INVESTIGATION OF SIGNAL TRANSDUCTION MECHANISMS REGULATING CD14 EXPRESSION AND SHEDDING BY MONOCYTE DERIVED MACROPHAGES

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HONG QIAN

INVESTIGATION OF SIGNAL TRANSDUCTION MECHANISMS REGULATING CD14 EXPRESSION AND SHEDDING BY MONOCYTE DERIVED MACROPHAGES

by

© Hong Qian, M.B.

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ABSTRACT

CD14 expression and shedding are important in the regulation of cellular responses to lipopolysaccharide (LPS) and concomitant production of cytokines and related immunological and pathophysiological changes. The rapid shedding of CD14 from monocytes, subsequent to contact with LPS, has led us to hypothesize that signalling events through the CD14 receptor complex are involved in the activation of enzymes responsible for the cleavage and loss of CD14 from the cell surface. This then ultimately regulates cellular responses to lipopolysaccharide. This hypothesis was tested using a number of signal transduction modulating agents to inhibit or activate secretions of the CD14 signalling pathway. Besides LPS, two other natural ligands tumour necrosis factor α (TNF-α) and *formyl*-methionyl-leucyl-phenylalanine (*f* MLP) were investigated to observe if they also had a role in regulating CD14 expression or loss. TNF-α, a cytokine released rapidly following LPS contact with CD14 on monocytes, was suspected of having a negative feedback control on monocytes by blocking LPS signalling or increasing CD14 loss from the monocyte surface. The other ligand, *f* MLP, was known to have chemotactic activity for both monocytes and neutrophils. Although *f* MLP has its own receptor, it activates monocytes in

a number of ways that are similar but not identical to LPS and may share common signalling pathways. In these studies only early events, up to 4 hours, were investigated using FACS analysis to follow CD14 expression, and immunoprecipitation and 2D-SDS PAGE analysis of membrane and soluble CD14 to confirm the mechanism and characterize the products released from cells. It was found that the most active pharmacological agents, inducing loss of membrane CD14 from monocytes, were regulators of PKC or cytoplasmic calcium levels. It was also found that *f*MLP was able to induce a rapid loss of membrane CD14 expression from monocytes. TNF-α, on the other hand, had reverse effect and caused a rapid increase in CD14 expression by monocytes.

As a result of the initial investigations into the action of various signalling pathway inhibitors on CD14 expression and shedding, an interesting observation was made with calphostin C, a photoactivated protein kinase C inhibitor. This compound was found to induce 100% loss of CD14 expression from normal human peripheral blood mononuclear cells and the human monocytic leukaemia cell line, THP-1, through membrane vesicle shedding or apoptosis. Further investigation found that Ca^{2+} dependent K^{+} channels were also involved in the regulation of apoptosis induced by calphostin C. These data were presented at the Paris Conference on

Apoptosis in AIDS and CANCER (Qian,H., Liepins,A., Richardson,V.J., *p*113. Dec.2-4, 1993, Paris, France. Abstract attached to the following page).

Finally, ouabain, an inhibitor of Na^{+}/K^{+}-ATPase, was also found to induce a rapid and complete loss of membrane CD14. Using 2D-SDS-PAGE analysis, this did not appear to be due to apoptosis or shedding of CD14 into the media.

ROLE OF PROTEIN KINASE C AND Ca^{2+} DEPENDENT K^+ CHANNELS IN APOPTOSIS

H.Qian, A.Liepins and V.J.Richardson, Faculty of Medicine, Memorial University of NFLD, St.John's, Newfoundland, Canada, A1B 3V6

Calphostin C is a photoactivated protein kinase C inhibitor which induces a site-specific oxidative modification of PKC at the Ca^{2+}-induced hydrophobic region (Gopalakrishna, 1992). Exposure of human monocytic leukaemia THP-1 cells to calphostin C in the presence of light, at concentrations of 10 nM to 200 nM, triggered the apoptosis process as manifested by cell surface blebbing, nuclear DNA condensation and fragmentation. Kinetic studies showed that calphostin C induced apoptosis in THP-1 is time and dose-dependent. The involvement of PKC in the apoptosis process was further confirmed by PMA, a PKC activator. The induction of calphostin C-mediated THP-1 cell apoptosis was significantly inhibited by the addition of PMA (100 nM). This may be due to competitive binding to regulatory domain of PKC by PMA and calphostin C. Pre-incubation of THP-1 cells with quinidine, a Ca^{2+} dependent K^+ channel blocker, and 4-aminopyridine, a K^+ channel blocker, significantly delayed the induction of apoptosis in THP-1 cells by calphostin C. We also found that the susceptibility to calphostin C-induced apoptosis is not restricted to THP-1, but also occurs in other tumour cell lines such as the monoblastic leukaemia U-937, as well as in normal human peripheral mononuclear cells. Staurosporine, also a PKC inhibitor, failed to induce apoptosis in these cells. This may reflect the different species of PKC that are inhibited by calphostin C and staurosporine respectively.

In conclusion, our data indicate that PKC function is required for suppression of apoptosis in the above leukaemia cells and normal human peripheral blood leucocytes and that Ca^{2+} dependent K^+ channels may also be involved in the regulation of apoptosis induced by calphostin C.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

AMML	Acute myelomonocytic leukaemia
AMoL	Acute monocytic leukaemia
AO	Akadaic acid
4-AP	4-Aminopyridine
A23187	Calcium ionophore
BSA	Bovine serum albumin
CSF-1	Colony-stimulating factor 1
DAG	Diacylglycerol
DFP	Diisopropylfluorophosphate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELAM	Endothelial leukocyte adhesion molecules
Fc	Fragment crystallizable
FCS	Fetal calf serum
f MLP	*formyl*-methionyl-leucyl-phenylalanine
GAP	$p21^{ras}$-GTPase activating protein
G-CSF	Granulocyte-colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosyl-phosphatidylinositol
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular adhesion molecule-1
IEF	Isoelectric focusing
IgG	Immunoglobulin G
IgG $F(ab')_2$-PE	Phycoerythrin-conjugated IgG Fab fragments
IgM	Immunoglobulin M
IP_3	Inositol 1,4,5-trisphosphate
IL-1	Interleukin-1
IL-6	Interleukin-6
INF-γ	Interferon γ
kDa	Kilodalton
LBP	LPS binding protein
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
LT	leukotriene

mAb	Monoclonal antibody
MAP kinase	Mitogen-activated protein kinase
MAP kinase	Microtubule-associated protein kinase
M-CSF	Monocyte-macrophage colony-stimulating factor
MDM	Monocyte derived macrophage
NF-kB	Nuclear transcriptor factor-kappa B
NP-40	Nonidet P-40
PAF	Platelet-activating factor
PBM	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCD	Programmed cell death
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PGE_2	Prostaglandin E_2
PI	Propidium iodide
PI3K	Phosphatidyiinositol 3-kinase
pI	Isoelectric point
PI-PLC	Phosphatidylinositol-specific phospholipase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLC γ	Phospholipase C γ
PLD	Phospholipase D
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phanylmethylsufonyl fluoride
PT	Pertussis toxin
PTK	Protein tyrosine kinase
RPMI medium	Roswell Park Memorial Institute medium
sCD14	Soluble form CD14
SDS	Sodium dodecyl sulphate
2D-SDS-PAGE	2 Dimension-SDS-polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-Tetramethylethylenediamine
TNF-α	Tumour necrosis factor alpha

Chapter 1 Introduction

1.1 Function of mononuclear phagocytes

The immune system is a network of remarkable defense mechanisms that protect our bodies from invasion by microorganisms and cancer, in which mononuclear phagocytes play a major role. When microorganisms such as bacteria invade our tissues, phagocytes such as neutrophils and monocytes are chemotactically attracted to the infection site and immediately begin phagocytosing these foreign particles. Phagocytosed organisms are killed and then degraded within the phagocytes by a number of mechanisms, all of which are part of the innate immune process. In addition to this, macrophages are activated and may stimulate an acute inflammatory response through secretion of short-lived inflammatory mediators such as platelet-activating factor (PAF), prostaglandins (PG), leukotrienes (LT), reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI) including nitrite, nitric oxide and nitrogen dioxide, as well as inflammatory cytokines such as IL-1,IL-6 and TNF-α, some of which also serve to kill microbes and control the spread of the infection.

If the invasion by microorganisms persists, the inflammatory response will be supplemented and augmented by the elements of acquired immunity, in which macrophages play a central role in the development of both humoral and cell-mediated immune responses through antigen processing and presentation to specific immune effector cells and further cytokine secretion. Subsequently,

specific acquired immune responses, both humoral and cellular, are induced to specifically eradicate the invading pathogen. Macrophages are also involved in direct tumour cell killing, either by antibody independent processes involving cytokines or reactive intermediates such as nitric oxide (Fiers, 1991), or through an antibody mediated process (Koren, 1983).

When mononuclear phagocytes are activated by microorganisms, they begin to secrete a variety of cytokines and other factors including IL-1, IL-6, TNF-α, GM-CSF, G-CSF, M-CSF, INF-α, complement factors and hydrolytic enzymes. Some of these cytokines are also involved in the development of a local inflammatory response and are a major component of the body's defense mechanism. Mononuclear phagocytes can also cause inflammatory disease, if they produce excess cytokines. For example, sepsis is the most severe inflammatory disease clinically, in which the cytokine TNF-α is the primary mediator secreted by activated mononuclear phagocytes. It was recently estimated that the incidence of Gram-negative sepsis in the United States was 170,000 cases per year, with an associated mortality of more than 70,000 per year (Adams, 1992). Substantial effort has been devoted to understanding the precise regulation of mononuclear phagocyte activation during Gram-negative sepsis, in order to maintain host defenses while avoiding complications such as organ failure and death due to endotoxemia. Lipopolysaccharide (LPS) produced by Gram-negative bacteria has been implicated as the major contributing factor in the pathogenesis of

endotoxemia (Rietshel *et al.*, 1987).

1.2 Lipopolysaccharide (LPS) structure and biological activity

LPS is the principal lipid component of the outer membrane of all Gram-negative bacteria (Fig.1) and is one of the most potent activators of mononuclear phagocytes. It is an amphipathic molecule, having three distinct regions (Fig.2): (i) the polysaccharide O-antigen, which is immunodominant and confers serological specificity to each particular Gram-negative bacterium; (ii) the core oligosaccharide, composed of the outer core and inner core, which is more conserved than the "O antigen" between the different species of bacteria; (iii) the lipid A region containing a diphosphorylated glycosamine disaccharide acylated with characteristic hydroxylated and nonhydroxylated fatty acid chains. Maximal toxicity is associated with the lipid A component which is highly conserved within families of bacteria (Morrison *et al.*, 1992).

During the course of a Gram-negative infection, LPS is released from the outer membranes of bacteria into the circulation, where it subsequently is responsible for initiating a cascade of events. These include an initial release of cytokines, PG, LT, ROI, RNI with activation of the coagulation, fibrinolytic, and complement systems, eventually leading to the severe pathological sequelae of endotoxic shock (Bone, 1991). Endotoxic shock is characterized by fever, hypotension, hypoxia, acidosis and disseminated intravascular coagulation, often

leading to multisystem organ failure and death (Tracey *et al.*, 1988). For many years, it has been recognized that experimental administration of purified LPS mimics the symptoms elicited during Gram-negative sepsis, and mononuclear phagocytes are believed to be the major target cells (Volgel *et al.*, 1990, 1992; Manthey *et al.*, 1992). These cells respond to LPS by synthesizing and secreting a variety of cytokines such as IL-1, IL-6 and TNF-α, all of which play a deleterious role in septic shock (Morrison *et al.*, 1979).

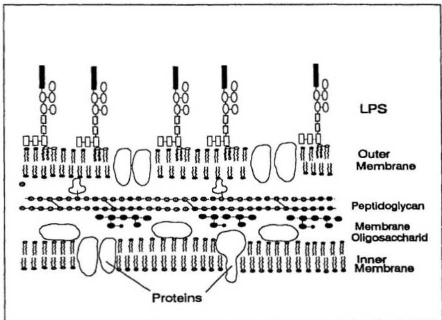


Figure 1. E.*coli* envelope organization.

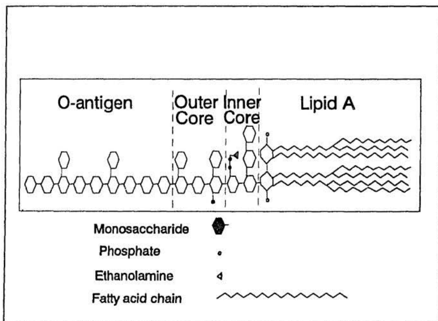


Figure 2. Lipopolysaccharide structure

1.3 CD14, a new candidate receptor for LPS

During the past two decades, a considerable volume of data has appeared concerning the interaction between mononuclear phagocytes and LPS. This has included LPS binding, uptake of LPS and the synthesis and secretion of cytokines (Morrison *et al.*, 1987; Schumann *et al.*, 1990). Binding of LPS to the phagocyte plasma membrane is the first essential step in this LPS-cell interaction. There are two different mechanisms by which LPS initially interacts with the cell's plasma membrane, namely specific and non-specific binding (Morrison, 1985). Non-specific interactions result from the binding of LPS to the plasma membrane phospholipid (Morrison, 1985). Whether this non-specific binding triggers cellular responses is still unknown. On the other hand, specific interactions result from the binding of LPS to receptors on the plasma membrane. Recently, several major receptors for LPS have been identified, including the 95 KDa CD18 found on leukocytes, the 80 KDa macrophage scavenger receptors RI/II and acetyl-low density lipoprotein receptor (acetyl-LDL), the 55 KDa CD14 found on human monocytes and macrophages and a 73 KDa membrane protein on monocytes, macrophages, lymphocytes, neutrophils and platelets (Halling *et al.*, 1992).

CD18 (also known as β_2 integrins or leukocyte integrins) binds particulate LPS when presented on the surface of bacteria or LPS-coated erythrocytes (Wright *et al.*, 1990) and participates in the phagocytosis of particles. The macrophage scavenger receptors, recently cloned by Freeman *et al.*(1990), may function to

remove endotoxin from the circulation and deliver it to lysosomes, where it can be metabolized to less active substances (Hampton *et al.*, 1991). Both these receptors mediate the disposal or removal of LPS without subsequent cytokine synthesis (Wright *et al.*, 1990). CD14 has been shown to function as a receptor for LPS and initiates the secretion of a variety of cytokines including TNF-α, IL-1, IL-6, and IL-8 (Dentener *et al.*, 1993). The following introduction will focus on the CD14 molecule. However, CD14 is not the only LPS receptor to be involved in cytokine secretion, as monocytes from patients with paroxysmal nocturnal haemaglobinuria, which lack CD14, have also been shown to be sensitive to LPS, though this is at much greater concentrations than when CD14 is present (Haziot *et al.*, 1988). It is now believed that a low affinity receptor, perhaps the 73 KDa protein mentioned above, may be involved in cytokine secretion. CD14 and the 73 KDa protein may form a receptor complex similar to that of many cytokine receptors such as IL-2 (Hatakeyama *et al.*, 1989) and IL-6 (Hibi *et al.*, 1990; Akira *et al.*, 1990). There is now some evidence that the 73 KDa protein is a lipid A specific receptor (Lei *et al.*, 1988).

1.3.1 A cell surface differentiation marker on monocytes/macrophages

CD14, a 55 KD glycoprotein, is a myeloid differentiation antigen expressed in abundance on the surface of monoblasts, more mature monocytes, and macrophages (Todd *et al.*, 1984; Griffin *et al.*, 1984; Goyert *et al.*, 1987), and in

trace amounts on activated granulocytes (Hogg *et al.*, 1987; Janaman *et al.*, 1989) as well as on human B cells (Labeta *et al.*, 1991). It was found that human breast cancer cells also have CD14 antigen on their surface (Calvo *et al.*, 1987). CD14 is not present in early stem cells such as the myelomonocytic HL-60 cell line or the monoblastic cell line U-937. However, these cells can be induced to differentiate into more mature monocyte-like cells which do express CD14 (Rigby *et al.*, 1984). More mature cells, like the monocytic cell line Mono Mac6, do express CD14 (Ziegler-Heitbrock *et al.*, 1988). Further maturation of these cell lines can either increase or decrease CD14 expression depending on the type of inducer (Ziegler-Heitbrock *et al.*, 1988). The majority of peripheral blood monocytes express a high level of CD14 ($CD14^{++}$), but a recently identified subpopulation expresses much lower levels of CD14 together with CD16, the FcγRIII molecule (Ziegler-Heitbrock *et al.*, 1988; Calvo *et al.*, 1987). CD14 is not only present in a membrane-bound form but also as a soluble or cell free CD14 (sCD14), which can be detected in normal human plasma (Maliszewski *et al.*, 1985; Bazil *et al.*, 1986), in the urine of nephrotic patients (Bazil *et al.*, 1986, 1989) and in the tissue culture supernatant of CD14 positive cells (Maliszewski *et al.*, 1985) suggesting that it can be shed or secreted. Further studies found that 70% of CD14 could be removed from the monocyte cell surface by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC), confirming also that CD14 is anchored to the membrane through a glycosyl-phosphatidylinositol (GPI) anchor (Haziot *et al.*, 1988).

1.3.2 Evidence of CD14 as a receptor for LPS

Recently, a variety of approaches have provided more direct evidence that the LPS binding to CD14 is more efficient when it is associated with the LPS binding protein (LBP) or with septin. Saturable and displaceable binding of ^{3}H-LPS or FITC-LPS to human monocytes in the presence of serum or purified LBP was shown to be completely inhibited by anti-CD14 mAb (Couturier *et al.*, 1991; Heumann *et al.*, 1992). A subclone of J774, a mouse macrophage that was hyporesponsive to LPS, was specifically deficient in CD14 expression, and failed to bind LPS (Hara *et al.*, 1990). Finally, LPS-crosslinking studies provided direct evidence that CD14 binds LBP-LPS complexes. A radioiodinated, photoreactive derivative of LPS (^{125}I-ASD-LPS) was used to crosslink proteins that bind LPS (Wollenerber *et al.*, 1985). When a low concentration of ^{125}I-ASD-LPS (5 ng/ml) was incubated with LBP and the THP-1 monocytic cell line, LBP and CD14 were the only major proteins crosslinked (Tobias *et al.*, 1993).

A functional role for CD14-mediated LPS binding was demonstrated using Gram-negative bacteria, LPS-coated particles, or highly purified preparations of LPS (Wright *et al.*, 1990). LPS-CD14 binding was enhanced markedly by the presence of LBP, a 60 kDa glycoprotein present in normal human serum at less than 0.5 μg/ml and rising up to 50 μg/ml after an acute-phase response (Tobias *et al.*, 1986). LBP binds to the lipid A moiety of the LPS molecule with an affinity of $\approx 10^{9}$ M^{-1} (Tobias *et al.*, 1989). Studies also suggested that LPS-induced

monocytic stimulation under physiological conditions, occurred when LPS formed a high-affinity complex with serum LBP and this complex then bound to cells via the CD14 molecule (Schumann *et al.*, 1990). Binding of LPS-LBP complex to macrophages via CD14 was shown to induce the production of tumour necrosis factor (TNF-α) (Bazil *et al.*, 1986), which is a primary mediator of endotoxic shock. Blockade of CD14 with monoclonal antibodies such as My 4 prevented synthesis of TNF-α by whole blood incubated with the physiological concentration of LPS (<1 ng/ml) (Wright *et al.*, 1990). Transfection of murine 70Z/3 B cells with the human CD14 gene resulted in a 1000-fold increase in their sensitivity to LPS when in the presence of LBP (Lee *et al.*, 1992). Moreover, transgenic mice, expressing human CD14 were shown to be hypersensitive to LPS (Ferrero *et al.*, 1993). The above evidence suggests that CD14 is a receptor for the LPS-LBP complex and involved in cytokine secretion.

In addition to LBP, "septin", a factor of at least two mixed protein species in normal human serum, also enhances monocyte responses to LPS. Septin is found in normal plasma at concentrations of less than 1 ng/ml (Wright *et al.*, 1992). Wright *et al.* also showed that septin binds to LPS and induces monocytes to secrete TNF-α. Induction of TNF-α by low concentrations of LPS (< 1 ng/ml) was completely blocked by anti-CD14 monoclonal antibodies in the presence of septin. This opsonic activity of septin in plasma appears distinct from LBP since it is not blocked by neutralizing antibodies against LBP. It has been reported that LBP is

present in low levels (~150 ng/ml) in the serum of healthy rabbits and is only abundant after induction of the acute phase response (Tobias *et al.*, 1989). In contrast, septin is present at very high titres in normal plasma and may be diluted more than 3000 fold before its activity is lost. This suggests that septin represents the principal means for opsonization of low levels of LPS for recognition by CD14 on monocytes or macrophages in a healthy host. The above evidence suggests that the response to sub-nanogram-per-millilitre concentrations of LPS depends on CD14. However, very high concentrations of LPS (>100 ng/ml) can still stimulate TNF-α synthesis in blood in which either LBP or CD14 was blocked with antibodies (Wright *et al.*, 1990). At a concentration of 10 μg/ml or more, LPS was also reported to be associated with intracellular compartments independent of the presence of serum (Kang *et al.*, 1990). Thus, macrophages seem to possess a second mechanism for responding to high concentrations of LPS that is independent of CD14.

1.4 CD14, a member of the diverse family of GPI-anchored proteins

Most membrane proteins are attached to the lipid bilayer by virtue of the hydrophobic portion of the protein. But late in 1985, a novel post-transcriptional modification mechanism was elucidated by which proteins could be anchored to membrane through covalent attachment to glycosyl-phosphatidylinositol (GPI) located in the lipid bilayer (Futerman *et al.*, 1985). This class of GPI-anchored

proteins has now been detected in a wide variety of eukaryotic cells (Table 1). They include hydrolytic enzymes, mammalian differentiation antigens, protozoal antigens, cell adhesion molecules and oncofoetal antigens (Low, 1989). These GPI-anchored proteins themselves have extreme structural and functional diversity, but their GPI-anchors retain a certain structural similarity. This anchoring mechanism may be vitally important in regulating the expression and shedding of these molecules. Moreover, this manner of attachment to the membrane would consequentially result in the interaction with other cell surface molecules or soluble ligands, due to the greater lateral mobility of the GPI-anchor than transmembrane-anchored proteins.

Table 1. Proteins with a glycosyl-phosphatidylinositol anchor (Low,1989)

Hydrolytic enzymes	
	Alkaline phosphatase
	5'-Nucleotidase
	Acetylcholinesterase
	Trehalase
	Alkaline phosphodiesterase I
	p63 protease (*Leishmania*)
	Renal dipeptidase
	Merozoite protease (*Plasmodium*)
	Aminopeptidase P
	Lipoprotein lipase
Mammalian antigens	
	Thy-1
	RT-6
	Qa
	Ly6
	Carcinoembryonic antigen (CEA)
	Blast-1
	CD14
Protozol antigens	
	Ssp-4 (*Trypanosoma*)
	Variant surface glycoprotein (*Trypanosoma*)
	Surface proteins (*Paramecium*)
	195-kDa antigen (*Plasmodium*)
Cell adhesion	
	LFA-3
	Heparan sulfate proteoglycan
	Neural cell adhesion molecule
	Contact site A (*Dictyostlium*)
	PH-20 (guinea pig sperm)
Miscellaneous	
	Decay-accelerating factor (DAF)
	130-kSa hepatoma glycoprotein
	34-kDa placental growth facotr
	Scrapie prion protein

1.4.1 Structure of GPI-anchors

Knowledge of the detailed structure of the glycosyl-phosphatidylinositol anchors largely derives from studies done with the variant surface glycoprotein (VSG) of the protozoal parasite *Trypanosoma brucei* (Ferguson *et al.*,1984, 1985; Schmitze *et al.*, 1987). The major features of this structure are illustrated in Figure 3 and are summarized as follows: (a) ethanolamine is amide-linked to the α-carboxyl group of the COOH-terminal amino acid of the protein; (b) a phosphodiester linkage between the hydroxyl of the ethanolamine and the 6-hydroxyl on mannose; (c) this mannose is the part of a branching glycan which contains mainly mannose residues and a branch containing galactose residues. A central mannose is then linked to a glucosamine residue with a free amino group at its reducing terminus. The presence of this unusual, sugar allows the structure to be selectively cleaved at this point by nitrous acid; (d) the glucosamine is glycosidically linked at C-1 position to the 6-hydroxyl on the inositol ring of phosphatidylinositol (Low *et al.*, 1988). Recently, evidence has shown that these major anchor structures are highly conserved in the GPI-linked portion, although there are some modifications found mainly in the galactose branching chain, variation in the fatty acid composition of the diglyceride (Ferguson *et al.*,1984) and the presence or absence of acylation of the inositol ring by fatty acid chains (Roberts *et al.*, 1987).

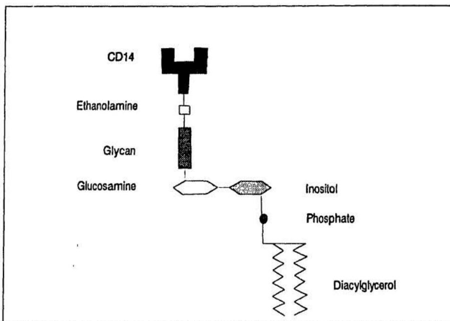


Figure 3. Structure of glycosyl-phosphatidylinositol anchor

Most proteins anchored to membrane via GPI-linkage are sensitive to degradation by bacterial phosphatidylinositol-specific phospholipases C (PI-PLC) resulting in the release of protein from the membrane. The advantages to the cell are that GPI-anchors may play a dynamic and versatile role in the regulation of cell surface protein expression or in the shedding of bioactive proteins into the circulation or extracelluar milieu. Regulation of CD14 expression or shedding may also occur via post-translational attachment to or enzymic cleavage of the GPI-structure.

1.4.2 Evidence of CD14 as a GPI-anchored protein

It was predicated, from the cDNA sequence, that the amino acid sequence of CD14 would contain a typical hydrophobic leader peptide of 17 amino acids (Goyert *et al.*, 1988). No absolute consensus sequence motif for GPI attachment has been confirmed, and six different amino acids have been found at the carboxyl terminus of the 19 or so proteins sequenced with covalently attached GPI anchors (Ferguson, 1988). A decrease in the cell surface expression of CD14 was observed after treatment of monocytes with PI-PLC (Goyert *et al.*, 1988). Sensitivity to PI-PLC confirmed that CD14 is anchored via GPI-linkage. It was also found by Goyert *et al.* (1988) that the monocytes of patients with paroxysmal nocturnal haemoglobinuria, a disease characterized by the lack of expression of all GPI-linked proteins, failed to express CD14. Low and Saltiel (1988) found that proteins linked to membrane via a GPI anchor possess, in the nascent protein, a sequence of amino acids at the C-terminal of relatively hydrophobic amino acids. This is also true for CD14, in which

the C-terminal amino acid sequence is (Ser, Gly) Thr-Val-Leu-COOH (Bazil *et al.*, 1989). All of the above evidence support CD14 as a GPI-anchored protein.

1.5 Soluble-form CD14 (sCD14)

1.5.1 An antagonist for monocyte response to LPS ?

Apart from the membrane-bound form of CD14 as a receptor for the complex of LPS-LBP or LPS-septin, a soluble form, sCD14, is found in normal human serum at a concentration of 2-6 μg/ml (Bazil *et al.*, 1986; Schütt *et al.*, 1992) and in even higher concentrations in some pathological conditions such as post-polytraumatized and severely burned patients (Kruger *et al.*, 1991), and in septicemia patients, in which an increased level of sCD14 correlates with the acute-phase C-reactive protein (Kruger *et al.*, 1991). Recent *in vitro* studies have shown that native sCD14 and recombinant soluble CD14 (rsCD14) can inhibit the response of monocytes to LPS:LBP (Schütt *et al.*, 1992). Schütt *et al.* provided evidence that sCD14 reduced LPS-inducible monocyte activation in a dose-dependent manner. Haziot *et al.* (1992) showed that native and rsCD14, isolated from the urine of a patient with nephrotic syndrome and from *Baculovirus* cultures, respectively, can bind LPS:LBP. More recently, Haziot et al. (1993) showed that the recombinant sCD14 competes very effectively with cell surface CD14 for LPS, and is a potent inhibitor of LPS-induced TNF-α production in whole blood. Thus, this evidence suggests that sCD14 may act as an antagonist for monocyte response to LPS. But this antagonistic LPS effect requires concentrations of sCD14 10-fold higher than normal human serum levels

(Schütt *et al.*, 1992; Haziot *et al.*, 1992; Haziot *et al.*, 1993). Indeed, in polytraumatized and in severely burned patients, sCD14 in serum starts to increase within the first 6 days post trauma and remains elevated during the first 14 days (Kruger *et al.*, 1991). It is possible that elevated levels of serum sCD14 in these patients indicate a natural protective mechanism against excessive monocyte cytokine production. Therefore, sCD14 was viewed as a possible therapy in prevention of endotoxic shock. However, this therapy must be viewed with caution as sCD14 can also act as an agonist for LPS-induced responses by endothelial cells.

1.5.2 An agonist for endothelial/epithelial cell responses to LPS ?

Recent evidence has shown that sCD14 was at least partly, if not entirely, produced by shedding of membrane CD14 following monocyte or macrophage activation in response to a physiologically activating agent such as bacterial LPS (Bazil *et al.*, 1991). *In vivo* circulating sCD14 may also result from shedding by activated monocytes/macrophages. In the presence of a physiological concentration of sCD14, both bovine and human endothelial cells, which do not express CD14 on their surface, were shown to respond to LPS (Beekhuizen *et al.*, 1991). Patric *et al.* (1992) was able to show that anti-CD14 antibodies can inhibit the serum-dependent response of bovine and human endothelial cells to LPS. Furthermore, Frey *et al.* (1992) demonstrated that immunodepletion of sCD14 from serum prevented human umbilical vein endothelial cell response to LPS and that the response was restored by the addition of sCD14. Pugin *et al.* (1993) supported that sCD14 was required for

the activation of endothelial cells and, in addition, found that epithelial cells were activated by LPS. Pugin also provided evidence that the LPS-LBP complexes transfer LPS to sCD14 and he proposed that the LPS-sCD14 complexes then bind to epithelial or endothelial cell receptor(s) yet to be identified. Haziot's (1993) data also supported this proposal since it was found that endothelial cells were activated by high concentrations of LPS in the presence of rsCD14 alone. In low concentrations of LPS (5 and 10 ng/ml), the rsCD14-stimulated activation was strongly enhanced by LBP. In addition, they showed that LPS bound to rsCD14 directly in the presence of low concentrations of LPS, and that binding was enhanced by the presence of LBP. It was proposed that sCD14 is responsible for the effects of LPS on epithelial and endothelial cells, potentially leading to the activation and perhaps the dysfunction of these tissues in the pathophysiology of endotoxic shock when sCD14 levels are elevated above normal.

1.6 CD14 and LPS-induced signalling events in monocytes and macrophages

One of the initial steps of the immune response to bacterial endotoxin is the binding of LPS to one of the host cell surface CD14 receptors. This interaction induces an oxidative burst (Schütt *et al.*, 1988), enhancement of adherence of monocytes (Lauener *et al.*, 1990), the activation of the nuclear transcription factor NF-κB, as well as the secretion of cytokines such as TNF-α (Wright *et al.*, 1990), IL-1 (Schütt *et al.*, 1988; Couturier *et al.*, 1992), IL-6 and IL-8 (Denterner *et al.*, 1993) by macrophages. CD14 is subsequently released from the cell surface (Bazil *et al.*,

1991). Antibodies against CD14 prevent LPS-LBP complexes from binding to macrophages (Wright *et al.*, 1990; Kitchens *et al.*, 1992), block the LPS-induced activation of the NF-κB transcription factor (Kitchens *et al.*, 1992; Bagasra *et al.*, 1992), and block TNF-α and IL-1ß production (Couturier *et al.*, 1992; Kitchens *et al.*, 1992). Thus, cell surface CD14 appears to play a very important role in LPS-induced monocyte/macrophage activation.

Since CD14 is GPI-anchored to the cytoplasmic membrane and, therefore, lacks an intracellular domain that could directly transduce signals into the cytoplasm, current data provide clues as to how CD14 might mediate transmembrane signalling. First, GPI-anchored proteins have increased lateral mobility in the membrane (Low *et al.*, 1988), which may facilitate establishing interactions with transmembrane molecules that provide the signal transduction function. Indeed, data from Štefanová and her co-workers (1991) support this signalling model. They co-precipitated protein tyrosine kinase (PTK) activity with several GPI-anchored proteins, including CD14. More recently, they further identified that the PTK p53/p56^{lyn} is coupled to CD14 in human monocytes (Štefanová *et al.*,1993). P53/p56^{lyn} is a member of the *src*-related tyrosine kinase family. The p53/56^{lyn}, encoded by the *lyn* gene, is expressed in haematopoietic cells of myeloid lineage such as macrophages, monocytes and platelets, and of lymphoid lineage, *i.e.*, B lymphocytes (Yamanashi *et al.*, 1989). It is present in membrane fractions but not in the cytoplasmic or nuclear fractions (Yamanshi *et al.*, 1989) and therefore, like *Src*, it may be myrisoylated. These findings suggest that the PTK p56^{lyn} has a specialized function at the plasma membrane level. Indeed, like

other members of *src*-related tyrosine kinases, lyn has also been implicated in signal transduction. It was shown that lyn is involved in transmembrane signalling associated with the IL-2 receptor (Kobayashi *et al.*, 1993), the high affinity IgE receptor (FcεRI) (Eiseman *et al.*, 1992), and with the B-cell antigen receptor (Yamanshi *et al.*, 1991).

In monocytes, lyn was reported to be involved in LPS-stimulated monocyte activation (Štefanová *et al.*, 1993). It was also shown that $P53/56^{lyn}$ was the only PTK detected in LPS-activated or in resting monocytes (Štefanová *et al.*, 1993), and may be physically associated with CD14. However, other *src*-related PTK such as $p58/64^{hck}$ and $p59^{c\text{-}fgr}$ may be activation-inducible and associated with CD14 following LPS triggering (Štefanová *et al.*, 1993). Štefanová *et al.* also demonstrated an increase in CD14-associated kinase activity, and an increase in phosphorylation of the PTK substrate enolase *in vitro* following LPS stimulation. After stimulation for 15 minutes, a relative decline was observed in CD14-precipitated $p53/p56^{lyn}$, which may be caused by the shedding of CD14 from the surface of activated monocytes. These data suggest that LPS binding to CD14 induced an increase of CD14-associated $p53/56^{lyn}$ tyrosine phosphorylation. Furthermore, Štefanová *et al.* found that herbimycin A, a *src*-related tyrosine kinase inhibitor, could block LPS-induced production of TNF-α,IL-1α and IL-6 as well as the shedding of CD14. Anti-CD14 antibodies could inhibit both protein tyrosine phosphorylation and TNF-α production induced by ng/ml concentrations of LPS (Weinstein *et al.*, 1993).

The above evidence suggests that CD14 signalling might occur directly or

indirectly via protein phosphorylation mediated by p53/56^{lyn}, which may lead to the secretion of cytokines by LPS-activated monocytes. When CD14 was transfected into LPS-responsive 70Z/3 pre-B cells which lack CD14, the LPS sensitivity of the cells was increased without altering the qualitative response (Lee *et al.*, 1992). Transfection of CD14 into LPS non-responsive chinese hamster ovary cells by contrast did not confer LPS responsiveness (Tobias *et al.*, 1992). These results suggest that transfected CD14 functions cooperate with the endogenous LPS signalling apparatus resulting in an increased cellular responsiveness to LPS. This is analogous to what is found with many cytokine receptors such as the IL-1 and IL-6 receptors each having at least a two subunit receptor, one for ligand binding and the other for transmembrane signalling (Akira *et al.*, 1990; Hatakeyma *et al.*, 1989; Hibi *et al.*, 1990).

The biochemical events between the LPS-induced activation of CD14-associated p53/56^{lyn}, and TNF-α secretion in monocytes and macrophages, is still unclear. However, indirect evidence provides some clues. Evidence from co-immunoprecipitation studies have shown that the *src* family protein tyrosine kinase, associates with down-stream effector molecules such as PLCγ, GAP (p21^{ras}-GTPase activating protein) and PI3-K (phosphatidylinositol 3-kinase) in a variety of cell types (Cichowshi *et al.*, 1992; Weber *et al.*, 1992; Yamanashi *et al.*, 1992). More recently, it has been shown by Pleiman *et al.* (1993) that the amino-terminal 27 residues within the unique domain of p56^{lyn} mediate association with PLCγ2, MAPK (microtubule-associated protein kinase) and GAP. Binding to PI3-K is mediated through the Src

homology 3 (SH3) domains of the Src family kinase. Although there is no direct evidence showing that p53/56^{lyn} is associated with these downstream effector molecules in monocytes and macrophages, it was found that p53/56^{lyn} is associated with PLCγ2, GAP and PI3-K in B-cell lysate and that the amount of detectable kinase activity increases with anti-IgM stimulation (Pleiman *et al.*, 1993; Yamanshi *et al.*, 1992). These effector molecules propagate signals by, for example, PI3-K, hydrolysis of PI 4,5-biphosphate (PLCγ1 and PLCγ2) and by possible activation of p21^{ras} activity (GAP). Hydrolysis of phosphatidylinositol by PLCγ1 and PLCγ2 generate the secondary messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), that possess potent effector functions themselves. IP_3 activates the release of Ca^{2+} from stores in the endoplasmic reticulum. DAG functions by recruiting PKC to the membrane, where it can be activated. Actually, in the murine macrophage cell line RAW 264.7, two LPS-induced tyrosine phosphoproteins of 41 KDa and 44 KDa have been identified as isoforms of the mitogen-activated protein (MAP) kinases (Weinstein *et al.*, 1992). MAP kinase is one of a family of serine/threonine protein kinases (Sturgill *et al.*, 1991) which has been shown *in vitro* to phosphorylate and activate a 90 KDa ribosomal S_6 protein kinase (Chung *et al.*, 1991; Sturgill *et al.*, 1988) and the c-*jun* transcription factor (Pulverer *et al.*, 1991). Since many of the responses triggered by LPS in macrophages depend on transcription and translation, LPS activation of MAP kinases could be a critical part of the mechanism by which LPS induces responses in macrophages. But the identity of the LPS-activated tyrosine kinase that phosphorylates MAP kinase is yet to be identified.

In addition to the role of PTK, PKC also has been implicated in LPS macrophage activation. In the murine macrophages, it was found that LPS induced phosphorylation of p65 exclusively at a serine residue (Shinomiya *et al.*, 1991), indicating a contribution by a LPS inducible serine kinase. LPS or LPS-derived lipids have been shown to activate PKC (Pripc *et al.*, 1987; Wright *et al.*, 1984). Thus, p65 could be phosphorylated by PKC in LPS-stimulated macrophages. The degree of the p65 phosphorylation in macrophages stimulated with LPS or lipid A, correlated well with IL-1 production (Shinomiya *et al.*, 1991), indicating p65 may play a crucial role in macrophage activation. The serine/threonine kinase (PKC) which phosphorylates p65 has not yet been identified. It was also reported that PMA, a PKC activator, could induce a pattern of protein phosphorylation which resembles that induced by LPS (Weiel *et al.*, 1988; Shinomiya *et al.*, 1991). These findings indicate a possible role for PKC-directed protein phosphorylation in mediating responses to LPS. This possibility is consistent with the documentation of increased DAG in LPS-treated cells. Of the five distinct phosphoproteins produced in macrophages in response to LPS, the 68 KDa PKC substrate has recently been termed the myristolyated alanine rich C kinase substrate, or MARCKS (Seykora *et al.*, 1991). It is specifically phosphorylated in LPS-treated macrophages and as a consequence is redistributed from the cytoplasm to the plasma membrane.

The ability of LPS to prime cells to release metabolites of arachidonate has also been linked to the altered phosphorylation of this protein (Aderem *et al.*, 1988). More recently, it was reported that PMA at concentrations between $3x10^{-10}$ M to $3x10^{-9}$ M,

or okadaic acid, which inhibits phosphatase I and IIa could augment LPS-induced TNF-α production in human monocytes (Coffey *et al.*, 1992). Staurosporine, a PKC inhibitor, completely inhibited LPS induction of TNF-α and reduced IL-6 mRNA in human monocytes, but had no effect on IL-1β, indicating that LPS can activate PKC but also that this kinase is important in the LPS induction of TNF-α and IL-6, but not IL-1β. A PKA inhibitor, H89, inhibited LPS-induced IL-6 mRNA synthesis but had no effect on IL-1 or TNF-α (Geng *et al.*, 1993), suggesting that IL-6 induction in monocytes is both PKA and PKC regulated following LPS stimulation. In contrast, the PTK inhibitor, herbimycin A, inhibited production of all three cytokines, IL-1, IL-6 and TNF-α and blocked LPS activation of NF-κB. The PTK signalling event could therefore be an earlier signalling event than PKC or PKA. Whether PKC or PKA are downstream molecules of PTK remains to be identified. Interestingly, the release of arachidonic acid metabolites from LPS-treated macrophages was also inhibited by herbimycin A, suggesting that the activation of PLA_2 and phospholipid hydrolysis (which produce arachidonic acid) may also be linked to tyrosine kinase activity (Weinstein *et al.*, 1991).

Following the early intracellular events such as the activation of PTK induced by LPS binding to cell surface receptor (CD14) on monocytes and macrophages, unidentified downstream effector molecules are activated which lead to gene expression and cytokine production. As mentioned, herbimycin A was found to reduce the LPS activation of NF-κB, which is a transcription factor involved in the expression of cytokine genes such as IL-6 and TNF-α, indicating that PTK is an important early

event. It was reported that LPS induces three fold increases in the rate of transcription of the TNF-α gene (Han *et al.*, 1990). Treatment of macrophages with LPS results in nuclear protein binding to the NF-κB enhancer. Large DNA fragments as well as smaller oligonucleotides containing the exact sequences from several different regulatory sites in the TNF-α gene complex were shown to bind to LPS-sensitive nuclear factors (Collart *et al.*, 1990; Drouet *et al.*, 1990; Shakhov *et al.*, 1990). These activities were NF-κB-like as indicated by the ability of such complexes to compete with unlabelled oligonucleotides containing defined NF-κB sequence motifs (Drouet *et al.*, 1990; Narumi *et al.*, 1992). The mechanism of LPS activation of NF-κB is not well known. It is speculated that upon cell activation by LPS, IκB is phosphorylated, probably through a PKC mechanism in which a putative protein kinase, operative in LPS-treated monocytes/macrophages, remains to be identified. The NF-κB heterodimer then dissociates from IκB and translocates to the nucleus, where it binds upstream of specific genes such as NF-κB enhancers and the Y box, which activate TNF-α gene transcription.

G-proteins are a family of receptor-associated signal transduction molecules that have been implicated in the control of a variety of metabolic processes. It has been reported that binding of the LPS-serum complex to CD14 of human neutrophils causes the translocation to the membrane of the inhibitory G-protein, $G_{i\alpha 2}$ (Kozo *et al.*, 1992). It was also shown that pre-treatment of C3He/FeJ mouse peritoneal macrophages with pertussis toxin (PT), a specific inhibitor to uncouple the G_i-protein-mediated signal transduction (Codina *et al.*, 1990), markedly enhanced LPS-induced

TNF-α production (Zhang *et al.*, 1993), implicating that PT-sensitive G-protein-mediated signal transduction as an important regulatory event in LPS-dependent macrophage activation.

In cells stimulated with LPS, there is also evidence that the Na^+/H^+ pump is activated (Adams, 1992). In addition to Ca^{2+} and Na^+/H^+ exchange, modulation of Na^+/K^+ exchange may also be involved in the control of LPS-induced gene expression (Ohmori *et al.*, 1991). Prostaglandin E_2, produced in response to LPS, may activate adenylate cyclase (AC) via a nucleotide regulatory protein coupled to cell surface receptors, and the elevation in intracellular cAMP can also modulate numerous intracellular events via protein kinase activation (Hamilton *et al.*, 1992). However, the above data remain to be clarified further as does the interrelationship between CD14, lyn, the lipid A receptor and G protein signalling. The following model of the signalling cascade, modified from Hamilton *et al.*, summarizes the data discussed above into a unifying model of LPS signalling (Fig.4).

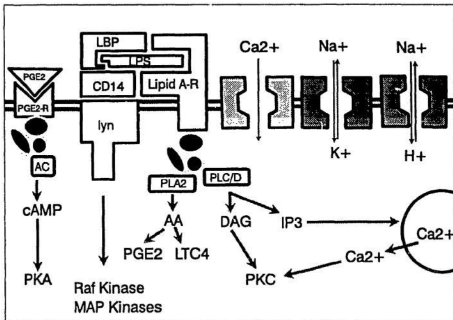


Figure 4. Membrane signal transduction model

1.7 Role of CD14 in disease processes

In addition to CD14 as a myeloid differentiation antigen marker and as a receptor for the LPS-LBP complex, it has been found that CD14 may play a role in either identifying or characterizing several diseases. It appears that CD14 may be a useful marker to assist in the diagnosis of certain leukaemias and in assessing prognosis. Anti-CD14 mAb can be used to identify mature cells of monocytic lineage in myelomonocyte leukaemia. CD14 is also a prominent marker found in acute monocytic leukaemia (AMoL) and acute myelomonocytic leukaemia (AMML). In the former, CD14-positive AMoL cells produce greater amounts of TNF-α and its presence may indicate a poorer prognosis for both AMML and AMoL (Griffin *et al.*, 1983).

In inflammatory disease, CD14 expression has been studied in alveolar macrophages of patients with sarcoidosis. Bronchoalveolar lavage cells, which are normally CD14 negative, show an increase in CD14 staining of the alveolar macrophages in sarcoidosis patients (Hance *et al.*, 1985). Following therapy, CD14 expression decreases to control levels, indicating successful anti-inflammatory intervention. Similar findings have been obtained in extrinsic allergic alveolitis (EAA) which exhibits an increase of CD14 expression as well as a rise in soluble CD14 in patient plasma (Pfonte *et al.*, 1993).

In septicemia patients, sCD14 was also found to increase and this correlates with levels of the acute-phase C-reactive protein (Kruger *et al.*, 1991).

The increased sCD14 may be related to the decreased expression of CD14 on blood monocytes in such sepsis patients (Fingerle *et al.*, in press). Cells from these patients exhibit a decreased functional response to LPS, but the consequences of this decreased responsiveness are currently unknown.

More recently, it has been demonstrated that expression of CD14 is necessary for LPS-induced augmentation of HIV-1 production in monocytes/macrophages. Blockade of CD14 dramatically ablates LPS-induced stimulation of HIV-1 replication (Bagasra *et al.*, 1992). Thus, modulation of CD14 expression may be implicated in AIDS. The transcription factor NF-κB may be the link here as there is an NF-κB consensus sequence on HIV-1 and several other viruses (Müller *et al.*, 1993).

1.8 Genetics and molecular biology of CD14

1.8.1 Isolation cloning and sequencing of CD14

Complementary DNA, coding for membrane CD14, has been cloned and sequenced by Goyert *et al.* (1988). The amino acid sequence predicted from the nucleotide sequence corresponds to a polypeptide consisting of an N-terminal extracelluar domain of 333 residues, a stretch of 17 hydrophobic and neutral amino acids (potentially serving as a membrane-spanning segment) and four C-terminal, possibly cytoplasmic, residues. The p.edicted N-terminal amino acid sequence by Goyert is in good agreement with that determined directly for one of the soluble

forms by Bazil *et al.* (1986).

1.8.2 Chromosomal localization of the CD14 gene

Analyses of cDNA and genomic clones of CD14 showed that the gene encoding CD14 is located on the long arm of chromosome 5 (5q23-q31), a region known to encode several myeloid-specific growth factors or growth factor receptors, including interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), colony-stimulating factor (CSF-1), CSF-1 receptor (CSF-1R) and the platelet-derived growth factor receptor (PDGF-R) (Goyert *et al.*, 1988). The mapping of the CD14 gene to this region of chromosome 5, its expression preferentially by mature myeloid cells, and its deletion in the malignant cells of patients having myeloid leukaemia and a deletion of 5q suggest that the CD14 antigen may also serve as a receptor important for myeloid differentiation (Goyert *et al.*, 1988).

1.8.3 Regulation of CD14 expression

CD14 expression on the surface of monocytes is dependent on the rates of CD14 loss and synthesis. Surface CD14 molecules may be lost either through internalization or shedding process. It has been reported by Bamezai *et al.*(1992) that following the addition of specific antibodies, two GPI-anchored proteins, Ly-6A.2 (TAP) and Thy-1, could be internalized by murine T lymphocytes via a

mechanism that is distinct from that used by several transmembrane proteins which are endocytosed via clathrin-coated pits and vesicles. But it has been demonstrated by Bazil & Strominger (1991) that down-modulation of surface CD14, induced by anti-CD14 mAb or its fragment $F(ab')_2$, which are known to activate monocytes, is caused by shedding rather than internalization. Shedding will be discussed in more detail later in this section.

The other factor influencing CD14 expression is the rate of synthesis of CD14 by monocytes. The synthesis of the polypeptide is regulated by nuclear transcription factors, the stability of CD14 mRNA, and by postranscriptional modification (glycosylation and attachment of the GPI-anchor). There does not appear to be any evidence available in the literature on factors affecting CD14 mRNA stability and little is known about the nuclear transcriptional factors involved in CD14 expression in monocytes. It was reported by Fernero and Goyert (1988) that the human CD14 gene contains a consensus motif for the SP-1 transcription factor. This motif is located in the 5' upstream region of 100 nucleotides. In the mouse, more of the 5' region has been sequenced (Matsuura *et al.*, 1989). Here, consensus motifs for PU-1, INF-γ activation factor, c/EBP (CAAT enhancer binding protein) and AP-1 can be identified (at positions -598, -461, -397 and -253, respectively), and it is possible that similar motifs may be detected in the human gene when more sequence data become available. In mature monocytes and macrophages, CD14 expression could be regulated by nuclear transcription factors

which may be activated or suppressed through physiological activating agents leading to a direct effect on CD14 antigen synthesis. IL-4 was reported by Laurner and his co-workers (1990) to down-regulate CD14 expression in human monocytes, which was first detectable after 24 to 36 hours of incubation with IL-4, and was almost complete after 4 days in culture. Further experiments showed that IL-4 dependent down-regulation of CD14 was not due to increased shedding and /or secretion, but resulted from decreased transcription of CD14 mRNA. Stimulation of human monocytes with IL-6 for 48 hours significantly increased cell surface CD14 density, as detected by mean fluorescence (MFI) but not by the percentage of $CD14^+$ cells. The mechanism of up-regulation of CD14 expression has not been investigated. The cytokine IFN-γ was also found to down-modulate the expression of CD14 on monocytes and macrophages *in vitro* after 15 to 45 hours of IFN-γ treatment (Landmann *et al.*, 1992). These data suggest that macrophage responsiveness to LPS may be regulated by cells releasing these cytokines. Thus, cytokine-mediated down-modulation of CD14 may be a mechanism for deactivating macrophages during the late stages of an immune response to a bacterial infection.

1.8.4 Regulation of CD14 shedding

In addition to a 55 KDa membrane-bound CD14 (mCD14) on monocytes, soluble CD14 (sCD14) is also found in human serum, urine, and in culture

medium. At least 99% of the CD14 found in the blood of humans is in the soluble form and only 1% is found on the surface of phagocytic cells (Wright, 1990). This evidence strongly suggests that membrane-bound CD14 could be shed into the circulation or medium. In 1991, Bazil & Strominger reported that *in vitro* CD14 was released from human monocytes spontaneously or rapidly following treatment with PMA. The protein released from PMA-treated monocytes migrated as a single band, with an apparently smaller molecular weight of 48 KDa in SDS-PAGE. Spontaneously released soluble CD14 from monocytes treated with PI-PLC *in vitro* was shown to have a molecular weight of 53 KDa, which was close to that of membrane-bound CD14. Soluble CD14 isolated from urine or serum appeared to be a mixture of the two forms having bands at approximately 48 KDa and 53 KDa.

The mechanisms of CD14 shedding have still to be elucidated. Goyert and her coworkers (1988) first reported that an *in vitro* spontaneously released form of CD14 lacks enthanolamine, indicating that sCD14 has no GPI-anchor. This raises the possibility that sCD14 is enzymatically cleaved and either released directly from a GPI anchor or is cleaved from the nascent hydrophobically anchored peptide. The metabolic pathway leading to the attachment of a GPI anchor to the protein is not fully understood and there is no direct evidence to prove this hypothesis. However, Fatemi and his colleagues (1987) did find that T cell mutants that have a defect in the GPI anchor attachment release large quantities of Thy-1 into culture supernatant. Therefore, the mechanism for escaping GPI

attachment can not be ruled out.

Secondly, it was found that treatment of monocytes with PI-PLC does not result in complete removal of membrane CD14. So, the PI-PLC resistant CD14 molecules could be anchored by a hydrophobic peptide transmembrane domain. This was shown for LFA-3 (Dustin *et al.*, 1987; Seed *et al.*, 1987; Wallner *et al.*, 1987) and for FcγRIII (CD16) where both GPI-anchored and transmembrane-anchored forms were identified and two mRNA were isolated for the two species. However, for CD14, only one mRNA was found (Goyert *et al.*, 1988). Furthermore, a single CD14 cDNA transfected into neuroglioma cells was found to result in both a membrane form and a smaller soluble form of CD14 in the supernatant. It was therefore unlikely that sCD14 was derived entirely from a transmembrane anchored CD14.

Thirdly, sCD14 may result from enzyme cleavage including such enzymes as PI-PLC, PLC/PLD, and protease. Evidence from Bazil and Strominger (1991) seems to support an endogenous protease as partly responsible for the spontaneous and the PMA-induced CD14 shedding. They showed that the sizes of the sCD14 shed from the cell surface spontaneously and after monocyte activation by PMA were identical, but clearly smaller than that of the membrane-bound or PI-PLC cleaved CD14. Serine protease inhibitors DFP and PMSF could also partly inhibit PMA-induced CD14 shedding. *In vitro* experiments seem to rule out phospholipase cleavage for the spontaneous and PMA-induced CD14

shedding, but definitive proof of the nature of these enzymes will require the complete characterization and identification of the cleavage sites of the various shed molecules. *In vivo*, the CD14 shedding process may be more complex. At least, CD14 shedding may be the result of a combination of phospholipase and protease, because two major soluble CD14 forms were found in the serum of healthy donors and in the urine of nephrotic patients (Bazil *et al.*, 1986, 1989). The smaller sCD14 has the same molecular weight as that shed *in vitro*, indicating that the CD14 shedding which occurs *in vivo* is perhaps the result of protease cleavage. The larger sCD14 form has the same molecular weight as that of PI-PLC treated-monocytes or close to the molecular weight of membrane-bound CD14 on monocytes *in vitro*, suggesting that a phospholipase may also be responsible for cleavage of CD14 *in vivo*.

The enzymatic activity responsible for shedding could be increased by transduction of an activating signal via membrane CD14 (Lauener *et al.*, 1990). It has been shown that GPI-anchored CD16 (Huizinga *et al.*, 1988, 1990) and the peptide-anchored TNF-receptor (Porteau *et al.*, 1990) from the cytoplasmic membrane of human neutrophils were also shed following cell activation. The consequences of TNF-receptor shedding was shown to regulate the function of neutrophils. *In vitro* studies also showed that about half of the mCD14 is shed from monocytes within 3 hours of LPS exposure (Bazil & Strominger, 1991). This evidence suggests that LPS may initiate a signal through CD14 to activate

enzyme(s) necessary for the cleavage of mCD14, but it remains to be determined which enzymes are activated following LPS treatment. Subsequently, CD14 shedding from the surface of monocytes may function as an efficient regulatory mechanism, which is able to blunt monocyte/macrophage response to LPS after primary stimulus, and regulate cytokine production. Moreover, sCD14 was found to function as a co-ligand with LPS in the activation of endothelial and epithelial cells (Haziot *et al.*, 1993; Pugin *et al.*, 1993).

1.9 Purpose of this dissertation

From the review of the literature, it is evident that sCD14 plays an important role in both vascular inflammatory and endotoxic responses. Consequently, modulation of CD14 expression and shedding may regulate the functions of mononuclear phagocytes and possibly endothelial and epithelial cell responses to LPS *in vivo*. However, little is known about the factors that determine the levels of CD14 on mononuclear phagocytes or what regulates its shedding. The objectives of this thesis were: i) to study CD14 expression and CD14 shedding induced by various signalling pathway inhibitors and / or activators, and; ii) to obtain clues as to what regulatory signals may be involved in the regulation of these two phenomena.

Figure 4 is the LPS-induced membrane signal transduction model as proposed by Hamilton *et al.* (1992). Figure 5 summarizes the experimental

strategy to be employed using various inhibitors and / or activators. LPS activates mononuclear cells and leads to the secretion of cytokines through the LPS-CD14 complex. Two molecules from this membrane complex involved with signal transduction are $p53/56^{lyn}$, a tyrosine kinase, and a G-protein coupled signalling molecule which activates a cascade of events involving phospholipases to produce IP_3 and DAG. The PGE_2 receptor is also implicated in the signal modulation. These signals lead to the activation of secondary signalling processes involving protein kinase A, other protein kinases (such as MAP kinase), and release arachidonic acid metabolites that activate PKC. These intracellular pathways lead to the regulation of gene expression and the activation of mononuclear cells. LPS ligation to CD14 not only has been shown to induce cytokine production, but also has been shown to result in CD14 shedding. A signal following LPS binding appears to influence the CD14 shedding process. Therefore, various LPS/CD14-signalling pathway inhibitors (shaded boxes in Fig.5) and activators (blank boxes in Fig.5) were used to investigate the involvement of these pathways in the regulation of CD14 expression and shedding.

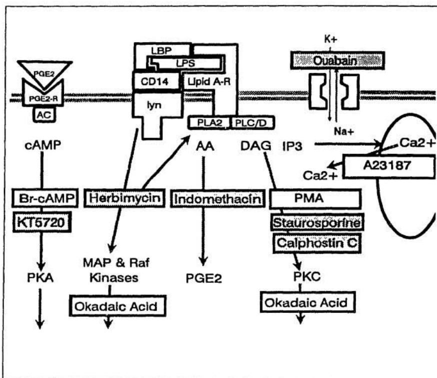


Figure 5. The experimental strategy. Various compounds which either inhibit (shaded boxes) or stimulate (blank boxes) signalling pathways were empolyed to investigate the involvement of LPS/CD14-signalling pathways in the regultion of CD14 experession and shedding.

Chapter 2 Materials and Methods

2.1 Induction of CD14 expression in THP-1 cells

Materials:

(1). $1\alpha,25(OH)_2D_3$ (called $VitD_3$ or cholecalciferol, Sigma Chemical Co.) was dissolved in 95% ethanol at $5x10^{-3}$ M (stock concentration) and kept at -20°C in the dark. $VitD_3$ was further diluted with media to working concentrations before use.

(2) Transforming growth factor-ß1 (TGF-ß1) was purchased from Sigma Chemical Co. 1 µg of TGF-ß1 was dissolved in 0.5 ml of 4 mM HCl containing 1 mg/ml BSA at 2 µg/ml (stock concentration) and kept at -70°C. Stock was diluted to 1 µg/ml of TGF-ß1 before use.

(3) Human recombinant tumour necrosis factor-α (rTNF-α) was supplied by Cedarlane Laboratories, Ontario, Canada. 10 µg of rTNF-α was dissolved in 100 µl PBS at 100 µg/ml (stock concentration), aliquoted and kept at -70°C. Stock was further diluted to a working concentration of 1.0 µg/ml with fresh RPMI-1640 medium before use.

THP-1 cells

THP-1 cells are human leukaemic cells, which were derived from the peripheral blood of a one year old male with acute monocytic leukaemia. THP-1

cells have Fc and C3b receptors and lack surface and cytoplasmic immunoglobulin, but THP-1 cells can be induced by PMA or $VitD_3$ to differentiate into macrophage-like cells (Tsuchiya, 1980).

THP-1 cells were purchased from ATCC and were grown in suspension in RPMI-1640 medium supplemented with 10% fetal bovine serum, $2x10^{-5}M$ mercaptoethanol, 100 U/ml penicillin and 100 μg/ml of streptomycin at 37°C in a humidified 5% CO_2 incubator. RPMI-1640 medium, fetal bovine serum, and antibiotics were obtained from GIBCO BRL (Ontario, Canada). THP-1 cells were grown in 75 cm^2 tissue culture flasks (Becton Dickinson and Company, NJ, U.S.A). The cells were passaged when their densities reached about $5x10^6$ cells/ml. The THP-1 cells were harvested during the logarithmic growing stage by centrifugation and used for experimental work. The number of cells and their viability were determined by light microscopy using a haemocytometer. For viability and cell counts, an equal volume of cell suspension was mixed with 0.1% trypan blue in phosphate-buffered saline, pH7.4 (PBS). Cells were washed with fresh RPMI-1640 medium before use in the experiments.

Protocol for induction of CD14 expression in THP-1 cells

CD14 is a myeloid differentiation marker which is expressed in mature myeloid cell lineage such as monocytes and macrophages, but not in immature

monocytic cells such as THP-1 and HL-60 (promyelocytic leukaemic cells). However, these immature leukaemia cells can be induced to differentiate into mature macrophage-like cells in the presence of $1\alpha,25(OH)_2D_3$. The differentiation was augmented by the addition of TGF-ß1 and TNF-α (Morikawa *et al.*, 1990). The following protocol was developed to induce THP-1 cells to express high levels of CD14 molecules on their surface.

(1) The logarithmic growing THP-1 cells were harvested by centrifugation.

(2) The cells were counted and adjusted to 10^5 cells/ml with fresh RPMI-1640 medium.

(3) 2 ml of 10^5 cells/ml THP-1 cells were added into each well of 6-well tissue culture plates (Becton Dickinson and Company, NJ, U.S.A.).

(4) The control wells were set up respectively as followed:

ethanol used as solvent for $VitD_3$ alone;

$8x10^{-8}$ M $VitD_3$ alone;

$8x10^{-8}$ M $VitD_3$ plus 1.0 ng/ml TGF-ß1;

$8x10^{-8}$ M $VitD_3$ plus 2.0 ng/ml rTNF-α;

1.0 ng/ml TGF-ß1 plus 2.0 ng/ml rTNF-α

Above control wells were only set up for the first time to confirm that three of above reagents together induced THP-1 cells maximum differentiation.

(5) $VitD_3$, TGF-ß1 and rTNF-α were added into each well to concentrations of $8x10^{-8}$ M, 1.0 ng/ml, 2.0 ng/ml respectively.

(6) These cells were incubated at 37°C under 5% CO_2 in a humidified incubator for 4 to 5 days. Between days 2 and 3, THP-1 cells which were in suspension started to become adherent with macrophage-like morphology. By day 4 or day 5, more and more THP-1 cells in suspension became adherent.

(7) After 5 days induction, the THP-1 cells in suspension were discarded. The adherent THP-1 cells were harvested using vigorous pipetting and the help of a rubber policeman if necessary.

(8) Cells were counted and viability determined using trypan blue exclusion. Viability was always greater than 90%.

2.2 L929 bioassay for TNF-α

Materials:

(1) L929 tumorigenic murine fibroblasts were kindly provided by Mr.Shawn Payne.

(2) Medium consisted of Eagle's Minimal Essential Medium (MEM) (GIBCO, N.Y., USA) supplemented with 2 mM glutamine, 5% FCS, 25 mM Hepes, and 10 U/ml penicillin, 100 µg/ml streptomycin.

(3) Actinomycin D (1 µg/ml) (Sigma Chemical Co.).

(4) Crystal violet (Sigma Chemical Co.): 0.5% crystal violet in 20% ethanol/PBS

(5) Sodium citrate: 0.1 M sodium citrate in 50% ethanol

(6) Human recombinant TNF-α standard (Sigma Chemical Co.): specific activity 2.7×10^7 units/ml (EC_{50}=0.18 ng/ml)

Procedure of L929 bioassay

The L929 cytotoxicity assay was performed based on the method described by Meager *et al.* (1989) with some modification. L929 cells were seeded at 1.25×10^4 cells/well in 200 µl of culture medium in 96-well flat bottom microtitier trays (Flow, Rockville, MD) and incubated for 24 h in 5% CO_2 atmosphere at 37°C. Spent medium was removed and equal volumes of TNF-α containing dilutions were added to wells. Control wells contained a known dilution of TNF-α. Trays were reincubated for 20 h followed by addition of 50 µl/well 0.5% crystal violet solution in PBS. After incubating 30 min at room temperature, dye containing medium was removed and the wells were washed three times with tap water and then 50 µl of 0.1 M sodium citrate in 50% ethanol was added to dissolve the crystal violet. Absorbance of wells at 595 nm microplate reader (Bio-Rad Co.). Percentage of cytotoxicity was calculated using the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{negative control - test sample}}{\text{negative control}} \times 100\%$$

2.3 Isolation of human monocytes

2.3.1 Principle of isolation of monocytes

Density gradient centrifugation is one of the common methods for cell

separation, in which a discontinuous density gradient or medium is used to isolate a homogenous population of cells from a heterogenous mixture of cells. Cells can be separated solely on the differences in density through density gradient centrifugation. In human peripheral blood, lymphocytes and monocytes are separated from granulocytes and erythrocytes by buoyant density centrifugation over a layer of Ficoll-Hypaque (density=1.077 g/ml). After centrifugation, the agglutinated red cells and granulocytes sediment to form a pellet, platelets remain suspended in the plasma and only lymphocytes, and monocytes remain at the interface between the Ficoll-Hypaque and plasma layers. Monocytes can be further separated from the other mononuclear cells by using the adherent properties of monocytes to adhere to a culture plate plastic surface.

2.3.2 Protocol for monocyte isolation

Materials:

(1) Sterile PBS or saline

(2) Ficoll-Hypaque (1.077 g/ml) (Sigma Co.)

(3) RPMI-1640 media (GIBCO. BRL)

(4) 0.1% trypan blue in saline

Isolation procedure:

The following protocol was used:

(1) 20 ml of sterile PBS or saline was added to 20 ml of heparinized normal human peripheral blood. Occasionally up to 50 ml of blood were used and the procedure was scaled up.

(2) 10 ml of diluted blood was very carefully layered on top of 3 ml of Ficoll-Hypaque (1.077 g/ml) in a 15 ml centrifuge tube.

(3) These tubes were spun for 30 min at 400xg (1400 rpm) at room temperature.

(4) The layer of white mononuclear cells was collected from the interface layer with a pasteur pipette into another test tube.

(5) At least 4 volumes of serum free RPMI-1640 medium were added to the mononuclear cells and mixed.

(6) The samples were spun at 200xg (1000 rpm) for 10 min.

(7) The supernatants were discarded.

(8) The cell pellet was resuspended with serum free RPMI-1640 medium and washed again as step 6 and 7.

(9) The cell pellet was resuspended with complete RPMI-1640 medium.

(10) Cells were counted and viability determined with trypan blue.

(11) To get monocytes, mononuclear cell suspensions (5×10^6 cells/ml) were incubated at 37°C for 1 hour in a 5% CO2 atmosphere in 100x20 mm tissue culture plastic petri dishes.

(12) After the incubation was completed, non-adherent cells were discarded and adherent cells were incubated for 15 minutes in freshly added medium.

(13) The cells adhering to the dishes were then extensively washed and resuspended by vigorous pipetting and use of a rubber policeman.

(14) Their viability was > 90% as assessed by trypan blue exclusion. Peripheral blood mononuclear cells yield approximately 5 to 10% monocytes by this isolation procedure.

2.4 Immunofluorescence technique

2.4.1 Direct and indirect immunofluorescence methods

Generally, immunofluorescence methods can be divided into two types: direct and indirect. In the direct immunofluorescence technique, conjugated antiserum is added directly to the tissue section or viable cell suspension. Unbound antibody is removed by washing, and the sample is examined by microscopy or FACS. The indirect immunofluorescence method is more widely used than the direct method. Here the sample is incubated with an unconjugated antibody, washed and incubated with a fluochrome-conjugated anti-immunoglobulin antibody. The second antibody thus reveals the presence of the first.

A major advantage of indirect immunofluorescence is that only a single fluorescent anti-immunoglobulin antibody is necessary to detect many first antibodies; and therefore it is not necessary to conjugate each new antibody individually to a fluorescent marker. The indirect technique is also more sensitive as it results in brighter fluorescence than the direct method. This is because the

anti-immunoglobulin contains antibodies to many epitopes on the immunoglobulin molecule, the use of fluorescent anti-immunoglobulin amplifies many-fold the fluorescent signal.

A disadvantage of indirect method is that the long procedure may increase non-specific background staining and therefore an isotype matched control is used to determine the level of background staining. This is important with cells, such as mononuclear cell, which naturally bind immunoglobulin via Fc or complement receptors.

2.4.2 Protocol for indirect immunofluorescence

Materials:

(1) Washing buffer (standard azide buffer) consisting of:

1%FCS, 0.1%NaN_3 in PBS

(2) Blocking buffer consisting of :

1:10 human serum 2%BSA, 0.1%NaN_3 in PBS

(3) Ab or Ab-PE conjugate dilution buffer consisting of :

0.5%BSA, 0.1%NaN_3 in PBS

(4) Anti-CD14 (IgG2a) mAb (IMO2), Isotype IgG2a control and B-phycoerythrin-conjugated AffiniPure $F(ab')_2$ Fragment Donkey anti-Mouse IgG (H+L) were purchased from BIO/CAN Scientific Company; Goat anti-mouse IgG (H+L)-FITC conjugate was purchased from Sigma Co. HLA-Class I Bw6/32 was kindly

provided by Ms.Jane Gamberg.

(5) Pharmacological reagents:

E.coli LPS, PI-PLC, PMA, ouabain, A23187, *f* MLP, calphostin C, indomethacin, and staurosporine were purchased from Sigma Chemical Company. KT5720, and okadaic acid was purchased from Kamiya Biomedical Company (CA, USA).

Procedure for indirect immunofluorescence method

All antibodies were employed at optimal dilutions, and analyzed in comparison to an appropriate isotype control. Cells were incubated at 4°C for 30 minutes with 10% human serum in blocking buffer to minimize nonspecific binding caused by the Fc portion of the antibody. After washing with washing buffer, cells were then incubated at 4°C for 30 minutes with anti-CD14 monoclonal antibody or an irrelevant control mAb of the same isotype. After 2 washes, cells were incubated with 100 μl of PE-labelled donkey $F(ab')_2$ anti-mouse IgG diluted 1:40 for another 30 minutes at 4°C. After 2 washes, cells were fixed in 2% paraformaldehyde and stored at 4°C until analyzed by FACS.

2.5 Detection of CD14 expression on monocytes by FACS

The stained cells were analyzed by a fluorescence activated cell sorter (FACStar, Becton Dickinson) equipped with an argon laser. The laser was

operated at 50 MW at 488nm and forward and side light scatter signals were used to gate on monocytes. Fluorescence signals were directed by a 560-nm short-pass dichroic mirror to the green photomultiplier equipped with a 530/30-nm bandpass filter and the red photomultiplier equipped with a 575/26-nm bandpass filter. At least $1x10^5$ cells were analyzed per sample, and histograms acquired were analyzed with the LYSYS II software package on a Hewlard Packard 340 computer. If there was overlap between staining of the specific MAb and the irrelevant control mAb of the same isotype at the same concentration, a substraction mode program was applied to correct for this overlap.

2.6 Characterization of the molecular weight of membrane and soluble CD14

2.6.1 Cell labelling with [^{35}S]-methionine and supernatant preparations

Materials:

(1) Minimal essential medium lacking L-methionine (D-MEM), glutamine and fetal calf serum was purchased from GIBCO/BRL.

(2) L-[^{35}S] methionine was purchased from Amersham International, Bucks.

(3) 0.22-nm filters were supplied by Millipore, Bedford, MA, USA.

(4) Microconcentrator-30s were purchased from Amicon, Beberly, MA, USA.

(4) Lysis buffer: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% (w/v) NP-40, 0.02% sodium azide, 2 mM PMSF (phenylmethyl-sulfonyl fluoride, Sigma).

(5) Protein A Sepharose CL-4B (Phamacia, Sweden).

Method:

The metabolic labelling of cells was performed as previously described (Goyert *et al.*, 1986). 10^7 isolated monocytes (see section 2.5) were cultured for 18 hours in 2 ml D-MEM medium lacking L-methionine and supplemented with 5% fetal calf serum, 1% L-glutamine and 1 mCi of L[^{35}S]-methionine. The radiolabeling was terminated by washing the cells three times in cold phosphate-buffered saline (PBS) containing 0.02% sodium azide. The culture supernatant from labelled cells (labelling media) was recovered for analysis of soluble CD14, filtered through 0.22 nm filter, and ultracentrifuged at 100,000xg for 30 minutes. NP-40 detergent and PMSF were then added at a final concentration of 0.5% (w/v) and 2 mM, respectively. The labelling media were exchanged for PBS/NaN_3 by three successive volume concentrations and volume restorations using PBS-0.02% NaN_3 in Amicon microconcentrators-30, and then exchanged with lysis buffer three times. Finally, the culture supernatant was concentrated 10 folds using microconcentrator 3 (Amicon. USA) for the immunoprecipitation experiments.

2.6.2 Protein solubilization and Immunoprecipitation

Method:

The labelled cells were lysed for 30 minutes at 4° C at a ratio of 1×10^7 cells/ml lysis buffer. The insoluble material was removed by centrifugation at 10,000xg for 30 minutes. The cell lysate (solublilzed protein pool) was recovered

for the immunoprecipitation experiments, about 5×10^6 labelled cells were used for each immunoprecipitation.

The immunoprecipitation technique was previously described (Goyert *et al.*, 1986). Briefly, labelled cell lysates and concentrated culture supernatants were incubated with an appropriate mAb for 1 hour with continual rotation at 4°C. Parallel lysates containing an irrelevant monoclonal antibody and an anti-HLA-Class I (W6/32) mAb were included as a negative and a positive control. This incubation was continued for a further 2 hours following the addition of 50 µl 50% protein A-Sepharose CL-4B. The resulting immune complexes were washed three times with lysis buffer and the immunoprecipitated antigens were eluted (1h at 50°C) in isoelectric focusing buffer (see later).

2.6.3 2-D-SDS-polyacrylamide gel electrophoresis (2D-SDS-PAGE)

Mini-Protein II 2-D cell (Bio-Rad) was used for this experiment.

First dimension--capillary isoelectric focusing (IEF)

Solutions for the first dimension:

(1) Acrylamide/Bis (30%T/5.4C)

(2) First dimension sample buffer: 8 M Urea (reduced from the BioRad specifications of 10 M), 2.0% Triton X-100, 5% ß-mercaptoethanol, 2.0% Bio-Lyte® pH 3.9/9.2 ampholyte.

(3) First dimension sample overlay buffer: 8 M Urea, 1% Bio-Lyte® 3.9/9.2

ampholyte, 0.1% Bromophenol blue.

(4) First dimension gel monomer solution: 8M urea, 4% acrylamide, 20% Triton X-100, 2% Bio-Lyte® 3.9/9.2 ampholyte, 0.01% ammonium persulfate, 0.1% TEMED.

(5) Upper chamber buffer: 100 mM NaOH

(6) Lower chamber buffer: 10 mM H_3PO_4

Procedure for IEF:

For 2D gel electrophoresis, pH gradient electrophoresis (isoelectric focusing) was used for the first dimension according to the Bio-Rad instruction manual. Briefly, the capillary tubes were carefully cast with first dimension gel monomer solution and subjected to pre-electrophoresis to form a pH gradient. After pre-electrophoresis, eluted antigens were loaded at the cathode in this capillary tube gel with 6 µl 2D-SDS-PAGE standard markers as a internal control and subjected to isoelectric focusing for 6 h at 750 volts. During IEF, proteins are separated in terms of their pI values. Capillary tubes with gels were stored in a polystyrene cooler box at -20°C until subjected to 2D-SDS-PAGE.

Second Dimension---SDS-PAGE

Electrophoresis in 10% polyacrylamide gels with sodium dodecyl sulphate (SDS) was performed as originally described by Laemmli (1970).

Materials:

(1) Acrylamide/bis acrylamide (30%T, 2.67%C).

(2) 10% SDS

(3) 10% Ammonium persulphate (Sigma Chemical Co.)

(4) N,N,N', N'-Tetramethylethylenediamine (TEMED). (GIBCO BRL.)

(5) 1.5 M Tris-HCl, pH 8.8

(6) 0.5 M Tris-HCl, pH 6.8

(7) 5X Running buffer: 9.0 g Tris, 43.2 g glycine and 3.0 g SDS were dissolved to 600 ml of deionised water and stored at 4°C. 60 ml 5X stock was diluted with 240 ml distilled water for one electrophoresis run.

(8) 2X Sample buffer: 1 ml 0.5 M Tris-HCl buffer, pH 6.8, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 2-mercaptoethanol, and 0.2 ml of 0.05% bromophenol blue were mixed together, and made up with deionised water to a final volume of 8 ml.

(9) Molecular weight markers: prestained high range protein molecular weight standards (GIBCO BRL).

Method:

(1) For preparation of the separating polyacrylamide gel (lower gel), 3 ml of 30% acrylamide/bis, 2.5 ml of 1.5 M Tris-HCl, pH 8.8, 100 µl of 10% SDS, 50 µl of 10% ammonium persulfate and 4.35 ml of deionised water were mixed and degassed for 10 minutes. 5 µl TEMED was added to the mixture immediately

before the gel was poured into a vertical mini-slab gel with 0.5 mm spacer (Bio-Rad Laboratories). Deionised water was layered on top of the gel to form a level gel surface. Polymerization was allowed to proceed for 30 to 45 minutes after which the water layer was poured off and gels rinsed with deionised water.

(2) For preparation of the stacking polyacryamide gel (upper gel), 1.3 ml of 30% acryamide/bis, 2.5 ml of 0.5 M Tris-HCl, pH 6.8, 6.1 ml of distilled water, 100 µl of 10% SDS, 50 µl of 10% ammonium persulfate were mixed and degassed for 10 minutes. 10 µl of TEMED was added to the mixture before pouring over the lower gel. A plastic comb was carefully inserted into the upper gel to form sample wells.

(3) Sample preparation and gel electrophoresis were performed as follows: The capillary tube gel from IEF was extruded from the tube by using a gel ejector, equilibrated with SDS sample buffer and loaded on top of the stacking gel. Prestained protein markers were diluted 1:20 with sample buffer to 0.05 µg/µl and boiled for 5 minutes. Then 0.075 µg of prestained protein marker were loaded into the well. Electrophoresis was carried out at constant voltage of 125 volts for 45 minutes to 1 hour at room temperature. After 2D-SDS-PAGE, the slab gels were silver stained. The approximate molecular weight of the examined protein spots ([^{35}S]-methionine labelled) was determined from the prestained high range protein molecular weight standards and 2D-SDS-PAGE protein standard markers run in parallel and internally.

2.6.4 Autoradiography

When radioactive material was run on SDS-PAGE, the protein bands were visualised by autoradiography after drying the gel onto a piece of 3MM filter paper. Gels were dried at 60°C under vacuum in a gel dryer, then exposed to a Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester,NY, USA) at -70° for 1 or 2 days or longer when necessary. Exposed film was then developed in an automatic X-Omat processor.

2.6.5 Silver staining

Silver staining is a highly sensitive method for detecting proteins in polyacrylamide slab gels. After 2D-SDS-PAGE, the slab gels were stained using a Bio-Rad silver stain kit according to the instruction manual. After silver staining, the protein spots were visualized in the gels.

2.7 Characterization of apoptosis

2.7.1 Detection of apoptosis by light and electron transmission microscopes

Cells obtained and treated as described in the experiments were examined morphologically by light microscopy or fixed by Karnovsky fixative before examining chromatin changes by JEOL JEM-1200EX electron transmission microscopy.

2.7.2 Flow cytometric analysis of apoptosis

Materials:

(1) Dimethyl sulfoxide (DMSO) (Sigma Chemical Co.)

(2) Calphostin C (Sigma Chemical Co.), a photo-activated protein kinase C inhibitor. 1 mg of calphostin C was dissolved in 1 ml sterilized DMSO to a final stock concentration of 1 mg/ml and dispensed in small aliquots and stored at -20°C. When used, it was diluted with fresh RPMI-1640 medium to the required working concentration.

(3) PMA (phorbol 12-myristate 13-acetate, Sigma Chemical Co.) 1 mg PMA was dissolved in 1 ml of DMSO to stock a concentration of 1 mg/ml and dispensed into small aliquots and stored at -20°C in the dark. It was diluted with fresh RPMI-1640 medium to the required working concentration as needed.

(4) Quinidine, a Ca^{2+}-dependent K^{+} channel blocker (Sigma Chemical Co.) Fresh working concentration of quinidine was made with RPMI-1640 medium prior to each experiment.

(5) 4-Aminopyridine (4-AP), a K^{+} channel blocker (Sigma Chemical Co.) Fresh working concentration of 4-AP was made with RPMI-1640 medium every time before use.

(5) Washing buffer: PBS containing 1% FCS and 0.1% NaN_3

(6) Ethanol: absolute

(7) RNAase (Sigma Chemical Co.)

(8) Propidium iodide (Sigma Co.): 5 mg propidium iodide (PI) was dissolved in 1 ml of PBS to a stock concentration of 5 mg/ml. Before use, PI was diluted to the required working concentration of 50 μg/ml.

Method:

Conventional methods used for identification of dead cells cannot be used to determine the percentage of apoptotic cells or recognize the apoptotic cells in a heterogeneous cell population. A flow cytometric method was used for investigating cell DNA content during apoptosis. The extent of apoptosis was evaluated by following changes in cell DNA content. The changes in DNA content of the cells during apoptosis were due to DNA fragmentation and loss (Afanasyve *et al.*, 1993). This loss of DNA can be measured by using fluorescent DNA stains such as propidium iodide. Cells at different stages of the cell cycle can also be observed. A shift in the fluorescence spectrum to the left (loss of DNA) can be quantified by FACS analysis.

(1) About 10^6 cells from each experiment were pelleted and washed once with standard azide buffer (1% FCS, 0.1% NaN_3 in PBS).

(2) They were resuspended in 150 μl standard azide buffer and fixed by adding 200 μl absolute ethanol for 1 hour or overnight at 4°C.

(3) Cells were then centrifuged, washed in 2 ml standard azide buffer twice and resuspended in 150 μl of standard azide buffer.

(4) To eliminate RNA fluorescence artifacts, 150 µl RNAse (1 mg/ml in PBS) was added to 150 µl of each sample and incubated at 37° C for 30 minutes.

(5) After RNA was digested, 300 µl of 50 µg/ml propidium iodide in PBS was added and incubated at 37°C for 20 minutes.

(6) After incubation, the cells were kept at 4°C in the dark until analyzed by FACS.

2.7.3 DNA extraction

Materials:

(1) Cells: THP-1 cells, HL-60 cells and human peripheral mononuclear cells.

(2) Hypotonic detergent buffer

10 mM Tris-HCl, pH 7.5

2 mM EDTA

0.2% Triton X-100

(3) RNAase A (Sigma, Co.). DNAase's free RNAase A was prepared by dissolving the enzyme at a concentration of 1 mg/ml in PBS, then heating to 100°C for 15 minutes. Heated RNAase A was allowed to cool slowly to room temperature, then dispensed into aliquots and stored at -20°C.

(4) Saturated NaCl (6 M)

350 g NaCl was dissolved in 1 litre distilled water at room temperature.

(5) Absolute ethanol and 70% ethanol

(6) TE buffer consisting of 10 mM Tris-HCl, pH 7, 0.2 mM EDTA

(7) Pronase (Sigma Co.): Pronase is a mixture of serine and acid proteases isolated from *Streptomyces griseus.* To eliminate contamination with DNAase and RNAase, pronase was used following self-digestion. Self-digested pronase was prepared by dissolving 20 mg powdered pronase in 1 ml of 10 mM Tris-HCl (pH 7.5), 10 mM NaCl to a final concentration of 20 mg/ml, and incubated at 37°C for 1 hour. After self-digestion, pronase was dispensed in small aliquots in tightly capped tubes and stored at -20°C.

Method:

DNA was extracted from cells based on the method described by Faustino Mollinedo *et al.* (1993) and Miller *et al.* (1988) with some modifications.

(1) About 4 to $6x10^6$ THP-1 cells for each experiment were washed with phosphate buffered saline.

(2) Cells were lysed with 300 µl hypotonic detergent buffer at 4°C for 30 min.

(3) Cell organelles were removed by centrifugation at 12,000xg for 20 min, and the supernatant, containing the DNA released into the cell cytoplasm due to DNA fragmentation, was incubated with RNAase A (75 µg/ml) at 37°C for 1 hour.

(4) After this incubation, 200 µg/ml pronase was added in the presence of 0.5% SDS and the incubation was continued at 37°C for 1 hour.

(5) After digestion was completed, to precipitate protein, 150 µl of saturated NaCl (approximately 6M) was added to each tube and the tubes shaken vigorously for

15 seconds, followed by centrifugation at 2500 rpm for 15 minutes.

(6) The precipitated protein pellet was left at the bottom of the tube and the supernatant containing the DNA fragments was transferred to another 15 ml polypropylene tube.

(7) To precipitate DNA, exactly twice the sample volumes of absolute ethanol was added and the tubes inverted several time and stored at -20°C for 24 hours.

(8) The DNA fragments were recovered by centrifugation at 12,000xg at 0°C for 1 hour.

(9) To wash out remaining protein, pellets were washed with 70% (v/v) ethanol twice and resuspended in 20 μl of 10 mM Tris-HCl, pH 7.5, 0.2 mM EDTA.

2.7.4 Electrophoresis of DNA in agarose gel

Electrophoresis in 1% agarose gels with 0.5 μg/ml ethidium bromide was performed as described by Sambrook *et al.* (Molecular cloning, a laboratory manual, 1986).

Materials:

(1) Agarose

(2) TBE buffer (electrophoresis buffer): TBE electrophoresis buffer from 10x stock concentration of TBE buffer (54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA, pH 8.0 in 1000 ml distilled water).

(3) Ethidium bromide (Sigma Co.): To visualize DNA in agarose gels, the fluorescent dye ethidium bromide (Sharp *et al.*, 1973) is added. This agent contains a planar group that intercalates between the stacked bases of DNA. Since the fluorescent yield of ethidium bromide: DNA complexes is much greater than that of unbound dye, small amounts of DNA can be detected in the presence of free ethidium bromide in the gel. 10 mg ethidium bromide was dissolved in 1 ml distilled water to a final concentration of 10 mg/ml, and stored at room temperature in dark bottles. The dye was incorporated into the gel and the electrophoresis buffer at a concentration of 0.5 μg/ml.

(4) Gel-loading buffer:(6x)

0.25% bromophenol blue

0.25% xylene cyanol FF

30% glycerol in distilled water

This was made up as 10 ml and stored at 4°C. When used, it was diluted to the appropriate concentration as required.

Method:

(1) The edges of a clear,dry, glass plate were sealed with autoclave tape so as to form a mold. The mold was set on a horizontal section of bench.

(2) Sufficient TBE electrophoresis buffer was prepared to fill the electrophoresis reservoir.

(3) 1 g powdered agarose was added into 100 ml of TBE buffer in an Erlenmeyer flask and heated to dissolve the agarose.

(4) The solution was allowed to cool to 60°C, and ethidium bromide is added to a final concentration of 0.5 μg/ml and mixed thoroughly.

(5) The comb 0.5 to 1.0 mm was positioned above the plate .

(6) The warm agarose solution was poured into the mold. The gel slabs were between 3 mm and 5 mm thick. Be sure that no air bubbles are under or between the teeth of the comb.

(7) After the gel was completely polymerized (around 30 minutes at room temperature), the comb was carefully removed and the gel mounted in the electrophoresis tank.

(8) Enough TBE electrophoresis buffer was added to cover the gel to a depth of about 1 mm.

(9) Samples of approximately 6 μg of DNA were mixed with 20 μl of gel-loading buffer, the mixture was loaded into the slots of the submerged gel using a micropipette.

(10) The lid of the gel tank was closed and attached to the electrical leads. The gel was run at 75 volts, allowing the DNA to migrate toward the anode (red lead).

(11) The gel was run until the bromophenol blue and xylene cyanol FF had migrated to 0.5 cm from the bottom of the gel (usually 6 cm migration).

(12) The electric current was turned off, and the leads and lid were removed from

the gel tank. Gels were examined using ultraviolet light and photographed to keep as a record.

Chapter 3 CD14 Studies

3.1 Introduction

Most of the data presented here, were obtained using human monocyte derived macrophages (MDM) from fresh peripheral blood from a number of volunteers (see methods for isolation procedure). Initially several human cell lines: HL-60, U937, THP-1 and Mono Mac 6 were also explored for their suitability as a CD14 shedding model. This proved to be rather fruitless as CD14 expression by these cell lines was found to be too low for this type of study. Although CD14 could be induced by treatment with vitamin D_3, phorbol esters and IFN-γ (Rigby *et al.*, 1984, 1985; Rossi *et al.*, 1987; Trinchier *et al.*, 1987; Ball *et al.*, 1984), they were still found unsuitable, because THP-1 cells could be induced to differentiate and to express higher levels of surface CD14 only by combined treatment with $VitD_3$, TNF-α and TGF-β_1. The numbers of cells required for these studies was too large to maintain an adequate supply of cells at a reasonable cost. Therefore, it was decided to use MDM.

Some of the preliminary data and the apoptosis studies were obtained using the human THP-1 cell line and we draw your attention to this fact. Wherever THP-1 cells were used, suitable reference to the cell line has been made.

3.2 Blocking of TNF-α production with anti-CD14 mAb

Preliminary studies were made to establish that previously reported phenomena could be reproduced in our hands. One of these was the dependence of TNF-α production on CD14 at low LPS concentration. This study was made using THP-1 cells.

THP-1 cells are monocytic leukaemia cells which express a relatively low level of membrane CD14 compared to normal human monocytes. When THP-1 cells are treated with 10 ng/ml LPS for 3 h, TNF-α is produced and this can be detected by L929 bioassay. TNF-α production by these cells was shown to be completely inhibited by pre-incubation of the cells with 10 ng/ml of anti-CD14 mAb (IMO2) for 30 min. TNF-α production was not blocked by an irrelevant isotype matched control mAb (Fig.6). These data confirm the model of CD14 as a receptor for LPS and its involvement in the production of TNF-α.

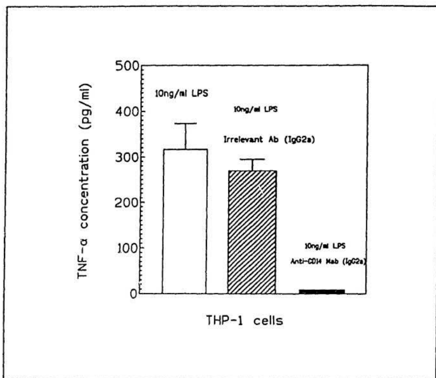


Figure 6. Inhibition of LPS-induced TNF-α production in THP-1 cells by CD14 antibodies (IMO2). 10 ng/ml LPS with 10 μg/ml of IMO2 (black bar), or irrelevant isotype matched control antibodies (striped bar) or without Ab (blank bar) were added to THP-1 cells. Supernatants were harvested after 4 h and assayed for TNF-α level by the L929 assay. Data are presented as mean values ± SD of triplicate samples (n=3).

3.3 Establishment of optimal conditions for immunofluorescence staining

3.3.1 Direct labelling studies

In preliminary studies, direct immunofluorescence staining was tested for its suitablity to detect CD14, as it is less time consuming compared with indirect labelling. The results using phycoerythrin-CD14 mAb conjugate, shown in Fig.7 (A,B) could not distinguish between specific PE-CD14 binding and nonspecific binding (PE-McA690, an isotype matched antibody). In contrast to direct labelling, indirect labelling gave a measurable ratio of specific to nonspecific binding (Fig.7 C,D).

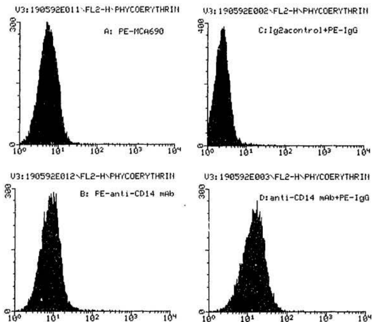


Figure 7. Comparison of direct and indirect labelling. THP-1 cells were incubated with a 1:10 dilution of human serum at 4°C for 30 min, then labelled with phycoerythrin-anti-CD14 mAb conjugate (histogram B) or phycoerythrin-isotype matched control antibody (PE-McA690) (histogram A) at 4°C for 30 min or direct labelled with anti-CD14 mAb or isotype matched control antibody, followed by the addition of a 1:40 dilution of PE-labelled donkey $F(ab')_2$ anti-mouse IgG at 4°C for

3.3.2 Blocking of non-specific binding by human serum diluted 1:10

Antibodies may bind non-specifically to monocytes and macrophages via the Fc receptors on these cells. In the experimental system, the second antibody used was PE-labelled donkey $F(ab')_2$ anti-mouse IgG . Theoretically, blocking with donkey serum would be the best to minimize non-specific cross reactivity. Unfortunately, donkey serum was not available. Therefore, in preliminary experiments, mouse and human sera were screened as alternative candidates in an antibody blocking experiment. Human monocytes were pre-incubated with the serum (GIBCO, BRL. Canada) in BSA/PBS at 4°C for 30 min. Of the two species of sera, the human, gave the highest ratio of specific to non-specific binding to human monocytes. 96% $CD14^+$ cells were detected with human serum blocking, only 17% $CD14^+$ cells with mouse serum blocking (Fig.8). Subsequently, it was found that a 1 in 10 dilution of human serum was the optimal concentration to block non-specific binding and thus a 1:10 dilution of human serum was used in all the following studies.

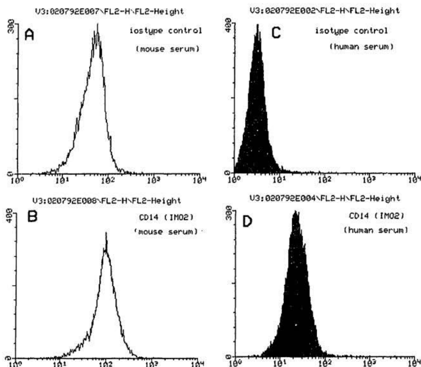


Figure 8. Comparison of mouse and human sera in blocking experiment. Human monocytes were blocked by mouse serum (Histogram A and B) or human serum (Histogram C and D) at 4°C for 30 min, followed by adding anti-CD14 mAb (Histogram B and D) and isotype matched control (Histogram A and C) and 1:40 donkey anti-mouse PE-labelled $IgG(Fab')_2$ at 4°C for 30 min. 96% $CD14^+$ cells were detected with human serum blocking (D), only 17% $CD14^+$ cells with mouse serum blocking (B).

3.3.3 Selection of antibody specific for CD14

Two clones of anti-CD14 mAbs, IMO2 (IgG2a) and 3C10 (IgG2b) were tested to identify cell surface CD14 on isolated human monocytes. After comparing specific anti-CD14 mAb binding with isotype matched control mAb, IMO2 was found to give the highest ratio of specific to non-specific binding (Fig.9). Therefore, the IMO2 monoclonal antibody was used in subsequent experiments. The working concentration of IMO2 chosen was 2 μg per $5x10^5$ isolated monocytes as recommended by the supplier.

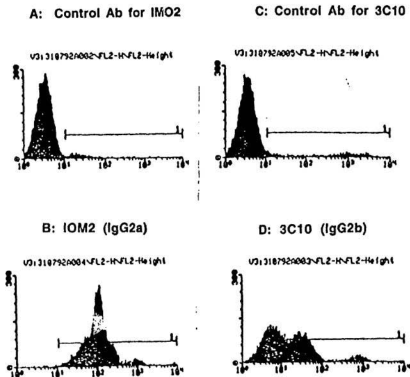


Figure 9. Comparison of specific binding of two clones of anti-CD14 mAb. Human monocytes were blocked by 1:10 human serum as described in materials and methods, followed by adding IMO2 (anti-CD14 mAb, IgG2a isotype) (B), 3C10 (anti-CD14 mAb, IgG2b isotype) (D), or isotype matched control (C for IgG2b, A for IgG2a), respectively. Finally, samples were labelled by 1:40 PE-labelled donkey $F(ab')_2$ anti-mouse IgG at 4°C for 30 min and analyzed by FACS.

3.3.4 Selection of fluorescence-antibody conjugate

FITC-labelled rabbit anti-mouse IgG (Sigma Co.) and PE-labelled donkey $F(ab')_2$ anti-mouse IgG (Bio/Can Sci.Co.) were tested. The PE-labelled donkey $F(ab')_2$ anti-mouse IgG was found to give the highest specific and relatively low non-specific fluorescence intensity (histograms C and D in Fig.10). Due to the high FITC-IgG non-specific binding to THP-1 cells, even after blocking, FITC-labelled rabbit anti-mouse IgG was not used. Several studies (not shown) were made in order to select the optimal concentration of PE-$F(ab')_2$; 1:40 was used as the working concentration for all further studies.

In addition, isotype-matched control antibodies (IgG2a) were set up in each experiment to determine background binding.

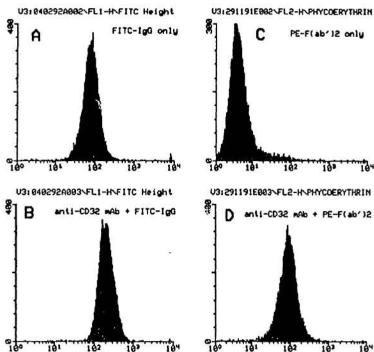


Figure 10. Selection of fluorescence-Ab conjugate. THP-1 cells (10^6/sample) were blocked with human serum, followed by treatment with anti-FcRII specific mAb (anti-CD32) (Histogram B and D) or without antibody (Histogram A and C). Finally, cells were stained with 1:40 FITC-labelled rabbit anti-mouse IgG2 (Histogram A,B), or 1:40 PE-labelled donkey $F(ab')_2$ anti-mouse IgG (Histogram C,D).

3.3.5 Analysis of FACS data and expression of FACS results

Cells were analyzed on a fluorescence activated cell sorter (FACStar, Becton Dickinson). Forward scatter (cell size) and side scatter (granularity) were measured to distinguish lymphocytes and monocytes. The gated human monocytes and macrophages were then analyzed for their fluorescence. Two major parameters were studied. These were percent positive cells and mean fluorescence intensity (MFI). The first of these was calculated as percentage of cells with fluorescence greater than the isotype control. The second parameter, mean fluorescence intensity (MFI) is an indicator of the level of CD14 expression by cells. This is a variable dependent on the efficiency of antibody labelling and laser performance, and can only be compared within batches analyzed on the same day. Because of the variability of MFI, intergroup variation in CD14 expression levels could not readily be made. This must be kept in mind when MFI data from different donors are presented. Percent positive cells is therefore the best parameter for inter-batch comparison, while MFI and % positive cells are both good indicators of intra-batch variations.

3.4 CD14 expression by normal human monocyte derived macrophages

In this study, blood from 14 healthy volunteers was used to investigate CD14 expression. Of the recovered monocytes, 60% to 91% expressed CD14 with a mean ± SEM of 78.84% ± 2.37 (n=14). Individual donor variation in CD14 expression by monocytes was also determined in two of the donors, these values were 72% ± 7.69 (n=5) and 80 %± 6.11 (n=3) respectively. Most volunteers in this study had approximately 85% CD14 positive monocytes, as shown in Figure 11.

The majority of primary blood monocytes express a high level of CD14 ($CD14^{++}$). However, recent studies identified a novel subset of blood monocytes, which expresses low levels of CD14 and high levels of CD16 (FcγRIII) on the cell surface (Passlick *et al.*, 1989). These $CD14^{+}/CD16^{+}$ cells were also found to produce lower levels of TNF-α, IL-1 and IL-6 in response to LPS stimulation (Ziegler-Heitbrock *et al.*, 1992). It was reported by Ziegler-Heitbrock *et al.* (1992) that about 13% of the monocyte population were weakly $CD14^{+}$ cells. Association of CD14 with CD16 was not evaluated in this thesis.

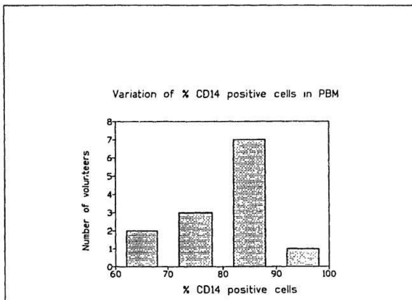


Figure 11. Variation of %CD14⁺ cells in PBM isolated from 14 donors used. Human monocytes were treated with monoclonal anti-CD14 Ab (IMO2) or isotype matched control (background), then labelled with phycoerythrin (PE)-F(ab')$_2$ as described in materials and methods, followed by FACS analysis. Background was subtracted from all the data presented above. The number of volunteers versus %CD14⁺ cells is plotted as a histogram.

3.5 Down-modulation of surface CD14 after monocyte stimulation by PMA

Both % positive cells and MFI decreased rapidly after incubation with the phorbol ester, PMA (phorbol-12-myristate-13-acetate) as assessed by indirect immunofluorescence analysis (Fig.12). Two different PMA doses, 10 ng/ml and 100 ng/ml, were used in preliminary studies. 100 ng/ml PMA only gave a slightly higher CD14 loss than that caused by 10 ng/ml PMA (data not shown). 10 ng/ml was selected for all subsequent studies. Data from the time response curve for one volunteer (Fig.13) show that CD14 loss had reached a maximum by 3 hours, and that 10 ng/ml PMA was sufficient to give a 70% loss of CD14 from the cell surface. As a consequence of this finding, a 3 hour exposure was selected for all subsequent studies with PMA. Using a single time point, data were gathered from a number of individuals. Loss of %CD14^{+} cells following PMA treatment ranged from 2% to 74% with a mean of 22% (Table 2). CD14 density as indicated by MFI on monocytes also decreased, following PMA treatment, with a mean fluorescence intensity (MFI) loss of 23%. At the same time, the expression of HLA-Class I (W6/32) used as a positive control in all studies was not significantly changed after PMA treatment, about 90% of the cells were positive and MFI was approximately 500.

Evidence from Bazil & Strominger (1991) previously confirmed that the down-modulation of surface CD14 induced by PMA was caused by shedding rather than internalization. They also showed that PMA-induced CD14 shedding

appeared to be the result of proteolytic cleavage rather than a phospholipase cleavage, as soluble CD14 isolated from the media had an apparently smaller molecular weight than membrane bound CD14.

Table 2. Effect of PMA on CD14 expression on human monocytes

cell surface molecule		untreated*	PMA*	P value#
CD14	positive %	70.78±3.9	55±5.3	$P<0.0001$
	MFI	204±38	157±27	$P=0.0128$
HLA-class I (W6/32)	positive %	91.2±2.73	87.2±2.72	$P> 0.1$
	MFI	537±129	509±127	$P=0.38$

* : The data represent the mean ± SEM of 14 experiments for CD14 and 10 experiments for HLA.

#: P value was assessed by a two-tailed Student's t-test.

PMA: Human monocytes were treated with 10 ng/ml PMA in 5% CO_2 incubator at 37°C for 3 hours.

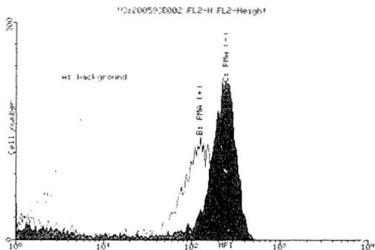


Figure 12. PMA-down modulation of CD14 expression on monocytes. Monocytes were treated with 10 ng/ml PMA at 37°C for 3 h (B) or medium only (C), followed by the addition of monoclonal anti-CD14 Ab or isotype matched control mAb and labelled with PE-F(ab')$_2$. The positive CD14 cells and MFI are presented. There is no significant change in isotype control (A) before and after PMA treatment.

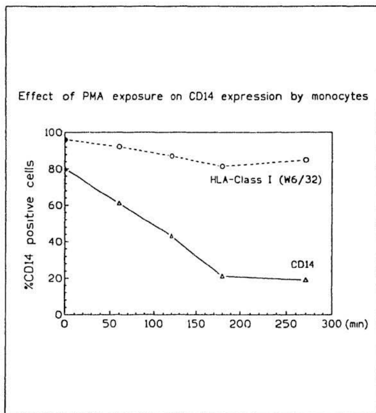


Figure 13. Time course curve of monocyte response to PMA. Monocytes were treated with 10 ng/ml PMA for different time periods, labelled with antibodies and PE-F(ab')$_2$ as described in materials and methods. CD14 and HLA-class I (W6/32) expression were analyzed by FACS.

3.6 Sensitivity of CD14 to phosphatidylinositol-specific phospholipase

CD14 is a member of the family of GPI-linked membrane glycoproteins. In order to determine the sensitivity of membrane bound CD14 to phosphatidylinositol-specific phospholipase (PI-PLC) cleavage, isolated monocytes were treated with PI-PLC from *Bacillus cereus*, as described in materials and methods. Results of one representative group from four independent experiments are shown in Figure 14. PI-PLC treatment led to the removal of 60% to a maximum of 80% of the CD14. A similar result was also found with induced-differentiated THP-1 cells, indicating that 20 to 40% CD14 appeared to be resistant to PI-PLC cleavage. These data are consistent with the data reported by Goyert *et al.*(1988). Increasing the concentration of PI-PLC and / or the length of the incubation had no further affect on CD14 loss. However, it did lead to a decrease in cell viability, as determined by trypan blue exclusion (data no shown). Interestingly, a previously unreported observation was made. The treatment of monocytes with PI-PLC in the presence of 10 ng/ml PMA resulted in 100% loss of CD14 from the monocyte cell surface (Fig.15). Expression of HLA-Class I (W6/32) molecules, which are anchored to the cell membrane through a transmembrane domain rather than a GPI anchor, was not significantly altered either after PI-PLC or the combination of PI-PLC and PMA and remained at 85% of cells positive for HLA-class I. The MFI shown in Fig.15D is lower than controls, because PI-PLC can also remove other PI-linked antigens including CD16 (FcγRIII)

that may be involved in some of the non-specific binding also.

It is believed that the major reason for GPI-anchor resistance to PI-PLC is a modification of the inositol ring (R). For example, two GPI-anchored proteins, the human erythrocyte acetylcholinesterase (AChE) (Low *et al.*, 1977), and human alkaline phosphatase (ALP) (Wong, 1992), were reported to be relatively resistant to PI-PLC cleavage due to the esterification of the 2-OH on the inositol ring with palmitic acid (Roberts *et al.*, 1987) and the resulting acylation of the inositol ring in the GPI anchor (Wong, 1992), respectively.

It would appear from the data obtained (Fig.14) that approximately 20% of the CD14 molecules are resistant to this enzyme and, therefore, may be in this acylated form or may lack the GPI-anchor. In three out of four studies, PMA alone was found to be less efficient than PI-PLC at inducing CD14 loss. In 13 out of 14 studies, shedding was usually only 20% to 40% of the membrane CD14 following PMA treatment, although in one study, a loss of 75% was detected. The above data suggest that the PMA and PI-PLC treatments may result in the cleavage of different species of CD14 or that PMA treatment results in the conversion of a PI-PLC resistant to a PI-PLC sensitive form of CD14.

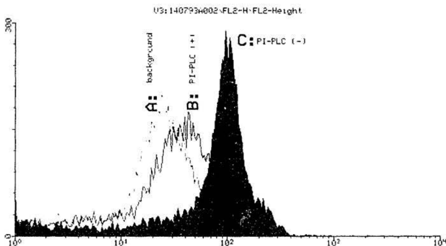


Figure 14. FACS analysis of PI-PLC-treated human monocytes. Peripheral blood monocytes, isolated by density gradient centrifugation and adherence, were treated with PI-PLC (2 units/ml) for 3 hour. Control monocytes were incubated under the same conditions without enzyme. Cells were treated with antibodies as described and analyzed by FACS. The $CD14^+$ percentage and mean fluorescence intensity was 70% and 120 respectively for the untreated cells (C) and 14% and 90 respectively for the PI-PLC treated cells (B). The fluorescence intensity of the antibody control (A) and Class I HLA (W6/32) (not shown) did not change significantly after PI-PLC treatment. The x-axis shows log fluorescence and the y-axis shows cell number. The frequency of positive cells and MFI are presented.

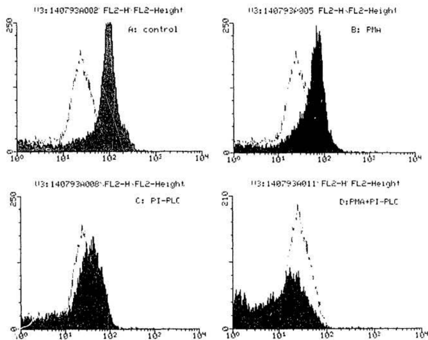


Figure 15. Additive effect of PI-PLC and PMA on CD14 expression loss on human monocytes. Monocytes were incubated in medium with 10 ng/ml PMA (B), 2 units PI-PLC (C), PMA plus PI-PLC (D) or 10% FCS RPMI-1640 medium only (A) at 37°C for 3 h as described in materials and methods. CD14 expression was measured by indirect immunofluorescence using monoclonal anti-CD14 Ab (IMO2) (solid histogram) or isotype matched mAb (outline histogram).

3.7 Additive down-modulation effect of A23187 with PMA on CD14 expression by monocytes

The effects of the calcium ionophore A23187 were investigated on CD14 expression by human monocytes. A23187 treatment of cells induces the release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm. It is known that Ca^{2+} plays a role in the activation of PKC and acts through a separate binding site to PMA. As shown in Figure 16, treatment of cells with A23187 alone was found to induce CD14 loss from PBM membranes. This effect was also found to be additive to that observed by the addition of PMA (Fig.16D). It therefore appears that PKC isoenzymes possessing both PMA and Ca^{2+} binding sites, may be involved in the signalling pathway resulting in loss of membrane CD14.

Similar results were also observed when differentiated THP-1 cells were treated with A23187. It was observed that 83% of CD14 was lost from the THP-1 cell surface following treatment with 500 ng/ml A23187 for 3 hours.

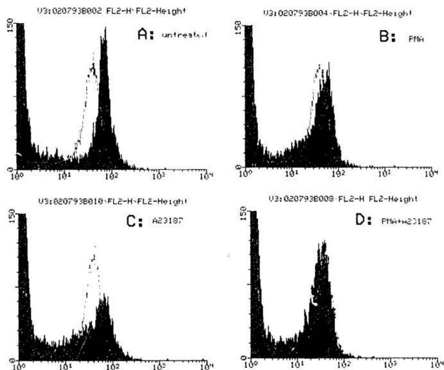


Figure 16. Additive down-modulation of CD14 expression on monocytes by A23187 with PMA. Monocytes were treated with the indicated reagents or medium only at 37°C for 3 h, then labelled with monoclonal anti-CD14 Ab (solid histogram) or isotype control antibody (outline histogram). %CD14 positive cells is dramatically decreased to 4% after combined PMA plus A23187 treatment (D).

3.8 Down-modulation of monocyte surface CD14 expression by LPS

Regulation of CD14 expression by LPS was investigated in the following studies. It was found that incubation of monocytes with 10 ng/ml or 100 ng/ml LPS for 3 h resulted in loss of surface CD14 as shown in Fig.17. A time course study, Fig.18, showed that stimulation of monocytes with 10 ng/ml LPS for 3 hours resulted in a maximum of 22% reduction in CD14$^+$ cells. CD14$^+$ expression gradually started to recover after 3 h. While percentage HLA$^+$ cells remained unaffected by LPS treatment.

We also observed that the reduction in MFI due to CD14 shedding induced by LPS varied form donor to donor and in some cases was hardly detectable. This may reflect different sensitivities of donor's mononuclear cells to LPS *in vivo*. Furthermore, our data show that loss of CD14 did not occur immediately but only after a delay of 1 h following LPS treatment, then CD14 expression started to recover by 4 h. Factors influencing CD14 loss after LPS exposure therefore must be slowly induced and be transient: lasting 2-3 h in these studies. Kinetics with PMA induced loss of CD14$^+$ cells in comparison (see Fig.13) lacked this delay. However, maximum loss of CD14 still occurred at 3 h. It was shown by Bazil & Strominger (1991) that surface CD14 loss induced by PMA or LPS is due to a process of shedding. Evidence has also been published showing that LPS activates PKC in monocytes and macrophages (Coffey *et al.*, 1992). One may speculate that LPS-induced CD14 shedding also occurs through activation of PKC.

The delay-phase observed with LPS induced shedding may reflect the slower activation of PKC or time delay due to the release of the secondary or tertiary messenger molecules or cytokines. To investigate a possible feedback mechanism of regulation, PBM were incubated with TNF-α. The data obtained indicated that it was very unlikely that the loss of cell surface CD14 by PBM following LPS treatment is secondary to the secretion of TNF-α, but does not entirely exclude the possibility. The results shown in Fig.17 show that the addition of TNF-α to PBM increased, not decreased the expression of CD14 under the conditions used in this investigation.

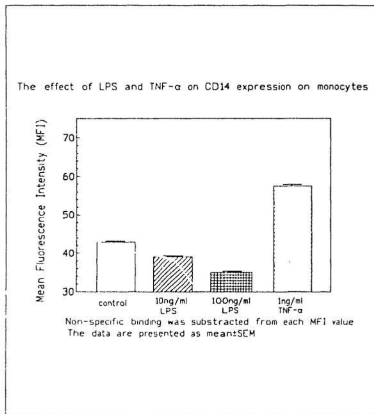


Figure 17. The effect of LPS and TNF-α on CD14 expression on monocytes. Monocytes were treated with or without 10 ng/ml LPS, 100 ng/ml LPS or 1 ng/ml TNF-α respectively for 3 h at 37°C, then blocked, treated with anti-CD14 mAb or isotype matched control and labelled with PE-F$(ab')_2$ as described in materials and methods. Non-specific binding is subtracted from each value. The data are presented as mean ± SEM (n=4).

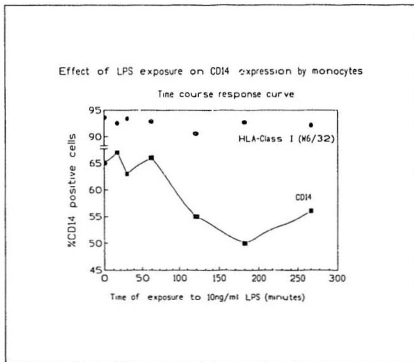


Figure 18. Time course response curve of monocytes to LPS. Monocytes were exposed to 10 ng/ml LPS over a 4 h period time, followed by blocking, antibody addition (anti-CD14 mAb, anti-HLA class I or isotype matched control) and labelling with PE-F(ab')$_2$ as described in materials and methods. CD14 expression was analyzed by FACS. Non-specific binding was subtracted from specific binding.

3.9 The effect of a protein phosphatase Inhibitor on monocyte CD14 expression

The data above have shown that PMA could induce CD14 shedding from the monocyte surface, indicating that PKC may be involved in the shedding process. The action of PKC may be enhanced by preventing dephosphorylation mediated by phosphatases. Okadaic acid (OA), a selective inhibitor of serine/threonine protein phosphatases (PP1 and PP2A) (Suganuma *et al.*, 1988; Haystead *et al.*, 1989) was used to study the role of these phosphoproteins in the regulation of PMA-induced CD14 loss. Treatment of monocytes with 10 nM OA only, a concentration known to augment TNF-α production in response of human monocyte to LPS (Coffey *et al.*, 1992), in 5% CO_2 at 37°C for 3 hours resulted in a decrease of 41% (MFI) CD14 on human PBM and a 10% decrease in %CD14 positive cells (Fig.19). The upper right panel solid histogram in Fig.19 also shows that there are two peaks after OA treatment. The presence of these two peaks suggests that there may be two different populations of CD14 positive monocytes, that respond differently to OA. When monocytes were treated with OA in the presence of PMA, only a single population of CD14 positive monocytes was observed as shown by the presence of a single peak (the bottom right solid histogram in Fig.19). Slight augmentative effects on CD14 loss were observed when monocytes were treated with OA plus PMA (Fig.19, the bottom right histogram). These results suggest that PKC activity, stimulated by PMA, may not

be the substrates for serine/threonine phosphateses 1 and 2A.

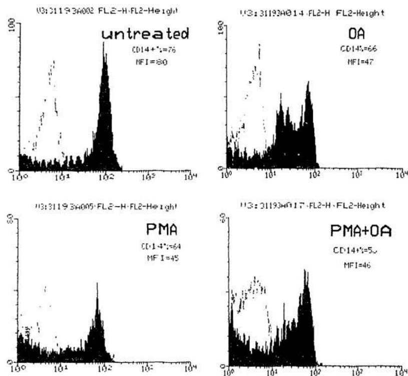


Figure 19. The effect of okadaic acid on CD14 expression on human monocytes. Monocytes were treated with or without 10 nM okadaic acid or 10 ng/ml PMA, or okadaic acid plus PMA at 37°C for 3 hours. Solid histograms represent cell surface CD14 expression. Outline histograms are background controls.

3.10 Effect of *f* MLP on CD14 expression by human monocytes

Formyl-methionyl-leucyl-phenylalanine (*f* MLP), a component from bacterial cell walls, is a monocyte chemotactic peptide. Monocytes and neutrophils have been reported to possess a *f* MLP-receptor (Murphy *et al.*, 1991). The treatment of human monocytes from three different donors for 4 hours with 100 ng/ml *f*MLP, a concentration which is known to enhance LPS-evoked TNF-α production by monocytes (Coffey *et al.*,1992), led to maximum of a 28% reduction in % $CD14^+$ cells and 23% loss in CD14 density on the monocyte membrane surface (Table 3). There was no effect on HLA-class I (W6/32). The mechanism of *f* MLP-induced CD14 loss on human monocytes is unknown. However, in human neutrophils, *f* MLP has been shown to stimulate receptor-mediated activation of G proteins with G protein-mediated activation of phospholipases (PLC and PLD) (Koo *et al.*, 1983), and PLA_2 (Murphy *et al.*, 1991) and subsequent generation of second messengers, such as DAG, Ca^{2+}, arachidonic acid, all of which can activate PKC. Similar signalling and activation of PKC may exist in the monocytes and may lead to CD14 loss. Evidence to support this was observed when monocytes were treated with *f* MLP plus PMA. The amount of CD14 lost was only just slightly higher than that observed from PMA alone (data not shown), indicating that *f* MLP had no significant augmentative effect with PMA on CD14 loss. This could be explained by PMA and *f* MLP working through a shared common mechanism.

Recently, it has been shown that stimulation of human neutrophils with *f*

MLP induced tyrosine phosphorylation and activation of two distinct mitogen-activated protein-kinases (MAPK I and II) (Torres *et al.*, 1993). MAPK was also shown to be phosphorylated on tyrosine following LPS triggering of murine macrophages (Weinstein *et al.*, 1992). In our studies, it was found that treatment of human monocytes with *f* MLP led to greater CD14 loss than that observed following treatment with LPS. However, only three volunteers were screened using *f* MLP and further studies would help to support these findings.

Table 3. Effect of *f* MLP on CD14 expression on human monocytes.

source donor	treatment	% Ag positive cells/MFI*		
		Isotype control	anti-CD14 Mab	anti-HLA Mab
N12	untreated	1.0	69/138	91/261
	f MLP	1.8	50/106 (28↓/23↓)	88/296
LA18	untreated	0.8	68/77	99/120
	f MLP	0.5	66/70 (3↓/9↓)	99/139
SR19	untreated	0.3	80/37	96/64
	f MLP	0.5	73/35 (9↓/5↓)	96/61

f MLP: 100 ng/ml for 4 hrs.
*: Values in parentheses are the percentage change from original values.

Monocytes were treated with indicated reagents for 4 h at 37°C, CD14 expression and MFI were decreased after *f* MLP treatment as indicated above. There was no change in the HLA Class I (W6/32) expression after the addition of *f* MLP.

3.11 The effect of Indomethacin on monocyte CD14 expression

As reviewed in the introduction, it is known that LPS induces activation of CD14-associated protein tyrosine kinase p53/56^{lyn} (Štefanová *et al.,* 1993). Herbimycin A blocked LPS-induced cytokine production, CD14 shedding and prostaglandin production (Weinstein *et al.* 1991; Štefanová *et al.*, 1993). These observations led us to investigate the indirect effect of prostaglandins on CD14 expression by using indomethacin, a cycloxygenase inhibitor. The results from two experiments both showed some reduction of CD14 MFI, 16% and 15% respectively (Table 4). The result from one of two experiments also showed a 25% reduction in % CD14^{+} cells. It is not clear from these data as to what role, if any, prostaglandins have, in the regulation of CD14 loss and further studies should be performed to obtain more convincing evidence of their effects.

Table 4. The effect of indomethacin on CD14 expression by monocytes

source donor	treatment	% Ag positive cells/MFI		
		isotype control	anti-CD14 mAb	anti-HLA mAb
N12	none	1.0	69/138	91/261
	Indomethacin	2.1	52/117 (25↓/15↓)	91/219 (0/19↓)
V20	none	1.5	86/157	99/319
	Indomethacin	1.5	87/132 (1↑/16↓)	99/308

Indomethacin: 1 μM for 3 h incubation.

Values in parentheses are the percentage change from original values.

Monocytes were treated with indicated reagents for 3 h at 37°C, % CD14 positive cells and MFI were decreased after indomethacin treatment as indicated above. There was no change in HLA Class I (W6/32) expression after the addition of indomethacin.

3.12 The effect of protein kinase inhibitors on human monocyte CD14 expression

Three protein kinase inhibitors were studied for their effects on CD14 expression. These were: staurosporine, calphostin C and KT5720. Staurosporine has been shown to inhibit the proteolytically generated catalytic domain of PKC (Tamaoki *et al.*, 1986). It is also known to inhibit other protein kinases including phosphorylase kinase, cAMP-dependent protein kinase, Ca^{2+}/calmodulin-dependent protein kinase and tyrosine kinase (Reugg *et al.*, 1989). We found that monocytes treated with 10 ng/ml PMA together with 20 nM staurosporine for 3 h, did shed CD14 indicating that staurosporine was not able to prevent PMA activated signalling (Fig.20). This may be explained by slow effects of staurosporine on preventing PKC activity or by staurosporine's broadly active inhibitory effects.

Compared with staurosporine, calphostin C is known to be a more specific inhibitor of PKC. After light activation, it interacts with the regulatory domain of PKC (Kobayashi *et al.*, 1989). The effects of calphostin C were studied in both PMB and THP-1 cells. Almost 100% of the CD14 expressed by PBM was lost following 3 h incubation with 50 nM calphostin C in the presence of ordinary fluorescent light, but HLA-class I molecules (W6/32) were also decreased significantly (Fig.21). It was difficult to interpret these results as the activation of PKC, *i.e.* by PMA, LPS or Ca^{2+} rather than inhibition of PKC was believed to cause loss of CD14. It now appeared that the inhibition of PKC could also lead to a more

rapid and greater CD14 loss than PKC activation. Cell viability in these studies as determined by trypan blue exclusion, was not affected, but both cell size and cell granularity were found to be reduced greatly after treatment with calphostin C (Fig.22). Subsequently, this phenomenon was studied in much greater detail and it was discoverd that loss of CD14 was the result of induction of apoptosis in both THP-1 cells and PBM, and the subsequent sheoding of apoptotic vesicles (see chapter 4).

KT5720 is a cAMP-dependent protein kinase inhibitor (Yamada *et al.*, 1989). Treatment of differentiated THP-1 cells with 40 nM KT5720 for 24 h caused only a slight loss of CD14 expression, dropping 7% in % CD14 positive cells and 11% in MFI. However, this observation was following a 24 h incubation time rather than the 3 hour used for the other studies. Data from these experiments should therefore be viewed with this limitation in mind.

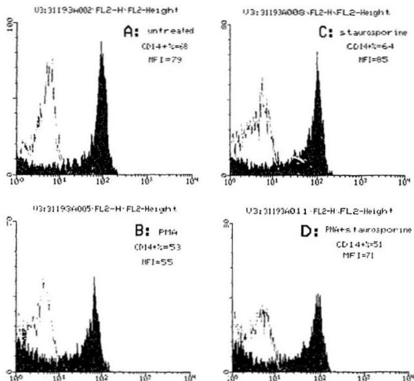


Figure 20. The effect of staurosporine on PMA-modulated CD14 expression. Monocytes were exposed to medium only (A), or 10 ng/ml PMA (B), or 20 nM staurosporine (C) or both (D) for 3 h at 37°C in the 5% CO_2 atmosphere, followed by the addition of isotype control antibodies or anti-CD14 mAb (IMO2) and stained by PE-IgG $F(ab')_2$. CD14 expression is represented in solid histograms and background control is represented in unshaded histograms. % $CD14^+$ cells and the level of CD14 expression (MFI) are given with each histogram.

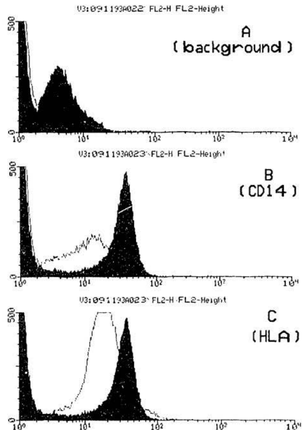


Figure 21. The effect of calphostin C on monocytes. Monocytes were incubated with medium only (shaded himstogram) or exposed to 50 nM calphostin C (unshaded histogram) in the presence of light for 3 h at 37°C, followed by blocking, antibody addition: isotype control mAb (A), anti-CD14 mAb (B), anti-HLA class I (C), and labelling with PE-F(ab')$_2$ as described in materials and methods.

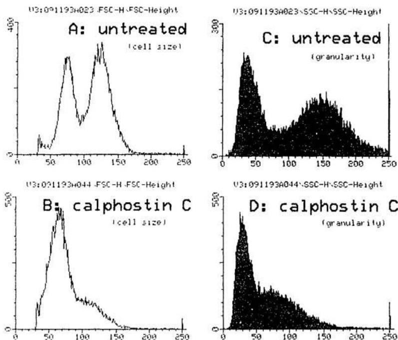


Figure 22. The effect of calphostin C on cell size and granularity. Monocytes were incubated with medium only (A and C) or with 50 nM calphostin C (B and D) at 37°C for 3 hours. Cell size (A and B) and granularity (C and D) were analysed by using forward scatter (FSC) and side scatter (SSC) FACS, respectively.

3.13 The effect of ouabain on CD14 expression on monocytes

Ouabain, a cardiotonic steroid, is a well known inhibitor of Na^+/K^+-ATPase (Seader *et al.*, 1980). It acts primarily on the K^+-binding sites located at the extracelluar side of the plasma membrane to inhibit the enzyme competitively with respect to K^+ (Choi *et al.*, 1977; Oishi *et al.*, 1991). Ouabain has been reported to enhance TNF-α and IL-1 production in monocytes through transcriptional mechanisms (Ohmori *et al.*, 1991) and it was this observation which led us to investigate the effects of ouabain on CD14 expression.

Normal human monocytes were treated with ouabain at various concentrations as shown in the dose response curve (Fig.23) and for different periods of time as shown in the time course study (Fig.24). Cell viability, determined by trypan blue staining, was always more than 90%. Flow cytometric analysis showed that ouabain caused a rapid loss of CD14 expression on human monocytes, but did not significantly affect HLA-Class I (W6/32) expression except at high dose (Fig.25). The kinetic studies show that a 3 hour treatment with 1 mM ouabain caused maximum loss (100%) of CD14 expression on human monocytes (Fig.24). Dose response studies showed that the IC_{50} value for ouabain was 200 µM and that 1 mM was the optimal concentration required to obtain maximum CD14 loss from the cell surface (Fig.23). It was also found that 1 mM ouabain affected HLA expression (Fig.25). Changes in cellular granularity as determined from cytometric analysis were also noted (Fig.26), but no changes in cell size or

apoptotic vesicles could be observed by using microscopy.

It has been shown by Bazil & Strominger (1991) that treatment with PI-PLC leads to the shedding of a 56 KDa MW CD14 species while treatment with PMA leads to the shedding of two molecular weight species 56 KDa or 48 KDa. It is believed that the lower molecular weight species is shed through a proteolytic cleavage of the CD14-GPI anchored molecule. It is unknown how the 56 KDa species arises but this may be due to phospholipase cleavage of the GPI-anchor.

To help elucidate the mechanisms of membrane CD14 loss and the molecular weights of the CD14 molecules shed following ouabain treatment, cells were labelled with [^{35}S] methionine, and both the membrane and the supernatant were investigated using anti-CD14 mAb immunoprecipitation. Shed CD14 molecules were analyzed by 2D-SDS-PAGE as described in materials and methods. Membrane CD14 was readily immunoprecipitated from cytoplasmic membrane preparations of untreated cells (Fig.27A), but could barely be detected in the membrane fractions from ouabain treated cells (Fig.27D). CD14 could still be detected in the membrane fractions from PMA treated cells (Fig.27C). Cells incubated with PI-PLC had little or no membrane CD14. All these data agree with the FACS analysis. The culture media from 3 h treated [^{35}S] labelled monocytes were also analyzed for immunoprecipitable CD14. There was no detectable sCD14 in the medium from control cells or ouabain or PMA-treated monocytes under the conditions used, but sCD14 from the medium of PI-PLC treated

monocytes was readily detected. This sCD14 had a molecular weight of approximate 53 KDa and a pI between 4.5 to 5.0 (Fig.28). The data from FACS and 2D-SDS-PAGE show that there is no mCD14 on the PBM cell surface following ouabain treatment. But sCD14 molecules were also not deteced in the media from ouabain treated cells. There are three possible explanations. First, CD14 on monocytes may have been internalized into the cytoplasm and if this were the reason, electron microscopic analysis using gold labelled antibody or FACS analysis of permiabilized cells could provide more direct evidence. Secondly, CD14 could have been shed into the medium, but destroyed by proteolysis, such that anti-CD14 mAb could not recognize the CD14 epitope and thus failed to immunoprecipitate the protein. Thirdly, the quantity of sCD14 may have been too low to detect. The latter explanation is not supported by the detection of spontaneously shed CD14 from overnight [^{35}S] labelled monocytes with approximate molecular weight of 53 KDa and a pI of 4.8, in which more monocytes ($2x10^7$) and a longer incubation time were used (Fig.29). It was also possible to detect PI-PLC cleaved CD14 after a 3 hr incubation and therefore the immunoprecitation had worked for this control. Thus internalization and or CD14 distruction are the only possible explanation.

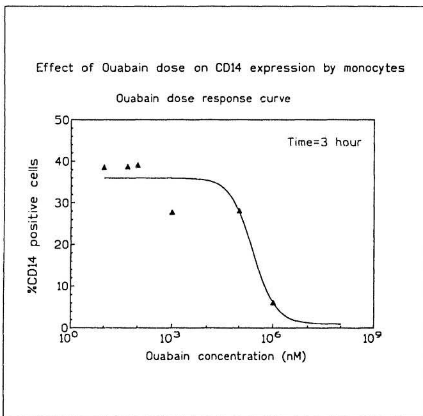


Figure 23. The effect of ouabain dose on CD14 expression by monocytes. Ouabain dose response curve (IC_{50}≈200 μM).

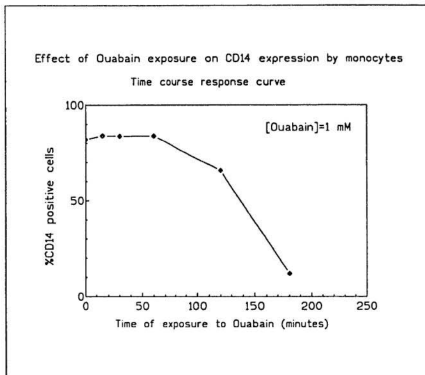


Figure 24. Time course response curve of monocytes to ouabain treatment.

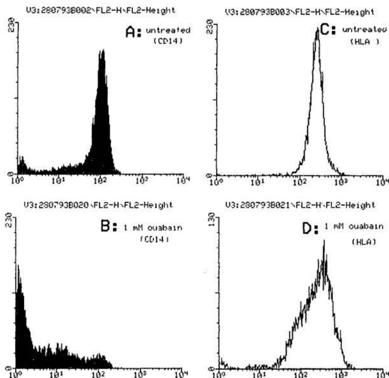


Figure 25. The effect of ouabain on monocytes analyzed by FACS. Monocytes were incubated with medium (A and C), or treated with 1 mM ouabain (B and D) at 37°C for 3 h, then labelled with anti-CD14 mAb (left panel, A and B) or anti-class I HLA (W6/32) mAb (right panel, C and D). CD14 molecules were dramatically lost after ouabain treatment (B), HLA class I molecule were not lost but were effected by ouabain treatment (D).

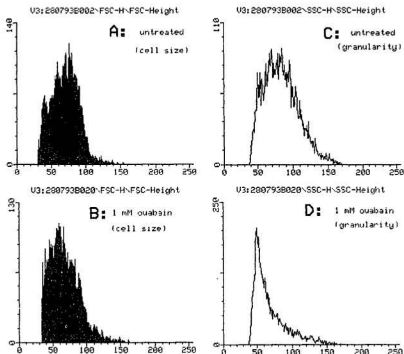


Figure 26. The effect of ouabain on monocyte size and granularity. Monocytes were incubated with medium only (A and C) or with 1 mM ouabain (B and D) for 3 h at 37°C. Cell size (left panel, A and B) and granularity (right panel, C and D) were analysed by using forward scatter (FSC) and side scatter (SSC) FACS, respectively.

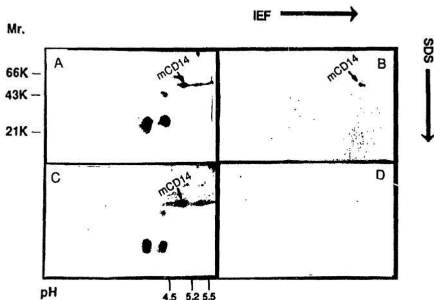


Figure 27. 2-D-SDS-gel electrophoresis of mCD14. Monocyte derived macrophages from untreated (A, the left upper panel), 1 mM ouabain treated (B, the right upper panel), 10 ng/ml PMA-treated (C, the bottom left panel) or 2 units PI-PLC (D, the bottom right panel) treated cells were lysed, mCD14 was prepared and subjected to 2D-SDS gel electrophoresis as described in materials and methods. After electrophoresis, gels were silver stained to indentify internal MW and pI markers, and subsequently autoradiographied. mCD14 MW and pI value were determined from the 2D-SDS-markers run with samples. mCD14 had disappeared after PI-PLC treatment (D).

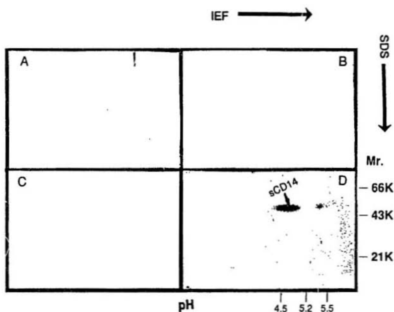


Figure 28. 2-D-SDS-gel electrophoresis of sCD14. sCD14 was immunoprecipitated from the medium from untreated (A, the left upper panel), 1 mM ouabain treated (B, the right upper panel), 10 ng/ml PMA-treated (C, the left bottom panel) or 2 units PI-PLC (the right bottom panel) treated monocyte derived macrophages and subjected to 2D-SDS gel electrophoresis as described in materials and methods. After electrophoresis, gels were silver stained to observe internal MW and pI markers and subsequently autoradiographied. sCD14 MW and pI values were determined from the 2D-SDS-marker running with samples.

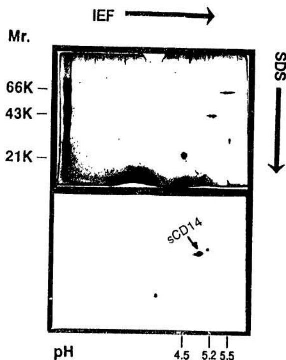


Figure 29. 2-D-SDS-gel electrophoresis of spontaneously released CD14. $2x10^7$ monocyte derived macrophages were labelled with $[^{35}S]$-methionine overnight, the medium was collected and sCD14 prepared and subjected to 2D-SDS gel electrophoresis as described in materials and methods. Top panel, silver stain pattern shows 2D-SDS-PAGE markers; bottom panel shows autoradiographic image of the 2-D gel. Arrow indicates sCD14. MW and pI values determined from 2D-SDS-PAGE marks.

3.14 Summary and conclusion

Summary:

The experimental results are summarized in Table 5. The two pharmacological agents actively involved in regulating CD14 membrane expression were calphostin C and ouabain.

Calphostin C, discussed later in this thesis, was found to induce apoptosis and lead to shedding of apoptotic vesicles and a 100% loss of CD14 expression. Ouabain was also found to induce 100% loss of membrane CD14 within 3 hours. Upon examination by light microscopy and 2D-SDS-PAGE, ouabain-induced mCD14 loss did not appear to be due to apoptosis or shedding of CD14 into the media and may therefore have been due to CD14 internalization. Although no loss of viability in cells was observed, changes in the FACS pattern observed with MHC Class I and granularity of the cells lead us to believe that profound membrane and cellular changes were taking place under the conditions under which CD14 loss was observed. Smaller effects on CD14 expression were observed with both PMA and calcium ionophore. These effects were shown to be additive leading to almost 100% loss of CD14 by 3 hours. Only slight changes in % $CD14^{+}$ cells, less than 25%, were observed with indomethacin, okadaic acid and the PKA inhibitor KT5720.

The natural ligands investigated were TNF-α, LPS and *f*MLP. TNF-α was found to increase CD14 expression, while LPS induced a measurable loss of CD14

and *f*MLP induced up to 30% loss of CD14 positive cells. More work studying the CD14 loss induced by *f* MLP could confirm if this was due to shedding or not.

Table 5. Summary of effects of agents on CD14 membrane expression

Agents	Decrease in CD14⁺ cell number	Incerease in CD14⁺ cell number
TNF-α		+
LPS	+	
f MLP	++	
Staurosporine	0	0
Calphostin C	(+++)	
PMA	++	
Okadaic Acid	+	
KT5720	+	
A23187	++	
Ouabain	+++	
PMA+A23187	+++	
Indomethacin	+	
PI-PLC	+++	
PMA+PI-PLC	+++ (100%)	

"+": 10-25% change of CD14 positive cell number
"++": 25-75% change of CD14 positive cell number
"+++": > 75% change of CD14 postive cell number
"0": no change of CD14 postive cell number

Conclusion:

It appears that the major effectors inducing loss of membrane CD14 from monocytes are regulators of PKC (PMA and calphostin C) or cytoplasmic calcium levels (A23187). PMA induced shedding has been shown by others to result from the proteolytic cleavage of CD14.

*F*MLP appears to cause a rapid loss of CD14 from monocytes. *F*MLP also looks as if it may be an interesting model to investigate further; no direct conclusions were reached as to how or why *f*MLP was able to result in CD14 loss. Indirect evidence may implicate some shared signalling pathways between LPS and *f*MLP, perhaps involving Ca^{2+} or PKC activated pathways.

TNF-α does <u>not</u> appear to act as a negative feedback inhibitor regulating CD14 shedding under the conditions examined. However, it does appear that TNF-α act as a positive feedback regulator resulting in an increase in membrane CD14 expression by monocytes. This phenomena would have to be studied further in respect of agents that are known to regulate TNF-α release.

Ouabain induces a rapid and complete loss of CD14 from monocyte membranes at concentrations previously used by others to enhance LPS induced TNF-α production. LPS and CD14 may be internalized and perhaps enhance TNF-α production.

The small effects of okadaic acid, KT5720 and indomethacin may be the result of cross talk between signalling pathways. As such these pathways appear

to play only a minor part in the signalling leading to CD14 loss.

Staurosporine was ineffective, but was not tested over a wide range of concentrations. Thus, its lack of activity would have to be studied further to be verified.

Chapter 4 Apoptosis Studies

Introduction

4.1 Programmed cell death (PCD) or apoptosis

Programmed cell death or apoptosis is a widespread, morphologically distinct process of cell death. It occurs during normal embryonic development of vertebrates and invertebrates, as well as during metamorphosis, hormone-dependent atrophy of tissues (Kerr *et al.*, 1972; Wyllie *et al.*, 1980), and deletion of auto-reactive T cells in the thymus (Duvall *et al.*, 1986). Kerr and Searle (1972) proposed the term "apoptosis" (from the Greek: falling off) to name this form of cell death. The term apoptosis is sometimes considered synonymous with programmed cell death and it implies a lethal genetic program. For example, as B-cell populations develop, about 95 cells out of every 100 cells will die for any of a variety of reasons such as faulty gene rearrangement, self-destructive receptor expression, or lack of stimulation (Deenen *et al.*, 1992). Apoptosis is also observed in malignant tumours (Sarraf *et al.*, 1986). A number of agents including gamma-radiation (Skalka *et al.*, 1976), toxic chemicals (McConkey *et al.*, 1988), cytotoxic T cells (Liepins *et al.*, 1978; Moss *et al.*, 1991) also kill their target cells by programmed cell death. PCD is widely regarded as a suicidal cell response since cell death appears to result from the induction of an active biological process within the cell (Wyllie *et al.*, 1980; McConkey *et al.*, 1990).

4.2 Morphological characteristics of apoptosis

PCD or apoptosis can be recognized by characteristic changes in morphology including membrane vesicle formation (cell surface blebbing) and shedding from the cell surface, a marked reduction in cell volume, loss of plasma membrane microvilli, and nuclear DNA condensation. Finally, cells break down into membrane-bound apoptotic bodies in which cytoplasmic organelles appear to be randomly distributed and most of them have a nuclear component (Wyllie *et al.*, 1980). These apoptotic bodies are sealed and maintain their osmotic gradients. Therefore, there is no spilling of intracellular contents, and thus no inflammation. There is evidence which suggest that the apoptotic cell strengthens its membranes against the risk of lysis by the activation of cross-linking enzymes such as transglutaminase (Fesus, 1991). Apoptotic bodies are eliminated through phagocytosis by neighbouring cells. Significant alterations in membrane composition occurs in apoptotic cells which may also aid their recognition and engulfment by the phagocytosing cells (Wyllie *et al.*, 1984; Duvall *et al.*, 1985). Macrophages are thought to play a major role in the removal of apoptotic bodies (Kerr *et al.*, 1987).

4.3 Biochemical characteristics of apoptosis

The best defined biochemical event in apoptosis involves nuclear DNA fragmentation. When the morphological changes of apoptosis are observed,

internucleosomal DNA fragmentation is almost always detected by agarose gel electrophoresis where it appears as a typical ladder pattern (multiples of 180-200 base pair subunits) (Wyllie *et al.*, 1980; Arends *et al.*, 1990).

Most studies indicate that there is an intracellular elevation in Ca^{2+} which is required for apoptosis (McConkey *et al.*, 1990) followed by the activation of a putative endonuclease responsible for the internucleosomal cleavage of DNA (Arends *et al.*, 1990). McConkey *et al.* (1990) have shown that a Ca^{2+}, Mg^{2+}-dependent endonuclease activity in thymocyte nuclei is rapidly lost following treatment of cells with either actinomycin D or cycloheximide. Recently, Arends *et al.* (1990) demonstrated that treatment of isolated nuclei with micrococcal nuclease leads to the pattern of chromatin condensation associated with apoptosis, thus supporting the view that an endogenous endonuclease is responsible for both DNA fragmentation and nuclear condensation. However, a specific endonuclease with internucleosomal cleavage activity has not yet been purified from eukaryotic cells.

In apoptosis, the cells actively participate in the self-destructive process, which requires metabolic energy (Alison and Sarraf., 1992). It has been reported that the process of cytotoxic T cell mediated tumour target cell killing occurs through an apoptotic mechanism and is associated with elevated oxygen consumption rates (Nathan *et al.*1982).

4.4 Possible signal transduction pathways for apoptosis

The Ca^{2+}/phospholipid-dependent protein kinase (PKC) has been demonstrated to participate in intracellular signalling processes in many cell types (Nishizuka *et al.*, 1984). PKC-mediated phosphorylation of numerous protein substrates is associated with a wide range of biological effects, including induction of cellular proliferation and differentiation (Craven *et al.*, 1988), activation of nuclear transcription factors and cell-surface receptors (Brach *et al.*, 1992), and tumour promotion (Rahmsdorf *et al.*, 1990). PKC is expressed in mammalian systems as a family of diverse serine-threonine kinases, consisting of at least 9 isoforms differing in both substrate specificity and dependence upon Ca^{2+} availability. Differential activation of PKC isoforms has been postulated to account for the divergent actions of different enzyme activators (Nishizuka *et al.*, 1988). PKC not only plays a physiological role for cellular regulation (Pelosin *et al.*, 1987), it has also recently been reported that this protein kinase is involved in signal transduction pathways that lead to apoptosis. The role of PKC in the induction of apoptosis is not clear due to conflicting reports. For example, Tomei *et al.* (1988) was able to show that the PKC activator PMA can prevent apoptosis in C3H-10T1/2 cells after serum removal or exposure to ionizing radiation. This result supports the idea of suppression of apoptotic cell death through activation of protein kinase C. However, the observation from Mercep *et al.* (1989), showed that PMA, either alone or in conjunction with Ca^{2+} ionophore, induced apoptosis

in cells of lymphoid origin, and that inhibition of PKC by exposure to H7, an inhibitor of PKC, prevented glucocorticoid-induced apoptosis in murine thymocytes (Ojeda *et al.*, 1990), suggesting that PKC activation promoted this process. It is conceivable that these conflicting findings on the role of PKC in the regulation of apoptosis may reflect cell type-specific responses or, alternatively, the influence of undefined factors or perhaps different isoforms of PKC.

Several lines of evidence indicate that PKC also modulates cell surface membrane ion conductance by phosphorylating ion channel proteins, ion pumps and ion exchange proteins suggesting that ion channel function may also be involved in the apoptosis process (Levitan, 1985).

Ion channels are integral membrane proteins through which ions can passively flow down their electrochemical gradient at rates exceeding 10^6 ions/sec (Lecar *et al.*, 1985; Gallin *et al.*, 1986). Shifts in the resting potential of cell-surface membranes may result from the activation of specific ion channels which are thought to be involved in a variety of cellular functions including nerve conduction, muscle-cell contraction and relaxation, cell proliferation, protein synthesis (DeCoursey *et al.*, 1984; Panet *et al.* 1985; Deutsch *et al.* 1986; Meldolesi *et al.*, 1987), as well as in processes associated with cell injury through apoptosis (Trump *et al.*, 1979; Schlichter *et al.*, 1986; Liepins *et al.*, 1987). K^+ channel activity has been reported to be involved in the delivery of the "lethal hit" by cytotoxic T-lymphocytes (Farber *et al.*, 1981; Fukushima *et al.*, 1984) and

human NK cells (Schlichter *et al.*, 1986). Other studies using target cells loaded with ^{86}Rb, have suggested the presence and function of K^+ channels in tumour cells undergoing T-cell mediated lysis (Henney *et al.*,1973; Ferluga *et al.*, 1974; Martz *et al.*, 1976; Sanderson *et al.*, 1981). Liepins *et al.* in 1985 and 1987 demonstrated that incubating cells first at 0° and then warming them to 37° induced apoptosis. Interestingly, two classic K^+ channel blockers, tetraethylammonuium (TEA) and 4-aminopyridine (4-AP), also inhibited, in a dose dependent manner, the membrane vesicle formation and shedding process as well as nuclear DNA fragmentation and membrane permeability changes. Furthermore, they showed that the temperature shift induced membrane vesicle formation and shedding was indistinguishable from that occurring during alloimmune T-lymphocyte-mediated injury of tumour cells. This evidence suggests that the function of K^+ channels are required for tumour cell susceptibility to the low temperature induced apoptosis. Thus, K^+ channel activity appears to be required for both the triggering or delivery of lytic signals from the effector cells, as well as for the target tumour cells to initiate a self-destructive cascade of events.

The observation that calphostin C led to CD14 loss from monocyte membrane and the subsequent observation that this might be an apoptotic process led to the present studies to elucidate the role of PKC and also ion channel function in the process of apoptosis. Our findings demonstrated that calphostin C, which is a highly specific inhibitor of PKC, was sufficient to induce apoptosis in

human monocytic leukaemia THP-1 cells as well as normal human peripheral mononuclear cells. Staurosporine, a potent, but less specific PKC inhibitor, failed to induce apoptosis in these cells. Further studies found that quinidine, a Ca^{2+}-dependent K^{+} channel inhibitor, significantly prevented calphostin C-mediated THP-1 apoptosis, indicating that Ca^{2+}-dependent K^{+} channels are involved in the regulation of apoptosis.

4.5 Induction of apoptosis by a PKC inhibitor, calphostin C, in THP-1 cells

4.5.1 Morphological features of calphostin C-mediated THP-1 cell apoptosis

Cells of the human monocytic leukaemia cell line, THP-1, were exposed to calphostin C, a light acitivated and highly specific PKC inhibitor. Concentrations ranging from 10 nM to 100 nM and different time periods were used. The results from one representative experiment of several independent experiments are shown in Figure 30. THP-1 cells start to bleb after 1 hour in the presence of 100 nM calphostin C only when exposed to light. Controls treated with calphostin C in the dark did not undergo blebbing. In parallel studies, nuclear condensation was also observed using transmission microscopy following calphostin C treatment in the presence of light, but not in the dark.

Figure 30. Morphological features of THP-1 cells undergoing apoptosis. THP-1 cells were exposed to 100 nM calphostin C for 2 h in the absence (A,C) or presence (B,D) of light as described in materials and methods. Cells blebbing are observed in B but not in A when using a light microscope. Apoptosis cells were fixed and examined by transmission microscopy. Nuclear condensation was observed in D, but not in C.

A

B

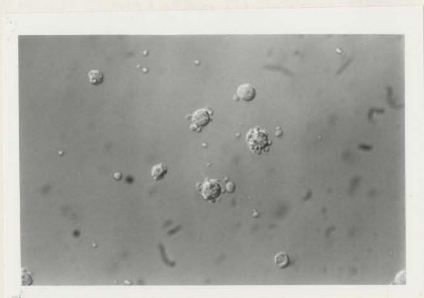
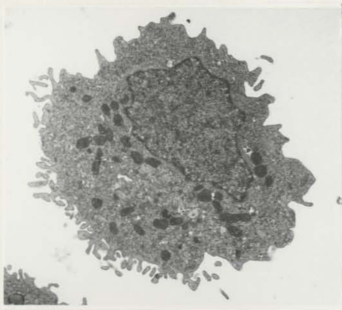


Figure 30

C



D

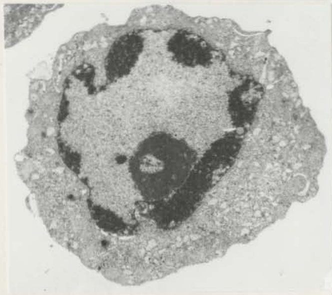


Figure 30

4.5.2 Dose and time dependent effect of calphostin C-mediated THP-1 apoptosis

Recently, flow cytometric analysis has been widely used in quantifying apoptosis. Normally, the histogram of DNA fluorescence in cells resembles that shown in Fig.31, in which A and B peaks represent cells in G_0/G_1 phase and in G_2/M phase, respectively, the plateau area between two peaks represents cells in S phase (Shapiro, 1988). Cells undergoing apopotosis will show as a discrete peak (A_0) in the area marked M_1 on Figure 31 displaying reduced fluorescence with respect to the G_0/G_1 (peak A on Fig.31) (Telford *et al.*, 1992).

By using flow cytometric analysis of propidium iodide-stained THP-1 cells, it was found that calphostin C mediated apoptosis in a dose-dependent manner over the range from 35 nM to 105 nM. Results of one representative experiment quantifying cell DNA content using the propidium iodide staining technique and flow cytometric analysis, is shown in Fig.32. The percentage of apoptosis recorded in the A_0, or apoptosis peak, was observed to increase with increasing calphostin C concentration from 34% to 51%. Figure 33 shows that calphostin C mediated apoptosis increased with the length of time (2-6 h) in the presence of ordinary fluorescent light. The percentage of cells undergoing apoptosis increased up to 28%. Figure 33 also shows that THP-1 cells were more subject to undergo apoptosis in serum free medium, where a 4 h incubation of cells with calphostin C lead to 34% apoptosis.

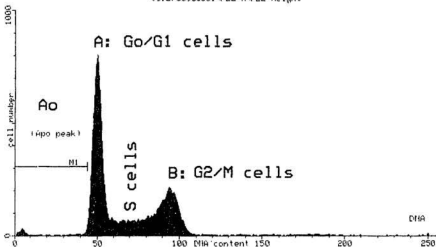


Figure 31. The histogram of DNA fluorescence in THP-1 cells. Under normal circumstances, all non-replicating normal diploid cells in the G_0 and G_1 phases of the cell cycle in the same eukaryotic organism have the same DNA content as shown in peak A (this quantity is sometimes expressed as 2C). DNA synthesis during S phase of the cell cycle results in an increase in cellular DNA content as shown in the plateau area, which reaches 4C at the end of S phase and remains at this value during the G_2 phase and during mitosis (M) as shown in peak B. Apoptotic cells are found in the A_0 region having less than 2C DNA content as shown in M_1 area.

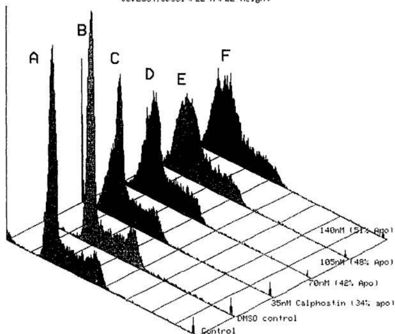


Figure 32. Dose-dependence of calphostin C-mediated THP-1 cell apoptosis. THP-1 cells were exposed to different concentrations of calphostin C ranging from 35 nM (C), 70 nM (D), 105 nM (E) to 140 nM (F) or DMSO solvent control (B) or media only (A), for 3 hours. Then THP-1 cells were fixed with 70% ethanol, stained with propidium iodide, and analyzed by FACS. Apoptosis increased with the calphostin C concentration as shown in the histograms.

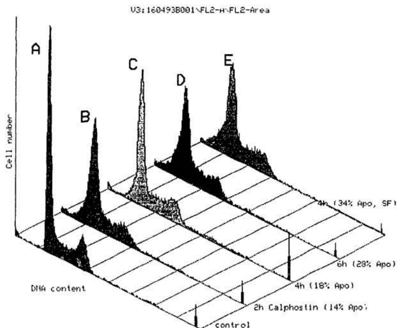


Figure 33. Time-dependence of THP-1 cell apoptosis induced by calphostin C. THP-1 cells were exposed to 10 nM calphostin C in the presence of ordinary fluorescent light for different periods of time as indicated in the histograms, then fixed by 75% ethanol and stained with PI as described in materials and methods, and finally analyzed by FACS. Apoptosis increased over the time period, 14% apoptosis for 2 h (B), 18% apoptosis for 4 h (C) and 28% apoptosis for 6 h (D). Histogram E shows that apoptosis also occurred in serum-free medium with calphostin treatment.

4.5.3 Detection of DNA cytoplasmic fragments by agarose gel electrophoresis in calphostin C treated THP-1

Another critical event in apoptosis is the activation of Ca^{2+}-dependent endonucleases which cleave DNA into multiples of 180 to 200 bp internucleosomal DNA fragments. Figure 34 shows this typical ladder-like pattern of DNA fragments, extracted from the cytoplasmic fraction, in the light-activated calphostin C-treated THP-1 cells (lane 4 and 5), and this was not observed when cells were kept in the dark (lane 6) or in untreated THP-1 cells (lane 2) and DMSO treated THP-1 cells (lane 3).

Figure 34. Oligonucleosome-sized DNA fragmentation following calphostin C treatment. THP-1 cells were incubated with medium alone (lane 2) or with solvent control (DMSO) (line 3), or with 50 nM (lane 5) or 100 nM (lane 4) calphostin C in the presence of light and 100 nM calphostin C (lane 6) in the dark. Cytoplasmic DNA was extracted and subjected to 10% agarose gel electrophoresis as described in materials and methods. 123 bps ladder sizes of molecular weight markers are applied in lane 1. DNA fragments were observed only in calphostin C and light treated cells (lines 4 and 5).

4.6 Induction of apoptosis by calphostin C in PBM

In view of the fact that THP-1 is a transformed cell line, it was decided to also look at PBM. In the literature, there have been no reports on the effects of calphostin C on normal PBM. Our studies found that calphostin C can effectively trigger normal human PBM to undergo apoptosis. Isolated normal human PBM, treated with calphostin C in the presence of light (33 watts) for 2 hours, underwent apoptosis. Because normal human PBM are contaminated with platelets, it is more difficult to observe PBM morphological changes by light microscopy. Consequently, the DNA content of calphostin C-treated cells were evaluated by FACS analysis following staining with propidium iodide. PBM treated with 50 nM or 100 nM calphostin C showed significantly less 2C DNA content than that of control PMB (Figure 35). After four similar experiments, it was confirmed that PBM were more sensitive to calphostin C induced apoptosis than THP-1 cells. The DNA content of these cell as measured by FACS, showed that treatment with 100 nM calphostin C induced 78% apoptosis compared to 48% apoptosis in THP-1 cells in the same period of time and under the same experimental conditions (Figure 36). The characteristic DNA fragment ladder was also found in calphostin C-treated PBM cells as shown in Fig.38, lane 8 after agarose gel separation.

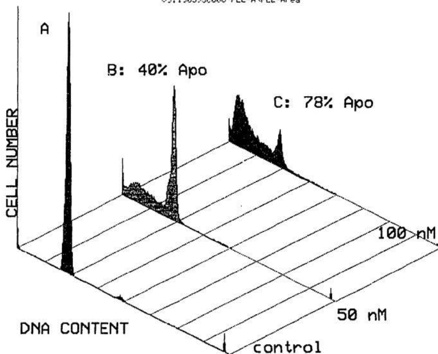


Figure 35. DNA content of normal and apoptotic PBM. PBMs were treated with 50 nM (B) or 100 nM calphostin C (C) or medium only (A) in the presence of ordinary fluorescent light for 2 hours in 5%CO_2 at 37°C, then fixed with 70% ethanol and stained with PI overnight as described in materials and methods. DNA frequency histograms analyzed by FACS show 48% apoptosis for 50 nM calphostin C treatment (B), and 78% apoptosis for 100 nM calphostin C treatment (C).

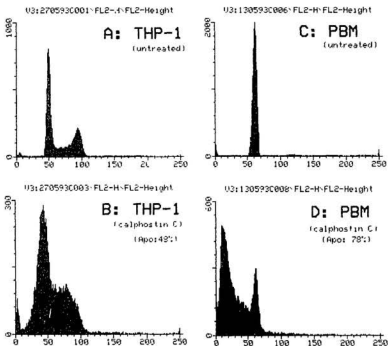


Figure 36. Different sensitivity of THP-1 cells and PBMs to calphostin C. THP-1 cells and PBMs were incubated with or without calphostin C for 3 hours in the presence of light. After 3 hours, cells were fixed and stained with PI, then analyzed by FACS. PBMs showed 78% apoptosis (D). THP-1 cells showed only 48% apoptosis (B).

4.7 Effect of PMA, 4-aminopyridine and quinidine on calphostin C-induced THP-1 apoptosis

THP-1 cells were pre-treated with PMA, a PKC activator, quinidine, a Ca^{2+}-dependent K^+ channel blocker or 4-aminopyridine (4-AP), a classical K^+ channel blocker, respectively for 1 hour, prior to 50 nM calphostin C treatment for 3 hours. The results from one representative set of 3 independent experiments are shown in Fig. 37. Flow cytometric analyses (Fig.37) showed 16% apoptosis, 17% apoptosis, and 28% apoptosis in PMA, quinidine and 4-AP pre-treated cells respectively versus 61% apoptosis in THP-1 cells treated with calphostin C alone. These results indicate that PMA and quinidine and to a lesser extent 4-AP could significantly delay calphostin C-induced apoptosis in THP-1 cells. Cytoplasmic DNA from treated cells was extracted and then subjected to 10% agarose gel electrophoresis. After electrophoretic separation, the ethidium bromide stained DNA gel was photographed using UV illumination. Fig.38 shows that the degree of DNA fragmentation correlates with percentage induction of apoptosis from the FACS analysis in Fig. 37.

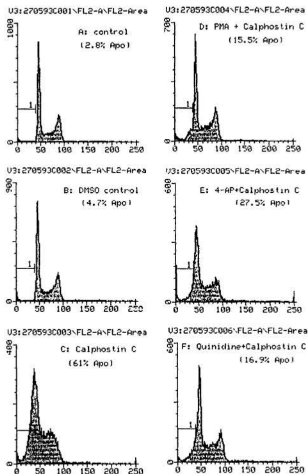


Figure 37. DNA histograms of THP-1 cells treated with the indicated reagents pre-treatment, followed by 50 nM calphostin C treatment for 3 h in the presence of light. Percentage of apoptosis is 2.9% for untreated THP-1 (A); 4.7% for solvent (DMSO) control treatment (B); 61% for 50 nM 3 h calphostin C treatment in the presence of light (C); 15.6% for 1 h pre-treatment with 50 nM PMA (D), and 27% for 1 h pre-treated with 10 nM 4-AP (E); 17% for 1 h pre-treated with 0.5 mM quinidine.

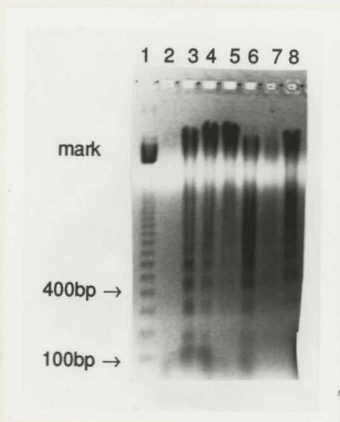


Figure 38. DNA fragmentation in THP-1 and PBM: THP-1 cells with no pretreatment (lane 3) or with 1 h pretreatment with 100 ng/ml PMA (lane 4); with 1 mM quinidine (lane 5) for 1 h, followed by 3 h 50 nM calphostin C treatment; untreated THP-1 cells (lane 2). PBMs were treated with 50 nM calphostin C (lane 8) or without calphostin C (lane 7) for 3 h in the presence of light. After 3 h, cytoplasmic DNA was extracted and subjected to 10% agarose gel electrophoresis as described in material and methods. 100 bps ladder sizes of molecular weight markers are applied in lane 1. The degree of DNA fragmentation in lane 5 with 1 h pre-quinidine treatment following 3 h calphostin C incubation is significantly less than in lane 3 with calphostin C treatment only.

4.8 Discussion:

Calphostin C is a specific inhibitor of PKC. It interacts with the regulatory domain of the PKC and inhibits phorbol dibutyrate binding to PKC (Pelosin *et al.*, 1984). The present findings demonstrate that exposing THP-1 cells or PBM to calphostin C was sufficient to induce DNA fragmentation, which is characteristic of apoptosis and cell death. The data also confirm that the inhibition of PKC activity by calphostin C, in intact cells, is light-dependent (Bruns *et al.*, 1991; Kobayashi *et al.*, 1989). The pre-treatment of THP-1 cells with PMA resulted in significant inhibition of calphostin C-mediated apoptosis. Staurosporine, however, failed to induce apoptosis in THP-1 cells and human PBMs. Staurosporine not only inhibits PKC, but also inhibits PKA, CaM-Kinase II (Ruegg *et al.*, 1989). It is reasonable to assume that either different isotypes of PKC are affected or that the non-specific inhibitory activity of staurosporine may account for this finding. In the literature, there is no previously reported evidence for PKC inhibitor-mediated PBM apoptosis. The data also demonstrate that normal PBM are more sensitive to induction of apoptosis by calphostin C than THP-1 cells under the same experimental conditions. These results imply that there is a different PKC requirement for the survival of leukaemia and normal cells.

PKC has been shown to activate the Na^+/H^+ antiporter (Rosoff *et al.*, 1984). Although there is no direct evidence showing that PKC can modulate K^+ ion channel function through phosphorylation, it has been reported that different ion

channels could be phosphorylated by different kinases in several cell systems (Levitan, 1985). Our studies demonstrated that pre-treatment of THP-1 cells with quinidine, a Ca^{2+}dependent K^+ channel blocker, significantly inhibited calphostin C-mediated apoptosis in THP-1 cells, indicating that the Ca^{2+}-dependent K^+ channels are involved in the regulation of apoptosis induced by calphostin C.

In summary, the results suggest that PKC activity is required for the suppression of apoptosis in THP-1 cells and PBM and that K^+ channels are also involved in the regulation of PKC inhibitor-mediated apoptosis.

References

Adams,D.O. (1992) LPS-initiated signal transduction pathways. In: Morrison D.C., Ryan,J.L. eds. Macrophages. Bacterial endotoxic lipopolysaccharides. New York: CRC Press, pp.285-310

Afanasyev,V.N., Korol,B.A., Matylevich,N.P., Pechatnikov,V.A. and Umansky,S.R. (1993) The use of flow cytometry for the investigation of cell death. *Cytometry* 14:603-609

Akira,S., Hirano,T., Taga,T. and Kishimoto,T. (1990) Biology of multifunctional cytokines:IL-6 and related molecules (IL-1 and TNF). *FASEB* J.4:2860

Alison,M.R. and Sarraf,C.E. (1992) Apoptosis: a gene-directed programme of cell death. *J Royal College Physicians London* 26:25-35

Arends,J.J., Morris,R.G. and Wyllie,A.H. (1990) Apoptosis: the role of the endonuclease. *Am.J.Path* 136:593-608

Bagasra,O., Wright,S.D., Seshamma,T., and Oakes,J.W. (1992) CD14 is involved in the control of human immunodeficiency virus type 1 expression in latently infected cells by lipopolysaccharide. *Proc.Natl.Acad.Sci.* USA 89:6285-6289

Bamezai,A., Goldmacher,V.S. and Rock,K.L. (1992) Internaliztion of glycosyl-phosphatidylinositol (GPI)-anchored lymphocyte proteins II. GPI-anchored and transmembrane molecules internalize through distinct pathway. *Eur.J.Immunol.* 22:15-21

Bazil,V., Horejsi,V., Baudys,M., Kristofova,H., Strominger,J.L., Kostka,W. and Hilgert,I. (1986) Biochemical characterization of a soluble form of the 53-kDa monocyte surface antigen. *Eur.J.Immunol.* 16:1583-1589

Bazil,V., Baudys,M.,Hilgert,I., Stefanova,I , Low,M.G., Zbrozek,J. and Horejsl.V. (1989) Structural relationship between the soluble and membrane-bound forms of human monocyte surface glycoprotein CD14. *Mol.Immunol.* 26:657-662

Bazil,V., and Strominger,J.L. (1991) Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. *J.Immunol.* 147:1567-1574

Beekhuizen,H.I., Bockland,A.J., Corsel-van Tilburg,F.K. and Furth van, R. (1991) CD14 contributes to the adherence of human monobytes to cytokines-

stimulated endothelial cells. *J.Immunol.* 147:3761

Beekhuizen,H., Blokland,I., Tilburg,A.J.C. (1991) CD14 contributes to the adherence of human monocytes to cytokine-stimulated endothelial cells. *J.Immunol.* 147:3761-3767

Boe,R., Gjertsen,B.T., Vintermays,O.K., Houge,G., Lanotte,M. and Doskeland,S.O. (1991) The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cell. *Exp. Cell Res.* 195:237-246

Bone,R.C.(1991) The pathogenesis of sepsis. *Ann.Int.Med.* 115:457-469

Brach,M.A., Herrmann,F. and Kufe,D.W. (1992) Activation of the AP-1 transcription factor by arabinofuranosylcytosine in myeloid leukemia cell. *Blood* 79:728-734

Braun-Breton,C., Rosenberry,T.L., Da Silva,L.P. (1988) Induction of the proteolytic activity of a membrane protein in Plasmodium falciparum by phosphatidylinositol-specific phospholipase C. *Nature* 332:456-459

Bruns,R.F., Miller,D., Merriman,R.L., Howbert,J.J., Heath,W.F., Takahashi,I., Tamaoki,T. and Nakano,H. (1991) Inhibition of protein kinase C by calphostin C is light-dependent. *Biochem. Biophy. Res. Commun.* 176:288-293

Calvo,F., Martin,P.M., Jabrane,N. (1987) Human beast cancer cells share antigens with myeloid monocytes lineage. *Br.J.Cancer.* 56:15-19

Castagna,M., Takai,Y., Kaibuchi,K., Sano,K., Kikkawa,U., and Nishizuka,Y. (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J.Biol.Chem.* 257:787-7851

Chung,J., Chen,R.H., Blenis,J. (1991) Coordinate regulation of pp90^{rsk} and a distinct protein-serine/threonine kinase activity that phosphorylates recombinant pp90^{rsk} *in vitro. Mol.Cell Biol.* 11:1868-1874

Cichowski,K., McCormick,F. and Brugge,J.S. (1992) p21^{ras} GAP association with Fyn,Lyn and Yes in thrombin activated platelets. *J.Biol.Chem.* 267:5025-5028

Coffey,R.G., Weakland,L.L. and Alberts,V.A. (1992) Paradoxic stimulation and inhibition by protein kinase C modulating agents of lipopolysaccharide evoked

production of tumour necrosis factor in human monocytes. *Immunology* 76:48-54

Cohen,J.J. and Duke,R.C. (1984) Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cells death. *J.Immunol* 132:38-43

Collart,M.A., Baruerle,P., Vassali,P. (1990) Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kB-like motifs and of constitutive and inducible forms of NF-kB. *Mol.Cell Biol.* 10:1498-1506

Conzelmann,A., Riezman,H., Desponds,C. (1988) A major 125-kd membrane glycoprotein of Saccharomyces cerevisiae is attached to the lipid bilayer through an inositol-containing phospholipid. *EMBO J.* 7:2233-2240

Couturier,C., Jahns,G., Kazatchkine,M.D. and Hawffner-Cavaillon,N. (1992) Membrane molecules which trigger the production of interleukin-1 and tumor necrosis factor-α by lipopolysaccharide-stimulated human monocytes. *Eur.J.Immunol.* 22:1461-1466

Couturier,C.N., Haeffner-Cavaillon,M.C. and Kazatchkine,M.D. (1991) Binding sites for endotoxin (lipopolysaccharides) on human monocytes. *J.Immunol.* 14:1899

Craven,P.A. and DeRubertis (1988) Role of activation of protein kinase C in the stimulation of colonic epithelial proliferation by unsaturated fatty acids. *Gastroenterology.* 95:676-685

Cross,G.A.M. (1990) Glycolipid anchoring of plasma membrane proteins. *Ann.Rev.Cell.Biol.* 6:1-39

Debets,J.M.H., Kampmeriger,R., Van der Linden,M.P.M.H., Buurman,W.A. and Vander Linden. (1989) Plasma tumor necrosis factor and mortality in critically ill septic patients. *Crit.Care Med.*17:489

DeCoursey,T.E., Chandy,K.G., Gupta,S and Cahalan,M.D. (1984) Voltage-gated K^+ channels in human T lymphocytes: A role in mitogenesis? *Nature* 307:465

Deenen,G.J., Van Balen,I. and Opstelten,D (1990) In rat B lymphocyte genesis sixty percent is lost from the bone marrow at the transition of nondividing pre-B cells to sIgM$^+$ B lymphocyte the stage of Ig light chain gene expression. *Eur.J.Immunol* 20:557-564

Dentener,M.A., Bazil,V., Von Asmuth,E.J.U., Ceska,M. and Buurman,W.A. (1993) Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-α, IL-6 and IL-8 release by human monocytes and alveolar macrophages. *J.Immunol.* 150:2885-2891

Deutsch,C., Krause,D. and Lee,S.C. (1986) Voltage-gated potassium conductance in human T-lymphocyte stimulated with phorbol esters. *J.Physiol.* 372:405

Draberova,L. and Draber,P. (1993) Thy-1 glycoprotein and src-like protein-tyrosine kinase p553/p56^{lyn} are associated in large detergent-resistant complexes in rat basophilic leukemia cells. *Proc.Natl.Acad.Sci.* USA 90:3611-3615

Drouet,C., Shakhove,A.N., Jongeneel,C.V. (1991) Enhancers and transcription factors controlling the inducibility of the tumor necrosis factor alpha promoter in primay macrophages. *J.Immunol.* 147:1694-1700

Dustin,M.L., Selvaraj,P., Mattaliano,R.J., Springer,T.A. (1987) Anchoring mechanisms for LFA-3 cell adhesion glycoprotein at membrane surface. *Nature* 329:846-848

Duvall,E., and Wyllie,A.H. (1986) Death and the cell. *Immunol.Today* 7:115-119
Farago,A and Nishizuka,Y (1190) Protein kinase C in transmembrane signalling. *FEBS* 268:350-354

Faustino Mollinedo,Rosa Martinez-Dalmau and Manuel Modolell. (1993) Early and selective induction of apoptosis in human leukaemic cells by the alkyl-lysophospholipid et-18-OCH$_3$. *Biochm.Biophy.Res.Commun. 192:603*

Eiseman,E. and Bolen,J.D. (1992) Engagement of the high-affinity IgE receptor activates *src* protein-related tyrosine kinase. *Nature* 355:78-80

Farber,J.L., Chien,K.R. and Mittnatcht,S. (1981) The pathogenesis of irreversible cell injury in ischemia. *Am.J.Pathol.* 102:271

Fateml,S.H., Haas,R., Jentoft,N., Rosenberry,T.L. and Tartakoff,A.M. (1987) The glucophospholipid anchor of Thy-1. Biosynthetic labeling experiments with wild-type and class E Thy-1 negative lymphomas. *J.Biol.Chem.* 262:4728

Ferguson,M.A.J. and Cross,G.A.M. (1984) Myristylation of the membrane form of a *Typanosoma Brucei Variant Surface Glycoprotein. J.Biol.Chem.* 259:3011-15

Ferguson,M.A.J., Haldar,K. and Cross,G.A.M. (1985) *Typanosoma Brucei Variant Surface Glycoprotein* has a *sn*-1,2-dimyristyl glycerol membrane anchor at its CooH terminus. *J.Biol.Chem.* 260:4963-68

Ferguson,M.A.J. (1988) Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. *Ann.Rev.Biochem.* 57:285-320

Ferluga,J.O. and Allison,A.C. (1974) Observations on the mechanism by which T-lymphocytes exert cytotoxic effects. *Nature.* 250:673

Ferrero,E., Hsieh,C.L., Francke,U. and Goyert,S.M. (1990) CD14 is a member of the family of leucine-rich proteins and is encoded by a gene synthetic with multiple receptor genes. *J.Immunol.* 145:331-336

Ferrero,E., Jiao,D., Tsuberi,B.Z., Tesio,L., Rong,G.W., Haziot,A. and Goyert,S.M. (1993) Transgenic mice expressing human CD14 are hypersensitive to lipopolysaccharide. *Proc. Natl.Acad.Sci.*USA 90:2380-3494

Fesus,L. (1991) Apoptosis fashions T and B cell repertoire. *Immunol.Lett* 30:277-282

Forbes,I.J., Zalewski,P.D., Giannakis,C., Cowled,P.A. (1992) Induction of apoptosis in chronic lymphocytic leukaemia cells and its prevention by phorbol ester. *Exp. Cel. Res.* 198:367-372

Freeman,M., Ashkenas,J., Rees,D.J.G., Kingsley,D.M., Copeland,N.G., Jemkins,N.A., and Krieger,M. (1990) An ancient ,highly conserved family of cysteine rich protein domains revealed by cloning of type I and type II murine macrophage scavenger receptors. *Proc.Natl.Acad.Sci.* USA 87:8810-8814

Frey,E.A., Miller,D.S., Jahr,T.G., Sundan,A., Bazil,V., Espevik,T., Finlay,B.B. and Wright,S.D. (1992) Soluble CD14 participates in the response of cells to lipopolysaccharide. *J.Exp.Med.* 176:1665-1671

Fukushima,Y., Hagiwara,S. and Henkart,M. (1984) Potassium current in clonal cytotoxic T lymphocytes from the mouse. *Nature* 250:673

Futerman,A.H., Low,M.G., Ackerman,K.E., Sherman,W.R., Silman,I. (1985) Identification of covalently bound inositol in the hydrophobic membrane-anchoring domain of *Torpedo* acetylcholinesterase. *Biochem.Biophys.Res.Commun.* 129:312-317

Gallin,E.K. (1986) Ionic channels in leukocytes. J.Leuk.Biol. 39:241

Gally,P., Jongeneel,C.V., Barras,C., Bunnler,M., Bumgartner,J-D., Glauser,M.G. and Heumann,D. (1993) Short time exposure to lipopolysaccharide is sufficient to activate human monocytes. *J.Immumol.*150:5086-5093

Geng,Y., Zhang,B.P. and Lotz,M. (1993) Protein tyrosine kinase activation is required for lipopolysaccharide induction of cytokines in human blood monocytes. *J.Immunol.* 151:6692-6700

Gopalakrishna,R.,Chen,Z.H.,Gundimeda,U.(1992) Irreversible oxidative inactivation of protein kinase C by photosensitive inhibitor calphostin C. *FEBS* 314:2,149-154.

Griffin,J.S., Mayer,R.J., Weinstein,H.J. (1983) Surface maker analysis of acute myeloblastic leukemia: indentification of differentiation-associated phenotypes. *Blood* 62:557-563

Goyert.,S.M., Ferrero,E.M., Seremetis,S.V., Winchester,R.J., Silver,J. and Mattison,A.C. (1986) Biochemistry and expression of myelomonocytic antigens. *J.Immunol.* 137:3909-3914

Goyert.,S.M., Ferrero,E.M. (1987) Biochemical analysis of myeloid antigens and cDNA expression of gp55(CDw14), *In Leucocyte Typing III* McMichael,A., Beverley,P.C.L., Cobbold et al., eds Springeer-Verlag, New York, p.613

Goyert,S.M., Ferrero,E., Rettig,J.W., Yenamandra,A.K., Obata,F., Le Beau,M.M. (1988) The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptor. *Science* 239:497

Griffin,J.D., and Schlossman,S.F. (1984) Expression of myeloid differentiation antigens in acute myeloblastic leukaemia. *In Leucocyte Typing*, Dermard,A., Boumsell,L., Dausset,J., Milstein,C., and Schlossman, eds. Springer-Verlag, New York, p. 404

Halling,J.L., Hamill,D.R., Lei,M.G. and Morrison,D.C. (1992) Indentification and characterization of lipopolysaccharide-binding proteins on human peripheral blood cell populations. *Infection and Immunity.* 60:845-852

Hamilton,T.A., Ohmori,Y., Tebo,J.M., Narumi,S. and Tannenbaum,C.S. (1992) Transmembrane and Intracellular signaling events in lipopolysaccharide-stimulate

macrophages. In: *Bacterial endotoxic lipopolysaccharides. Vol.1 Molecular Biochemistry and Cellular Biology.* (eds by Morrison and Ryan) *p* 83-96

Hampton,R.Y., Golenbock,D.T., Penman,M., Krieger,M., and Raetz,C.R.H. (1991) Recognition and plasma clearance of endotoxin by scavenger receptors. *Nature (London)* 352:342-344

Hance,A.J., Douches,S., Winchester,R.J., Ferrans,V.J. and Crystal,R.G. Characterization of mononuclear pagocyte subpopulations in the human lung by using monoclonal antibodies changes in alveolar macrophage phenotype associated with pulmonary sarcoidosis. (1985) *J.Immunol.*134:284-292

Hara,K.S., Amano,F., Nishijima,M., Akamatsu,Y. (1990) Isolation of a lipopolysaccharide (LPS)-resistant mutant, with defective LPS binding, of cultured macrophage-like cells. *J.Biol.Chem.* 265:6606-10

Hatakeyama,M., Tsudo,M., Minamoto,S., Kono,T., Doi,T., Miyata,T., Miyssaka,M., and Taniguchi,T. (1989) Interleukin-2 receptor ß chain gene: generation of three receptor forms by cloned human α and ß chain cDNA's. *Science* 244:551

Haystead,T.A., Sim,A.T., Carling,D., Hornor,R.C., Tsukitani,Y., Cohen,P., Hardie,D.G. (1989) Effects of the tumor pomotor okadaic acid on intracelluar protein phosphorylation and metabolism. *Nature.* 337:78-81

Haziot,A., Chen,S., Ferrero,E., Low,M.G., Silber,R. and Goyert,S.M. (1988) The monocytes differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. *J.Immunol.* 141:547-552

Haziot,A. and Goyert,S.M. (1989) Biosynthesis of CD14. *Blood* 74s:335a (Abstr.)

Haziot,A., Rong,G.W., Tsuberi,B. and Goyert,S.M. (1992) Soluble CD14 inhibits the activation of monocytes induced by LPS. *J.Cell.Biochem.Suppl.*16C:163 (*Abstr.*)

Haziot,A., Rong,G.W., Silver,J. and Goyert,S.M. (1993) Recombinant soluble CD14 mediates the activation of endothelial cells by lipopolysaccharide. *J.Immunol.*151:1500-1507

Hefta,S.A., Hefta,L.J., Lee,T.D., Oanzton,R.J., Shively,J.E. (1988) Carcinoembryonic antigen is anchored to membranes by covalent attachment to

a glycosylphosphatidylinositol moiety: identification of the ethanolamine linkage site. *Proc.Natl.Acad.Sci.*USA 85:4648-4652

Henney,C.S. (1973) On the mechanism of T-cell mediated cytolysis. *Transplant Rev.* 17:37

Heumann,D.P., Gallay,P., Barras,C., Zaech,P., Ulevitch,R.J., Tobias,P.S., Glauser,M. and Baumgartner,J.D. (1992) Control of lipopolysaccharide (LPS) binding and LPS-induced tumor necrosis factor secretion in human peripheral blood monocytes. *Eur.J.Immumol.*22:1461

Hibi,M., Murakami,M.,J., Saito,M., Jirano,T., Taga,T., and Kishimoto,T. (1990) Molecular cloning and expression of an IL-6 signal transducer, gp130. *Cell (Cambridge, Mass)* 63:1149

Hirohata,S., and Oka,H. (1993) Modulation of T cell production of interferon-γ by human monocytes: effect of engagement of CD14 on monocytes. *Cellular Immunol.* 151:270-282

Hogg,N. and Horton,M.A. (1987) Myeloid antigens: new and previously defined clusters. *In Lerkocyte Typing* III *White Cell Differentiation Antigens* (Edited by MaMichael,A.) p. 576. Oxford University Press, Oxford.

Huber,A.R., Kunkel,S.L., Tudd,R.F. and Weiss,S.J. (1991) Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science* 254:99

Huizinga,T.W.J., van der Schoot,C.E., Jost,C., Klassen,R. Kleijer,A.E.G., von dem Borne,A.E.G.Kr., Roos,D. and Tetteroo,P.A.T. (1988) The PI-linked receptor FcR III is released on stimulation of neutrophils. *Nature* 333:667

Huizinga,T.W., de Haas,M., Kleijer,M., Nuijens,J.H., Roos,D. and von de, Borne,A.E.G.Kr. (1990) Soluble Fcγ receptor III in human plasma originates from release by neutrophils. *J.Clin.Invest.* 86:416

Jaraman,Y. and Hogg,N. (1989) Expression of CD14 molecules by human neutrils. *In 4th Int. Conf.Human Leucocyte Differentiation Antigens* (Edited by Knapp W.) Abstract. *Tissue Antigens* 33:199

Kang,Y.H., Dwivedi,R.S., and Lee,C.H. (1990) Ultrastructural an immunocytochemical study of the uptake and distribution of bacterial lipopolysaccharide in human monocytes. *J.Leuk.Biol.* 48:316

Kanter.P., Leister,K.J. Tomel,L.D., Wenner,C.E. (1984) Epidermal growth factor and tumour promoters prevent DNA fragmentation by different mechanisms. *Biochem.Biophy.Res.Commun.* 118:392-399.

Kerr,J.F.R., Wyllie,A.H., Currie,A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br.J.Cancer.* 26:239-257

Kerr,J.F.R. and Searle,J. (1972) A suggested explanation for the paradoxically slow growth rate of basal cell carcinomas that contain numerous mitotic figures. *J.Pathol.* 107:41-44

Kerr,J.F., Searle,J., Harmon,B.V. and Bishop,C.J. (1987) Apoptosis. In perspectives on Mammalian cell death (Potten,C.S., ed) pp.93-128, *Oxford university press, Oxford, England*

Koren,H.S. (1983) Macrophage-mediated antibody-dependent cellular cytotoxicity. Immunology series. Vol.21 Marcel Dekker, INC press

Kobaysashi,E., Nakano,H., Morimoto,M. and Tamaoki,T. (1989) Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochm.Biophy.Res.Commun* 159:548-553

Kobayashi,N., Kono,T., Hatakeyama,M., Minami,Y., Miyazaki,T., Perlmutter,R.M. and Taniguchi,T. (1993) Function coupling of the *src*-family protein tyrosine kinase $p59^{fyn}$ and $p53/56^{lyn}$ with the interleukin 2 receptor: Implications for redundance and pleiotropism in cytokine signal transduction. *Proc.Natl.Acad.Sci.* USA 90:4201-4205

Kozo,Y., Becker,E.L. and Sha'afi,R.I. (1992) Lipopolysaccharide and serum cause the translocation of G-protein to the membrane and prime neutrophils via CD14. *Biochm.Biophy.Res.Commun.* 183:1280-1286

Kitchens,R.L., Ulevitch,R.J. and Munford,R.S. (1992) Lipopolydaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway. *J.Exp.Med.* 176:485-494

Krüger,C., Schütt,C., Obertacke,U., Joka,T., Müller,F.E., Knöller,J., Köller,M. (1991) Serum CD14 levels in polytraumatized and severely burned patients. Clin. *Exp.Immunol.*85:297-301

Labeta,M.O., Lanndmann,R., Obrecht,J.P. and Obrist,R. (1991) Human B cells express membrane-bound and soluble forms of the CD14 myeloid antigen. *Mol.Immunol.* 28:115-122

Labeta,M.O., Durieux,J-J, Fernandez,N., Herrmann,R. and Ferrara,P (1993) Release from a human monocyte-like cell line of two different soluble forms of the lipopolysaccharide receptor, CD14. *Eur.J.Immunol.* 23:2144-2151

Landmann,R., Fisscher,A.E. and Obrecht,J.P. (1992) Interferon-γ and interleukin-4 down-regulate soluble CD14 release in human monocytes and macrophages. *J.Leuk.Biol.* 52:323-330

Lauener,R.P., Geha,R.S., and Vercelli,D. (1990) Engagement of the monocyte surface antigen CD14 induces lymphocyte function-associated antigen-1/intercellular adhesion molecule-1-dependent homotypic adhesion. *J.Immunol.* 145:1390-1394

Lee,J.D., Kato,K., Tobias,P.S., Kirland,T.N. and Ulevitch,R.J. (1992) Transfection of CD14 into 70/3 cells dramatically enhances the sensitivity to complexes of lipopolysaccharide (LPS) and LPS binding protein. *J.Exp.Med.* 175:1697-1705

Lei,M.L., Morrison,D.C. (1988) Specific endotoxic lipopolysaccharide-binding proteins on murine splenocytes. I. Detection of lipopolysaccharide-binding sites on splenocytes and splenocyte subpopulations. *J.Immunol.* 141:996-1005

Le Bel,D., Beattie,H. (1988) The major protein of pancreatic zymogen granule membranes (GP-2) is anchored via covalent binds to phosphatidylinositol. *Biochem.Biophys.Res.Commun.* 154:818-823

Leca,H. (1985) Gated ionic channels and the mechanism of excitability. *Fed.Proc.* 44:2941

Lei,M.,G., Chen,T.Y., and Morrison,D.C. (1990) Lipopolysaccharide/lipid A receptors on lymphocytes and macrophages. *Intern.Rev.Immunol.* 6:223-235

Levitan,I.B. (1985) Phosphorylation of ion channels. *J.Memb.Biol.* 87:177

Liepins,A., Faanes,R.B., Choi,Y.S. and de Harven,E. (1978) A lymphocyte mediated lysis of tumor cells in the presence of alloantiserum. *Cell Immunol.* 36:331

Liepins,A. and Younghusband,H.B. (1985) Low temperature induced cell surface membrane vesicle shedding is associated with DNA fragmentation. *Exp. Cell Res.* 161:525

Liepins,A. and Younghusband,H.B. (1987) A possible role for K⁺ channels in tumor cell injury. Membrane vesicle shedding and nuclear DNA fragmentation. *Exp. Cell Res.* 169:385

Low,M.G., and Finean,J.B. (1977) Non-lytic release of acetylcholinesterase from erythrocytes by a phosphatidylinositol-specific phospholipase C. *FEBS Lett.* 82:143-

Low,M.G., and Saltiel,A.R. (1988) Structural and functional roles of glycosyl-phosphatidylinositol in membranes. *Science* 239:268-275

Low,M.G. (1989) Glycosyl-phosphatidylinositol:a versatile anchor for cell surface proteins. *FASEB* 3:1600-1608

Lue,K-H., Lauener,R.P., Winchester,R.J., Geha,R.S. (1991) Engagement of CD14 on human monocytes terminates T cell proliferation by delivering a negative signal to T cells. *J.Immunol.* 147:1134-1138

Maliszewski,C.R., Currier,j., Fisher,J., Smith,R.J. (1985) Monoclonal antibodies that bind to the My23 human myeloid cell surface molecule: Epitope analysis and antigen modulation studies. *Mol.Immunol.* 24:17

Maliszewski,C.R., Ball,E.D., Graziano,R.F., Fanger,M.W. (1985) Isolation and characterization of MY23, a myeloid cell-derived antigen reactive with the monoclonal antibody AML-2-23. *J.Immunol.* 135:1929-1936

Maliszewski,C.R., Currier,j., Fisher,J., Smith,R.J. (1987) Monoclonal antibodies that bind to the My23 human myeloid cell surface molecule:epitope analysis and antigen modulation studies. *Mol.Immunol.*,24:17-25

Manthey,C.L., Vogel,S.N. (1992) The role of cytokines in host responses to endotoxin. Revs Med Microbiol. *pp* 72-79

McCarthy,J.B., Wahl,S.M., Rees,J., Olsen,C.E., Sanndberg,A.L. and Wahl,L.M. (1980) Mediation of macrophage collagenase production by 3'-5' cyclic adenosine monophosphate. *J.Immunol.* 124:2405-2410

McConkey,D.J., Hartzell,P., Duddy,S.K., Hakansson,H., and Orrenius,S. (1988) 2,3,7,8-Tetrachlorodibenzo-p-dioxin kills immature thymocytes by Ca^{2+} -mediates endonuclease activation. *Science* 242:256-259 .

McConkey,D.J., Hartzell,P., Jondal,M., and Orrenius,S. (1989) Inhibition of DNA fragmentation in thymocytes and isolated thymocyte nuclei by agents that stimulate protein kinase C. *J. Biol.Chem.* 264:13399-13402.

McConkey,D.J., Hartzell,P. and Orrenius,S. (1990) Rapid turnover of endogenous endonuclease activity in thymocytes: effects of inhibitors of macromolecular synthesis. *Arch.Biochem.Biophys.* 278:284-287

McConkey,D.J., Orrenius,S, and Jondal,M. (1990) Cellular signalling in programmed cell death (apoptosis). *Immunol.Today* 11:120-121.

Meager,A., Leung,H. and Woolley,J. (1989) Assays for tumour necrosis factor and related cytokines. *J.Immunol.Methods* 116:1-17

Meldrum,E., Parker,P.J. and Carozzi,A. (1991) The PtdIns-PLC superfamily and signal transduction. *Bichimica et Biophysica Acta* 1092:49-71

Mercep,M., Noguchi,P.D., Ashwell,J.D. (1989) The cell cycle block and lysis of an activated T cell hybridoma are distinct processes with different Ca^{2+} requirements and sensitivity to cyclosporine A. *J Immunol* 142:4085-4092

Miller,S.A., Dykes,D.D., and Polesky,G.F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 16:1215.

Morrison,D.C., and Ryan,J.L. (1979) Bacterial endotoxins and host immune responses. *Adv Immunol.* 28:293-450

Morrison,D.C. (1985) Nonspecific interactions of bacterial lipopolysaccharides with membranes and membrane components. In: *Cellular Biology and Endotoxin, Berry,L.J.*(ed) Elsevier, New York, *pp* 25-55

Morrison,D.C., and Ryan,J.L. (1987) Endotoxins and disease mechanisms. *A.Rev.Med.* 38:417-432

Morrison,D.C., and Ryan,J.L. (1992) Bacterial endotoxic lipopolysaccharides. CRC Press. *Vol.1 Molecular Biochemistry and Cellular Biology.*

Moss,D.J., Burrows,S.R., Baxter,G.D., and Lavin,M.F. (1991) T cell-T cell killing is induced by specific epitopes: Evidence for an apoptotic mechanism. *J.Exp.Med.* 173:681-686

Müller,J.M., LömsZiegler-Heitbrock,H.W., and Baeuerle,P.A. (1993) Nuclear factor kappa B, a mediator of lipopolysaccharide effects. *Immunobiol.* 187:233-256

Murphy,P.M. and McDermottt,D. (1991) Functional expression of the human formyl peptide receptor in *Xenopus* oocytes requires a complementary human factor. *J.Biol.Chem.* 266:12560-12567

Nathan,C.F., Mercer-Smith,J.A., Desantis,N.M. and Palladino,M.A. (1982) Role of oxygen in T-cell mediated cytolysis. *J.Immunol.* 129:2164

Nathan,C.F. (1987) Secretory products of macrophages. *J.Clin.Invest.*79:319

Narumi,S., Tebo,J.M., Finke,J.H., Hamilton,T.A. (1992) IFN-γ and IL-2 cooperatively activate NF-kB in murine peritoneal macrophages. *J.Immunol.* 150:204-213

Nelipovich,P.A., Nilonova,L.V. and Umansky,S.R. (1988) Inhibition of poly (ADP-ribose) polymerase as a possible reason for activation of Ca^{2+} /Mg^{2+}-dependent endonuclease in thymocytes of irriadiated rats. *Int.J.Radiat.Biol.* 53:749-756

Nicoletti,I., Migliorati,G., Pagliacci,M.C., Grignani,F. and Riccardi,C. (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J.Immunol.Method* 139:271-279.

Nishizuka,Y. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* 334:661-665

Nourshargh,S., Perkins,S.J.A., Showell,H.J., Matsushima,K., Williams,T.J. and Collins,P.D. (1992) A comparative study of the neutrophil stimulatory activity in vitro and pro-inflammatory properties in vivo of 72 amino acid and 77 amino acid IL-8. *J.Immunol.* 148:106

Ohmori,y., Reynolds,E., Hamilton,T.A. (1991) Modulation of Na^{+}/K^{+} exchange potentiates LPS-induced gene experssion in murine peritoneal macrophages. *J.Cell Physiol.* 148:96-105

Panet,R., Amir,I., Atlan, H. and Panet,A. (1985) Control of K+ influx in 3T3 cells transformed by a conditional mutant of Rous sarcoma virus. *J.Biol.Chem.* 260:15752

Passlick,B., Flieger,D. and Ziegler-Heitbrock,H.W.L. (1989) Indentification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood.*74:2527-2534

Patric,D., Betts,J., Frey,E.A., Prameya,R., Dorovini-Zis,K. and Finlay,B.B. (1992) Haemophilus influenzae lipopolysaccharide disrupts confluent monolayers of bovine brain endothelial cells via a serum-dependent cytotoxic pathway. *J.Infect.Dis.* 165:865-872

Pelosin,J.M., Keramidos,M., Souvignet, C., Chambaz, E.M. (1984) Differentiated inhibition of protein kinase C subtypes. *Biochem Biophy Res Commun* 169:1040-1048

Pleiman,C.M., Clark,M.R., Gauen,L.K., Winitz,S., Coggeshall,K.M., Johnson,G.L., Shaw,A.S. and Cambier,J.C. (1993) Mapping of sites on the Src family protein tyrosine kinases $p55^{blk}$, $p59^{fyn}$, and $p56^{lyn}$ which interact with the effector molecules phospholipase C-γ2, microtubule-associated protein kinase, GTPase-activating protein, and phosphytidylinositol 3-kinase. *Mol.Cel.Biol.*13:5877-5887

Porteau,F. and Nathan,C. (1990) Shedding of tumor necrosis factor receptors by activated human neutrophils, *J.Exp.Med.* 172:599

Pripc.V., Weiel,J.E., Somers,S.D. DiGuiseppi,J.,Gonias,S.L., Pizzo,S.V., Hamilton,T.A., Herman,B. and Adams,D.O. (1987) Effects of bacterial lipopolysaccharide on the hydrolysis of phosphatidylinositol-4,5-bisphosphate in murine peritoneal macrophages. *J.Immunol.* 139:526-33

Pugin,J., Schürer-Maly,C-C., Letturcq,D., Moriarty,A., Ulevitch.R.J. and Tobias,P. (1993) Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proc.Natl.Acad.Sci.* USA 90:2744-2748

Pulverer,B.J., Kyriakis,J.K., Avruch,J., Nikolakaki,E., Woodgett,J.R. (1991) Phosphorylation of c-jun mediated by MAP kinases. *Nature* 353:670-674

Raetz,C.R.H. (1990) Biochemistry of endotoxins. *Annu.Rev.Biochem.*59:129

Rahmsdorf,H.J. and Herrlich,P. (1990) Regulation of gene expression by tumor promotors. *Pharmacol Ther* 48:157-188

Rajotte,D., Haddad,P., Haman,A., Cragoe,E.J., Jr.§, and Hoang,T. (1992) Role of protein kinase C and the Na^+/H^+ antiporter in suppression of apoptosis by granulocyte macrophage colony-stimulating factor and interleukin-3. *J. Biol.Chem.* 24 267:9980-9987.

Rietschel,E.T., Brade,L., Brandenburg,K., Flad,J.de Jong-Leuveninck,H.D., Kawahara,K., Linder,B., Loppow,H., Luderitz,T., Schade,U., Seydel,U., Sidirzyk,Z., Tacken,Aa., Zahringer,U. and Brade,H. (1987) Chemical structure and biological activity of bacterial and synthetic lipid A. *Rev.Ifect.Dis.* 9:S527-S536

Rigby,W.F.C., Shen,E.D., Ball,E.D., Guyre,P.M. and Fanger,M.W. (1984) Differentiation of a human monocytic cell line by 1,25-dihydroxyvitamine D_3 (Calcitrol): a morphologic, phenotypic and functional analysis. *Blood* 64:1110

Roberts,W.L., Kim,B.H., Rosenberry,T.L. (1987) Differences in the glycolipid membrane anchores of bovine and human erythrocyte acetylcholinesterases. *Proc. Natl.Acad. Sci* USA 84:7817-21

Rosoff,P.M., Stein, L.F., Cantley,L.C. (1984) Phorbol diesters induce diffferentiation in a pre-B-lymphocyte cell line by enhancing Na^+/H^+-exchange. *J Biol. Chem.* 265:17323-17333

Sack,T.L., Gum,J.R., Low,M.G., Kim,Y.S. (1988) Release of carcinoembryonic antigen from human colon cancer cells by phosphatidylinositol-specific phospholipase C. *J.Clin.Invest.* 82:586-593

Sarraf,C.E., and Bowen,I.D. (1986) Kinetic studies on a murine sarcoma and an analysis of apoptosis. *Br.J.Cancer.* 54:989-998

Saunders,J.W. (1966) Death in embryonic systems. *Science* 154:604-612

Schmitz,B., Klein,R.A. and Duncan,I.A. (1987) MS and NMR analysis of the cross-reacting determinant glycan from *Trypanosoma Brucei* MITat 1.6 variant specific glycoprotein. *Biochem.Biophys.Res.Commun.* 146:1055

Schumann,R.R., Leong,S.R., Flaggs,G.W., Gray,P.W., Wright,S.D., Mathison,J.C., Tobias,P.S., and Ulevitch,R. (1990) Structure and function of lipopolysaccharide binding protein. *Science* 249:1429-1431

Schütt,c., Ringel,B., Nausch,M., Bazil,v., Hořeješi,V., Neels,P., Walzel,H., Jonas,L., Siegl,E., Friemel,H. and Plantikow (1988) Human monocyte activation induced by anti-CD14 monoclonal antibody. *Immunol.Lett.* 19:321-328

Schütt,C., Schilling,T., Grumwald,U., Schoenfeld,W. & Kruger,C. (1992) Endotoxin-neutralizing capacity of soluble CD14. *Res.Immunol.* 143:71-78

Sellins,K.S. and Cohen,J.J. (1987) *J.Immunol.* 139:3199-3200

Shakhov,A.V., Collart,M.A., Vassalli,P., Nedospasov,S.A., Jongeneel,C.V. (1990) kB-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor α gene in primary macrophages. *J.Exp.Med.* 171:35-47

Shapiro,H.M.(1988) Practical flow cytometry (2nd Edition) Alan R.Liss, Inc.

Shinomiya,H., Hirata,H. and Nakano,M. (1991) Purification and characterization of the 65-kDa protein phosphorylated in murine macrophages by stimulation with bacterial lipopolysaccharide. *J.Immunol.* 146:3617-3625

Simmons,D., Tan,S., Tenen,D.G., Nicholson-Weller,A.,and Seed,B. (1989) Monocytes antigen CD14 is a phospholipid anchored membrane protein. *Blood* 73:284-289

Skalka,M., Matyasova,J. and Ceskova,M. (1976) DNA in chromatin of irradiated lymphoid tissues degrades *in vivo* into regular fragments. *FEBS Letter* 72:271-274

Starnes,F., Perce,H.M.K., Tewari,A., Yim,J.H., Zou,J., and Abrams,J.S. (1990) Anti-IL-6 monoclonal antibodies protect against lethal Escherichia coli infection and lethal tumor necrosis factor-α challenge in mice. *J.Immumol.* 145:4185

Štefanová,I., Horejsi,V., Ansotegui.I.J., Knapp,W., Stockinger,H. (1991) GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* 254:1016-1019

Štefanová,I., Corcoran,M., Horak,E., Wahl,L., Bolen,J., and Horak,I. (1993) Lipopolysaccharide induces activation of CD14-assocated protein tyrosine kinase

p53/p56^{lyn} *J.Biol.Chem.* 268:20725-20725

Sturgill,T.W., Ray,L.B., Erikson,E., Maller,J.L. (1988) Insulin-stimulated MAP-2 kinase phosphorylates and activated ribosomal protein S6 kinase II. *Nature* 334:715-718

Suganuma,M., Fujiki,H., Suguri,H., Yoshizawa,S., Hipota,M., Nakayasu,M., Ozika,M., wakamatsu,K., Yamada,K. and Sugimura,T. (1988) Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. *Proc.Natl.Acad.Sci.*USA. 85:1768-71

Sundler,R., Alberts,A.W., and Vagelos,P.R. (1978) Ebzymatic properties of phosphatidylinositol inositophosphogydrolase from *Bacillus cereus. J.Biol.Chem.* 253:4175-4179

Telford,W.G., King,L.E. and Fraker,P.J. (1992) Comparative evaluation of several binding dyew in the detection of apoptosis-associate chromatin degradation by flow cytometry. *Cytometry* 13:137-143

Thomas,P.M. and Samelson,L.E. (1992) The glycophosphatidylinositol-anchored Thy-1 molecules interactions with the p60^{fyn} protein tyrosine kinase in T cells. *J.Biol.Chem.* 267:12317-12322

Tobias,P.S., Soldau,K., and Ulevitch,R.J. (1986) Isolation of a lipopolysaccharide-binding acute reactant from rabbit serum. *J.Exp.Med.* 164:77-793

Tobias,P.S., Soldau,K. and Ulevitch,R.J.(1989) Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. *J.Biol.Chem.* 264:10967-10871

Tobias,P.S., Soldau,K., Hatlen,L., Schumann,R., Einhorn,G., Mathison,J. and Ulevitch,R. (1992) Lipopolysaccharide binding protein. *J.Cell Biochem.* (suppl 16C):151

Tobias,P.S., Soldau,K., Kline,L., Lee,J.D., Kato,K., Martin,T.P. (1993) Cross-linking of lipopolysaccharide (LPS) to CD14 on THP-1 cells mediated by LPS-binding protein. *J.Immunol.* 150:3011-3021

Todd,RF., Alvarez,P.A., Brott,D.A. and Liu,D.Y. (1985) Bacterial lipopolysaccharide, phorbol acetate, and muramyl dipeptide stimulate the

expression of human monocyte surface antigen. *Mol.Immunol.* 135:3869

Todd,R.F., Bhan,A.K., Kabawat,S.E., and Schlossman,S.F. (1984) Human myelomonocytic differentiation antigens defined by monoclonal antibodies. *In Leucocyte Typing.* Bermard,A., Bounmse,L., Daisset,J., Milstein,C., and Schlossman,S.F. eds. Springer-Verlag, New York, p.424

Tomei,D., Kanter,P., Wenner,C.E. (1988) Inhibition of radiation-induced apoptosis *in vitro* by tumor promoter. *Biochem.Biophys.Res.Commun.* 155:324-31

Torres,M., Hall,F.L. and O'Neill,K. (1993) Stimulation of human neutrophils with formyl-methionyl-leucyl-phenylalanine induces tyrosine phosphorylation and activation of two distinct mitogen-activated protein-kinases. *J.Immunol.* 150:1563-1578

Tracey,K.J., Lowry,S.F. and Cerami,A. (1988) Cachectin/TNF mediates the pathophysiological effects of bacterial endotoxin/lipopolysaccharide (LPS), p77-88. *In* Buller,H.R., van Deveter,S.J.H. and Struck,A (ed.), Bacterial endotoxins: pathophysiological effects, clinical significance and pharmacological control. Alan R.Liss,Inc., New York.

Ugolini,V.G., Nunez,R.G., Smith,G.S., and Capi,J.D. (1980) Initial characterization of monoclonal antibodies against human monocytes. *Proc.Natl.Acad.Sci.*USA 77:6764

Vogel,S.N., Hogan,M.M. (1992) Role of cytokines in endotoxin-mediated host response. In: Oppenheim,J.J., Shevach,E.R. eds. Immunophysiology. New York: Oxford University Press, *pp* 238-58

Vogel,S.N. (1992) The *Lps* gene: insights into the gentetic and molecular basis of LPS responsiveness and macrophage differentiation. In: Beutler,B. ed. Tumor necrosis factors: the molecules and their emerging role in medicine. New York: Raven Press, *pp* 485-523

Weber,J.R., Bell,G.M., Han,M.Y., Pawson,T. and Imboden,J.B. (1992) Association of the tyrosine kinase Lck with phospholipase C-γ after stimulation of the T cell antigen receptor. *J.Exp.Med.* 176:373-379

Wallner,B.P., Frey,A.Z., Tizard,R., Mattaliano,R.J., Hession,C., Sanders,M.L., Dustin,M.L. and Springer,T.A. (1987) Primary structure of lymphocyte function-associated 3 (LFA-3). The ligand of the T lymphocyte CD2 glycoprotein.

J.Exp.Med. 166:923

Weinstein,S.W., June,C.H. and De Franco,A.L. (1991) LPS stimulated protein tyrosine phosphorylation in human macrophages involved of CD14. *J.Leucocyte.Biol.* (Suppl.1)11:41 (*Abstrct*)

Weinstein,S.L., Gold,M.R. and Defranco,A.L. (1991) Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages. *Proc.Natl.Acad.Sci. USA* 88:4148-4152

Weinstein,S.L., Sanghera,J.S., Lemke,K., De Franco,A.L. and Pelech,S.L. (1992) Bacterial lipopolysaccharide induce tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages. *J.Biol.Chem.* 267:14955-14962

Weinstein,S.L., June,C.H. and DeFranco,A.L. (1993) Lipopolysaccharide-induced protein tyrosine phosphorylation in human macrophages is mediated by CD14. *J.Immunol.* 151:3829-3838

Wightman,P.D. and Raetz,C.R.H. (1984) The activation of protein kinase C by biologically active lipid moieties of lipopolysaccharide. *J.Biol.Chem.* 259:10048-10052

Wollenweber,H.W. and Morrison,D.C. (1985) Synthesis and biochemical characterization of a photoactivatable, iodinatable, cleavable bacterial lipopolysaccharide derivative. *J.Bio.Chem.* 260:15068-74

Wong,Y.W. and Low,M.G. (1992) Phospholipase resistance of the glycosyl-phosphatidylinositol membrane anchor on human alkaline phosphatase. *Clin.Chem.* 38:2157-2525

Wright,S.D. and Jong,M.T.C. (1986) Adhesion-promoting receptors on human macrophages recognize *E. coli* by binding to lipopolysaccharide. *J.Exp.Med.* 164:1876-1888

Wright,S.D., Ramos,R.A., Tobias,P.S., Ulevitch,R.J., Mathison,J.C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 249:1431-1433

Wright,S.D. (1991) CD14 and immune response to lipopolysaccharide. *Science* 32:1321-1322

Wright,S.D., Ramos,R.A., Patel,M. and Miller,D.S. (1992) Septin: A factor in plasma that opsonizes lipopolysaccharide-bearing particles for recognition by CD14 on phagocytes. *J.Exp.Med.* 176:719-727

Wyllie,A.H., Kerr,J.F.R. and Currie,A.R. (1980) Cell death: the significance of apoptosis. *Int.Rev.Cytol.* 68:251-306.

Wyllie,A.H. (1980) Gluococorticoid-induced thymocyte apoptosis is a associated with endogenous endonuclease activation. *Nature* 284:555-556

Yamanashi,Y., Mori,S., Yoshida,M., Kishimoto,T., Inoue,K., Yamaoto,T. and Toyoshima,K. (1989) Selective expression of a protein-tyrosine kinase, $p56^{lyn}$, in hematopoietic cells and association with production of human T-cell lymphotropic virus type I. *Proc.Natl.Acad.Sci.* USA. 86:6538-6542

Yamanashi,Y., Kakiuchi,J., Mizuguchi,J., Yamamoto,T and Toyoshima,K. (1991) Association of B cell antigen receptor with protein tyrosine kinase Lyn. *Science* 251:192-194

Yamanshi,Y., Fukui,W., Wongsasant,W., Kinoshitata,Y., Ichimori,K., Toyoshima,K. and Yamamoto,T. (1992) Activation of Src-like protein-tyrosine kinase Lyn and its assocaition with phosphatidylinositol 3-kinase upon B-cell antigen receptor-mediated signaling. *Proc.Natl.Acad.Sci.* USA 89:1118-1122

Yamada,K. and Ishii,A. (1989) " KT5720 a new selective inhibitor of PAF-induced platelet responses without receptor antagonism ". *Jpn.J.Pharmacol* 49

Yasui,K., Becker,E.L. and Shaafi,R.I. (1992) Lipopolysaccharide and serum cause the translocation of G-protein to the membrane and prime neutrophils via CD14. *Biochem.Biophy.Res.Comm.* 183:1280-1286

Zhang,X. and Morrison,D.C. (1993) Pertussis toxin-sensitive factor differentially regulates lipopolysaccharide-induced tumor necrosis factor and nitric oxide production in mouse peritoneal macrophages. *J.Immunol.* 150:1011-1018

Ziegler-Heitbrock,H.W.L, Ströbel,M., Kieper,D., Fingerle,G., Schlunck,T., Oetersmann,I., Ellwart,J., Blumenstein,M. and Haas,J. (1992) Differential expression of cytokines in human blood monocyte subpopulations. *Blood* 79:503-511

Ziegler-Heitbrock,H.W.L. and Ulevith,R.J. (1993) CD14: cell surface receptor and differentiation marker. *Immunol.Today.* 14:121-125

Ziegler-Heitbrock,H.W.L., Thiel,E., Futterer,A. (1988) Establishment of human cell line (Mono Mac 6) with characteristic of mature monocytes. *Int.J.Cancer* 41:456-461

Ziegler-Heitbrock,H.W.L., Passlick,B. and Flieger,D. (1988) *Hybridoma.* 7:521-527