ *et al* 2001; Nieuwland R *et al* 1997; Nieuwland R *et al* 2002), which contribute to haemostasis by stimulation of cytokine secretion and tissue factor expression in endothelial cells (Boulanger CM *et al* 2001; Jimenez JJ *et al* 2003). PMVs contain several signalling proteins and bioactive lipids including sphingosine 1-phospahte (S1P) and arachidonic acid (AA)

and are said to be involved in signal transduction. They can activate certain signalling pathways in human cells such as P38 and mitogen-activated protein kinase (MAPK) (Boettner DR *et al* 2008). Other PMV fractions were reported to induce cellular growth, chemotaxis, apoptosis and the outgrowth of transplanted haematopoetic stem cells (Baj-Krzyworzeka M *et al* 2002; Janowska-Wieczorek A *et al* 2001) as well as the inhibition of endothelium-dependent vasodilatation (Boulanger CM *et al* 2001; Van Wijk MJ *et al* 2002).

PMVs can directly activate and stimulate cells to produce inflammatory substances or mediators such as cytokines (Barry OP *et al* 1998; Barry OP *et al* 1997; Mesri M *et al* 1999). PMVs bearing antigens of their cell of origin can transfer these surface molecules to other cell types and in so doing they may alter the biological activity of the recipient cells (Li MO *et al* 2003). In addition, at least *in vitro* microparticles mediate intercellular interactions (Barry OP *et al* 1998; Forlow SB *et al* 2000; Huber J *et al* 2002). Also, subpopulations of PMVs isolated from human plasma expose C1q, C3 and C4, strongly suggesting their direct involvement in activation of the complement system (Nauta AJ *et al* 2002). Currently, the relation between cellular PMVs and C-reactive protein (CRP) is being studied. This acute phase protein is known to bind to membranes and, in the membrane-bound form, may activate the classical pathway of the complement system, ultimately leading to vascular damage (Diamant M *et al* 2002).

Although elevated levels of PMV subpopulations are present in the circulation of patients with inflammatory disease, both of infectious and autoimmune origin, any causal link between PMVs and inflammatory processes cannot readily be established, because cytokines trigger cells, thereby stimulating the release of PMVs, whereas PMVs trigger cells to produce and release cytokines (Berckmans

RJ *et al* 2001; Mesri M *et al* 1999). Therefore, it is as yet unclear whether cellular PMVs are a cause or a consequence of inflammatory processes and associated vascular damage. PMVs may also expose adhesion-cell molecules, and specifically adhere to for example endothelial cells thereby stimulating these cells to produce various intermediates, such as E-selectin and tissue factor (Baj-Krzyworzeka M *et al* 2002; Barry OP *et al* 1998; Rauch U *et al* 2000).

PS acts as a marker for cells that are identified as injured and ready for phagocytosis. However because injured cells have a window of opportunity to be repaired, PMVs have to be shed from the cells when the repair is successful by probably getting rid of the phagocytotic signal (Bevers EM *et al* 1982, Butikofer P *et al* 1989). PMVs help cells resist death by eliminating the membrane attack complex (MAC) of complement from their surface so inhibiting apoptosis of polymorphonuclear leucocytes (Boettner DR *et al* 2008).

Chemotactic responsiveness of human haematopoitic stem/progenitor cells as well as increased survival of these cells is enhanced by platelet-derived PMVs (Baj-Krzyworzeka M *et al* 2002; Barry OP *et al* 1999). PMVs also play a role in organ defence systems such as stress response, inflammation and regeneration as well as in modulation of vascular tone, angiogenesis and stem cell engraftment (Baj-Krzyworzeka M *et al* 2002). The role of PMVs in the inhibition of phagocytosis of apoptotic cells is also being looked into.

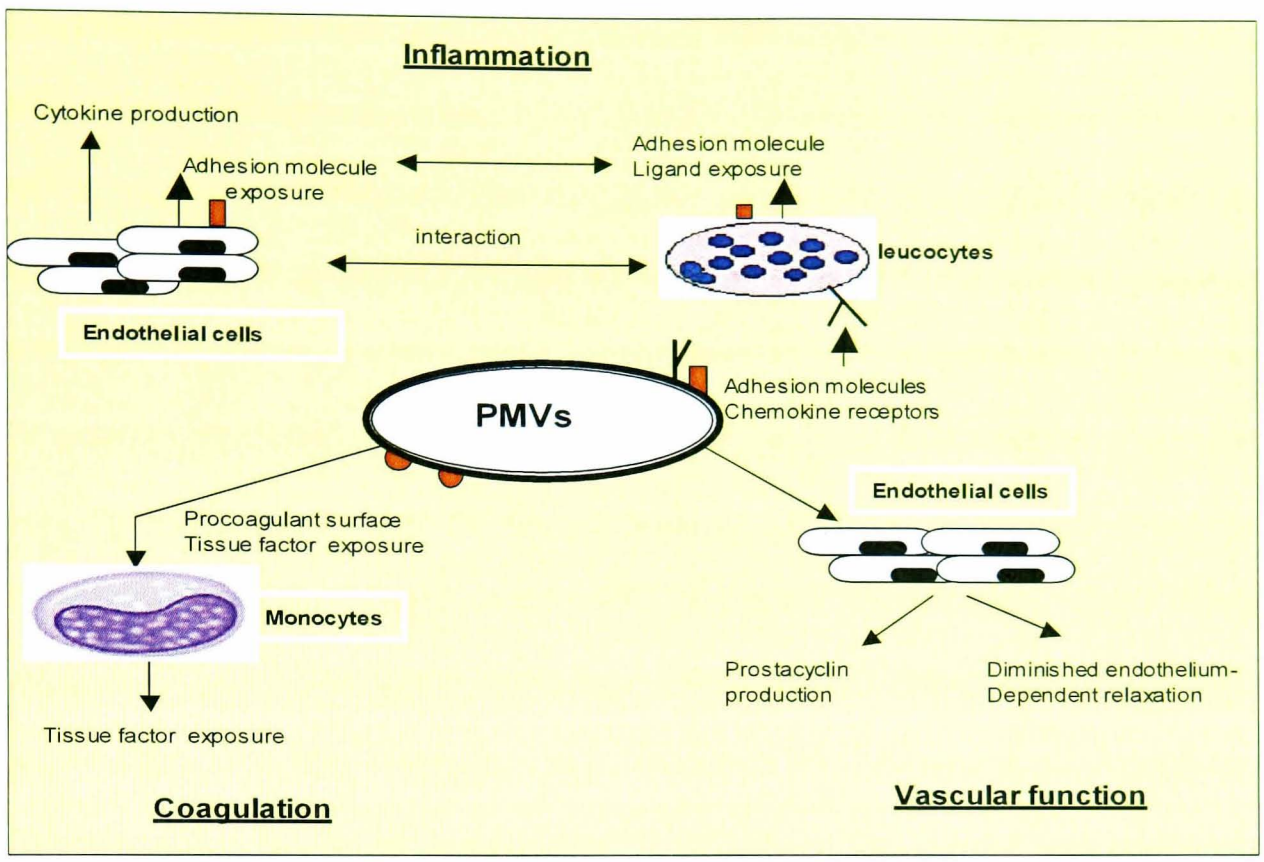


Fig. 3 Schematic representation of functions attributed to microparticles.

1.10 PMVs and diseases

Although circulating basal level PMVs can be found in the blood of normal individuals, increased numbers of circulating PMVs have been identified in individuals with certain diseases, including hypertension, prothrombotic states such as thrombotic thrombocytopenic purpura, the antiphospholipid antibody syndrome (SLE), multiple sclerosis, cerebral malaria and many diseases associated with inflammation (Berckmans RJ *et al* 2001; Boulanger CM *et al* 2001). PMVs can be taken as a sign of cellular dysfunction and serve as general indicators of cell injury, stress, thrombosis and inflammation although their role in the various diseases whether as direct contributors or merely a reflection of the disease remains to be determined (Berckmans RJ *et al* 2001; Boettner DR *et al* 2008). However the presence of PMVs provides an important tool in measuring the protective effect of therapeutic intervention in a non-invasive manner as well as acting as a marker or indicator of severity of disease states (Berckmans RJ *et al* 2001).

The quantity, cellular origin and composition of PMVs are dependent on the type of disease. For example, monocyte, platelet and endothelial-cells derived PMVs are found in septic patients (Brechot N *et al* 2008). Platelet-derived PMVs are increasingly present in patients with peripheral artery disease, myocardial infarction, atherosclerosis, diabetic retinopathy, paroxysmal nocturnal haemoglubinuria and those undergoing ischemic events (Boulanger CM *et al* 2001; Everett H *et al* 1999). Recent studies indicate that PMVs are able to decrease nitric-oxide-dependent vasodilation, increase arterial stiffness, promote inflammation, and initiate thrombosis at their PS-enriched membrane, which highly co-expresses tissue factor (Diamant M et al 2002). PMVs are also known to be elevated in acute coronary syndromes, end-stage renal disease and all conditions associated with endothelial injury or dysfunction.

Elevated levels of circulating platelet, monocyte, or endothelial-derived PMVs are associated with most of the cardiovascular risk factors and appear indicative of poor clinical outcome (Diamant M *et al* 2002; Janowska-Wieczorek A *et al* 2001). In addition to being a valuable hallmark of vascular cell damage, PMVs are at the crossroad of atherothrombosis processes by exerting direct effects on vascular or blood cells (Boulanger CM *et al* 2001). Under pathological circumstances, circulating PMVs would support cellular cross-talk leading to vascular inflammation and tissue remodeling, endothelial dysfunction, leukocyte adhesion, and stimulation (Boulanger CM *et al* 2001. At sites of vascular injury, P-selectin exposure by activated endothelial cells or platelets leads to the rapid recruitment of PMVs bearing the P-selectin glycoprotein ligand-1 and blood-borne tissue factor (TF), thereby triggering coagulation (Janowska-Wieczorek A *et al* 2001; Jimenez JJ *et al* 2003). PMVs accumulating in the lipid core of the atherosclertotic plaque are thus a major determinant of its thrombogenecity (Lucas AD *et al* 2001). In fact one of the main

goals of research is to present PMVs within the atherosclerotic plaque, sequestered PMVs constitute the main reservoir of TF activity, promoting coagulation after plaque erosion or rupture (Mesri M *et al* 1999).

Elevated levels of PMVs have been observed in cerebral malaria (CM) where their procoagulant property helps in the sequestration of activated blood cells (notably monocytes/macrophages, parasitized erythrocytes, and platelets) in cerebral vessels consequent to immune responses in the host (Combes *et al* 2005). Experiments carried out with the ATP-binding cassette transporter A1 (*ABCA1*) knockout mice showed a reduction in the capacity of cells to vesiculate, resulting in complete resistance to cerebral malaria of the *ABCA1*$^{-/-}$ mice. The finding that *ABCA1* deletion confers a complete protection against CM, associated to an impaired PMVs production, orientates toward new therapeutic approaches to the search for candidate genes controlling vesiculation (Piccin A *et al* 2007). With vesiculation (PMV release) being implicated in atherosclerosis and CM, one of the main goals of research is to prevent both PMV release and PS exposure at the cell surface. Thus, lower PMV levels are likely to reduce the incidence of CM and thrombotic diseases (Boettner DR *et al* 2008).

Although the prognostic potential of circulating PMVs is still in its infancy, the different studies being carried out clearly demonstrate that their detection and quantification is an interesting and potentially valuable tool to appreciate in disease states (Iwata M *et al* 2006). Elucidation of the PMV composition and the mechanisms involved in exertion of their effects will supply a lot of evidence that will enable us to develop intervention strategies for prevention and treatment of most diseases associated with PMV release (Nieuwland R *et al* 2002; O'Brien JR 1955).

1.11 Acute promyelocytic leukemia (APL)

1.11.1 Background

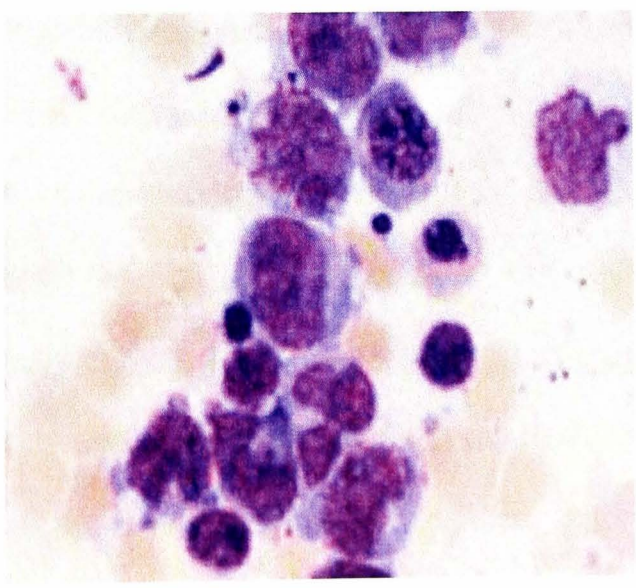
Acute promyelocytic leukemia (APL) is a unique subtype of the acute myelogenous leukemia (AML) with distinct cytogenetics, clinical features, and biologic characteristics (Breccia M *et al* 2008). It is the most malignant form of acute leukaemia caused by an arrest of leukocyte differentiation at the promyelocyte stage that leads to excess promyelocytes in the bone marrow and peripheral blood (Soignet SL *et al* 1998). APL with translocation between chromosomes 15 and 17 t(15;17) which is the classification given to the disease by the World Health Organization (WHO) as it is due to translocation between chr15 and 17 t(15;17) is also classified as the M3 variant of AML in the internationally accepted French-American-British (FAB) Classification system (Avvisati G *et al* 2002). It is consistently associated with the haemorrhagic diathesis disorder that resembles but is not identical to Disseminated Intravascular Coagulation (DIC) (Chen Z *et al* 1993).

As a result of this disorder, there is in APL a pronounced tendency to haemorrhage, that can manifest itself as petechiae, small ecchymosis, epistaxis, bleeding in the mouth, haematuria, bleeding from venipuncture and bone marrow sites and girls and women who are menstruating may have menometrorrhagia (Daniel MT *et al* 1993). The haemorrhagic diathesis may precede the diagnosis of leukaemia by 2-8 weeks. Two features that are unique to APL are the remission of the disease obtained with retinoic acid (RA) treatment, whose mechanism of action consists of inducing the APL blasts to differentiate terminally, and the presence in the APL blasts of an anomalous protein, the PML/RARα protein- a mutant of one of the retinoic acid receptors (Warrell RP Jr *et al* 1993).

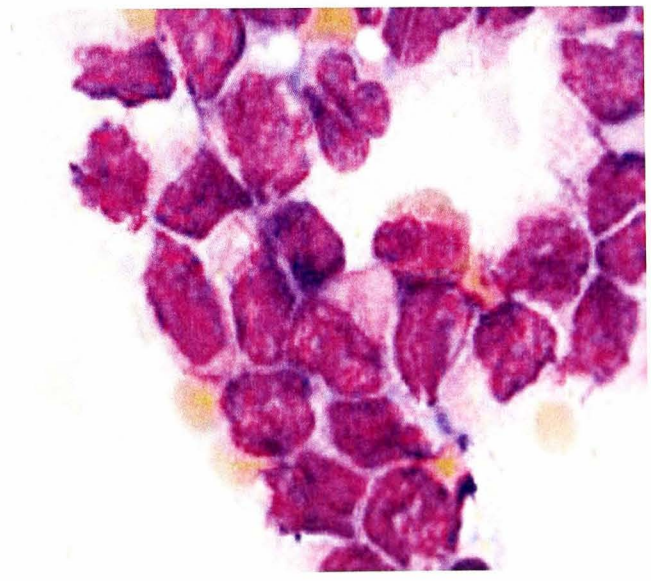
1.11.2 Epidemiology of APL

Acute promyelocytic leukemia represents 5-8% of AML in adults and 5-15% of all adult leukaemias and affects both males and females equally. There are approximately 30,800 cases of acute leukaemia diagnosed yearly of which about 1000 are APL (Pecorino L. 2008). The median age is approximately 40 years, which is considerably younger than the other subtypes of AML (70 years). About 21,700 patients die of leukaemia yearly but it is not clear how many of these patients die from APL, however, without proper medicine and treatment, the disease is fatal (Warrell RP Jr *et al* 1993). APL has a high rate of relapse with conventional chemotherapy but responds to differential therapy combined with chemotherapy (Soignet SL *et al* 1998). The discovery and elucidation of the molecular pathogenesis for APL has led to the first and only targeted therapy for leukaemia and over the past 50 years, APL has transformed from a highly fatal disease to a highly curable one (see 1.16.1.6).

Fig. 4 Microscopic images of acute promyelocytic leukaemia



Acute promyelocytic leukaemia



Hypergranular subtype of acute promyelocytic leukaemia

1.11.3 Pathophysiology of APL

APL is mostly defined by its cytogenetic properties and characterized by a balanced chromosomal translocation between chr15q22 and chr17q21 involving the promyelocytic leukemia (*PML*) gene on chr15 and the retinoic acid receptor-alpha (*RARα*) gene on chr17 [t(15;17)(q22;q12)] (Douer D *et al* 2003). The PML gene, which encodes a growth suppressing transcription factor, is disrupted by the break in chr15 whilst the break in chr17 interrupts the RARα gene that regulates myeloid differentiation (Paietta E *et al* 1994). The translocation creates a PML-RARα fusion gene leading to the formation of an abnormal fusion or hybrid protein with altered functions that arrests the maturation of myeloid cells at the promyelocytic stage (reduces terminal cell differentiation) causing an increased proliferation of promyelocytes as the fusion protein binds with enhanced affinity to sites on the cell's DNA, blocking transcription and differentiation (Chen Z *et al* 1993). It does so by enhancing interaction of nuclear co-repressor (NCOR) molecule and histone deacetylase (HDACL) (Douer D *et al* 2003; Rambaldi A 2003).

The RARα gene encoded by the long arm of chr17 is mainly expressed in haematopoietic cells and as stated has an important role in regulating gene expression (Paietta E *et al* 1994; Rambaldi A 2003). In the absence of retinoic acid, the RARα gene is bound by nuclear co-repressor factor, which causes transcriptional repression, but in the presence of retinoic acid however, the genes are activated and terminal differentiation of promyelocytes occurs, as the RAR receptor is dependent on retinoic acid for regulation of transcription (Chen Z *et al* 1993; Rambaldi A 2003). In APL, the fusion gene product between the two chromosomes causes the retinoic acid receptor gene to bind more tightly to the nuclear co-repressor factor and therefore the gene is not able to be activated with physiological doses of retinoic acid. Although the chromosomal translocation

involving *RARα* is believed to be the initiating event of APL, additional mutations are required for the development of leukaemia (Rambaldi A 2003; Douer D *et al* 2003).

There are three possible isoforms caused by these translocations. The breakpoint in chr17 is consistently found in intron 2, but varies in chromosome 15. The 3 breakpoints on the PML gene can occur at intron 3 (L form), intron 6 (S form), and exon 6 (V form) (Breccia M *et al* 2008). It has been reported that the S form is associated with a shorter remission duration and overall survival compared with the L form. In about 5% of cases, there are alternative rearrangements of chr17q21 with other gene partners. These include promyelocytic zinc finger (PZLF) t(11;17)(q23;q21), nucleophosmin (NPM) t(5;17)(q35;q12-21), nuclear mitotic apparatus (NuMa) t(11;17)(q13;q21), and STAT5b (17;17)(q11;q21). It is important to note that the nature of the fusion partner significantly impacts the disease characteristics and response to therapy (Douer D *et al* 2003). For example, APL with PLZF-RAR alpha is not sensitive to retinoic acid or as sensitive to chemotherapy. Also about 40% of APL cases also express additional chromosomal abnormalities (trisomy 8 and isochromosome 17) that do not appear to impact the overall prognosis (Fenaux P *et al* 1997).

1.11.4 Signs and symptoms of APL

The majority of signs and symptoms of APL are also seen with the other cases of AML (Fenaux P *et al* 1997; Tallman MS et al 2008). They are therefore non-specific and include fatigue, weakness, dyspnoea related to anaemia, minor infections and fever related to leucopoenia, a tendency to bleed caused by thrombocytopenia or coagulopathy and enlargement of spleen which may cause minor abdominal discomfort (Breccia M *et al* 2008; Fenaux P *et al* 1997). There is usually pancytopenia with low levels of red blood cells, granulocytes, monocytes and

platelets due to the accumulation of promyelocytes in the bone marrow, thus preventing it from producing normal healthy cells. About 10-30% of cases present with leukocytosis though.

Most patients of APL present with coagulopathy, which has been described as resembling Disseminated Intravascular Coagulation (DIC) with associated hyperfibrinolysis. APL has been associated with low levels of plasminogen, alpha2-plasmin inhibitor, and plasminogen activator inhibitor 1 found in fibrinolytic states (Tallman MS *et al* 2008; Breccia M *et al* 2008).. There is also increased expression of annexin II, a receptor for plasminogen and plasminogen-activating factor on the surface of leukaemic promyelocytes, which can lead to overproduction of plasmin and fibrinolysis. It is important to treat the coagulopathy as a medical emergency as in 40% of untreated patients, pulmonary and cerebral haemorrhages can occur and it also takes 5-8 days for coagulopathy to improve with treatment (Tallman MS *et al* 2008). Physical examination can reveal pallor, petechiae, and areas of ecchymoses, there can be bleeding from the gums, and a slow murmur can be heard with severe anemia. Patients can also present with neurologic deficits or headaches if there is CNS involvement (Breccia M *et al* 2008; Tallman MS *et al* 2008).

1.11.5 Diagnosis of APL

Acute promyelocytic leukemia can be distinguished from other types of AML based on morphologic examination of a bone marrow aspirate or biopsy as well as finding the characteristic chromosomal rearrangement (Soignet SL *et al* 1998). Definitive diagnosis however requires testing for the *PML-RARα* fusion gene and this may be done by PCR, fluorescent *in situ* hybridization (FISH), or conventional cytogenetics of peripheral blood or bone marrow (Breccia M *et al* 2008; Tallman MS *et al* 2008).

Monitoring for relapse using PCR tests for *PML/RARα transcript* allows early re-treatment which is successful in many instances (Fenaux P *et al* 1997).

1.11.6 Current therapies (Differentiation therapy) for APL

Differentiation therapy is a cancer therapy technique in which the malignant cell is regarded as having escaped the normal controls of cell growth and differentiation and pathologically arrested at an early stage of differentiation, retaining the ability to proliferate and so multiply at an abnormally fast rate (Dimberg *et al* 2002; Ades L *et al* 2005). The malignant cells are treated with agents that remove the block on differentiation and allow the cells to resume the process of maturation and differentiate along more normal lines until they eventually lose their ability to divide and replicate (Bombara C *et al* 1992; Hmama Z *et al* 1999). Differentiation therapy does not destroy the cancer cells, but restrains their growth and allows the application of more conventional therapies such as chemotherapy to eradicate those (Piccin *et al* 2007). One advantage is that differentiation agents tend to have less toxicity than conventional cancer treatments (Ades L *et al* 2005).

Until recently, chemotherapy and haematopoietic stem-cell transplantation were the only therapeutic options available in acute leukaemia and the idea of restoring normal differentiation in primitive leukaemic cells seemed unrealistic until the effect of all-*trans*-retinoic acid (ATRA) in APL was shown (Huang ME *et al* 1988; Warrell RP *et al* 1993). ATRA was the first differentiation agent found to be successful in APL treatment and most APL patients are now treated first with ATRA, which causes the promyelocytic to differentiate by acting on the PML–RAR fusion gene, to lift the block on differentiation thus decreasing promyelocytes proliferation (Huang ME *et al* 1988; Frankel SR *et al* 1992). ATRA induces a complete remission in about 70% of APL cases and is the prototype of a differentiation therapy agent.

As described above, in APL, there is an accumulation of leukaemic blasts carrying the t(15;17) translocation that fuses the PML gene to the RARα gene (He LZ *et al* 1993; Fenaux P *et al* 1993). This chimeric gene encodes the PML–RARα fusion protein, which has a crucial role in the pathogenesis of APL by interfering with factors essential for the differentiation of myeloid precursors (Huang ME *et al* 1988; He LZ *et al* 1993). To function properly, the normal proteins PML and RARα must join (dimerize) with another PML protein, in the case of PML, or a retinoid X receptor protein (RXR) in the case of RARα (Huang ME *et al* 1988). However, the abnormal PML–RARα hybrid in APL cells binds to PML and RXR, thereby preventing them from linking up with their natural partners (He LZ *et al* 1993).

PML, which acts as a growth suppressor and is normally localized in distinctive nuclear bodies, is displaced in the presence of PML–RARα and this plays a role in the proliferation of blasts cells (He LZ *et al* 1993). RARα is a DNA-binding transcription factor that, in conjunction with its cofactor, RXR, regulates myeloid differentiation; interference with the formation of RARα–RXR dimers by the fusion protein PML–RARα is what blocks differentiation at the promyelocytic stage (He LZ *et al* 1993).

Retinoic acid (RA) may act as a regulator of differentiation at various stages of cellular development (Tallman MS *et al* 1997). The recent discovery that RA interacts with nuclear receptors related to the steroid and thyroid hormone receptors, a family of proteins that function as ligand–dependent transcription factors, suggests that the morphogenetic functions of RA may result at least in part, from activation of developmentally regulated genes by RA receptor (RAR) complexes (Martinez MC *et al* 2006; Li MO *et al* 2006). There are three retinoic acid receptors (RAR), RAR–alpha, RAR–beta and RAR–gamma and each receptor

isoform has several splice variants: two–for alpha, four–for beta and two–for gamma (Sham RL *et al* 1995). The RAR's are nuclear receptors with activity similar to the steroid receptors such as oestrogen or progesterone and other nuclear receptors: PRAR: LXR and FXR.

All-*trans* retinoic acid (ATRA) is the natural ligand for RAR (Li MO *et al* 2006). Like the steroid receptors, when activated, RARs dimerize usually forming heterodimers, translocate to the cell nucleus, and bind to RAR response elements on DNA, which elicits expression or transrepression of gene products. 9–cis retinoic acid is the natural ligand of another nuclear hormone receptor termed the retinoid x receptor (Sham RL *et al* 1995).

ATRA has caused APL blasts to differentiate into polymorphonuclear leukocytes *in vitro* in *PML–RARα* transgenic mice (Sham RL *et al* 1995). Moreover, complete-remission rates of about 90 percent with ATRA have been observed in phase 2 studies of patients with APL and the response was not due to cytotoxicity but the differentiation of leukaemic blasts (Tallman MS *et al* 1997; Li MO *et al* 2006). A major effect of ATRA on APL cells appears to be the degradation of the fusion protein PML–RARα and in contrast to classic anthracycline–cytarabine chemotherapy, ATRA also improves the characteristic coagulopathy of APL and does not induce aplasia of the bone marrow. However, as known, ATRA can cause the potentially fatal retinoic acid syndrome condition (Fenaux P *et al* 1993). Furthermore, cytotoxic chemotherapy alone can cure 30 to 40 percent of the patients who achieve complete remission, but virtually all patients treated with ATRA alone subsequently relapse.

Also, ATRA and other agents with the capacity to induce differentiation of leukaemic blasts *in vitro* have not shown efficacy *in vivo* in acute leukaemias other than APL but in many types of acute leukaemia, however, disruption of genes involved in cell proliferation and differentiation has been observed (Tallman MS *et al* 1997; Fenaux P *et al* 1993). The paradigm of APL therefore gives hope that agents targeting those genes especially by down-regulating fusion proteins will be able to restore differentiation of other types of leukaemic blasts (Tallman MS *et al* 1997).

1.11.7 MicroRNA (miRNA) reexpression as differentiation therapy in cancer

MicroRNAs are small, noncoding RNAs about 22–23 nucleotides long, shown to regulate gene expression at translational and posttranslational levels (Wang H *et al*. 2008). miRNAs mostly bind to the 3′ untranslated region of target genes and inhibit gene expression translationally and/or by destabilizing the target mRNA (Campelovier P *et al* 2006). miRNAs, encoded in intergenic/intronic noncoding regions once thought not to be useful DNA, contribute to differentiation of cells when expressed in a tissue-specific manner and play an important role in maintaining tissue-specific functions and cellular differentiation (Ratajczak J *et al* 2006). Such miRNAs are responsible for tissue integrity and homeostasis, behaving functionally to maintain order in specific tissues, by stringently regulating the expression of their target genes, especially oncogenes (Ciarapica R *et al*. 2009).

With respect to cancer, miRNAs are typically downregulated in tumours and it has been found that inhibiting miRNA biogenesis tends to enhance tumorigenesis (Hunter MP *et al* 2008). Downregulation may be achieved through mutation or by epigenetic silencing of the miRNA, resulting in loss of tissue-specific miRNA synthesis and overexpression of pro-proliferation genes (oncogenes) (McCarthy JJ 2008; Campelovier P *et al* 2006). Even though a few miRNAs are overexpressed in

cancer and seem to function or can act as oncogenes themselves, many investigators have proposed that reexpression of specific miRNAs may have therapeutic anti-cancer value as greater numbers have been shown to be downregulated in cancer and have the potential to act as tumour suppressors (Ratajczak J *et al* 2006). Both miRNA reexpression and downregulation therefore have anti-tumour effects since silencing an oncogenic miRNA could allow reexpression of tumour suppressor genes, while reexpressing a tumuor suppressor miRNA could downregulate multiple oncogenes (Mishra PJ *et al* 2009; Shen ZX *et al* 1997).

The discovery of miRNAs therefore has added an entirely new dimension to anti-tumour therapeutic approaches, as the potential of noncoding and inactive RNAs as drugs for cancer patients is both intriguing and compelling (Nasser MW *et al*. 2008; Campelovier P *et al* 2006). Since miRNA expression seems to be altered in many human diseases, including cancer, the miRNA revolution has already set the stage for the so called miRNA reexpression therapy and a model describing miRNA reexpression as differentiation therapy in cancer has been described (He L *et al* 2004). Tissue-specific tumour-suppressing miRNAs have the potential to promote the redifferentiation of tumour cells to their normal counterparts and solid malignancies to their original tissue types (Shen ZX *et al* 1997; Campelovier P *et al* 2006). In fact reexpression, to physiological levels, of tissue-specific miRNAs that are lost in cancer can induce the differentiation of cancer cells. miRNAs regulate a vast number of genes and pathways including oncogenes and tumour suppressors, which are targets of cancer drugs currently used for treatment (Wachtel M *et al* 2006).

1.11.8 Advantages of miRNA therapy

miRNAs are easy to synthesize and can potentially target any gene (Mishra PJ *et al* 2009). One major advantage is that miRNA reexpression can influence the expression of hundreds of genes involved in many cellular pathways and that it can target an array of different gene products. In fact, both the sense and antisense strands of miRNAs might target different mRNAs (Lim LP *et al* 2005). However, as with any such drug, the advantage of hitting a broad spectrum of targets is a potential disadvantage but miRNAs are evolutionarily conserved, and targeting the upregulation or downregulation of a tissue-specific tumour suppressor miRNA or oncogenic miRNA, respectively, to its physiological level may have fewer side effects than when using siRNAs (Chuang JC *et al* 2007; Calin GA *et al* 2006).

miRNA therapy also has some disadvantages, including delivery limitations, instability, and off-target effects (Kumar MS *et al* 2007). A major obstacle to effective miRNA-based therapy is the requirement for successful delivery, because unlike many other drugs, miRNAs do not freely diffuse into cells. miRNA therapy may therefore require special delivery approaches to achieve the desired effect (Datta J *et al* 2008; Garzon R *et al* 2006). Also, small RNAs tend to be unstable and might be degraded upon entering a cell. Another factor is that in the case of miRNA reexpression therapy of cancer, preventing miRNA expression from exceeding physiological levels also represents a therapeutic challenge (Zhang B *et al* 2007. Ultimately, miRNA-based gene therapy has many of the same disadvantages as gene therapy (Mishima Y *et al* 2009) but developing smarter/safer ways to deliver stable miRNAs/miRNA inhibitors into cells should go a long way toward overcoming the majority of these limitations, and preclinical models will be extremely helpful in this regard (Hammond SM *et al* 2006; Negrini M *et al* 2007)

Although successful use of miRNA as therapeutic agents represents a substantial challenge, their therapeutics also carry great expectations based on the ability of a single miRNA to specifically target multiple oncogenes, many of which are being individually targeted by drugs already in use or under preclinical development (Chen JF *et al* 2006; Silber J *et al* 2008). Cancer however remains a complex disease and patients with the same diagnosis will harbour gene mutations and polymorphic variants in a myriad of permutations. Therefore, incorporation of personalized medicine approaches will continue to be advisable (Fabbri M *et al* 2007; Taulli R *et al* 2009).

1.11.9 Prognosis for APL

ATRA can induce a complete remission in most patients with APL by causing the APL-blasts to mature. However, ATRA cannot eliminate the leukaemic clone and is therefore used in combination with chemotherapy including an anthracycline drug (Chomienne C *et al* 1990). Survival is better with the combination of ATRA and chemotherapy than chemotherapy alone in newly diagnosed APL, because ATRA + chemotherapy makes for a slightly higher rate of complete remissions while allowing significantly fewer relapses. Maintenance treatment with ATRA, and possibly with low-dose chemotherapy, further reduces the incidence of relapse (Huang ME *et al* 1988).

The prognosis for APL depends on a number of factors including the white blood cell count at the time of diagnosis, etc. Overall, more than 90% of patients with newly diagnosed APL today can achieve complete remission, and about 75% can be cured by the combination of ATRA and chemotherapy (Frankel SR *et al* 1992). In patients who relapse after remission, treatment may include arsenic trioxide which is

currently being evaluated for treatment of relapsed / refractory disease. Studies have shown arsenic reorganized nuclear bodies and degrade the mutant PML-RAR fusion protein (Fenaux P *et al* 1993). Arsenic also increases caspase activity which then induces apoptosis and does reduce the relapse rate for high risk patients. After stable remission is induced, the standard of care is to undergo 2 years of consolidation chemotherapy with Methotrexate, Mercaptopurine, and ATRA. Also allogeneic bone marrow or stem cell transplantation are the preferred treatment options for relapsed or refractory disease (Fenaux P *et al* 1993).

The advent of ATRA therapy revolutionized the treatment of APL and markedly improved the prognosis (Shen ZX *et al* 1997). However, ATRA syndrome is a serious side effect of ATRA treatment and includes fever, respiratory distress, and hypotension, usually with an increase in leukocyte counts, which may be instigated by cytokines released from leukaemic blasts after exposure to ATRA (Tallman MS *et al* 1997).

1.12 Apoptosis

Apoptosis is the process of programmed cell death that may occur in multicellular organisms (Cohen JJ *et al* 1992; Eguchi K 2001). It begins when a cell receives internal or external signals that trigger the activity of proteolytic caspases and proceeds through a series of characteristic or morphological stages typically including rounding-up of the cell, retraction of pseudopodes, reduction of cellular volume (pyknosis), chromatin condensation (Kerr JF 1965), nuclear fragmentation (karyorrhexis), plasma membrane blebbing (but maintenance of its integrity until the final stages of the process), chromosomal DNA fragmentation and ends with the death of the cell (Kerr JF 1965; O'Rourke MG, Ellen KA 2000). Apoptosis differs from necrosis in that the processes of disposal of cellular debris associated with

apoptosis do not damage the organism as do necrosis (Brechot *et al* 2008; Brüne B 2003). In general, apoptosis confers advantages during an organism's life cycle. For example, the differentiation of fingers and toes in a developing human embryo occurs because cells between the fingers apoptose, thus enabling the digits to separate (Cohen JJ *et al* 1992).

Between 50 billion and 70 billion cells die each day due to apoptosis in the average human adult and for an average child between the ages of 8 and 14, approximately 20 billion to 30 billion cells die a day (Cohen JJ *et al* 1992; Collazo C *et al* 2006). In a year, this amounts to the proliferation and subsequent destruction of a mass of cells equal to an individual's body weight (Cohen JJ *et al* 1992). In addition to its importance as a biological phenomenon, defective apoptotic processes have been implicated in an extensive (Collazo C *et al* 2006) variety of diseases including cancer, autoimmune disease, viral infection, AIDS, cardiovascular disease, neurodegenerative disorders, osteoporosis and ageing (Eguchi K 2001; Giles KM *et al* 2000). Excessive apoptosis causes hypotrophy, such as in ischemic damage, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer (Eguchi K 2001).

1.12.1 Processes involved in apoptosis

The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (*extrinsic inducers*) or intracellularly (*intrinsic inducers*) and signals may positively or negatively induce apoptosis (Chen G, Goeddel DV 2002; Chen EH 2003). Extracellular signals may include toxins (Brüne B *et al* 2003), hormones, growth factors, nitric oxide (Brüne B 2003) or cytokines, and therefore must either cross the plasma membrane or transduce to effect a response whilst intracellular signaling is initiated through response to a stress.

The binding of nuclear receptors by glucocorticoids (Popov SG *et al* 2002), heat, radiation, nutrient deprivation, viral infection , hypoxia (Popov SG *et al* 2002) and increased intracellular calcium concentration (Chiarugi A *et al* 2002) as a result of damage to the cell membrane can all trigger the release of intracellular apoptotic signals. An extrinsic pathway has also been noticed in several toxin studies that have shown that an increase in calcium concentration within a cell caused by drug activity has the ability to cause apoptosis via a calcium binding protease calpain (Fesik SW, Shi Y 2001). The binding and subsequent initiation of apoptosis by a molecule is termed positive, whereas the active repression of apoptosis by a molecule is termed negative.

Before the actual process of cell death is carried out by enzymes, apoptotic signals must cause regulatory proteins to start off the death pathway (Chen G, Goeddel DV 2002). This step allows apoptotic signals to cause cell death, or the process to be stopped, should the cell no longer need to die. Several proteins are involved, but two main methods of regulation have been identified that target mitochondria functionality, or that directly transduce the signal via adaptor proteins to the apoptotic mechanisms (Dejean LM *et al* 2006). A number of cellular components, such as poly ADP ribose polymerase, may also help regulate apoptosis (Goeddel DV *et al* 2002).

1.12.2 Mitochondrial regulation of apoptosis

The mitochondria are essential to multicellular life and without them, a cell ceases to respire aerobically and quickly dies, a fact exploited by some apoptotic pathways (Laurent M *et al* 2006). Apoptotic proteins that target mitochondria affect them in different ways (Dejean LM *et al* 2006). They may cause mitochondrial swelling through the formation of membrane pores, or they may increase the permeability of

the mitochondrial membrane and cause apoptotic effectors to leak out (Popov SG *et al* 2002). There is also a growing body of evidence that indicates that nitric oxide is able to induce apoptosis by helping to dissipate the membrane potential of mitochondria so making them more permeable (Dejean LM *et al* 2006).

Mitochondrial proteins known as SMACs (second mitochondria-derived activator of caspases) are released into the cytosol following an increase in permeability. SMAC binds to inhibitor of apoptosis proteins (IAPs) and deactivates them, preventing the IAPs from arresting the apoptotic process and therefore (Connor J *et al* 1992; Diamant M *et al* 2002) allowing apoptosis to proceed. IAPs also normally suppress the activity of caspases (a group of cysteine proteases) (Fesik SW, Shi Y 2001; Laurent M *et al* 2006), which carry out the degradation of the cell. Therefore the actual degradation enzymes can be seen to be indirectly regulated by mitochondrial permeability (Dejean LM *et al* 2006).

Cytochrome c is also released from mitochondria due to formation of the mitochondrial apoptosis-Induced Channel (MiAC) in the outer mitochondrial membrane, (Dejean LM *et al* 2006; Laurent M *et al* 2006). The formation of MiAC has a regulatory function as it precedes morphological change associated with apoptosis (Popov SG *et al* 2002). Once cytochrome c is released it binds with Apoptotic protein c activating factor 1 (Apaf-1) and ATP, which then bind to *pro-caspase-9* to create a protein complex known as an apoptosome (Laurent M *et al* 2006). The apoptosome cleaves the pro-caspase to its active form of caspase-9, which in turn activates the effector caspase-3 (Fesik SW, Shi Y 2001). MiAC is itself subject to regulation by various proteins, such as those encoded by the mammalian Bcl-2 family of anti-apoptopic genes. Bcl-2 proteins are able to promote or inhibit apoptosis either (Laurent M *et al* 2006; Murphy KM *et al* 2000) by direct action on

MiAC or indirectly through other proteins. It is important to note that the actions of some Bcl-2 proteins are able to halt apoptosis even if cytochrome c has been released by the mitochondria (Murphy KM *et al* 2000; Popov SG *et al* 2002).

1.12.3 Direct signal transduction.

Two theories of the direct initiation of apoptotic mechanisms in mammals have been suggested: the TNF-induced (tumour necrosis factor) model and the Fas-Fas ligand-mediated model, both involving receptors of the TNF receptor (TNFR) family coupled to extrinsic signals (Fig. 5) (Chen G, Goeddel DV 2002; Wajant H 2002, Wajant H 2009). TNF is a cytokine produced mainly by activated macrophages, and is the major extrinsic mediator of apoptosis. Most cells in the human body have two receptors for TNF: TNF-R1 and TNF-R2 (Chen G, Goeddel DV 2002; Diaz C *et al* 1996). The binding of TNF to TNF-R1 has been shown to initiate the pathway that leads to caspase activation via the intermediate membrane proteins TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD) (Goeddel DV *et al* 2002; Wajant H 2002). Binding of this receptor can also indirectly lead to the activation of transcription factors involved in cell survival and inflammatory responses (Wajant H 2009; Werlen G *et al* 2003). The link between TNF and apoptosis shows why an abnormal production of TNF plays a fundamental role in several human diseases, especially in autoimmune diseases (Goeddel DV *et al* 2002; Rauch U *et al* 2000).

The Fas receptor (also known as Apo-1 or CD95) binds the Fas ligand (FasL), a transmembrane protein which is part of the TNF family (Chen G, Goeddel DV 2002; Wajant H 2002). The interaction between Fas and FasL results in the formation of the death-inducing signaling complex (DISC), which contains the FADD, caspase-8 and caspase-10 (Mesri M *et al* 1999; Wajant H 2009). In some types of cells (type I),

processed caspase-8 directly activates other members of the caspase family, and triggers the execution of apoptosis (Diaz C *et al* 1996; Mesri M *et al* 1999). In other types of cells (type II), the Fas-DISC starts a feedback loop that spirals into increasing release of pro-apoptotic factors from mitochondria and the amplified activation of caspase-8 (Murphy KM *et al* 2000; Wajant H 2002).

Following *TNF-R1* and *Fas* activation in mammalian cells a balance between pro-apoptotic and anti-apoptotic members of the *Bcl-2* family is established, (Cheng EH 2003; Polster BM *et al* 2004). This balance is the proportion of pro-apoptotic homodimers that form in the outer-membrane of the mitochondrion (Susin SA *et al* 1999). The pro-apoptotic homodimers are required to make the mitochondrial membrane permeable for the release of caspase activators such as cytochrome c and SMAC (Murphy KM et al 2000; Susin SA *et al* 1999). Control of pro-apoptotic proteins under normal cell conditions of non-apoptotic cells is incompletely understood, but it has been found that a mitochondrial outer-membrane protein, VDAC2, interacts with BAK to keep this potentially lethal apoptotic effector under control (Susin SA *et al* 1999). When the death signal is received, products of the activation cascade displace VDAC2, and BAK is able to be activated (Cheng EH 2003; Dejean LM *et al* 2006). There also exists a caspase-independent apoptotic pathway that is mediated by apoptosis-inducing factor (AIF).

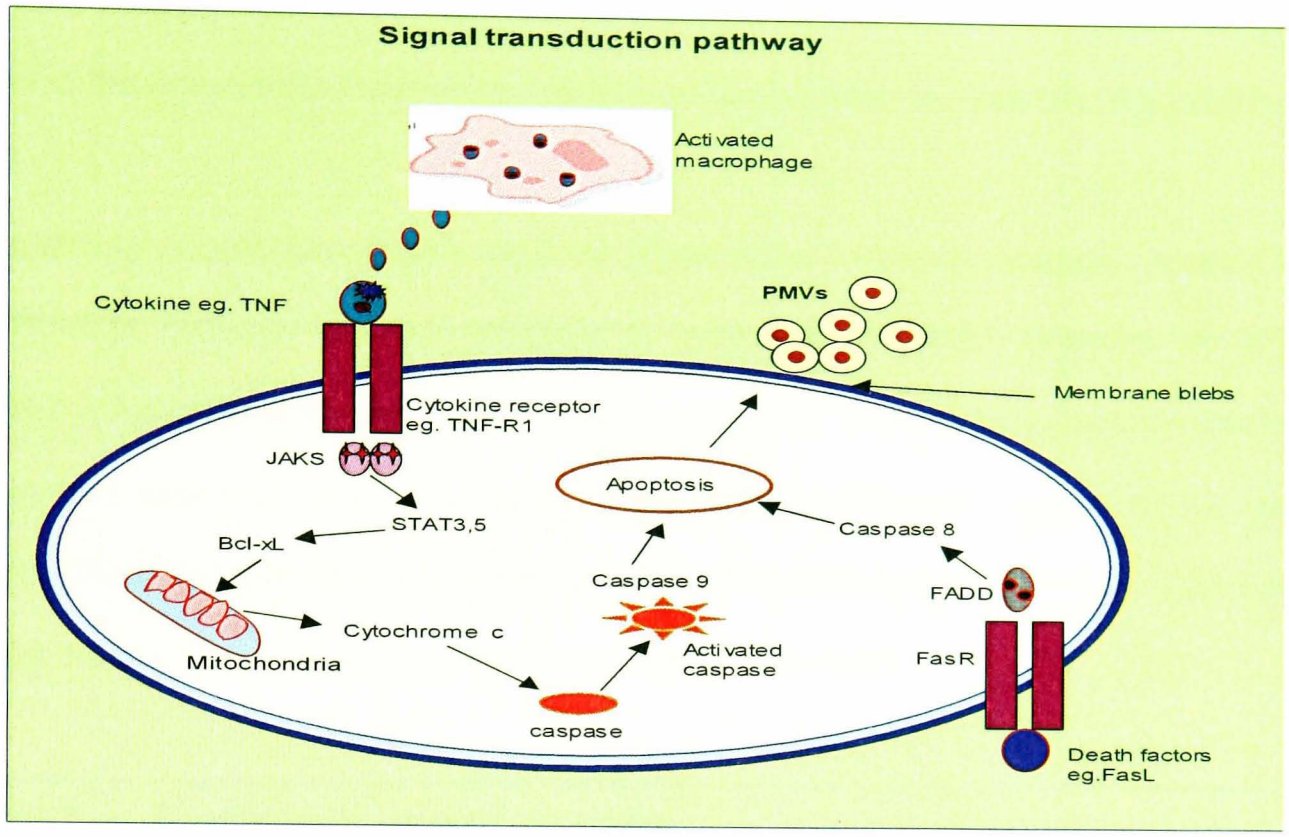


Fig. 5 Overview of signal transduction pathways.

1.12.4 Execution (cell death)

Many pathways and signals lead to apoptosis, but there is only one mechanism that actually causes the death of a cell (Diaz C *et al* 1996; Goeddel DV *et al* 2002). After a cell receives an apoptosis-inducing stimulus, it undergoes organized degradation of cellular organelles by activated proteolytic caspases. A cell undergoing apoptosis shows a characteristic morphology. The cell shrinks and rounds up because of the breakdown of the proteinaceous cytoskeleton by caspases; the cytoplasm appears dense, and the organelles appear tightly packed; chromatin undergoes condensation into compact patches against the nuclear envelope; the nuclear envelope (Mesri M *et al* 1999) becomes discontinuous and the DNA inside it is fragmented (karyorrhexis). The nucleus then breaks into several discrete chromatin bodies or nucleosomal units due to the degradation of DNA, the cell membrane

shows irregular buds known as blebs and the cell breaks apart into several vesicles called the apoptotic bodies, which are then phagocytosed (Goeddel DV *et al* 2002).

Apoptosis progresses quickly and its products are quickly removed, making it difficult to detect or visualize (Chiarugi A, Moskowitz MA 2002; Grønlien HK *et al* 2002). During karyorrhexis, endonuclease activation leaves short DNA fragments, regularly spaced in size. These give a characteristic "laddered" appearance on agar gel after electrophoresis and such tests for DNA laddering differentiate apoptosis from ischemic or toxic cell death (Vandivier RW *et al* 2006)

1.12.5 Functions or uses of apoptosis

1.12.5.1 Cell termination

Apoptosis occurs when a cell is damaged beyond repair, infected with a virus, or undergoing stressful conditions such as starvation (Chiarugi A, Moskowitz MA 2002; Cohen JJ *et al* 1992). Damage to DNA from ionizing radiation or toxic chemicals can also induce apoptosis via the actions of the tumour-suppressing gene *p53* (Cohen JJ *et al* 1992; Nagata S 2000; Wang XW *et al* 1995). The "decision" for apoptosis can come from the cell itself, from the surrounding tissue, or from a cell that is part of the immune system (Eguchi K 2001; Freyssinet JM *et al* 1999). In these cases apoptosis functions to remove the damaged cell, preventing it from sapping further nutrients from the organism, or halting further spread of viral infection (Chiarugi A, Moskowitz MA 2002). Apoptosis also plays a role in preventing cancer. If a cell is unable to undergo apoptosis because of mutation or biochemical inhibition, it continues to divide and develop into a tumour. Another example involves infection by papillomaviruses which causes a viral gene to interfere with the cell's p53 protein, an important member of the apoptotic pathway (Berckmans RJ *et al* 2001; Everett H

and McFadden G 1999). Such interference in the apoptotic capability of the cell is believed to play a role in the development of cancer (Thompson CB 1995).

1.12.5.2 Homeostasis

Homeostasis involves a complex series of reactions, an ongoing process inside an organism that calls for different types of cell signaling whereby any impairment can cause a disease (Gregory DC and Devitt A 2004; Hopkinson-Woolley *et al* 1994). In the adult organism, the number of cells is kept relatively constant through cell death and division. Cells must be replaced when they malfunction or become diseased, but proliferation must be offset by cell death (Kerr JF *et al* 1972; Rosen A *et al* 1999). This control mechanism is part of the homeostasis required by living organisms to maintain their internal states within certain limits (Rosen A *et al* 1999). Homeostasis is achieved when the rate of mitosis (cell division) in the tissue is balanced by cell death (Thompson CB 1995). If this equilibrium is disturbed, one of two potentially fatal disorders occurs. Either the cells divide faster than they die, effectively resulting in tumour development or the cells divide slower than they die, causing cell loss (Giles KM *et al* 2000; Gregory DC and Devitt A 2004).

1.12.5.3 Development

During development, apoptosis is tightly regulated and different tissues use different signals for inducing apoptosis (Collazo C *et al* 2006; Cohen JJ *et al* 1992). Development of an organ or tissue is often preceded by the extensive division and differentiation of a particular cell, the resultant mass is then "pruned" into the correct form by apoptosis (Freyssinet *et al* 1999; Giles KM *et al* 2000). Unlike necrosis, cellular death caused by injury, apoptosis results in cell shrinkage and fragmentation (Everett H and McFadden G 1999). Such shrinkage and fragmentation allow the cells to be phagocytosed and their components reused without releasing potentially

harmful intracellular substances such as hydrolytic enzymes into the surrounding tissue (Gregory DC and Devitt A 2004; Kerr JF *et al* 1972).

During vertebrate embryo development, structures called the notochord and the floor plate secrete a gradient of the signaling molecule (Shh), and it is this gradient that directs cells to form patterns in the embryonic neural tube (Teodoro JG, Branton PE 1997; Thompson CB 1995). Cells that receive Shh in a receptor in their membranes called patched1 (Ptc1) survive and proliferate (Vandivier RW *et al* 2006). In the absence of Shh, one of the ends of this same Ptc1 receptor (the carboxyl-terminal, inside the membrane) is cleaved by caspase-3, an action that exposes an apoptosis-producing domain (Teodoro JG, Branton PE 1997).

The development of B-lymphocytes and T lymphocytes in a human body is a complex process that creates a large pool of diverse cells and subsequently eliminates those potentially damaging to the body (Collazo C *et al* 2006; Eguchi K 2001; Everett H and McFadden G 1999). In T cells, apoptosis is initiated by the withdrawal of survival signals (Giles KM *et al* 2000). Cytotoxic T cells are able to directly induce apoptosis by opening up pores in the target's membrane and releasing chemicals that bypass the normal apoptotic pathway (Gregory DC and Devitt A 2004). The pores are created by the action of secreted perforin, and the granules contain granzyme B, a serine protease that activates a variety of caspases by cleaving aspartate residues Collazo C *et al* 2006). Dying cells that undergo the final stages of apoptosis display phagocytotic molecules, such as phosphatidylserine, on their cell surface (Gregory DC and Devitt A 2004;Kerr JF *et al* 1972), which mark the cell for phagocytosis by cells possessing the appropriate receptors, such as macrophages (Gregory DC and Devitt A 2004).

1.13 Phagocytosis

Phagocytosis is a fundamental cellular process by which certain living cells (leukocytes) of the immune system called phagocytes ingest or engulf and kill other cells (marked for removal), infectious micro-organism and other 'foreign' matter or particles (Vandivier RW et al 2006; Werlen G *et al* 2003). Phagocytosis is a specific form of endocytosis and is therefore distinct from other forms of endocytosis such as pinocytosis, which is the vesicular internalization of various liquids (Tidball JG *et al* 2007). Phagocytosis involves the engulfment of extracellular targets by wrapping of pseudopodia supported by cytoskeletal elements around them and internalizing them into vacuoles called phagosomes which then fuse with a lysosome, forming a phagolysosome (Parham P 2009). The phagocytosed or engulfed particles are then digested in an oxidative burst by lysosomal enzymes and the hydrolyzed products assimilated by absorption into the cytoplasm through the vacuolar wall as the waste products are excreted from the cell (Gregory DC and Devitt A 2004; Parham P 2009). In some forms of animal life, such as amoebas and sponges, phagocytosis is a means of feeding whereas in higher animals it is chiefly a defensive reaction against infection and invasion of the body by foreign substances (antigens) (Parham P 2009).

The particles commonly phagocytosed by leukocytes include bacteria, dead tissue cells, protozoa, various dust particles, small mineral particles, pigments, and other minute foreign bodies (Ishimoto H *et al* 2008; Parham P. 2009). In humans and in vertebrates generally, the most effective phagocytic cells are two kinds of leukocytes: the macrophages (large phagocytic cells) and the neutrophils (a type of granulocyte) (Vandivier RW *et al* 2006). The macrophages occur especially in the lungs, liver, spleen, and lymph nodes, where their function is to free the airways, blood, and lymph of bacteria and other particles. Macrophages are also found in all

tissues, and the monocyte, a precursor of the macrophage, is found in the blood (Wajant H *et al* 2009). The smaller phagocytes are chiefly neutrophils that are carried along by the circulating blood until they reach an area of infected tissue, where they pass out through the blood vessel endothelial cells and lodge in that tissue (St. Pierre BA,Tidball JG 1994). Both macrophages and neutrophils are drawn toward an area of infection or inflammation by means of chemotatic agents off by the infectious agent and the infected tissue or by a chemical interaction between the infectious agent such as bacteria and the complement system of blood serum proteins (Lucas AD, Greaves DR 2001). Neutrophils, however may also engulf particles after colliding with them by chance.

Before phagocytosis is accomplished, the phagocyte and the particle must adhere to each other, the possibility of which depends largely on the chemical nature of the surface of the particle (Parham P 2009). If the phagocyte cannot adhere directly, blood proteins can form a surface film on the particle or bacteria to which phagocytes adhere, and phagocytosis follows (Brechot *et al* 2008; Lucas AD, Greaves DR 2001). The speed with which a phagocytic cell ingests a particle varies somewhat with the size of the particle, small particles, such as bacteria being ingested almost instantaneously whilst larger objects, such as clumps of bacteria or tissue cells including apoptotic cells, being phagocytosed by a more prolonged response of the leukocyte often requiring opsonisation (Ishimoto H *et al* 2008). The cell flows around the object until it has been completely engulfed and the engulfed object is thus enclosed within the membrane-bound vacuole (phagosome) (Tidball JB *et al* 1999). The phagocyte then digests the ingested particle with hydrolytic enzymes, which are contained within membrane-enclosed sacs, the lysosomes found within the cell (Chen G, Goeddel DV 2002; Tidball JB *et al* 2007). The phagocytic enzymes are secreted into the vacuole in which digestion takes place.

Macrophages excel at phagocytosis and their engulfment of pathogens initiates the innate immune response, which in turn orchestrates the adaptive response (Tidball JG *et al* 1999; Vandivier RW *et al* 2006). In order to discriminate between infectious agents and self, macrophages have evolved a restricted number of phagocytic receptors, like the mannose receptor, that recognize conserved motifs on pathogens (Kerr JF 1965). Pathogens are also phagocytosed through complement receptors after relatively nonspecific opsonization with complement proteins such as C3b or iC3b and via Fc receptors after specific opsonization with antibodies (Krombach F *et al* 1997). All these receptors induce rearrangements in the actin cytoskeleton that lead to the internalization of the particle (Kerr JF 1965). However, important differences in the molecular mechanisms underlying phagocytosis by different receptors are now being appreciated (Krombach F *et al* 1997). These include differences in the cytoskeletal elements that mediate ingestion, vacuole maturation, and inflammatory responses.

1.13.1 Steps involved in phagocytosis

There are a number of distinct steps involved in phagocytosis. This section summarizes some of these seps including activation and chemotaxis of the phagocyte, attachment of the phagocyte to the cell or particle and ingestion and destruction of the cell or particle by the phagocyte.

1.13.1.1 Activation of the phagocyte

Upon activation by inflammatory mediators such as bacterial products, complement proteins, inflammatory cytokines, and prostaglandins (Grønlien HK, Berg T, Løvlie AM 2002) resting, circulating phagocytes produce surface glycoprotein receptors that increase their ability to adhere to the inner surface of capillary walls, enabling

them to squeeze out of the capillary and be attracted to the site of infection (Kerr JF *et al* 1972). In addition, they produce endocytic pattern-recognition receptors (EPRR) that recognize and bind to pathogen-associated molecular patterns (PAMPs) which are components of common microbial molecules such as peptidoglycan, teichoic acids, lipopolysaccharide, and mannose that are not found in human cells (Lucas AD, Greaves DR 2001). They also exhibit increased metabolic and microbicidal activity by increasing their production of ATPs, lysosomal enzymes and lethal oxidants.

1.13.1.2 Chemotaxis of Phagocytes

Chemotaxis is the movement of phagocytes toward an increasing concentration of a chemo attractant such as bacterial factors (bacterial proteins, capsules, LPS, peptidoglycan, teichoic acids) (Kerr JF *et al* 1972), complement proteins C5a, chemokines (chemotactic cytokines such as interleukin-8 secreted by various cells), fibrin split products, kinins, and phospholipids released by injured host cells (Lucas AD, Greaves DR 2001). This leads to the aggregation of the phagocytes in affected tissue to carry out phagocytosis.

1.13.1.3 Attachment of the phagocyte to the marked cell or particle

Attachment of cells and particle is necessary for ingestion and appears to be a relatively passive phenomenon based mainly on physicochemical interaction between the surfaces of the particles and the cell. It may be enhanced or not. There may also be extracellular trapping with neutrophil extracellular traps (NETs) (Kerr JF *et al* 1972).

1.13.1.4 Enhanced attachment

Enhanced attachment or opsonisation involves the attachment of particles to phagocytes by way of an immunoglobulin, the complement proteins C3b and C4b produced during the complement activation and acute phase proteins such as mannose-binding lectin (MBL) and C-reactive protein (CRP) (Lee YJ *et al* 1999). Enhanced attachment is much more specific and efficient than unenhanced.

1.13.1.5 Unenhanced attachment

Unenhanced attachment is the innate recognition of pathogen-associated molecular patterns or PAMPs (Boettner DR *et al* 2008) by means of endocytic pattern-recognition receptors, which are found on the surface of phagocytes and promote the attachment of microorganisms to phagocytes leading to their subsequent engulfment and destruction. e.g. scavenger receptors and mannose receptors.

1.13.1.6 Extracellular trapping with neutrophil extracellular traps (NETs)

In response to certain pathogen-associated molecular patterns such as LPS, and certain cytokines such as IL-8, neutrophils release chromatin and antimicrobial granular proteins (Ishimoto H *et al* 2008); these neutrophil extracellular traps (NETs) consisting of extracellular fibres and degranulated antimicrobial factors bind to bacteria, prevent them from spreading, and kill them.

1.13.1.7 Ingestion of the cell or particle by the phagocyte

Ingestion of particles seems to be an active, energy-dependent event in which the machinery of cell motility plays an important role (Parham P 2009). Following attachment, polymerisation and then depolymerisation of actin filaments the phagocytes sends pseudopods out to engulf the particle and place it in an endocytic vesicle - the phagosome. The phagosome of ingested material is then fused with the

lysosome to form the phagolysosome or perixosome depending on the material, leading to degradation (Parham P 2009).

1.13.1.8 Destruction of the cell or particle

Phagocytes contain membranous sacs called lysosomes produced by the Golgi apparatus that contain various digestive enzymes, microbicidal chemicals, and toxic oxygen radicals (Scotts RS *et al* 2001). The lysosomes fuse with the phagosomes containing the ingested particle or cell and are destroyed. The destruction can be through oxygen or non-oxygen dependent system (Savill J *et al* 2003).

1.13.1.9 The oxygen-dependent system: production of reactive oxygen species (ROS)

The cytoplasmic membrane of phagocytes contains the enzyme oxidase, which converts oxygen into superoxide anion (O^{2-}) (Parham P. 2009). This can combine with water by way of the enzyme dismutase to form hydrogen peroxide (H_2O_2) and hydroxyl (OH) radicals (Wajant H 2002; Parham P 2009). In macrophages, nitric oxide (NO) can combine with H_2O_2 to form peroxynitrite radicals (Werlen G *et al* 2003; Wang X *et al* 2003). In the case of neutrophils, but not macrophages, the H_2O_2 can then combine with chloride (Cl^{2-}) ions by the action of the enzyme myeloperoxidase (MPO) to form hypochlorous acid (HOCL), and singlet oxygen (Shacter E *et al* 2000). Collectively, these oxidizing free radicals are called reactive oxygen species (ROS) and are very microbicidal because they are powerful oxidizing agents which oxidize most of the chemical groups found in proteins, enzymes, carbohydrates, DNA, and lipids breaking down cytoplasmic membranes (St. Pierre BA, Tidball JG 1994).

Oxidase also acts as an electron pump that brings protons (H^+) into the phagosome lowering the pH within the phagosome so that when lysosomes fuse with the phagosome, the pH is correct for the acid hydrolases, like elastase, to effectively break down cellular proteins (Krombach F *et al* 1997; Tidball JG *et al* 1999). In addition to phagocytes using this oxygen-dependant system to kill microbes intracellularly, neutrophils also routinely release these oxidizing agents, as well as acid hydrolases, for the purpose of killing microbes extracellularly which however also wind up killing the neutrophils themselves as well as some surrounding body cells and tissues as mentioned above (Tidball JG *et al* 1999).

1.13.1.10 The oxygen-independent system

Some lysosomes contain defensins, cationic peptides that alter cytoplasmic membranes, lysozyme, an enzyme that breaks down peptidoglycan, lactoferrin, a protein that deprives bacteria of needed iron, cathepsin G, a protease that causes damage to microbial membranes, elastase, a protease that kills many types of bacteria, bactericidal permeability increasing protein (BPI), proteins used by neutrophils to kill certain bacteria by damaging their membranes, collagenase (Ishimoto H *et al* 2008) and various other digestive enzymes that exhibit antimicrobial activity by breaking down proteins, RNA, phosphate compounds, lipids, and carbohydrates.

1.14 Recognition and phagocytosis of cells undergoing apoptosis

The normal fate of cells undergoing apoptosis is recognition, uptake and degradation of the intact dying cell by phagocytes so that dead cells are removed before their membrane integrity is breached and their contents leaked into the surrounding tissue (St. Pierre BA, Tidball JG 1994). This prevents exposure of tissue to toxic enzymes, oxidants and other intracellular components such as

proteases and caspases (Vandivier RW *et al* 2006). The phagocytes that play an important role in the clearance of dying and dead cells are the macrophages and are typically, and perhaps simplistically, viewed as the professional phagocytes of apoptotic cells. They are the primary phagocytes responsible for clearance of apoptotic cells in most organs (Savill J *et al* 1993) and very often the apoptotic cell and its fragments are visible in phagocytic vacuoles within the macrophage cytoplasm.

Cellular clearance by this mechanism is fast, efficient, injury-limiting, and often associated with actively anti-inflammatory responses as cells dying purposefully by programmed cell death or apoptosis are thought to be phagocytosed by mechanisms that fail to incite inflammatory or immune reactions (Cohen JJ *et al* 1992; Gregory DC and Devitt A 2004). By contrast, macrophage responses to necrotic cells, including secondarily necrotic cells derived from uncleared apoptotic cells, are perceived as pro-inflammatory (Gregory DC and Devitt A 2004). Indeed, persistence of apoptotic cells as a result of defective apoptotic-cell clearance has been found to be associated with the pathogenesis of both systemic and organ-specific autoimmune disease (Eguchi K 2001).

One of the features of an apoptotic cell is the presentation of a variety of intracellular molecules on the cell surface that mark them for disposal, such as Calreticulin, Phosphatidylserine (from the inner layer of the plasma membrane), Annexin A1 and oxidised LDL (Schroit AJ *et al* 1984; Tidball JG *et al* 1999). These molecules are recognised by receptors on the cell surface of the macrophage such as the Phosphatidylserine Receptor (PSR) (Zhou Z 2007), or by soluble (free floating) receptors such as Thrombospondin 1, Gas-6 and MFG-E8, which then themselves bind to other receptors on the macrophage such as CD36 and Alpha-V Beta-3

Integrin (Ishimoto H *et al* 2008; Ziegler U & Groscurth P 2004). It is conceivable that recognition mechanisms may be ordered in a hierarchy of 'back-ups', each recognising cells at different stages of the death program. However, a full understanding of this complex process will require definition of recognition mechanisms which operate *in vivo* in higher organisms (Krombach F *et al* 1997).

The processes of apoptosis and phagocytosis work together to perform central roles in processes such as embryogenesis, mature tissue homeostasis, elimination of infected, aged, and injured cells, cellular immunity, and resolution of inflammation (Cohen JJ *et al* 1992; Hopkinson-Woolley *et al* 1994). Thus, engulfment of apoptotic cells by phagocytes is not limited to safe packaging and disposal of unwanted cells and their contents. This process clearly plays an important regulatory role in the immune system, highlighting significance of macrophage recognition and clearance of apoptotic cells that lead to their efficient removal (Chiarugi A Moskowitz MA 2002; Cohen JJ *et al* 1992; Giesen PLA *et al* 1999). Perhaps not surprisingly, however, the immunological consequences of a particular mode of cell death or the effects of failed clearance are not as straightforward as they might be perceived (Cohen JJ *et al* 1992).

1.15 Phagocytosis assay

A variety of assays have been developed to quantify phagocytic activity. These include: direct microscopic visualization, spectrophotometric evaluation of phagocytized paraffin droplets containing dye, scintillation counting of radiolabeled bacteria, fluorometric, and flow cytometric analysis of fluorescent particles (Jy W *et al* 2004). The flow cytometric assay offers the advantage of rapid analysis of thousands of cells and quantification of the internalized particle density for each

analyzed cell. The assay may be performed with purified leukocyte preparations or anticoagulated whole blood.

In fluorometric, and flow cytometric analysis the method of choice in this work, fluorescent particles or cells that have been opsonized with serum or specific IgG are mixed with phagocytic leukocytes at 37°C and continuously mixed to optimize the cell–particle interaction. The reaction is stopped by the addition of ice-cold medium, and the free particles are washed away from the leukocytes by centrifugation. The cells are resuspended in cold medium and analyzed.

1.16 Promonocytes/monocytes and macrophages

1.16.1 Monocytes

Originally, monocytes and macrophages were classified as cells of the reticulo-endothelial system (RES) (Dimberg A *et al* 2002). Later the mononuclear phagocyte system (MPS) was proposed, and monocytes and macrophages became basic cell types of this system. Their development starts in the bone marrow and passes through the following steps: stem cell - committed stem cell - monoblast - promonocyte - monocyte (bone marrow) - monocyte (peripheral blood) - macrophage (tissues) (Hmama ZD *et al* 1999). The blood or circulating promonocytes are young immature cells that are supposed to be quarantined in the bone marrow but may appear in the peripheral system due to leukaemia (Ades LS *et al* 2005).

Upon migration into tissues, monocytes undergo further differentiation (at least one day) to become multifunctional tissue macrophages (Gregory JL *et al* 2006; Flieger OA *et al* 2003). Monocytes are generally, therefore, considered to be immature macrophages. Also, it can be argued that monocytes represent the circulating

macrophage population and should be considered fully functional for their location, changing phenotype in response to factors encountered in specific tissue after migration (Gregory JL *et al* 2006). Monocytes are not very abundant in the peripheral circulation, accounting for only 1-6% of all nucleated blood cells but can perform phagocytosis using intermediary (opsonising) proteins such as antibodies or complement that coat the pathogen, as well as by binding to the microbe directly via pattern-recognition receptors that recognize pathogens (Flieger OA *et al* 2003).

1.16.2 Macrophages

Macrophages are generally a population of ubiquitously distributed mononuclear phagocytes responsible for numerous homeostatic, immunological, and inflammatory processes (Martinez MC *et al* 2006). Their wide tissue distribution makes these cells well suited to provide an immediate defence against foreign elements prior to leukocyte migration. Because macrophages participate in both specific immunity via antigen presentation and IL-1 production and nonspecific immunity against bacterial, viral, fungal, and neoplastic pathogens, it is not surprising that macrophages display a range of functional and morphological phenotypes (Sham RL *et al* 1995).

Macrophages can be divided into normal and inflammatory macrophages. Normal macrophages includes macrophages in connective tissue (histiocytes), liver (Kupffer's cells), lung (alveolar macrophages), lymph nodes (free and fixed macrophages), spleen (free and fixed macrophages), bone marrow (fixed macrophages), serous fluids (pleural and peritoneal macrophages), skin (histiocytes, Langerhan's cells) and in other tissues (de Almeida MC *et al* 2000; Sham RL *et al* 1995). Inflammatory macrophages are present in various exudates. Various specific markers, e.g. peroxidase activity, may characterize them and since they are derived

exclusively from monocytes, they share similar properties (Morgan BP *et al* 1985). The macrophage population in a particular tissue may be maintained by three mechanisms: influx of monocytes from the circulating blood, local proliferation and biological turnover. Under normal steady-state conditions, the renewal of tissue macrophages occurs through local proliferation of progenitor cells and not via monocyte influx (Bombara C and Ignotz RA 1992).

Macrophages are versatile cells. Besides acting as phagocytic scavengers, they secrete a wide variety of signalling cytokines (monokines) that are vital to the immune response (Fadok VA *et al* 1998). Whenever it encounters certain inflammatory mediators or other signals of tissue distress, the cell undergoes a process known as macrophage activation, in which it rapidly increases its metabolic rate, motility and phagocytic activity (Lehmann LE *et al* 2005). Activated macrophages are somewhat larger than their inactive counterparts, owing mainly to an increase in cytoplasmic volume and are much more efficient at killing bacteria and other pathogens (Waclavicek W *et al* 2001). Originally, it was thought that tissue macrophages were long-living cells but more recently, however, it has been shown that depending on the type of tissue, their viability ranges between 6 and 16 days.

1.17 Promonocyte/monocyte differentiation to macrophage

Under normal circumstances, promonocytes develop to monocytes which undergo a process of differentiation following their accumulation into extravascular spaces (Abedin MJ *et al* 2003) However, the precise external signals that control differentiation of peripheral blood monocyte to tissue macrophage are incompletely defined. Monocytes leave the bone marrow and circulate continuosly in the peripheral blood until they migrate through the vessel walls to reach tissues in the process of margination and emigration (Forrest ARR *et al* 2010)

During margination, which is in three phases, the monocyte collides with the vessel wall, allowing P and E selectin molecules from the endothelium to bind cell surface mucins that bear the appropriate carbohydrate sidechains (selectin-mediated phase). Leukocyte chemotatic factors such as chemokines (monokines) from the endothelium or tissues then bind to receptors on the monocyte and trigger the activation phase (Flieger, O *et al* 2003).

Intergrins (Mac-1 and leukocyte functional antigen 1 or LFA-1) on the cell surface enable them to bind to specific glycoproteins (ICAM-1 and VCAM-1) on the endothelium which lead to stable, long-lasting molecular contact that prevent further movement of the monocyte (intergrin-mediated phase) (Daigneault M *et al* 20100). The cells stop rolling along the vessel wall, flatten out against the endothelium and actively squeeze themselves between endothelial cells to migrate out of the venule and into a tissue (emigration) (Abedin MJ *et al* 2003).

Once they reach a tissue, perhaps in response to monocyte colony-stimulating factor (M-CSF), granulocyte-monocyte colony-stimulating factor (GM-CSF), (Daigneault M *et al* 20100), monocyte chemoattractant protein-1 (MCP-1), and/or IL-3, they differentiate into macrophages by growing in size and increasing their lysosomal compartment, the amount of hydrolytic enzymes, the number and size of mitochondria, and the extent of their energy metabolism (Abedin MJ *et al* 2003).

1.18 Systemic Lupus erythematosus (SLE)

Lupus erythematosus is a connective tissue and chronic inflammatory disease (Freedberg *et al* 2003), that occurs when the body's immune system gets out of control and attacks its own cells, tissues and organs instead of foreign bodies causing damage and dysfunction. Inflammation caused by lupus can affect many

different body systems, including joints, skin, kidneys, blood cells, heart, nervous system and lungs (Massarotti EM 2008). Some patients with lupus have very mild disease, which can be treated with simple medications, whereas others can have serious, life-threatening complications (Hodkinson B *et al* 2009; Harrison's internal medicine, 17th ed. 2006).

Lupus is more common in women than men, and for reasons that are not precisely understood, its peak incidence is after puberty (Massarotti EM 2008). Most often, it develops in people between 18 to 45 years old but can affect people of all ages. While lupus is a chronic illness, it is characterized by periods when the disease activity is minimal or absent (remission) and when it is active (relapse or flare) (Asanuma Y *et al* 2003). The outlook for patients with lupus today is much better than years ago because of greater awareness and more accurate tests leading to earlier diagnosis and treatment as well as more effective and safer medications (Rahman A and Isenberg DA 2008).

1.18.1 Causes of SLE

SLE has no one specific cause. There are however a number of environmental triggers and genetic susceptibilities that can cause it to start (Hemminki K *et at* 2009). These lead to the body's immune system producing antibodies against itself, particularly against proteins in the cell nucleus (Mendoza-Pinto C *et al* 2009). SLE is the prototypical autoimmune disease with all the key components of the immune system involved in the underlying mechanisms according to (Rahman A *et al* 2008; Syuto T *et al* 2009). From an evolutionary perspective, according to Crow (Crow MK. *et al* 2008), the population must have enough genetic diversity to protect itself against a wide range of possible infection; some genetic combinations result in autoimmunity but the immune system must have a balance (homeostasis) between

being sensitive enough to protect against infection, and being too sensitive and attacking the body's own proteins (autoimmunity) (Asanuma Y *et al* 2003).

1.18.2 Genetics of SLE

The first mechanism leading to SLE may arise genetically even though the disease does not run in families (Hahn BH *et al* 2003). Research indicates that SLE may have a genetic link but as yet no single, causal, gene has been identified (Roman MJ *et al* 2003). Instead, multiple genes appear to influence a person's chance of developing lupus when triggered by environmental factors. The most important genes are located in the HLA region on chromosome 6, where mutations may occur randomly (*de novo*) or may be inherited.

HLA class I, class II, and class III are associated with SLE, but only class I and class II contribute independently to increased risk of SLE (Neuwelt CM *et al* 2009). Other genes which contain risk variants for SLE are *IRF5*, *PTPN22*, *STAT4*], *CDKN1A*, *ITGAM*, *BLK*, *TNFSF4* and *BANK1*. Some of the susceptibility genes may be population specific (Honczarenko K *et al* 2008) There is ongoing research in trying to identify the individual genes, the proteins they produce, and their role in the immune system as each protein is a link on the autoimmune chain, and researchers are trying to find drugs to break each of those links (D'Cruz DP *et al* 2006).

1.18.3 Signs and symptoms of SLE

SLE is one of several diseases known as "the great imitators" because it often mimics or is mistaken for other illnesses (Martens HA *et al* 2009; Yang W 2009). It is a classical item in differential diagnosis because its symptoms vary widely and come and go unpredictably. Diagnosis can thus be elusive, with some people suffering unexplained symptoms of untreated SLE for years (Zielonka TM *et al* 2008;

Anderson LA *et al* 2009). Common initial and chronic complaints include fever, malaise, joint pains, myalgias, fatigue, and temporary loss of cognitive abilities but because they are so often seen with other diseases, these signs and symptoms are not part of the diagnostic criteria for SLE (Kanta H *et al* 2009). When occurring in conjunction with other signs and symptoms however, they are considered suggestive.

1.18.4 Pathophysiology of SLE

One manifestation of SLE is abnormalities in apoptosis and clearance of apoptotic cells. Apoptosis is increased in monocytes and keratinocytes as expression of Fas by B and T cells is increased with a correlations between the apoptotic rates of lymphocytes and disease activity (Yazdany J *et al* 2008). Even though the exact mechanisms for the development of SLE are still unclear as the pathogenesis is a multifactorial event, impaired clearance of dying (apoptotic) cells is a potential pathway for the development of this systemic autoimmune disease beside the discussed causations (Foocharoen C *et al* 2009). This is a result of reduced or deficient phagocytic activity and scant serum components in addition to increased apoptosis.

Monocytes isolated from whole blood of SLE sufferers show reduced expression of CD44 surface molecules involved in the uptake of apoptotic cells (Danchenko N *et al* 2006). Most of the monocytes and tingible body macrophages (TBM), which are found in the germinal centres of lymph nodes, even show a definitely different morphology; they are smaller or scarce and die earlier. Serum components like complement factors, CRP, and some glycoproteins are decisively important for an efficiently operating phagocytosis but with SLE, these components are often missing, diminished, or inefficient.

1.18.5 Defective clearance of apoptotic cells in SLE

The clearance of apoptotic cells is an important function in multicellular organisms (Casciola-Rosen LA *et al* 1994). Normally, apoptotic cells are swiftly removed by phagocytosis due to surface changes induced in the early phase of apoptotic process and it induces neither inflammation nor an immune response (Voll RE *et al* 1997; Fadok VA *et al* 2005). The early recognition and engulfment of apoptotic cells is necessary to avoid the cells entering into the late stages of apoptosis and finally to secondary necrosis (Rahman A. *et al* 2008). Necrotic (uncleared apoptotic) cells release nuclear fragments as potential autoantigens constituting endogenous danger signals that are subsequently presented to antigen-presenting cells. Thus, over-riding peripheral tolerance mechanisms and triggering an autoimmune response by autoreactive nucleosome T-cells. This leads to the production of autoantibodies, such as antichromatin autoantibodies, which through a complex formation with chromatin become deposited in basement membranes, especially in the skin and the kidney (Dieker JW *et al* 2002). Apoptotic cell/material removal therefore has to be very efficient to avoid accumulation of apoptotic debris, which has been implicated in the aetiopathogenesis of SLE. Also, it has determined that the primary clinical and pathological manifestations of SLE are consequences of local inflammation events initiated by widespread immune-complex deposition in various tissues (Yazdany *et al* 2008).

The role of apoptosis in the development of SLE is supported by several mouse models with an abnormal function of factors involved in apoptosis. Interference with the expression of Fas and Fas Ligand and other apoptotic factors leads to accumulation of apoptotic cells/blebs/chromatin resulting in breaking of tolerance with ultimately the formation of anti–nuclear antibodies and lupus-like glomerulonephritis (Seshasayee D *et al* 2003). During apoptosis, proteins, DNA and

RNA are modified by cleavage through proteases, caspases and endonucleases. In addition, specific apoptosis induced post-translational modifications of autoantigens can take place, which include methylation, phosphorylation, ubiquitination, and transglutamination (Yan M *et al* 2001). Nuclear autoantigens (chromatin) targeted in SLE are clustered in blebs at the surface of apoptotic cells (Radic M *et al* 2004). Normally, phagocytes will quickly remove those apoptotic cells and blebs long before they could have released their modified contents but in SLE this processes is disturbed (Seshasayee D *et al* 2003) leading to modified nuclear autoantigens exposure to the immune system which are recognised as non-self antigens.

Recognition of apoptotic cells is a complex process involving many phagocytic receptors, bridging molecules and markers on the apoptotic cell which points up the extreme importance of a solid and efficient recognition and clearance system (Salmon JE *et al* 1984). The early apoptotic process includes chromatin condensation and also surface changes, most notably the appearance of phosphaditylserine (PS) at the outside, but maintaining cell membrane intergrity (Scott RS *et al* 2001). This and other changes provide the signals to phagocytic cells that express a number of receptors able to bind apoptotic cells, including the PS receptor, CD14, the C1q receptor, the vitronectin receptor and others (Manfredi AA *et al* 1998-a). The lipopolysaccharide (LPS) receptor, CD14, normally induces a pro-inflammatory response to invading organisms. However, docking of apoptotic cells results in the opposite effect, an anti-inflammatory reaction (Hart SP *et al* 2004). There are several putative candidates whose increased presence could lead to a decrease in apoptotic cell phagocytosis including antiagonists to PS such as PMVs which has been implicated in the modulation of apoptotic cell clearance (Manfredi AA *et al* 1998-b).

PS act as a good recognition signal for phagocytes as it is recognized by several receptors of the phagocytic cells and is an indispensable and essential condition for the future engulfment of apoptotic cells (Fadok VA *et al* 2001). It has been suggested that PS is exposed on apoptotic and necrotic cells in a clustered fashion and therefore these dying cells are taken up, in contrast to viable cells, which may also expose PS, but at a lower density (Appelt U *et al* 2004). Importantly, PS-mediated phagocytosis of apoptotic cells suppresses inflammatory signals like IFN-δ, TNF-α and nitric oxide, and also triggers the production of TGFβ an anti-inflammatory cytokine (Gaipl US *et al* 2001). Apoptotic cell uptake can be inhibited by PMVs, which binds preferentially and with very high affinity to PS receptor (Callahan MK *et al* 2000) and can very efficiently disrupts the PS-dependent recognition of apoptotic or dying tumour cells during clearance and thus enhances the immune response against these cells (Hart SP *et al* 2004). Evidence of important molecules involved in the clearance process collected from knockout mouse models shows that accumulation of apoptotic cells in tissues together with an immune response against DNA-containing complexes represents a central pathogenic process in the development and acceleration of SLE (Botto M *et al* 1998; Napirei M *et al* 2000).

A working group has shown the accumulation of apoptotic debris in germinal centres (GC) of patients with SLE (Baumann I *et al* 2002). In normal lymph nodes, apoptotic nuclei can almost exclusively be detected inside tingible body macrophages (TBM), which often displayed an altered morphology in patients with SLE where non-ingested apoptotic nuclei were often found outside TBMs. Sometimes nuclear debris is observed to be attached to the surfaces of follicular dendritic cells (FDC), which normally retain complement-opsonised immune complexes on their surfaces, promoting affinity maturation (Baumann I *et al* 2002). However, in SLE, clearance

deficiency leads to the accumulation of apoptotic material on the FDC, which may provide short-term survival signals for autoreactive B cells. In 2007, Hepburn, et al (Hepburn AL *et al* 2007) reported that a high proportion of bone marrow from patients with SLE contained apoptotic debris and the presence of not cleared apoptotic material at those sites of B-cell selection may explain the loss of B-cell tolerance and the induction of T-cell help against autoantigens (Strasser A *et al* 1991).

Apoptosis and SLE Apoptosis not only plays a critical role in removing damaged cells, but is also very important in inducing and maintaining self-tolerance. Tolerance of self antigens requires the deletion of autoreactive T- and B-cells by apoptosis and, therefore, defects in inducing apoptosis could lead to the persistence of autoreactive T- or B-cells (Strasser A *et al* 1991). Taken together, apoptosis and clearance of apoptotic cells/material are key processes in the aetiology of SLE and depending on the context, phagocytosis of apoptotic cells may lead to a pro-inflammatory response or an anti-inflammatory response, which is decisive in the development of autoimmunity and may sustain inflammatory conditions.

1.18.6 Treatment of SLE

Being a chronic disease with no known cure, the treatment of SLE is symptomatic. In essence, this involves preventing flares and reducing their severity and duration when they occur (Rahman A *et al* 2008). Currently, medication is the main form of treatment. Treatment for a lupus anticoagulant is usually undertaken in the context of documented thrombosis, such as extremity phlebitis or dural sinus vein thrombosis (Sabahi R *et al* 2006). Patients with a well-documented (i.e., present at least twice) lupus anticoagulant and a history of thrombosis are considered

candidates for indefinite treatment with anticoagulants. Patients with no history of thrombosis and a lupus anticoagulant should probably be observed.

Current evidence suggests that the risk of recurrent thrombosis in patients with an antiphospholipid antibody is enhanced whether that antibody is measured on serological testing or functional testing. The Sapporo criteria specify that both serological and functional tests must be positive to diagnose the antiphospholipid antibody syndrome. Miscarriages may be more prevalent in patients with a lupus anticoagulant and these can be prevented with the administration of low molecular weight heparins (LMWHs), and thrombosis is treated with anticoagulants (LMWHs and warfarin).

1.19 Aims of thesis

The issue of standardization is very relevant to the study of all cell-derived PMVs and this thesis aim to provide evidence that forms the main characteristics of PMVs and which can be used as basis to establish reliable and reproducible methods of isolating and categorising PMVs. Given the important role TGF-β1 plays in the regulation of cellular proliferation, and its autocrine inhibition of proliferation in the acute promyelocytic leukaemia (APL) cell line, HL-60, we asked whether any growth regulation and differentiation of promonocytic leukaemia cells could in part be influenced by PMVs bearing TGF-β1, that are released from cells by the action of known differentiation therapeutics. It has also been established that both PMVs and ACs express PS on the outer leaflet of their membrane, and PS is a marker for phagocytosis of ACs by macrophages. We therefore set out to ascertain whether PMVs inhibits the phagocytosis of the ACs by blocking the PS receptor on macrophages and if this has a role in SLE in which there is increased levels of PMVs as well as persistent apoptosis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents and Solutions

RPMI – RPMI 1640 medium + 2.05 mM L – Glutamine, from Hyclone Laboratory Inc, USA.

Foetal Bovine Serum (FBS) – Pre prepared.

Phosphate Buffered Saline (PBS) – One tablet dissolved in 200 ml of distilled water and autoclaved, From Sigma - Aldrich

PMA – Phorbol 12-Myristate 13-Acetate, dissolved in absolute ethanol according to manufacturer's protocol, from Sigma-Aldrich.

Fixing solution - 2.25 ml glacial acetic acid mixed with 0.25 ml formaldehyde stock solution (37%), and 2.5 mL H_2O.

Fluorescein isothiocyanate (FITC) dye – Pre-prepared according to manufacturer's protocol, Sigma-Aldrich

Hoescht dye – Pre-prepared according to manufacturers protocol, from Sigma Aldrich

Normal Human Serum (NHS) – Pre-prepared stock solution (100%) from Sigma Aldrich.

EDTA Mixture – 10mM EDTA added to 1 part of PBS and 1 part of complete growth medium.

NBT solution - To prepare 0.2% Nitro Blue Tetrazolium substrate, one tablet (0.01 g) dissolved in 5ml of PBS in a test tube wrapped in a foil and placed on a mixer to dissolve. Maintain light protected until use.

Anti-Phosphatidylserine receptor – Supplied as a solution with a concentration of 1.0 - 1.5 mg/ml, from Sigma Aldrich UK.

$CaCl_2$ – 1.47g CaCl2 dissolved in 70 ml purified water and adjusted up to 100 ml with distilled water. Solution then filter sterilized through a pre-washed 0.2µM filter unit.

EDTA - ethylenediaminetetraacetic acid. To prepare a 0.5 M EDTA (pH 8.0), dissolve 95.05 g of Na_2EDTA in ~400 ml H_2O. Adjust pH to 8.0 with 6 N NaOH. Then bring volume to 500ml and autoclave.

EGTA- Ethylene glycol-bis [β-aminoethyl ether]-*N,N,N',N'*-tetraacetic acid. To prepare a 1M solution of EGTA, add 250 g of EGTA and 53 g of NaOH to 400 mL H_2O. Adjust the pH to 7.0 with HCl. Adjust total volume to 660 mL with H_2O. Filter, autoclave, and store at 4°C for up to 1 month.

ProteoSeek™ Albumin/IgG Removal Kit- From Pierce Biotechnology, Inc., Rockford, IL, USA.

Melon™ Gel IgG Spin Purofication Kit- From Thermo Scientific.

Running Buffer (SDS-PAGE) – Tris-Glycine buffer (10x): 144 g glycine in 1000 ml water, pH 8.3; 30g Tris (tris (hydroxymethyl)-aminomethane). Dilute stock solution x10 then add 3ml 20% SDS to 600ml of diluted stock solution.

Sample Buffer (SDS-PAGE) – 20% SDS (in water) (6ml); glycerol (3ml); 1M Tris-HCl, pH 6.8 (2.4ml); distilled water (15.6ml); few grains of bromopohenol blue.

Transfer Buffer (Western Blotting) – 100ml running buffer (SDS-PAGE) without SDS; 200ml absolute ethanol. Adjust to 1 litre with water.

2.2 Equipment

Fluorescence Activated Cell Sorting (FACS) – Guava Easycyte from Guava technologies Inc.

Fluorescent Microscope – Olympus 1X81 motorised inverted research microscope equipped with UCMAD3 monochromatic camera, from Olympus Life and Material Science, Europa GMBH – Germany.

Centrifuge – Eppendorf 5810R, Rotor number A-4-62,

Micro-centrifuge – Eppendorf 5417R, Rotor number FA 45-24-11

Stericup Millipore Filter – Pre sterilised vacuum – driven disposable filtration system with a pore size rating of 0.22μm, from Millipore Corporation, USA.

2.3 Cell lines

2.3.1 HL-60 promonocytic cells

The HL-60 cell line is a leukaemic cell line that was established in 1977 from a 36 year old Caucasian female patient with acute myeloid leukaemia diagnosed later as acute promyelocytic leukaemia. The cells largely resemble promyelocytes but can be induced to differentiate terminally *in vitro* and have been used for laboratory research on how certain kinds of blood cells are formed. The HL60 cell genome contains an amplified *c-myc* proto-oncogene with corresponding high *c-myc* mRNA levels in undifferentiated cells that declines rapidly following induction of differentiation. HL-60 cells proliferate continuously in suspension culture in nutrient medium supplemented with fetal bovine serum, L-glutamine, HEPES and antibiotic chemicals and it represents an attractive cell model for studies of differentiation in general, and human myeloid cell differentiation in particular. Proliferation occurs through the transferrin and insulin receptors, on the cell surface with the requirement for insulin and transferrin being absolute.

HL-60 cells have a growth doubling time that can vary from 20 - 45 hours, depending on the subline and all sublines also display a myeloblastic/promyelocytic morphology which is consistent with their origins. They stain positively with periodic acid-Schiff reagent and occasionally for acid phosphatase, characteristics of mature *in vivo* granulocytes. Compounds such as dimethyl sulfoxide (DMSO), retinoic acid, 1, 25-dihydroxyvitamin D_3, Phorbol 12-myristate 13-acetate (PMA) and Histamine can induce HL-60 to differentiate to monocytic, macrophage-like and eosinophil

phenotypes. The HL-60 cultured cell line provides a continuous source of human cells for studying the molecular events of myeloid differentiation and the effects of physiologic, pharmacologic, and virologic elements on this process.

2.3.2 THP1-monocytic cells

The THP 1 cell line was obtained from the Health Protection Agency (HPA) Culture Collections (formerly known as European Collection of Cell Cultures (ECCAC)). This cell line is a human monocytic leukaemic cell line that is used as a novel model in the regulation of macrophage specific genes, human monocyte-to-macrophage differentiation in monocytic leukaemia and the regulation of complement components. The cell line is ideal to use in *in-vitro* experiments as the cells' behaviour is similar to native monocyte-derived macrophages once they have terminally differentiated, as compared to other similar cell types, such as the U937 (promonocytic), KG-1 (myelolastic), HL-60 (promyelocytic) etc.

2.3.3 Jurkat cells

Jurkat cells are an immortalised line of T lymphocyte cells which is employed to analyse acute T cell leukaemia, T cell signaling, and the expression of assorted chemokine and cytokine receptors susceptible to viral entry, particularly HIV. They were established in the late 1970s from the peripheral blood of a 14 year old boy with T cell leukaemia. The ability of Jurkat cells to produce IL-2 makes them quite useful in scientific research and derivatives can be obtained that have been mutated to lack certain genes. Jurkat cells can be transfected and are therefore useful for studies of blood proto-oncogene expression, apoptosis and cell survival, although their main use is to determine the biochemical mechanism of differential susceptibleness of cancers to drugs and radiotherapy. The cell line used for the experiments in this thesis was the J45.01 cell line obtained from HPA Culture

Collections UK. The cells were adopted for the production of apoptotic cells required for the phagocytosis assay as well as to induce the production of PMVs.

2.4 Cell culture and peripheral blood monocyte isolation

All cells used in this study were maintained in growth medium containing RPMI 1640 supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were occasionally maintained for a week in growth medium supplemented with 1% kanamycin at 37°C in a humidified atmosphere of 5% CO_2 (all reagents purchased from Sigma-Aldrich). Exponentially growing cells were counted and viability determined using the Guava EasyCyte flow cytometer (ViaCount assay, Guava Technologies). Throughout, after 3 days in culture, cells were split 1:4 and only cultures with at least 95% viability were used. After 3 months, cells were recovered from original frozen stocks. For experiments, HL-60 cells were grown in a similar growth medium supplemented with 5% FBS. Throughout, cell morphology was monitored and images were collected using a 1X81 motorized inverted fluorescence microscope (Olympus Corporation). Peripheral blood monocytes were isolated from whole blood using a two-step technique essentially using Ficoll-Hypaque of density 1.070g/ml and then a Percoll gradient of density 1.064g/ml. This technique gave a high yield of monocytes with a minimum contamination with platelets and red blood cells.

2.4.1 Cryopreservation of cells

Cells were stored for the duration of this project frozen under liquid nitrogen. Whenever cells were frozen actively growing (exponential phase) cells were used. To freeze cells, they were first harvested by centrifugation (160*g* for 10min at 4°C. They were then counted and adjusted to $1x10^7$ cells/ml. The cell suspension was

added dropwise to an equal volume of cryoprotective solution (fetal bovine serum, 50%; dimethy sulphoxide [DMSO], 20%; tissue culture medium, 30%). The mixture was cooled at a rate of 1°C/min (by placing in a polystyrene box in a -80°C freezer overnight) and then plunged into liquid nitrogen. DMSO as cryoprotectant was not used if over 3 months old to avoid the accumulation of toxic peroxides. To recover frozen cells, the cryotube containing frozen cells was thawed rapidly to 37°C in a water bath. Cells were then washed three times in tissue culture medium by centrifugation (160*g* for 10 min at room temperature).

2.5 FACS analysis (Flow Cytometry)

All the experiments that involved FACS analysis were done on a Guava EasyCyte flow cytometer as described below.

2.5.1 Guava Viacount

This was done to count the cells in culture and to measure their viability. In order to do this 50µl of cell suspension to be analysed was added to 150µl of Guava Viacount reagent in two separate wells of a 96 well plate. One well was used to adjust the settings and the other used for the count.

2.5.2 Guava Express Plus

This was done to measure the relative FITC fluorescence of labelled cells as well as verifying and counting PMVs with Annexin V. 200µl of the labelled cell sample was added to a single well of a 96 well plate. As a control 200µl of unlabelled cells was added to a separate well. A third well was prepared containing 200µl of unlabelled cells used for adjustment. The plate was then loaded in the machine and analysed.

In the case of quantifying PMVs the sample was diluted 1:40 and the result obtained multiplied by the dilution factor.

2.5.3 Guava Nexin Assay

This assay was used primarily to detect and measure percentage apoptosis of PMA induced Jurkat cells. 100µl of the apoptotic cells (PMA induced Jurkat cells) were added to 100µl of Guava Nexin reagent in a well of a 96 well plate. For control, 100µl of viable Jurkat cells was added to 100µl of Guava Nexin reagent in a separate well. A third well was also prepared the same way as the control for adjustment. The plate was then incubated at room temperature on a shaker for 30 minutes in the dark by covering with foil. After incubation the plate was then loaded into the machine and analysed.

The Guava Nexin assay relies on a two-dye approach: Annexin V-PE to detect PS expressed on the external membrane of apoptotic cells and 7-AAD cell impermeant dye which is an indicator of membrane structural integrity (indicator of late stage apoptosis and dead cells).

2.6 Fluorescent Microscopy Analysis

For fluorescent microscopy, all experimental samples were placed into plates containing coverslips by centrifugation (200*g*, 5min, using an A-2-DWP rotor, in a 5804R centrifuge, Eppendorf) and fixed with 4% paraformaldehyde at 37°C for 10 min. For PMV release, cells were labelled with annexin V AlexFluor 488 (1µg/10^6 cells), washed 3x with RPMI and after stimulation for 30 min fixed. Plates were gently washed twice with PBS and coverslips mounted on microscope slides with DAPI-VECTASHIELD medium (Vector Laboratories Inc. Burlingame, CA) for fixed cells, and mounting medium (Agar Scientific, Essex, UK) for PMVs. Images were

collected using a fluorescence microscope (1X81 motorized, inverted fluorescence microscope, Olympus Corporation).

2.7 PMV induction from cells

2.7.1 Differential centrifugation

In experiments to induce PMV release, cells with a viability of ≥95% were used. Cells ($1x10^6$) were washed twice at 160*g* for 5min and resuspended in 1ml of fresh pre-warmed RPMI 1640. 2mM $CaCl_2$ was then added to the cell suspension. The requisite amounts of the various inducing agents (10% normal human serum [NHS], aPLA plasma or SLE plasma, PMA and PMVs) were then added and the cell preparation incubated at 37°C in a water bath (with shaking) for 30 minutes. After incubation, the reaction was stopped by placing the tubes on ice. In order to remove cells, the cell suspension was centrifuged at a low speed of 160*g* for 5 min. The supernatant was then collected and transferred to another tube and centrifuged at a higher speed (4000*g*, 60 minutes) to remove any cell debris. The resultant supernatant was sonicated in a sonicating water-bath (Townson and Mercer Ltd, Croydon) for 5 x 1 min prior to centrifugation, in order to disperse aggregated exosomes. Supernatant was centrifuged at 25,000*g* for 90 min to pellet PMVs. The supernatant was discarded and the pellet containing the PMVs resuspended in 200µl PBS. The PMV samples (diluted 1:40 in PBS) were then quantified using Guava Express plus (see section 2.5.2).

2.7.2 Filtration

Here, after centrifugation at high speed (4000*g*, 60 minutes), the supernatant was filtered using a sterile 0.22µm pore size filter (Stericup Millipore filter, see section 2.2). As PMVs similar or larger than the pore size would be deposited on the membrane, it was carefully removed and cut into smaller pieces and resuspended in

about 1-5 ml of PBS (depending on the size of the sample). The tube and content was then sonicated as above followed by 1 minute of vortexing to detach PMVs from the membrane. After vortexing, the supernatant was transferred to a 1.5ml eppendorf tube and ultracentrifuged at 25,000*g* for 45 minutes. After ultracentrifugation the supernatant was discarded and the pellet containing the PMVs treated as above. This is also the procedure for the separation of exosomes from PMVs where a mixture is passed through the Millipore filter and exosomes are collected in the filtrate.

2.8 PMV induction from HL-60 cells

Following the above protocol, HL60 cells ($1x10^5$), in a 12-well plate in triplicate were treated with bacterial products fMLP (10μM in the presence of 2mM $CaCl_2$ at 37°C for 1h), calcium ionophore (15μg/ml in the presence of 2 mM $CaCl_2$ at 37°C for 18h) and NHS (10%), heat-inactivated NHS (HI), C9-depleted NHS, EGTA treated NHS (5% for 30 min at 37°C in the presence of 2mM $CaCl_2$). Cells treated with fMLP were either wild type HL60, DMSO-differentiated HL60 or fMLP receptor- (FPR1-) transfected HL60 (the latter two therefore only expressing FPR1). Heat-inactivated NHS (HI) was prepared by heating to 56°C for 20 min. Complement C9 was depleted from NHS by use of a polyclonal anti-C9 antibody and 5mM EGTA was added to the NHS treatment to abrogate PMV release. Cells were also treated with 1mM of glyburide and 200μM of calpeptin to cause the abrogation of PMV release under 5% NHS.

2.9 Purification and characterization of PMVs from conditioned medium

PMVs were isolated as stated above from conditioned medium from cells ($1x10^6$) stimulated with 10% NHS, 0.1μM PMA, 10μM histamine or 1μM ATRA at 37°C for

30 min, unless otherwise stated. The PMVs were quantified on a Guava EasyCyte flow cytometer using ExpressPlus software, or stained with annexin-V-FITC to determine surface PS. The protein concentration of PMVs was determined using the BCA protein assay kit (Pierce), according to the manufacturer's instructions. To confirm the size range of PMVs isolated, and to standardise the flow cytometer (Guava EasyCyte) settings for counting HL-60 derived PMVs, Megamix fluorescent sizing beads (Biocytex, France) calibrated to 0.5 and 0.9μm were used, according to the manufacturer's instructions.

2.10 Sucrose density gradient cenrifugation

The sucrose density gradient was created by gently and progressively overlaying less dense or lower concentrations (1.02g/ml) of sucrose on higher concentrations (1.25g/ml) in a centrifuge tube. A steady application of the solutions yields the most reproducible gradient therefore care was taken in the preparation. Firstly the centrifuge tube was held upright in a tube stand. Next a yellow (200μl) pipettor tip was placed on the end of a blue (1000μl) pipettor. Both snugly fitting tips are held steady by a clamp stand and the end of the yellow tip is allowed to make contact with the inside wall of the tube. Now the sucrose solutions of about 1ml each were placed inside the blue tip and gravity allowed to feed the solutions into the tube slowly and steadily. Once the 1.25g/ml solution drained into the tube, the next solution was then loaded into the blue tip which flowed down the inside of the tube and layered on top of the 1.25g/ml solution. This procedure was continued with the rest of the solutions up to the 1.02g/ml with enough space left at the top of the tube upon which about 0.2ml of released HL-60 microvesicles, a mixture of PMVs and exosomes was layered. Having applied the sample to the top of the gradient, the tube was then centrifuged for 1h at 200,000g. Fractions (10x1ml) were collected from the top and then 200μl aliquots removed for determination of density and for

analysis by flow cytometry for markers of exosomes (Lamp1) and PMVs (annexin V-positivity).

2.11 SDS-PAGE and Western blotting

Protein extracts were prepared and either analysed by SDS-PAGE (2.11.1) or by Western blotting (2.11.2). Reduced samples (with 20 or 50 mM DTT) and nonreduced samples were prepared in SDS-PAGE loading buffer. PMVs for Western analysis was conducted by homogenizing the PMVs in a Dounce homogenizer in 150µl of RIPA (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) in the presence of protease inhibitors. After centrifugation (14 krpm for 10 min), the supernatant was respun as above. For lysates, PMVs were suspended in 0.75 ml of lysis buffer (PBS with 1% (v/v) Nonidet P-40 and 5 mM EDTA (pH 8). After adding protease inhibitors (0.2mM PMSF, 4mM aminoethylbenzenesulfonylfluoride (Pefabloc), 1mM NaF, 5µg/ml aprotinin, 4mM benzamidine) (all Sigma-Aldrich), the PMVs were mixed gently and incubated on ice for 10 min. After low-speed centrifugation (250*g*; 5 min), the supernatant was respun (25,000*g*; 25 min), and the supernatant was aliquoted and stored (-80°C). PMV lysates were centrifuged (25,000*g* for 30 min), and supernatants were used freshly. Before running samples they were diluted 2:1 in sample buffer (3 ml glyereol; 1.5 ml 20% (w/v) SDS; 3 ml 2-Mercaptoethanol; 1% (w/v) bromophenol blue diluted in 0.5M Tris base, pH6.8), then boiled for 5 min to denature the proteins.

2.11.1 SDS-PAGE

Having assembled the glass plates according to the manufacturer's instructions (Bio-Rad, Mini Protean II) acrylamide solution was prepared to generate a 10% gel (10 ml acylamide/bisacrylamide [30 %/0.8 %]; 7.5 ml Tris-HCl 1.5 M, pH 8.7; 12.08 ml distilled water; 0.3 ml 10% SDS). After adding APS (0.1ml of a 10 % solution)

and TEMED (N, N, N', N'-tetramethylethylenediamine) (0.02ml), the mixture was swirled vigorously and poured between the plates. This was carefully overlaid with isobutanol to prevent oxygen inhibition of polymerization. Upon polymerization, removal of isobutanol and washing, the stacking gel is prepared (1.3ml of acrylamide/bisacrylamide [30 %/0.8 %); 2.5ml Tris-HCl, 0.5 M, pH 6.8); 6.07ml distilled water; 0.1ml 10% SDS; 0.02ml 10% APS and 0.01ml TEMED. This mixture was poured onto the polymerised stacking gel together with the comb and left to polymerise. After removal of the comb, running buffer was added into the electrophoresis tank, bubbles removed from the gel and the samples (1-10μg) loaded using a long narrow tip. The gel was run with a constant voltage (up to 15V/cm) until the bromophenol blue had reached the bottom of the gel.

2.11.2 Western blotting

Having separated proteins by SDS-PAGE, a PVDF membrane was soaked in absolute ethanol for 1 min. In a tray of transfer buffer (TB) the following were laid: SDS-PAGE gel; 2 porous pads; 2 pieces of thick filter paper; PVDF membrane cut to the size of the gel. After 10 min, onto the cathode electrode, soaked with TB were placed the porous pad, filter paper, gel, PVDF membrane, second filter paper, second porous pad and finally the anode electrode. The sandwich was placed in the transfer tank and run for 1h at RT at 0.65 mA/cm^2. The membrane was then blocked in Tris-buffered saline with 3% non-fat milk for 2h at RT. The primary antibody (10 μg/ml or 1:1000 for anti-P-Smad 2 [Cell Signalling Technology]) was added for 1h at RT, the membrane washed with TBS-0.1% Tween20 (6x10 min washes). The secondary HRP antibody (anti-rabbit) was then added (1:5000 dilution) for 1h at RT. After washing detection was by chemiluminescence using the ECL kit (Amersham) using a UVP ChemDoc-It system (UVP systems, UK). Positive expression was

semi-quantified using ImageJ software to calculate the mean intensity of the antigen.

2.12 Transmission Electron Microscopy and Negative Staining

HL-60 cells ($5x10^6$/ml) either stimulated (5% NHS) or not (control) were fixed in 0.1M fixative solution (3% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.2) and incubated at RT for 1 h. Samples were post-fixed by incubation at 0°C for 1 h in 1% osmium tetroxide solution (1:1 mixture of 2% osmium tetroxide (Sigma-Aldrich) and 0.2M sodium cacodylate buffer), and block stained in 1% aqueous uranyl acetate. Samples were resuspended in 1% hot agarose and dehydrated in an ascending ethanol series, (from 70% to 100%, absolute ethanol, v/v, Sigma-Aldrich) and washed twice with propylene oxide (Agar Scientific, Essex, UK). Sample was infiltrated with a 1:1 mixture of propylene oxide: Agar resins (mixture of 4.8g agar resin, 3.6g methyl nadic anhydride, 1.9g dodecenyl succinic anhydride and 0.2g benzyl dimethylamine, Agar Scientific, Essex, UK) and left rocking overnight at RT. Infiltrated samples were embedded in capsules using applicators and polymerised at 60°C for 24h. Ultra thin sections were cut on a Leica Ultracut R ultra microtome and stained in Reynolds Lead Citrate. The sections were examined on a Jeol JEM – 1200 Ex II Electron Microscope. For negative staining, microvesicles isolated without prior sonication were stained with 2% aqueous uranyl acetate or 2% phosphotungstic acid pH 6.8 plus aqueous Bacitracin. Samples were placed on 400 mesh copper grids with a pioloform support film (Grids and Pioloform powder from Agar Scientific), and pre-treated with 1% aqueous Alcian Blue 8GX for 10min. Digital images were recorded using the AMT digital camera previously described for the examination of stained ultra thin sections.

2.13 Volunteer selection and sample processing

Blood samples were collected from twenty four healthy volunteers of Caucasian decent (eleven males, thirteen females). Sixteen of the volunteers were between the ages of 18 to 25, four were between 25 and 40 and four between 50 and 75years. The samples were taken with informed consent under Institutional Review Board approval from the North West London NHS Trust – Northwick Park Hospital. To minimize the effect of daily variations within the group, the samples from a given subject were taken at approximately the same time, within a thirty-minute window, in the morning for each one of them. Other variables were not controlled for in order to better mimic the variability in typical human plasma samples (age, fasting, illness, medication, etc.). To better examine the longitudinal variation and minimize the chance of an individual providing samples while experiencing an underlying condition such as a cold, volunteers' were given physical examinations and asked to fill out confidential disclosure forms. Each individual was assigned an identification number to blind the samples and ease the experimental design. After venipuncture, plasma was obtained by centrifugation for 10 min at 2000g at RT and PMVs analysis carried out as described earlier (2.7).

2.14 Growth Inhibition Assays

HL-60 cells in the exponential phase were washed twice with RPMI 1640 and resuspended in growth medium containing 5% FBS. Cells were seeded into 12-well plates at $1x10^5$ cells/well in triplicate. Different concentrations of PMVs, or a range of differentiation/ proliferation inhibiting agents (PMA, ATRA, histamine) were added to each well (except controls) and plates were incubated at 37°C for three days. On the days indicated, nonadherent cells were removed and counted by ViaCount assay on a Guava EasyCyte flow cytometer. In some experiments, HL-60 cells were

incubated with 30µg PMVs alone or in the presence of the TGF-β receptor antagonist, SB-431542 (10µM) or with 25µg/ml affinity purified, neutralising rabbit anti-TGF-β1 (Genway).

2.15 Differentiation Assays

Adherence of cells was determined following treatment with 30µg of PMVs. On the day of the experiment, $1x10^5$ cells/well were seeded into 12-well culture plates in triplicate. On each of the indicated days, the non-adherent cells were transferred into new plates and incubated at 37°C in a 5% CO_2 incubator. The wells were then washed twice with serum-free RPMI 1640 and the attached cells released with trypsin/EDTA (Sigma-Aldrich). The trypsinised cells, now in suspension were collected by centrifugation (200*g*, 5min), stained with ViaCount reagent and counted by flow cytometry. To investigate differentiation, HL-60 cells were seeded into 12-well plates in triplicate. Cells were left untreated (control) or treated either with 30µg PMVs or with PMA (0.1µM) and incubated at 37°C in a humidified atmosphere of 5% CO_2 for three days. After this time, cells, still in suspension were removed and the plates washed three times with RPMI 1640. Cells attached to the bottom of the plates were immuno-labelled at 4°C for 1h with the antibodies, anti-CD14-FITC, anti-CD11b-PE and anti-DC-SIGN-FITC with IgG-FITC and IgG-PE isotypes being used as controls. After three washes with PBS, cells were placed in PBS, 1% BSA and relative fluorescence determined using the FLUOstar Omega microplate reader (BMG Labtech, UK).

2.16 Measurement of intracellular calcium

These measurements were made before and after addition of histamine, ATRA and PMA with or without 25µM of the calcium chelator BAPTA-AM (Sigma). After 30min pre-treatment with the agents in phenol red-free RPMI 1640 (Gibco/Invitrogen), cells

($1x10^6$/ml) were resuspended in physiological salt solution (PSS) containing 130mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM HEPES and 1% BSA. Essentially the cells were loaded with 2μM fura-2-AM (Sigma, made up in aceone) with continual stirring and fluorescence monitored on a spectrofluorimeter at 505nm upon excitation at 340 and 380nm every second. Intracellular calcium was calculated using the equation: [Ca2+]i =Kd[R-Rmin)/Rmax-R)] where R is the ratio of the emission intensities measured at 505 nm on excitation at 340 and 380nm. Rmin was obtained at zero [Ca2+]I (by addition of 10mM EGTA) and Rmax was obtained by lysing the cells with 0.1% Triton-X-100 thus allowing Fura-2 measurement in the maximum external calcium concentration of 1mM. The temperature of the experiment was 37±1°C and Kd for Fura-2 is 224nM.

2.17 Nitroblue Tetrazolium (NBT) assay

Nitroblue tetrazolium (NBT) (Sigma Aldrich) was used to determine intracellular O_2^- as a measure of differentiation. For this assay, cells seeded at ~$5x10^5$ cells/ml in 24-well plates were treated for 1h at 37°C with 1mg/ml NBT and 4μg/ml PMA. After washing in PBS the cell pellet was dissolved in 100μl DMSO and the absorbance read at 570nm on a spectrophotometer.

2.18 Quantitation of TGF-β1 by ELISA

PMVs were isolated from HL-60 cells stimulated with 10% NHS at 37°C for 30 min. The concentration of TGF-β1 was measured by using 30μg of protein in ELISA kits (R&D Systems) according to the manufacturer's instructions. In some experiments, PMVs ($1x10^5$/ reaction), in triplicate, from HL-60, THP-1, Jurkat and MCF-7 cells were lysed before measuring TGF-β1 levels.

2.19 Cell cycle analysis

HL-60 cells ($1x10^6$) in cold PBS were fixed in cold 70% ethanol. After washing in PBS, cells were stained with propidium iodide (50µg/ml) for 1h at 4°C and analysed using a FACSCalibur flow cytometer (BD Bioscience San Jose, CA, USA) according to the manufacturer's instructions. The percentage of the population in G0/G1, S and G2/M phases of the cell cycle was determined using CellQuest software (BD Bioscience) and represented in GraphPad Prism 5.0 (San Diego CA, USA).

2.20 Fluorescence microscopy of PMV-cell interaction using R18-(octadecyl rhodamine-) labelled PMVs

HL-60 derived PMVs were labelled with octadecyl Rhodamine chloride (R18) by treating PMVs with 1.37mM of an ethanolic solution of R18 (containing a final concentration ≤ 1% v/v) for 1 h at RT in the dark. R18-labelled PMVs were separated from unincorporated R18 by ultracentrifugation at 100,000*g* for 1h followed by dialysis in 14 kDa dialysis tubes at 4°C in HEPES/NaCl buffer. The ratio of R18 to PMVs was estimated measuring the fluorescence before and after removal of the unincorporated probe. HL-60 cells were incubated with R18-labelled PMVs for 10 min at 37°C, followed by three washes in PBS. The cells were fixed in 4% paraformaldehyde in PBS for 15 min. The cells were then washed five times and mounted on slides with Vectashild mountain medium (Vector Laboratories). For fluorescence microscopy, an Olympus IX81 inverted microscope, equipped with a monochromatic camera U-CMAD3, was used. Images were subsequently coloured using the Cell^M imaging software (Olympus).

2.21 Monocyte-to-macrophage differentiation

Before inducing differentiation, THP1 cells were first washed as mentioned in section 2.4 and then analysed by FACS in order to find out the cell count and

viability. Cells were only used after demonstrating a viability of over 94%. 1 x 10^5 cells (in complete growth medium) were then added per well in a 24 well plate adjusting the final volume to 500µl per well using complete growth medium. 1µM of PMA was then added to each well to induce differentiation of THP1 cells to macrophages (after Preliminary experiments 0.1µM of PMA having been established as representing an optimal concentration for THP1 differentiation). The 24 well plates were then incubated in a 37°C humidified 5 % CO_2 incubator for three days for the differentiation to take place. Macrophages were then washed 3x in PBS to remove PMA. Differentiation was confirmed by expression of surface markers CD11b/CD14 and the ability to generate reactive oxygen species (ROS) via NADP oxidase using the nitro blue tetrazolium (NBT) (Sigma-Aldrich) assay (section 2.16, above).

2.22 FITC labelling Jurkat cells and induction of apoptosis

FITC dye (prepared as per the manufacturer's protocol [Sigma-Aldrich]) was added (1µg/10^6 cells) to the cell suspension and incubated on a shaker at room temperature for 90 minutes in the dark. After incubation, the cells were washed three times with PBS at 400*g* for 5 minutes and re-suspended in RPMI ready for use. A small sample of the labelled cells was then tested for FITC staining by FACS analysis. The cells were only used after obtaining fluorescence greater than 70%. 15µM of PMA was then added to a 1ml cell suspension containing 7 x 10^6 FITC-labelled Jurkat cells in RPMI, which was incubated at 37°C for 1 hour in the dark. After three washes with PBS and resuspension in 2ml PBS, flow cytometry (Guava Nexin® assay) was carried out to confirm apoptosis.

2.23 Flow cytometric analysis of apoptosis

To see whether PMV-releasing HL-60 cells were undergoing apoptosis, cells were stained with annexin-V (AnV) and 7-amino-actinomycin D (7-AAD) (Guava Nexin® Reagent). Annexin V and 7-AAD positive cells were then quantified using a Guava EasyCyte flow cytometer, over the course of 1h. To see if adding PMVs to cells rendered them apoptotic, these measurements were made 24, 48 and 72h after addition of PMVs. This assay was also used to detect and measure percentage apoptosis of PMA-inducedJurkat cells.

2.24 Opsonisation of Apoptotic cells with NHS

7 x 10^6 of FITC labelled apoptotic cells (ACs) were suspended in 2ml of PBS. 20 % of NHS was then added to the cell suspension for opsonisation. The tube was then covered with foil and incubated at 37^0C in a water bath for 30minutes. After incubation the cells were washed three times with PBS at 400*g* for 5 minutes and then re–suspended in the required volume of RPMI.

2.25 Phagocytosis assay

FITC-labelled ACs (7 x 10^6 in 2ml PBS) were opsonised with 20% NHS for 30min at 37°C and then washed three times in PBS. The 24-well plates containing macrophages (1x10^5) were kept on ice during addition of the various combinations of ACs and PMVs and then incubated at 37°C for 18h in a CO_2 incubator. After incubation, the plates were placed on ice for 2min and coverslips with adherent macrophages washed three times to remove non-phagocytosed cells. Hoescht dye (0.2µg/ml final concentration) was added to wells designated for fluorescence microscopy and 5mM EDTA added to detach macrophages where they were needed for flow cytometry using the Guava EasyCyte flow cytometer (Guava Express Plus). The plate was then incubated at 37ºC for a further 30 minutes. After

washing to remove unbound excess dye, 100µl of methanol was added for 5 min at room temperature to fix the cells for microscopy. The wells were then washed once with PBS to remove excess methanol and then refilled with 250µl of PBS for microscopy.

For determining phagocytosis by microscopy, 500 macrophages were counted per well, noting the number of macrophages that had internalised at least one AC. The average was then taken and a phagocytic index calculated (expressed as a mean percentage of macrophages that had phagocytosed at least one AC). The fluorescence microscope was equipped with a UCMAD3 monochromatic camera, and images taken for fluorescence were coloured with the Cell^M software (Olympus).

2.26 Spin-column procedure for IgG antibody purification from plasma

This was carried out as described in the manufacturer's instructions. Briefly, after equilibrating the Gel IgG Purification Support in Purification Buffer (PB), the Purification Support slurry was washed with PB in a spin column twice. With the bottom cap in place, 100 - 500µl serum was added with mixing and the purified antibody collected by centrifugation.

2.27 IgG removal from plasma

A spin column containing 170µl of a slurry of immobilized Cibacron Blue/Protein A gel was prepared. The SLE plasma was diluted to 75µl with Binding /Wash Buffer, added to the column with vortexing and left on a mixer for 10 min. Filtrate was then collected from the column by removing the bottom plug and then 75µl Binding/Wash buffer collected in the same tube by centrifuging at 10,000 *g* for 1 min. The sample obtained was now free of albumin and IgG.

CHAPTER 3

CHARACTERISATION OF PLASMA MEMBRANE-DERIVED VESICLES RELEASED FROM THE HUMAN PROMYELOCYTIC LEUKAEMIA CELL LINE, HL-60

3.1 INTRODUCTION

The importance of PMVs in physiological processes such as inflammation, haemostasis, angiogenesis, blood coagulation etc cannot be overemphasized and as such in recent years there has been a great surge of scientific interest in trying to ascertain its main functions. However, researchers often state that interpreting results is difficult due to a lack of proper characterisation of PMVs as a result of less or no standardization in the pre and post analytical context (Jy W *et al* 2004; Shah MD *et al* 2008). There is currently a large amount of variation between investigators with regard to the pre-analytical steps employed, with key differences being centrifugation and sample storage conditions, which often leads to result variability. Also, a strict definition of PMVs has not yet been fully established (Burnier L *et al* 2009).

Since 2003, an international consortium of scientists has been trying to address this issue and some progress appears to have been made in the form of forums detailing various methods and attempts to establish a core laboratory in charge of sample distribution. This has coupled with a recent increase in publication of studies focusing on the measurement of PMVs and other subtypes. But still, proper standardization of preparation and detection methods as well as characterisation is yet to be achieved. As already stated, PMVs, are extracellular vesicles released from the plasma membrane of blood cells following a rise in cytosolic Ca^{2+} levels, causing membrane protein aggregation resulting from the action of transaminases and cytoskeletal changes due to the breakdown of major cytoskeletal proteins (Anderson LA *et al* 1977; Salzer U *et al* 2002). As well as proteolysis, phospholipid redistribution also occurs, with phosphatidylserine (PS) being externalised (Bucki R *et al* 1998).

It has been suggested that PS exposure on PMVs is a signal for their removal by the reticuloendothelial system as it was found that almost 50% of PMVs were rapidly removed from the circulation by liver Kupffer cells in a PS-dependant manner in rat models (Bosman GJ *et al* 2005; Willekens FL *et al* 2005). PS exposition in the outer membrane leaflet could also explain the role of PMVs in events such as phagocytosis and thrombosis. PMV release has also been associated with cell ageing (Rumsby MG *et al* 1977), numerous disease states (Knowles DW *et al* 1997) and *in vitro* mechanisms such as spectrin oxidation (Wagner GM *et al* 1987) and Adenosine Triphosphate (ATP) depletion (Lutz HU *et al* 1977).

The purpose of this work is to be able to properly analyse, categorise and characterise PMVs to provide credence to PMV research, and to draw attention to the challenge of standardization whilst highlighting some characteristics of PMVs that have not yet been considered despite the increased presence of PMVs in a number of pathological states, such as sickle cell disease and in atherosclerotic plaques (Van Beers EJ *et al* 2009). This work also aims to provide evidence that will highlight why standardization is seen by many researchers as an issue of utmost importance (Dignat-George F *et al* 2009) and which characteristics clearly define PMVs from other vesicular bodies.

This will be done by inducing HL60 promonocytes to produce PMVs which will be analysed using methods such as flow cytometry and transmission electron microscopy (TEM) to determine the presence of PS, lipid raft content sizing by comparison to fluorescent calibration beads, as well as quantifying levels in some healthy volunteers. Some factors contributing to result variability and the technical hurdles that need to be overcome in order to establish standardized reagents and

assays for PMVs will also be looked at. A critical approach will be adopted, with possible methodological pitfalls being highlighted and discussed where necessary.

Hopefully, in the coming years, as more work is done on PMVs and as this exciting field continues to grow, investigators will adopt a more collaborative effort. In essence, there has to be more inter-laboratory cooperation to establish a reliable and reproducible standard for preparing samples and quantifying PMVs (Ardoi SP *et al* 2007; Enjeti AK *et al* 2007) since investigators of PMVs at the moment continue to adopt a wide range of methods to isolate and quantify PMVs.

3.1.1 Previous research on PMVs characterisation

Some work on the release of PMVs that made a significant impact was carried out between 1976 and 1980 (Allan D *et al* 1976; Allan D *et al* 1977; Allan D and Thomas P 1980; Lutz HU *et al* 1977). These studies focused on PMV production in storage and found that when the intracellular Ca^{2+} concentration was increased by the addition of calcium ionophore A23187 or when the red cells were ATP deficient, PMVs budded from the cell membrane. The PMVs were depleted in the internal membrane lipid phosphatidylcholine and cytoskeletal proteins spectrin and actin. Glycophorin, band 3 and acetylcholinesterase levels though were found to be the same as the intact membranes (Cole WF *et al* 1978; Cole WF *et al* 1979; Rumsby MG *et al* 1977).

In the last few years numerous quantitative studies published on PMVs in both healthy and diseased subjects has laid the groundwork for PMV testing to enter the mainstream of clinical testing, which some investigators believe could occur in the not too distant future (Horstman LL *et al* 2004). Currently however, a normal physiological range for PMVs is yet to be established and it is believed this work

amongst others being carried out at the Cellular and Molecular Immunology Research Centre (CMIRC) will go a long way to solving this problem.

3.1.2 Standardisation of PMVs isolation and characterisation

Recently, some leading investigators in the field of PMVs work established a working group on vascular biology with an aim of investigating and understanding in more detail, the detection, function, measurement and clinical significance of PMVs. Standardization of PMVs was highlighted as a high priority topic, setting a benchmark for the future work on PMVs. A consensus was reached amongst the participating members that a network should be established, aimed at standardizing PMV detection, partly in response to the increase in publication but primarily because of their potential as a pathogenic marker. A definition for PMVs was later established which has been adopted in a recent review (Enjeti AK *et al* 2007). Defining PMVs though is challenging due to their diversity and is open to debate, with different definitions sometimes being adopted by different groups (Shet AS *et al* 2003; Shah MD *et al* 2008). The use of calibrated microbeads of known sizes has strongly been recommended.

3.1.3 Analytical variables

PMVs are mostly defined in terms of annexin V binding to externalized PS (Ahn YS *et al* 2004; Mallat Z *et al* 2000; Shah MD *et al* 2008). However, Ahn et al (Ahn YS *et al* 2004) have postulated that not all PMVs are positive for annexin V binding which means that some investigators could therefore be underestimating the total number of PMVs. The majority of PMVs do however, express PS (Sweeney J *et al* 2009), with one study finding that 87% of PMVs bound annexin V.

The problem of counting a fraction of PMVs was raised by a working group on vascular biology at a recent meeting where questions were asked as to whether focusing on the top of the PMV iceberg was representative of the clinically significant markers being sought. There have also been concerns with measuring PMV numbers by acetyl cholinesterase activity, which has been done by one group (Frenkel EJ *et al* 1986), but a 2004 review stated that there is currently not enough evidence to suggest that all PMVs have this characteristic (Horstman LL *et al* 2004).

3.1.4 PMVs analysis by flow cytometry

Flow cytometry is the most commonly used technique to detect PMVs. Some investigators identify PMVs with a forward angle light scatter smaller than the 1-1.5 μm latex beads used as an internal standard (Shet AS *et al* 2003). Other investigators also employ a lower size limit of 100 nm. However, any particles detected by the flow cytometer under this size are not considered true PMVs (Boulanger CM *et al* 2006). Using the upper size limit criteria could also lead to an underestimation of true PMV numbers as some investigators consider PMVs to be up to 2 μm in size (Piccin A *et al* 2007).

At the moment, different flow cytometers are used to analyse PMVs and therefore it may be possible that the flow technology and instrument settings as well as interpretation of results could impact on PMV measurement. For example, laser alignments can differ and different instruments capture PMVs in different ways. Bench top flow cytometers such as FACSCalibur have a fixed laser in constant alignment needing no human adjustment whereas FACSVantage SE uses removable lasers, which need manual adjustment (Becton, Dickinson and Company, 2002).

3.1.5 PMVs counts in plasma

Although some studies have shown contrasting PMVs counts based on the mode of analysis, some are comparable with others. A recent study detected 169 (61-308) Red cell PMVs (RPMVs/µl plasma) (Willekens FL *et al* 2008) which is similar to other studies that reported counts of 28 (13-46) and 176 (87-222) RPMVs/µl of plasma respectively (Berckmans RJ *et al* 2001; Hron G *et al* 2007). Surprisingly, these comparable results were obtained despite different centrifugation methods being adopted. It is difficult if not impossible to determine why these counts can be similar despite the differences in sample preparation.

These findings raise the possibility that the way in which samples are prepared may not necessaryly contribute to result variability as much as first thought, there is however substantial evidence suggesting that sample preparation does indeed affect counts (Dignat-George F *et al 2009*; Shah MD *et al* 2008; Simak J and Gelderman P 2006). Recently, a team looked at standardizing PMV counting on 3 flow cytometers (Cytomics FC500) in different laboratories using 0.5 and 0.9 µm fluorescent beads as standards and found that long term reproducible counts can be achieved between instruments (CV <12%). This study also highlighted the heterogeneity of methods employed by investigators using flow cytometry (Robert S *et al* 2008).

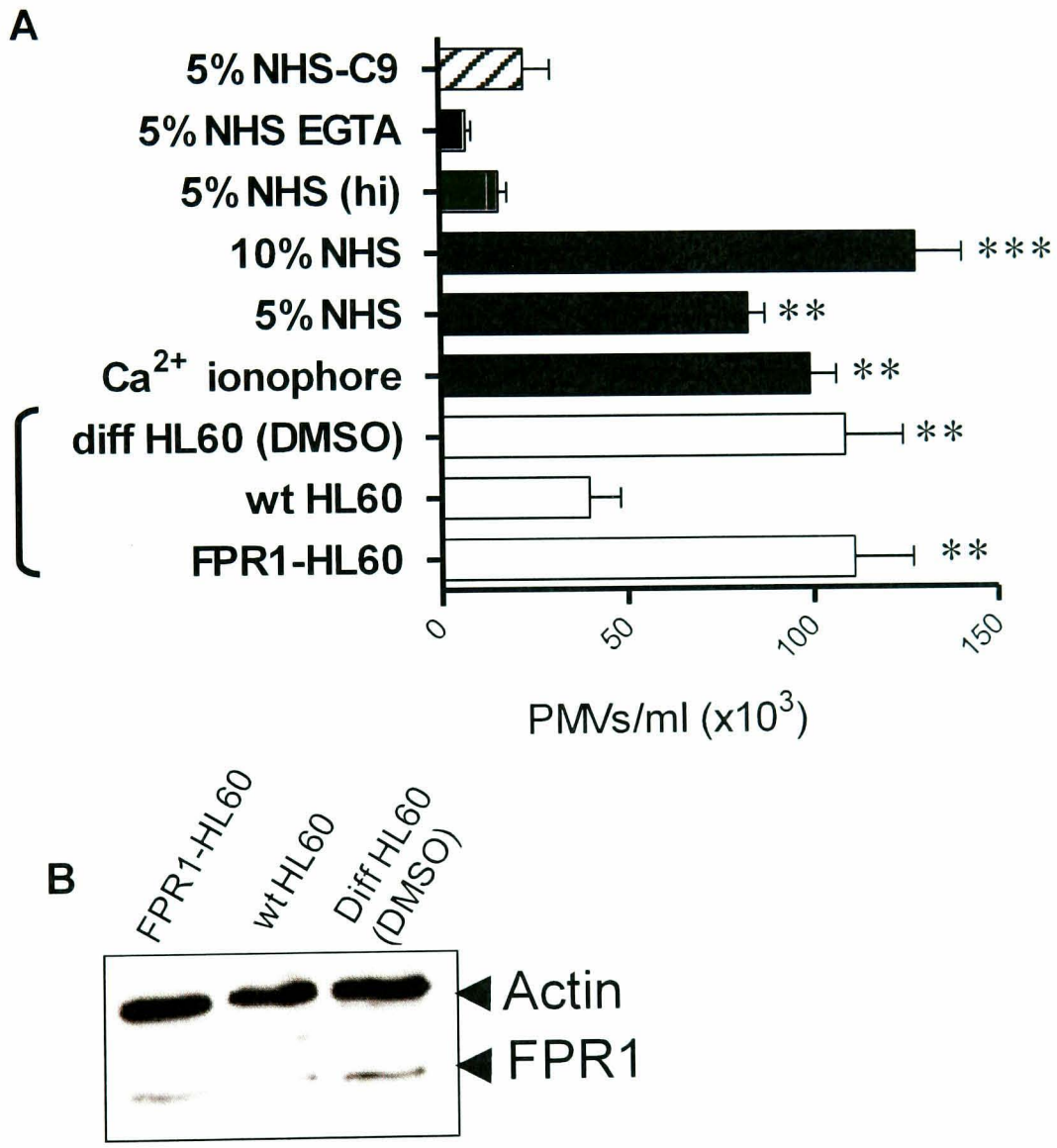
3.2 PMV release and isolation

3.2.1 Plasma Membrane-derived Vesicles are released by the action of sublytic complement on HL60 cells and upon treatment with fMLP or calcium ionophore

Plasma Membrane-derived Vesicles (PMVs) have been used in a host of studies but the stimuli used to release them from parent cells have varied throughout. In the work presented, sublytic complement, in the form of 5% NHS at 37°C for 30 min was initially used. As shown in Fig 6A, there was a dose response with increasing NHS, 5% NHS releasing approximately $9x10^4$ PMVs/ml and 10%, just over $1x10^5$ PMVs/ml. To indicate the involvement of sublytic complement (membrane attack complex) deposition (and increased $[Ca^{2+}]_i$) heat-inactivated NHS (56°C for 20 min) and C9-depleted NHS were found to abrogate PMV release. The need for raised $[Ca^{2+}]_i$ was then shown by blocking PMV release after calcium chelation using EGTA (Fig. 6A). Two agents which increased $[Ca^{2+}]_i$ were also used, calcium ionophore and fMLP. As the fMLP receptor (FPR1) is absent in wild type (wt) HL60 cells (Fig. 6B), they were differentiated with DMSO toward the neutrophil lineage which does express FPR1 (Fig. 6B). It was then found that differentiated HL60 cells and FPR1 transfectants (kind gift from S. Lange) did respond to fMLP to release PMVs unlike the wild type control (Fig. 6A).

Fig. 6

PMVs are released from HL-60 cells by Stimulation with sublytic complement or fMLP or LPS



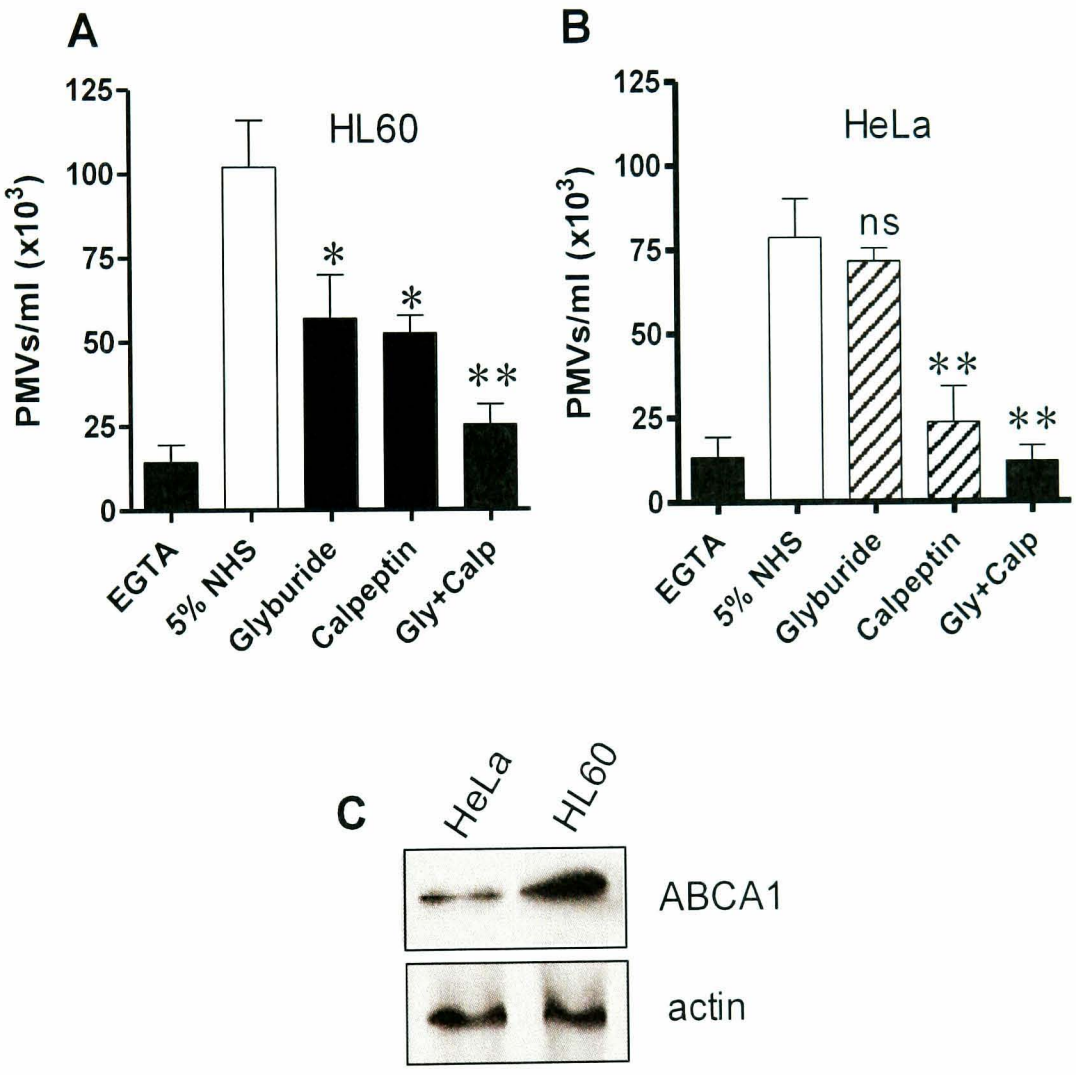
HL60 cells ($1x10^5$), in a 12-well plate in triplicate were treated with bacterial products fMLP (10 μM in the presence of 2 mM $CaCl_2$ at 37°C for 1h) and calcium ionophore (15 μg/ml in the presence of 2 mM $CaCl_2$ at 37°C for 18h). Cells treated with fMLP were either wild type HL60, DMSO-differentiated HL60 or fMLP receptor- (FPR1-) transfected HL60 (the latter two therefore only expressing FPR1) as shown in an immunolbot, B. Treatments with NHS were for 30 min at 37°C in the presence of 2 mM CaCl2. Heat-inactivated NHS (HI) was prepared by heating to 56°C for 20 min. Complement C9 was depleted from NHS by use of a polyclonal anti-C9 antibody. Treatment of NHS with 5mM EGTA was also used to abrogate PMV release.

3.2.2 ABCA1 transporter plays a role in PMV formation in HL60 cells but not HeLa cells

It has been reported, principally in the studies of George E. Grau (Combes V *et al* 2005) that ABCA1 knockout mice have reduced levels of plasma PMVs. We wanted to see whether inhibition of ABCA1 would reduce microvesiculation (PMV release) from HL60 cells. Glyburide is a specific inhibitor of ABCA1 and preincubation of HL60 cells reduced microvesiculation by 50%. Separate treatment of HL60 cells with calpeptin which inhibits calpain-mediated cleavage of the cytoskeleton reduced microvesiculation by similar levels and there was a synergistic effect when the two were combined (Fig. 7A). Interestingly HeLa cells which express very low levels of ABCA1 protein (Remaley, *et al* 2001) and which we confirmed by immunoblotting (Fig. 7C) were not susceptible to glyburide inhibition of PMV release, unlike calpeptin treatment.

Fig. 7

PMV release from HL60 cells can be Abrogated by inhibition of the ABCA1 Transporter and by inhibition of calpain



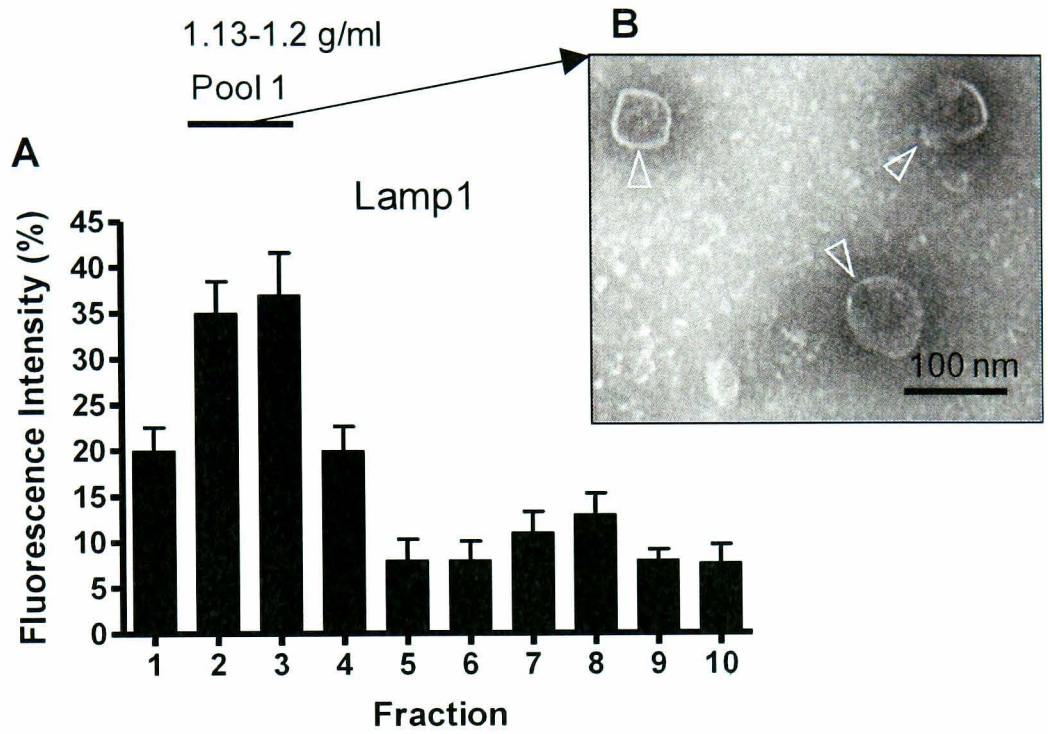
(A) HL60 cells ($1x10^5$), in a 12-well plate in triplicate were treated with sublytic complement. Partial inhibition of PMV release occurred after treatment with XuM glyburide and XuM calpeptin. Complete inhibition comparable to that achieved by Ca^{2+} chelation with EGTA was achieved upon treating with glyburide plus calpeptin (Gly+Calp). When HeLa cells were similaly treated (B) glyburide had no effect on PMV release levels but calpeptin did. An immunoblot of a HeLa cell lysate and an HL60 cell lysate shows high expression of the ~220 kDa ABCA1 transporter on HL60 cells but lower expression on HeLa cells, with actin levels used as a control.

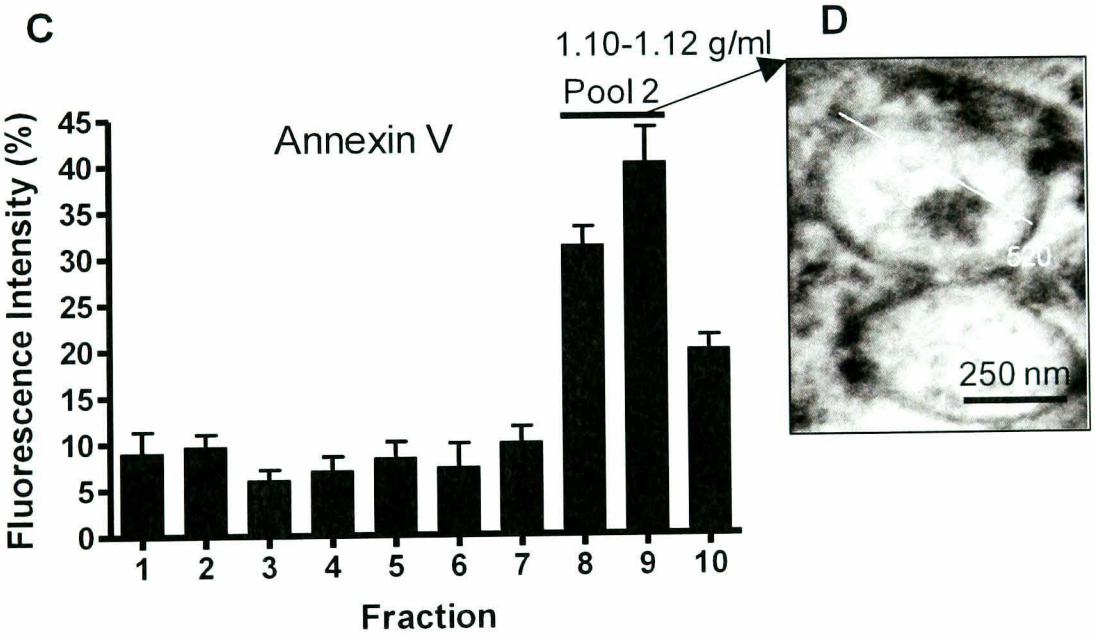
3.2.3 Isolation of Plasma Membrane-derived Vesicles and exosomes by sucrose density gradient centrifugation

Various methods have been used in the isolation of PMVs which sometimes co-purify exosomes as well as other vesicles. In order to differentiate PMVs from other vesicular bodies (exosomes), we employed a sucrose density gradient centrifugation method which allowed us to isolate the vesicles according to size and by staining them with their respective surface markers to identify each vesicle. As shown by surface markers (Lamp 1 as representative of exosomes and Annexin V binding to PS, as representative of PMVs) exosomes were isolated in the lower, more dense fractions (Fig. 8A), whilst PMVs were isolated from the higher, least dense fractions (Fig. 8C). This protocol clearly highlights the density difference between PMVs and exosomes, the exosomes having pooled into fractions ranging in sucrose density from 1.13-1.2 g/ml (Fig. 8A) and the PMVs in fractions of 1.10 - 1.12 g/ml (Fig. 8C). This protocol also highlights the size difference, the population of exosomes typically ranging in size from 50 – 100 nm in diameter, as seen by transmission electron microscopy (Fig. 8B) the PMVs averaging at 500 nm in diameter (Fig. 8D). Having used sucrose gradients to clearly define the two populations of microvesicles, it was then possible to devise a novel method to obtain as pure as possible populations of either exosomes or PMVs.

Fig. 8

Isolation of PMVs and PMVs using a sucrose density gradient centrifugation





Sucrose gradients were prepared as described in materials and methods to give a range of densities from 1.02-125 g/ml and released HL-60 microvesicles, a mixture of PMVs and exosomes (0.2 ml) was layered on top of the gradient which was then centrifuged for 1h at 200,000 *g*. Fractions (10x1ml) were collected from the top and then 200 μl aliquots removed for determination of density and for analysis by flow cytometry for markers of exosomes (Lamp 1) (A) and shown by TEM (B). PMVs were detected (C) as annexin V-positive and confirmed by TEM (D).

3.2.4 A novel protocol to separate exosomes and PMVs involving sonication and filtration

As the most common isolation protocol involves differential centrifugation, PMV isolates are usually contaminated with exosomes and vice versa. Some of the confusion in the literature as to whether plasma membrane-derived vesicles or exosomes have been described is due to isolation of contaminating microvesicle populations. To avoid any confusion in this study, a novel protocol was devised which is summarised in Fig. 9A.

To start with fairly pure populations of PMVs or exosomes, pools 1 and 2 from a sucrose density gradient prepared as described before were used. As it was known that exosomes tend to clump together to make clusters that may reach the average diameters of PMVs, sonications (5x1 min) in a sonicating water bath were carried out on half of the pooled fractions before filtering through a 0.22μm pore size filter. By way of control, unsonicated samples were passed directly through a similar filter. The rationale for this protocol was that breaking up exosome clumps would overcome exosome contamination of PMV preparations. In this way PMVs, which are largely greater than 220nm in diameter would remain on the filter and the smaller exosomes pass through the filter collecting in the filtrate. PMVs caught on the filter would then be recovered by washing off from the filter. Complement Receptor 1 (CR1) was used as a marker for microvesicles, whether PMVs or exosomes and this way it was possible to monitor their isolation. As shown in Fig. 9B, both vesicles were identified by CR1 fluorescence in pool 1 (93%) & 2 (88%). CR1 fluorescence was again taken after filtration and as can be seen in Fig. 9B there was a big drop in fluorescence in pool 2 filtrate (24%) as compare to pool 1 filtrate (93%) indicating that pool 1 probably had more vesicles that were capable of

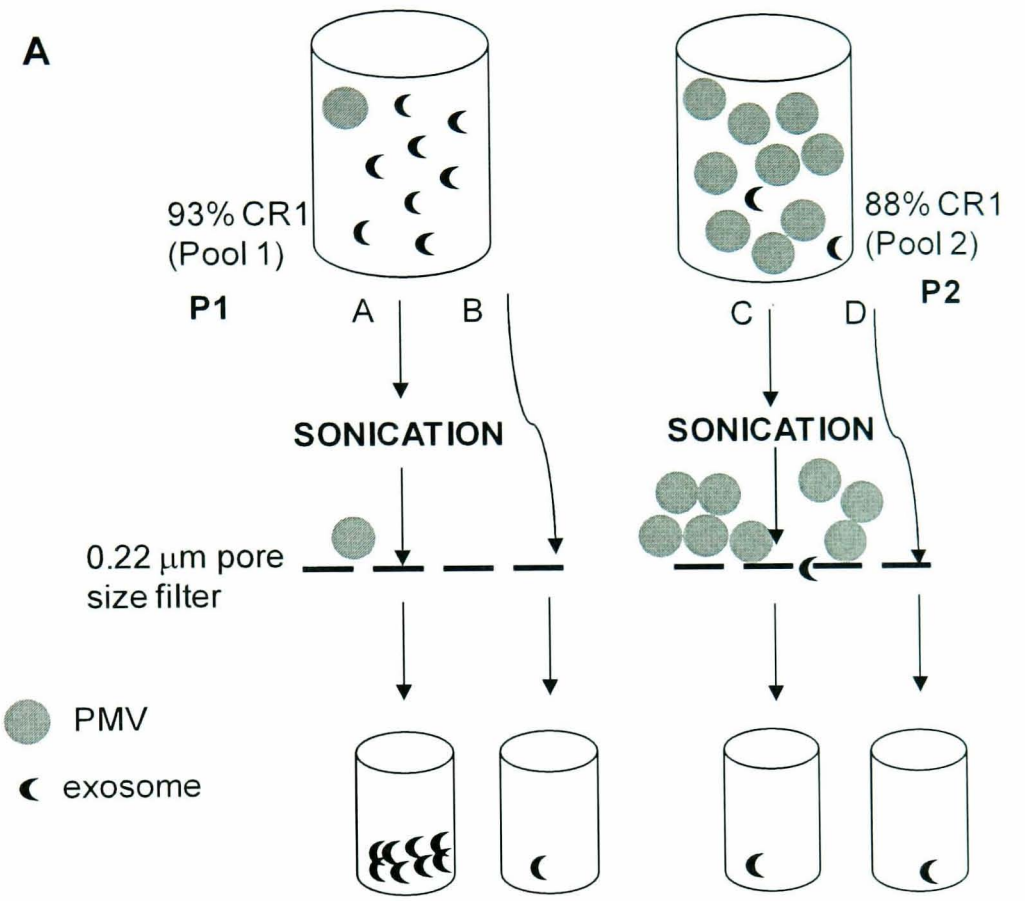
passing through the pore and which are likely to be exosomes. This still emphasize the importance of the size difference between the two vesicles.

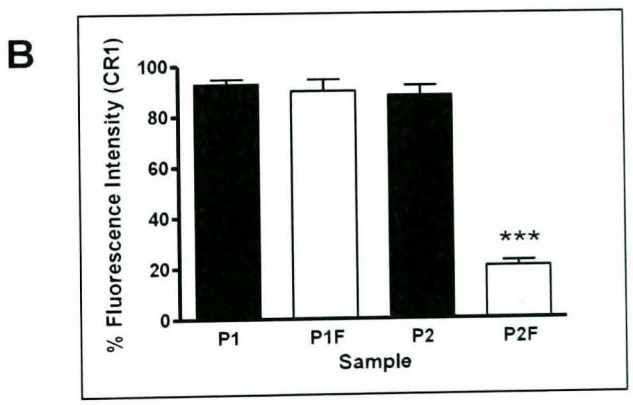
3.2.5 Comparison of the protein profile of PMVs isolated by sonication and filtration with PMVs and exosomes isolated by differential centrifugation

PMVs are known to carry surface or membrane proteins of their parent cell, which are characteristic of the parental cell from which they are derived. These properties aid the identification of PMVs from a mixed population, and also help predict their likely physiological roles. It was therefore important to see whether the method of isolation has any impact on the protein profile of PMVs. A fairly crude comparison was carried out using SDS-PAGE and silver staining and the results shown in Fig. 10. PMVs isolated using the two different methods employed in this thesis showed similar protein profiles indicating that as long as the PMVs come from the same cellular source, the method of isolation, sonication followed by filtration or differential centrifugation, does not affect the protein content of the PMVs. The exosome profile was however different from that of the PMVs, as their constituents are different, the vesicles being quite different in nature and shed through a different mechanism.

Fig. 9

Isolation of microvesicles (PMVs + exosomes) and flow chart illustrating novel method for separation by sonication and filtration

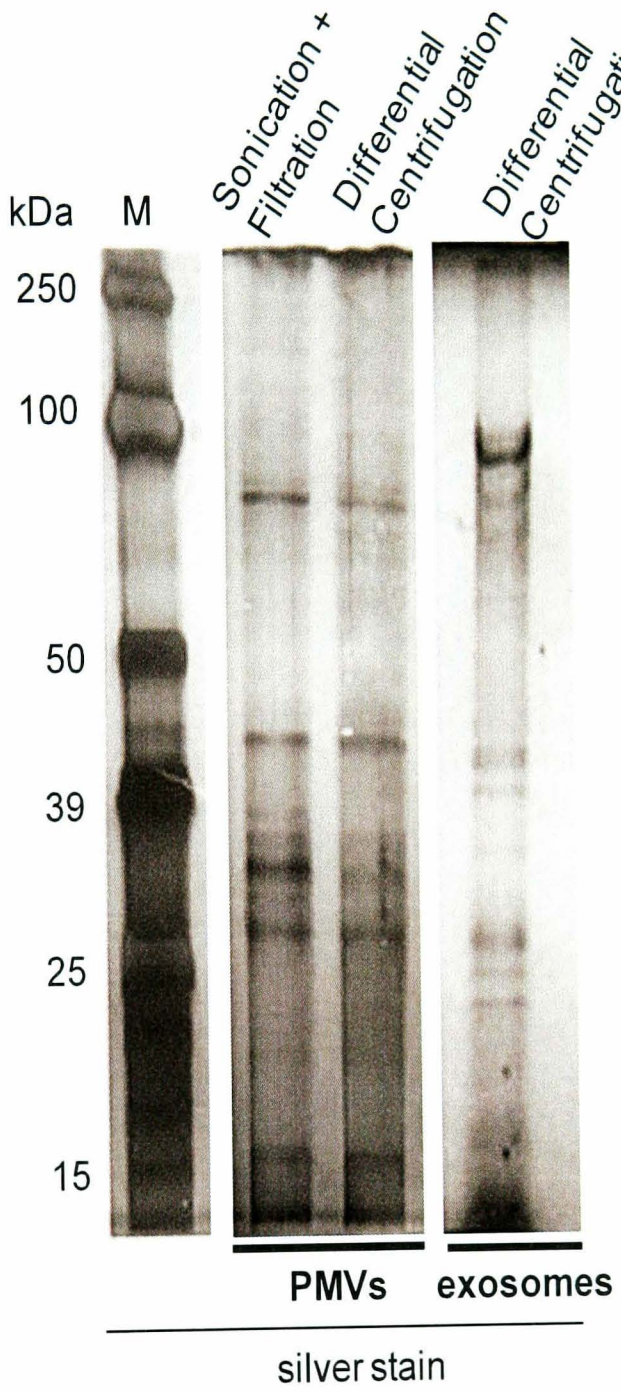




A, schematic showing novel isolation protocol for PMVs. Having recovered pool 1 (1.13-1.2 g/ml) fractions from a sucrose denstiy gradient (largely containing exosomes) and pool 2 (1.10-1.20 g/ml) (largely containing exosomes) sonication in a sonication water bath breaks up clumps of exosomes (pool 1) so that exosomes can be collected in the filtrate (0.22 μm pore size) (P1F). By washing collected PMVs from the filter when pool 2 is sonicated and filtered, PMVs can be purified from exosomes.In B exosomes and PMVs are identified by CR1 fluorescence The drop in fluorescence in P2F but not of P1F implies that pool 1 must have had mostly smaller vesicles (exosomes) because have passed through the 0.22 um pore size filter and that pool 2 mostly had larger vesicles (PMVs).

Fig. 10

Protein profile comparison of PMVs isolated by sonication+ filtration with PMVs and exosomes isolated by differentiation centrifugation



Lysates of PMVs isolated by filtration and of PMVs isolated by differential centrifugation were compared by SDS-PAGE and siver staining; both isolation protocols resulted in PMVs with a similar protein profile. Exosomes isolated by differential centrifugation were also included for comparison.

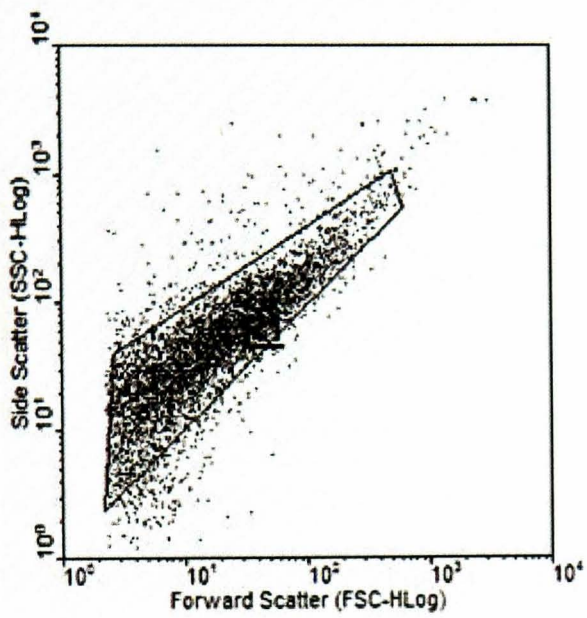
3.2.6 Flow cytometer distrubution for PMVs whether from HL-60 promonocytes or primary peripheral blood monocytes show a typical forward-/side- scatter distribution

PMVs can also be identified by their forward-/side- scatter appearance on flow cytometer. We wanted to compare the forward-/side- scatter distribution of PMVs derived from HL-60 promonocytic and primary monocytic cells stimulated with fMLP. As can be seen from Fig. 11A and B, the forward/side scatter distribution of the PMVs from both cell types shows a similar pattern in line with the classical appearance of PMVs on FACS and comparable to those obtained by others in similar experiments. This, in combination with other properties such as the type of surface markers detected give credence to the fact that throughout these experiments, PMVs were being obtained.

Fig. 11

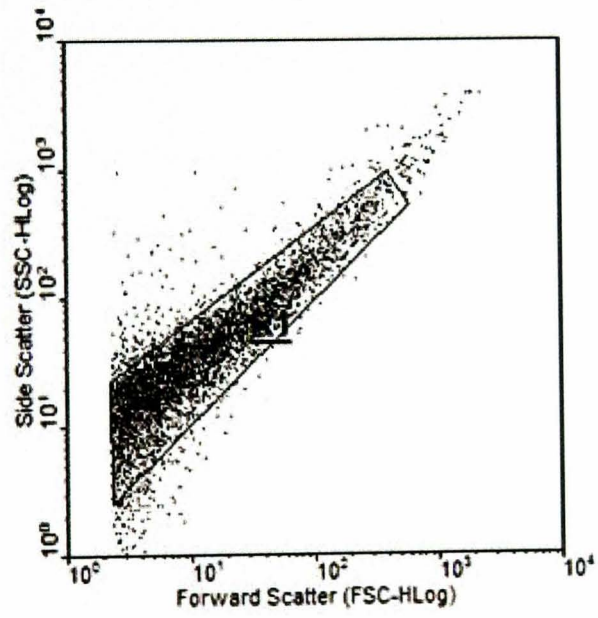
Typical forward-/side- scatter flow cytometer distrubution for plasma membrane-derived microvesicles (PMVs) from HL-60 promonocytes and primary peripheral blood monocytes

A



HL-60 PMVs

B



Monocyte PMVs

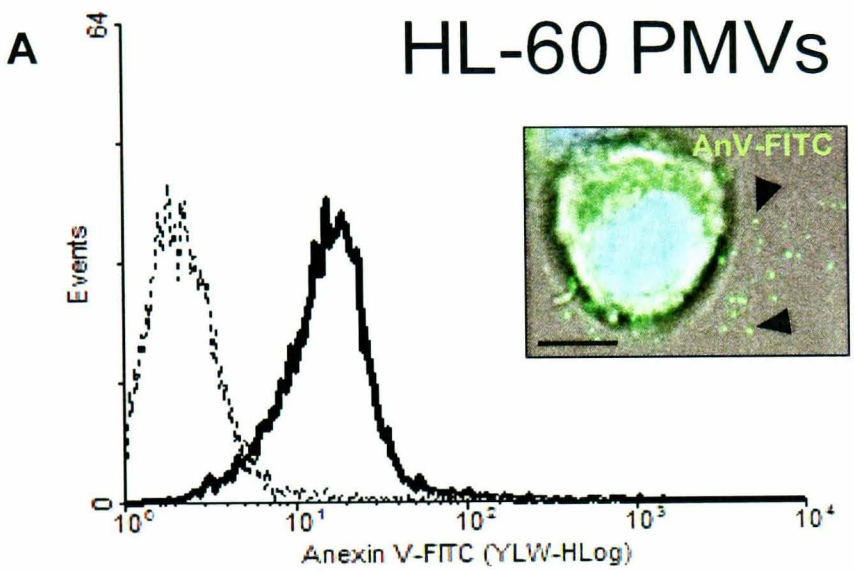
HL60 cells were stimulated to microvesiculate by treating with fMLP. PMVs were isolated by filtration as described previously and subjected to flow cytometric analysis using the Guave EasyCyte. Presented are typical forward/side scatter dot plots of the isolated PMVs from HL60 cells (A) and peripheral blood monocytes (B).

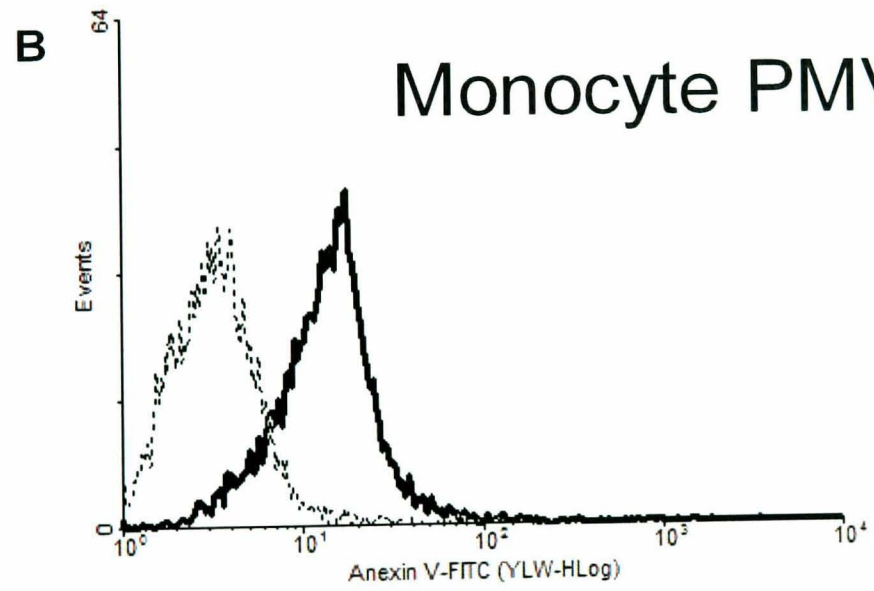
3.2.7 HL-60 cells and primary peripheral blood monocytes express surface phosphatidylserine (PS) as evidenced by the positivity for Annexin V (AnV) binding

PMVs during their formamtion expose phosphatidylserine (PS) on their surface and this has become the archetypal marker of identification of PMVs as well as being linked to their role in some *in vivo* effects. In the experiment shown in Fig. 12, PMVs obtained from HL-60 promonocytes and peripheral monocytes were stained with FITC-labelled annexin V (AnV) for the identification of PS. PMVs from both cell types showed AnV positivity indicated by a positive shift in the histogram of relative fluorescence of FITC compared to unlabelled PMVs treated the same way (control) on GRN HLog scale (Fig. 12A and B). The positive shift to the right, indicating AnV binding suggests that the PMVs express PS on their surface. As mentioned before, PS is an important identification characteristic on the surface of PMVs that is exposed on the outer leaflet of the plasma membrane when the parent cells' membrane asymmetry starts to be lost. This important phospholipids is the means by which PMVs mediate many of their biological effects, some of which have been investigated in this thesis (chapter 5). Inset in Fig 12A is an immunofluorescence microscopy image of an AnV FITC-labelled cell shedding PMVs that have also picked up the FITC label.

Fig. 12

PMVs from HL-60 cells and primary peripheral blood monocytes express surface phosphatidylserine (PS) as evidenced by the positivity for Annexin V (AnV) binding on the Guava EasyCyte flow cytometer





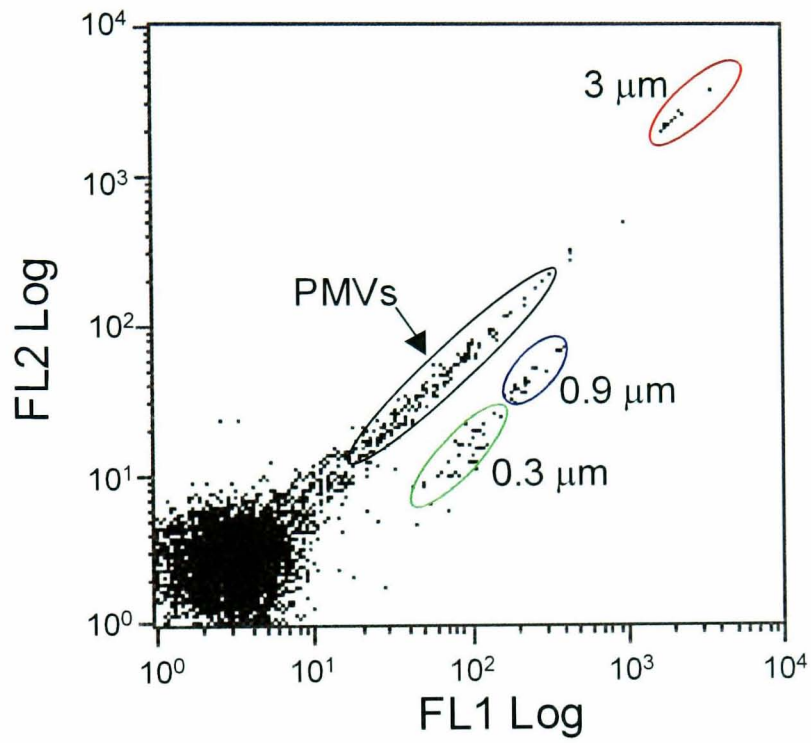
HL60 cells (A) and primary monocytel-derived PMVs (B) were stained with FITC-labelled Annexin V (AnV), and both confirmed to express surface phosphatidylserine (PS). Inset in A is presented an HL60 cell releasing PMVs (stained as Annextin V-FITC positive).

3.2.8 PMVs from HL-60 cells can be sized on a flow cytometer, using fluorescent calibration beads

In an attempt to standardize PMV counting on flow cytometers in different laboratories and to set a proper and acceptable method for PMV analysis, the use of 0.5 and 0.9 μm fluorescent beads as standards has been recommended as it has been found that long term reproducible counts can be achieved between instruments (CV <12%) when such beads are used. This is to help combat the effect of the different methods employed by investigators using flow cytometry (Robert S *et al* 2008). Throughout this study, fluorescent calibration beads (Magimix) were used to size the PMVs and typical distribution sizes between 0.3 - 0.9μm were obtained. This size is identical to the normal ranges set by a host of researchers working in the field of PMVs. As a result, such calibration gives guidance as to how to set the gating for the flow cytometer such that too small (exosome) or too large vesicles, which may not be PMVs, are not considered as PMVs.

Fig. 13

PMVs from HL-60 cells sized on a flow cytometer using fluorescent calibration beads



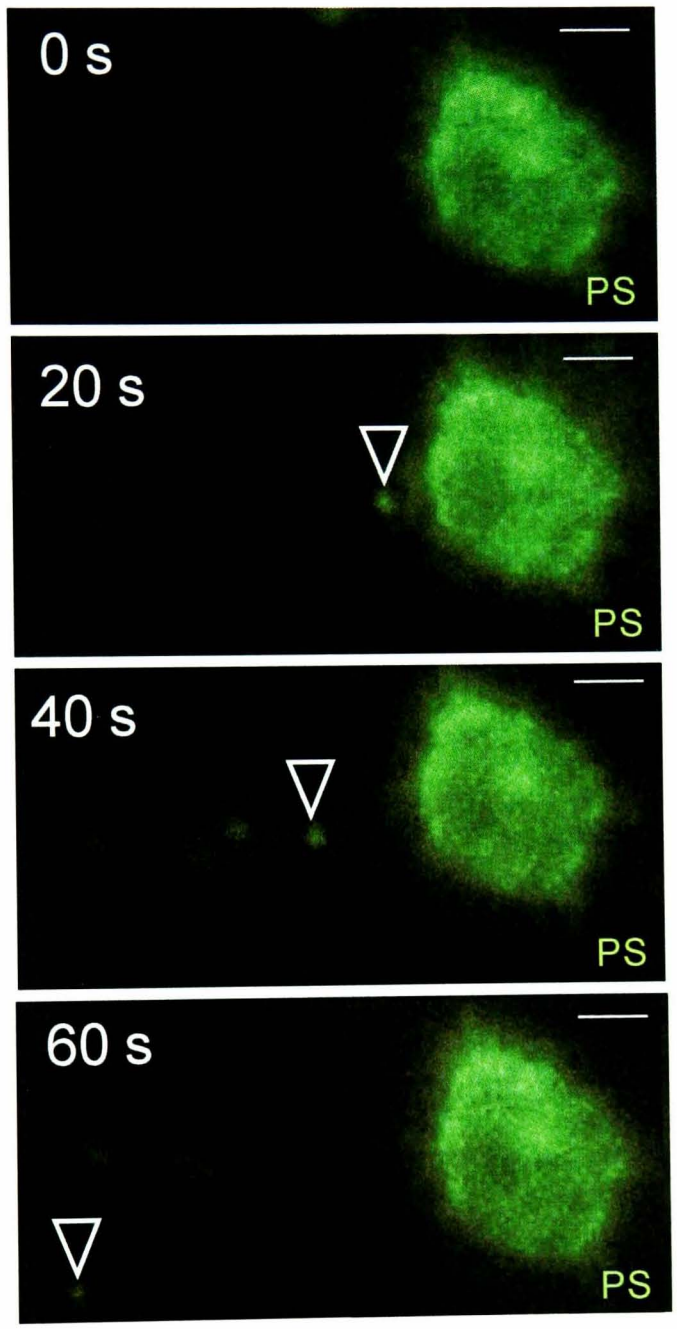
HL60 PMVs were mixed with Magimix fluorescent sizing beads and applied To the Guava EasyCyte flow cytometer. The PMVs which gave a typical distribution were sized at between 0.3-0.9 μm.

3.2.9 Annexin V positive Plasma Membrane-derived Vesicles are released by HL-60 cells upon treatment with sublytic complement

From earlier work carried out in this thesis (3.2) and by others found that PMV release can be stimulated by sublytic complement (C5b-9) or Membrane Attack Complex (MAC) deposition. We wanted to see whether cells that have picked up AnV FITC label through binding PS will release fluorescent PMVs. A time lapse experiment of AnV-FITC-labelled HL-60 promonocytes stimulated with sublytic complement in the form of NHS showed AnV FITC positive PMVs budding off the cells as captured by time-lapse immunofluorecence microscopy. At time zero, no PMVs were seen. Then after 20s a PMV is seen budding off which continues to move away from the parent cell over the ensuing 40s. Having shown the release of PS-positive PMVs, it can only be speculated as to how the negatively charged PMVs reach target cells and mediated intercellular communication. For example it may be unlikely that PMVs interact with apoptotic cells, which are themselves negatively charged due to PS exposition.

Fig. 14

HL60 cells release AnV-FITC positive PMVs after stimulation with sublytic complement



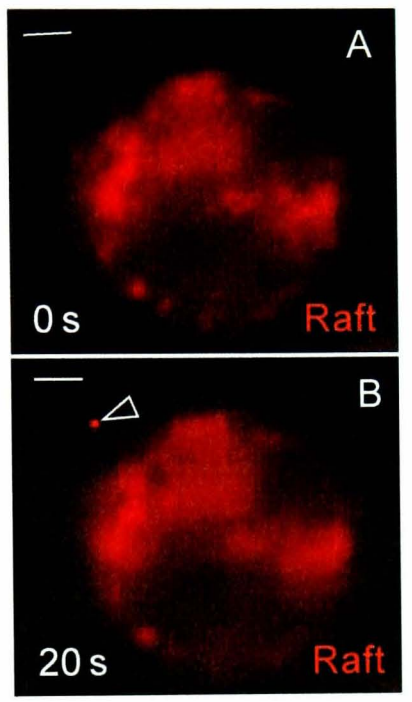
HL60 cells, pre-labelled with AnV-FITC to identify exposed PS (phosphotidylserine) were induced to microvesiculate by coating with sublytic complement. The release of PS-positive PMVs was captured by time-lapse immunofluorescence microscopy. Bar represents 5 µm.

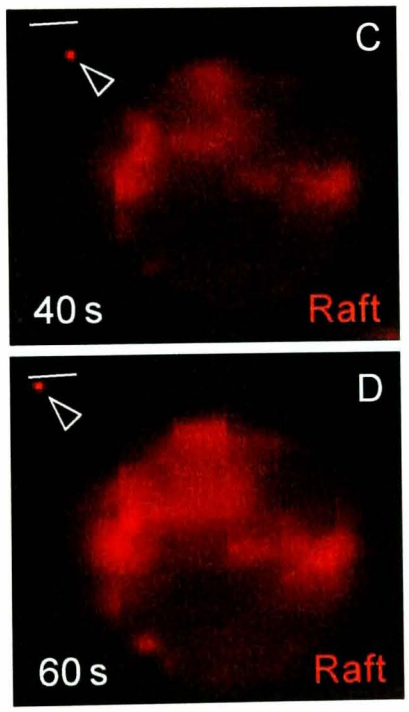
3.2.10 Vybrant Alexa 594 cholera toxin B-positive Plasma Membrane-derived Vesicles are released by HL-60 cells upon treatment with sublytic Complement

It is believed that PMVs are released from lipid raft-rich regions of the cell membrane and that this explains the observed enrichment in PMVs of lipid rafts and lipid-raft associated proteins such as GPI-anchored proteins. This implies that if the lipid raft regions on the cell are stained and the PMVs bud off, they can be identified through immunofluorescent detection of such lipid rafts. Experiment were therefore undertaken to demonstrate this characteristic of PMV shedding. As can be seen in Fig 15, (images from time-lapse immunofluorecence microscopy), in A, which is at time zero, no PMVs are seen to be budding off. In B, after 20 secs, a Vybrant Alexa 594 cholera toxin B-positive PMV is seen.

Fig. 15

HL60 cells release Vybrant Alexa 594 cholera toxin B-positive PMVs after stimulation with sublytic complement





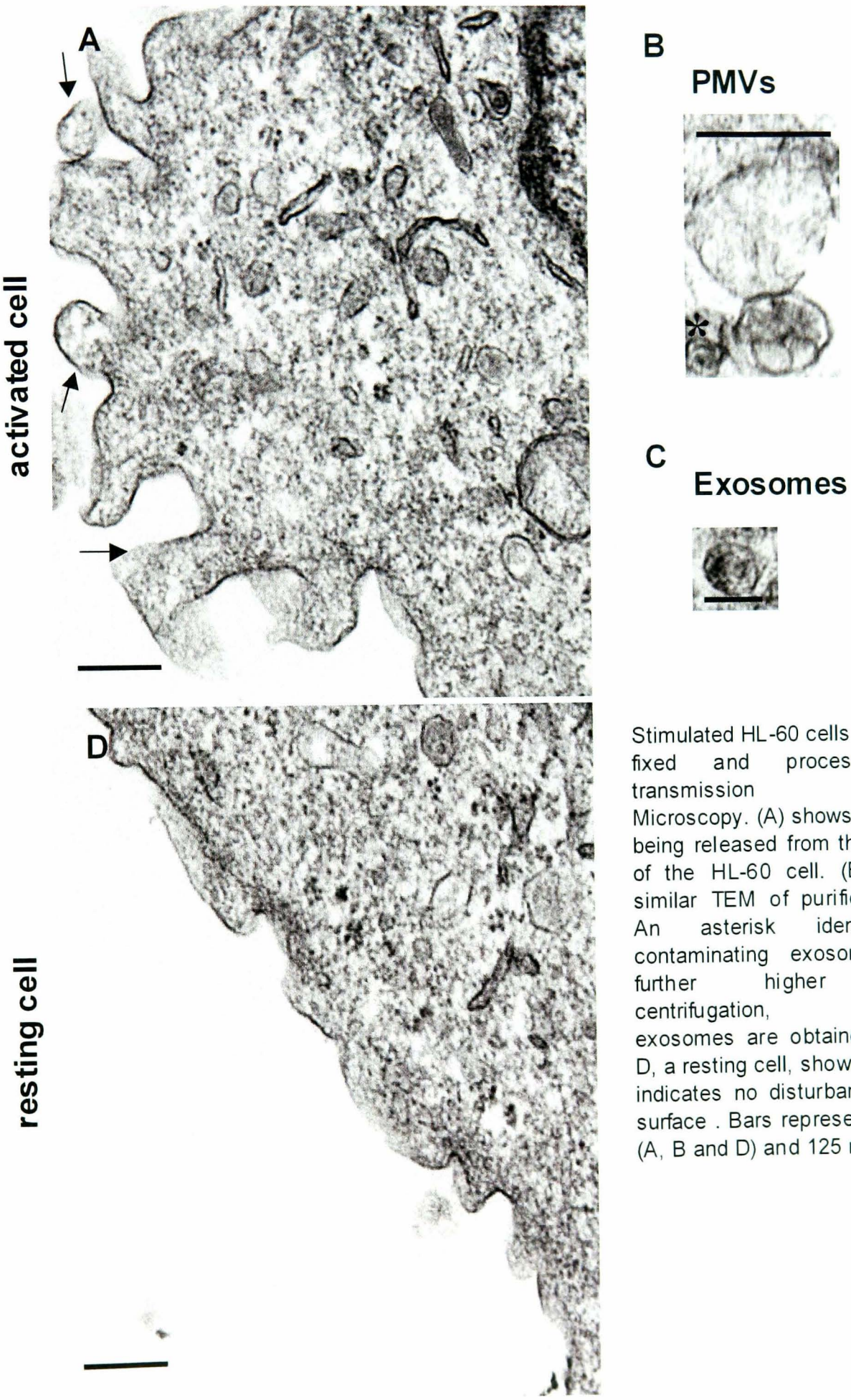
HL60 cells, pre-labelled with Vybrant® Alexa 594 cholera toxin B. The release of lipid raft-positive PMVs was captured by time-lapse immunofluorescence microscopy. Bar represents 2 µm.

3.2.11 The release of PMVs from the surface of HL-60 cells and PMVs and exosomes isolated by differential centrifugation is shown by Transmission electron microscopy (TEM)

In a resting stage, the various phospholipids that form the bilayer are distributed asymmetrically and the cell retains a smooth surface appearance (Huber J *et al* 2002). However, when cells are stimulated, and start to release PMVs, this asymmetrical phospholipid distribution is disturbed as the cellular surface begins to bleb and release PMVs that carry away some of the membrane phospholipids for example PS. Fig. 16A shows PMVs being released from the surface of a stimulated HL-60 promonocyte in comparison to a resting (unstimulated) cell (Fig. 16D), which without stimulation shows quite a uniform cellular surface. Under stimulation, the surface protrusions begin to be seen on the cell and PMVs begin to bud off (Fig. 16A). Fig. 16B and C respectively show PMVs and the smaller exosomes, purified by differential centrifugation, which once more emphasizes the size difference between these two types of microvesicle. In a similar experiment (Fig 17), not only were released PMVs (~400 nm in diameter) visualised by TEM of the HL-60 cell surface, but also exosomes (≤ 100 nm in diameter), identified by their typical 'saucer shape' morphology.

Fig. 16

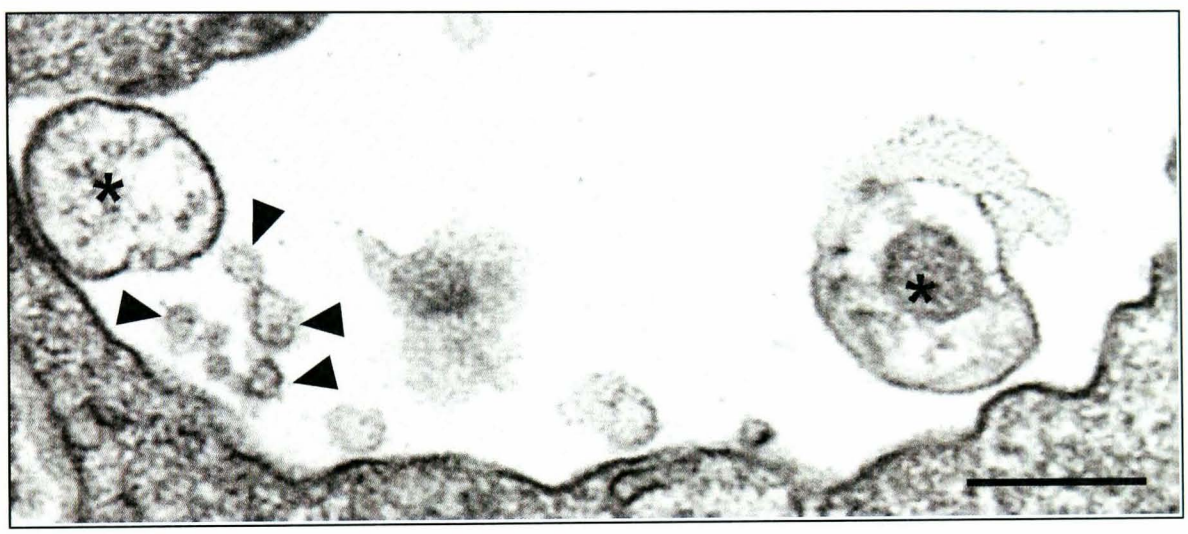
Transmission electron microscopy showing release of PMVs from the suface of HL-60 cells and differentially purified PMVs and exosomes



Stimulated HL-60 cells were fixed and processed for transmission Electron Microscopy. (A) shows PMVs being released from the surface of the HL-60 cell. (B) Shows similar TEM of purified PMVs. An asterisk identifies a contaminating exosome. After further higher speed centrifugation, purified exosomes are obtained (C). In D, a resting cell, shown by TEM, indicates no disturbance at the surface . Bars represent 250 nm (A, B and D) and 125 nm (C).

Fig. 17

Transmission electron microscopy showing release of PMVs and exosomes from the suface of HL-60 cells



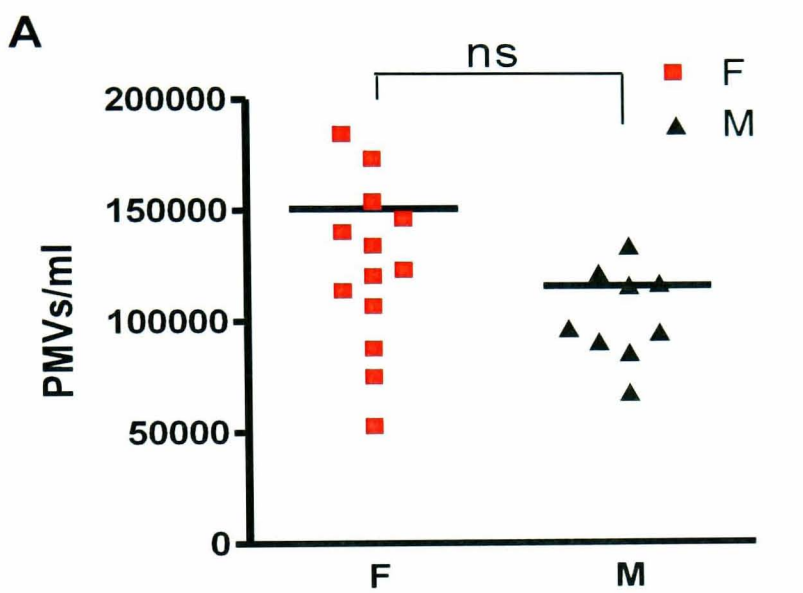
HL60 cells stimulated with sublytic complement release ~500 nm diameter vesicles (PMVs, asterisked) and some ~100 nm diameter exosomes as shown by TEM. Scale bar represents 400 nm.

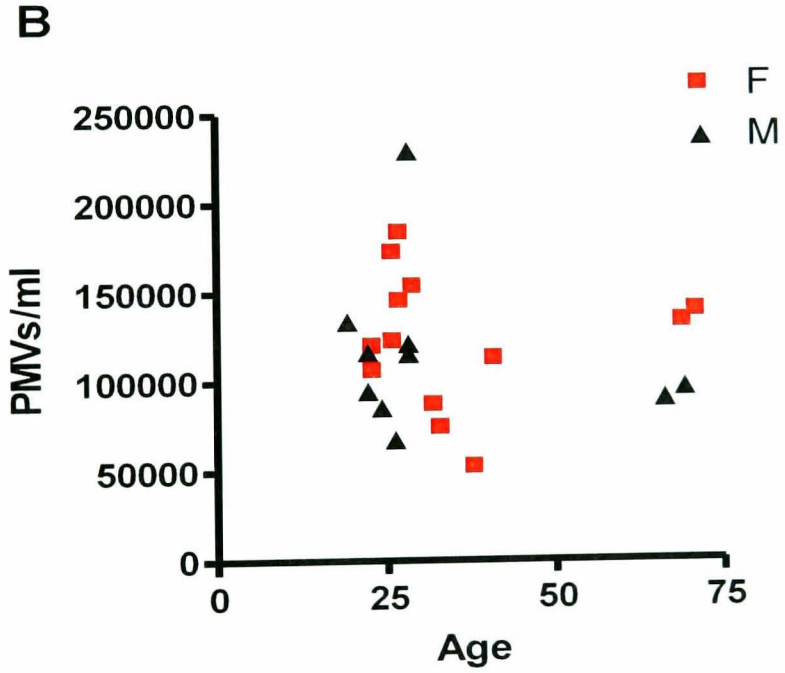
3.2.12 Quantitation of plasma levels of PMVs in healthy volunteers

Numerous quantitative studies have been published on PMVs in both healthy subjects and in pathological states (Horstman LL *et al* 2004). Currently however, a normal physiological range for PMVs is yet to be established and if PMV testing is to enter the mainstream of clinical testing as a diagnostic tool, then it is important that normal physiological levels are established so that when their levels are increased, it can be put down to cellular alterations in a disease condition. Fig. 18A and B represents an estimation of levels of PMVs from 24 healthy volunteers of different backgrounds (age/sex). The levels of PMVs in females were higher than the levels in males even though the range is also larger in the female population than the males. With regards to age, not much comparison could be made, since not many older people were sampled. As PMV levels in normal individuals are relatively low as compared to disease states, the experiment tried to establish a normal range of plasma PMV levels. The average level of PMVs was ascertained at $1.3x10^5$ PMVs/ml.

Fig. 18

PMV plasma levels in healthy volunteers





Plasma levels of PMVs (PMVs/ml) were determined for 24 healthy volunteers and the results compared with volunteer age and sex. N=23.

3.3 DISCUSSION

The role of Plasma Membrane-derived Vesicles (PMVs) in helping maintain a delicate balance between health and disease has gained a great deal of interest in recent times in the scientific community (Toth B *et al* 2007). Apart from playing a role in various physiological functions such as thrombosis, inflammation, angiogenesis and vasoconstriction (Berckmans RJ *et al* 2005; Kim HK *et al* 2004; Boulanger CM *et al* 2001). PMVs have been linked with a wide range of clinical conditions such as diabetes, atherosclerosis, cardiovascular and other autoimmune diseases (Nomura S *et al* 2008) (Chironi G *et al* 2006; Mallat Z *et al* 1999). It is therefore important that analysis and estimation of PMVs is done correctly in order to portray the right picture as to whether they play a part in any disease. This would then allow us to determine if they can be used as a diagnostic tool.

Findings from the study attempted to establish typical characteristics of PMVs which could be a bench mark for PMVs analysis as we move to the era of using PMVs in diagnosing diseases. Firstly using a modified isolation protocol, it was important to show a typical flow cytometer forward-/side- scatter distrubution or dot plot for PMVs which was similar to the dot plot that has been described by others in previous studies and to therefore confirm that the vesicles isolated were PMVs rather than cell debris. Obtained PMVs were also analysed for their phosphatidyl serine (PS) content which is found on the outer leaflet of their plasma membrane and which has become a pertinent feature by which PMVs are identified and which also helps to explain some of the roles in physiological events that PMVs play. As the results show, the PMVs expressed surface PS as evidenced by the positivity for Annexin V (AnV) binding on the Guava EasyCyte flow cytometer.

Fluorescent calibration beads were also used to size up the PMVs on the flow cytometer whereby the PMVs were mixed with Magimix fluorescent sizing beads and analysed. The PMVs, which gave a typical distribution, were sized at between 0.3 - 0.9μm in diameter, which falls within the expertent normal range (0.1 to 1μm) quoted by various researchers. Then HL60 cells were pre-labelled with AnV-FITC to identify exposed PS on PMVs upon realease after induction by sublytic complement. The release of PS-positive PMVs was captured by time-lapse immunofluorescence microscopy, which confirmed the notion that PMVs released carry PS from their parent cell. When the cells were pre-labelled with Vybrant® Alexa 594 cholera toxin B for the identification of lipid raft in a similar manner as for PS labelling, lipid raft-positive PMVs were observed giving credence to the fact that PMVs production originates from lipid raft regions of the plasma membrane and therefore is released with some of the rafts.

Transmission electron microscopy was also used to show the release of PMVs and to establish a clear difference between PMVs and exosomes which have been described as 5'-nucleotidase activity-containing vesicles ranging in size from 30 to 90nm. In contrast to PMVs, they are preformed membrane vesicles, which are stored in cellular compartments named multivesicular bodies (MVB) and secreted when the multivesicular bodies fuse with the cell membrane. The result shows PMVs being released from the surface of the HL-60 cell. Similar TEM carried out of purified PMVs and Exosomes, the latter obtained after further higher seed centrifugation.

Plasma levels of PMVs in healthy volunteers were next analysed, and the results compared according to age and sex of the volunteers. Females appear to have a wider range of PMVs with a variation between 5.0 - 20.0 x 10^4 PMVs/μl and a

median of about 15.0 x 10^4 PMVs/µl. The males however, seemed to have a narrower range and in terms of age, since only a few older people were measured, a clear differentiation could not be made, although the younger age group appeared to have a variable range as well.

These findings have in effect shed light as to what the average size of PMVs is, molecules they may be carrying from their parent cell, the site on the cell membrane they originate from, and based on their content, which pathological activities they are likely to be involved in.

Since knowing the characteristics of PMVs are so important, there have been various studies undertaken in this regard in trying to come out with a set of standards by which they can be measured, such as the appropriate centrifuge speed, sample removal and storage, flow cytometry (types and mode of operation) amongst others as there seem to be considerable variability regarding pre-analytical variables. In the case of sampling for instance, there is a general consensus that citrate is the preferred anti-coagulant (Jy W *et al* 2004) because of its ability to chelate calcium (Gritters M *et al* 2005), a necessary prerequisite for PMV release. Despite this consensus, one group studying red blood and endothelial cell PMVs used ethylenediaminetetraacetic acid (EDTA) (Nomura S 2004).

But a recent review has questioned the suitability of EDTA as an anticoagulant for PMV analysis because of its ability to extract calcium which is necessary for the structural integrity of surface markers and to induce platelet activation through CD62P, which could result in higher PMV counts (Enjeti AK *et al* 2007; Horstman LL *et al* 2004). Another issue is the effect of storage temperature and duration on PMV numbers. A group of researchers quantified PMVs in stored red cell concentrates

and reported a 20-fold increase from 3,371 ± 1,181 PMVs/µl at day 5 to 64,858 ± 37,846 PMVs/µl at day 50 (Rubin O *et al* 2008). By contrast, another group found that PMV counts decrease during storage because when PMVs were aliquoted and stored overnight at 4°C, at -20°C and after storage for 1 month at -80°C, lower PMV concentrations were observed (Shah MD *et al* 2008).

Centrifugation conditions are not standardized and without having a comparable standard, conflicting PMV counts are common (Horstman LL *et al* 2004). Different, centrifugation conditions have been postulated with some being (160*g* for 20 min), (2 x 1,500*g* for 20 min) and (13,000*g* for 2 min (Shah MD *et al* 2008). Van Beers *et al* used an initial centrifugation of 20 minutes at 1,550*g* to remove cells and after storage at -80°C for an unknown duration, submitted their sample to 2 x 30 minute centrifugations at 18,890*g* that resulted in the formation of a PMV pellet. Shet AS *et al* used a somewhat different protocol that consisted of an initial centrifugation of 1,500*g* for 10 minutes followed by 13,000*g* for 10 minutes to obtain platelet-free plasma. We conducted our centrifugation at 160g for 5min, followed by 4,000g for 1hr and then 25,000g for 90 minutes.

It is also important to note that flow technology and its incorrect implementation could lead to result variability as well and therefore requires standardisation. It was recently suggested that flow cytometry methods and settings need to be presented in full in publications to make results both reproducible and easier to interpret. To this effect, a study conducted by a large cohort of scientists has provided guidelines for the correct presentation of flow cytometry methods within the literature (Lee JA *et al* 2008). This is so that results can be better interpreted and compared. The

guidelines suggest that the manufacturer and model be declared along with the fluidics configuration, optical configuration and electronic configuration.

An important area worthy of discussion is that of suitable controls for PMV estimation, if this procedure is going to be used as a diaginostic tool. For example, one study that looked at RPMVs in patients infected with *falciparum* malaria failed to include the baseline characteristics of both the healthy volunteers and the patients (Nantakomol D *et al* 2008). There may have been differences between the groups regarding age, race and clinical history that may have affected the results and so including these characteristics weakens the impact of such studies. Without a body of work defining the optimum analytical conditions of any assay procedure, their suitability will continue to be determined via consensus as opposed to a standard based on scientific evidence, making any results obtained difficult to interpret unless reference standard methods are developed (Dignat-George F *et al* 2009; Horstman LL *et al* 2004).

3.3.1 Conclusion

More collaborative effort is needed to establish reliable and reproducible standards. While the PMV counts obtained by most research groups are similar, the centrifugation, storage and some procedural conditions are different and therefore make it difficult to accept results. A meeting of scientists has however announced the validation of numerous flow cytometers, which is a huge step in the right direction. This, coupled with a recent study providing guidelines for the correct reporting of flow methods is evidence that good progress is being made and researchers focusing on the study of PMVs need to come to a consensus regarding sample processing and analysis to make interpreting results less of a challenge.

CHAPTER 4

PLASMA MEMBRANE-DERIVED VESICLES INDUCE THE DIFFERENTIATION OF THE PROMONOCYTE CELL LINE, HL-60: POSSIBLE ROLE OF PMVs RELEASE IN DIFFERENTIATION THERAPY IN APL

4.1 INTRODUCTION

Acute promyelocytic leukaemia (APL) a subtype of the acute myelogenous leukemia (AML) from which the promonocytoid leukaemia HL-60 cell line is derived, represents 5-8% of AML in adults and 5-15% of all adult leukaemias. The various approaches to treat APL in which blast cells are unable to mature into adult cells include differentiation, apoptosis induction and cytotoxic agent therapy. Differentiation therapy, works by inducing differentiation of leukaemic promonocytes into mature non-replicative cell types that are undergoing cell cycle arrest and eventually undergo apoptosis (Dimberg A *et al* 2002). Retinoids such as all-*trans*retinoic acid (ATRA) which are used in differentiation therapy to treat promyelocytic leukaemia (APL) (Ades L *et al* 2005) specifically target neoplastic cells leaving normal mature cells unaffected. Other inducers of differentiation (to granulocytes as well as monocyte/macrophage) in myeloid leukaemia cells include vitamin D3 (Hmama Z et al 1999), the phorbol ester, 12-Otetradecanoylphorbol-13-acetate (TPA) also commonly known as phorbol 12-myristate 13-acetate (PMA) and calcium ionophore (A23187) (Waclavicek W *et al* 2001).

Microvesiculation is a ubiquitous cellular mechanism, which occurs as a result of exocytosis, to release exosomes (between 50-100nm) (Simons M and Raposo G 2009) or by direct release of vesicles from the cell surface membrane, referred to, in this study as Plasma Membrane-derived Vesicles, PMVs (0.1-1μm) (Hugel B *et al* 2005). Various changes in cell physiology are involved in the release of cellular PMVs (reviewed elsewhere (Ratajczak J 2006) but an increase in intracellular calcium and a loss of lipid asymmetry in the plasma membrane are always the initial sign of microvesiculation (Ratajczak J 2006). *In vitro*, PMV release can be initiated by sublytic complement deposition and the calcium ionophore A23187. Unlike apoptotic bodies, which are derived from damaged cells, PMVs, released from

healthy viable cells are smaller in size and do not contain damaged DNA. Instead, PMVs carry microRNA (Hunter MP *et al* 2008), mRNA, numerous membrane proteins, lipids and cytoplasmic constituents, characteristic of their parental cell (Piccin A *et al* 2007) and being able to transmit such proteins between cells, are important mediators of intercellular communication.

In experiments using THP-1 cells, microRNAs have been shown to be involved in monocytic differentiation (Forrest ARR *et al* 2010) so it may be pertinent to HL-60 differentiation that PMVs carry microRNAs (Hunter MP *et al* 2008). In other studies involving the chronic myeloid leukaemia (CML) cell line, K-562, PMVs carrying hedgehog (Hh) proteins could induce the differentiation towards the megakaryocytic lineage (Eken C *et al* 2008). Most recently PMVs derived from embryonic stem cells (ESC) were found to carry Wnt-3, which is involved in hematopoietic differentiation, and such PMVs were shown to reprogramme hematopoietic progenitor cells (Ratajczak J 2006). This evidence, that PMVs, albeit from ESC, are involved in differentiation in the hematopoietic environment in the bone marrow, thus suggests possible PMV involvement in leukaemia myeloid development, and possibly monocytic hematopoiesis, and needs to be investigated further.

PMA, which is a polyfunctional diterpene phorbol ester, has been widely used as a tumor promotor in cancer research as it can induce human promonocytic/monocytic cells to differentiate to macrophages. It is understood that before initiating cell differentiation, PMA treatment first induce an inhibition of cell growth mostly at G1-phase of the cell cycle (Daigneault M *et al* 2010). This is done via a complex mechanism associated with the modulation of the expression of several cell cycle regulators, initiated by the cellular generation of reactive oxygen species (ROS).

Although PMA is quite toxic, it has shown a very good anti-leukemic activity in humans as a differentiator of human leukemia cells in various experiments.

Vitamin D3 was discovered in the 1980s in both *in vivo* and *in vitro* experiments as not only able to reduce the unregulated growth of malignant cells by promoting apoptosis but also it promoting differentiation of immature cells to mature ones thereby preventing new cells from becoming cancerous(Hmama Z *et al* 1999). There is a lot of evidence that simple vitamin D has helped prevent cancer and that adequate vitamin D nutrition may help retard the growth of cancer by blocking metastasis and angiogenesis.

Calcium ionophores are compounds that increase the permeability of cellular membrane barriers to calcium ions by functioning as mobile ion carriers or channel formers (Allan D *et al* 1980). They contain hydrophobic regions conferring lipid solubility and hydrophilic ion-binding regions that delocalise the charge of the ion to shield it from the hydrophobic regions of the membrane lipid bilayer. Calcium ionophore is used in laboratories to increase intracellular Ca^{2+} levels in intact cells and have been mentioned as being able to cause cellular differentiation (Allan D *et al* 1981).

In addition to PMA and ATRA, inflammatory mediators such as histamine, and bacterial products fMet-Leu-Phe (fMLP) and lipopolysaccharide (LPS) can also induce microvesiculation, providing cells have the cognate receptors (Abedin MJ *et al* 2003). Since inducers of PMV release have been described in connection with possible therapies for acute monocytic leukaemia, it was deemed imperative in this study to see whether PMVs themselves could induce cell cycle arrest/terminal differentiation (the hallmark of differentiation therapy) in the promonocytic leukaemia

cell line, HL-60 (Yu LM *et al* 2006). This was important in view of the recent findings that microvesicles, albeit from neutrophils (termed ectosomes) were shown to interfere with the maturation of immature dendritic cells (Eken C *et al* 2008). Furthermore, early success of ATRA in APL has not been followed up with new drugs and resistance can develop with all existing therapies (Garay E *et al* 2007; Campelovier P *et al* 2006).

This work aims to show principally whether PMVs can initiate differentiation/stop proliferation of HL-60 cells. From surveying the literature, such PMV-mediated differentiation would likely be a multifactorial process involving several proteins and microRNAs. Amongst the many factors PMVs harbour and numerous PMV-associated cytokines revealed by proteomics studies, is the multifunctional transforming growth factor-β1 (TGF-β1), which is found principally associated with platelet PMVs (Garcia BJ *et al* 2005) and which inhibits the proliferation of various cell types *in vitro* (Li MO *et al* 2006). Given the important role TGF-β1 plays in the regulation of cellular proliferation, and its autocrine inhibition of proliferation in the APL cell line, we asked whether any growth regulation and differentiation of promonocytic leukaemia cells could in part be influenced by PMVs bearing TGF-β1, that are released from cells by the action of known differentiation therapeutics. If so, this would be the first report of TGF- β1 carried on the surface of a PMV that was deliverable as a functional signalling molecule.

4.2 PMVs and HL-60 promonocyte differentiation

4.2.1 PMV release from the promonocytic cell line, HL60 is greater than from primary peripheral blood monocytes

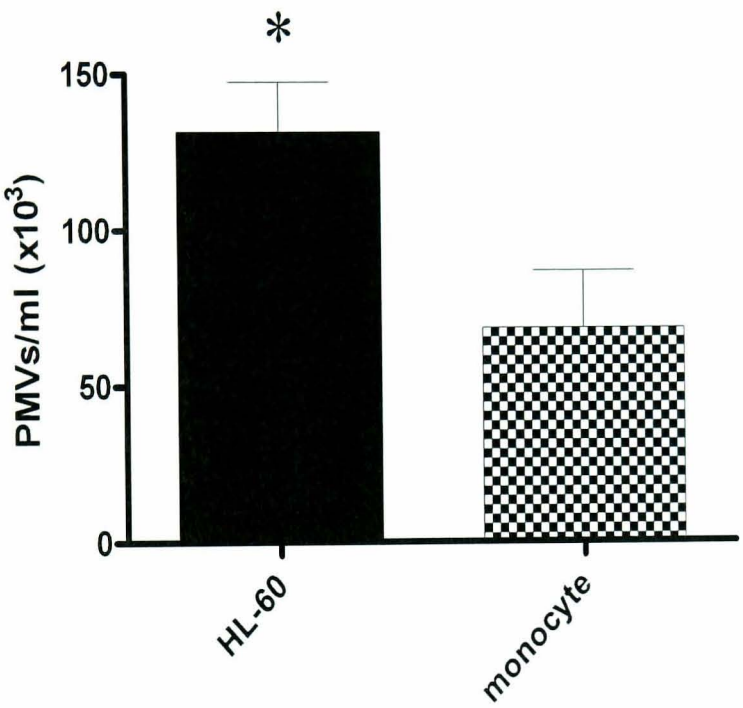
Although basal levels of PMVs are found in normal individuals, increased vesiculation and therefore PMVs have been identified in disease states (Berckmans RJ *et al* 2001) especially cancer. PMV release was therefore quantified to see if HL-60 cells being leukaemic would release more PMVs than primary peripheral blood monocytes. The experiment was carried out as per the protocol in the methods section. As Fig. 19 shows, production of PMVs induced from $1x10^5$ HL-60 cells with sublytic complement deposition in the form 5% NHS was significantly increased (about $120x10^3$ PMVs/ml) as compared to that from the primary peripheral blood monocytes (about $70x10^3$ PMVs/ml). This shows that leukaemic promonocytes are indeed able to release greater numbers of PMVs than normal cells.

4.2.2 Inducers of monocyte- to- macrophage differentiation inhibit the proliferation of HL-60 cells

The concept that exit from the cell cycle into G0/G1 is a necessary, or at least frequent prelude to differentiation, has led to the examination of the relationship between cellular proliferation and differentiation in the sense that if cells exit the growth cycle prior to terminally differentiating, then a reduction of proliferation must accompany any differentiation. To this point we thought it prudent to examine the effect of different differentiation inducers on the proliferation of HL-60 cells. Three such inducers of monocyte-to-macrophage differentiation (PMA, ATRA and histamine) were thus used in this experiment. Compared to untreated control (Fig. 20), which resulted in increasing growth over 72h, the addition of PMA halted proliferation within 24h. There was then no increase up to 48h and a slight decline to 72h. Compared with control, treatment with histamine or ATRA, significantly reduced proliferation over the same time period.

Fig. 19

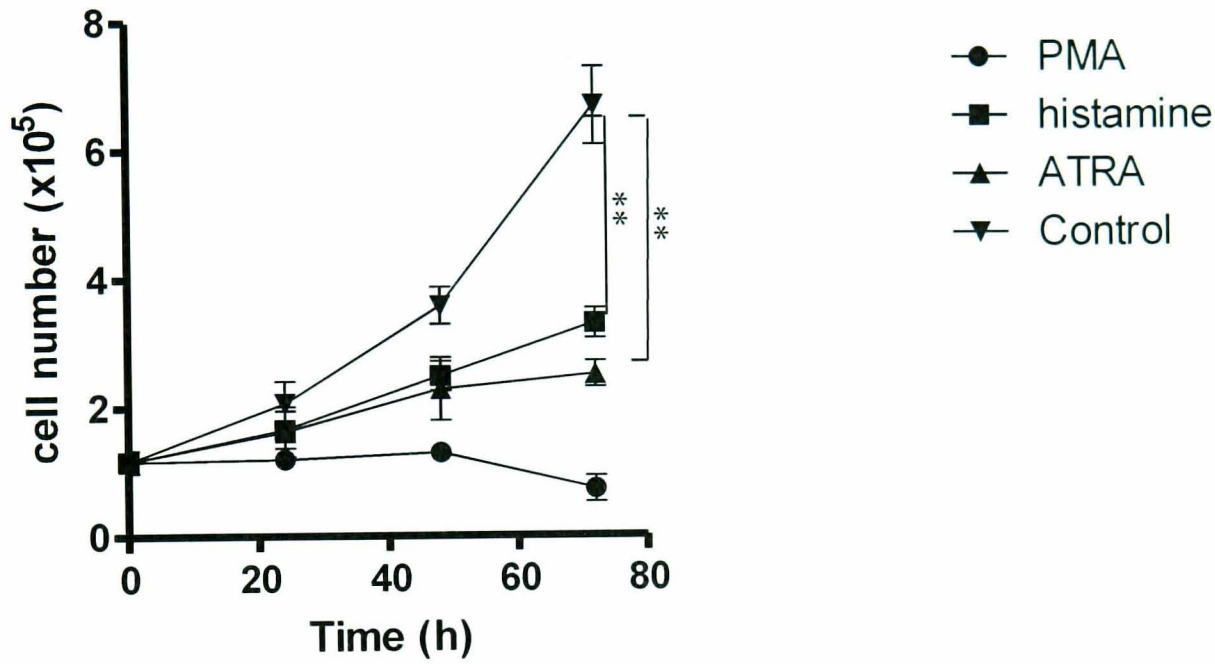
PMV release from promonocytic cell line, HL60 and Primary peripheral blood monocytes



HL-60 cells and monocytes ($1x10^5$) were treated with 5% NHS for 30 min at 37°C.Released PMVs were purified by differential centrifugation and sonication and quantified using the the Guava EasyCyte flow cytometer.

Fig. 20

The effect of inducers of monocyte-to-macrophage differentiation on the proliferation of HL-60 cells



HL-60 cells seeded at a starting concentration of $1.1x10^5$ cells/ml were grown in 24-well plates. From the outset, cells, plated out in triplicate for each condition, were either not treated (control), or treated with histamine (10 μM), ATRA (1 μM) or PMA (0.1μM). Viable cells were then counted on the Guava EasyCyte flow cytometer using a ViaCount assay after 24, 48 and 72h.

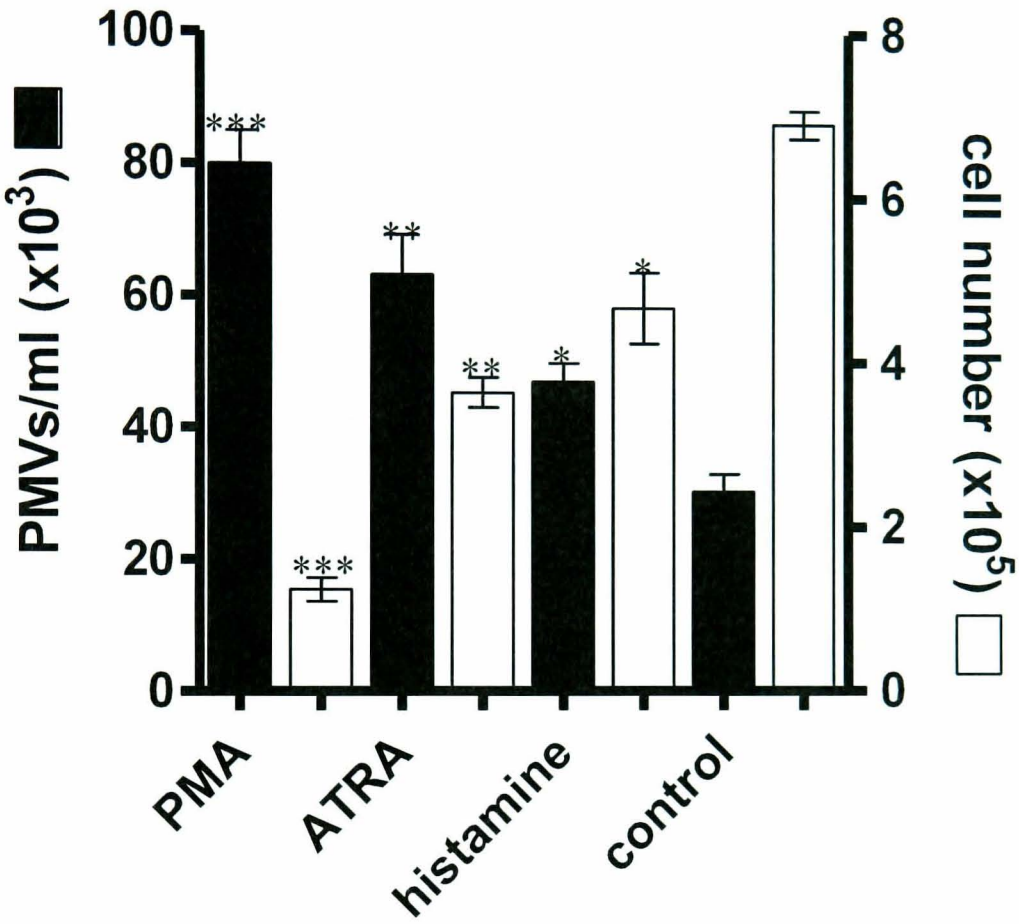
4.2.3 Monocyte/macrophage differentiation inducing agents cause a Ca^{2+}- mediated release of PMVs

4.2.3.1 ATRA, PMA and histamine have differing capacities to induce PMV release from HL-60 cells and differing affects on HL-60 proliferation

Experiments have shown that increased PMV release from cells occurs through activation of such cells by various stimuli. What is not clear is whether this process affects the proliferation or growth rate of these cells. This prompted an investigation of the effect of ATRA, PMA and histamine on the release of PMVs from HL-60 cells and on their proliferation. As can be seen from Fig 21, PMA (at 0.1μM) was able to induce the release of a greater level of PMVs, ~80x 10^3 PMVs/ml, but also a great decrease in cellular proliferation, ~18 x10^3 PMVs/ml. This was followed by ATRA (1μM) with about 65 x10^3 PMVs release and 50 x10^3 proliferations that is more PMVs release than proliferation. In the case of histamine (10μM), proliferation (60 x10^3) was more than PMVs (35 x10^3) release similar pattern to the control, only the control had greater than 80 x10^3 of proliferating cells. There was a clear inverse relationship between the release of PMVs and cellular proliferation in that the agents that produced more PMVs (PMA and ATRA) caused less cells to proliferate and histamine that could not halt proliferation did not cause much PMVs release.

Fig. 21

The effect of ATRA, PMA and histamine on release of PMVs from HL-60 cells and on their proliferation



HL-60 cells were treated with ATRA (1 μM), PMA (0.1μM) or histamine (10 μM). Cell number and released numbers of PMVs/ml released on day 3 were monitored. There is an apparent inverse correlation between cell number and PMVs/ml released

4.2.3.2 The monocyte/macrophage differentiation inducing agents histamine, ATRA and PMA increase $[Ca^{2+}]_i$ in HL-60 cells

Agents that induce cellular differentiation inhibits proliferation to varying degrees and may also cause PMVs release which is mediated by a rise in cytosolic calcium concentration. To show that these differentiating/proliferation inhibiting agents are inducing PMV release mediated after a rise in $[Ca^{2+}]_i$, spectrophotometric measurements of $[Ca^{2+}]_i$ using Fura-2-AM were made after addition of ATRA, PMA or histamine. As Fig. 22 shows, a sharp increase of intracellular calcium was observed in HL-60 cells within 60 s of the agents being added as compared to the cells that were not treated. This confirms that the differentiation agents used, ATRA, histamine and PMA cause a release of PMVs that is mediated by increases in intracellular calcium.

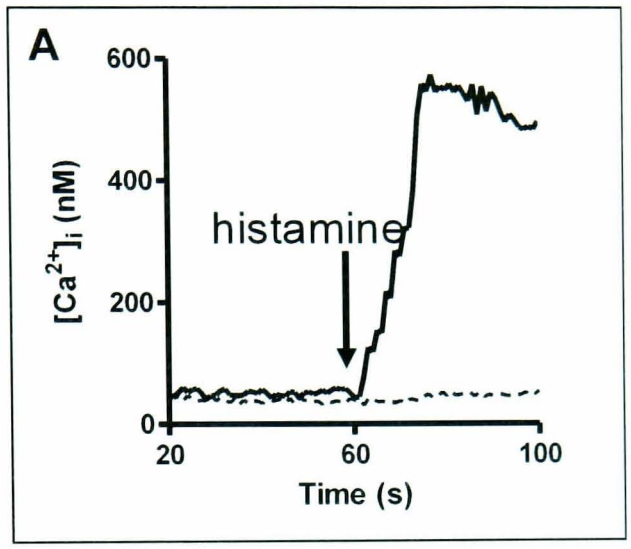
4.2.4 HL-60 cells treated with PMVs differentiate along the monocyte/macrophage lineage

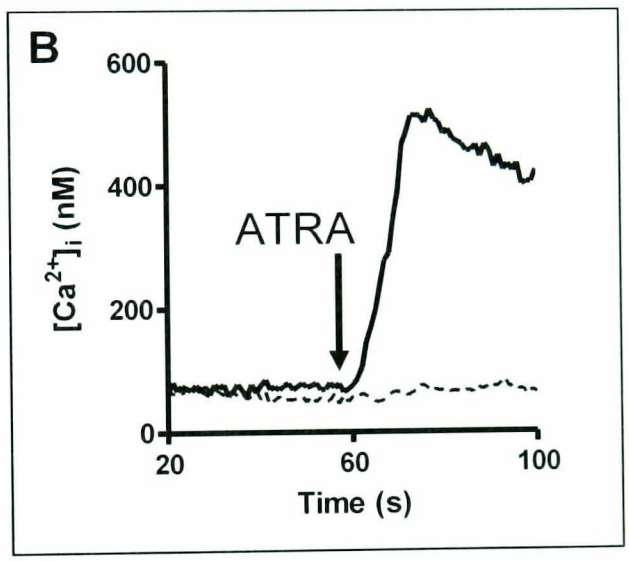
4.2.4.1 HL-60 cells treated with PMVs for 24 or 72 h have an increased capacity to reduce NBT, as a measure of myeloid differentiation

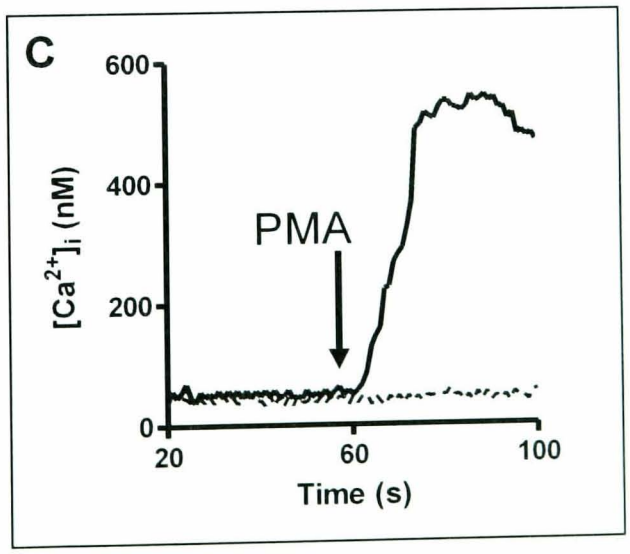
In this experiment, NBT reduction capacity was measured as an indicator of superoxide and therefore of HL-60 cellular differentiation. From Fig. 23, it can be seen that the reduction of the blue formazan salt which was measured spectrophotometrically (A_{560}) at time 0h was almost similar whether cells had been exposed to PMVs or not. After 24h however, there was a higher increase in NBT reduction where cells had been treated with PMVs than without, and this became even more pronounced after 72h.

Fig. 22

Addition of differentiaiton inducing agents histamine, ATRA and PMA cause a sharp increase in $[Ca^{2+}]_i$ in HL-60 cells



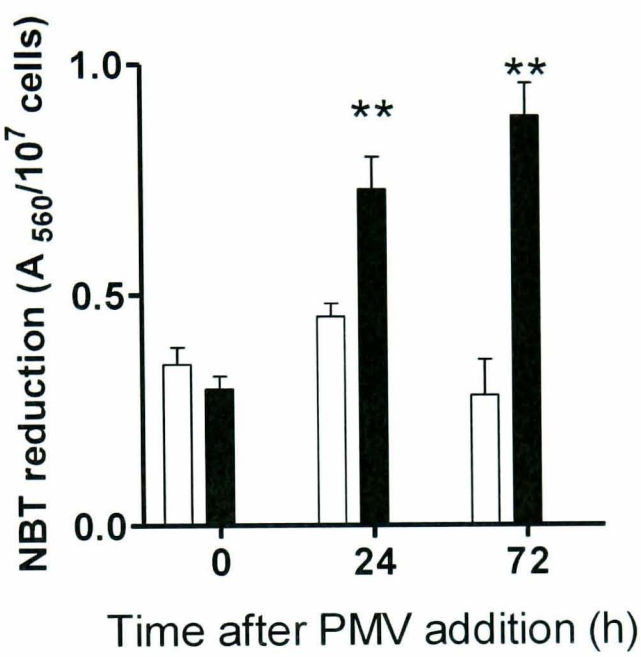




The differentiating agents, histamine (A), ATRA (B) and PMA (C) were added to cultures of HL60 cells. Using Fura-2AM and a spectrophotometer, the concentration of intracellular calcium $[Ca^{2+}]_i$ was determined and in all cases found to increase rapidly within ~30 mins. Dotted line represents control, untreated cells.

Fig. 23

HL60 cells treated with PMVs for 24 or 72h have an increased reduction capacity of NBT, as a measure of superoxide production and myeloid differentiation



NBT (nitroblue tetrazolium) reduction assay to show differentiation of HL60 cells. $1x10^6$ cells were treated with PMVs for 24 and 72h. At these time points they were stimulated with 1 μM PMA and incubated with 1 mg/ml NBT for 30 min at 37°C. They were then dissolved in DMSO and measured spectrophotometrically (A650) for reduction of the blue formozan salt. Clear bars represent untreated, control HL60 cells and filled bars HL60 cells treated with 30 μg PMVs.

4.2.4.2 HL60 cells treated with PMVs show a dose-dependent increase in adherence after 24h

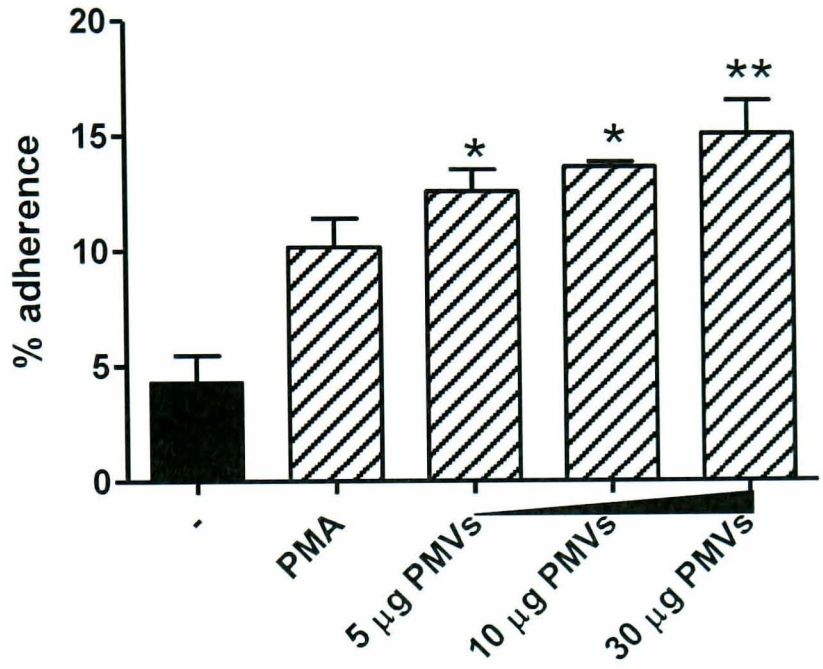
The ability of cells to adhere to specific surfaces is vital in the process of proliferation and differentiation as it elicits a multitude of intracellular events, which occur in response to signals that originate at the surface of the cell and are then propagated by diverse biochemical pathways (Hynes R0 and Lander AD 1992). Cellular differentiation is accompanied by adherence, and to show that cells may have differentiated, it is also important to demonstrate adherence. As seen in Fig. 24, PMVs were able to cause HL-60 cells to adhere to the culture plate in a dose-dependent manner with increasing amounts of PMV ranging from 5 to 30µg. PMA which is an efficient inducer of terminal differentiation was used as a positive control and showed a doubling of adherence albeit less than that achieved using 5µg PMVs.

4.2.4.3 PMV treatment of HL-60 cells induces increased expression of CD11b and CD14

When cells are differentiated along the monocyte/macrophage lineage, they show increased surface expression of CD14 and CD11b. If differentiation is along the dendritic cell lineage, then they express DC-SIGN (CD209). HL-60 cells were induced to differentiate using 30µg of PMVs or PMA by way of positive control for monocyte/macrophage differenation, and tested for surface expression of CD14 and CD11b by measuring Mean Fluorescence Intensity. As can be seen in Fig. 25A & B, there was a significant increase in expression of CD14 and CD11b, (also shown in Fig. 25D-F) compared to untreated controls. From Fig. 25C, the expression of DC-SIGN (CD209), a marker of differentiation along the dendritic cell lineage was negligible in the experiments with PMVs but showed a minimal expression level upon PMA treatment.

Fig. 24

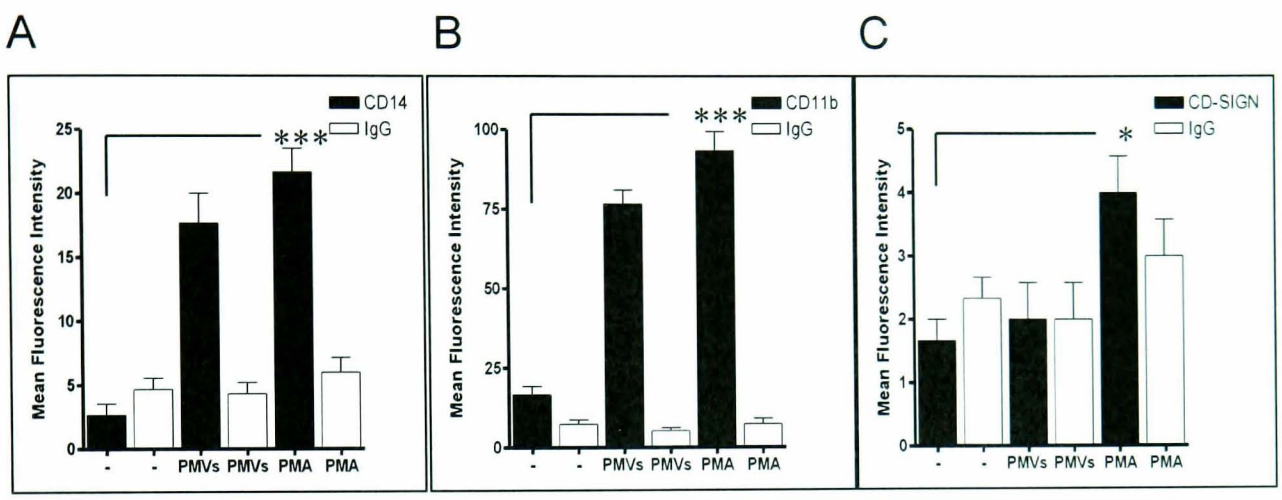
HL60 cells treated with PMVs show a dose-dependent Increase in adherence

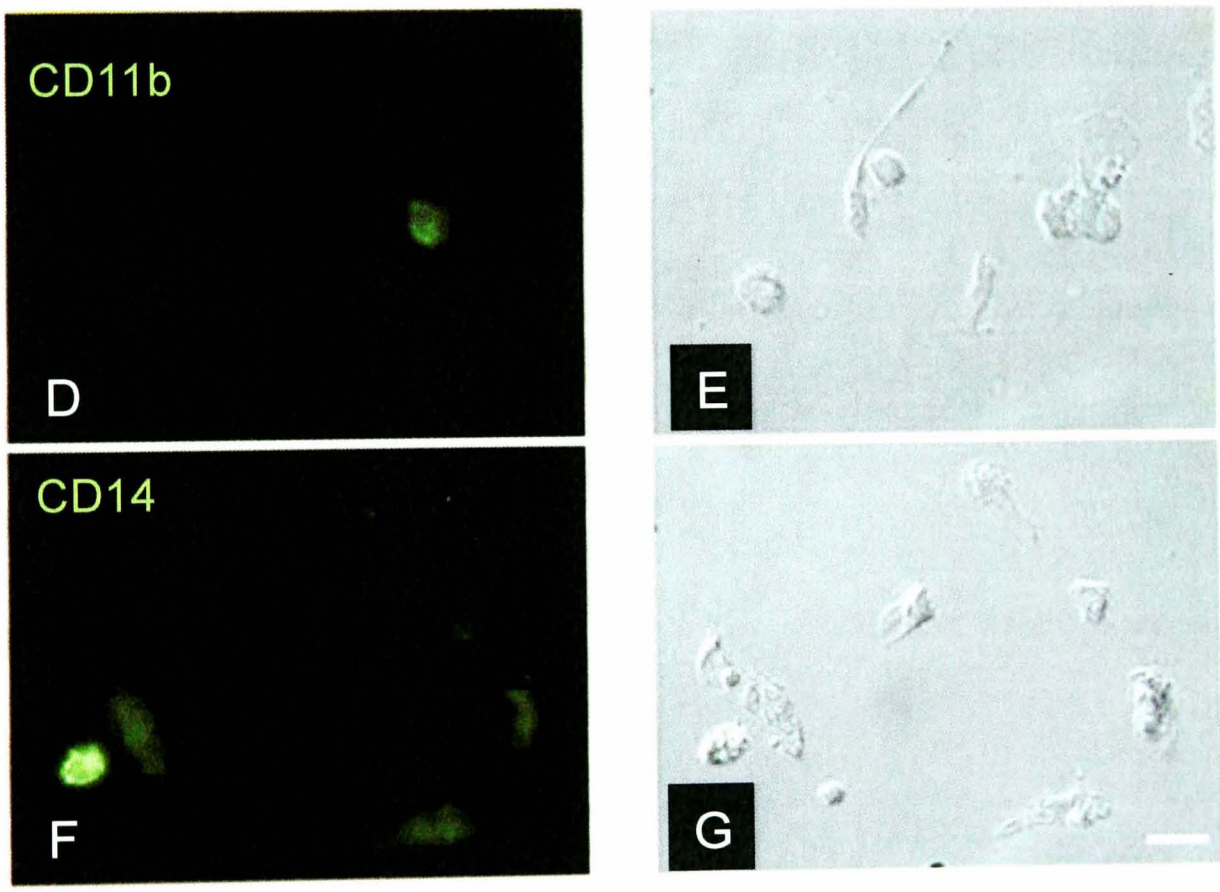


HL60 cells ($1x10^5$/ml) were treated with 5, 10 and 30 μg PMVs in a 24-well plate, in triplicate and after 24h the percentage adherence determined. The positive control cells were treated with PMA to induce terminal differentiation.

Fig. 25

PMVs induce the differentiation of HL60 cells as measured By the expression of CD11b and CD14





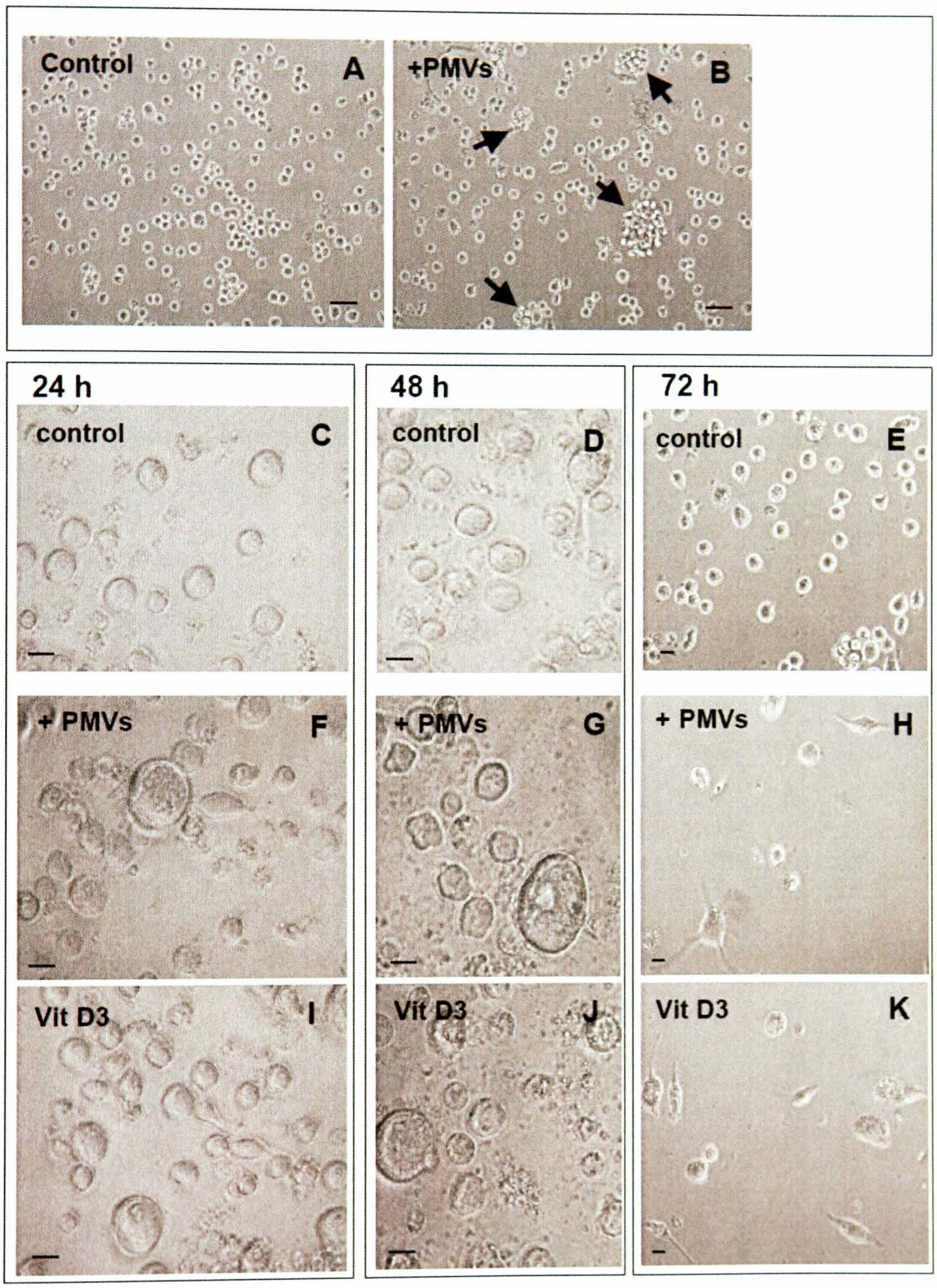
HL-60 cells were treated with PMVs (30 µg) or PMA. Mean Fluorescence Intensity as a measure of surface expression levels was monitored. In the case of CD14 (A) and CD11b (B), as indicators of differentiation along the monocyte/macrophage lineage, there was a significant increase in expression, compared to untreated controls. However, expression of DC-SIGN (CD209) as a measure of differentiation along the dendritic cell lineage was negligible (C). Differentiating HL-60 cells expressing CD11b and CD14 are also shown (in D-F) by Immunoflourescence microscopy, bright fields (BF) in (E) and (G). Bar, 10 µm

4.2.4.4 Morphological changes of HL-60 cells induced upon treatment with PMVs

When cells are induced to undergo differentiation, dramatic changes in the morphology occur which can be monitored as a sign of differentiation. For example, cells can become increasingly irregular in shape with membrane extensions resembling pseudopodia. Upon treatment of HL-60 cells with PMVs or vitamin D3, rapid changes in cell morphology, could be observed within 12h, the cells showing formation of aggregates (Fig. 26A and B). After 24h and 28h, the cells began to show signs of developing pseudopodia (Fig. 26F, G, respectively) as with vitamin D3 (Fig. 26I, J) compared to control. By 72h there were clear signs of differentiation (Fig. 26H for PMV treatment and K for vitamin D3 treatment. Controls are shown in Fig. 26C, D, and E.

Fig. 26

Morphological changes of HL-60 cells upon treatment with PMVs



Treatment of HL-60 cells with PMVs or vitamin D3 resulted in rapid changes in cell morphology, within 12 h the cells showing formation of aggregates (A and B). 24 h after treatment with PMVs (30 µg) the cells begin to show signs of developing pseudopodia (F, G); similarly upon vitamin D3 (I, J) treatment, compared to control. By 72h there were clear signs of differentiation (H for PMV treatment and K for vitamin D3 treatment). Controls are shown in C, D, and E. Bars A, B, 20 Tm. Bars C-K, 10 µm.

4.2.4.5 PMV treatment of HL-60 cells causes phosphorylation of the TGF-β/Smad signaling pathway

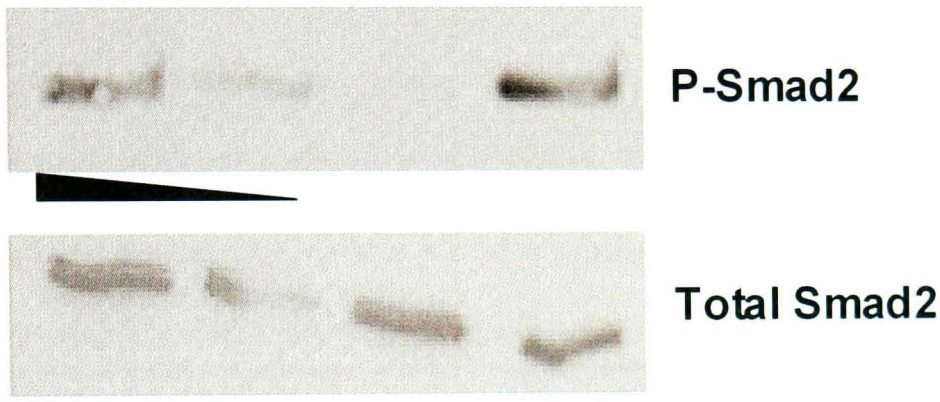
Depending on the stimulus, cellular differentiation is mediated through different signaling. In resting cells, Smad2 is present in its unphosphorylated form but is highly phosphorylated (P-Smad2) when induction of cellular differentiation is through the monocyte/macrophage pathway as opposed to the neutrophil or dendritic pathways. An experiment was carried out to show whether PMV-mediated differentiation of HL-60 is along the monocyte/macrophage pathway, which is accompanied by the phosphorylation of the TGF-β/Smad signalling pathway. If the differentiation was along any other pathway, then Smad2 would not be phosphorylated. As shown in Fig. 27A, increased expression of P-Smad2 was detected when cellular differentiation was caused by PMVs and vitamin D3 as compared to the negative control (no addition of PMVs) or upon treatment with ATRA, which induces differentiation along the neutrophil pathway. At the same time we can see almost equal expression of the unphosphorylated form (total Smad2) in all the tests. An increased expression of P-Smad2 upon differentiation with PMVs was also visualized by immunofluorescence microscopy, Fig. 27B.

To give an estimation of P-Smad2 immunoreactivity in addition to the data in Fig. 27A, obtained by immunoblotting of HL-60 lysates before and after addition of PMVs, image J software was employed to semi-quantifiy the expression of P-Smad2 on cells as obtained by immunoflourescence microscopy (Fig. 27B). Essentially this was used to calculate the area percentage and mean intensity of P-Smad2 immunoreactivity in the nucleus, obtained in ten microscopic fields of view. The mean, taken and illustrated graphically using GraphPad Prism 5.0, in Fig. 27C, implies an increase in phosphorylated Smad2 expression in HL-60 cells upon treatment with PMVs.

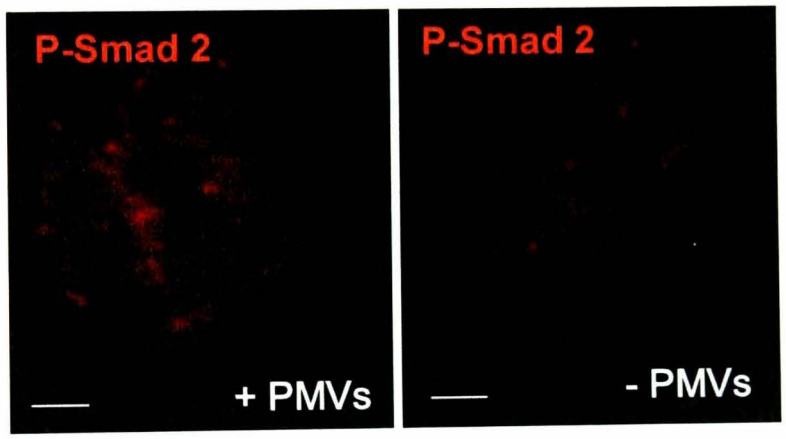
Fig. 27

Stimulation of HL60 cells to differentiate along the Monocyte/macrophage pathway by treatment with PMVs or vitamin D3 causes phosphorylation of the TGF-β/Smad signalling pathway

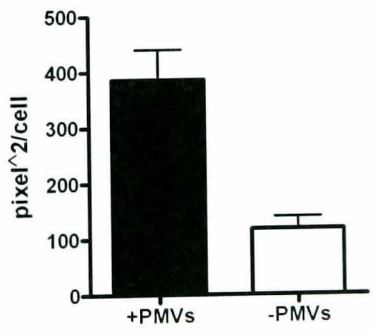
A



B



C



HL-60 cells treated with PMVs or differentiated along the monocyte/macrophage lineage by treatment with vitamin D3 have increased levels of phosphorylated Smad2. The level of expression of Smad 2 (total Smad2) was used as a control for the level of phosphorylated Smad2 (P-Smad2) This compares with negligible expression (control, no PMVs) and upon ATRA treatment (differentiation to neutrophils) (A). In (B), P-Smad 2 expression was also increased in HL-60 cells after treatment with PMVs, as seen by immunoflourescence microscopy. Bar represents 2 μm. (C) shows the P-Smad2 immunoreactivity estimated by ImageJ software.

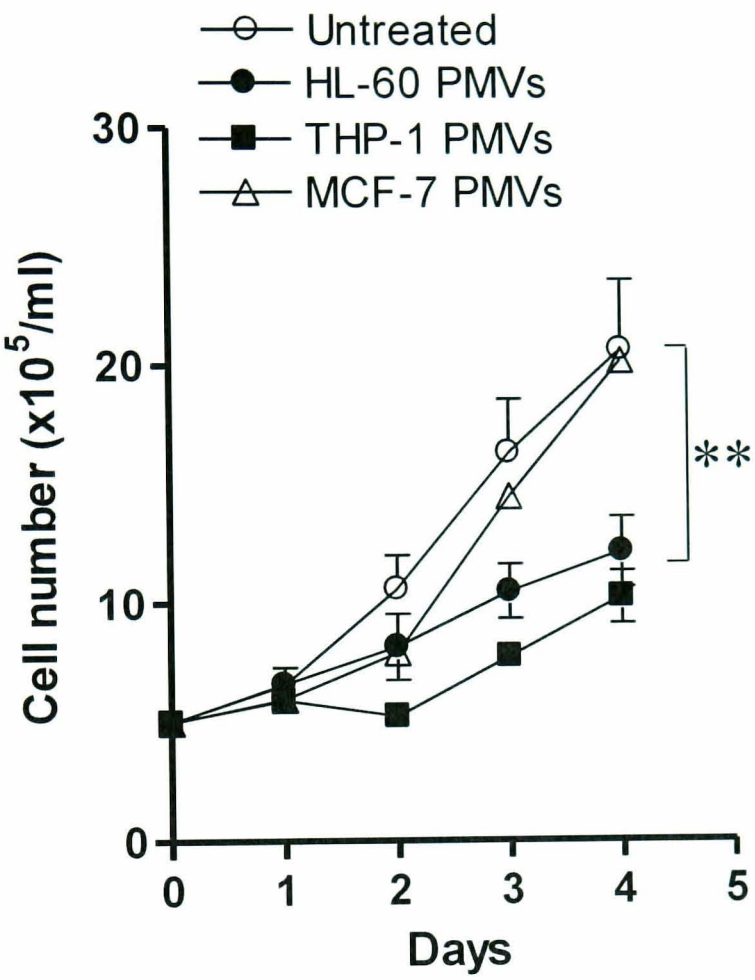
4.2.5 TGF-β1-bearing PMVs reduce the proliferation of HL-60 cells

4.2.5.1 PMVs from HL60 or THP-1 cells but not from MCF-7 cells significantly reduce the growth rate of HL-60 cells

Previously (section 4.3) it was shown that inducers of monocyte/macrophage differentiation stimulate the release of PMVs from HL-60 cells and that these increasing levels of released PMVs seem to result in decreased proliferation levels. It was noted that these PMVs would have been from only one cell type, HL60, so it was decided to treat HL-60 cells with PMVs isolated from different cell types, HL-60, THP-1 or MCF-7, or else left untreated. The cells were incubated at 37°C and quantified each day by flow cytometry. From Fig. 28, PMVs from HL-60 and THP-1 cells were more effective at reducing the growth rate than MCF-7 (breast cancer cell line) PMVs which resulted in normal growth close to control levels. As a result, it was clear that it was neither the release process or the PMVs themselves, regardless of which cell they were derived from that was important in inducing differentiation, but that the protein or some other factor (such as micro RNA) content of the PMVs may be important.

Fig. 28

Addition of PMVs from HL60 or THP-1, but not from MCF-7 cells significantly reduces the growth rate of HL-60 cells



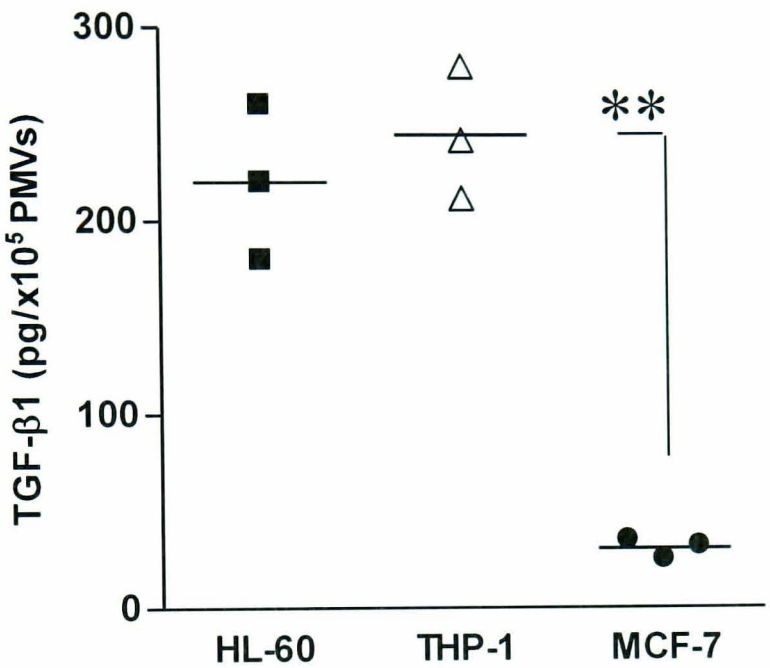
Application of PMVs (30 µg) from HL-60, THP-1 and MCF-7 cells to HL-60 cells ($5x10^5$ cells/ml) was monitored using the Guava EasyCyte flow cytometer for its effect on the growth of HL-60 cells over 5 days.

4.2.5.2 PMVs from different cell types carry different levels of TGF-β1

PMVs contain cell surface or membrane proteins as well as cytoplasmic components from their cell of origin. As PMVs are increasingly being described as mediators of intercellular communication and because of their observed effect in this thesis on promonocyte replication and terminal differentiation, it was appropriate to measure TGF-β1 which is known to play an important role during the differentiation of myeloid cells by inhibiting cellular proliferation. In this experiment PMVs were isolated from HL-60, THP-1 and MCF-7 cells, lysed with triton-X-100 (1%) and then applied to an ELISA system (R & D Systems) to measure the concentration of TGF-β1. The ELISA measurements show in Fig. 29 that PMVs obtained from both promonocytic cell types, HL-60 and THP-1, carry higher levels of TGF-β1 than those from breast cancer cell line, MCF-7.

Fig. 29

HL60 and THP-1 cells carry higher levels of TGF- β1 than MCF-7 cells



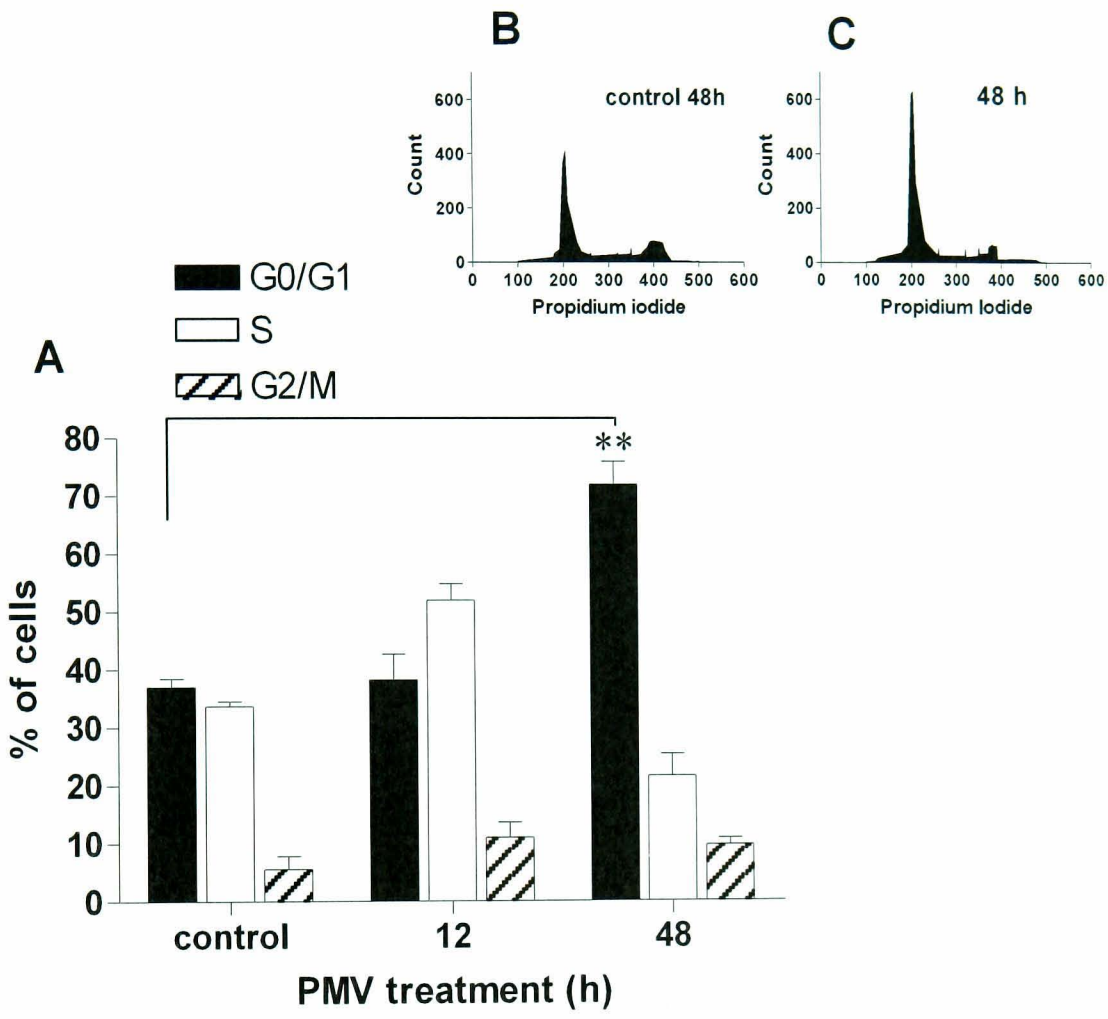
PMVs were isolated from HL-60 (promonocyte), THP-1 (promonocyte) and MCF-7 cells (breast cancer), lysed with triton-X-100 (1%) and then applied to an ELISA system (R & D Systems) to measure the concentration of TGF-β1

4.2.5.3 PMV treatment of HL-60 cells causes them to exit the cell cycle at G0/G1

For growing cells to differentiate, they need to undergo a growth arrest at some point usually the G0/G1 stage. It was observed, in section 4.5.1, that PMV-treated HL-60 cells had significantly reduced cell numbers after 3 days in culture. If therefore PMVs were causing the differentiation of HL-60 cells, they would first of all arrest the cells (at G0/G1) and then induce differentiation. To show whether this was the case, 30µg of HL-60 PMVs were added to HL-60 cells and propidium iodide (PI) staining analysed using a Guava EasyCyte flow cytometer. As Fig. 30A shows in comparison to controls, more cells were calculated to be in the S stage followed by those in G0/G1 and G2/M after 12 h. However, after 48 h almost all the cells were found to be in the G0/G1 stage the time frame at which differentiation begins to occur and when proliferation begins to diminish compared to untreated controls (section 4.5.1). Fig. 30B and C shows the level of PI staining after 48 h for control (B) and upon PMV addition, which are summarised in Fig. 30A.

Fig. 30

HL60 cells treated with PMVs exit the cell cycle at G0/G1



HL60 cells were treated with 30 μg of HL60 PMVs and after 12 and 48h analysed for their stage in the cell cycle by measuring propidium iodide staining in a Guava EasyCyte flow cytometer. A, shows a summary bar chart of the % of cells calculated as being in any particular stage of the cell cycle. B and C are representative plots indicating degree of propidium iodide staining at 48 h without prior addition of PMVs (B) Or following addition of 30 μg of HL60 PMVs .

4.2.5.4 The reduction in proliferation of HL-60 cells upon treatment with promonocyte PMVs is dependent on TGF-β1 delivered by the PMVs

Having suggested a role for TGF- β1 carried by PMVs in the reduction of cellular proliferation of HL-60 cells, we wanted to confirm this by using an antagonist that would abrogate this effect. Addition of SB-431542 an antagonist of TGF-βR-mediated signalling did indeed reversed the growth inhibition mediated by PMVs (Fig. 31). Addition of anti-TGF-β1 to HL-60 cells but not of the control Rb IgG, also abolished the inhibitory effect of PMVs significantly and restored the growth rate of the cells to almost control levels.

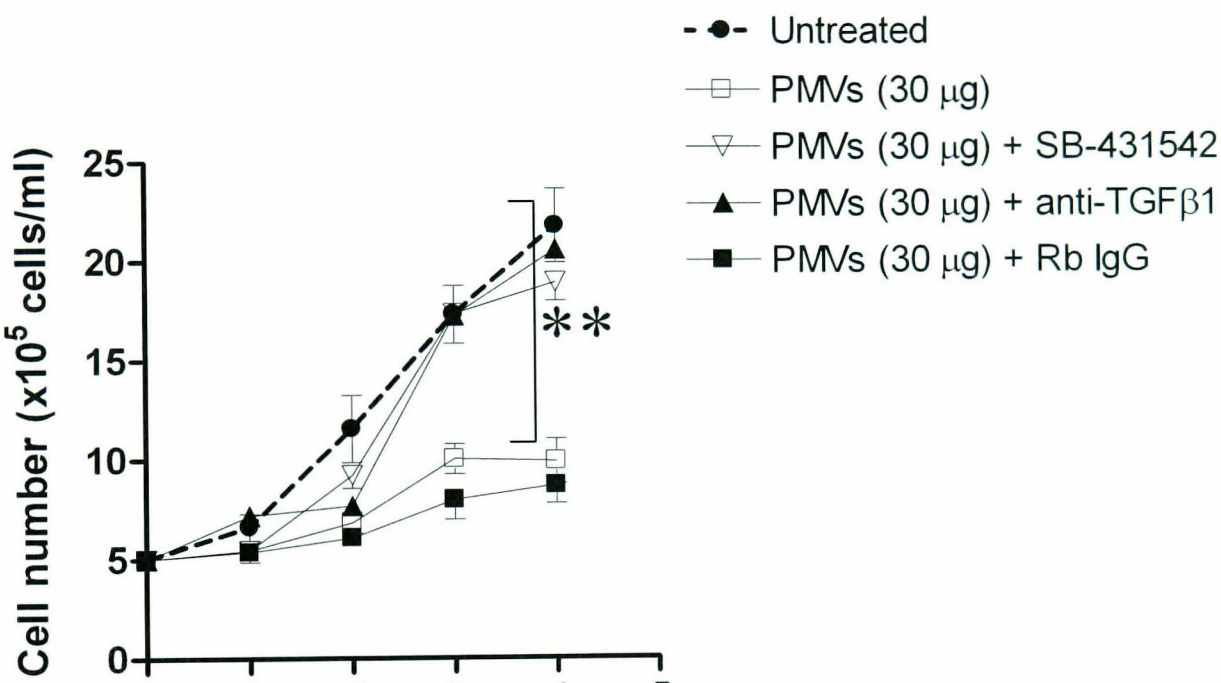
4.2.6 PMVs fuse with HL-60 cells

4.2.6.1 PMVs from Octadecyl rhodamine (R18) labelled HL-60 cells bind to the surface of HL-60 cells

PMVs transferred from one cell to another are believed to attach to the surface of the recipient/host cell and to undergo fusion (del Conde I *et al* 2005). In an attempt to show this for HL-60 cells, HL-60 PMVs were first labelled with octadecyl rhodamine (R18) (Fig. 32A). This label was apparently then transferred upon surface attachment to unlabelled HL-60 cells as shown in Fig. 32B. This suggests contact although not necessarily membrane fusion (lipid mixing). In Fig. 32C, we see control, unlabelled HL-60 cells that show no fluorescence. R18-labelled HL-60 cells, on the other hand, have uniform, strong surface labelling with R18 (Fig. 32D).

Fig. 31

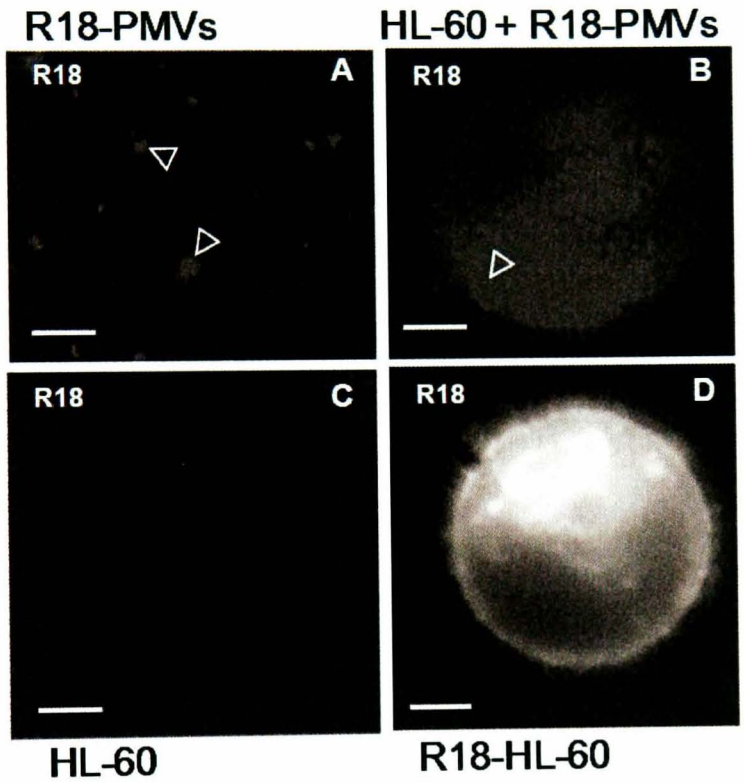
The reduction in proliferation of HL-60 cells upon addition of 30 μg PMVs can be reversed by pretreatment with the TGF-βR signalling antagonist SB 431542 or anti-TGF-β1



Application of HL-60-PMVs (30 μg) to HL-60 cells ($5x10^5$ cells/ml) in culture caused a marked reduction in the proliferation of HL60 cells which could be reversed by addition of 10 μg/ml anti-TGF-β1 or the TGF-βR antagonist, SB-431542.

Fig. 32

Octadecyl rhodamine (R18) labelled HL-60 PMVs bind to the surface of HL-60 cells



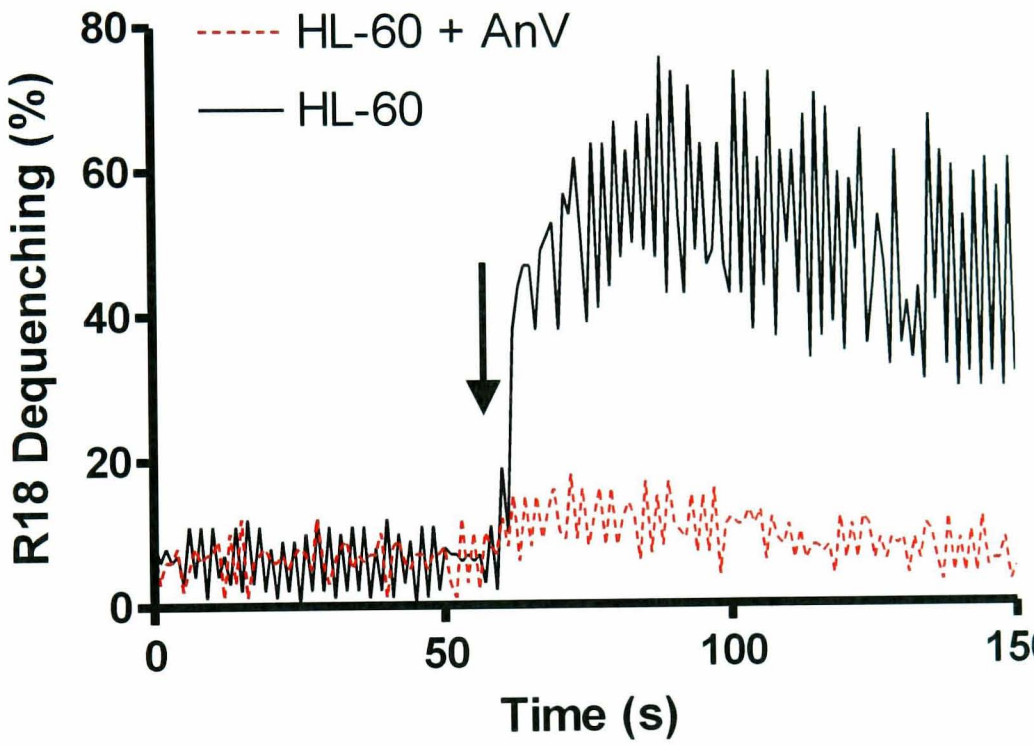
Addition of octadecyl rhodamine- (R18-) labelled PMVs, highlighted with arrowheads in (A) to HL-60 cells results in a transfer (arrowhead and surrounding difuse labelling of R18) to recipient unlabelled HL-60 cells (B). Control unlabelled HL-60 cells show no fluorescence (C). Control, R18-labelled HL-60 cells have a uniform, strong surface labelling with R18 (D). Bar, 2 μm.

4.2.6.2 R18-labelled PMVs added to HL-60 cells results in lipid mixing as a measure of membrane fusion

As a measure of membrane fusion to demonstrate the successful transfer of labelled PMVs to unlabelled cells, a 'lipid mixing' experiment was carried out. Lipid mixing upon addition of octadecyl rhodamine- (R18-) labeled PMVs to HL-60 monoyctes is indicated by rapid dequenching as determined spectrophotometrically (Fig. 33). This is probably mediated through PS interactions as preincubation of HL-60 cells with AnV abrogated the fusion.

Fig. 33

The addition of R18-labelled PMVs to HL-60 cells results in lipid mixing as a measure of membrane fusion



R18-labelled HL-60 PMVs were added to HL-60 cells. The R18 dequenching shows lipid mixing as a measure of membrane fusion. Lipid mixing is probably mediated through PS interactions as preincubation of HL-60 cells with AnV blocked fusion.

4.3 DISCUSSION

PMVs release by various cells has been implicated in a wide range of biological activities both from *in vitro* and *in vivo* studies (Piccin A *et al* 2007) with the most highlighted function being that of intercellular communication (signal transduction) (Ratajczak J *et al* 2006; Pap E *et al* 2009). This work has shown that the release of PMVs from cells is achieved through treatment with either agents used in differentiation therapy or stimulation with the deposition of sublytic complement. The studies herein also demonstrated that PMVs (isolated from HL-60 and THP-1 cells) carry TGF-β1 on their membrane surface. These TGF-β1-bearing PMVs can modulate the growth rate of HL-60 promonocytes *in vitro,* without inducing apoptosis, the cells exiting the cell cycle at G0/G1. In addition, dramatic changes in the morphology of PMV-treated cells were observed as they became increasingly irregular in shape with membrane extensions resembling pseudopodia.

When HL-60 cells and monocytes were treated with 5% NHS for 30 min at 37°C and released PMVs quantified, it was found that the promonocytic HL-60 cells released more PMVs than primary peripheral monocytes. Also, inducers of monocyte-to-macrophage differentiation such as histamine (10μM), ATRA (1μM) and PMA (0.1μM) had reduced proliferation of HL-60 cells whilst inducing differentiation. The agent that caused most differentiation such as PMA also caused the greatest number of cells to die. This trend was followed by histamine and then ATRA. Further experiments also showed an apparent inverse correlation between cell number and PMVs/ml released after treating HL-60 cells with ATRA (1μM), PMA (0.1μM) and histamine (10μM).

HL60 cells treated with HL-60 PMVs for 24 or 72h showed an increased capacity to reduce NBT, as a measure of superoxide production and myeloid differentiation, meaning that the PMVs were able to cause significant differentiation of the HL-60 cells. Similarly experiments measuring Smad2 expression showed increased levels of phosphorylated Smad2 in comparison with negligible expression for control (no PMVs) showing that the signalling pathway being stimulated is likely to be TGFβ1. Also HL60 cells treated with PMVs showed a dose-dependent increase in adherence to the culture plate when percentage adherence was determined after 24 hours.

HL-60 and THP-1 cells were shown to carry higher levels of TGF-ß1 than MCF-7 from ELISA measurements of TGF-β1 isolated from HL-60, THP-1 and MCF-7 cells and so when those PMVs were added to HL60 cells, they significantly reduced the growth rate as compared to PMVs from MCF-7 cells carry negligible TGF-ß1. In effect the HL-60 cells were induced to undergo differentiation and by so doing caused cell cycle arrest, so reducing cell number. This reduction of cellular proliferation of the HL-60 cells by the TGF-ß1 containing PMVs was however, able to be reversed by the addition of TGF-ßR signalling antagonist SB 431542 or anti-TGF-ß1.

The concept that surface TGF-β1 can stimulate TGF-β-mediated signalling on cell-to-cell contact or by implication in this study by PMV-cell contact is not new and has been described in T cells (Wahl SM and Chen W 2005). Regulatory T cells ($CD4^+CD25^+$) express active TGF-β1, which interacts with TGF-β receptor on effector cells, thereby mediating immunosuppression (Nakamura K *et al* 2001). Similarly, surface-bound, albeit latent TGF-β, on the surface of colorectal cancer (CRC) cells has also been implicated in immunosuppression following cell-cell

contact (Baker K *et al* 2008). Although activation may occur through various mechanisms, upon cell contact, latent TGF-β may be activated by alpha V integrins, and then mediating Smad signalling through the TGFβRI and TGFβRII receptors; MAPK and PI3K/Akt pathways may also be modulated.

Several inhibitors of TGF-β1 signalling have been reported as potential therapeutics in cancer immunotherapy. Amongst these is the TGF-β receptor type I (TβRI) antagonist, SB-431542, a selective inhibitor of endogenous activin, of TGF-β1 signalling (Yingling JM *et al* 2004), and of resulting phosphorylation of Smads. The tumor suppressor functions of TGFβ1 have also been reversed using SB-431542, in studies using the colon cancer-derived FET cells (Halder SK *et al* 2005) and the antagonist has been shown to inhibit the ligand-dependent growth of HT-29 colon cancer cells.

We have demonstrated TGF-β1-mediated involvement in this study by showing that SB-431542 almost completely abrogated the growth inhibition due to PMV-treatment of cells. That TGF-β1, on the surface of PMVs, could mediate such a change was confirmed by also reversing growth inhibition in the presence of anti-TGFβ1. To ascertain the relative importance of PMV release after stimulation with ATRA, PMA or histamine, in terms of limiting proliferation, it would be interesting to treat promonocytic cell lines with these agents following pretreatment with calpeptin, which specifically blocks calpain-mediated PMV release.

In another experiment, the addition of differentiation inducing agents histamine, ATRA and PMA caused a sharp increase in $[Ca^{2+}]_i$ in HL-60 cells signifying an attempt to produce PMVs by the HL-60 cells. Transfer of octadecyl rhodamine (R18) from R18-labelled HL-60 PMVs to recipient unlabelled HL-60 cells suggested

contact although not necessarily membrane fusion (lipid mixing). By contrast control R18-labelled HL-60 cells had uniform, strong surface labelling with R18. In Lipid mixing experiments, upon addition of R18-labeled PMVs to HL-60 promonoyctes, lipid is indicated by rapid dequenching as determined spectrophotometrically. This was probably mediated through PS interactions as preincubation of HL-60 cells with AnV abrogated the fusion.

Besides inducing cell cycle arrest, PMVs also induced the differentiation of HL60 cells as measured by the expression of CD11b and CD14. HL-60 cells were treated with PMVs (30μg) or PMA and mean fluorescence intensity as a measure of surface expression levels monitored. In the case of CD11b and CD14, as indicators of differentiation along the monocyte/macrophage lineage there was a significant increase in expression, compared to untreated controls. However expression of DC-SIGN (CD209) as a measure of differentiation along the dendritic cell lineage was negligible. Differentiating HL-60 cells expressing CD11b and CD14 were also shown by immunoflourescence microscopy. Morphological changes of HL-60 cells upon treatment with PMVs or vitamin D3 resulted in rapid changes in cell morphology. Within 12 hours, the cells showed formation of aggregates and after 24 hours they began to show signs of developing pseudopodia compared to control. By 72 hours there were clear signs of differentiation

4.3.1 Conclusion

TGF-β1 is unlikely to be the only factor that PMVs may convey to induce differentiation and so other factors carried by PMVs with the potential to induce differentiation, should now also be looked at, including Gal-3 and monocyte microRNAs, such as miRNA-155, shown to be involved in myeloid differentiation (Forrest ARR *et al* 2010). In view of the need to expand the limited range of

therapies in myeloid leukaemias, it might be prudent to look at other PMV-releasing agents, as potential alternative drugs in differentiation therapy against APL. There is every confidence that with more work over time, all questions regarding the role of PMVs on promonocyte/monocyte-macrophage differentiation and its use as an alternative treatment for promonocytic leukaemia would be answered.

CHAPTER 5

PMVs INHIBIT THE PHAGOCYTOSIS OF APOPTOTIC CELLS: ROLE IN SYSTEMIC LUPUS ERYTHEMATOSUS

5.1 INTRODUCTION

Phagocytosis is a well-coordinated sequence of events and a fundamental cellular process by which phagocytes of the immune system engulf and kill other cells or extracellular targets that are marked for removal. The phagocytes have a plethora of receptors to find and ingest these extracellular targets and clear them from tissues (Parham P 2009). They carry out this ubiquitous process by wrapping cytoskeletally supported pseudopodia around the targets and internalizing them into vacuoles called phagosomes which then fuse with a lysosome, forming a phagolysosome (Gregory DC and Devitt A 2004; Parham P 2009). Digestion of the engulfed or phagocytosed particles in an oxidative burst by lysosomal enzymes occurs leading to hydrolyzed products which are assimilated by absorption into the cytoplasm through the vacuolar wall. Waste products resulting from the process are then excreted from the cell (Gregory DC and Devitt A 2004).

A number of distinct steps such as activation and chemotaxis of the phagocyte, binding or attachment of the phagocyte to the cell or particle, internalisation and digestion/destruction of the cell or particle by the phagocyte are involved in phagocytosis. One main point however is, before phagocytosis is accomplished, the phagocyte and the particle to be ingested must adhere to each other, an act that depends largely on the morphological and biochemical nature of the cell or particle surface (Vandivier RW *et al* 2006; Parham P 2009). If the phagocyte cannot adhere directly, then some proteins from the blood can form a surface film on the particle or bacterium to which phagocytes adhere, and phagocytosis follows. Macrophages and neutrophils are the most effective phagocytic leukocytes both of which are drawn toward an area of infection by means of substances (chemoattractants) given off by the invading object and the infected tissue or by a chemical interaction between the

infectious agent and the complement system of blood serum proteins (Vandivier RW *et al* 2006).

When cells become apoptotic, they need to be removed from the blood system. This is achieved through a form of phagocytosis called efferocytosis, ('burying of dead cells') (Abid-Hussein MN *et al* 2003), a mechanism, which does not provoke inflammatory or immune reactions and an essential feature of the immune system. Necrotic and secondary necrotic cells, derived from uncleared apoptotic cells (ACs) due to defective phagocytosis are by contrast proinflammatory and associated with autoimmune disease (Balasubramanian K *et al* 1997). In order for ACs not to progress to necrosis and burst and release their content into the blood stream so initiating or enhancing autoimmune disease, any blood component such as Plasma membrane-derived vesicles (PMVs) that can cause phagocytosis to be defective needs to be investigated; hence the work described involving PMVs and their interference in this process.

PMVs released from the plasma membrane (PM) of most cells (Albert ML *et al* 2000) carry PM-associated proteins and a host of intravesicular proteins including cytokines, growth factors and acute phase proteins, as well as messenger RNA (mRNA) and microRNA (miRNA) (Mishima, Y *et al* 2009). There is a range of stimuli that result in PMV release but key amongst these is an increase in intracellular calcium and the exposition of phosphatidylserine on the outer leaflet of the releasing cell and the resultant PMV. The presence of basal levels of PMVs is common in healthy individuals, and is estimated, in peripheral blood, to range between 5 - 50 μg/ml ($2x10^5$ – $2x10^6$ PMVs/ml).

PMVs play a role in various physiological functions and have been linked with a wide range of clinical conditions (Barry OP *et al* 1998), particularly where there is persistent apoptosis coupled with defective clearance of apoptotic cells (Barry OP *et al* 1997; Berckmans RJ *et al* 2001). Whether PMVs are direct contributors of such diseases or are a reflection of the disease remains to be determined. However, this provides an important tool in measuring the protective effect of therapeutic intervention in a non-invasive manner and a knowledge of PMV levels in plasma may serve as an indicator of cell stress and injury and thus, in the future, help monitor disease treatment (Berckmans RJ *et al* 2002).

Systemic Lupus Erythematosus (SLE) is a prototypic autoimmune disease and the most common form of the lupus disease in which there is a deficient phagocytosis of ACs. The resulting secondary necrosis leads to production of autoantibodies such as antiphospholipid antibodies (aPLA), which can further block phagocytosis and enhance disease progression. In SLE patients with secondary antiphospholipid syndrome (APS) there are increased numbers of plasma PMVs (Bettina T *et al* 2007). Similarly, in patients with aPLA it is believed that aPLA-mediated endothelial injury or activation leads to increased PMV release (Boulanger CM *et al* 2006). Because both PMVs and ACs express phosphatidylserine (PS) on the outer leaflet of the plasma membrane, and as PS is a marker for phagocytosis of ACs by macrophages, we set out to ascertain if the PMVs have any significant effect on this process, essentially to see whether the PMVs inhibit the phagocytosis of ACs by competing for the PS receptor on macrophages.

SLE often harms most of the body systems including blood vessels and the liver. The course of the disease is unpredictable, with periods of illness (*flares*) alternating with remissions. Like lupus in general, it occurs more often in women than in men,

especially between the ages of 15 and 50, and is more common in those of non-European descent. SLE is treatable through addressing its symptoms, mainly with corticosteroids such as cortisol which control carbohydrate, fat and protein metabolism and are anti-inflammatory by preventing phospholipid release, decreasing eosinophil action and a number of other mechanisms (Hodkinson B *et al* 2009). Immunosuppressants such as cortisone, azathioprine and cyclosporine are also used. SLE can be fatal, although with recent medical advances, fatalities are becoming increasingly rare with survival for people approximately 95% at five years, 90% at 10 years, and 78% at 20 years. Currently there is no definitive cure.

SLE has no one specific cause. There are however a number of environmental triggers and genetic susceptibilities that can cause it to start (Hemminki K *et al* 2009). These lead to the body's immune system producing antibodies against itself, particularly against proteins in the cell nucleus (Mendoza-Pinto C *et al* 2009). SLE is the prototypical autoimmune disease with all the key components of the immune system involved in the underlying mechanisms according to (Rahman A and Isenberg DA 2008; Syuto T *et al* 2009). From an evolutionary perspective, a population must have enough genetic diversity to protect itself against a wide range of possible infection; some genetic combinations result in autoimmunity but the immune system must have a balance (homeostasis) between being sensitive enough to protect against infection, and being too sensitive and attacking the body's own proteins (autoimmunity) (Asanuma Y *et al* 2003).

5.2 PMVs and phagocytosis of apoptotic cells

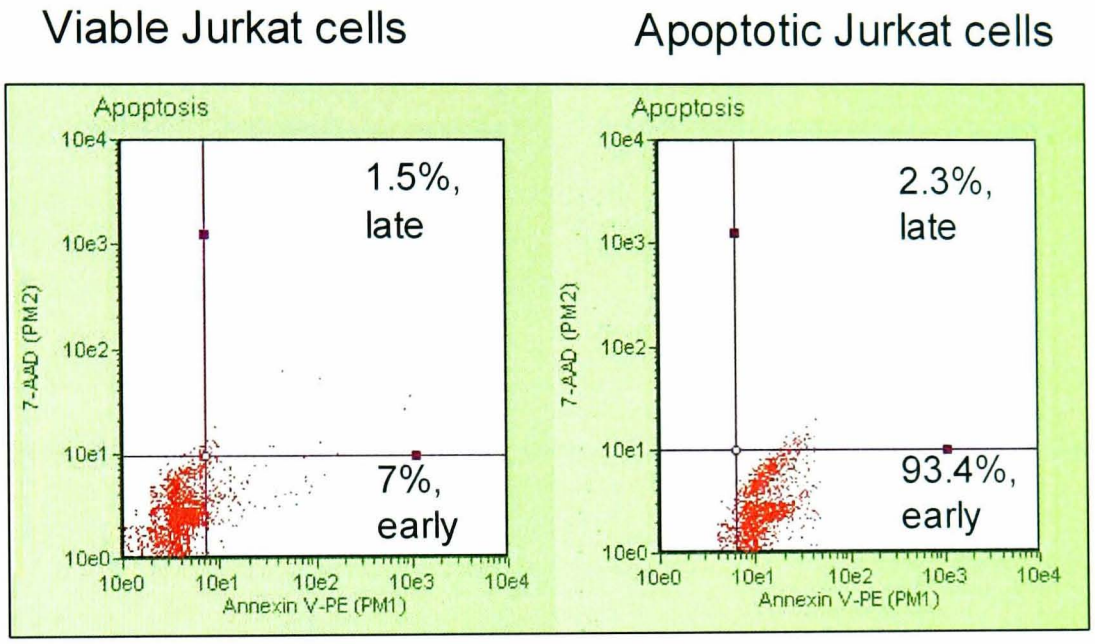
5.2.1 Induction of early apoptosis in Jurkat cells

To be able to show phagocytosis of apoptotic cells (AC), early apoptotic cells were produced from FITC-labelled Jurkat cells by inducing the cells with 0.1 μM PMA for 3 days. The cells were then washed 3x with PBS to remove extra PMA and apoptosis measured by FACs using the Guava Nexin assay to measure the level of staining with annexin V as an indicator of early apoptosis and with 7-AAD as a measure of late apoptosis. Fig. 34A is a plot of the FACS analysis showing untreated viable cells and apoptotic cells. The lower left quadrant indicates viable cells, the lower right quadrant early stage apoptosis, the upper right quadrant late stage apoptosis and the upper left quadrant the number of dead cells. In the first dot plot which is for untreated FITC labelled control Jurkat cells, 90.6% of cells were viable, 7.0% in early stage apoptosis, 1.5% in late stage apoptosis and 0.9% of the cells were dead. In the plot of FITC labelled Jurkat cells treated with 0.1 μM PMA 3.7% of cells were viable, 93.4% cells were in early stage apoptosis, 2.3% in late stage analysis, and 0.6% of the cells were dead. The % of FITC-labelled apoptotic cells was judged to be 88% as represented in the histogram obtained from the Guave EasyCyte flow cytometer.

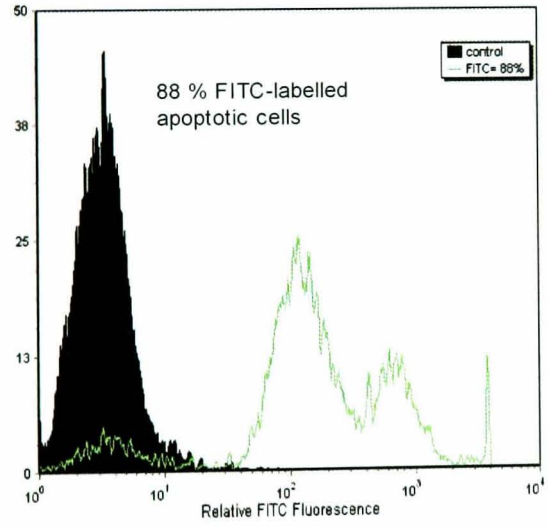
Fig. 34

Induction of early apoptosis in Jurkat cells

A



B



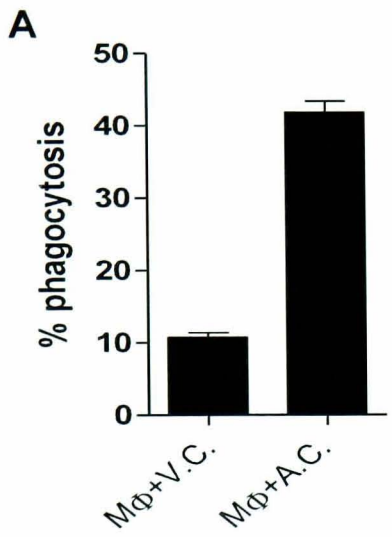
Jurkat cells were treated with 0.1 μM PMA for 3 days. To determine apoptosis the cells were treated with the Nexin assay (Guava, Millipore) to measure the level of staining with Annexin V as an indicator of early apoptosis and with 7-AAD as an indicator of late apoptosis (A). Apoptotic cells were then stained with FITC, giving levels of 88% labelling (B).

5.2.2 Phagocytosis of FITC-labelled apoptotic Jurkat cells by THP-1-derived macrophages

Phagocytosis assays were carried out to quantify the phagocytosis by macrophages of apoptotic cells. The aim was to establish whether PS is necessary for the phagocytosis of apoptotic cells (AC). An experiment to show this was performed with viable Jurkat cells (PS^-) and apoptotic Jurkat cells (PS^+) and both analysed by FACS (Guava Express plus) and fluorescence microscopy. The phagocytosis index was calculated as the percentage of macrophages containing at least one ingested apoptotic Jurkat cell. As can be seen in Fig. 35A, the percentage phagocytosis of AC (43%) was higher than that of viable cells (VC) (~10%). Fig. 35B is a representative fluorescent image showing phagocytosed AC (identified by a green FITC fluorescence) and also indicated by arrow heads. Essentially this experiment showed that the phagocytosis of apoptotic Jurkat cells is significantly increased as compared to that of viable Jurkat cells at 37°C (**, $p < 0.05$). This implies that phosphatidylserine is necessary for phagocytosis as only AC have PS on their surface to facilitate their uptake and without PS, only a few of the VC were phagocytosed.

Fig. 35

THP-1-derived macrophages phagocytose FITC-labelled apoptotic Jurkat cells



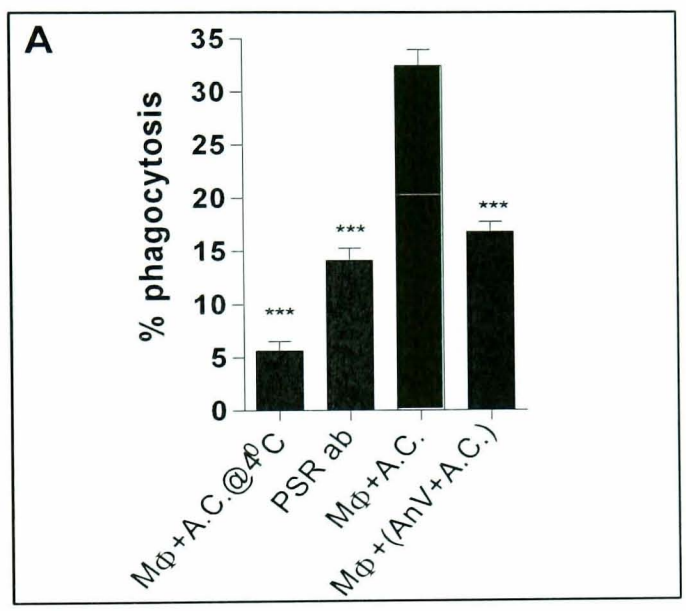
THP-1-derived macrophages were fed FITC-labelled viable and apoptotic Jurkat cells (A). The % phagocytosis was determined by microscopy, a representative field for phagocytosis of apoptotic cells being shown in B.

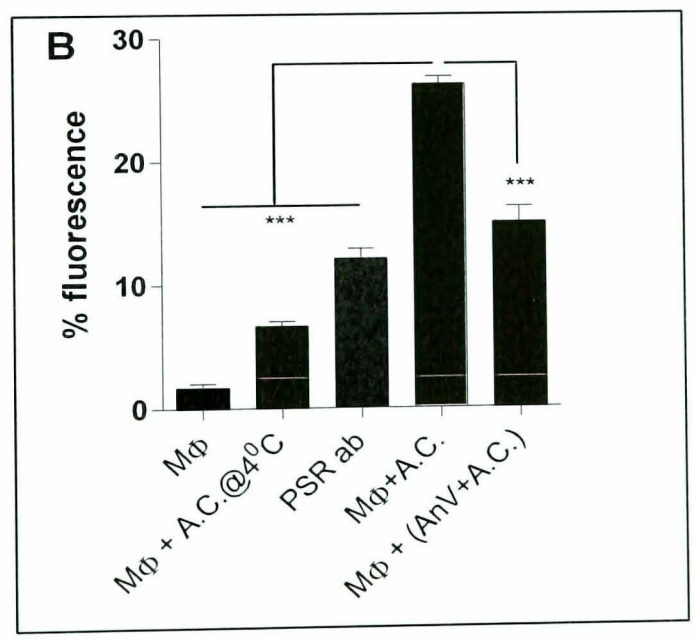
5.2.3 Demonstration that the phagocytosis of FITC-labelled apoptotic Jurkat cells is mediated through the interaction of phosphatidylserine on apoptotic cells and phosphatidylserine receptor on macrophages

Phosphatidylserine (PS) is thought to be the main factor by which, through an interaction with PS receptor (PSR) on macrophages, AC are phagocytosed. To show this, macrophages were preincubated with PS receptor antibodies to see if they could competitively compete with the PS on ACs and the receptor on the macrophages. From Fig. 36 A and B, it was observed that this caused a great reduction in the phagocytosis index of the apoptotic cells. A similar effect was seen when the AC were pre-incubated with AnV which through binding the PS on the ACs was able to out compete the PS-PSR interaction between AC and macrophages. Meanwhile, phagocytotic activity was unaffected in the experiment with no inhibitors thus confirming the importance of PS and PSR in the uptake and phagocytosis of AC.

Fig. 36

The phagocytosis of FITC-labelled apoptotic Jurkat cells is mediated through the interaction of phosphatidylserine on PMVs and PSR on macrophages





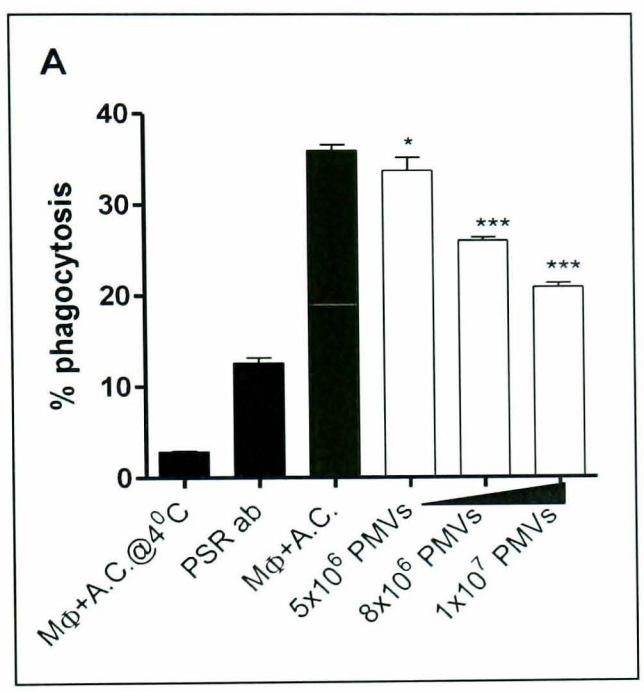
Phagocytosis of 4°C or in the presence of phosophatidylserine receptor-blocking antibody, or using AnV to block PS on ACs abrogates phagocysosis

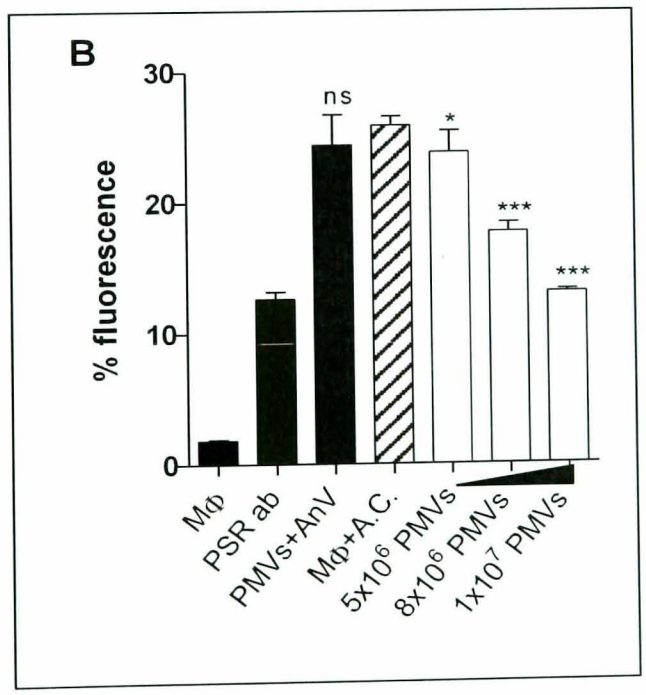
5.2.4 Can the additon of PMVs inhibit the phagocytosis of FITC-labelled apoptotic Jurkat cells?

PMVs carry PS on the outer leaflet of the plasma membrane as do AC, and as AC are phagocytosed through a PS-PSR interaction, it was conceivable that the presence of PMVs may have an effect on their uptake. To determine this, we carried out phagocytosis assays in the presence of increasing concentrations of PMVs, PMVs blocked with AnV, or AC blocked with PSR and assays carried out at 4°C were analysed microscopically and by FACs. Fig. 37 A and B shows the effect of PMVs on phagocytosis of AC. PMVs ($5x10^6$, $8x10^6$ and $1x10^7$) were added to $5x10^6$ ACs in a ratio of 1:10, 1:16 or 1:20 and it was seen that s can be seen, that increasing concentrations of PMVs reduced the phagocytosis of the AC and that at 4°C little or no phagocytosis occurred. This indicates that PMVs at high concentrations could have an inhibitory effect on the uptake of AC by macrophages and indeed that PMVs show a dose dependent inhibitory effect on the phagocytosis of ACs. As a negative control, the PS receptor antibody also was able to inhibit phagocytosis significantly (p value of 0.001) compared to the assay without PMVs and the negative control.

Fig. 37

The phagocytosis of FITC-labelled apoptotic Jurkat cells can be inhibited dose dependently by addiiton of PMVs





PMVs ($5x10^6$, $8x10^6$ and $1x10^7$) added to $5x10^6$ ACs in a ratio of 1:10, 1:16 or 1:20 respectively dose-dependently inhibit phagocytosis of ACs.

5.3 SLE plasma and PMV release

5.3.1 Do antiphospholipid antibody (aPLA) plasma samples induce PMV release?

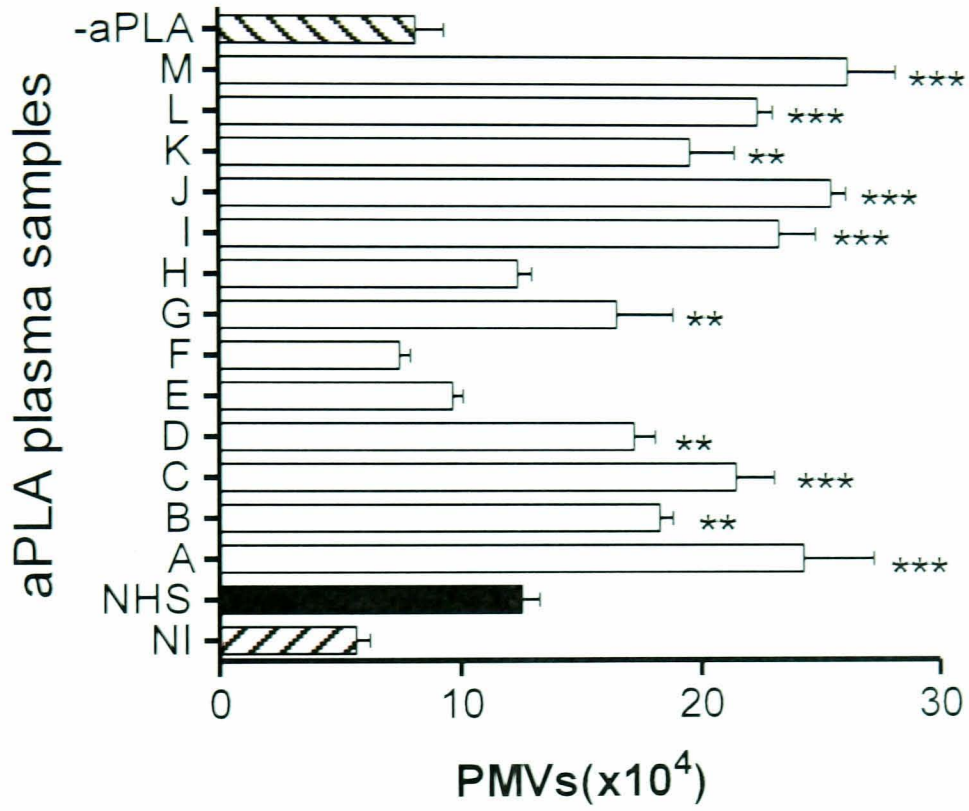
From other, detailed work in the lab, and as outlined earlier in chapter 3 (section 3.2) it was found that PMV release can be stimulated by sublytic complement (C5b-9) or Membrane Attack Complex (MAC) deposition and that Classical Pathway-mediated deposition of sublytic MAC is initiated by antibody binding. The aim now was to show whether the anti-phospholipid antibodies (aPLA) in Systemic Lupus Erythematosus (SLE) stimulate PMV release. An experiment was therefore carried out with plasma from SLE patients that had been tested qualitatively and shown to contain aPLA (versus those without aPLA) to induce PMV release from apoptotic or viable Jurkat T cells. From Fig. 38, it can be seen that PMV release induced using plasma from SLE patients was significantly increased as compared to that induced with non-SLE patients plasma (-aPLA), NHS and also the experiment with no induction. This implied that the anti-phospholipid antibodies (aPLA) in SLE stimulate PMV release, and that the raised levels of PMVs in plasma of SLE patients may accentuate the disease by inhibiting phagocytosis of AC in the way we have shown.

5.3.2 Do SLE plasma samples have increased levels of PMVs?

Increased numbers of PMVs have been identified in individuals with certain diseases including SLE, the quantity and composition dependent on the type of disease. The following experiments were carried out to estimate quantitatively the level of PMVs in plasma of SLE patients with aPLA using Guava ExpressPlus. From Fig. 39, it can be seen that almost all the samples known to contain aPLA showed increased amounts of PMVs as compared to those from non-SLE patients and therefore containing no aPLA.

Fig. 38

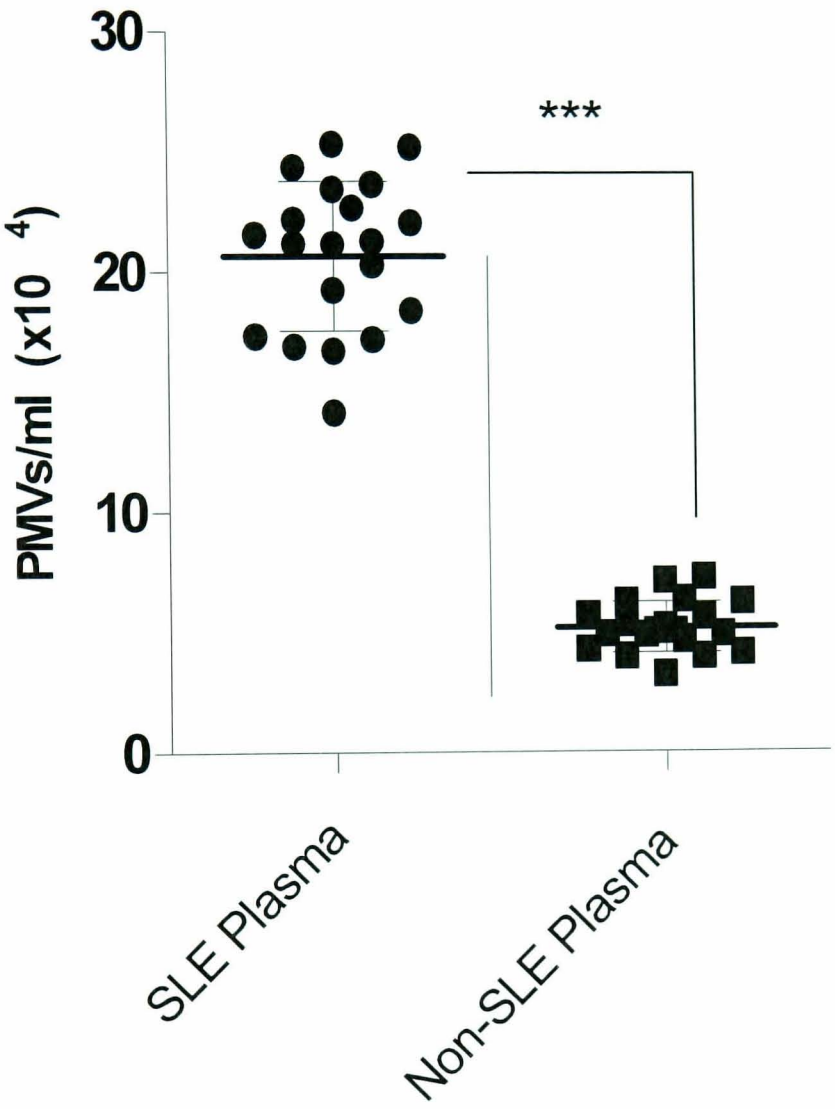
aPLA plasma samples induce the release of PMVs



A cohort of aPLA plasma samples, compared to NHS and plasma without aPLA show a general raised level of PMVs released from Jurkat cells.

Fig. 39

SLE plasma samples have increased levels of PMVs



SLE plasma levels of PMVs are higher than those of non-SLE plasma, n=20.

5.3.3 The effect of depletion of aPLA autoantibodies from SLE plasma on the level of PMVs released from Jurkat cells

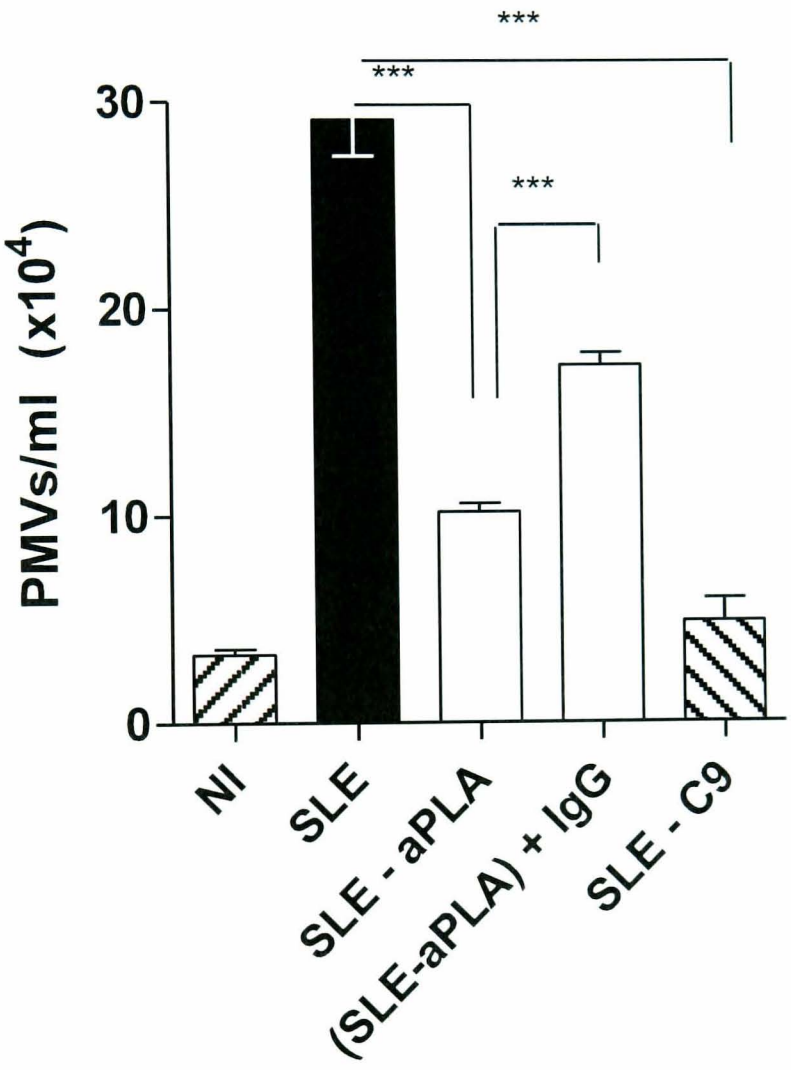
Having shown that the presence of aPLA causes the release of PMVs, it was necessary to confirm that these antibodies are required for the release of PMVs. Therefore, SLE plasma was depleted of aPLA or complement C9 using an IgG removal kit and anti-C9 and used for PMV induction. The depletion of the antibodies caused a reduction in PMV release from early apoptotic jurkat cells. Restoration with IgG only partially restored the induction effect showing that the presence of the aPLA antibodies was the reason the plasma were able to induce the release of PMVs. The additional need for complement factors was shown by the significant reduction of PMV release upon C9 depletion (Fig. 40).

5.3.4 The number of PMVs released was increased by the effect of adding aPLA autoantibodies from SLE plasma to NHS

As in the previous experiment, NHS was depleted of immunoglobulin or complement C9 and then tested for the ability to induce PMV induction. The depletion of immunoglobulin caused a reduction in PMV release from early apoptotic jurkat cells and their replenishment in the form of aPLA restored the capacity to induce PMV release (Fig. 41) confirming that the presence of the antibodies was the reason the plasmas were able to induce the release of PMVs.

Fig. 40

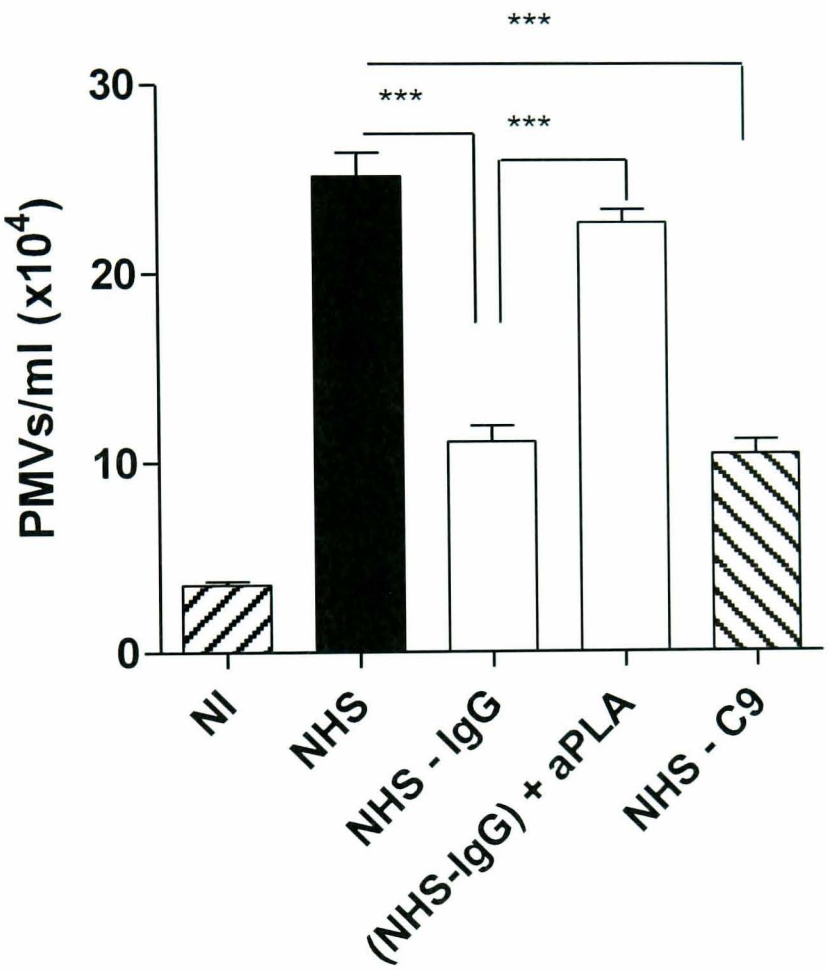
Depletion of aPLA autoantibodies from SLE plasma reduces the level of PMVs released



Depletion of aPLA or complement C9 from SLE plasma reduces PMV release from early apoptotic Jurkat cells and this is only partially restored by adding non-specific, control IgG (1 mg/ml).

Fig. 41

Addition of aPLA autoantibodies from SLE plasma to NHS increases the number of PMVs released



Depletion of IgG or complement C9 reduces PMV release and this is partially restored upon addition of purified aPLA (1mg/ml).

5.4 DISCUSSION

The effective removal of apoptotic cells (ACs) by phagocytes is not just limited to safe removal of injured, aged or infected cells but is crucial to processes ranging from embryogenesis, tissue homeostasis, and cellular immunity to the resolution of inflammation (Albert ML *et al* 2000). It has also been well established that in certain clinical conditions such as cystic fibrosis, as well as chronic autoimmune diseases (Boulanger CM *et al* 2001; Brechot N *et al* 2008) there is a defect in the clearance of ACs. Recently there has been a rise in the interest of the role of PMVs in physiological conditions (Brüne B 2003) and on this basis, this investigation reports that PMVs may play a role in the reduction of phagocytosis of ACs by macrophages. Various studies have shown that PMVs express high concentrations of PS on the outer leaflet of the plasma membrane when they are released via blebbing and shedding following the loss of phospholipid membrane asymmetry on the parent cell (Butikofer P *et al* 1989).

Apoptotic cells also express PS on the outer leaflet, this being one of the main signals on ACs for phagocytosis by macrophages (Brüne B 2003). Blocking of PS interaction with PS receptor on phagocytes will likely inhibit the phagocytosis of ACs significantly (Mattson MP 2003; Chargaff E *et al* 1946). These studies also showed there to be a significant reduction in phagocytosis of ACs after incubating macrophages with a PS receptor antibody whilst enrichment of cell membranes with exogenous PS results in the phagocytic clearance of Jurkat cells (Chen G, Goeddel DV 2002). On the basis of these findings, an explanation for the dose-dependent reduction in phagocytosis by PMVs observed could be that PS on PMVs in binding to PS receptors (PSRs) on macrophages directly competes with PS on ACs. This was confirmed by blocking the PS on PMVs with annexin V, which abrogated PMVs' inhibition of AC phagocytosis.

Experiments carried out included the induction of early apoptosis in Jurkat cells, which were used for the subsequent phagocytosis assay. The first phagocytosis assays was carried out to establish the fact that PS is necessary for the phagocytosis of ACs and the assay was performed with viable (PS^-) and apoptotic (PS^+) Jurkat cells. The results showed that there was a significant increase ($p = 0.0013$) in the mean percentage phagocytosis of apoptotic cells (43%) compared to viable cells (~10%) as shown in Fig 35A. The use of phsophatidylserine receptor-blocking antibody and AnV to block PSR and PS on macrophage and ACs respectively both abrogated the phagocytosis emphasising the fact that the phagocytosis of the apoptotic Jurkat cells is mediated through the interaction of phosphatidylserine on ACs and PSR on macrophages.

The next experiment showed that increasing concentration of PMVs reduced the phagocytosis of the ACs, which was observed via FACS and further confirmed by microscopy. This indicates that PMVs have a dose effect on the phagocytosis of ACs and that at high concentrations show a higher inhibitory effect on the uptake of the ACs by the macrophages *in vitro*. aPLA plasma samples were also shown to have a general raised level of PMVs released from Jurkat cells as compare to NHS. SLE plasma levels of PMVs were also higher than those of non-SLE plasma when samples from volunteers were analysed. However, the depletion of aPLA or complement C9 from SLE plasma reduced PMV release from early apoptotic Jurkat cells and this is only partially restored by adding non-specific, control IgG (1 mg/ml). Also depletion of IgG or complement C9 from NHS reduces PMV release and this is partially restored upon addition of purified aPLA (1mg/ml) to the NHS.

Our findings do have certain clinical implications. It has been well established that increased apoptosis occurs in a variety of clinical settings such as during

chemotherapy, and radiation therapy (Cheng EH 2003; Chiarugi A, Moskowitz MA 2002) as well as certain autoimmune diseases such as rheumatoid arthritis, and SLE mainly due to a defect in apoptosis (Hermann M *et al* 1998). In addition to this, ACs themselves have been shown to produce PMVs which in turn can induce further PMV production (Cohen JJ *et al* 1992). In these cases, the already high level of ACs would rise still further as more AC-derived PMVs would reduce the phagocytosis of ACs leading to their secondary necrosis, thereby intensifying inflammation, cell injury and worsening the disease prognosis.

If defective phagocytosis in SLE might be associated with increased PMV levels, then anti-phospholipid antibody (aPLA) or SLE plasma samples would be expected to have elevated levels of PMVs. Such raised levels of PMVs could themselves be due to the presence of increasing levels of sensitizing autoantibodies such as aPLA and sublytic complement (Boettner DR *et al* 2008). In general, as expected, we found raised PMV levels in both aPLA plasma (Fig. 38, n=13) and SLE plasma (Fig. 39, n=20) compared to normal controls. To test whether the aPLA component of SLE plasma samples was able to stimulate PMV release from early ACs through the stimulation of sublytic complement, we were able to show that removal of aPLA reduced PMV production, which was then only partially replenished with 1 mg/ml human IgG (Fig 40). That the SLE-induced PMV release from ACs was due to complement activation was confirmed by depletion of complement C9 (Fig 40).

5.4.1 Conclusion

This study has shown firstly that high concentrations of PMVs significantly reduce the phagocytosis of ACs by competing with the normal PSR-PS mediated phagocytosis of ACs. Secondly raised plasma PMV levels are symptomatic of SLE and may well play a role in disease progression. Whilst autoantibodies such as

aPLA may help stimulate PMV release through sublytic complement, this will need further investigation. Where other particular cell types, undergoing apoptosis and needing appropriate and efficient removal to avoid inflammatory damage occur, our findings on the role of PMVs in the PS-dependent removal of ACs become pertinent. For example, in neural circuit development, axon pruning is essential because apoptotic neurons need to be efficiently removed by glial phagocytes (Connor J *et al* 1992). Together our findings add to the novel roles being described for PMVs in normal cell physiology and disease.

CHAPTER 6

DISCUSSION

6.1 PMV characterisation

PMVs shed from both normal and cancerous cells may serve as a means of intercellular communication as they carry proteins, lipids and nucleic acids derived from the host cell (Piccin A *et al* 2007; Ahn YS *et al* 2004). Their isolation and analysis from blood samples has the potential to provide information about the state and progression of malignancy in terms of cancer. PMVs are also likely to prove of great clinical importance as biomarkers for a variety of disease states in other words of potential diagnostic value as well as a therapeutic tool (Ratajczak J *et al* 2006). However, a standardized protocol for isolation has not yet been agreed upon. It is often unclear what the content of the isolates are and whether the isolated PMVs, were present *in vivo* or whether they were created during the isolation procedure. Vesicular structures, which are sized from 1 μm down to 50nm, are present in isolates of many body fluids. Isolates of PMVs sometimes contain different populations that differ in size and shape, which indicates that methods of isolation and determination of the number of PMVs in the peripheral blood need to be elaborated and improved upon (Halder SK *et al* 2005).

A difficulty in the study of PMVs has been comparing results between laboratories. Since PMVs exist as heterogeneous species and express several different cell surface markers, which can vary according to the cell of origin and depending on the stimulus that caused their release, it is unlikely that any single marker will efficiently label all PMVs (Nantakomol D *et al* 2008). Thus, the need for precise and reproducible quantification is necessary if PMVs are to be a reliable marker for diagnosis (Jy W *et al* 2004). To date, the measurement and detection of levels of PMVs in various conditions have not translated into therapeutic or diagnostic strategies in the management of disease conditions in which they have been shown to be relevant (Gritters M *et al* 2005). However they have helped to shift the

understanding of the pathophysiological mechanisms of several diseases such as sickle cell and thrombotic thrombocytopenic purpura. The detection of chronically elevated levels of circulating PMVs in patients with sickle cell disease provides an insight into the chronic endothelial attack that characterises this condition, and may provide an important tool in measuring the protective effects of therapeutic interventions in an early and non-invasive manner (Allan D *et al* 1980).

6.2 Methods of PMV analysis

6.2.1 Flow cytometry

Although several methods for analysis of PMVs have been reported, conventional Flow-cytometry and ELISA are the most widely adopted, with flow cytometry (FACS) the commonly used method for analysing the number, size, and properties of PMVs (Shet AS *et al* 2003). With flow cytometry, it is possible to determine the number of events (forward scattering) and the density of the events (side scattering) allowing quantification of the PMVs identified in each case (Boulanger CM *et al* 2006). Antibodies to cell surface molecules allow the identification of specific PMV subpopulations as in the case of FACS analysis of cells, but because of the size of PMVs, the amount of any surface marker is drastically reduced in comparison to intact cells, thereby limiting detection. Again because of the sizes, detection is limited by 'noise' in the flow cytometer as conventional FACS instruments are mostly designed to measure cells, which are 10-to 100-fold greater in diameter than PMVs (Piccin A *et al* 2007). In addition to proteins, the presence of phosphatidylserine on the outer leaflet of the PMV plasma membrane allows binding of annexin V, which is also used to identify and enumerate the PMVs via FACS.

Some investigators identify PMVs with a forward angle light scatter smaller than the 1-1.5 μm latex beads most instruments use as an internal standard (Shet AS *et al* 2003) whilst others also employ a lower size limit of 100 nm and any particles detected by the flow cytometer under this size are not considered true PMVs leading to underestimation (Boulanger CM *et al* 2006). Using the upper size limit criteria could also lead to an underestimation of true PMV numbers as some investigators consider PMVs to be up to 2 μm in size (Piccin A *et al* 2007). As different flow cytometers are used to analyse PMVs, it may be possible that the flow cytometer technique and the way they are used in terms of interpreting results could have a greater part to play in PMV analysis. For example, laser alignments can differ and different instruments capture PMVs in different ways. Bench top flow cytometers such as FACSCalibur have a fixed laser in constant alignment needing no human adjustment whereas FACSVantage SE uses removable lasers, which need manual adjustment (Becton, Dickinson and Company, 2002).

For capture, FACSCalibur systems use a device referred to as a catcher tube to sort PMVs. The catcher moves in and out of the sample stream to collect a population of desired PMVs, with a maximum rate of 300 per second (Becton, Dickinson and Company, 2002). The laser alignment and stream velocity are fixed, making the time it takes for PMVs to travel from the laser to the catcher tube constant. Conversely, the FACSVantage SE isolates a cell of interest by vibrating the stream. The sample stream vibrates and separates the sample into drops, with the distance between drops being fixed. When the sheath velocity and the vibration speed are constant, the pattern of drop formation is fixed, allowing the distance between drops to be calculated followed by PMV isolation (Becton, Dickinson and Comapny, 2002). Even though no known studies appear to have used FACSVantage SE to analyse PMVs, highlighting such differences alerts investigators prior to undertaking experiments

that flow technology and its incorrect implementation could lead to erroneous results.

6.2.2 Solid phase binding assays

An alternative method for identifying PMVs involves binding assays in a solid-phase or microtitre plate format. In this approach, antibodies to cell surface molecules can capture particles for subsequent detection by another antibody or a functional assay (Dignat-George F *et al* 2009; Horstman LL *et al* 2004). While such assays can assess the total amount of PMV-related material in a specimen, they cannot provide information on the number or size of particles. PMVs analysis can be impacted not only by type of assay, but also by the manner in which sample (blood) is collected and processed, including sampling site, needle diameter, centrifugation, re-suspension and washing of the isolated PMV pellet (Lee YJ *et al* 2008). Also it is important to ensure that platelets are removed, since they interfere with PMV detection, giving false positive signals. At present, PMV analysis is constrained by lack of standardized PMV assays (Reich CF *et al* 2009; Hind E *et al* 2010).

6.3 Importance of proper PMV level estimation

PMVs constitute a dynamic circulating storage pool by themselves, able to induce vascular responses to pro-apoptotic, inflammatory, or thrombotic stimuli. Therefore, the clinical background associated with elevated PMVs levels (Beyer C *et al* 2010; Morgan BP *et al* 1985) or accelerated clearance should be taken into close consideration in deciphering their multiple effects. For instance, misleading quantification or phenotypes could result from accelerated degradation by secretory phospholipase A2 (Bianchi SM *et al* 2008), interactions with the vascular wall (Herrman M *et al* 1998), or trapping in cell–cell aggregates or within the thrombus (Baumann I *et al* 2002). An additional complexity recently observed was

that circulating PMVs may bear antigens from different cellular origin, pointing to multiple transcellular PMVs-mediated exchanges (Cocucci E *et al* 2010). Antigens specifically expressed during cell activation could prove useful in identifying the various pathways of PMV release and discriminating underlying pathologies and associated damages. The circulating levels of PMVs may have a direct pathophysiological role in the development of several diseases and thus quantitation of PMV levels may serve as an early diagnostic screening tool for those diseases (Piccin A *et al* 2007).

Although increased PMVs, compared to healthy controls, have been associated with increased severity of diseases such as β-thallasaemia and Sickle Cell (Pattanapanyasat K *et al* 2004), there is only evidence to support a circumstantial association at this stage as studies are only quantitative and do not look into the specific role of PMVs in disease states and their contribution to pathogenesis. Hopefully, more experiments that focus on the function of PMVs in disease states will emerge in the future to give concrete evidence of the role PMVs play in diseases. A recent study on erythrocyte PMVs and other circulating PMV subtypes in sickle cell disease found that they did not detect Tissue Factor$^+$ (TF$^+$) PMVs, monocyte (CD14$^+$) PMVs (MPMVs) or endothelial cell (CD 144$^+$, CD146$^+$, CD62E$^+$) PMVs (EPMVs) in their samples (Van Beers *et al* 2009). Contrastingly, another group did not just detect TF$^+$ PMVs, EPMVs and MPMVs, but concluded that the observed increase gave weight to the hypothesis that sickle cell disease is an inflammatory state with endothelial cell and monocyte activation along with abnormal vessel wall activity (Shet AS *et al* 2003). Unsurprisingly, both groups used different methods for PMV isolation (Allan D *et al* 1982).

6.4 PMVs and their constituents

Owing to the plasticity of the lateral organization of the plasma membrane into raft domains, known to segregate particular proteins and lipid species, a given stimulus can be expected to elicit the release of PMVs that carry microRNA (Hunter MP *et al* 2008), mRNA, numerous membrane proteins, bioactive lipids and cytoplasmic constituents, characteristic of their parental cell which are implicated in a variety of fundamental processes (Piccin A *et al* 2007). PMVs also carry the majority of non-conventionally secreted proteins released into culture supernatants, and potentially carry them within the PMVs themselves (Mackenzie A *et al* 2001; Flieger O *et al* 2003) helping in the export of proteins lacking a signal peptide (Nickel W 2005; MacKenzie A *et al* 2001) as an alternative to conventional protein export. Amongst these, epimorphin, fibroblast growth factor 1 and 2 (FGF-1 and FGF-2), macrophage migration inhibitory factor (MIF) and galectin 3 (Gal-3) are all transported to the plasma membrane via the adenosine triphosphate cassette transport channel (ABCA1) needed for the release of PMVs or by exocytosis of exosomes (Flieger O *et al* 2003).

The potential of PMVs to transmit proteins between cells play an important part in intercellular communication and the maintenance of homeostasis under physiological conditions, or initiation of deleterious processes in the case of excess or when carrying pathogenic constituents. Most recently PMVs derived from embryonic stem cells (ESC) were found to carry Wnt-3, which is involved in hematopoietic differentiation, and such PMVs were shown to reprogramme hematopoietic progenitor cells (Ratajczak J *et al* 2006).

The ability of cells stimulated by sublytic complement to release PMVs has been demonstrated in this thesis by work showing that these PMVs carry surface molecules such as TGF-β1 with which they in turn cause promonocytic/monocytic cells to differentiate along the monocyte/macrophage differentiation lineage. PMVs can therefore be considered a disseminated storage pool of bioactive effectors, the nature and proportion of the latter accounting for duality, more particularly evidenced in vascular disease, inflammation, and immunity.

6.5 PMVs as a differentiation agent (Differentiation therapy)

The significance of the local effect of PMVs on different cell types and in physiological conditions has recently attracted growing attention since a great variety of biological consequences of the action of PMVs in the immune response and inflammation have been revealed (Gasser O and Schifferli J 2004). For example, the presence and type of stimulatory signals determine whether promonocytes/monocytes acquire dendritic cell or macrophage characteristics and functions and in this study the effect of PMVs on the induction of differentiation of promonocytes into macrophages was clear.

In recent times much research has been carried out on differentiation agents as cancer therapies whereby they are able to differentiate the immature promonocytic forms into mature ones to halt their proliferation and so the leukaemia (Tallman MS *et al* 2008). The work described herein was to find out how PMVs induce differentiation of promonocytes to macrophages and so prevent their proliferation and whether this could provide the underlying principle by which PMVs may be used for treatment of myeloid leukaemia.

Since ATRA, PMA and histamine are all involved in cellular differentiation, and therefore leukaemia treatment, we also investigated their effect on the terminal differentiation of HL-60 to monocytes/macrophages. NBT assay with statistical analysis again confirmed these agents (two of which are already in use in cancer therapeutics) to be good inducers of differentiation. Until recently, the idea of restoring normal differentiation in primitive leukaemic cells seemed unrealistic until the effect of ATRA in APL was shown. This is because chemotherapy and hematopoietic stem-cell transplantation were the only therapeutic options available in acute leukaemia but most APL patients are now treated first with ATRA since it became the first differentiation agent found to be successful in APL treatment. The differentiation process was observed through a change of morphology, appearance of cell surface markers such as CD14 and CD11b, which are known typical macrophage markers and an NBT assay.

It was also shown that attachment of PMVs to HL-60 cells is PS mediated, as with contact (and fusion) of monocyte-derived PMVs with platelets, (del Conde I *et al* 2005), but the attachment/fusion is not a prerequisite for the observed differentiation. Although contact with or phagocytosis of apoptotic cells and PMVs by macrophages has been shown to release TGF-β1, at levels of 80 pg/ml (Fadok VA *et al* 1998) and 350 pg/ml respectively, importantly, such release and autocrine-like action of TGF- β1 on the HL-60 cells, resulting in the observed reduction in proliferation (and consequent differentiation), is unlikely to have occurred in this study, as low TGF- β1- carrying MCF-7-derived PMVs were unable to elicit significant reductions in proliferation (Gasser O and Schifferli J 2004).

Macrophages can be identified by specific expression of a number of proteins including CD14 and CD11b. CD14 acts as a co-receptor (along with the Toll-like

receptor TLR 4 and MD-2) for the detection of bacterial lipopolysaccharide (LPS) and although LPS is considered its main ligand, CD14 also recognizes other pathogen-associated molecular patterns (Fadok VA *et al* 1998). CD11b on the other hand is a 170 kDa α_M or Integrin alpha M (ITGAM) subunit of CD11b/CD18 heterodimer (Mac-1, αMß2 integrin or CR3). CD11b functions as a receptor for complement (iC3b), fibrinogen, or clotting factor X (Gasser O and Schifferli J 2004). It mediates inflammation by regulating leukocyte adhesion and migration and has been implicated in several immune processes such as phagocytosis, cell-mediated cytotoxicity, chemotaxis and cellular activation. Even though CD11b is directly involved in causing the adhesion and spreading of cells, it cannot mediate cellular migration without the presence of the β2 (CD18) subunit.

The discovery of tissue-specific microRNAs (miRNAs) has also provided further proof that there are more agents capable of causing cellular differentiation (Calin GA, Croce CM 2006). The very nature of their action, i.e. the ability to simultaneously extinguish the expression of a multitude of genes and negate their functions, immediately suggested therapeutic promise. miRNAs are expressed in a tissue-specific manner and play an important role in maintaining tissue-specific functions and cellular differentiation with respect to cancer, where they are often located in genomic unstable regions and therefore are typically downregulated (Chuang JC, Jones PA 2007). They are able to restore the myogenic differentiation program and then block the tumorigenic phenotype, and even possess the power to shift global mRNA expression patterns with respect to differentiation signatures (Calin GA, Croce CM 2006).

Differentiation therapy has shown a great deal of promise but has its shortfalls since ATRA and other agents with the capacity to induce differentiation of leukemic blasts

in vitro have not shown efficacy *in vivo* in acute leukemias other than APL even though in many types of acute leukemia, disruption of genes involved in cell proliferation and differentiation has been observed (Shen ZX *et al* 1997). The successful treatment of APL with differentiation therapy however, gives hope that agents targeting those genes especially by down-regulating fusion proteins will be able to restore differentiation of other types of leukemic blasts in the near future.

6.6 Effect of PMVs on phagocytosis of apoptotic cells

Differentiated cells eventually lose their ability to divide and replicate, become apoptotic and die. Apoptosis is therefore a conserved cellular process that functions to target cells for phagocytosis by macrophages leading to their removal from the body (Kerr JF 1972). This physiological event is of fundamental importance because it ensures that cells are removed before plasma membrane integrity is lost and cytosolic molecules are released into the extracellular milieu. Leakage of intracellular molecules is thought to cause the induction of potentially harmful inflammatory responses (Sauter B *et al* 2000; Voll RE *et al* 1997). Indeed, most autoimmune diseases may have aetiologies associated with impaired uptake of apoptotic cells (Bott M *et al* 1998; Herrmann M *et al* 2000).

A number of molecules have been identified as being involved in the timely phagocytosis of apoptotic cells by macrophages. On the surface of the macrophage, receptors specific for phosphatidylserine (PS) (Fadok VA *et al* 2000), CD14 (Devitt A *et al* 1998), CD91 (Ogden CA *et al* 2001), and scavenger ligands (Bird DA *et al* 1999), have been implicated as important mediators of macrophage recognition of apoptotic cells. Integrins such as CD11b/CD18 heterodimer on the surface of macrophages appear to promote adhesion (Finnemann SC *et al* 1999; Moffatt OD *et al* 1999) and generate signals necessary for the phagocytosis of apoptotic cells. In

addition, members of the complement system (Gershov D *et al* 2000; Mevorach D *et al* 1998) thrombospondin and protein β2 glycoprotein I in serum have been demonstrated to bind apoptotic cells and facilitate their removal.

6.7 Phagocytosis – Role of phosphatidylserine

The only phagocytosis-associated change known to occur on the apoptotic cell surface is the translocation of PS from the inner leaflet to the outer leaflet of the plasma membrane (Fadok VA *et al* 1992; Verhoven B *et al* 1995). This event often requires caspase activation and is observed early during apoptosis hence the use of early apoptotic cells in these studies (Martin SJ *et al* 1995). Substantial evidence indicates that PS is a key recognition moiety that targets many types of apoptotic cells for phagocytosis. Recently, Fadok and colleagues demonstrated that the introduction of PS into the external leaflet of the plasma membrane of healthy cells could target cells for phagocytosis by macrophages (Fadok VA *et al* 2001). This finding suggests that PS expression on the surface of cells is sufficient for ensuring their removal by macrophages. However, it does not preclude the possibility that other elements on the apoptotic cell surface may also promote phagocytosis either in combination with PS or on their own.

We carried out this study to determine if the blocking of the link between the externalized PS on the surface of PMA-treated Jurkat ACs and its receptor (PSR) on the surface of macrophages by PS bearing PMVs correlated with the inability of the cells to be phagocytosed by the macrophages. These experiments required several steps including the differentiation of monocytes to macrophages, induction of PMVs from Jurkat cells, FITC labelling and apoptosis induction of Jurkat cells and a series of phagocytosis assays using PMVs as well as different inhibitory agents.

The monocyte cell line was used to derive macrophages for the phagocytosis assay, as the macrophages derived from this cell line resemble native monocyte–derived macrophages. After the incubation period (see section 2.7), the wells were analysed under an Olympus inverted fluorescent microscope. More than 90% of cells were adherent to the bottom of the well, cellular proliferation had decreased and morphological changes typical of macrophages were noticed. These include a macrophage like appearance with a large irregular shape, occurrence of a large number of intracellular vacuoles, and the presence of pseudopodia which is known to be a classic characteristic of macrophages as shown in Fig. 26.

6.8 Phagoctosis assay

The next stage of the experiment was the induction of PMVs from Jurkat cells. It is known that PMVs are released by cells as part of normal cell function, however, the production is fairly low and experiments done by other members of CMIRC (data not shown) have shown that the release of PMVs can be increased by exposing Jurkat cells to 2mM $CaCl_2$ and 5% NHS for half an hour. Following incubation, PMVs were isolated by ultracentrifugation and quantified via FACS (refer to section 2.6 for the procedure in detail). From the FACS analysis (refer to section 2.5.2), an FSC vs. SSC dot plot was observed that was similar to the dot plot described in this thesis (Fig. 11) and by others in previous studies.

To visualise the phagocytosis of ACs they needed to be labelled. However from preliminary experiments (data not shown) we noticed that by labelling apoptotic cells directly, the background fluorescence was too high and the overall labelling was not effective. The washing process after labelling was also very stressful on the apoptotic cells and cells were being lost in large numbers. It was therefore decided to label the cells before inducing apoptosis. Data obtained from FACS analysis

showed that cells incubated with the FITC dye gave 88% positive events compared to the control (unlabelled Jurkat cells), which gave 2.9% positive events on the GRN Hlog scale (Fig. 34B) under the same settings. This therefore indicates that 85% of Jurkat cells were successfully labelled with the FITC dye, which was maintained through apoptosis induction and opsonisation. After successfully labelling the Jurkat cells with FITC, the next step was to induce apoptosis (refer to section 2.9) and following incubation, the cells were washed and analysed via FACS (Guava Nexin, Section 2.5.3). Results from FACS analysis showed 93.4% of the Jurkat cells treated with 0.1μM PMA had undergone early apoptosis (2.3% late stage) (Fig 34A).

Having obtained macrophages, PMVs and labelled opsonised ACs, phagocytosis assays were then carried out. To establish that PS is necessary for the phagocytosis of ACs, viable jurkat cells (-PS) and apoptotic jurkat cells (+PS) were anaylsed both by FACS (Guava Express plus) and fluorescence microscopy. In the microscopy analysis, 500 macrophages were counted per well while noting the number of macrophages that had internalised at least one apoptotic cell. The average was then taken and a phagocytosis index calculated (mean percentage of macrophages that had phagocytosed at least one apoptotic cell). The results showed that there was a significant increase ($p = 0.0013$) in the mean percentage phagocytosis of ACs (43%) compared to wells with viable cells (~10%) as shown in Fig. 35A. Fig. 35B represent fluorescent microscopy pictures showing FITC-labelled apoptotic cells (green) phagocytosed by Hoescht dye-labelled macrophages (blue).

The next phagocytosis assay was performed whilst varying temperature, and in the presence of PSR antibodies and AnV. FACS and microscopy analysis showed that very little phagocytosis occurred at 4°C as expected. PSR antibodies also led to decreased phagocytosis through occupation of PS receptors on the macrophages

and prevention of ACs attachment to the macrophages via the PSR. An experiment with AnV also showed a decrease in phagocytosis due to its occupation of PSR binding sites, having attached to PS on PMVs. The Phagocytosis index in the assay without any inhibition was however quite high as there was no interruption between the PS on the apoptotic cells and their receptors (PSR) on the macrophages.

When PMVs were added to the phagocytosis assay, lower concentrations of PMVs did not show a significant effect on phagocytosis, but increasing the concentration of PMVs reduced the phagocytosis of the AC by FACS and further confirmed by microscopy. A possible explanation for this effect could be that at lower concentrations PMVs are not able to effectively compete with ACs probably due to size or inability to occupy all the receptors on the macrophages and ACs are phagocytosed almost as normal. However at high concentrations, the macrophages are saturated with PMVs as they are more readily exposed to the PS on the abundant PMVs thereby reducing the clearance capacity of the macrophages and thus uptake of ACs leading to effective inhibition of phagocytosis. This indicates that PMVs have dose-dependent effect on the phagocytosis of ACs.

6.9 PMVs and PS

Various studies have shown that PMVs express high levels of PS on the outer leaflet of the plasma membrane when they are released via blebbing and shedding following phospholipid membrane asymmetry rearrangement from the parent cell (Piccin A *et al* 2007). On the other hand, ACs have also been shown to express PS on the outer leaflet that is considered to be one of the primary phospholipids through which they are phagocytosed by macrophages (Fadok VA *et al* 2000). Inhibition of PS on ACs has been shown to inhibit phagocytosis significantly, therefore confirming the fact that PS plays an important role in phagocytosis (Fadok Va *et al*

2000; Fadok VA 1998). Further to this, Kagan *et al* 2002 have also shown that enrichment of the cell membrane of various viable cell lines including Jurkat cells with exogenous PS resulted in the clearance of these cells via phagocytosis (Kagan VE *et al* 2000; Kagan VE *et al* 2002). Interestingly, we observed a significant reduction in phagocytosis of ACs on incubating macrophages with a PS receptor antibody.

6.10 SLE- role of PMVs

Other studies have shown or established the presence of high concentrations of ACs in certain disease conditions including rheumatoid arthritis, Systemic Lupus Erythematosus and in clinical settings such as during chemotherapy and radiation therapy primarily due to increased production and a defect in clearance or phagocytosis of ACs (Herrmann M *et al* 2000). High levels of PMVs have also been detected in most of these disease conditions and therefore the findings of our study suggest that the high concentrations of PMVs in these disease conditions probably reduces the clearance capacity of resident macrophages hence reducing the uptake of ACs which in turn may undergo secondary necrosis thereby potentiating further inflammation and worsening of the diseases.

As stated above, accumulation of ACs is seen in established SLE with correlations between the rates of apoptosis of cells and the disease activity (Herrmann M *et al* 2000). It is therefore believed that the pathogenesis of the disease may be due to impaired clearance of dying (apoptotic) cells and because PMVs have been shown to inhibit phagocytosis of ACs, their importance in SLE cannot be ignored and requires a thorough analysis. To investigate this, some experiments were carried out which comprised the quantitation of PMVs in the plasma of a number of SLE patients and induction of PMVs from Jurkat T cells using these plasma and purified

aPLA from them. This was to show that plasma of SLE patients with aPLA can induce the production of PMVs which in turn inhibits the removal of cells that are undergoing apoptosis, leading to the persistence of ACs with the resulting immunological problems.

As our results showed, the amounts of PMVs detected from SLE patient plasma are almost 10-fold higher than in plasma from non-SLE subjects. This gives credence to the theory that SLE patients normally have higher (above physiological) amounts of PMVs circulating in their system (Herrmann M *et al* 2000). Using those same plasma, production of PMVs was induced from jurkat T cells which was significantly increased as compared to that induced with non-SLE patients' plasma (-aPL), NHS and also an experiment with no inducing agent. These experiments were repeated using EGTA (not shown) as an inhibitor of PMV production to ensure that it is the aPLA in the SLE patient plasma that was causing the production of the PMVs. When EGTA was used as an inhibitor, the production of PMVs was significantly reduced in the SLE and non-SLE containing plasma as well as the NHS, meaning that the plasma components actually cause the release of PMVs and if this process is inhibited, PMV production also stops.

These were followed by experiments where SLE plasma and NHS was depleted of IgG and complement component C-9 using ProteoSeek™ Albumin/IgG Removal Kit and Human anti-C9 antibody (see section 2.11). The Kit removes human plasma albumin and the major subclasses of gamma globulin (IgG) from plasma. The IgG and C-9 depleted SLE plasma and NHS were then used for PMVs induction. As seen in Fig. 40 & 41, IgG and C-9 depleted SLE plasma and NHS produced less PMVs as compare to complete/intact SLE plasma and NHS. However, when NHS IgG was added to aPLA depleted SLE plasma, induction capacity was almost

restored. A similar effect was seen when purified aPLA was added to IgG depleted NHS.

These results go to confirm that it is the presence of the antibodies (aPLA in the SLE plasma and IgG antibody in the NHS) that is causing the induction of the PMVs as was found in other work carried out at CMIRC that PMV release can be stimulated by sublytic complement (C5b-9) or Membrane Attack Complex (MAC) deposition mediated by classical Pathway complement activation which is initiated by antibody binding. This is proven by the fact that the removal of the antibodies brought about decreased production of PMVs whilst their restoration subsequently led to almost normal induction.

Several mechanisms are thought to be operative in the pathogenesis of autoimmune diseases, against a backdrop of genetic predisposition and environmental modulation. Some of the important mechanisms are: T-cell bypass, T-cell-B-cell discordance, aberrant B cell receptor-mediated feedback, molecular mimicry, idiotype cross-reaction, cytokine dysregulation, dendritic cell apoptosis and epitope spreading or epitope drift. A person's sex also seems to have some role in the development of autoimmunity and it has also been suggested that the slight exchange of cells between mothers and their children during pregnancy may induce autoimmunity. An interesting inverse relationship exists between infectious diseases and autoimmune diseases. In areas where multiple infectious diseases are endemic, autoimmune diseases are quite rarely seen. The reverse, to some extent, seems to hold true. The hygiene hypothesis attributes these correlations to the immune manipulating strategies of pathogens. Certain chemical agents and drugs can also be associated with the genesis of autoimmune conditions, or conditions that simulate autoimmune diseases. The most striking of these is the drug-induced lupus

erythematosus. Usually, withdrawal of the offending drug cures the symptoms in a patient.

6.11 Challenges

While carrying out this project some limitations were experienced. One of the greatest shortcomings was to decide on the appropriate concentrations of PMVs to be used. Finding the concentration for *in vitro* experiment proved arduous, as it had to be physiological. This information was very hard to find from the general literature, as it has not yet been established; figures obtained from this study suggest the average level to be $1.5x10^4$ PMVs/µl of plasma. Nevertheless, increasing the concentrations to a pathological level was another difficult task as they have not yet been clearly established either. Another limitation to the project was the novelty of the topic itself, meaning little in the way of standard protocols.

6.12 Further research

As this topic is at its early stages further extensive research is required to verify findings. Possibilities include: repeating the experiment with established physiological and pathological concentrations of PMVs to see the effects on phagocytosis. Also, repeating the experiment with ACs and PMVs induced from different cell lines and looking at their effect. PMVS from cells that has already being made apoptotic could also be used. Also, one important study that can be carried out and which could have a very significant clinical impact would be to study the effects of high concentrations of PMVs on the uptake of parasites via phagocytosis; parasites such as *Trypanasoma cruzi* and *Leishmania donovani* can reside within phagocytes without being affected by the phaogolysosome (Cestari *et al* 2008), since such organisms can induce PMV release from macrophages.

6.13 Conclusion

Over the past few years the scientific community in areas allied to immunology and medicine has taken a great interest in the role of Plasma Membrane-Derived Vesicles (PMVs) in helping maintain the delicate balance between health and disease. Apart from playing a role in various physiological functions such as thrombosis, inflammation, angiogenesis and vasoconstriction (Berckmans RJ *et al* 2001), PMVs have been linked with a wide range of clinical conditions such as diabetes, cardiovascular, atherosclerosis as well as other autoimmune diseases (e.g SLE). However, they have not been properly characterised, nor is there a standardised method of isolation and quantification acceptable to all researchers in the field. It is based on this that we set out to work on their characterisation so as to obtain an acceptable procedure that will set a benchmark for their isolation and quantification so that their presence in disease states can be attributed to the condition or otherwise.

With proper characterisation of PMVs, there will be confidence in the approach of using it as a diagnostic tool and possibly as a therapeutic due to its ability to induce cell cycle arrest of monocytic leukaemic cells. We have demonstrated that PMVs carry TGF-β1 on their membrane surface and can modulate the growth rate of promonocytes without inducing apoptosis and then induce their differentiation. This could be the underlying principle by which PMVs may be used for treatment of leukaemias as a form of differentiation therapy.

It has also been well established that there is a defect in the clearance of ACs in patients with clinical conditions where high levels of PMVs have been detected (Herrmann M *et al* 2000). The studies presented herein gives an indication that externalised PS on ACs is one if not the main signal for uptake by macrophages and

also high concentrations of PMVs significantly reduce the phagocytosis of ACs by competing with the PS on the ACs for their receptors on the macrophages as seen in the laddering (dose-dependent) pattern of PMV inhibition observed from the analysis of data generated in this study. On the basis of this information it can be concluded that PMVs indeed inhibit the phagocytosis of ACs and do so dose dependently. It implies therefore that in disease conditions where their high presence lead to persistence of the disease, blockade of vesiculation and shedding of PMVs could bring the condition under control.

REFERENCES

Abdallah M, B'Chir Hamzaoui S, Bouslama K. *et al.* (2005). Acute pancreatitis associated with hemophagocytic syndrome in systemic lupus erythematous: a case report. *Gastroenterol. Clin. Biol.* 29, 1054-1056.

Abedin, MJ, Y Kashio, M. Seki, K. Nakamura and M. Hirashima. (2003). Potential roles of galectins in myeloid differentiation into three different lineages. *J. Leukoc. Biol.* 73, 650-656.

Abid-Hussein MN, Meesters EW, Osmanovic N, *et al.* (2003) Antigenic characterization of endothelial cell-derived microparticles and their detection ex vivo. *J Thromb Haemost.* 1,2434–43.

Ades, L, S. Chevret, S. De Botton, *et al.* (2005). Outcome of acute promyelocytic leukemia treated with all-*trans*-retinoic acid and chemotherapy in elderly patients: the European group experience. *Leukemia* 19, 230-233.

Ades L, Raffoux E, Chevret S, et al. (2004). Is Ara-C required in the treatment of newly diagnosed APL? Results of a randomized trial (APL 2000). *Blood.* 104,391-397

Al-Nedawi, K, B. Meehan, J. Micallef, *et al.* (2008). Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat. Cell Biol.* 10, 619 – 624.

Albert ML, Kim JI and Birge RB. (2000). Alphavbeta5 intregrin recruits the crkII-Dock180-rac1 complex for phagocytosis of apoptotic cells. *Nat. Cell Biol.*, 2, 899-905.

Alimonti JB, T. Blake Ball and Keith R. Fowke (2003) "Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS". *J Gen Virology* 84: 1649–61.

Allan D, Billah M.M., Finean J.B. and Michell R.H. (1976). Release of diacylglycerol-enriched vesicles from erythrocytes with increased intracellular (Ca^{2+}). *Nature* 261, 58-60.

Allan D. and Michell R.H. (1977) Calcium ion-dependent diacylglycerol accumulation in erythrocytes is associated with microvesiculation but not with efflux of potassium ions. *Biochem. J.* 166, 495-499.

Allan D., Thomas P. and Limbrick R. (1980). The isolation and characterization of 60nm vesicles ('nanovesicles') produced during ionophore A23187-induced budding of human erythrocytes. *Biochem. J.* 188, 881-887.

Allan D and Thomas P. (1981) Ca^{2+}-induced biochemical changes in human erythrocytes and their relation to microvesiculation. *Biochem. J.* 198, 433-440.

Altman, A. (2001). Acute tumor lysis syndrome. *Semin. Oncol.* 28, 3-8.

Anderson D.R., Davis J.L. and Carraway K.L. (1977). Calcium-promoted changes of the human erythrocyte membrane: involvement of spectrin, transglutaminase, and a membrane-bound protease. *J. Biol. Chem.* 252, 6617-6623.

Anderson LA, Pfeiffer RM, Landgren O, *et al.* (2009). "Risks of myeloid malignancies in patients with autoimmune conditions". *Br. J. Cancer* 100, 822–8.

Ahn Y.S., Jy W., Jimenez J.J., and Horstmann L.L. (2004). More on: cellular microparticles: what are they bad or good for? *J Thromb Hemost* 2, 1215-1216.

Appelt U, Sheriff A, Gaipl US, *et al.* (2004). Viable, apoptotic and necrotic monocytes expose phosphatidylserine: cooperative binding of the ligand Annexin V to dying but not viable cells and implications for PS-dependent clearance. *Cell Death Differ* 12, 194-6

Ardoin S.P., Shanahan J.C., and Pisetsky D.S. (2007). The role of microparticles in inflammation and thrombosis. *Scand. J., Immunol.* 66, 159-165.

Asanuma Y., Annette Oeser, B.S., Ayumi K. Shintani, *et al.* (2003). "Premature coronary-artery atherosclerosis in systemic lupus erythematosus". *New England Journal of Medicine* 349, 2407–2414.

Asou N, Adachi K, Tamura J, et al. (1988). Analysis of prognostic factors in newly diagnosed acute promyelocytic leukemia treated with all-trans retinoic acid and chemotherapy. *J Clin Oncol.* 16,78–85.

Aupeix K, Hugel B, Martin T, *et al.* (1997). The significance of shed membrane particles during programmed cell death in vitro, and in vivo, in HIV-1 infection. *J Clin Invest* 99,1546–54.

Avvisati G, Petti MC, Lo Coco F, *et al.* (2002). Induction therapy with idarubicin alone significantly influences event-free survival duration in patients with newly diagnosed hypergranular acute promyelocytic leukemia: final results of the GIMEMA randomized study LAP 0389 with 7 years of minimal follow-up. *Blood.* 100:3141–3146.

Awasaki, T. R. Tatsumi, K. Takahashi, *et al.* (2006). Essential role of the apoptotic cell engulfment genes draper and ced-6 in programmed axon pruning during Drosophila metamorphosis. *Neuron* 50,855-867.

Baj-Krzyworzeka M., Majka M., Pratico D. *et al.* (2002). Platelet-derived microparticles stimulate proliferation, survival, adhesion, and chemotaxis of hematopoietic cells. *Exp Hematol* 30, 450-459.

Baker, K., P. Raut, and J.R. Jass. (2008). Colorectal cancer cells express functional cell surface-bound TGF-β. *Int. J. Cancer* 122, 1695-1700.

Balasubramanian K., Chandra J. and Schroit AJ. (1997). Immune clearance of phosphatidylserine-expressing cells by phagocytes. The role of beta2-glycoprotein I in macrophage recognition. *J. Biol. Chem.,* 272,31113-31117.

Barry OP, FitzGerald GA. (1999) Mechanisms of cellular activation by platelet microparticles. *Thromb Haemost* 82,794–800.

Barry OP, Pratico D, Savani RC, FitzGerald GA. (1998) Modulation of monocyte–endothelial cell interactions by platelet microparticles. *J Clin Invest* 102,136–44.

Barry OP, Pratico D, Lawson JA, FitzGerald GA. (1997) Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. *J Clin Invest* 99,2118–27.

Baumann, I. W. Kolowos, R.E. Voll, B. *et al.* (2002). Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. *Arthritis Rheum.* 46, 191-201.

Bednarski, Jeffrey J. (2003). Attenuation of autoimmune disease in fas-deficient mice by treatment with a cytotoxic benzodiazepine. *Arthritis & Rheumatism* 48, 172-183

Berckmans R.J., Neiuwland R., Boeing A.N. *et al.* (2001). Cell-derived microparticles circulate in healthy humans and support low-grade thrombin generation. *J Thromb Hemost* 85, 639-646.

Berckmans RJ, Nieuwland R, Böing AN, *et al.* (2002) Microparticles from synovial fluid support coagulation exclusively via a factor VII-dependent mechanism. *Arthritis Rheum* 46,2857–66.

Berckmans, R.J., R. Nieuwland, M.C. Kraan, *et al.* (2005). Synovial microparticles from arthritic patients modulate chemokine and cytokine release by synoviocytes. *Arthritis Res. Ther.* 7, R536-R544.

Berden JH, Licht R, van Bruggen MC, Tax WJ. (1999). Role of nucleosomes for induction and glomerular binding of autoantibodies in lupus nephritis. *Curr Opin Nephrol Hypertens* 8, 299–306

Bernard J, Weil M, Boiron M, *et al.* (1973). Acute promyelocytic leukemia: results of treatment by daunorubicin. *Blood.* 41,489–496.

Bettina Toth, Christianne A. R. Lok, Anita Boing, *et al.* (2007). Microparticles and Exosomes: Impact on Normal and Complicated Pregnancy, *American Journal of Reproductive Immunology* 58, 389–402.

Beutler E., Kuhl W., and West C. (1982). The osmotic fragility of erythrocytes after prolonged liquid storage and after reinfusion. *Blood* 59, 1141-1147.

Beutler E., and West C. (1983). Storage of red cell concentrates in CPD-A2 for 42 and 49 days. *J Lab Clin Med* 102, 53-62.

Bevers EM, Wiedmer T, Comfurius P, *et al.* (1992). Defective Ca (2+)-induced microvesiculation and deficient expression of procoagulant activity in erythrocytes from a patient with a bleeding disorder: a study of the red blood cells of Scott syndrome. *Blood* 79, 380-388.

Bevers EM, Comfurius P, van Rijn JL, *et al.* (1982). Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem* 122,429–36.

Beyer, C. D.S. Pisetsky. (2010). The role of micoparticles in the pathogenesis of rheumatic diseases. *Nat. Rev. Rheumatol.* 6,21-29.

Bianchi, S.M. L.R. Prince, K. McPhillips, *et al.* (2008). Impairment of apoptotic cell engulfment by pyocyanin, a toxic metabolite of *Pseudomonas aeruginosa*. *Am. J. Respir. Crit. Care Med.* 177, 35-43

Bird DA, Gillotte KL, Horkko S, *et al.* (1999). Receptors for oxidized low-density lipoprotein on elicited mouse peritoneal macrophages can recognize both the modified lipid moieties and the modified protein moieties: implications with respect to macrophage recognition of apoptotic cells. *Proc. Natl. Acad. Sci. USA,* 96, 6347-6352 .

Biró É., Nieuwland R., and Sturk A. (2004). Measuring circulating cell-derived microparticles. *J Thromb Haemost* 2,1843-1844.

Boettner DR, Huston CD, Linford AS, *et al.* (2008). "Entamoeba histolytica phagocytosis of human erythrocytes involves PATMK, a member of the transmembrane kinase family". *Jpn. J. Infect. Dis*. 61, 294–7.

Bosman G.J., Willekens F.L., and Werre J.M. (2005). Erythrocyte aging: A more than superficial resemblance to apoptosis? *Cell Physiol Biochem* 16, 1-8.

Botto M, Dell'Agnola C, Bygrave AE, *et al.* (1998). Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. *Nat. Genet.,* 19, 56-59.

Boulanger C.M., Amabile N., and Tedgui A. (2006). Circulating microparticles: a potential prognostic marker for atherosclerotic vascular disease. *Hypertension* 48,180-186.

Boulanger CM, Scoazec A, Ebrahimian T, *et al.* (2001). Circulating microparticles from patients with myocardial infarction cause endothelial dysfunction. *Circulation* 104,2649–52.

Brechot N. Elisa Gomez, Marine Bignon *et al.* (2008). "Modulation of macrophage activation state protects tissue from necrosis during critical limb ischemia in thrombospondin-1-deficient mice". *PLoS ONE*. 3, e3950.

Breccia M, Latagliata R, Carmosino I, *et al.* (2008). "Clinical and biological features of acute promyelocytic leukemia patients developing retinoic acid syndrome during induction treatment with all-trans retinoic acid and idarubicin". *Haematologica* 93, 1918–20.

Brüne B (2003). "Nitric oxide: NO apoptosis or turning it ON?". *Cell Death Differ.* 10, 864–9.

Brune, M., S. Castaigne, J. Catalano, *et al.* (2006). Improved leukaemia-free survival after postconsolidation immunotherapy with histamine dihydrochloride and interleukin-2 in acute myeloid leukaemia: results of a randomized phase 3 trial. *Blood* 108, 88-96.

Bucki R., Bachelot-Loza C., Zachowski A., *et al.* (1998) Calcium induces phospholipid redistribution and microvesicles release in human erythrocyte membranes by independent pathways. *Biochemistry 3*7,15383-15391.

Burnier L., Fontana P., Kwak B.R., and Angelillo-Scherrer, A. (2009). Cell-derived microparticles in haemostasis and vascular medicine. *J Thromb Haemost.* 101, 439-451.

Burnett AK, Grimwade D, Solomon E, on behalf of the MRC Adult Leukemia Working Party, *et al.* (1999). Presenting white blood cell count and kinetics of molecular remission predict prognosis in acute promyelocytic leukemia treated with all-trans retinoic acid: result of the randomized MRC trial. *Blood.* 93,4131–4143.

Butikofer P, Kuypers FA, Xu CM, *et al.* (1989). Enrichment of two glycosyl-phosphatidylinositol-anchored proteins, acetylcholinesterase and decay accelerating factor, in vesicles released from human red blood cells. *Blood* 74,1481–5.

Calin, G.A., Croce, C.M. (2006). MicroRNA signatures in human cancers. *Nat. Rev. Cancer.* 6,857-866

Callahan MK, Williamson P, Schlegel RA. (2000). Surface expression of phosphatidylserine on macrophages is required for phagocytosis of apoptotic thymocytes. *Cell Death Differ* 7,645–53.

Campelovier, P., V. Pautre, M. Elatifi, *et al.* (2006). Resistance to phorbol ester-induced differentiation in human myeloid leukaemia cells: a hypothetic role for the mRNA stabilization process. *Leukaemia. Res.* 30,1407-1416.

Card R.T., Mohandas N., and Mollison P.L. (1983). Relationship of post-transfusion viability to deformability of stored red cells. *Br J Haematol* 53, 237-40.

Casciola-Rosen LA, Anhalt G, Rosen A. (1994). Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J Exp Med* 179, 1317–1330

Chargaff E, West R. (1946). The biological significance of the thromboplastic protein of blood. *J Biol Chem* 166,189–97.

Chen G, Goeddel DV. (2002). "TNF-R1 signaling: a beautiful pathway". *Science* 296 1634–5.

Chen, J.F., Mandel,EM., Thomson, M. *et al.* (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat. Genet.* 38,228-233.

Cheng EH (2003). "VDAC2 inhibits BAK activation and mitochondrial apoptosis". *Science* 301, 513–7.

Chen Z, Brand NJ, Chen, A. *et al.* (1993). "Fusion between a novel Krüppel-like zinc finger gene and the retinoic acid receptor-alpha locus due to a variant t(11;17) translocation associated with acute promyelocytic leukaemia.". *EMBO J* 12,1161–7

Cheson BD, Bennett JM, Kopecky K, *et al.* (2003). Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. *J Clin Oncol.* 21,4642–4649.

Chiarugi A, Moskowitz MA. (2002). "PARP-1—a perpetrator of apoptotic cell death?". *Science* 297, 259–63.

Chironi, G. A. Simon, B. Hugel, *et al.* (2006). Circulating leukocyte-derived microparticles predict subclinical atherosclerosis burden in asymptomatic subjects. *Arterioscler. Thromb. Vasc. Biol.* 26, 2775-2780.

Chomienne C, Ballerini P, Balitrand N, et al. (1990) All-trans retinoic acid in promyelocytic leukemias. II. In vitro studies: structure-function relationship. *Blood*; 76,1710-1717

Chuang, J.C., Jones, P.A. (2007). Epigenetics and microRNAs. *Pediatr. Res.* 61,24R-29R.

Ciarapica, R., Russo G, Verginelli F. *et al.* (2009). Deregulated expression of miR-26a and Ezh2 in rhabdomyosarcoma. *Cell Cycle.* 8,172-175.

Cocucci, E. G. Racchetti, J. Meldolesi, (2010). Shedding microvesicles: artefacts no more. *Trends Cell Biol.* 15, 43-51.

Cohen JJ, Duke RC, Fadok VA, Sellins KS. (1992). Apoptosis and programmed cell death in immunity. *Annu Rev Immunol* 10,267–293.

Cole W.F., Rumsby M.G., Langster G.H., and Tovey L.A. (1979). Changes in the inhibition of specific agglutination by plasma due to microvesicles released from human red cells during storage for transfusion. *Vox Sang* 37, 73-77.

Cole W.F., Rumsby M.G., Langster G.H., and Tovey L.A. (1978). The release of erythrocyte membrane antigens to the plasma as membrane microvesicles during the storage of human blood for transfusion. *Biochem Soc Trans* 6,1375-1378.

Combes V., Coltel N., Alibert M., *et al.* (2005). ABCA1 Gene Deletion Protects against Cerebral Malaria Potential Pathogenic Role of Microparticles in Neuropathology. *Am J Pathol,* 166,295–302

Connor J, Pak CH, Zwaal RF, Schroit AJ. (1992). Bidirectional transbilayer movement of phospholipid analogs in human red blood cells Evidence for an ATP-dependent and protein-mediated process. *J Biol Chem*; 267, 19412–7.

Crow MK. (2008). Collaboration, genetic associations, and lupus erythematosus. *N Engl J Med.* 358,956-61.

Cyrelys Collazo, Osmani Chacón, Orlando Borrás. (2006). "Programmed cell death in plants resembles apoptosis of animals", *Biotecnología Aplicada* 23, 1–10,

Danchenko N, Satia JA, Anthony MS. (2006). "Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden". *Lupus* 15, 308–18.

Daniel MT, Koken M, Romagne O, *et al.* (1993) PML protein expression in hematopoietic and acute promyelocytic leukemia cells. *Blood*; 82,1858-1867.

Daigneault, M., J.A. Preston, H.M. Marriott, *et al.* (2010). The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS One* 5,1-10.

Datta, J., Huban K., Mohd W. *et al.* (2008). Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis. *Cancer Res.* 68,5049-5058.

de Almeida, M.C., A.C. Silva, A. Barral, and M.B. Netto. (2000). A simple method for human peripheral blood monocyte isolation. *Mem. Inst. Oswaldo Cruz* 95, 221-223.

Dejean LM, Martinez-Caballero S, Manon S, Kinnally KW. (2006). "Regulation of the mitochondrial apoptosis-induced channel, MAC, by BCL-2 family proteins". *Biochim. Biophys. Acta* 1762,191–201.

del Conde, I, C.N. Shrimpton P. Thiagarajan, and J.A. Lopez. (2005).Tissue-factor-bearing microvesicles arise from lipid rafts and fuse withactivated platelets to initiate coagulation. *Blood* 106,1604-1611.

Denzer K., M.J. Kleijmeer, H.F. Heijnen, *et al.* (2000). Exosome: from internal vesicle of the multivesicular body to intercellular signaling device, *J. Cell Sci.* 113, 3365–3374.

Devitt A, Moffat OD, Raykundalia C, *et al.* (1998). Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature,* 392, 505-509.

Diamant M, Nieuwland R, Berckmans RJ, *et al.* (2002). Elevated numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type 2 diabetes. *Circulation*; 106,2442–7.

Diaz C, Schroit AJ. (1996). Role of translocases in the generation of phosphatidylserine asymmetry. *J Membr Biol* 151,1–9.

Dieker JW, van der Vlag J, Berden JH. (2002). Triggers for antichromatin autoantibody production in SLE. *Lupus* 11,856–864

Dignat-George F., Freyssinet J.-M., and Key N.S. (2009). Centrifugation is a crucial step iPMVacting microparticle measurement. *Platelets* 20, 225-226.

Dimberg, A., F. Bahram, I. Karlberg, *et al.* (2002) Retinoic acid-induced cell cycle arrest of human myeloid cell lines is associated with sequential down-regulation of

c-Myc and cyclin E and posttranscriptional up-regulation of p27 (Kip1). *Blood* 99, 2199–2206.

Distler, J.H. L.C. Huber, A.J. Hueber, *et al.* (2005). The release of microparticles by apoptotic cells and their effects on macrophages. *Apoptosis* 10, 731-741

D'Cruz DP, Khamashta MA, Hughes GR. (2007). "Systemic lupus erythematosus". *Lancet* 369 , 587–96.

D'Cruz DP (2006). "Systemic lupus erythematosus". *BMJ* 332, 890–4.

Douer D, Santillana S, Ramezani L, *et al.* (2003). "Acute promyelocytic leukaemia in patients originating in Latin America and is associated with an increased frequency of the bcr1 subtype of the PML/RARalpha fusion gene". *Br. J. Haematol.* 122, 563–70

Douer D, Tallman MS. (2005) Arsenic trioxide: new clinical experience with an old medication in hematological malignancies. *J Clin Oncol.* 23,2396–2410

Eguchi K. (2001). Apoptosis in autoimmune diseases. *Intern Med* 40,275–284.

Eken, C., O. Gasser, G. Zenhaeusern, *et al.* (2008). Polymorphonuclear neutrophil-derived ectosomes interfere with the maturation of monocyte-derived dendritic cells. *J. Immunol.* 180, 817-824.

Enjeti A.K., Lincz L.F., and Seldon M. (2007). Detection and measurement of microparticles: an evolving research tool for vascular biology. *Semin Thromb Hemost* 33, 771-779.

Estey E, Garcia-Manero G, Ferrajoli A, *et al.* (2006). Use of all-trans retinoic acid plus arsenic trioxide as an alternative to chemotherapy in untreated acute promyelocytic leukemia. *Blood*; 107,3469–3473.

Everett, H. and McFadden, G. (1999). "Apoptosis: an innate immune response to virus infection". *Trends Microbiol* 7, 160–5.

Fabbri, M., Iliopoulos D, Trapasso F. *et al.* (2007). MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc. Natl. Acad. Sci. U. S. A.* 104,15805-15810.

Fadeel, A (2002). Role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. *J. Immunol.* 169, 487-499.

Fadok VA, Bratton DL, Rose DM, *et al.* (2000). A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature,* 405, 85-90.

Fadok VA, Bratton DL, Frasch SC, *et al.* (1998). The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. *Cell Death Differ.* 5, 551-562.

Fadok VA, de Cathelineau A, Daleke DL, *et al.* (2001). Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *J. Biol. Chem.,* 276, 1071-1077.

Fadok VA, Warner ML, Bratton DL and Henson PM. (1998). CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor (alpha v beta 3). *J. Immunol.,* 161, 6250-6257.

Fadok VA, Voelker DR, Campbell PA, *et al.* (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.,* 148, 2207-2216.

Fadok, V.A., D.L. Bratton, A. Konowal, *et al.* (1998). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-Beta, PGE2 and PAF. *J. Clin. Invest.* 101, 890-898.

Faure V., Dou L., Sabatier F. *et al.* (2005). Elevation of circulating endothelial microparticles in patients with chronic renal failure. *J Thromb Haemost* 4, 566-573.

Fenaux P, Chomienne C, Degos L. (1997). Acute promyelocytic leukemia: biology and treatment. *Semin Oncol*; 24,92-102

Fenaux P, Le Deley MC, Castaigne S, *et al.* (1993). Effect of all transretinoic acid in newly diagnosed acute promyelocytic leukemia: results of a multicenter randomized trial. *Blood*; 82,3241-3249.

Fenaux P, Chastang C, Chevret S, *et al.* (1999). A randomized comparison of ATRA followed by chemotherapy and ATRA plus chemotherapy, and the role of maintenance therapy in newly diagnosed acute promyelocytic leukemia. *Blood* 94,1192–1200

Fesik SW, Shi Y. (2001). "Controlling the caspases". *Science* 294, 1477–8.

Finnemann SC, Rodriguez-Boulan E. (1999). Macrophage and retinal pigment epithelium phagocytosis: apoptotic cells and photoreceptors compete for alphavbeta3 and alphavbeta5 integrins, and protein kinase C regulates alphavbeta5 binding and cytoskeletal linkage. *J. Exp. Med.* 190, 861-874

Flieger, O., A. Engling, R. Bucala, *et al.* (2003). Regulated secretion of macrophage migration inhibitory factor is mediated by a non-classical pathway involving an ABC transporter. *FEBS Lett.* 551, 78–86.

Foocharoen C, Nanagara R, Salang L, *et al.* (2009). "Pregnancy and disease outcome in patients with systemic lupus erythematosus (SLE): a study at Srinagarind Hospital". *J Med Assoc Thai* 92, 167–74.

Forlow SB, McEver RP, Nollert MU. (2000). Leukocyte–leukocyte interactions mediated by platelet microparticles under flow. *Blood* 95,1317–23.

Forrest, A.R.R., M. Kanamaori-Katayama, Y. Tomaru, *et al.* (2010). Induction of microRNAs, mir-155, mir-222, mir-424 and mir-503, promotes monocytic differentiation through combinatorial regulation. *Leukemia* 24, 460-466.

Fox JE, Austin CD, Boyles JK, Steffen PK. (1990). Role of the membrane skeleton in preventing the shedding of procoagulant-rich microvesicles from the platelet plasma membrane. *J Cell Biol* 111, 483–93.

Frankel SR, Eardly A, Lauwers G, *et al.* (1992). The "retinoic acid syndrome" in acute promyelocytic leukemia. *Ann Intern Med* 117,292-296.

Freedberg IM., Terui T., Takahashi K. *et al.* (2003). The epidemiology of sunburn in the US population. *Fitzpatrick's Dermatology in General Medicine.* 6th ed., 127-48.

Frenkel E.J., Kuypers F.A., Op den Kamp J.A., *et al.* (1986). Effect of membrane cholesterol on dimyristoylphosphatidylcholine-induced vesiculation of human red blood cells. *Biochim Biophys Acta*. 855, 293-301.

Freyssinet J.M. (2003). Cellular microparticles: what are they bad or good for? *J Thromb Haemos*. 1, 1655-1662.

Freyssinet JM, Toti F, Hugel B, *et al.* (1999) Apoptosis in vascular disease. *Thromb Haemost*; 82,727–35.

Gaipl US, Kuenkele S, Voll RE, *et al.* (2001). Complement binding is an early feature of necrotic and a rather late event during apoptotic cell death. *Cell Death Differ* 8, 327–34.

Gasser, O., and J.A. Schifferli. (2004). Activated polymorphonuclear neutrophils *Immunol. Dis. 22*, 572–574.

Garay, E., R. Donnelly, X. Wang, and G.P. Studzinski. (2007). Resistance to 1,25D-induced differentiation in human acute myeloid leukaemia HL60-40AF cells is associated with reduced transcriptional activity and nuclear localization of the vitamin D receptor. *J. Cell. Physiol.* 213, 816-825.

Garcia, B.J., D.M. Smalley, H. Cho, *et al.* (2005). The platelet microparticle proteome. *J. Proteome Res.* 4: 1516-1521. seminate anti-inflammatory microparticles by ectocytosis. *Blood* 104, 2543-2548.

Garofalo, M., Condorelli, G., Croce, C.M. (2008). MicroRNAs in diseases and drug response. *Curr. Opin. Pharmacol.* 8,661-667.

Garzon, R., Fabbri, M., Cimmino, A., *et al.* (2006). MicroRNA expression and function in cancer. *Trends Mol. Med.* 12,580-587.

Gershov D, Kim S, Brot N, Elkon KB. (2000). C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. *J. Exp. Med.* 192,1353-1364.

Giesen PLA, Rauch U, Bohrmann B, *et al.* (1999). Blood-borne tissue factor: another view of thrombosis. *Proc Natl Acad Sci USA* 96,2311–5.

Giles KM, Hart SP, Haslett C, *et al.* (2000). An appetite for apoptotic cells? Controversies and challenges. *Br J Haematol* 109,1–12.

Goeddel, DV, Guoqing C. (2002). "Connection Map for Tumor Necrosis Factor Pathway". *Science*; 296, 1634-5.

Greenwalt T.J. (2006). The how and why of exocytic vesicles. *Transfusion* 46,143-152.

Gregory DC and Devitt A. (2004). The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically? *Immunology*; 113, 1–14.

Gregory, J.L., E.F. Morand, S.J. McKeown, *et al.* (2006). Macrophage migration inhibitory factor induces macrophage recruitment via CC chemokine ligand 2. *J. Immunol.* 177, 8072-8079.

Gritters M., Grooteman M.P., Schoorl M., *et al.* (2006). Citrate anticoagulation abolishes degranulation of polymorphonuclear cells and platelets and reduces oxidative stress during haemodialysis. *Nephrol Dial Transplant* 21,153-159.

Grønlien HK, Berg T, Løvlie AM. (2002). "In the polymorphic ciliate Tetrahymena vorax, the non-selective phagocytosis seen in microstomes changes to a highly selective process in macrostomes". *J. Exp. Biol.* 205, 2089–97.

Guerrero I, Ruiz i Altaba A. (2003). "Development. Longing for ligand: hedgehog, patched, and cell death". *Science* 301, 774–6.

Gumbiner, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 84, 345-357.

Hahn B.H. (2003). "Systemic lupus erythematosus and accelerated atherosclerosis". *New England Journal of Medicine* 349, 2379–2380.

Hahn, Bevra Hannahs. (2008). "Systemic Lupus Erythematosus." *Harrison's Principles of Internal Medicine*, 17th ed. Chapter 313.

Halder, S.K., R.D. Beauchamp, and P.K. Datta. (2005). A specific inhibitor of TGF-β receptor kinase, SB-431542, as a potent antitumor agent for human cancers. *Neoplasia* 7, 509-521

Hamilton, K.K., Hattori, R., Esmon, C.T. & Sims, P.J. (1990). Complement proteins C5b-9 induce vesiculation of the endothelial plasma membrane and expose catalytic surface for assembly of the prothrombinase enzyme complex. *The Journal Of Biological Chemistry* 265, 3809–3814.

Hammond, S.M. (2006). MicroRNAs as oncogenes. *Curr. Opin. Genet. Dev.* 16,4-9.

Harel-Meir M, Sherer Y, Shoenfeld Y. (2007). Tobacco smoking and autoimmune rheumatic diseases. *Nat Clin Pract Rheumatol.* 3, 707-15.

Hardisty RM, Hutton RA. (1966). Platelet aggregation and the availability of platelet factor 3. *Br J Haematol* 12, 764–76.

Hart SP, Smith JR, Dransfield I. (2004). Phagocytosis of opsonized apoptotic cells: roles for 'old-fashioned' receptors for antibody and complement. *Clin Exp Immunol* 135,181–5.

Hay, S. and Kannourakis, G. (2002). "A time to kill: viral manipulation of the cell death program". *J Gen Virol* 83, 1547–64.

He LZ, Tribioli C, Rivi R, et al. (1997). Acute leukemia with promyelocytic features in PML/RARα transgenic mice. *Proc Natl Acad Sci U S A* 94, 5302-5307.

He, L., Hannon, G.J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. *Nat. Rev. Genet.* 5, 522-531

Hemminki K, Li X, Sundquist J, Sundquist K. (2009). "Familial associations of rheumatoid arthritis with autoimmune diseases and related conditions". *Arthritis Rheum.* 60, 661–668.

Hepburn, AL, Lampert, IA, Boyle, JJ, *et al.* (2007). In vivo evidence for apoptosis in the bone marrow in systemic lupus erythematosus. *Ann Rheum Dis*; 66, 1106–1109.

Herrmann, M., R.E. Voll, O.M. Zoller, *et al.* (1998). Impaired phagocytosis of apoptotic cell material by monocytederived macrophages from patients with systemic lupus erythematosus. *Arthritis Rheum* 41, 1241-1250

Herrmann M, Voll RE, Kalden JR. (2000). Etiopathogenesis of systemic lupus erythematosus. *Immunol Today* 21, 424-426.

Hess C, Sadallah S, Hefti A *et al.* (1999). Ectosomes released by human neutrophils are specialized functional units. *J Immunol* 163, 4564.

Hillestad LK. (1957). Acute promyelocytic leukemia. *Acta Med Scand* 159,189–194

Hind, E., Ansa-Addo, E. A., Antwi-Baffour, S., *et al.* (2010) Red-Cell Derived Plasma Membrane-derived Vesicles: Result Variability and Standardization; *Biochem. Biophys. Res. Commun.* 280, 818–823.

Hochberg MC. (1991). "The history of lupus erythematosus". *Md Med J* 40, 871–3.

Hodkinson B, Musenge E, Tikly M. (2009). "Osteoarticular tuberculosis in patients with systemic lupus erythematosus". *QJM* 102, 321

Honczarenko K, Budzianowska A, Ostanek L. (2008). "Neurological syndromes in systemic lupus erythematosus and their association with antiphospholipid syndrome". *Neurol. Neurochir. Pol.* 42, 513–517.

Hopkinson-Woolley J, Hughes D, Gordon S, Martin P. (1994) Macrophage recruitment during limb development and wound healing in the embryonic and foetal mouse. *J Cell Sci* 107,1159–1167.

Horstman L.L., Jy W., Jimenez J.J., *et al.* (2004). New horizons in the analysis of circulating cell-derived microparticles. *Keio J Med* 53, 210-230.

Horstman LL, Ahn YS. (1999). Platelet microparticles: a wide-angle perspective. *Crit Rev Oncol Hematol* 30,111–42.

Hron G., Kollars M., Weber H., *et al.* (2007) Tissue factor-positive microparticles: cellular origin and association with coagulation activation in patients with colorectal cancer. *J Thromb Haemost* 97, 119-123.

Huber J, Vales A, Mitulovic G. et al. (2002) Oxidized membrane vesicles and blebs from apoptotic cells contain biologically activate oxidized phospholipids that induce monocyte–endothelial interactions. *Arterioscler Thromb Vasc Biol* 22,101–7.

Huang ME, Ye YC, Chen SR. et al. (1988). Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* 72, 567-576

Hugel B., Socié G., Vu T. *et al.* (1999). Elevated levels of circulating procoagulant microparticles in patients with paroxysmal nocturnal hemoglobinuria and aplastic anemia. *Blood* 93, 3451-3456.

Hugel, B., M.C. Martínez, C. Kunzelmann, and J.M. Freyssinet (2005). Membrane microparticles: two sides of the coin. *Physiology* 20, 22-27.

Hunter, M.P., N. Ismail, X. Zhang, *et al.* (2008). Detection of microRNA expression in human peripheral blood microvesicles. *PloS One* 3, 1-11

Hynes, R. 0. & Lander, A. D. (1992). *Cell* 68, 303-322.

Hyslop PA, Hinshaw DB, Halsey Jr WA *et al.* (1988). Mechanisms of oxidant-mediated cell injury. The glycolytic and mitochondrial pathways of ADP phosphorylation are major intracellular targets inactivated by hydrogen peroxide. *J. Biol. Chem.* 263, 1665-1675.

Inal, J.M. K.-M. Hui, S. Miot, *et al.* (2005). Complement C2 Receptor Inhibitor Trispanning: a novel human complement inhibitory receptor *J. Immunol* 174, 356-366.

Ishimoto H, Yanagihara K, Araki N, *et al.* (2008). "Single-cell observation of phagocytosis by human blood dendritic cells". *Jpn J Infect Dis* 61, 294–7.

Iwata, **M** D., Myerson, B Torok-Storb and RA Zager (1996). "An evaluation of renal tubular DNA laddering in response to oxygen deprivation and oxidant injury". *Proc Natl Acad Sci U S A* 93,2239–2244.

Janowska-Wieczorek A, Majka M, Kijowski J, *et al.* (2001) Platelet-derived microparticles bind to hematopoietic stem/progenitor cells and enhance their engraftment. *Blood.* 98, 3143-3149.

Jeha, S. (2001). Tumor lysis syndrome. *Semin. Hematol.* 38, 4-8.

Jenkins, G. (2008). The role of proteases in transforming growth factor-beta activation. *Int J Biochem Cell Biol.* 40, 1068-1078.

Jimenez JJ, Jy W, Mauro LM, *et al.* (2003). Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thromb Res* 109, 175–80.

Jimenez J.J., Jy W., Horstmann L.L., and Ahn Y.S. (2004). Measuring circulating cell-derived microparticles. *J Thromb Haemost* 2,1850-1851.

Jy W., Horstmann L.L., Jimenez J.J., and Ahn Y.S. (2004) Measuring circulating cell-derived microparticles. *J Thromb Haemost* 2,1842-1843.

Jy, W. M. Tiede, C.J. Bidot, *et al.* (2008). Platelet activation rather than endothelial injury identifies risk of thrombosis in subjects positive for antiphospholipid antibodies. *Thromb. Res.* 121, 319-325.

Kanta H, Mohan C. (2009). "Three checkpoints in lupus development: central tolerance in adaptive immunity, peripheral amplification by innate immunity and end-organ inflammation". *Genes Immun* 10, 390-6

Kagan VE, Gleiss B, Tyurina YY. *et al.* (2002). A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. *J Immunol* 169, 487-499.

Kagan VE, Fabisiak JP, Shvedova AA. *et al.* (2000). Oxidative signaling pathway for externalization of plasma membrane phosphatidylserine during apoptosis. *FEBS Lett,* 477, 1-7.

Kagan, V.E. B. Gleiss, Y.Y. Tyurina, *et al.* (2002). A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. *J. Immunol* 169 , 487-499.

Kerr, JF. (1965). "A histochemical study of hypertrophy and ischaemic injury of rat liver with special reference to changes in lysosomes". *J Pathol Bacteriol* 90, 419–35.

Kerr JF, Wyllie AH, Currie AR. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics". *Br J Cancer* 26, 239–57.

Khamashta MA. (2006). Systemic lupus erythematosus and pregnancy. *Best Pract Res Clin Rheumatol.* 20, 685-94.

Kim, H.K., K.S. Song, J.H. Chung, *et al.* (2004). Platelet microparticles induce angiogenesis in vitro. *Br. J. Haematol.* 124, 376-384.

Kimby E, Nygren P, Glimelius B, for the SBU-group. (2001). A Systematic Overview of Chemotherapy Effects in Acute Myeloid Leukaemia. *Acta Oncologica*; 40,231–252.

Knowles D.W., Tilley L., Mohandas N., and Chasis J.A. (1997). Erythrocyte membrane vesiculation: model for the molecular mechanism of protein sorting. *Proc Natl Acad Sci USA* 94,12969-12974.

Krahling S, Callahan MK, Williamson P, Schlegel RA. (1999). Exposure of phosphatidylserine is a general feature in the phagocytosis of apoptotic lymphocytes by macrophages. *Cell Death Differ* 6, 183-189.

Krombach F, Münzing S, Allmeling AM, *et al.* (1997). "Cell size of alveolar macrophages: an interspecies comparison". *Environ Health Perspect.* 105 Suppl 5: 1261–3.

Kumar, M.S., Lu, J., Mercer, K.L., *et al.* (2007) Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nat Genet* 39,673-677.

Kuypers, F.A, and K. De Jong. (2004). The role of phosphatidylserine in recognition and removal of erythrocytes. *Cell. Mol Biol* 50, 147-158.

Lane NE. (2006) Therapy Insight: osteoporosis and osteonecrosis in systemic lupus erythematosus. *Nat Clin Pract Rheumatol* 2, 562-569.

Laurent M. Dejean, Sonia Martinez-Caballero, Kathleen W. Kinnally. (2006). "Is MAC the knife that cuts cytochrome c from mitochondria during apoptosis?". *Cell Death and Differ* 13, 1387–5.

Lawrie A.S., Albanyan A., Cardigan R.A., *et al.* (2009) Microparticle sizing by dynamic light scattering in fresh-frozen plasma. *Vox Sang* 96, 206-212.

Lawrie A.S., Harrison P., Cardigan R.A., and Mackie I.J. (2008). The characterization and impact of microparticles on haemostasis within fresh-frozen plasma. *Vox Sang* 95,197-204.

Lee JA, Spidlen J, Boyce K, *et al.* (2008) MIFlowCyt: the minimum information about a flow cytometry experiment. *Cytometry A* 73, 926-930.

Lee YJ, Shacter E. (1999). Oxidative stress inhibits apoptosis in human lymphoma cells. *J Biol Chem* 274, 19792-19798.

Leist M, Single B, Castoldi AF. *et al.* (1997). Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med* 185, 1481-1486.

Lehmann, L.E., S.U. Weber, D. Fuchs. *et al.* (2005). Intracellular detection of macrophage migration inhibitory factor in peripheral blood leukocytes. *Free Radic Biol Med* 38, 1170-1179.

Lengfelder E, Reichert A, Schoch C, et al. (2000). Double induction strategy including high dose cytarabine in combination with all-trans retinoic acid: effects in patients with newly diagnosed acute promyelocytic leukemia. *Leukemia* 14,1362–1370

Lennon SV, Martin SJ, Cotter TG. (1991). Dose-dependent induction of apoptosis in human tumour. *Cell Prolif* 24, 203-214.

Lesèche, G., Wassef M., Mallat Z., et *al.* (2007). Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques. *J Am Coll Cardiol* 49, 772-777.

Li MO, R. A. Flavell, J. S. Levine. *et al.* (2003). "Phosphatidylserine receptor is required for clearance of apoptotic cells". *Science* 302, 1560–3.

Li, M.O., Y.Y. Wan, S. Sanjabi, *et al.* (2006). Transforming growth factor-β1 regulation of immune responses. *Annu Rev Immunol* 24, 99-146.

Lim, L.P., Nelson C. Lau, Philip Garrett-Engele *et al.* (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature.* 433,769-773.

Liu, F.T. (2005). Regulatory roles of galectins in the immune response. *Int mArch. Allergy Immunol.* 136, 385-400.

Liu, G. J. Wang, Y.J. Park, *et al.* (2008). High mobility group protein-1 inhibits phagocytosis of apoptotic neutrophils through binding to phosphatidylserine. *J Immunol* 181, 4240-4246.

Liu R., Klich I., Ratajczak J. *et al.* (2009). Erythrocyte-derived microvesicles may transfer phosphatidylserine to the surface of nucleated cells and falsely 'mark' them as apoptotic. *Eur J Haematol* 83, 220-229.

Lodish, Harvey; Arnold Berk, Chris A. Kaiser. *et al.* (2004). *Molecular Cell Biology.* New York: W.H. Freedman and Company. ISBN 0-7167-4366-3.

Lucas AD, Greaves DR. (2001). "Atherosclerosis: role of chemokines and macrophages". *Expert Rev Mol Med* 3, 1–18.

Lutz H.U., Liu S.C., and Palek J. (1977). Release of spectrin-free vesicles from human erythrocytes during ATP depletion. I. Characterization of spectrin-free vesicles. *J Cell Biol.* 73, 548-560.

MacKenzie, A., H.L. Wilson, E. Kiss-Toth *et al.* (2001). Rapid secretion of interleukin-1β by microvesicle shedding. *Immunity* 8, 825–835.

Madeleine Kihlmark, Gabriela Imreh and Einar Hallberg. (2001). "Sequential degradation of proteins from the nuclear envelope during apoptosis". *Journal of Cell Science* 114, 3643–53.

Mallat, Z. B. Hugel, J. Ohan. e*t al.* (1999) Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity. *Circulation* 99, 348-353.

Mallat Z., Benamer H., Hugel B. e*t al.* (2000) Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation* 101, 841-843.

Manfredi AA, Rovere P, Heltai S. *et al.* (1998). Apoptotic cell clearance in systemic lupus erythematosus. II. Role of beta2-glycoprotein I. *Arthritis Rheum* 41,215–23.

Manfredi AA, Rovere P, Galati G. *et al.* (1998). Apoptotic cell clearance in systemic lupus erythematosus. I. Opsonization by antiphospholipid antibodies. *Arthritis Rheum* 41, 205–14.

Mandelli F, Diverio D, Avvisati G, *et al.* (1997). Molecular remission in PML/RAR–positive acute promyelocytic leukemia by combined all-trans retinoic acid and idarubicin (AIDA) therapy. *Blood*, 90, 1014–1021.

Martinez MC, Tesse A, Zobairi F, Andrianstsitohaina R. (2005). Shed membrane microparticles from circulating and vascular cells in regulating vascular function. Am *J Physiol Heart Circ Physiol* 288, 1004–9.

Martinez, M.C., F. Larbret, F. Zobairi. *et al.* (2006). Transfer of differentiation signal by membrane microvesicles harbouring hedgehog morphogens. *Blood* 108, 3012-3020.

Martens HA, Nolte IM, van der Steege G, *et al.* (2009). "An extensive screen of the HLA region reveals an independent association of HLA class I and class II with susceptibility for systemic lupus erythematosus". *Scand. J. Rheumatol* 1–7.

Martin SJ, Reutelingsperger CP, McGahon AJ. *et al.* (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpressing of Bcl-2 and Abl. *J. Exp. Med.,* 182, 1545-1556.

Massarotti EM. (2008). "Managing musculoskeletal issues in lupus: The patient's input invited". *J Musculo Med* 67, 195–205

McCarthy, J.J. (2008). MicroRNA-206: the skeletal muscle-specific myomiR. *Biochim. Biophys. Acta.* 1779, 682-691

Medeiros PB, Febrônio MV, Bonfá E, *et al.* (2009). "Menstrual and hormonal alterations in juvenile systemic lupus erythematosus". *Lupus* 18, 38–43.

Mendoza-Pinto C, García-Carrasco M, Sandoval-Cruz H, *et al.* (2009). "Risk factors of vertebral fractures in women with systemic lupus erythematosus". *Clin. Rheumatol.* 28, 579.

Mesri M, Altieri DC. (1999). Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in JNK1 signaling pathway. *J Biol Chem* 274, 23111–8.

Merlino, G., Khanna, C. (2007). Fishing for the origins of cancer. *Genes Dev.* 21,1275-1279.

Merlino, G., Helman, L.J. (1999). Rhabdomyosarcoma--working out the pathways. *Oncogene.* 18, 5340-5348.

Mevorach D, Mascarenhas JO, Gershov D, Elkon KB. (1998). Complement-dependent clearance of apoptotic cells by human macrophages. *J Exp Med* 188, 2313-2320.

Mishra, P.J., Bertino, J.R. (2009). MicroRNA polymorphisms: the future of pharmacogenomics, molecular epidemiology and individualized medicine. *Pharmacogenomics.* 10, 399-416.

Mishima, Y., Cei Abreu-Goodger, Alison A. Staton. *et al.* (2009). Zebrafish miR-1 and miR-133 shape muscle gene expression and regulate sarcomeric actin organization. *Genes Dev.* 23, 619-632.

Miyazaki, Y., Nomura, S., Miyake, T. *et al.* (1996) High shear stress can initiate both platelet aggregation and shedding of procoagulant containing microparticles. *Blood* 88, 3456–3464.

Mizukoshi, E., M. Suzuki, A. Loupatov. *et al.* (1999). Fibrobloast growth factor-1 interacts with the glucose-egulated protein GFP75/mortalin. *Biochem J* 343, 461-466.

Moffatt OD, Devitt A, Bell ED. e*t al.* (1999). Macrophage recognition of ICAM-3 on apoptotic leukocytes. *J Immunol* 162, 6800-6810.

Morgan, B.P., A.K. Campbell (1985). The recovery of human polymorphonuclear leucocytes from sublytic complement attack is mediated by changes in intracellular free calcium. *Biochem J* 231, 205-208.

Morel O., Toti F., Hugel B. *et al.* (2006) Procoagulant microparticles: disrupting the vascular homeostasis equation? *Arterioscler Thromb Vasc Biol* 26, 2594-2604.

Mullier F., Dogné J.M., Bailly N. *et al.* (2009) Accurate quantification of microparticles by flow cytometry: important issues. J *Thromb Haemost* 7: Suppl 2 Abstract no. PP-MO-040.

Murphy KM, Ranganathan V, Farnsworth ML. *et al.* (2000). "Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells". *Cell Death Differ* 7, 102–11.

Myamoto S, Marcinkiewicz C, Edmunds LH Jr, Niewiarowski S. (1998) Measurement of platelet microparticles during cardiopulmonary bypass by means of captured ELISA forGPIIb/ IIIa. *Thromb Haemost* 80, 225–30.

Nagata, S. R. Hanayama, K. Kawane, (2010). Autoimmunity and the clearance of dead cells. *Cell* 140, 619-630.

Nagata S. (2000). "Apoptotic DNA fragmentation". *Exp Cell Res* 256, 12–8.

Nagata S, Suda T. (1995). Fas and Fas ligand: lpr and gld mutations. *Immunol Today* 16, 39–43

Nakamura, K., A. Kitani, and W. Strober. (2001). Cell contact-dependent immunosuppression by CD4+CD25+ regulatory T cells is mediated by cell surface-bound transforming growth factor β1. *J Exp Med* 194, 629-644.

Nantakomol D, Chimma P, Day NP. *et al.* (2008) Quantitation of cell-derived microparticles in plasma using flow rate based calibration. *Southeast Asian J Trop Med Public Health* 39,146-153.

Nantakomol D., Imwong M., Soontarawirat I. *et al.* (2009). The absolute counting of red cell-derived microparticles with red cell bead by flow rate based assay. *Cytometry B Clin Cytom* 76, 191-198.

Napirei M, Karsunky H, Zevnik B. *et al.* (2000). Features of systemic lupus erythematosus in Dnase1-deficient mice. *Nat Genet*; 25,177–81.

Nasser, M.W., J. Datta, G. Nuovo, *et al.* (2008). Down-regulation of micro-RNA-1 (miR-1) in lung cancer. Suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin-induced apoptosis by miR-1. *J Biol Chem* 283, 33394-33405.

Nauta AJ, Trouw LA, Daha MR. *et al.* (2002). Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. *Eur J Immunol* 32,1726–36.

Negrini, M., Ferracin, M., Sabbioni, S., Croce, C.M. (2007). MicroRNAs in human cancer: from research to therapy. *J Cell Sci* 120,1833-1840.

Neuwelt CM and Young RG. (2009). "Managing neuropsychiatric lupus: Top 10 clinical pearls". *J Musculo Med* 26,127-135

Nickel, W. (2005). Non-conventional secretory routes: direct protein export across the plasma membrane of mammalian cells. *Traffic* 6, 607-614.

Nieuwland R, Berckmans RJ, Rotteveel-Eijkman RC. *et al.* (1997). Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant. *Circulation;* 96,3534–41.

Nieuwland R, Sturk A. (2002). Platelet-derived microparticles. *Academic Press, Elsevier Science*; pp.255–65.

Nieuwland R., Berckmans R.J., McGregor S. *et al* (2000) Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. *Blood* 95, 930-935.

Nomura S. (2004). Measuring circulating cell-derived microparticles. *J Thromb Haemost* 2, 1847-1848.

Nomura, S. Y. Ozaki, Y. Ikeda. (2008). Function and role of microparticles in various clinical settings. *Thromb Res* 123, 8-23.

O'Brien JR. (1955). The platelet-like activity of serum. *Br J Haematol* 1, 223–8.

Ogden CA, deCathelineau A, Hoffmann PR. *et al.* (2001). C1q and mannose binding lectin engagement of cell surface calreticulin and cd91 initiates macropinocytosis and uptake of apoptotic cells. *J Exp Med* 194, 781-796.

O'Rourke MG, Ellem KA. (2000). "John Kerr and apoptosis". *Med J Aust* 173, 616–7 .

Oreskovic R.T., Dumaswala U.J., and Greenwalt T.J. (1992). Expression of blood group antigens on red cell microvesicles. *Transfusion* 32, 848-849.

Paietta E, Andersen J, Racevskis J, *et al.* (1994). Significantly lower Pglycoprotein expression in acute promyelocytic leukemia than in other types of acute myeloid leukemia: immunological, molecular and functional analyses. *Leukemia* 8, 968–973

Parham Peter. (2004). The Immune System, *Garland Science*, 2nd edition.

Pattanapanyasat K., Noulsri E., Fucharoen S. *et al.* (2004) Flow cytometric quantitation of red blood cell vesicles in thalassemia. *Cytometry B Clinical Cytom* 57, 23-31.

Pap, E., E. Pallinger, M. Pasztoi, and A. Falus. (2009). Highlights of a new type of intercellular communication: microvesicle-based information transfer. *Inflamm Res* 58,1-8.

Pecorino, L. (2008). *Molecular Biology of Cancer: Mechanisms, Targets and Therapeutics.* 2nd Ed. NewYork: Oxford University Press p.53.

Philippé J., De Logi E., and Baele G. (2004). Comparison of five different citrated tubes and their in vitro effects on platelet activation. *Clin Chem* 50, 656-658.

Piccin A., Murphy W.G., and Smith O.P. (2007). Circulating microparticles: pathophysiology and clinical implications. *Blood Rev* 21, 157-171.

Tanaka, M., Miwa K, Shinohara A. *et al* (2009) Apoptotic cell clearance by phagocytes. *Int. Congr. Ser.* 1285, 55-59.

Platt N, Suzuki H, Kurihara Y. *et al.* (1996). Role for the class A macrophage scavenger receptor in the phagocytosis of apoptotic thymocytes in vitro. *Proc. Natl. Acad. Sci. USA,* 93, 12456-12460.

Polster, B.M. Pevsner, J. and Hardwick, J.M. (2004). "Viral Bcl-2 homologs and their role in virus replication and associated diseases". *Biochim Biophys Acta* 1644, 211–27.

Popov SG, Villasmil R, Bernardi J, *et al.* (April 2002). "Lethal toxin of Bacillus anthracis causes apoptosis of macrophages". *Biochem Biophys Res. Commun* 293, 349–55.

Racek J., Herynková R., Holecek V. *et al.* (1997) Influence of antioxidants on the quality of stored blood. *Vox Sang* 72, 16-19.

Radic, M, Marion, T, Monestier, M. (2004). Nucleosomes are exposed at the cell surface in apoptosis. *J Immunol* 172, 6692–6700.

Rauch U, Bonderman D, Bohrmann B. *et al* (2000). Transfer of tissue factor from leukocytes to platelets is mediated by CD15 and tissue factor. *Blood*, 96,170–5.

Rahman A and David A. Isenberg. (2008). "Review Article: Systemic Lupus Erythematosus". *N Engl J Med* 358, 929–939.

Rambaldi, (2002) Leukemia: *The Curtis Center,* Philadelphia, PA, , p.531

Ratajczak, J., M. Wysoczynski, F. Hayek. e*t al. (*2006). Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. *Leukemia* 20, 1487-1495.

Ratajczak, J., K. Miekus, M. Kucia. e*t al.* (2006). Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* 20, 847-856.

Rao, P.K., Kumar, R.M., Farkhondeh, M. e*t al. (*2006). Myogenic factors that regulate expression of muscle-specific microRNAs. *Proc Natl Acad Sci USA* 103,8721-8726.

Reich C.F.3rd and D.S. Pisetsky, (2009) The content of DNA and RNA in microparticles released by Jurkat and HL-60 cells undergoing in vitro apoptosis *Exp Cell Res* 315, 760-768.

Remaley A.T., Stonik J. A., Demosky S.J. e*t al.* (2001). Apolipoprotein Specificity for Lipid Efflux by the Human ABCAI Transporter; *Biochemical and Biophysical Research Communications* 280, 818–823.

Robert S., Poncelet P., Lacroix R. *et al.* (2008). Standardization of platelet-derived microparticle counting using calibrated beads and a Cytomics FC500 routine flow cytometer: a first step towards multicenter studies? *J Thromb Haemost* 7,190-197.

Roman M.J., Beth-Ann Shanker, Adrienne Davis. e*t al.* (2003). "Prevalence and correlates of accelerated atherosclerosis in systemic lupus erythematosus". *New Eng J Med* 349, 2399–2406.

Rosen A, Casciola-Rosen L. (1999). Autoantigens as substrates for apoptotic proteases: implications for the pathogenesis of systemic autoimmune disease. *Cell Death Differ* 6, 6–12.

Rosing J, Speijer H, Zwaal RF. (1988). Prothrombin activation on phospholipid membranes with positive electrostatic potential. *Biochemistry* 27, 8–11.

Rozmyslowicz T., Majka M., Kijowski J. *et al.* (2003) Platelet- and megakarocyte-derived microparticles transfer CXCR4 receptor to CXCR4-null cells and make them susceptible to infection by X4-HIV. *AIDS* 17, 33-42.

Rubin O., Crettaz D., Canellini G. e*t al.* (2008) Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools. *Vox Sang* 95, 288-297.

Rumsby M.G., Trotter J., Allan D., and Michell R.H. (1977). Recovery of membrane micro-vesicles from human erythrocytes stored for transfusion: a mechanism for the erythrocyte discocyte-to-spherocyte shape transformation. *Biochem Soc Trans* 5,126-128.

Sabatier F, Darmon P, Hugel B. *et al.* (2008). Type 1 and type 2 diabetic patients display different patterns of cellular microparticles. *Diabetes* 51, 2840–5.

Salmon JE, Kimberly RP, Gibofsky A, Fotino M. (1988). Defective mononuclear phagocyte function in systemic lupus erythematosus: dissociation of Fc receptor-ligand binding and internalization. *J Immunol* 133, 2525–31.

Salzer U., Hinterdorfer P., Hunger U. e*t al.* (2002). Ca++-dependent vesicle release from erythrocytes involves stomatin-specific lipid rafts, synexin (annexin VII), and sorcin. *Blood* 99, 2569-2577.

Santos A. Susin, Eric Daugas, Luigi Ravagnan. e*t al.* (2000). "Two Distinct Pathways Leading to Nuclear Apoptosis". *J Exp Med* 192, 571–80.

Sanz MA, Martín G, Lo-Coco F. (2003) Choice of chemotherapy in induction, consolidation and maintenance in acute promyelocytic leukemia. *Baillieres Best Pract Res Clin Haematol* 16,433–451

Sanz MA, Martín G, Rayón C, *et al.* (1999). A modified AIDA protocol with anthracycline-based consolidation results in high antileukemic efficacy and reduced toxicity in newly diagnosed PML/RARα-positive acute promyelocytic leukemia. *Blood*, 94, 3015–3021.

Sanz MA, Tallman MS, Lo-Coco F. (2005). Tricks of the trade for the appropriate management of acute promyelocytic leukemia. *Blood* 105, 3019–3025

Sanz MA, Martín G, González M, *et al.* (2004). Risk-adapted treatment of acute promyelocytic leukemia with all-trans retinoic acid and anthracycline monochemotherapy: a multicenter study by the PETHEMA Group. *Blood* 103,1237–1243

Sanz MA, Fenaux P, Lo-Coco F. (2005). Arsenic trioxide in the treatment of acute promyelocytic leukemia. A review of current evidence. *Haematologica* 90,1231–1235.

Sauter B, Albert ML, Francisco L. *et al.* (2000). Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med* 191, 423-434.

Savill J, Dransfield I, Hogg N, Haslett C. (1990). Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. *Nature,* 343, 170-173.

Savill J, Fadok V, Henson P, Haslett C. 92007). Phagocyte recognition of cells undergoing apoptosis. *Immunol Today* 14,131–136.

Savill J, Fadok V. (2000). Corpse clearance defines the meaning of cell death. *Nature,* 407, 784-788.

Savill J, Gregory C, Haslett C. (2003). "Eat me or die". *Science* 302, 1516–7.

Savill J, Hogg N, Ren Y, Haslett C. (1992). Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J Clin Invest* 90, 1513-1522.

Schroit AJ, Tanaka Y, Madsen J, Fidler IJ. (1984). The recognition of red blood cells by macrophages: role of phosphatidylserine and possible implications of membrane phospholipid asymmetry. *Biol Cell* 51,227–38.

Schraufstatter IU, Hinshaw DB, Hyslop PA. *et al* (1986). Oxidant injury of cells. DNA strand-breaks activate polyadenosine diphosphate-ribose polymerase and lead to depletion of nicotinamide adenine dinucleotide. *J Clin Invest* 77, 1312-1320.

Schrijvers, D.M. G.R. De Meyer, M.M. Kockx. *et al.* (2005). Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 25, 1256-1261.

Scott RS, McMahon EJ, Pop SM. *et al* (2001). Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature,* 411, 207-211.

Seshasayee D, Valdez P, Yan M *et al.* (2003). Loss of TACI causes fatal lymphoproliferation and autoimmunity, establishing TACI as an inhibitory BLys receptor. *Immunity* 18,279–288

Shacter E, Williams JA, Hinson RM. *et al* (2000). Oxidative stress interferes with cancer chemotherapy: inhibition of lymphoma cell apoptosis and phagocytosis. *Blood,* 96, 307-313.

Shah M.D., Bergeron A.L., Jing-Fei D., and López J.A. (2008). Flow cytometric measurement of microparticles: pitfalls and protocol modifications. *Platelets* 19, 365-372.

Sham, R.L., P.D. Phatak, K.A. Belanger. *et al* (1995). Functional characteristics of mature granulocytes in a patient with acute promyelocytic leukemia treated with all-trans retinoic acid. *Leukemia Res.* 19, 505-511.

Shen ZX, Chen GQ, Ni JH, *et al.* (1997). Use of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia (APL). II. Clinical efficacy and pharmacokinetics in relapsed patients. *Blood* 89,3354-3360.

Shen ZX, Shi ZZ, Fang J, *et al.* (2004) All-trans retinoic acid/As2O3 combination yields a high quality remission and survival in newly diagnosed acute promyelocytic leukemia. *Proc Natl Acad Sci U S A* 101, 5328–5335.

Shet A.S., Aras O., Gupta K. *et al.* (2003). Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. *Blood* 102, 2678-2683.

Shet A.S. (2008). Characterizing blood microparticles: technical aspects and challenges. *Vasc Health Risk Manag* 4 769-774.

Silber, J., Lim DA, Petritsch C. et al. (2008). miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. *BMC Med* 6,14.

Sims PJ, Wiedmer T. (2001). Unraveling the mysteries of phospholipid scrambling. *Thromb Haemost* 86,266–75.

Simak J, and Gelderman M.P. (2006). Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers. *Transfus Med Rev* 20,1-26.

Simons, M., and G. Raposo. (2009). Exosomes – vesicular carriers for intercellular communication. *Curr Opin Cell Biol* 21, 575-581.

Soignet SL, Maslak P, Wang ZG, *et al.* (1998). "Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide". *N Engl J Med* 339, 1341–8.

Soignet S. L., Peter Maslak, Zhu-Gang Wang, *et al.* (1998).Complete Remission After Treatment of APL with Arsenic Trioxide, *N Engl J Med* 339,1341-1348.

St Pierre BA, JG Tidball (1994). "Differential response of macrophage subpopulations to soleus muscle reloading following rat hindlimb suspension". *Journal of Applied Physiology* 77, 290-297.

Strasser A, Harris AW and Cory S. (1991). bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* 67,889–899.

Susin SA, Lorenzo HK, Zamzami N, *et al.* (1999). "Molecular characterization of mitochondrial apoptosis-inducing factor". *Nature* 397, 441–6.

Sweeney J., Kouttab N., and Kurtis J. (2009). Stored red blood cell supernatant facilitates thrombin generation. *Transfusion* 49,1569-1579.

Syuto T, Shimizu A, Takeuchi Y, *et al.* (2009). "Association of antiphosphatidylserine/prothrombin antibodies with neuropsychiatric systemic lupus erythematosus". *Clin Rheumatol* 89, 398-401

Takaoka A, Hayakawa S, Yanai H. *et al.* (2003). "Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence". *Nature* 424, 516–23.

Tallman MS, Andersen JW, Schiffer CA. *et al* (2008).. All-trans-retinoic acid in acute promyelocytic leukemia. *N Engl J Med* 337,1021-1028.

Tallman MS, Altman JK (2008). "Curative strategies in acute promyelocytic leukemia". *Hematology Am Soc Hematol Educ Program* 391–9.

Tallman MS, Nabhan Ch, Feusner JH, et al. (2002). Acute promyelocytic leukemia: evolving therapeutic strategies. *Blood* 99,759–767.

Taulli, R., Francesca Bersani, Valentina Foglizzo. *et al.* (2009). The muscle-specific microRNA miR-206 blocks human rhabdomyosarcoma growth in xenotransplanted mice by promoting myogenic differentiation. *J Clin Invest* 119,2366-2378.

Taylor PR, Carugati A, Fadok VA. e*t al.* (2000). A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. *J Exp Med.*, 192, 359-366.

Teodoro, J.G. Branton, P.E. (1997). "Regulation of apoptosis by viral gene products". *J Virol* 71, 1739–46.

Thery, C., M. Boussac, P. Veron. *et al* (2001). Proteomic analysis of dendritic cellderived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J Immunol* 166, 7309–7318.

Thibert C, Teillet MA, Lapointe F. *et al* (2003). "Inhibition of neuroepithelial patched-induced apoptosis by sonic hedgehog". *Science* 301, 843–6.

Thompson, CB. (1995). "Apoptosis in the pathogenesis and treatment of disease". *Science* 267, 1456–62.

Tidball JG, Berchenko E, Frenette J (1999). "Macrophage invasion does not contribute to muscle membrane injury during inflammation". *J Leuko Biol* 65, 492-498.

Tidball JG, Wehling-Henricks M. (2007). "macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo". *J Physiol* 578, 327-336.

Toth B., Lok C.A., Boeing A. *et al.* (2007). Microparticles and exosomes: impact on normal and complicated pregnancy. *Am J Reprod Immunol* 58, 389-402.

Toti F. and Freyssinet J.M. (2005). An ABC for Scott syndrome? *Blood* 106, 396-397.

Turley, J.M., L.A. Falk, F.W. Ruscetti. *et al.* (1996) Transforming growth factor β1 functions in monocytic differentiation of hematopoietic cells through autocrine and paracrine mechanisms. *Cell Growth Differ.* 7, 1535-1544.

van Beers E.J., Schaap M., Berckmans J. *et al.* (2009). Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease. *Haematologica* 94, 1513-1519

Van Dieijen G, Tans G, Rosing J, Hemker HC. (1981). The role of phospholipid and factor VIIIa in the activation of bovine factor X. *J Biol Chem* 56, 3433–42.

Van Wijk MJ, Svedas E, Boer K. et al (2002). Isolated microparticles, but not whole plasma, from women with preeclampsia impair endothelium-dependent relaxation in isolated myometrial arteries from normal pregnant women. *Am J Obstet Gynecol* 187,1686–93.

Vandivier RW, Henson PM, Douglas IS. (June 2006). "Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease". *Chest* 129, 1673–82.

Verhoven B, Schlegel RA, Williamson P. (1995). Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J Exp Med* 182, 1597-1601.

Voll RE, Hermann M, Roth EA. *et al.* (1997). Immunosuppressive effects of apoptotic cells. *Nature,* 390, 350-351

Waclavicek, W., A. Berer, L. Oehler, J. *et al.* (2001). Calcium ionophore: a single reagent for the differentiation of primary human acute myelogenous leukaemia cells toward dendritic cells. *Br J Haematol* 114, 46-473.

Wachtel, M., Dettling M, Koscielniak E, *et al.* (2006). Subtype and prognostic classification of rhabdomyosarcoma by immunohistochemistry. *J Clin Oncol* 24,816-822.

Wagner G.M., Chiu D.T., Qju J.H. *et al.* (1987) Spectrin oxidation correlates with membrane vesiculation in stored RBCs. *Blood* 69, 1777-1781.

Wahl, S.M., and W. Chen. (2005). Transforming growth factor-□-induced regulatory T cells referee inflammatory and autoimmune diseases. *Arthritis Res. Therapy* 7, 62-68.

Walsh M, James M, Jayne D. *et al.* (2007). Mycophenolate mofetil for induction therapy of lupus nephritis: a systematic review and meta-analysis. *Clin J Am Soc Nephrol* 2,968-75.

Warrell RP Jr, de Thé H, Wang Z-Y, Degos L. (1993) Acute promyelocytic leukemia. *N Engl J Med* 329,177-189.

Wang X, Wu YC, Fadok VA. *et al.* (2003). "Cell corpse engulfment mediated by C. elegans phosphatidylserine receptor through CED-5 and CED-12". *Science* 302, 1563–1566.

Wang, X.W. Gibson, M.K. Vermeulen, W. e*t al.* (1995). "Abrogation of p53-induced Apoptosis by the Hepatitis B Virus X Gene". *Cancer Res* 55, 6012–6.

Wang, H., Garzon R, Sun H. *et al.* 2008. NF-kappaB-YY1-miR-29 regulatory circuitry in skeletal myogenesis and rhabdomyosarcoma. *Cancer Cell.* 14,369-381

Wajant H (2002). "The Fas signaling pathway: more than a paradigm". *Science* 296, 1635–6.

Wajant, H. Erman, A. and Jezernik, K. (2009) "Connection Map for Fas Signaling Pathway". *Science* 296, 1635-6

Warren BA, Vales O. (1972). The release of vesicles from platelets following adhesion to vessel walls in vitro. *Br J Exp Pathol* 53,206–15.

Werlen G, Hausmann B, Naeher D and Palmer E. (2003). "Signaling life and death in the thymus: timing is everything". *Science* 299, 1859–63.

Wiedmer, T. & Sims, P.J. (1991). Participation of protein kinases in complement C5b-9-induced shedding of platelet plasma membrane vesicles. *Blood* 78, 2880–2886.

Willekens F.L., Were J.M., Kruist J.K. *et al.* (2005) Liver 'kupffer cells rapidly remove red blood cell-derived vesicles from the circulation by scavenger receptors. *Blood* 105, 2141-2145.

Willekens F.L., Werre J.M., Groenen-Döpp Y.A. *et al.* (2008) Erythrocyte vesiculation: a self-protective mechanism? *Br J Haematol* 141, 549-556.

Wolf P. (1967). The nature and significance of platelet products in human plasma. *Br J Haematol*;13:269–88.

Wyllie AH, Kerr JF, Currie AR (1980). Cell death: the significance of apoptosis. *Int Rev Cytol* 68,251–306.

Yan M, Wang H, Chan B et al. (2001). Activation and accumulation of B cells in TACI-deficient mice. *Nat Immunol* 2, 638–643

Yang L, Mashima T, Sato S, *et al.* (2003). "Predominant suppression of apoptosome by inhibitor of apoptosis protein in non-small cell lung cancer H460 cells: therapeutic effect of a novel polyarginine-conjugated Smac peptide". *Cancer Res* 63, 831–7.

Yang W, Ng P, Zhao M, *et al.* (2009). "Population differences in SLE susceptibility genes: STAT4 and BLK, but not PXK, are associated with systemic lupus erythematosus in Hong Kong Chinese". *Genes Immun.* 10, 219-26.

Yano, Y., Kambayashi, J., Shiba, E. *et al.* (1994). The role of protein phosphorylation and cytoskeletal reorganization in microparticle formation from theplatelet plasma membrane. *Biochem J* 299, 303–308.

Yazdany J (2008). "Strategies for preventive care in systemic lupus erythematosus". *J Musculo Med.*

Yingling, J.M., K.L. Blanchard, and J.S. Sawyer. (2004). Development of TGF-β signaling inhibitors for cancer therapy. *Nat. Rev. Drug Discov.* 3, 1011-1022.

Yoshida S, Takeuchi T, Itami Y, *et al.* (2009). Hemophagocytic syndrome as primary manifestation in a patient with systemic lupus erythematosus after parturition. *Lupus* 32, 66-70.

Yu, L.M., D.X. Chen, Q.X. Zhou. *et al.* (2006). Effects of histamine on immunophenotype and notch signalling in human HL-60 leukemia cells. *Exp Biol Med* 231, 1633-1637.

Zhang, B., Pan, X., Cobb, G.P. and Anderson, T.A. (2007). microRNAs as oncogenes and tumor suppressors. *Dev Biol* 302,1-12.

Zhou Z. (2007). New phosphatidylserine receptors: clearance of apoptotic cells and more. *Dev Cell* 13, 759-760.

Zhu, L., and A. I. Skoultchi. 2001. Coordinating cell proliferation and differentiation. *Curr Opin Genet Dev* 11, 91-97.

Ziegler U & Groscurth P (2004). Morphological features of cell death. *News Physiol Sci* 19,124-128.

Zielonka TM, Demkow U, Zycinska K, *et al.* (2008). "Angiogenic activity of sera from patients with systemic autoimmune diseases in relation to clinical, radiological, and functional pulmonary status". *J. Physiol. Pharmacol.* 59 Suppl 6, 791–800.

Zwaal RF, Bevers EM. (1983). Platelet phospholipid asymmetry and its significance in hemostasis. *Subcell Biochem* 9,299–334.

Zwaal RF and Schroit AJ (1997). Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* 89, 1121- 1132.