THE SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN THE RESPIRATORY BURST OF MURINE PERITONEAL MACROPHAGES

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Master of Philosophy

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November 2000

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An investigation into the signalling pathways responsible for the activation of NADPH oxidase and the oxidative burst in murine peritoneal macrophages has been conducted. An array of key enzymes and second messengers responsible for this orchestration has been identified. The ligation of complement and sugar receptors by opsonised zymosan causes early activation of tyrosine kinases. The resultant protein tyrosine phosphorylation is crucial for the oxidative burst. Inhibition of tyrosine phosphatase, preventing subsequent tyrosine dephosphorylation validated this as the oxidative burst was augmented.

The phosphorylation of tyrosine residues is most likely to cause the activation of phospholipase Cγ yielding inositol 1,4,5 trisphosphate (IP3) and diacylglycerol (DAG). It is very likely that it also causes activation of phosphatidylinositol 3-kinase (PI-3K), for inhibition of this enzyme abrogates the oxidative burst. It is still obscure what the immediate downstream targets for this enzyme or its products are. The mobilisation of intracellular calcium stores and activation of protein kinase C (PKC) are activated by IP3 and DAG respectively. NADPH oxidase activation is dependent on both of these products. Obstructing calcium mobilisation or inhibition of PKC reduces the oxidative burst. In addition, the direct activation of PKC by phorbol myristate acetate (PMA) alone can evoke an oxidative burst, which is potentiated if calcium is raised using a calcium ionophore.

The initiation of this amplification pathway, via cell surface receptor ligation is also responsible for an influx of extracellular calcium. This is an essential prerequisite for the oxidative burst since the absence of extracellular calcium or blockade of calcium channels inhibits it.

A ligand operated sodium channel may be involved in the opening of these voltage operated calcium channels.

An increase in intracellular calcium activates calmodulin, whose response elements participate in the oxidative burst, for a calmodulin antagonist stifled superoxide

anion generation. The direct or indirect protein phosphorylation initiated by calcium-dependent kinases and PKC activate the NADPH oxidase enzyme complex. Elevation of intracellular cyclic AMP (c-AMP) and therefore presumably protein kinase A PKA activation produced a profound reduction in the oxidative burst elicited by opsonised zymosan. The protein phosphorylation responsible has not been identified but the findings suggest that there may be an element of cross-talk between different phagocyte receptors via their associated second messenger systems serving to control macrophage function.
These findings illustrate the multiple mechanisms required for the onset of NADPH oxidase activation and subsequent oxidative burst.

Murine peritoneal macrophage, Oxidative burst, Opsonised zymosan, Tyrosine kinase, Protein kinase C

DEDICATION

I dedicate this thesis to the memory of my parents Tom and Agnes Stirling and my late uncle and aunt, Andy and Helen Mackie. Their words of encouragement enabled me to realise my true potential.

ACKNOWLEDGEMENTS

First of all I would like to thank my supervisor Dr Alan Perris for his expert guidance and enthusiasm and for his constructive ideas. I would also like to extend my appreciation of his patience and understanding throughout the duration of my M.Phil. My gratitude also goes to Dr Nick Hartell for his invaluable assistance and dedication while working with his fluorescence microscopy system. I also thank Kevin Hughes for his inestimable assistance with computing.

LIST OF CONTENTS

LIST OF FIGURES AND TABLES

PAGE No

ABBREVIATIONS

AA	Arachidonic acid
ADCC	Antibody dependent cellular cytotoxicity
Ag	Antigen
APC	Antigen presenting cell
BIM	Bisindolylmaleimide
C3	Third component of complement
c-AMP	Cyclic adenosine monophosphate
CCD	Charged coupled device
CFU-GM	Colony forming unit
CR	Complement receptor
CTL	Cytotoxic T cell
DAG	Diacylglycerol
DMSO	Dimethylsulfoxide
EGTA	Ethylenedinitrilotetraacetic acid
FBS	Foetal bovine serum
FcγR	Fc gamma receptor
FMLP	Formylmethionylleucinephenylalanine
GAP's	Rac GTPase activating proteins
H_2O_2	Hydrogen peroxide
ICAM	Intercellular adhesion molecule
IFNγ	Interferon gamma
IL-1	Interleukin 1
IL-6	Interleukin 6
IP3	Inositol trisphosphate
IP4	Inositol 1,3,4,5 tetrakisphosphate
ITAM's	Immunoreceptor tyrosine-based activation motifs
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
Mϕ	Macrophage
MAF	Macrophage activation factors
Maleyl-BSA	Maleylated bovine serum albumin

ABBREVIATIONS

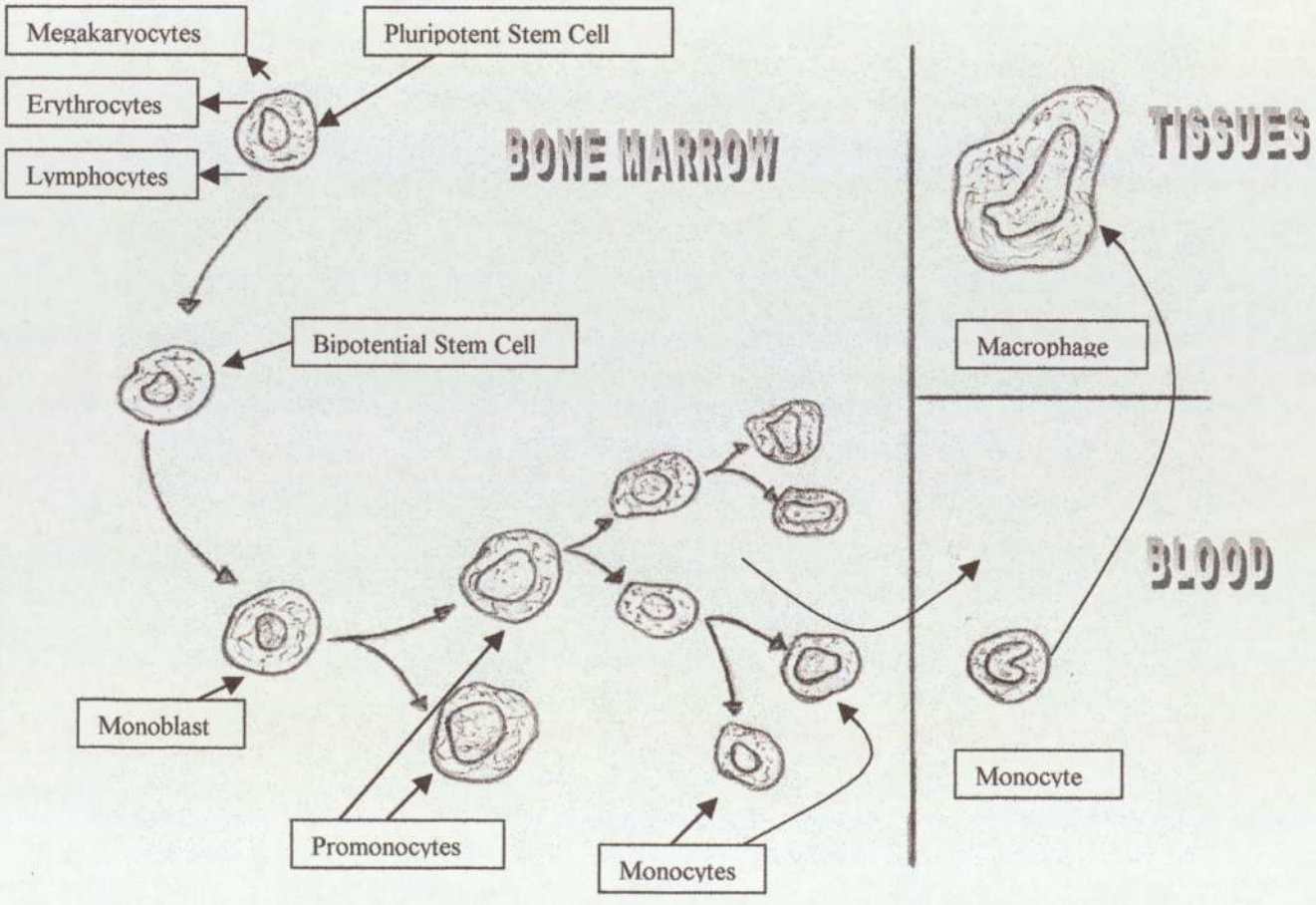
MAPK	Mitogen activated protein kinase
MARCKS	Myristoylated alanine-rich C-kinase substrate
M-CSF	Macrophage colony stimulating factor
MDP	Muramyl dipeptide
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
O_2^-	Singlet oxygen
OCL^-	Hypochlorite ion
OH^-	Hydroxyl ion
OZ, OPZ	Opsonised zymosan
PA	Phosphatidic acid
PAF	Platelet activating factor
PAK	p21 activated kinase
PAP-1	Phosphatidic acid phosphohydralase
PBS	Phosphate buffered saline
PI-3 Kinase	Phosphatidylinositol 3 kinase
PIP2	Phosphatidylinositol 4,5 bisphosphate
PIP3	Phosphatidylinositol 3,4,5 trisphosphate
PKC	Protein kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PTK	Protein tyrosine kinase
Rho-GDI	GDP-dissociation inhibitor
RLU's	Relative light units
SERCAS	Sarco-endoplasmic reticulum calcium ATP-ase
SH3	Src homology 3
SR	Scavenger receptor
SRBC	Sheep red blood cells
TNFα	Tissue necrosis factor alpha
Z	Zymosan

1. Introduction

1.1 The phylogeny of the phagocyte

The macrophage is the major differentiated cell of a phylogenetically primitive system of cells termed the mononuclear phagocyte system. They originate in the bone marrow, which not only contains resident macrophages but also the precursors known as monocytes, promonocytes and monoblasts, with the latter being the least mature as reflected in their morphology and ultrastructure. A common progenitor is thought to exist which gives rise to monocytes and neutrophils, and is termed the colony forming unit granulocyte macrophage (CFU-GM), and it is believed that this cell becomes committed to monocytic or granulocytic differentiation before the monoblast stage (Lewis et al 1991). The division of monoblasts and subsequent promonocytes gives rise to monocytes, which then travel in the peripheral blood and are distributed between circulating and marginating pools (Meuret et al 1973). The several developmental types are illustrated in (fig 1.0). However to become a macrophage the monocyte leaves the circulation and migrates into the extravascular tissues and fluids where it undergoes differentiation and remains for several months.

Fig 1.0 (Ontogeny of mononuclear phagocytes)



Extravasation is mediated by binding of a high molecular weight integrin adhesion molecule (CD11b, CD18) on the monocytes surface, to a receptor, intercellular adhesion molecule 1 (ICAM 1/CD54) present on vascular endothelial cells (Rothlein et al 1986; Dustin et al 1986). Expression of ICAM 1 is enhanced by cytokines such as interleukin 1 and interferon γ, increasing monocyte margination and migration especially to sites of inflammation. Having arrived at their target organs, monocytes differentiate into macrophages and can be found in a variety of locations throughout the body (Furth et al 1968). Although 95% of tissue macrophages are derived from the migratory monocyte, it is thought that the remaining quantity may be derived from the local division of mononuclear phagocytes in the tissues. These are not resident macrophages but have arrived in tissues and body cavities from the bone marrow within the previous 24 hrs (Kuby 1994)

1.1.2 Receptors on phagocytic cells involved in recognition

Phagocytosis of pathogens by macrophages initiates the innate immune response, which in turn orchestrates the adaptive response. In order to discriminate between infectious agents and self, macrophages have evolved a restricted number of phagocytic receptors and all of these receptors induce rearrangements in the actin cytoskeleton that lead to the internalisation of the particle. The purpose of this section is to give a general overview of receptors on the surface of macrophages associated with phagocytosis. Attention is focused on those receptors that either are associated with microbial recognition or have the potential to participate in this process. Initiation of the several functions of macrophages is controlled by ligand binding to these receptors.

1.1.3 Receptors for particles opsonized with immunoglobulin G

Amongst the earliest receptors to be identified were those which bind the Fc region of the IgG molecule and which bind the cleavage product of the third component of complement (C3) (Berken et al 1966). There are three distinct classes of receptors for this Fc portion of IgG and are collectively termed the Fcγ receptors (FcγRs) and individually designated as FcγRI, FcγRII, and FcγRIII. They provide a crucial link

between the phagocytic effector cells and the lymphocytes that secrete immunoglobulins. The Fcγ receptors are members of a large multigene family that mediate a series of diverse functions critical to cellular immune responses including phagocytosis, antibody dependent cellular cytotoxicity (ADCC), secretion of inflammatory mediators, generation of the respiratory burst and clearance of immune complexes (Unkeless et al 1988). The FcγRI, designated CD64, is a high affinity receptor binding IgG with an apparent affinity of approximately $1x10^{-8}M$ and can effectively bind immune complexes by multiple receptor ligand interactions (Unkeless et al 1988). The FcγRI receptors are glycosylated monomeric proteins with three immunoglobulin-like extracellular domains (and are limited in tissue distribution), being expressed primarily on cells of the monocyte/macrophage lineage, with resting cells expressing between 10,000-30,000 receptors/cell (Ravetch 1991). An example of a stimulator of the FcγRI receptor is interferon gamma (IFN-γ), which up regulates receptor expression on monocytes by as much as twenty fold (Schreiber et al 1984). FcγRI occupancy enhances phospholipase A_2 (PLA_2) activity and the related arachidonic acid cascade (Schreiber et al 1984).

The FcγRII receptors, designated CD32, are low affinity receptors for IgG and are glycosylated monomeric proteins with two IgG like extracellular domains. These receptors bind monomeric IgG poorly if at all, with an affinity some ten times lower than that of the FcγRI receptor (Mosser et al 1994), however they are widely distributed, they bind immune complexes readily, and they efficiently mediate the endocytosis of bound ligand. The FcγRII found on cells of myeloid and lymphoid lineage including monocytes, neutrophils, lymphocytes, and platelets. Murine macrophages express approximately 100,000 FcγRII receptors/cell whereas platelets (for instance) have approximately 1000 receptors/cell. FcγRII occupancy may also mediate elevation of cyclic AMP (Adams et al 1984).

The FcγRIII receptors (CD16) have low affinity for IgG. They are expressed on neutrophils, natural killer cells and tissue macrophages and are not expressed on undifferentiated macrophage precursors and expressed poorly if at all in freshly isolated macrophages. Monocyte-derived macrophages however begin to express FcγRIII after several days in culture (Unkeless et al 1988). Macrophages will recognise immune complexes carried in association with red blood cells, which bind the complexes via an FcγRIII occupancy (Stites et al 1991). The biology of the

individual FcγR's is complicated by the fact that there are different forms of each of these receptors. The functional diversity observed is a reflection of the structural heterogeneity, especially in the cytoplasmic portion of the FcγR. Thus a single FcγR expressed in two different cell types may mediate different biological functions. These observations imply that the function of specific classes of Fc receptors may be an important focus for regulatory control. The contribution of the phagocytic FcγR's in host defence is best inferred from numerous in vivo observations documenting the contribution of antibody to host defence, and there is clearly an accessory role for complement in many of these processes which will be subsequently discussed

1.1.4 Receptors for particles opsonized with complement

Leukocytes also recognise particles opsonized with complement. CR1 (CD35) and CR3 designated (CD11b/CD18) or Mac-1, recognise the activated third components of complement: C3b and C3bi respectively. Resting CR1 will not internalise bound C3b (Griffin et al 1987). Other signals derived from either T cells ie, bacterial LPS or phorbol ester treatment converts the CR1 into an endocytic receptor (Bohnsack et al 1986).

The CR1 in contrast to the FcγR's described earlier does not trigger a respiratory burst upon particle ligation nor does it lead to cellular prostaglandin synthesis (Aderem et al 1985). Thus the CR1 can be converted from a binding site on resting cells to a receptor capable of transmitting the cellular signals required for internalisation. Three different murine proteins with homology to the human complement receptors have been identified (Paul et al 1989; Holers et al 1992). One called Crry has no C3 binding activity but is homologous to CR1, whereas MCR1 and MCR2 express C3b/C4b and C3dg binding activity respectively. This emphasises that there are clear structural distinctions between the murine and human complement receptor, but both species share enough homology to achieve both binding and complement regulatory activity. In vitro, macrophage complement receptors in synergy with FcγR receptors or alone, have been shown to participate in the uptake of a number of bacterial species. It has been demonstrated that in the absence of exogenous opsonins, encapsulated yeast is almost totally resistant to

ingestion by murine macrophages whereas opsonisation in vitro with non-immune mouse serum showed subsequent phagocytosis via CR3 receptor (Cross et al 1997). Macrophage-colony stimulating factor (M-CSF) and interleukin-4 have also been shown to activate both CR1 and CR3 to become competent for phagocytosis in murine macrophages (Sampson et al 1991).

The Fab portion of IgG and fragments C3b and C3bi are known as opsonins (recognition molecules which enhance phagocytosis) and bind to specific sites on microbes (Kuby 1994). This opsonisation permits macrophages and other phagocytic cells via antibody and complement to bind to a diverse array of molecules on microbial surfaces, and macrophages employing a limited number of FcγR or CR to phagocytose them, although the two receptor types may employ different signals (Loegering et al 1991). As a consequence of this the immune system is able to eliminate potential pathogens by opsonising them with specific IgG and phagocytosing them via FcγR receptors. In addition to the Fcγ and C3b receptors, macrophages carry several additional specific receptors, which can also contribute to phagocytosis but also trigger a variety of other processes (Sharon 1984;Wright et al 1986; Ofek et al 1988; Speert et al 1988;) (See table 1 over).

Table 1

Macrophage receptors and the functional consequences of ligand-receptor binding. (Adapted from Lewis et al 1991)

Receptor	Function
Fcγ Receptors	Increases phagocytosis. Releases H_2O_2. Triggers the secretion of prostaglandin's and lysosomal enzymes. Releases reactive oxygen intermediates. Receptor numbers increased by α, β and γ interferon.
Complement receptor (CR1, CR3)	Increases phagocytic function. Induced by T cell derived lymphokine.
C_5A receptor	Mediates chemotaxis. Promotes IL-1 secretion.
Man-fuc-glunac receptor	Involved in binding and phagocytosing certain microbial elements.
Fmet-leu-phe receptor (FMLP)	The affinity of the receptor regulates response. High affinity mediates chemotaxis. Low affinity initiates the secretion of reactive oxygen intermediates, prostaglandin's and lysosomal hydrolases
Cytokine receptor e.g. Interferon γ	Enhances macrophage function by rendering them sensitive to second signals.
LPS receptor	Activates phospholipase C. Stimulates lysosomal hydrolase and neutral protease secretion.
α-2 macroglobulin receptor	Inhibits protease secretion.
Maleylated-bovine serum albumin receptor	Induces chemotaxis. Triggers protease secretion and tumouricidal function. Stimulates the production of prostaglandin E2.
Prostaglandin E2 receptor.	Diminishes phagocytic potential. Decreases secretion of interleukin-1 and tumour necrosis factor α.
Lactoferrin receptor	Inhibits macrophage colony stimulating factor (CSF) secretion from macrophages.

A unique recognition mechanism involved in the interaction of some microbes with macrophages has been described and involves the binding of bacterial lectins to the man/fuc/glunac receptor glycoproteins exposed on the surface of the macrophage, which is known as lectinophagocytosis.

1.1.5 Carbohydrates as recognition determinants on macrophages

The mannosyl/fucosyl/glunac (man/fuc/glunac) receptor is perhaps the best characterised and is involved in recycling mannosylated macromolecules from the circulation (Stahl et al 1980). It is known by this name, as it is also capable of recognising fucose and to a lesser extent, glunac. This receptor has been reported to have two primary biological roles. The first is being a scavenger type capable of removing released neutrophil granule glycoproteins at the site of inflammation and its other role is in the adsorption of certain bacteria, fungi and parasites (Sung et al 1983; Blackwell et al 1985; Kan et al 1988) to their surface via carbohydrates prior to phagocytosis. Binding to the mannose receptor generally results in efficient particle internalisation and this is usually accompanied by a brisk respiratory burst. The mannose receptor and the FcγR may be closely associated, for when macrophages were exposed to a mannose-coated substrate the functions and activities of both receptor types were lost (Sung et al 1985). This would indicate that under appropriate circumstances the mouse macrophage FcγR and man/fuc/glunac receptors can co-modulate. The phagocytic activity of murine peritoneal macrophages treated with glucose and mannose specific lectins (such as concanavalin A and lentil lectin), towards heterologous erythrocytes was shown to increase whereas peanut agglutinin (a galactose specific lectin), diminished phagocytic activity (Maldonado et al 1994). This suggests that a galactose-N-acetyl-galactosamine containing structure could participate as a negative modulator of phagocytosis and demonstrates the effect that various lectins can have on different cell surface carbohydrates in the regulation of macrophage activity.

Another important saccharide specific receptor present on the macrophage surface is the β glucan receptor, which ligates with β glucans, which originate on the cell walls of yeast, fungi and plants. It has been suggested that zymosan (an insoluble β

glucan) triggers H_2O_2 production in murine peritoneal macrophages via this β glucan specific receptor (Adachi et al 1993). It has recently been found that only particulate (zymosan/zymocel) and not soluble β glucans are able to activate murine peritoneal macrophages to produce H_2O_2 in vitro and that the activation signals were translocated through protein tyrosine kinase and phospholipase A_2 (Okazaki et al 1996). This would yield arachidonic acid, which may have important roles for phagocytic activity in monocytes (Tarsi-Tsuk et al 1990). To achieve maximal phagocytosis of unopsonised heat killed yeast, co-expression of both mannose and β glucan receptors is required in murine peritoneal macrophage and macrophage-like cell lines (Giaimis et al 1993). A sialoadhesin is also thought to be present on the murine peritoneal macrophage cell surface as they recognise sialosaccharide chains in mouse erythyrocytes that have been treated with oxidising agents (Beppu et al 1994), and that exposed β-galactosyl residues are capable of triggering the generation of reactive oxygen species (Aparna et al 1994).

So it is clear that lectin-carbohydrate interactions play a role in phagocytic activities such as the binding and ingestion of bacteria, yeast, cells, zymosan as well as sialidase treated erythyrocytes. For a brief review of some carbohydrate-lectin interactions see table 1.1 and 1.2.

Table 1.1 Interactions between lectins on the surface of phagocytic cells and sugars on particles or other cells.

Lectin of Macrophage	Surface carbohydrate	Sugar or particle	Effect	Reference
Man/fuc/glunac (mannose) receptor on macrophages.	Yeast mannans, Lysosomal-glycosidases.	Zymosan, Yeasts.	Phagocytosis of unopsonised heat killed Saccharomyctes cerevisiae.	**Giamis et al (1993).**
			Modulation of man/fuc/glunac receptor by binding to substrates coated with mannose containing oligosacharides.	
β Glucan receptor on macrophages.	Yeast β glucans.	Zymosan from yeasts.	Soluble β glucans shown to inhibit phagocytosis of heat killed yeast.	**Goldman et al (1994).**
			Zymosan triggered H_2O_2 production mediated by β glucan receptor.	**Adachi et al (1993).**
			Particulate β glucans (zymosan and zymocel) induced H_2O_2 production.	**Okazaki et al (1996).**
D-Galactose specific receptor on macrophages.	Sialosaccharide on mouse erythrocyte.		Attachment of mouse erythrocyte to mouse macrophage.	**Beppu et al (1994).**

Table 1.2 Interaction between sugars on the surface of phagocytic cells and lectins on the surface of other cells or particles

Lectin	Sugar specificity on macrophage	Cell Type	Effect	Reference
Concanavalin A	D-Mannose	Macrophage	Formation of large vacuoles on the cell surface which are subsequently phagocytosed	**Sharon (1984)**
Wheat germ agglutinin	N-acetyl-D-glucosamine Sialic acid	Macrophage		
Peanut agglutinin Soybean agglutinin	D-galactose N-acetyl-D-galactosamine N-acetylneuraminic acid	Macrophage		
Type 1 Fimbriae (Pili) From S.typhimurium	Mannose	Mouse peritoneal macrophage	Generation of oxidative burst	**Ofek et al (1988)**

1.1.6 The PAF receptor

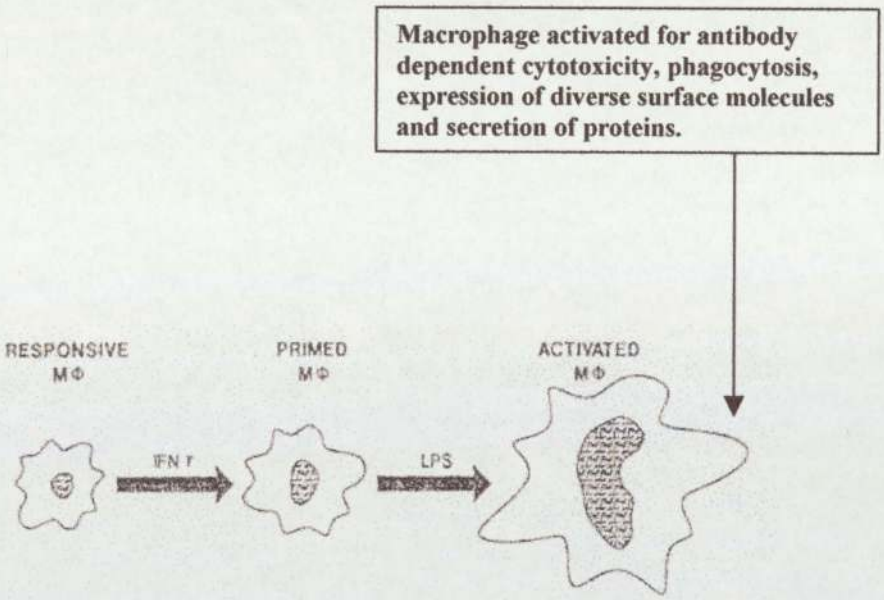
Macrophages also bear platelet activating factor (PAF) receptors. PAF is one of the multifunctional and physiologically active compounds involved in the process of extracellular and intracellular signalling. It is also a naturally occurring lipid mediator inducing locomotion, secretion of lysosomal enzymes, enhancement of metabolism and the generation of reactive oxygen intermediates. The breakdown products of polyphosphoinositides, subsequent changes in intracellular calcium, protein phosphorylation and chemotaxis have been observed in murine peritoneal macrophages treated with PAF (Prpic et al 1988). The direct and modulating action of PAF on intracellular calcium concentration [$(Ca^{2+})i$] has been studied using PAF and its structural analogues, and this has shown that Ca^{2+} is derived from the macrophage endoplasmic reticulum (Zinchenko et al 1997). Macrophage responses to PAF such as [$(Ca^{2+})i$] mobilisation and O_2^- production have been shown to be suppressed by pre-treatment with lipopolysaccharide or interferon γ and that this correlated with an alteration in PAF receptor number and binding affinity (Howard et al 1996), which provides evidence that IFNγ and LPS play a significant role in the regulation of macrophage responsiveness to other agents as well as in macrophage activation.

1.2 Macrophage activation

Development of competence for microbicidal and/or tumouricidal function has been designated as the activation of macrophages. In murine macrophages, lytic function requires two signals in a defined sequence. (Hamilton et al 1987). One belongs to a class of lymphokines operationally termed macrophage activating factors (MAF) and a secondary signal frequently supplied by bacterial cell wall products. The first signal lowers the dose requirement of the second. IFNγ is a potent MAF in vitro and in vivo and a second signal like LPS or the lipid A (active component of LPS), from bacterial cell walls will push primed macrophages to the activated stage (Figure 1.1). Macrophages thus activated by LPS produce a variety of active substances such as tissue necrosis factor alpha (TNFα), interleukin 1 (IL-1), interleukin 6 (IL-6), procoagulant activators, eicosanoids, reactive oxygen and nitrogen intermediates. LPS activation induces phosphorylation of various membrane and

cytosolic proteins. Thus a 65Kda protein (p65) becomes closely associated with PKC and calmodulin dependent kinases (Nakano et al 1990; Shinomya et al 1991).This indicates the existence of multiple pathways and different regulatory mechanisms used to transduce the LPS signal in the macrophages. In order for LPS to bind to the macrophage, a receptor for it must be in existence. Several LPS binding proteins (LBP) have been identified on the cell surface and proposed as LPS receptors. LBP is a trace plasma glycoprotein that binds to the lipid A moiety of LPS and can subsequently increase its affinity for CD14 on the Mϕ surface (Wright et al 1991).

Fig 1.1 (A model for macrophage activation) (From Uhing et al 1989).



Another bacterial cell component involved in macrophage activation is muramyl dipeptide (MDP), which is the minimal adjuvant from cell wall peptidoglycan required for activation. MDP causes a dose-dependent enhancement of attachment and spreading on plastic and glass surfaces, which is considered to reflect activation (Tanaka et al 1980). Peritoneal macrophages treated with MDP enhance O^{2-} modulation on stimulation with PMA or opsonised zymosan (Pabst et al 1980). Mouse macrophage monolayers that had been treated with MDP have also shown significant increases in the release of H_2O_2, O_2^- and IL-1 as compared to untreated

macrophages (Sodhi et al 1989). However, mouse macrophages were shown not to be activated in comparison to guinea pig and rat macrophages indicating a species dependency (Nagao et al 1990). This discrepancy may be due to different sensitivities of macrophages to the stimulatory activity of MDP. Various MDP analogues and derivatives also failed to stimulate murine peritoneal macrophages (Nagao et al 1992). Alternatively, lipopeptide derived analogues of MDP have been found to produce a cytotoxic activity in mouse peritoneal macrophage supernatants that are higher than stimulation with MDP alone (Breton et al 1992). It has also been shown that adherent macrophages incubated with a lipophylic derivative of MDP, exhibited rounding and extensive ruffling of the cell surface and generation of elevated levels of luminol dependent chemiluminescence in response to stimulation by zymosan (Masihi et al 1986).

Membrane specific receptors have been identified at the macrophage surface which interact with MDP (Silverman et al 1985) however, the number of these receptors are much lower here than located in the cytosolic compartment and cannot account for the biological activity of MDP. Interestingly, serotonin treatment modifies macrophage responsiveness to MDP suggesting that its function may subject in part to neural control indicating the possibility of a common signal transduction pathway for both (Silverman et al 1985).

Another agent known as maleylated bovine serum albumin (maleyl-BSA) can replace LPS in activating appropriately primed macrophages for tumouricidal function (Johnson et al 1982; Somers et al 1987; Johnstone et al 1987), suggesting that LPS and mal-BSA are both linked to phospholipase C. Specifically, binding of mal-BSA results in the accumulation of messages for and secretion of several proinflammatory gene products such as TNFα and IL-1 via ligation of the low affinity scavenger receptor (SR) on macrophages. These same stimuli also induce the expression of early response genes, which may be involved in macrophage activation. It has recently been seen that mal-BSA acts synergistically with IFNγ to initiate the production of nitric oxide in macrophages (Alford et al 1998).

It is evident that there is cooperation between different receptor types as they often jointly participate in given microbial recognition events. For instance, macrophages use receptors for both IgG and complement to phagocytose encapsulated bacteria. Often, the receptor to which the microbe binds orchestrates many of the subsequent intracellular events during phagocytosis by transducing specific cellular signals. Some receptors, for instance the man/fuc/glunac and Fcγ receptors are particularly well suited to direct particles to phagolysosomes and trigger a respiratory burst whereas other receptors; for example, the CR1, may not.

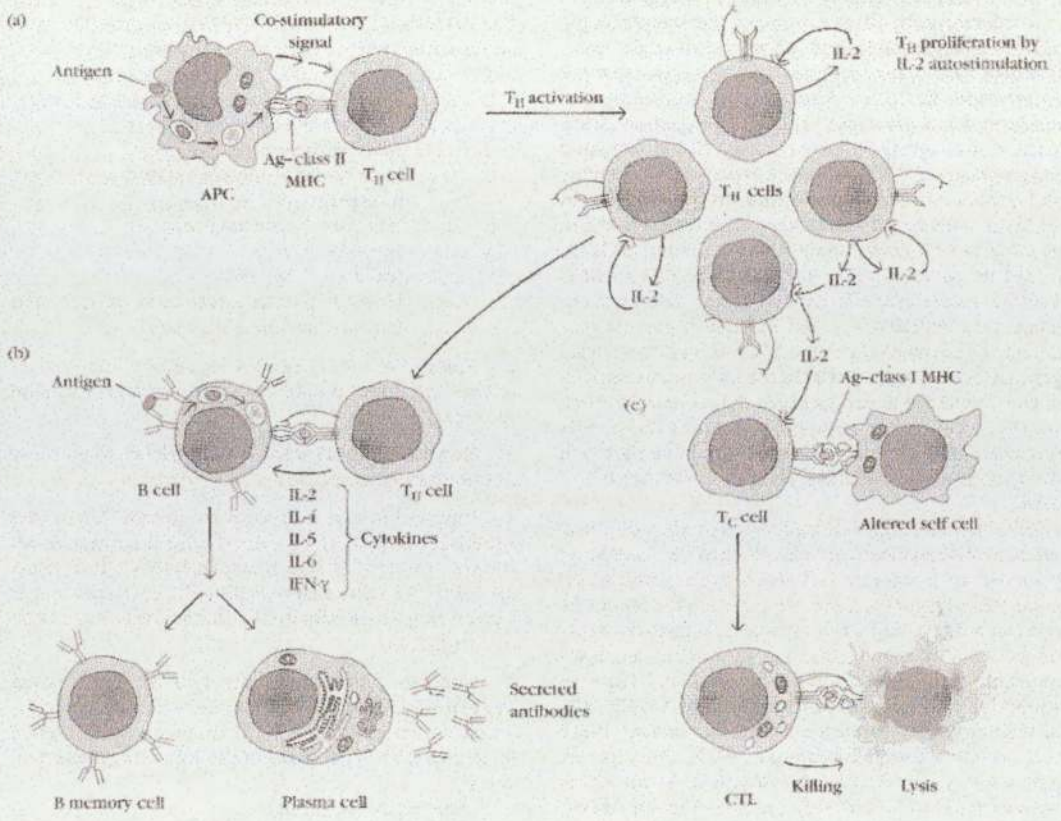
1.3 Antigen Processing and Presentation

An important function of the macrophage is its ability to engulf solid particulate matter. Under electron microscopy general features such as membrane bound lysosomes (phagolysosomes), in which ingested bacterial material in various stages of degradation and digestion can be seen (Lewis et al 1991). The prominent ruffling, locomotion, pseudopod formation and polarity of migration in response to chemotactic stimuli are all due to actin microfilaments immediately beneath the cell membrane (Lewis et al 1991). The ability of phagocytes to orchestrate a number of diverse functions such as the protection against invading microorganisms, removal of dead cells and tissue repair are carried out by phagocytic, pinocytotic, secretory and intracellular digestive processes. In addition, macrophages participate in various specific aspects of cellular and humoral immunity, initiating and facilitating immune responses against invading pathogens (Grey et al 1989). In the initial stage, antigen after being processed by the macrophage is presented to the T or B lymphocyte which are then subsequently activated and themselves secrete cytokines which further activate the macrophages and contribute to the local inflammatory reaction and to increased resistance to many microbial pathogens (see fig 1.2). Some bacterial cells escape detection by macrophages by using the cell surface receptor on the macrophage and multiply for a time within the vacuolar organelles avoiding antimicrobial agents of immune and non-immune origin in the surrounding milieu. However, these pathogens will eventually encounter a B cell with receptor antibodies able to bind to the epitopes on the bacterial cell surface. Once the B cell

binds it becomes sensitive to stimulatory signals from the macrophage and helper T cells. Intracellular processing of protein antigens by macrophages may allow the appearance of peptides of the cell surface in association with class 1 and class 2 MHC molecules for recognition by the appropriate (CD-8 expressing) or (CD-4 expressing) T cells respectively.

Fig 1.2 (Cellular interactions involved in the induction of an immune response) (From Kuby et al 1994)

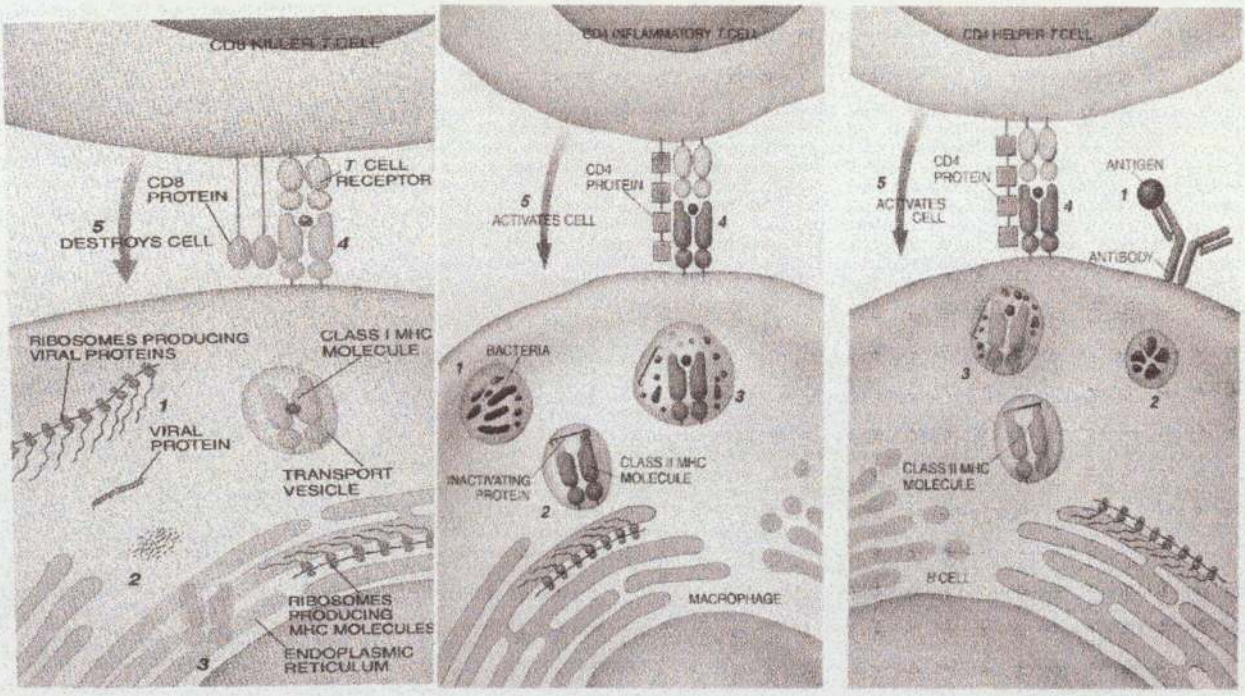
Activation and proliferation of T helper cells a) is required for generation of a humoral response b) and a cell mediated response to altered self cells c).
APC=antigen presenting cell, Ag=antigen, IL=Interleukin, IFNγ=Interferon gamma Tc/CTL=cytotoxic T cell, Th=helper T cell



Antigen processing and presentation to $CD4^+$ cells by antigen presenting cells including macrophages is a prerequisite for the recognition of most antigens by T cells (Werderlin et al 1988). Exogenous antigens are ingested by endocytosis,

transported to an acid cellular compartment and subjected to proteolytic fragmentation. Some of these antigen fragments bind to MHC class 2 molecules and are transported to the surface of the antigen-presenting cell. (see figure 1.3). Because of MHC class 2 molecules expressed in individual antigen presenting cells, each MHC molecule is capable of binding several different peptides (De lis et al 1986). Macrophages can also present peptides derived from internally synthesised endogenous antigens on MHC class 1 molecules. Accordingly, most peptides capable of binding to a particular MHC molecule share broad structural motifs detectable at the level of primary amino acid sequence (Sette et al 1989; Bjorkman et al 1987).

Fig 1.3 (Antigen processing and presentation) (From Janeway, Scientific American 1993).



Viral proteins produced by an infected Cell (1). are broken doen into peptides (2). The peptides are taken to the endoplasmic reticulum where class 1 MHC molecules form around them (3). Each complex goes to the cell surface. There it can be detected by a killer T cell which expresses a CD8 protein (4). The T cell then secretes compounds that destroy the infected cell (5).

The bacteria that infected a macrophage reside in the cells vesicle (1). A class 11 MHC molecule, produced in the endoplasmic reticulum is transported to the vesicle (2). A protein chain (black line) keeps the molecule inactive until it reaches the vesicle. In the vesicle the chain falls away, enabling the class 11 MHC molecule to bind to any peptides there (3). The complex then moves to the cell surface, where a so called inflammatory CD4 t cell binds to the peptide (4). The T cell then activates the macrophage, signaling it to destroy the material in its vesicle (5).

An antibody on the surface of a B cell serves as the B cells receptor. If the antibody discovers a foreign antigen in the bloodstream, it binds to it (1) and delivers the antigen to a vesicle inside the cell. The antigen is broken down into peptides (2). A class 11 MHC molecule , which is produced in the endoplasmic reticulum, migrates to the vesicle, where it grabs a peptide (3). The MHC molecule transports the peptide to the cell surface (4). A CD4 helper T cell binds to the antigen and makes molecules that tell the B cell to proliferate and to produce antibodies (5).

1.4 Phagocytosis and the respiratory burst

1.4.1 The process of phagocytosis

The zipper model of the mechanism of phagocytosis has been the dominant paradigm of the field over the past twenty years (Griffin et al 1974). Contact in FcγR mediated phagocytosis between the phagocyte and the particle guides the extension of a pseudopod to form together with a patch of the plasma membrane, a tight fitting phagosome. Advance of the pseudopod around the particle requires continual ligation of new receptors with this response proportional to particle size. The phagosome subsequently matures by fusing with late endosomes, and ultimately lysosomes to form a phagolysosome. Endosomes are dynamic tubulovesicular structures that interchange components with other endosomes, the golgi complex and the plasma membrane through budding and fusion events. The ingested material can now be degraded and killed with the help of hydrogen peroxide, oxygen free radicals, peroxidase, lysozyme and various hydrolytic enzymes such as β glucoronidase and cathepsin D. The digested contents of the phagolysosome are then eliminated in a process called exocytosis. (See fig 1.4).

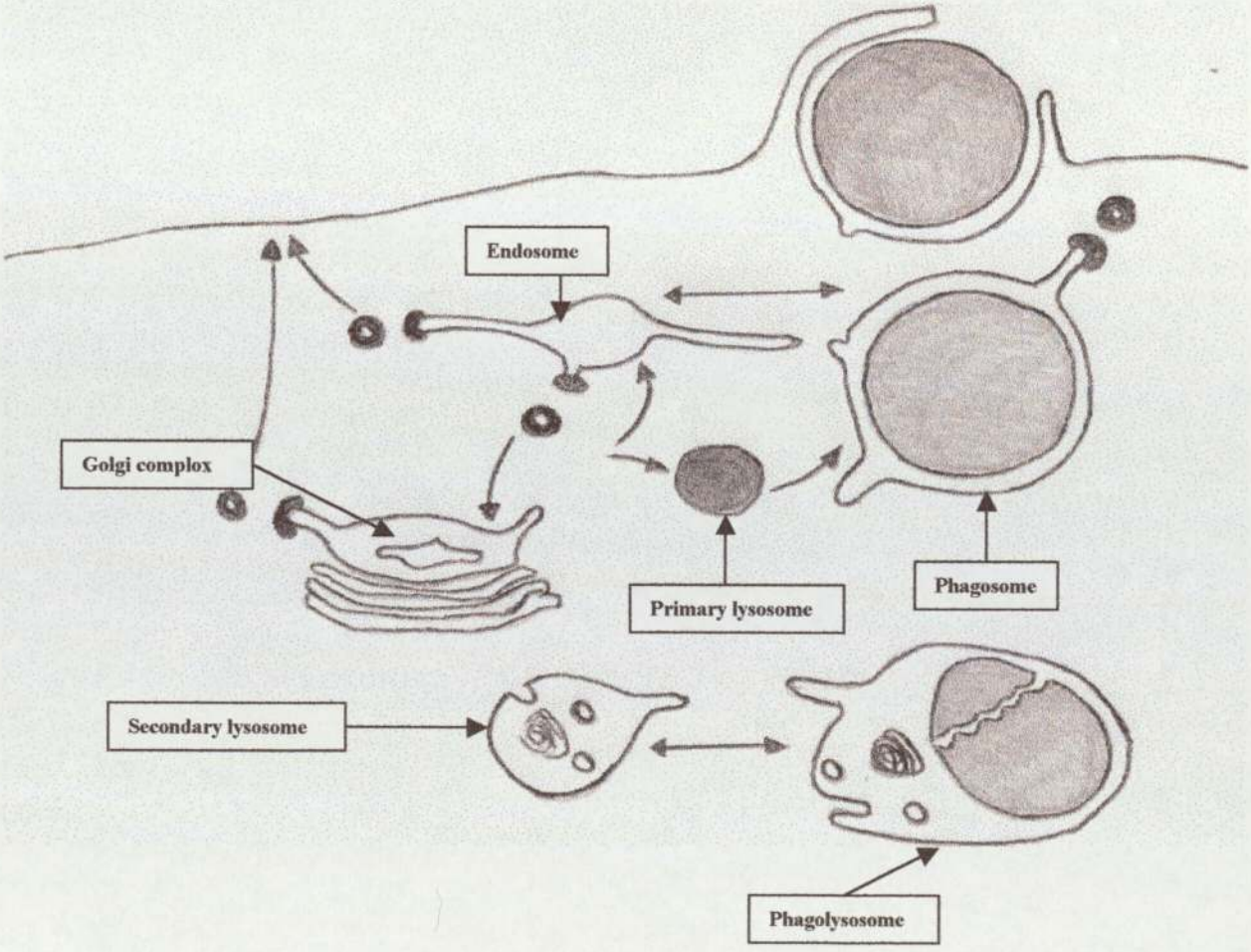
Complement receptor (CR) mediated phagocytosis is a relatively passive process that occurs by a variation of the classic zipper model, as complement opsonised particles appear to stick into the cells with elaboration of small, if any pseudopodia (Allen et al 1996). Moreover, the phagosome membrane is less tightly opposed to complement- opsonised particles, with point like contact areas seperating regions of looser membrane (Aderem 1999). These point like contact areas are enriched with a variety of cytoskeletal proteins including F-actin, vinculin, paxillin, α actinin and phosphotyrosine containing proteins with their formation being blocked by inhibitors of PKC, but not by inhibitors of protein tyrosine kinases. By comparison all of these proteins are diffusely distributed in phagosomes that have undergone FcγR mediated phagocytosis which is blocked by both PKC and protein tyrosine kinase inhibitors (Allen et al 1996). Thus the signals required for particle ingestion and the arrangement of cytoskeletal proteins on the phagosome surface vary depending on which phagocytic receptor is engaged.

An additional difference between FcγR-mediated and CR-mediated phagocytosis relates to their capacity to trigger the release of inflammatory mediators. FcγR-

induced phagocytosis is tightly coupled to the production and secretion of pro-inflammatory molecules such as reactive oxygen intermediates and arachidonic acid metabolites (Aderem et al 1985). By contrast, CR-mediated phagocytosis does not elicit the release of either of these classes of inflammatory mediators (Aderem et al 1985). During mannose-receptor mediated phagocytosis of zymosan, the actin cytoskeleton is mobilised around the nascent phagosomes and proteins such as F-actin, talin, PKC, MARCKS and myosin 1 are recruited (Allen et al 1996). However, in contrast to FcγR and CR-mediated phagocytosis, vinculin and paxillin are not recruited to mannose receptor phagosomes. This reinforces the notion that different phagocytic receptors send different signals to the actin cytoskeleton, which initiate a variety of different mechanisms for internalisation.

Fig 1.4 Phagocytosis (Adapted from Beron et al 1995)

(See over for diagram summary)



Summary of Fig 1.4 (Phagocytosis)

During phagocytosis, a particle is internalised and becomes sequestered in a membrane-bound organelle called a phagosome. The changes in phagosome composition during the maturation process involve extensive and selective membrane budding and fusion with several compartments of the endocytic and exocytic pathways. The newly formed phagosome exchanges membrane-bound and soluble components with early endosomes by means of tubular connections. The budding of transport vesicles from the phagosomal membrane also plats an important role from the transport of specific components. As a consequence of the inward and outward flux of components, the phagosome matures into a phagolysosome and becomes able to fuse with endocytic compartments (secondary lysosomes).
SINGLE ARROW = Vectorial transport among different compartments.
DOUBLE HEADED ARROW = Tubular connections allowing bi-directional flux of components between compartments.

1.5 The respiratory burst

The respiratory burst is a characteristic feature of neutrophils, eosinophils, monocytes and macrophages and is a consequence of the phagocytosis of microbes or the response to certain chemotactic mediators and is a highly specialised period of intense oxygen consumption.This usage is not linked to energy production but to the generation of powerful antimicrobial reactive oxygen species by the partial reduction of oxygen. This reaction is catalysed by activated NADPH oxidase, which causes the one electron reduction of O_2 to O_2^- (superoxide) at the expense of NADPH (Babior 1984).

$$O_2 + NADPH \xrightarrow{\text{NADPH OXIDASE}} O_2^- + NADP^+ + H^+$$

Most of this O_2^- reacts rapidly with itself, dismuting to produce oxygen and hydrogen peroxide.

$$2O_2^- + 2H^+ \xrightarrow{\text{SUPEROXIDE DISMUTASE}} H_2O_2 + O_2$$

At the same time, glucose is metabolised via the hexose-monophosphate shunt in order to regenerate the NADPH. These reactions take place in the cell membrane

and in the phagosome (internalised cell membrane). A glutathione dependent H_2O_2 detoxifying system is found in the cytoplasm of the phagocyte so that H_2O_2 is restricted to the extracellular region and the interior of the phagosome. H_2O_2 in fact is only weakly microbicidal and O_2^- is completely innocuous (Babior 1984), however they can be transformed to potent microbicidal species in a number of ways (see over).

1.5.1 Free radicals

The potent microbicidal oxidants of phagocytosis fall into two classes, firstly the oxidised halogens and secondly the oxidised radicals. The production of the former starts with the myeloperoxidase-catalysed oxidation of chloride to hypochlorite (OCl^-) by H_2O_2.

$$Cl^- + H_2O_2 \xrightarrow{\text{MYELOPEROXIDASE}} OCl^- + H_2O$$

Hypochlorite is the prototype for the oxidised halogens and is also a precursor for the chloramines, a group of microbicidal-oxidised halogens that are formed by the reaction between hypochlorite and ammonia or amine.

$$OCl^- + RNH_3 \longrightarrow RNH_2Cl + OH^-$$

The oxidising radicals on the other hand are formed in reactions involving O_2^-. The hydroxyl radical (OH^-) is thought to be made, by a metal catalysed reaction between oxygen and hydrogen peroxide, (known as the Haber-Weiss reaction) and is the principal toxic reactant formed along with singlet oxygen (O_2^-).

$$O_2^- + H_2O_2 \xrightarrow{\text{Fe or Cu}} OH^- + OH^- + O_2^-$$

Thus all of these reactions ultimately depend on the activation of the membrane bound enzyme NADPH-oxidase which is now given further scrutiny.

1.5.2 The Phagocyte NADPH-Oxidase

The NADPH-oxidase consists mainly of a multimolecular complex of proteins, all of which are necessary for O_2^- production. Associated with the plasma membrane is a cytochrome with unusual characteristics. It has a very low midpoint potential of –245mV (hence its name cytochrome b-245), although it is also called cytochrome b559 according to the wavelength of its α band of light absorption. This low midpoint potential enables it to react directly with oxygen to form superoxide. The cytochrome is a heterodimer consisting of two α membrane bound subunits, each with a molecular weight of 21Kda tightly bound to a β subunit which is 22Kda and these are termed $p21^{phox}$ and $gp91^{phox}$ respectively (Bokoch 1995). The membrane component of NADPH-oxidase has been isolated and characterised from macrophage membranes and it was concluded that it is identical to cytochrome b559 (Knoller et al 1991).

It has long been known that a flavoprotein is involved in electron transport from NADPH to oxygen and it is thought that $gp91^{phox}$ itself is this flavoprotein and has the NADPH binding site (Segal et al 1992). Cytochrome b559 contains two redox centres, FAD and heme, and electron flow from NADPH to oxygen is initiated by the interaction of cytochrome with two cytosolic components known as $p47^{phox}$ and $p67^{phox}$ and the small GTP-ases $p21^{rac1}$/$p21^{rac2}$ (Diatchuk et al 1997), which binds GTP with high affinity. $p21^{rac1}$ is more generally expressed than $p21^{rac2}$ and in vitro binding studies shows that it binds to $p67^{phox}$ (Dickman et al 1994), although it is not known whether this interaction plays a role in the activation of the oxidase either in a cell free system or in vivo.

1.5.3 The oxidase complex

Upon stimulation of a macrophage rac dissociates from its complex with rho-GDI (a regulatory protein and GDP-dissociation inhibitor) (Abo et al 1991), and translocates to the membrane simultaneously with $p47^{phox}$ and $p67^{phox}$. Oxidase activity has been reconstituted with recombinant $p47^{phox}$, $p67^{phox}$, $p21^{rac}$ and purified

recombinant flavocytochrome (Rotrosen et al 1992). Another cytosolic factor identified, is one termed $p40^{phox}$ which has been found to co-immunoprecipitate with $p67^{phox}$ and is considered to be specifically involved in the NADPH-oxidase because it forms a 1:1 complex with $p67^{phox}$, it has strong homology with $p47^{phox}$ and it translocates to the membrane in a flavocytochrome dependent manner (Wientjes and Segal 1995). $p40^{phox}$ is not required for activity in a cell free system and is possibly a bridge between the other cytosolic factors and the cytoskeleton. It may confer messages from signalling pathways to $p47^{phox}$ and $p67^{phox}$ or it may be a downregulator of the oxidase by occupying binding sites for $p47^{phox}$ and $p67^{phox}$, which are vacated upon activation. $p47^{phox}$ and $p67^{phox}$ both have two SH3 (src homology 3) domains and $p40^{phox}$ has one. These domains have been found to recognise a proline rich motif and such motifs are present in $p21^{phox}$, $p47^{phox}$ and $p67^{phox}$ giving numerous possibilities for protein-protein interactions. In vitro binding studies have indicated the following:

(i) $p40^{phox}$ binds to $p47^{phox}$ and $p67^{phox}$

(ii) $p47^{phox}$ and $p67^{phox}$ associate by an interaction between the proline rich domain on $p47^{phox}$ and the C terminal SH3 domain on $p67^{phox}$

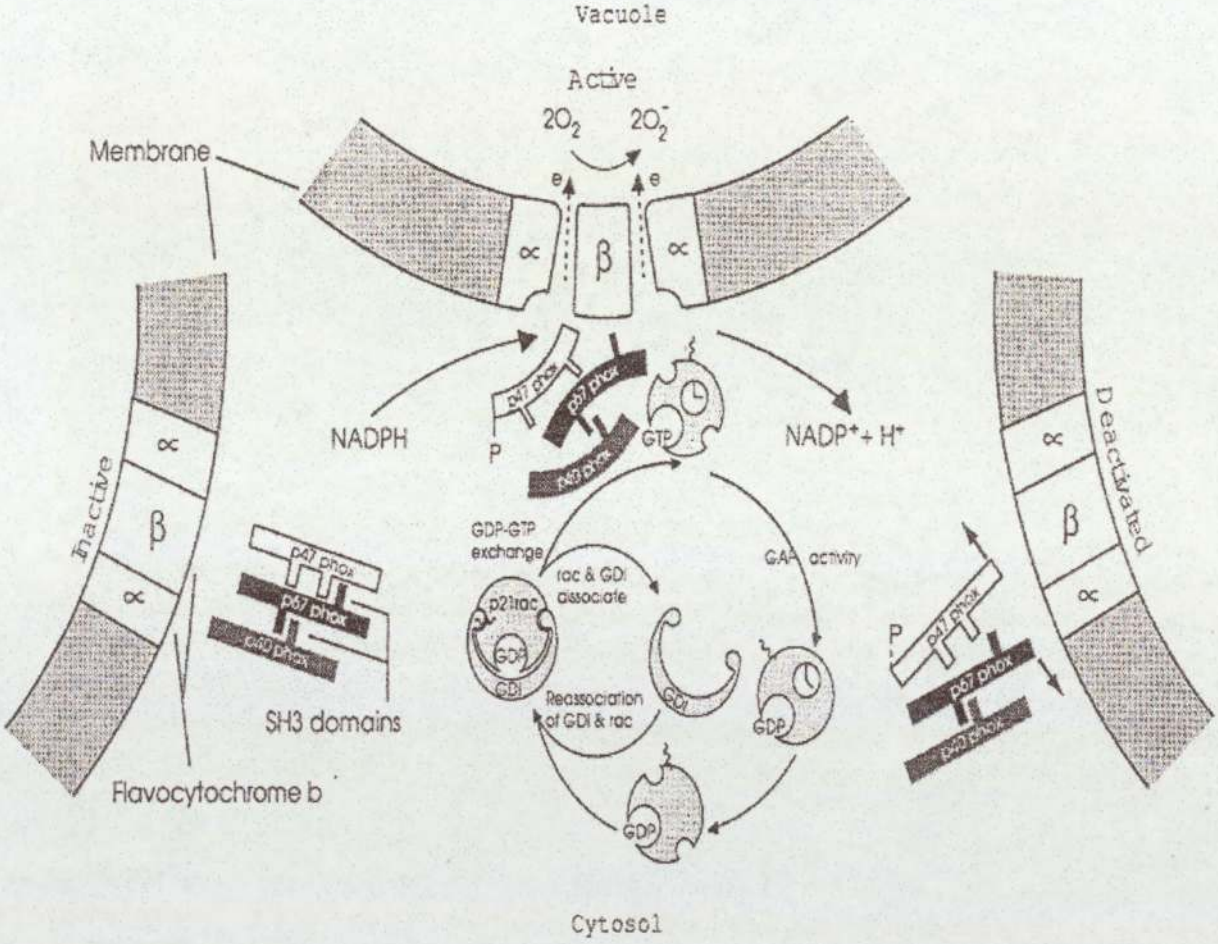
(iii) $p47^{phox}$ SH3 domains interact with a C terminal proline rich sequence in $p21^{phox}$ (Wientjes and Segal 1995).

It is well known that phosphorylation reactions are crucial for normal NADPH-oxidase activation as illustrated by the dramatic effects on O_2^- formation upon addition of inhibitors of protein kinases and phosphatases. It is of interest then that one target for activated $p21^{rac}$ has been identified as a kinase, termed pak-1 (Manser et al 1994). It might be therefore that rac regulates some of the key phosphorylation events that seem to be so important for O_2^- formation.

So in summary, activation of the NADPH-oxidase is associated with phosphorylation of the $p47^{phox}$ and $p67^{phox}$, subunits of the flavocytochrome and translocation of the cytosolic factors including $p21^{rac}$ to the membrane. $p21^{rac}$ appears to return to the cytosol, whereas the cytosolic phox proteins seem to remain

on the membrane even after oxidase activity has ceased. (see figure 1.5 for a schematic representation of the activation/deactivation process of the oxidase.

Fig 1.5 (Schematic model of the activation/deactivation process of the NADPH-oxidase) (From Wientjes et al 1995)



In order for NADPH-oxidase activity to manifest, p21rac must be in a GTP-bound state. Upon cell activation the p21rac (which is complexed with Rho-GDI), becomes associated with the plasma membrane and Rho-GDI dissociates this being controlled by the conversion of p21rac-GDP to rac-GTP. This process can occur because once rac is converted to the GTP bound form, it has a lower affinity for the Rho-GDI leading to the dissociation of this complex. P21rac then translocates to the membrane with kinetics similar to those of p47, p40 and $p67^{phox}$. The NADPH is thus reduced by this oxidase complex to $NADP^+ + H^+$ with the generation of superoxide (electron transport). Rac GTPase activating proteins (GAP's) then stimulate the conversion of Rac-GTP to the GDP bound inactive form, which leads to deactivation. So, the GTP/GDP bound state of Rac serves as a critical point for NADPH-oxidase activity in the intact phagocyte. The cytosolic phox proteins remain on the membrane even after activity has ceased.

1.5.4 Switching on/off of the respiratory burst

The understanding of the signal transduction pathways leading to the activation of the oxidase is far from complete. Macrophages display a wide variety of responses to agonists including shape changes, movement, phagocytosis, as well as the activation of the respiratory burst. It is therefore very difficult to dissect out those systems specifically related to the activation of the burst. The phagocytic signal transduction cascade can be broadly divided into three stages 1) Target binding to the phagocytic surface as previously discussed. 2) Proximal signaling events including tyrosine phosphorylation of the FcγR and activation of small molecular weight G-proteins and 3) more distal events including activation of protein kinase C. Protein tyrosine kinases are now recognised to be critical components in the regulatory pathways controlling many cellular functions and there is implicating evidence that tyrosine phosphorylation is an important signal for the activation of the phagocyte respiratory burst (see below).

1.6 Tyrosine kinases and the respiratory burst

Protein tyrosine kinases (PTK's) are enzymes, which phosphorylate tyrosine residues of protein substrates. A role for tyrosine phosphorylation in phagocyte stimulus response coupling was first suggested by Kraft et al (1987), who demonstrated the presence of tyrosine kinase activities in neutrophils and that these activities increased during myelocytic differentiation. Soon after, a number of groups reported that the chemotactic peptide FMLP and PMA, agents known to activate the respiratory burst induced tyrosine phosphorylation in phagocytes (Berkow et al 1989). There appears to be a high correlation between PKC activation, reactive oxygen species formation, enhanced tyrosine phosphorylation and the activation of PLA_2. In the intact cell protein tyrosine phosphorylation reflects a balance between phosphorylation and dephosphorylation processes. Thus vanadate, an inhibitor of protein tyrosine phosphatases enhances the PKC activation induced by zymosan (Goldman et al 1994). Further confirmation that PTK's are important for phagocytosis of particulate β glucans comes from genistein, which has been shown to generally inhibit the glucan-induced respiratory burst (Okazaki et al

1996). Vanadate generally promotes the zymosan induced respiratory burst indicating tyrosine kinase activation is an essential link in the chain between receptor and NADPH-oxidase activation. However, (Conde et al 1995) found the opposite.

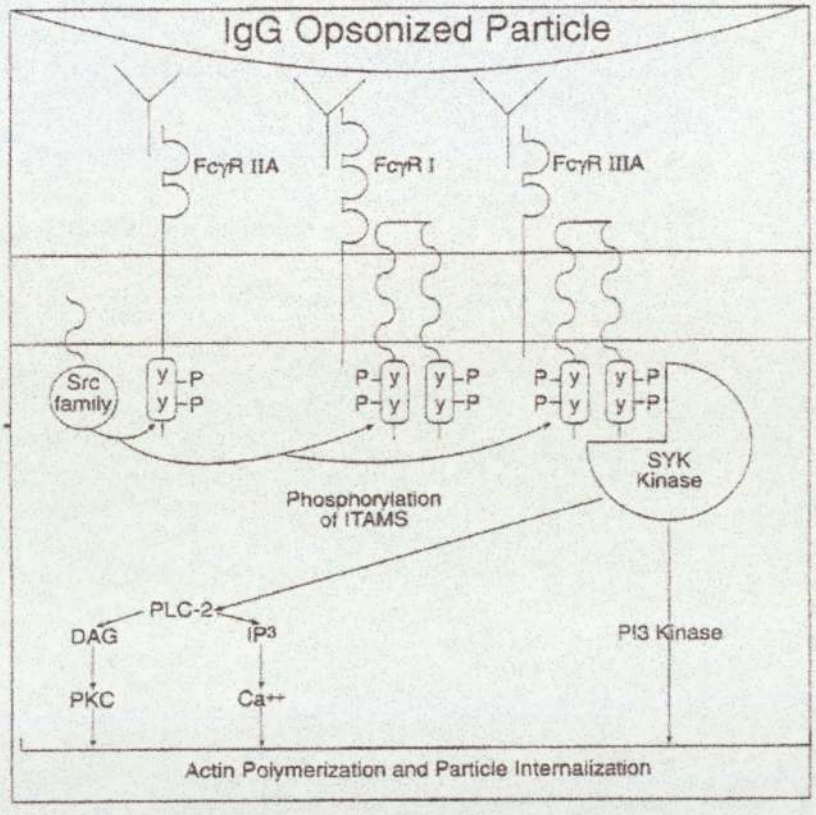
Signaling events necessary for FcγR responses are thought to be initiated by src and syk family kinases after FcγR cross-linking.

1.6.1 The syk and src family kinases

Tyrosine kinases of the src and syk families become activated and associated with specific recognition sequences known as immunoreceptor tyrosine-based activation motifs (ITAM's) contained within the intracellular domains of some of the FcγR subunits (Aderem et al 1999). Upon receptor clustering caused by particulate ligands, the tyrosine residues within the ITAM's become phosphorylated by a tyrosine kinase. It is likely that this initiating kinase is a member of the src family, since several src family members can be co-precipitated with FcγR I and II from a variety of cell lines (Jouvin et al 1994). Following phosphorylation of the tyrosine residues within ITAM's, another tyrosine kinase $p72^{syk}$ (syk) is recruited to these phosphorylated motifs where it activates syk kinase. This in turn triggers a plethora of pathways leading to cytoskeletal rearrangement by actin polymerisation and the release of inflammatory mediators. (See fig 1.6 over). This suggests that syk may be critical for coupling phagocytosis-promoting receptors to the cytoskeleton. It has been shown that macrophages derived from syk kinase deficient mice are defective in the phagocytosis of particles bound by FcγR. FcγR activation is also impaired in mice deficient in the src family kinases (Hck/Fgr/Lyn) (Crowley et al 1997). This observation provides additional strong evidence for a model of sequential activation of src and syk family tyrosine kinases by the FcγR's and possible involvement in the respiratory burst. How syk tyrosine kinase stimulates actin assembly is unknown although it is likely that a PI-3 kinase is involved.

Fig 1.6 Fcγ receptor signalling via ITAM domains (From Aderem et al 1999)

Receptor cross linking stimulates src family kinases to phosphorylate tyrosine (Y) residues within the ITAM domain of the FcγRIIA or within the dimerized γ subunits of FcγRI or FcγRIIIA. The tyrosine kinase syk is then recruited to the phosphorylated ITAM domain, and upon its activation, it is thought to mediate particle internilization by activating PI-3 kinase and phospholipase C.



1.6.2 The PI-3 Kinase

This kinase has been implicated in the regulation of endocytosis, intracellular membrane traffic and cell growth. It phosphorylates phosphoinositides at the D3 position of the inositol rings phosphatidylinositol, phosphatidylinositol 4 phosphate and phosphatidylinositol 4,5 bisphosphate (Greenberg 1995). PI3-kinase has shown to be responsible in a late step in macropinocytosis and phagocytosis of phagosome into intracellular organelles but not in the signalling for pseudopod extension, as the potent and irreversible PI3-kinase inhibitor wortmannin inhibited the endocytic step but did not appear to inhibit the formation of phagocytic cups (Araki et al 1996).

This suggests that actin polymerisation in the plasma membrane is not always coupled to pseudopod extension and distinct signals may be required for this function (Crowley et al 1997). Furthermore, wortmannin inhibited both increases in PI-3 kinase activity and particle ingestion (Ninomiya 1994). It has also been noted that there is striking similarity between the phagocytic defect in syk kinase-deficient cells and the stage at which phagocytosis was blocked by treatment of normal cells with wortmannin. In both cases, actin cups formed but could not close off to internalise the bound particle. It is therefore attractive to propose that the defect in syk kinase deficient macrophages results from a failure to activate a signalling pathway involving PI3-kinase. Syk kinase may be required to activate and/or to properly localise PI3-kinase activity during phagocytosis. Another group has hypothesised that one or more isoforms of PI3-kinase are required for maximal pseudopod extension but not phagocytosis, and that this kinase is needed for endocytic/exocytic membrane insertion as well (Cox et al 1999). How PI3-kinase may be acting in the phagocytic process is still to be fully elucidated. One possibility is suggested by the connection between PI-3 Kinase and small G-proteins involved in cytoskeletal rearrangements. The Rho family GTP-ases, rho and rac play important roles in the actin cytoskeleton to produce membrane ruffling, filopodia and stress fibre formation and all are essential for FcγR mediated phagocytosis (Cox et al 1997; Hackam et al 1997). A rapidly expanding body of evidence suggests that phospholipases play an integral role in phagocytosis by generating essential second messengers and these deserve some scrutiny (see over).

1.7 The Phospholipases

Phospholipases are hydrolytic enzymes that cleave phospholipids. The position of cleavage in the glycerol backbone identifies the phospholipase family, with the generation of unique products some of which have second messenger functions.

1.7.1 Phospholipase C

The oligomerisation of a G-protein coupled receptor is thought to be responsible for the activation of phospholipase C. Phospholipase C can be divided into four isoforms, PLCβ1 which is thought to be G-protein responsive, PLCγ1 and PLCγ2 which require tyrosine phosphorylation for activity and PLCδ whose activation mechanism is not fully understood. The products of PLCβ (inositol 1,4,5 trisphosphate IP3) and diacylglycerol DAG act as second messengers which initiate different intracellular activation pathways with different time courses and thus several secondary events, such as a rise in Ca^{2+} levels, serine/threonine kinase and tyrosine kinase activation. It has been demonstrated that when murine peritoneal macrophages are stimulated with zymosan, phospholipase C is activated via a G-protein-dependent or independent process leading to the production of DAG and IP3 (Moscat et al 1987).

1.7.2 Phospholipase D

This phospholipase catalyses the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline. PA can subsequently be converted to DAG through the action of an enzyme phosphatidic acid phosphohydralase (PAP-1) and thus promoting activation of protein kinase C (Lennartz et al 1999). Phagocytosis of complement opsonised zymosan and of IgG opsonised SRBC's, has been shown to activate PLD in macrophages and the inhibition of this process has been seen with PTK inhibitors genistein and herbimycin A (Kusner et al 1996; Kusner et al 1999). This demonstrates that PLD is activated during macrophage phagocytosis and that PTK's are involved in the stimulation of PLD. PLD activity in murine peritoneal

macrophages has also been shown to be sensitive to stimulation by zymosan and PMA (Meats et al 1993). A PA activated kinase has been described which phosphorylates the $p47^{phox}$ subunit of NADPH oxidase suggesting a possible role in the respiratory burst (Waite et al 1997). This finding is intriguing in light of data demonstrating that constitutively active PI-3K also leads to phosphorylation of $p47^{phox}$ (Didichenko et al 1996). Based on these results one could postulate a PI-3K $\rightarrow$ PIP_3 $\rightarrow$ PLD $\rightarrow$ PA $\rightarrow$ PA specific kinase $\rightarrow$ $P47^{phox}$ $\rightarrow$ NADPH-oxidase. A PI-3K $\rightarrow$ PLD pathway is also supported by studies demonstrating that PLD activity is enhanced in vitro by PI-4,5-bisphosphate (PIP_2) and PI-3,4,5-trisphosphate (PIP_3) (Exton1997).

PA has also been implicated in the mediation of a Ca^{2+} and PKC-independent release of arachidonic acid (AA) from macrophages by stimulating an AA selective phospholipase A_2 (PLA_2) (Fernandez et al 1994).

1.7.3 Phospholipase A_2 and Arachidonic acid

The liberation of arachidonic acid from membrane phospholipids is one of the earliest events that follow stimulation of macrophages with different agonists, with the subsequent metabolism of free AA into leukotrienes, prostaglandins and the thromboxanes which sustains the inflammatory response necessary for clearing pathogens (Lennartz et al 1999). It has been shown that peritoneal macrophages release AA when challenged by zymosan and that the phospholipase A_2 pathway controls AA release upon this stimulation with zymosan (Balsinde et al 1992). Further evidence has demonstrated the release of AA in murine macrophages in response to opsonised zymosan (Lloret et al 1996). Murine macrophages and related cell lines contain multiple phospholipase A_2 forms divided into three classes : the secreted Ca^{2+} dependent ($sPLA_2$), the cytosolic Ca^{2+}-dependent ($cPLA_2$) and the cytosolic Ca^{2+}-independent ($iPLA_2$) (Dennis 1994). It has recently been discovered that murine peritoneal macrophage adherence and spreading is dependent on the release of AA by at least two different PLA's, the $iPLA_2$ and the $cPLA_2$ indicating that Ca^{2+}-dependent and independent phospholipases are at least necessary for cell spreading (Teslenko et al 1997). A direct link between $cPLA_2$ and NADPH oxidase has been made using antisense oligodeoxynucleotides to downregulate $cPLA_2$

expression. This results in a decreased respiratory burst in response to zymosan (Dana et al 1998). Inositol phospholipid degradation and release of phospholipid bound AA can be induced in intact peritoneal macrophages by exposure to PMA a known activator of PKC suggesting the involvement of the PLA_2 pathway may be via PKC (Emilsson et al 1986; Wijkander et al 1989).

1.8 Protein Kinase C

PKC is a serine/threonine kinase and is very important in eliciting full physiological cellular responses through its synergistic action with calcium. The murine peritoneal macrophage has been found to express five PKC isoenzymes, (PKC α, β, δ, ε and ξ,) and that PKC α triggers arachidonic acid metabolism and eicosanoid synthesis when induced by zymosan giving a strong argument for PKC induces phosphorylation in the activation of PLA_2 (Huwiler et al 1993). As discussed earlier, phagocytes may recognise bacteria by lectin/carbohydrate interactions in the absence of opsonins. It has been shown that the lectin concanavalin A induced the release of arachidonic acid from murine peritoneal macrophages in a time and dose dependent manner and is highly dependent on Ca^{2+} availability, but appears to take place independently of PKC activation (Fernandez and Balsinde 1996). It has also been proposed that murine peritoneal macrophages could activate arachidonic acid release via a PKC independent pathway as reactive oxygen species O_2^- and H_2O_2 could be involved in the pathways that result in arachidonic mobilisation through phosphorylation and dephosphorylation processes, therefore stimulating specific signalling pathways (Martinez et al 1996).

The chelation of intracellular calcium has been shown not to affect the release of arachidonic acid from murine peritoneal macrophages upon stimulation with phorbol myristate acetate (PMA) or opsonised zymosan. However, the removal of calcium from the extracellular medium has shown a 30-50% decrease in arachidonic release from PMA and opsonised zymosan-stimulated macrophages indicating that

both calcium-dependent and independent mechanisms contribute to this process (Lokesh et al 1985). It can be seen that receptor agonists appear to initiate two distinct signal transduction sequences, one is Ca^{2+} dependent and leads to the activation of protein kinase C and the other is Ca^{2+} independent and does not involve PKC, and it has been shown that both sequences must be at their optimum for the activation of the respiratory burst response (Dewald et al 1988). It appears that arachidonic acid release from macrophages responding to both receptor-directed and soluble stimuli such as PMA can occur in the presence of extracellular calcium. It is possible that voltage gated Ca^{2+} channels may be required for the PLA_2 activation and corresponding arachidonic acid release, as verapamil (a voltage-dependent Ca^{2+} channel blocker) nearly abolished arachidonic acid release to both platelet activating factor (PAF) and zymosan (Fernandez et al 1991). Inhibitor studies in various cell types have demonstrated that arachidonic acid release and inositol 1,4,5 trisphosphate production are separately controlled and mediated through association of distinct G-proteins to PLA_2 and PLC respectively. This is shown by the coupling of zymosan and concanavalin A receptors on murine peritoneal macrophages to both polyphosphoinositide hydrolysis, and PLA_2 being inversely regulated by protein kinase activators (Balsinde et el 1990). The involvement of PKC in phagocytosis is tantalising since its major substrate the actin binding protein MARCKS (myristolated-alanine rich-C-kinase substrate), is rapidly phosphorylated during particle uptake. MARCKS and PKCα are recruited to the forming zymosan phagosome with kinetics similar to those of F-actin (Allen et al 1995). It is therefore proposed that PKC-dependent phosphorylation is an early signal required for zymosan phagocytosis and that MARCKS and PKCα have a role in phagosome maturation.

1.8.1 Intracellular crosstalk

The biochemical events involved in signal transduction facilitating phagocytosis can be seen to be a complex process involving many intracellular events. Fig 1.7 illustrates a hypothetical signaling pathway thought to be involved in the initiation of the respiratory burst. In the diagram FcγR ligation initiates a cascade of reactions, the most proximal which include phosphorylation of the receptor by protein tyrosine kinases (Greenberg 1995). Receptor associated PTK's phosphorylate PI-PLC-γ and assemble adaptor proteins (Grb2) and GTP exchange proteins (sos) for the docking of PI-3K and activation of low molecular weight G-proteins (Ras, Rac, Rho) (Sanchez-Majorada and Rosales 1998). The IP_3 and DAG products of PI-PLC-γ result in a rise in $[Ca^{2+}]i$ and activation of PKC to membranes, while DAG acts as a cofactor for a membrane associated PKC. Ras activation regulates a MAPK pathway for activation of PLA_2, which generates the AA necessary for the respiratory burst (Marshall 1995). Rac and Rho mediate membrane ruffling and stress fibre formation respectively and both are required for phagocytosis (Cox 1997; Hackam 1997). The polyphosphoinositide products of PI-3K have a plethora of effects, including PLD activation (Kusner 1996; Exton 1997), effects on membrane trafficking (ie membrane ruffling) (Barker et al 1995; Derman et al 1997), formation of endosomes (Li et al 1995), exocytosis of intracellular vesicles (Barker et al 1995), closures of phagosomes (Araki et al 1996) and activation of the novel PKC isoforms (Derman et al 1997; Palmer et al 1995).

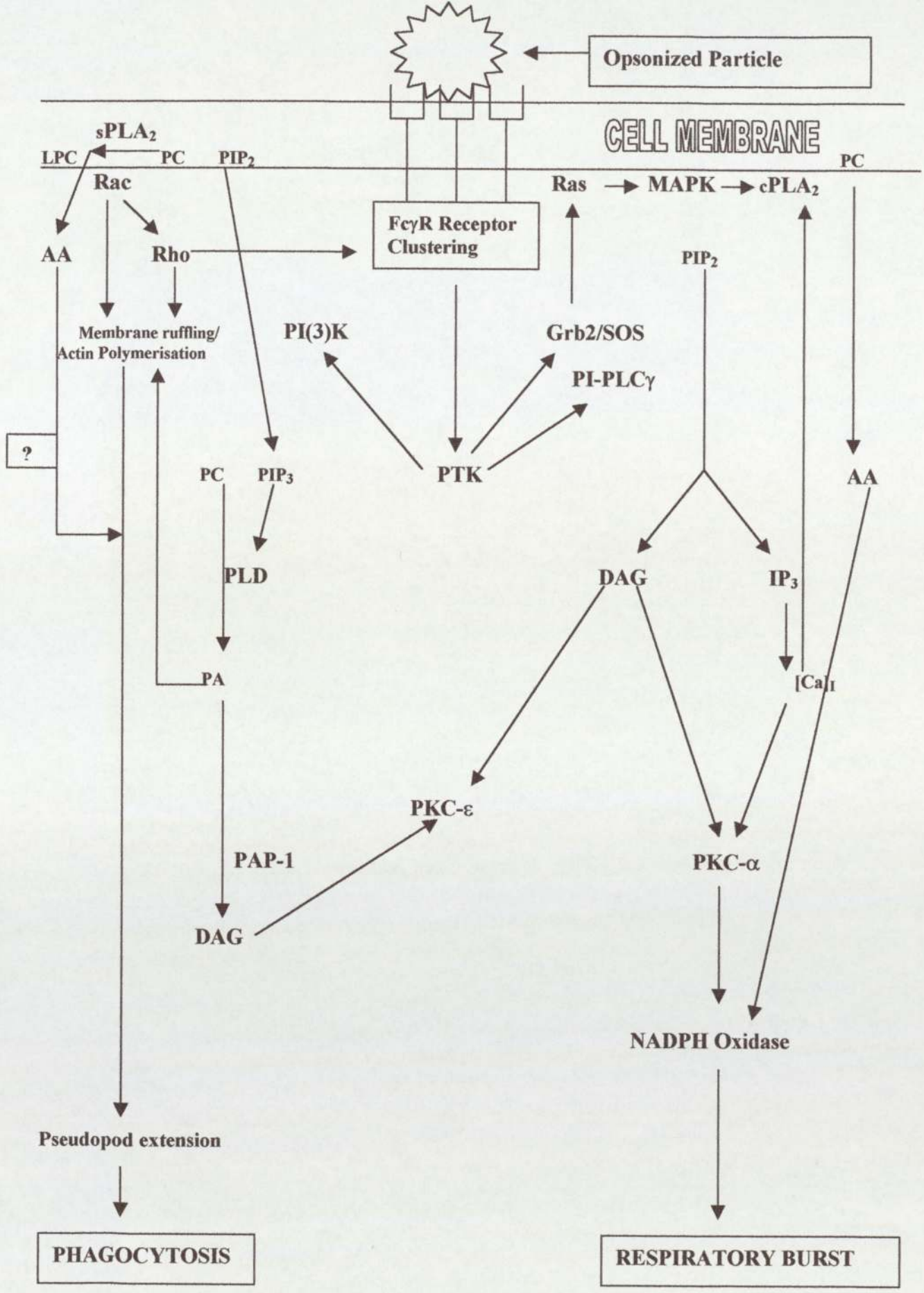


Fig 1.7 Phagocytic signal transduction
(Adapted from Lennartz 1999

1.8.2 Calcium mobilisation and phagocytosis

Intracellular calcium ($[Ca^{2+}]i$) is an important second messenger for a range of phagocytic functions including chemotaxis and phagocytosis itself. The possibility has been suggested that changes in the ($[Ca^{2+}]i$) by stimulation of C3bi and Fcγ receptors may play an important role as a signal mediator in the activation of the O_2^- production system and phagocytosis. There seems however to be conflicting reports of this as it was reported that a generalised or total cytoplasmic rise in ($[Ca^{2+}]i$) above resting levels did not occur in phagocytosing murine peritoneal macrophages under buffering conditions accepted as physiological and is therefore not necessary for phagocytosis (McNeil et al 1986). This involved populations of adherent peritoneal macrophages and it is possible that temporal asynchronous calcium transients in different cells might go undetected. However, it has been shown that when using single cells as well as population studies that there are two independent signalling mechanisms for phagocytosis in glycocholate elicited murine peritoneal macrophages. One is a ($[Ca^{2+}]i$) dependent and PKC-independent mechanism for Fcγ receptor mediated phagocytosis, and the other is an extracellular calcium and PKC-dependent mechanism for non-specific phagocytosis (Hishikawa et al 1991). In the absence of external calcium, the FcγR mediated calcium response has been seen only to be partially blocked suggesting that release of calcium from internal stores represents a substantial portion of the observed calcium signal, with a decrease in ($[Ca^{2+}]i$) inhibiting phagocytosis but also an increase in ($[Ca^{2+}]i$) using an ionophore also inhibiting phagocytosis (Young et al 1984). It has already been demonstrated that ($[Ca^{2+}]i$) increase is not necessary for the ingestion of single zymosan particles, as a rapid and transient elevation of ($[Ca^{2+}]i$) occurs during contact of a macrophage with zymosan, but following ingestion of the particle the ($[Ca^{2+}]i$)returns to levels indistinguishable from basal levels (Meagher et al 1991). When these macrophages were allowed to spread in a frustrated attempt to phagocytose the substrate without coming into contact with zymosan, bursts of repetitive spikes were observed (Meagher et al 1991). The significance of oscillations observed in the spreading macrophage is unknown except for assumptions that spreading is akin to frustrated phagocytosis. It seems clear that early ($[Ca^{2+}]i$) elevation achieved in particle contact is important in the phagocytic

event that follows. It is therefore important to understand the temporal relationship between the calcium pulse and events of functional significance, which it may mediate during phagocytosis. One such event may be phagosome-lysosome fusion, however kinetic analysis has shown that in macrophages, none of the steps leading from particle binding to eventual phagosome-lysosome fusion is regulated by ($[Ca^{2+}]i$) in a rate limiting way (Zimmerli et al 1996).

1.9 Aims

Although the identification of critical signalling molecules is progressing rapidly the challenge for the future is to understand the complex interplay between the enzymes and second messengers that result in the highly coordinated and specific process of phagocytosis. The purpose of this study is to try and establish a clearer picture to the intracellular pathways involved in the process of phagocytosis using mouse peritoneal macrophages as the in vitro model. In the sequence of events that lead to O_2^- generation, the precise roles for tyrosine phosphorylation/dephosphorylation, G-protein, PLC, PLA_2, PLD, PKC, Ca^{2+}, and PI-3 kinase are far from fully clarified. Moreover, signals from various bacterial components other than LPS have barely been investigated as is the case for neurotransmitters often found in nerve endings adjacent to macrophage virgin territory. Furthermore, the role of the adenylate cyclase/c-AMP system has not been conclusively defined.

2. Materials and methods

Mouse peritoneal Mϕ's and the human promonocytic cell line U937 were employed to investigate signalling pathways involved in the activation of the oxidative burst. Thus cells were exposed to particulate matter e.g. zymosan to elicit the release of reactive oxygen intermediates which in turn were measured by chemiluminescence. Opsonised zymosan-induction of U937's proved unsuccessful as no measurable response was observed by chemiluminescence. As a consequence, pre-incubation with interferon γ was employed to prime the U937's for a more enhanced response upon challenging by opsonised zymosan. This method proved to be expensive however, and was discontinued. It was therefore decided to use murine peritoneal macrophages as an in vitro model, as it was known that these macrophages did not require priming. Comparative studies between these two cell lines showed that the murine macrophages produced a more powerful and rapid oxidative burst.

2.1 Adherent cells

To prepare peritoneal macrophages for cell culture, adult male MF1 out-bred mice were killed by cervical dislocation under ether anaesthesia. The animal was layed on its back, swabbed with 70% ethanol and using sterile scissors and forceps an incision was made along the midline to remove some fur to expose the abdominal wall. A 10ml syringe and 0.5x16mm, 25G needle (Terumo) was then used to inject 10mls of pre-warmed supplemented sterile RPMI-1640 medium into the peritoneal cavity. The medium was supplemented with L-glutamine 2mM, 10% foetal bovine serum and antibiotic/antimycotic solution containing penicillin, streptomycin and amphotericin B at concentrations of 100IU/ml, 0.1mg/ml and 25μg/ml respectively, (Sigma). A gentle massaging of the abdominal wall was then undertaken for approximately 2 minutes to release Mϕ's into suspension. The medium was then removed using the same 10ml syringe and placed into a 15ml conical tube (Sarstedt). Another 10 mls of supplemented medium was injected in the same manner and the procedure repeated to remove as many macrophages as possible. The combined volume of both the lavages was around 15mls.

The cell suspension was then centrifuged at 1500rpm for 10 minutes and the resulting supernatant was removed. Pre-warmed supplemented medium was then added (5mls)

to the pellet, which was resuspended and dispensed into a 50x18mm petri dish (Sarstedt) containing 5mls of pre-warmed medium. The cell suspension contained in the petri dish was then incubated in a humidified incubator with a 95% air/5%CO_2 atmosphere for 24hrs to allow the macrophages to adhere.

The next day, non-adherent cells were removed from the petri dish by decanting the medium and washing the surface of the dish with 1ml of normal supplemented medium. The adherent peritoneal macrophages were then mechanically removed by adding 1ml of supplemented medium and then scraping the dish surface using a sterile scraper. (Orange Scientific). This 1ml suspension was then placed in a 15ml conical tube and centrifuged at 1500rpm for 10 mins. The cell pellet was then resuspended in another 1ml of supplemented medium and cell numbers assessed using a haemocytometer. Normally duplicate samples were counted and the yield was usually $1.5 - 2.5x10^6$ cells per ml.

2.1.1 Non-adherent cells

The human myelomonocytic cell line U937 (Sundstrom and Nilsson 1977) was maintained in supplemented RPMI-1640 medium. The cells were primed using 1ml of human recombinant interferon gamma (human rIFNγ) (R&D systems) at a concentration of 10,000 IU/ml by adding 300µls to a $75cm^2$ tissue culture flask (Nunc) containing 30mls of U937 cells seeded at a density of $1x10^5$/ml. This resulted in a final incubation concentration of 100IU/ml.

The cells were maintained by passaging once every 3-4 days by decanting them into a sterile 50ml universal (Sarstedt) and centrifuging for 10 minutes at 1200rpm. The resulting cell pellet was re-suspended in 5mls of medium and then counted using a haemocytometer. The cell number was adjusted to $3x10^6$/ml and 1ml of cells was added to 30mls of medium in a $75cm^2$ tissue culture flask. The U937 cells were thus seeded at a density of $1x10^5$/ml and incubated in a humidified incubator in a 5%CO_2/95 air atmosphere at 37^0C.

2.1.2 Freezing and thawing cells

Long-term storage of the U937's was achieved by freezing $1x10^7$ cells in 1ml of freezing medium (Sigma) containing 10% Dimethyl Sulphoxide (DMSO) in a 1ml

cryovial (Sarstedt). The cryovial containing the cells was then placed in a polystyrene box and covered with cotton wool and then stored for 24hrs at -70^0C. This was then transferred to a liquid nitrogen cell bank at -196^0C. During the resurrection of cells they were quickly thawed in a water bath at 37^0C, washed twice in fully supplemented medium and resuspended in 10mls of supplemented medium containing 20% FBS in a 25cm^2 tissue culture flask (Nunc) and placed in a humidified incubator as before. After the Mϕ's had fully re-established their normal growth regime, they were cultured in RPMI-1640 medium containing 10% FBS.

2.2 Microscopy

2.2.1 Phagocytic index and detection of phagosomes

Morphological indications of phagocytosis were assessed by mixing 300µl of Mϕ's (containing 1x10^6 cells) with 100µl of opsonised zymosan (5mg/ml). The cell/particulate mixture was gently centrifuged for 10 minutes (1500rpm).

After a 30-minute incubation in a water bath at 37^0C the cell particle mixture was washed twice in 5mls of un-supplemented medium and then resuspended in fully supplemented medium at a concentration of 5x10^5 cells/ml. 100µls of cells were then cytospun for 3 minutes at 300rpm. The slides were then removed and air dried for 10 minutes and then fixed with Diff-Quick (Baxter-Dade). The number of cells containing phagosomes was counted by independent observers under oil immersion at a magnification of x1250 using a Leitz microscope.

2.3 Assessment of respiratory burst using chemiluminescence

2.3.1 Mechanism of chemiluminescence

Chemiluminescence is a simple and reliable method of measuring the release of reactive oxygen species during the respiratory burst following phagocytosis. The oxidising species released by the murine peritoneal macrophages are highly active and react with susceptible biological or organic substrates to form excited compounds. These compounds relax to their ground state by photon emission. This energy release is in the form of light, which can be quantified at a peak wavelength of 425nm.

Chemiluminescence is thus defined as light produced from cells that has a chemical origin and is a function of phagocytic oxygenating activity (Stevens et al 1977). Native chemiluminescence can be amplified by the use of bystander molecules called chemiluminescent probes, which react with oxidising species to produce electronically excited products in high yield. Providing the number of bystander molecules is not limiting, chemiluminescence is directly related to the functional capacity of the macrophage present. A generalised equation for this reaction is as follows:

$$\underset{\text{reactant}}{\text{Reduced luminescent}} + H_2O_2 \longrightarrow \underset{\text{reactant}}{\text{Oxidised luminescent}} + \text{light (425nm)}$$

2.3.2 Chemiluminescent probe and assay preparation

The probe used in the experiments was lucigenin (Sigma), an acridinium salt which reacts specifically with superoxide (Allen et al 1986) to produce the electronically excited N-Methylacridone. Macrophage populations were harvested, re-suspended and counted as previously described. To each assay tube (Labtech) was added 100µl of (10^{-4}M) lucigenin, 100µl fully supplemented RPMI-1640 medium and 100µl of $1x10^6$ macrophages/ml. Preliminary experiments revealed this concentration gave maximum light generation. The final volume of each tube was always 500µl after the addition of activators, inhibitors and extra medium. Details of compounds utilised are described later. The tubes were then left for between 30-45 minutes in a water bath covered with foil at 37^0C to allow any spontaneous generation of light by the cells to abate. Readings were taken at regular time intervals using a Biorbit 1253 luminometer (Labtech) by gently shaking the tubes and placing them into the luminometers turret. The instrument reads the light production from the cells using a modified photomultiplier tube which it converts to millivolts and is then displayed on a digital readout in relative light units (RLU's). Experiments were initiated only after background levels were attained. Readings were taken after every 2-5 minute intervals over 30-45 minutes or as appropriate. The final results were expressed as (RLU) x 1000 per $1x10^5$ cells.

For inhibition studies, macrophages were pre-incubated with varying concentrations of inhibitors for 30, 60 or 120-minute intervals.

2.3.3 Cellular stimuli for chemiluminescence

The respiratory burst was activated by treating the cells with zymosan or opsonised zymosan, which is a preparation from the yeast cell wall of Saccharomyces cerevisiae. Alternatively, phorbol myristate aceteate (PMA) from (Sigma) was used which is a soluble chemical initiator of the respiratory burst

2.4 Measurement of cytosolic free calcium concentration using fluorescence

2.4.1 Fura-2-AM

To measure intracellular Ca^{2+} movements a fluorescent indicator dye was used known as Fura-2-AM. This indicator has been modelled on the widely used Ca^{2+}-selective chelator EGTA and it binds its target ion Ca^{2+} in a complex in which the metal ion is part of a ring like cage. See (fig's 2.1and 2.2) which represents the eight-coordinate tetracarboxylate chelation site for Ca^{2+} in the Fura 2 molecule.

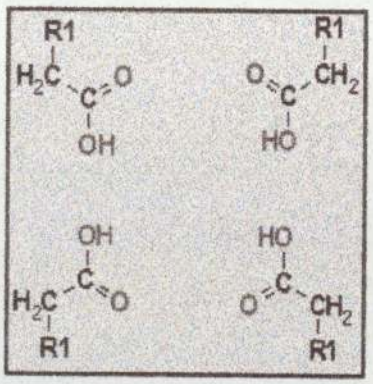


Fig 2.1 In this figure the chelation site is empty and ready to bind a calcium ion.

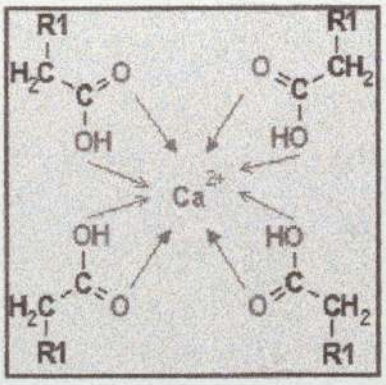


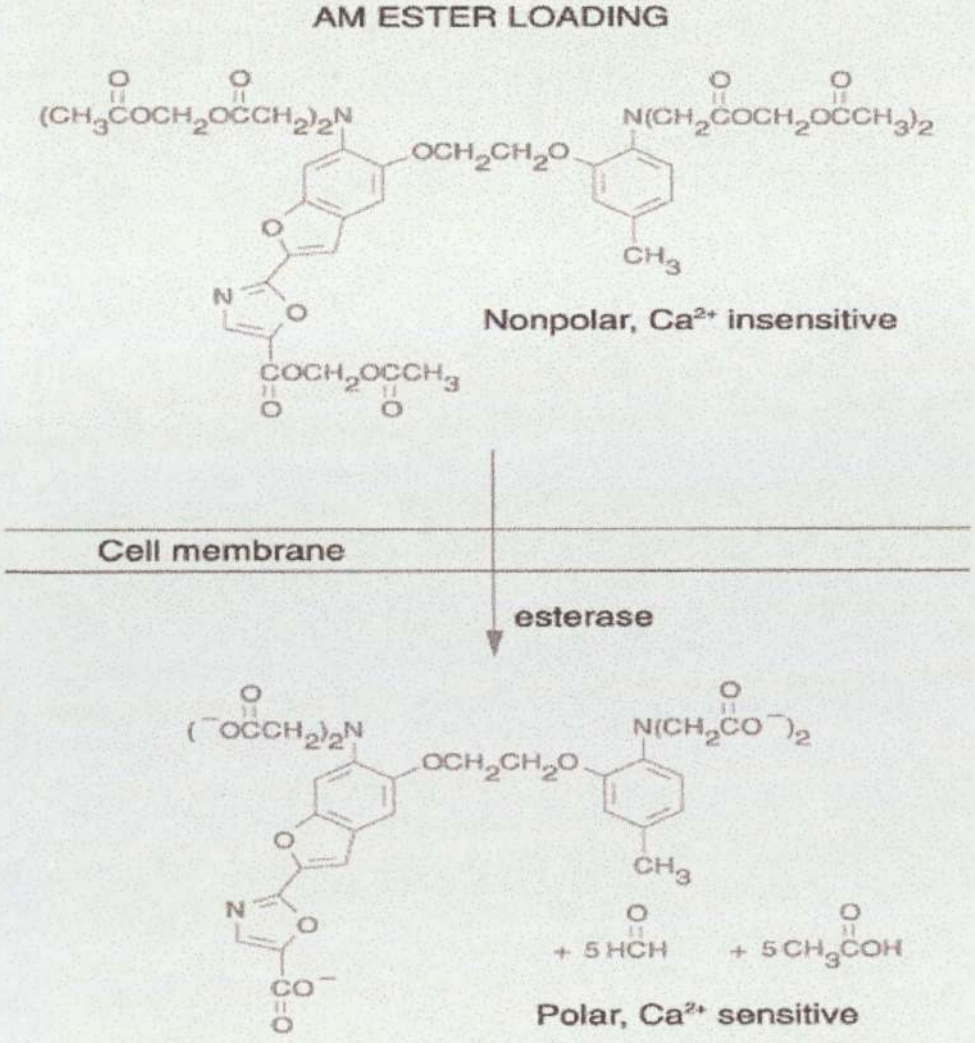
Fig 2.2 In this figure a calcium ion is shown chelated into the Fura-2 binding site.

As a chelating agent, Fura 2 is a highly charged molecule and as a result cannot penetrate the cell membrane. Modification of the carboxylic acids with acetoxymethyl (AM) ester groups results in an un-charged hydrophobic molecule that can permeate the cell membrane but is incapable of ion binding. Once inside the cell the AM ester bond is attacked by numerous non-specific endogenous esterases that hydrolyse the

liberation of acetic acid and formaldehyde (see fig 2.3). The esterases that mediate the conversion of the dye to the impermeable active form exist only inside the cell therefore once converted it is not possible for the active dye to escape from within the cell.

Fig 2.3 Ester loading

This is a schematic diagram of the processes involved in loading cells using membrane-permeant acetoxymethyl (AM) ester derivatives of fluorescent indicators, in this case fura-2. Note the generation of potentially toxic by products (formaldehyde and acetic acid).



2.4.2 Dual excitation of fura-2-AM

The fluorescence excitation maximum of Fura-2-AM shifts to a lower wavelength on binding of Ca^{2+} with negligible effect on the emission spectra, which allows Fura-2-AM to be used as a dual excitation indicator. For optimal separation of fluorescence due to the two forms of the indicator, Ca^{2+} free Fura-2-AM is usually monitored at 380nm and Ca^{2+} bound at 340nm.

2.4.3 Non-disruptive loading of fura-2-AM into macrophages

Murine peritoneal macrophages were harvested as previously described and seeded on to a large petri dish at a density between $1.0\text{-}1.5x10^6$ cells/ml. Prior to seeding, 5 9mm diameter x 2mm thick circular coverslips (Appleton Woods) were placed inside the petri dish in order to allow the cells to adhere to them overnight. On the day of loading, 20µl of a 1mM stock solution of Fura-2-AM in DMSO (Molecular Probes) was taken and mixed with 20µl of a 20% solution of pluronic F-127 in DMSO (Molecular Probes). The pluronic F-127 is a non-ionic surfactant polyol and was used as an additional non-toxic dispersion agent to facilitate cell loading. This mixture was then added to 960µl of cell incubation bathing medium in an Eppendorpf. This gave a final Fura-2-AM concentration of 20µM. A single coverslip and its adherent macrophages was then placed into a 50 x 18mm petri dish (Appleton Woods) and the 1ml of the Fura-2-AM/Pluronic F-127 mixture was added to the coverslip in the petri dish and incubated for 45 minutes in a humidified $5\%CO_2/95\%$ air incubator. The dish was gently shaken every 5 minutes to facilitate even loading. After incubation the coverslip was gently washed several times with bathing medium to remove any excess unloaded Fur-2-AM. The macrophages were now ready for fluorescent measurements.

2.4.4 Perfusion system

Glass coverslips were inserted on to a glass bottomed perspex chamber on the microscope stage. Small bore tubes at each end of the chamber allowed perfusion at about 2mls/minute. The input solution was gravity fed and a constant level of bathing solution was achieved by suction. A switchable syringe reservoir system was employed to allow the introduction of test agents into the system. All test agents were made up in bathing solution beforehand to final working concentrations and then put into the syringes ready for application.

2.5 Ratio-imaging of cytosolic calcium

2.5.1 Microscope

An epifluorescence microscope (Olympus Bx50WI) was used for imaging fluorescence emission from the macrophages. Firstly the macrophages were observed directly using transmission optics using a 40x-water immersion objective (0.8 N.A.). Next, a cluster of cells for observation was chosen and images were captured using a (Hammamatsu C4880) thermoelectrically cooled charge coupled device (CCD) camera.

2.5.2 Fluorescence illumination system

Excitation light was supplied with a monochromater (Till Photonics) with a xenon arc lamp light source. Light of 360 and 380 nm was applied alternatively and images of emission light above a wavelength were collected.

2.5.3 Image processing and temporal resolution

To minimise exposure times and maximise light capture levels digital binning was used. The procedure basically combined an array of adjacent pixels to form a larger super pixel prior to digitisation. For instance instead of an image comprising 640x480 pixels, a 4x4-pixel format was used resulting in an image of 160x120 pixels. A typical experiment was ready to proceed once a defined region of cells had been chosen on a coverslip and adjustment of exposure times and ratio settings were complete. The

number of images to be taken and the delay between images was also chosen depending on the duration of the experiment. The ranges selected were normally of 300 images at 20-second intervals. Before addition of test chemicals, a background level of fluorescence was taken in order to establish basal levels of Ca^{2+} within the cell. This was achieved by perfusing bathing solution over the cells for between 1-2 minutes. Data were collected using custom written automations using Openlab software (Improvision). Subsequent data analysis was achieved off line using custom written software using (Igor Pro).

2.6 Preparation of chemicals

Zymosan and Opsonised Zymosan

Zymosan A (Sigma) was opsonised by suspending 100mg in 10 mls of guinea pig complement (Gibco) in a 15ml sterile conical test tube (Sarstedt) and incubating at 37^0C for 30 minutes. This allowed the zymosan A to be coated in complement components e.g. C3. The preparation was then washed three times in phosphate buffered saline (ICN) and re-suspended in RPMI-1640 medium and stored at 4^0C. The zymosan was prepared in the same way by replacing the guinea pig complement with PBS.

Supplemented RPMI-1640 Medium

5mls of heat inactivated foetal bovine serum (FBS), 500µls of 200mM L-glutamine and 500µls of antibiotic/antimycotic solution containing 10,000 units of penicillin, 10mg streptomycin and 25µg of amphotericin B (all Sigma), were added to a 50ml universal

And RPMI-1640 was added to a volume of 50mls. This gave final working concentrations of 10% FBS, 2mM L-glutamine, 100units/ml penicillin, 0.1mg/ml streptomycin and 0.25µg/ml amphotericin B.

PBS

1 tablet was dissolved in 100mls of water to produce a physiological solution.

Lucigenin

The luminescent probe lucigenin (bis-N-methylacridinium nitrate was made up at a stock concentration of 10^{-3}M in PBS and stored in the fridge at 4^0C. On the day of an experiment a 1 in 10 dilution was made in PBS to give 10^{-4}M and used as the working preparation.

PMA, Ionomycin and FMLP

The phorbol ester, phorbol 12 –myristate 13-acetete (PMA) was used to activate the cells directly via protein kinase C (PKC). Stock solutions of PMA and its inactive analogue 4α-phorbol 12-13-didecanoate were prepared in DMSO at a concentration of 10^{-2}M as was the calcium ionophore Ionomycin and the chemotactic peptide FMLP. These solutions were then put into 0.5ml micro centrifuge tubes (Sarstedt) in 10μl aliquots and stored at -20^0C. Further dilutions were then made in RPMI-1640 medium prior to experimentation.

Forskolin

The adenylate cyclase activator forskolin (Sigma) was solubilised in DMSO at 10^{-2}M and stored as above.

Platelet Activating Factor

The phospholipid agonist and stimulator of G-protein linked cell surface receptors platelet activating factor (PAF) was dissolved in sterile RPMI-1640 medium at a stock concentration of 10^{-3}M and frozen in 0.5ml micro centrifuge tubes in aliquots of 50μls.

Inhibitors

The protein kinase C inhibitor bisindolylmaleimide I (BIM) (Sigma), protein tyrosine kinase inhibitor genistein and its inactive analogue daidzein (both Sigma) and the PI-3 kinase inhibitor wortmannin (Alexis Corporation) were all dissolved in DMSO at stock concentrations of 10^{-2}M. They were then aliquoted in 10μl amounts in 0.5ml micro centrifuge tubes and frozen at -20^0C. Further dilutions were made in RPMI-1640 medium as previously mentioned.

W7

W7 (Calbiochem) a calcium/calmodulin antagonist was dissolved in distilled water at a stock concentration of 10^{-3}M and frozen at -20^{0}C in 50µl aliquots.

Sodium Orthovanadate

The protein tyrosine phosphatase inhibitor sodium orthovanadate (Sigma) was firstly made up to a stock 100mM in 1M KOH. This was then diluted to 10mM by taking 5mls of the stock and adding 35mls of (KCl/MOPS) adjusting to a pH of 3.75 and adding distilled water to a final volume of 50mls. This was then stored in the fridge at 4^{0}C with a stability of several months. The experimental decavanadate was readjusted by removing 1ml of 10mM, adding 6mls of distilled water and adjusting to a pH of 7.5 using 1M KOH. This was then made up to a final volume of 10mls with distilled water. All procedures in the preparation of this compound were carried out on ice. The assay concentrations of decavanadate were made up in RPMI-1640 on ice until ready for use.

Amiloride and Benzamil

Amiloride and benzamil (Sigma) are blockers of the Na^{+}/H^{+} exchange pathways and were made up at stock concentrations of 10^{-3}M in RPMI-1640 medium and stored in aliquots of 50µls at -20^{0}C. Working concentrations were made in RPMI-1640 medium.

Thapsigargin

The compound thapsigargin (Calbiochem) is the most widely used inhibitor of sarco-endoplasmic reticulum Ca^{2+}ATP-ases (SERCAS) in mammalian cells and is a popular tool for elucidating the mechanisms of intracellular Ca^{2+} signaling. Ca^{2+} is released from the intracellular stores by preventing the pumps from counterbalancing the passive leak from the stores to the cytosol (Treiman et al 1998). Thapsigargin was made up at a stock concentration of 10^{-2}M in DMSO and aliqouted into 0.5ml micro-centrifuge tubes and stored at -20^{0}C until required. Working dilutions were made up in RPMI-1640 medium.

Fura-2-AM and Pluronic F-127

Fura-2-AM was provided as a 1mM stock in anhydrous DMSO and stored in a dessicator. Pluronic F-127 was provided as a 20% w/v emulsion in DMSO (both Molecular Probes).

Bathing Solution

145mM NaCl, 1mM Na_2HPO_4, 5mMKCl, 0.5mM $MgSO_4$, 5mM Glucose, 1mM $CaCl_2$ and 10mM Hepes was made up at a pH of 7.4.

EGTA

The Calcium chelator ethylenedinitrilo-tetracetic acid (EGTA) (Sigma) was dissolved in 1N NaOH and diluted to a working concentration of 2.5mM in PBS.

3. Results

3.1 Optimisation of experiments

The lucigenin-enhanced chemiluminescent method was used in all experiments investigating the respiratory burst in the murine peritoneal MΦ. Initially the most favourable assay conditions and methodology to provide the optimum response during the oxidative bursts were established. Further studies on the signal transduction pathways involved in the initiation of the oxidative burst could then be undertaken.

3.2 Statistical analysis

The level of significance of results was analysed utilising the Students paired t test.

3.3 Measurement of the respiratory burst in murine peritoneal MΦ

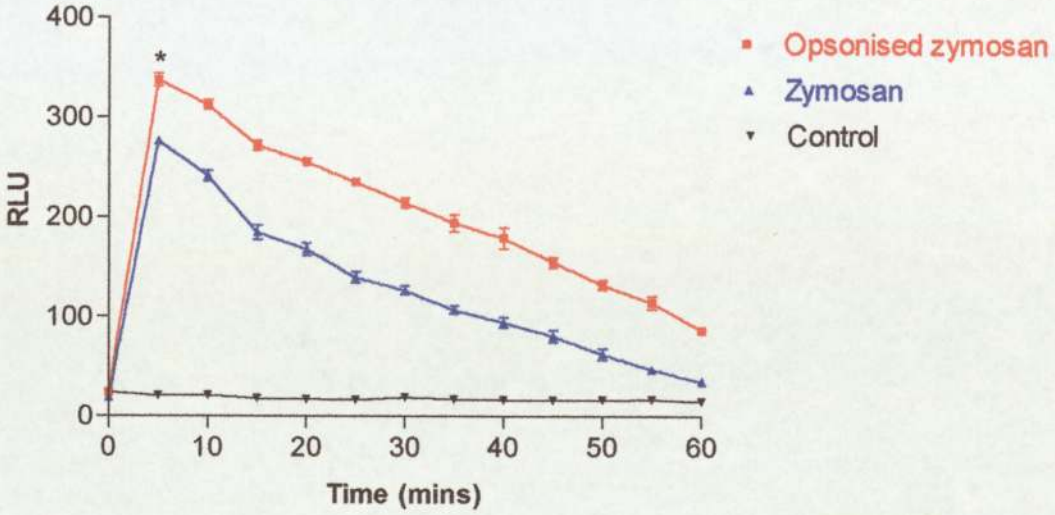
When murine peritoneal MΦ's were incubated with lucigenin and zymosan or opsonised zymosan at a selected dose of 2mg/ml an oxidative burst was produced (Fig.3.1).

The burst peaked after 5 minutes for both zymosan and opsonised zymosan with a subsequent steady decline to control values after about 60 minutes in both cases. The opsonised zymosan showed an enhanced response in comparison to the zymosan with peak values about 40% greater.

Dose-response curves were obtained to evaluate the optimum dose of zymosan or opsonised zymosan, which would be used for subsequent studies in MΦ activation (Figs 3.2a and 3.2b). At both 5 minutes (the max response) and 10 minutes after activation, a curve was evident for both zymosan and opsonised zymosan- elicited MΦ with the response beginning to level off at between 1.2mg-2.0mg/ml. The intensity of the respiratory burst was similar after 5 and 10 minutes at the specified dose range. The response to opsonised zymosan was about 35% greater than that to zymosan at both 5 and 10 minutes. A final assay concentration of 2mg/ml opsonised zymosan was therefore utilised in all subsequent experiments when required as an activator of the respiratory burst.

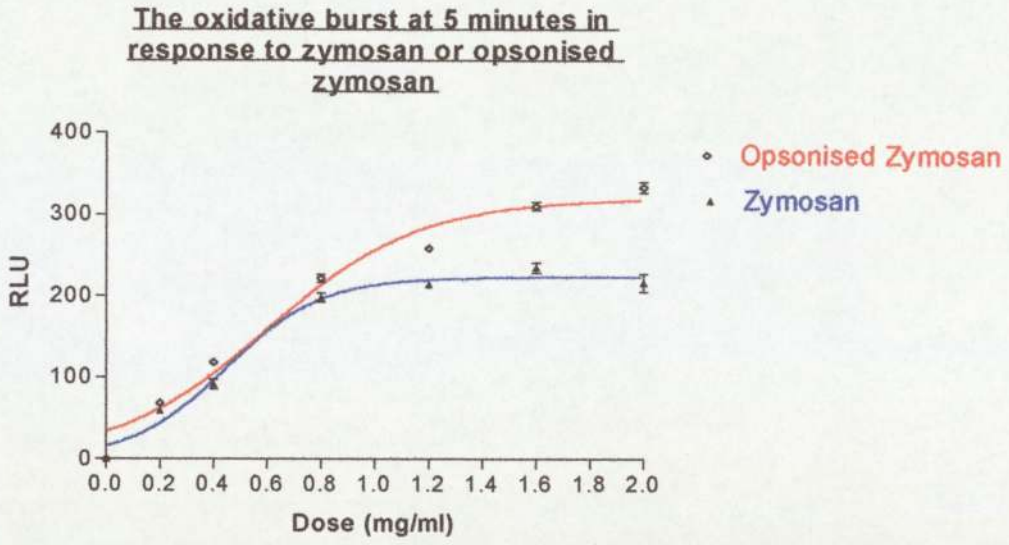
(Fig 3.1)

Respiratory burst of peritoneal M Φ challenged with zymosan or opsonised zymosan

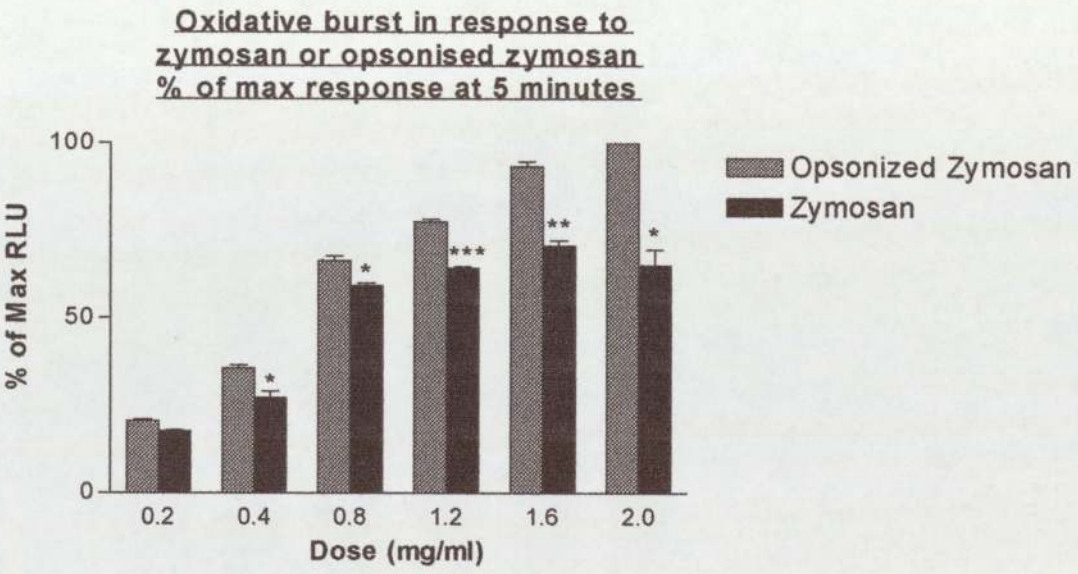


$1x10^5$ peritoneal MΦ's were incubated with zymosan or opsonised zymosan (2mg/ml) at time zero. The oxidative burst, expressed as relative light units (RLU), was assessed at 5-minute intervals thereafter. Values are derived from 3 independent experiments using cells from different mice. At the peak of the response the Mϕ's challenged with opsonised zymosan showed a significantly enhanced oxidative burst in comparison to zymosan ($p<0.05$) *.

(Fig 3.2a)



(Fig 3.2b)



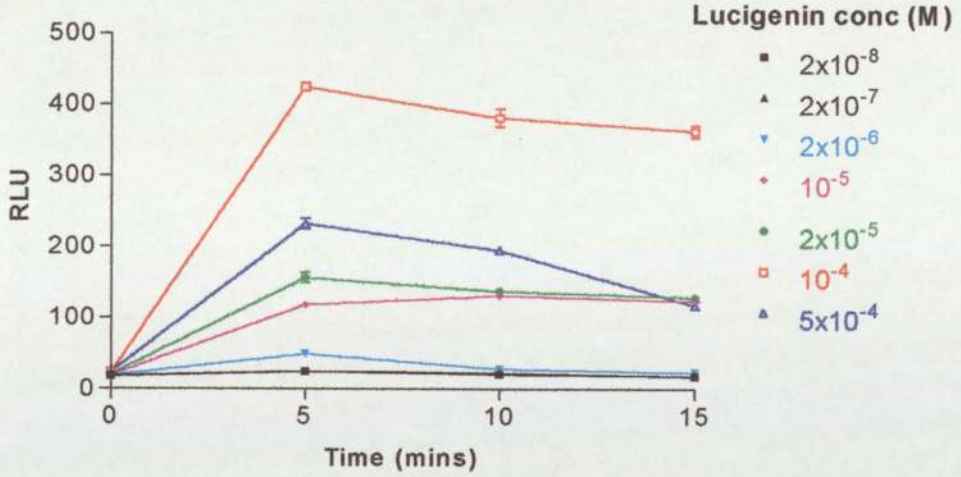
$1x10^5$ peritoneal MΦ's were incubated with Z or Opz at (0.2-2.0mg/ml) and RLU values measured after 5 minutes (Fig 3.2a). Points represent means +/- SEM derived from 3 independent experiments using cells from different mice. The % values of max response at 5 minutes were recorded (figs 3.2b). Zymosan showed a significantly reduced response in comparison to OpZ at the same concentrations ($p<0.05$)* ($p<0.01$)** ($p<0.001$)***.

3.4 Factors affecting chemiluminescence

To establish the optimum concentration of the chemiluminescent probe (lucigenin) the oxidative burst was measured using a range of concentrations from $2x10^{-8}$M to $5x10^{-4}$M/final cuvette concentrations. Maximum RLU values were obtained when Mϕ's were challenged in the presence of 10^{-4}M lucigenin (Fig 3.3); this concentration was adopted for all future experiments. Higher concentrations of lucigenin may be toxic since the magnitude of the burst was diminished at $5x10^{-4}$M lucigenin.

(Fig 3.3)

The effect of varying lucigenin concentrations on the respiratory burst elicited by opsonised zymosan

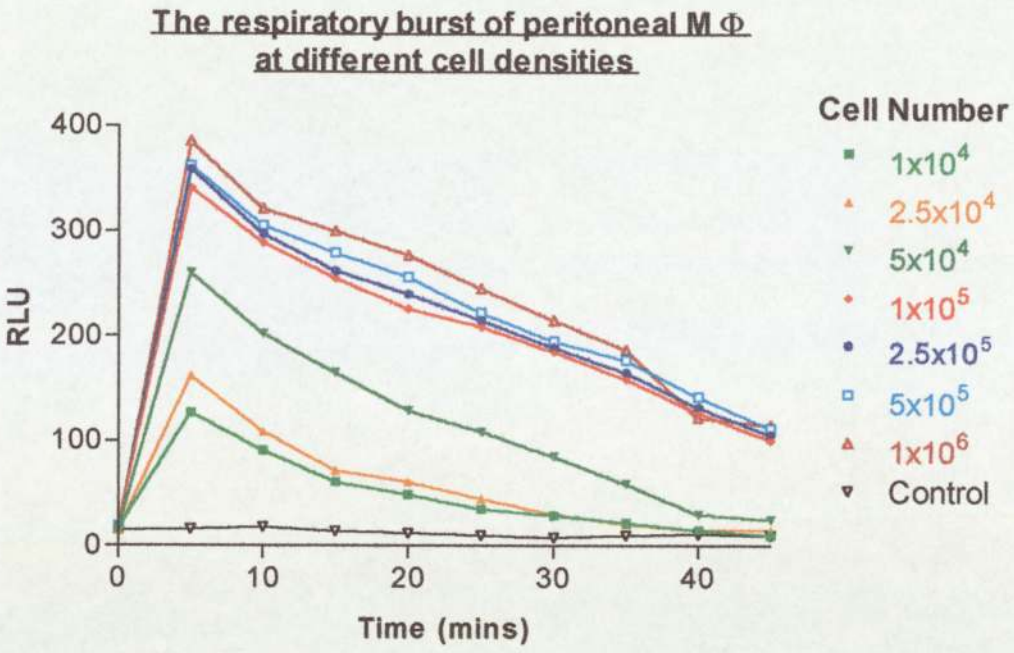


$1x10^5$ peritoneal MΦ's were incubated with OpZ (2mg/ml) in the presence of various concentrations of lucigenin. RLU values were measured every 5 minutes over a 15-minute period.

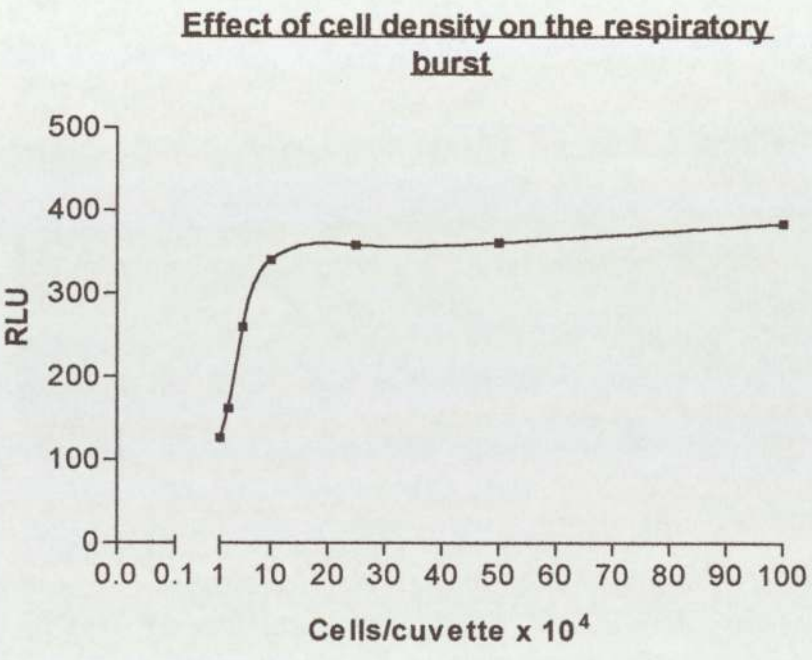
When Mϕ cell numbers were varied employing the previously established optimum concentrations of opsonised zymosan and lucigenin, RLU values progressively increased between $1x10^4$ and $1x10^5$ cells per cuvette (Fig 3.4a). Further increases in cellularity up to $1x10^6$ cells per cuvette resulted in little further apparent increase in response. Possibly the particle to cell ratio had become rate limiting or the lucigenin concentration was insufficient to capture all the H_2O_2 generated. The maximum respiratory burst of the Mϕ's after 5 minutes at different cell densities can be seen in

(fig 3.4b). In subsequent experiments therefore $1x10^5$ cells/cuvette was selected as the optimum cell density.

(Fig 3.4a)



(Fig 3.4b)

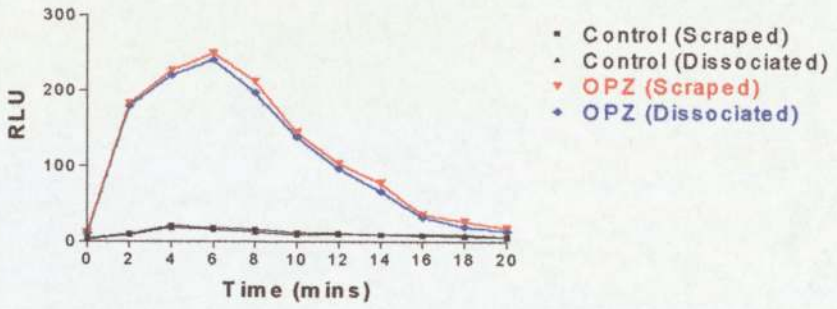


A range of peritoneal MΦ's between $1x10^4$-$1x10^6$/cuvette was incubated with OpZ (2mg/ml) and readings taken every 5 minutes for 40 minutes (Fig 3.4a). Values were derived from a single experiment. The RLU values of the maximum respiratory bursts (5 min) at varying cell densities are represented in (Fig 3.4b).

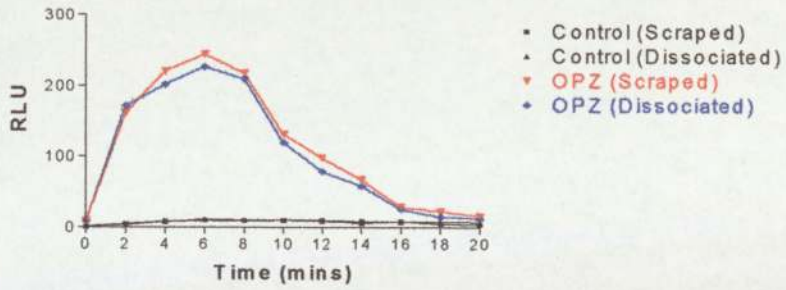
3.5 Harvesting technique

The experiments presented thus far used Mϕ, which had been freshly harvested after an overnight incubation. Thus they were detached from the plastic surface of the petri dish using a sterile mechanical scraper. Superficially this seemed crude and likely to cause trauma so their performance was compared to Mϕ released from their adherence using a cell dissociation solution. Cells freshly prepared using both techniques seemed to perform equally well when challenged with opsonised zymosan (Fig 3.5a). When the cells were preincubated at 37^0C for 30 or 60 minutes prior to opsonised zymosan application, the cells harvested by the two techniques were indistinguishable in their response. Some slight deterioration in response was noted with protracted preincubation (Fig's 3.5b and 3.5c). Neither technique seemed therefore to impose immediate or latent trauma upon the cells. Mechanical scraping was the harvesting technique used in all subsequent experiments on grounds of economy.

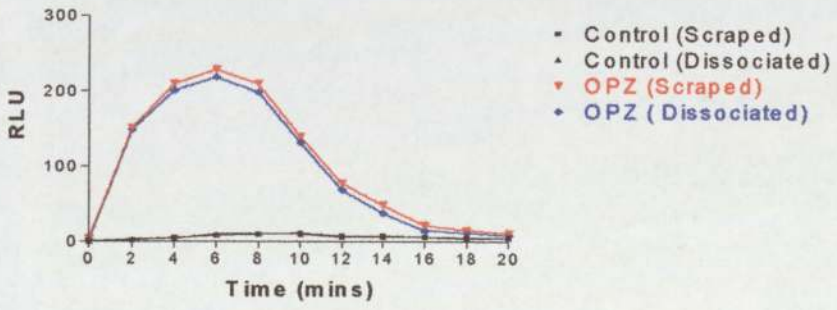
(Fig 3.5a) The respiratory burst of scraped or dissociated peritoneal MΦ 's without incubation



(Fig 3.5b) The respiratory burst of scraped or dissociated peritoneal MΦ 's at 30 mins



(Fig 3.5c) The respiratory burst of scraped or dissociated peritoneal MΦ 's at 60 mins



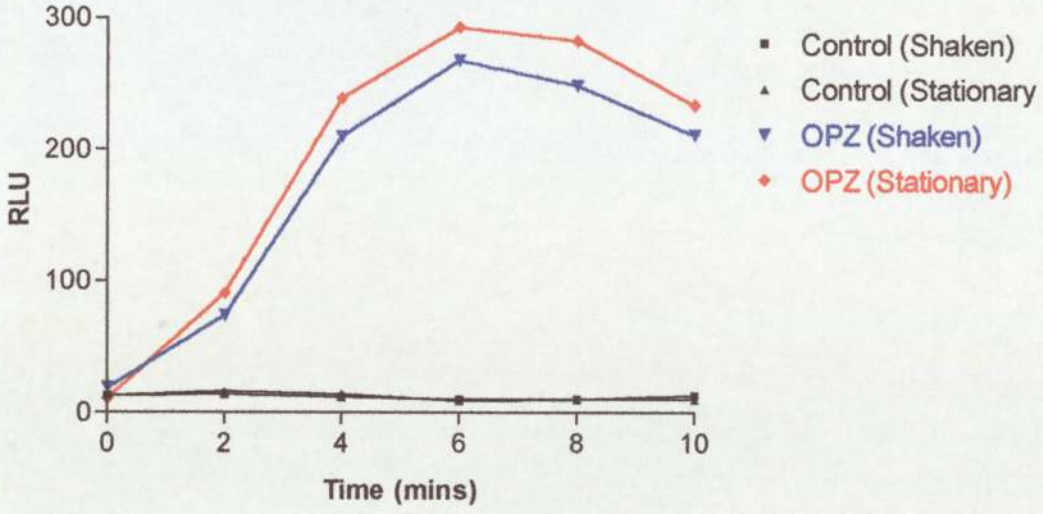
Peritoneal MΦ's were harvested by scraping or with cell dissociation solution and activated instantaneously (fig 3.5a) or after 30 or 60 minutes (figs 3.5b and 3.5c) with OpZ (2mg/ml) over a period of 20 minutes with readings taken every 5 minutes.

3.6 Agitation/oxygenation

Since future anticipated experiments would require the Mϕ's to be preincubated with a variety of inhibitors or antagonists it was important to try and ameliorate the time-dependent deterioration in performance during preincubation noted above (see Fig's 3.5a and 3.5c). During such a period cells would sediment and probably start to adhere to plastic surfaces and potentially become hypoxic. Consequently the performance of stationary Mϕ's was compared to those shaken at 2-minute intervals throughout the preincubation period to prevent adhesion and promote oxygenation. No significant differences in the magnitude of the oxidative burst in response to opsonised zymosan were noted between stationary and shaken cell preparations at any of the preincubation periods tested (Fig's 3.6a-d). A slight deterioration in the response to opsonised zymosan was noted for stationary and shaken cell preparations when the preincubation period extended to 60 minutes (compare Fig's 3.6a and 3.6d). Thus it would seem prudent to conduct and conclude further experiments within this time frame.

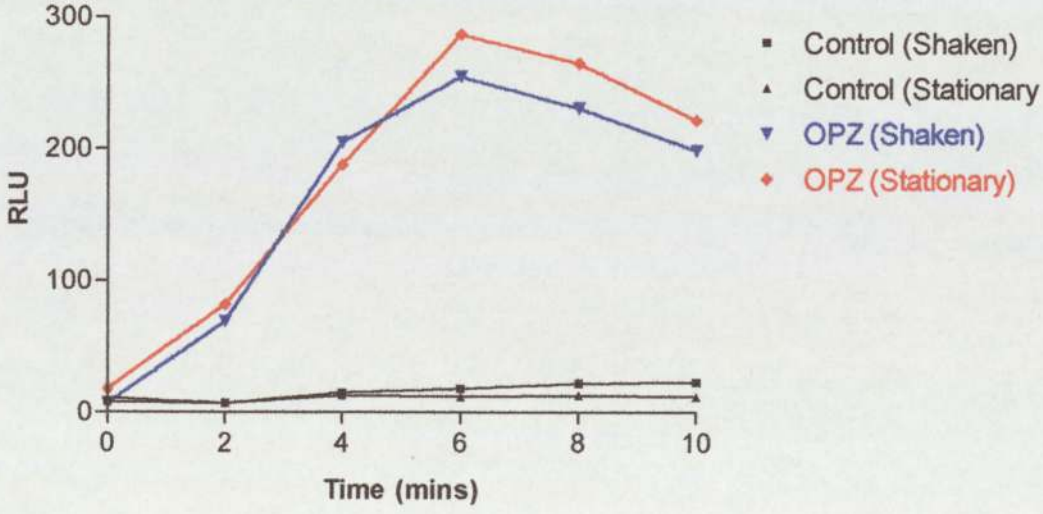
(Fig 3.6a)

Comparison of respiratory burst between shaken and stationary assay tubes after 15 mins pre-incubation at 37^{0}C

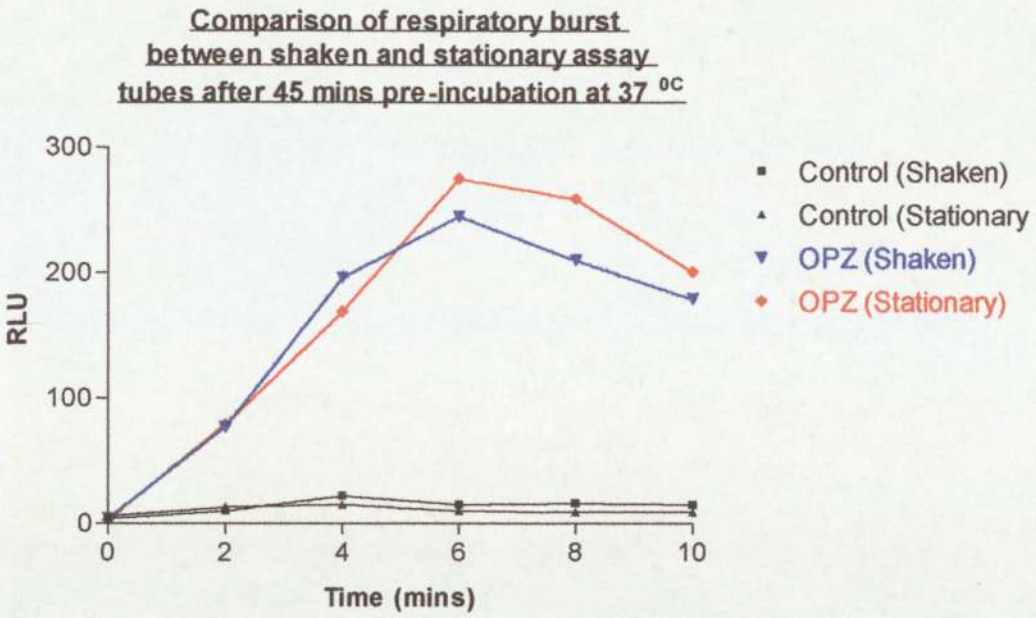


(Fig 3.6b)

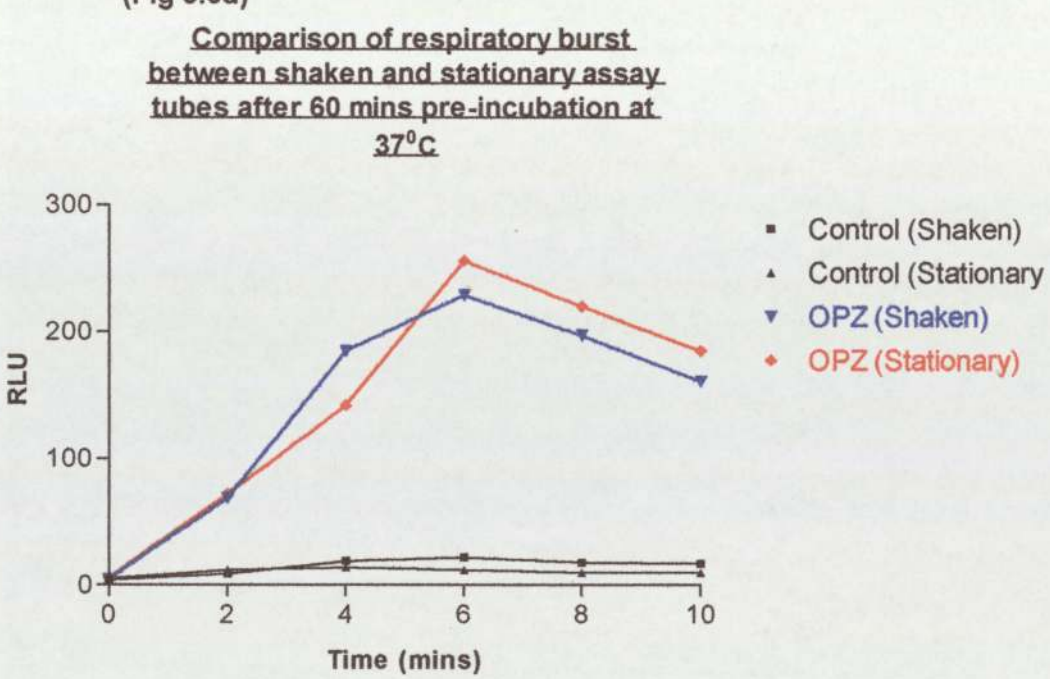
Comparison of respiratory burst between shaken and stationary assay tubes after 30 mins pre-incubation at 37^{0}C



(Fig 3.6c)



(Fig 3.6d)

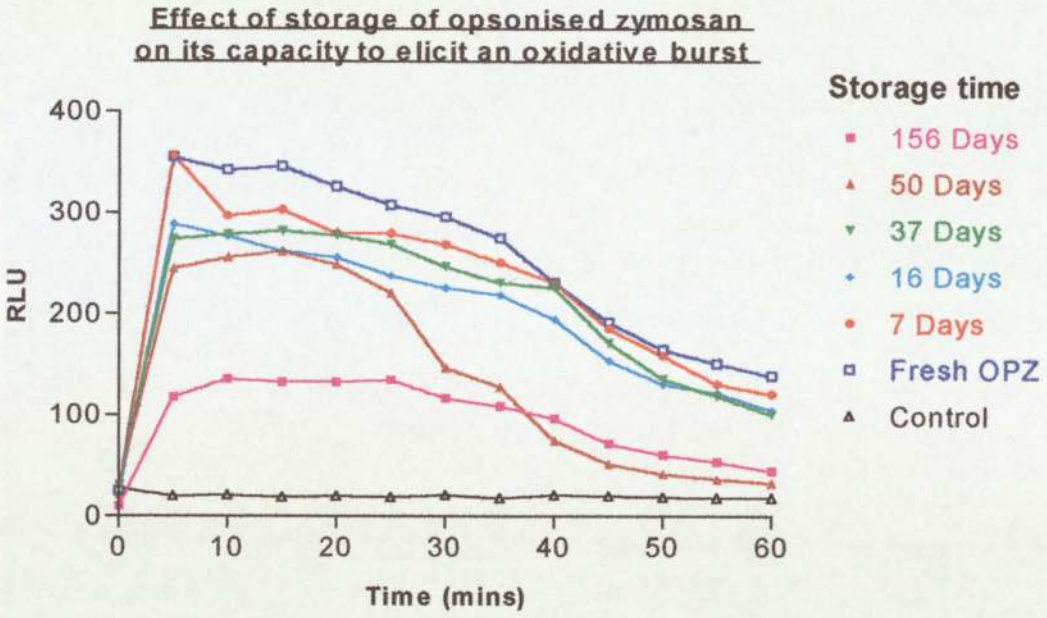


Peritoneal MΦ's were harvested and pre-incubated between 15-60 minutes and either manually shaken every 2 minutes or untouched. The MΦ' were then challenged with OpZ (2mg/ml) and readings taken every 2 minutes for ten minutes (figs 3.6a-3.6d). Values represent a single experiment using cells from the same mouse.

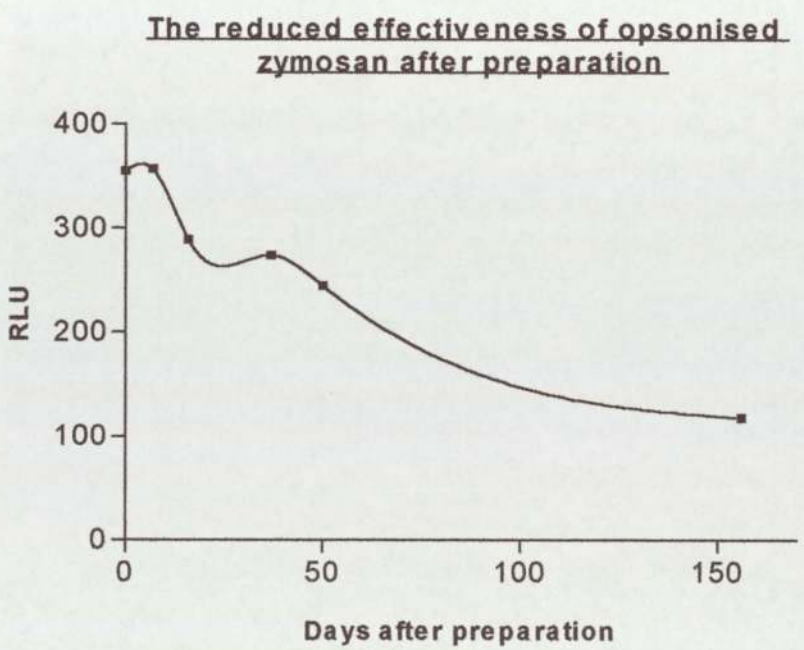
3.7 Longevity of opsonisation

Freshly prepared opsonised zymosan clearly elicited a greater burst than zymosan (Fig's 3.1 and 3.2) In some preliminary experiments these differences were not so apparent using zymosan/opsonised zymosan preparations which had been stored for protracted periods before use suggesting deterioriation might occur with storage. When this was investigated in a systematic manner storing opsonised zymosan at 4^0C for periods up to 156 days, it was clear that the magnitude of the peak oxidative burst declined with storage time (Fig's 3.7a and 3.7b).

(Fig 3.7a)



(Fig 3.7b)



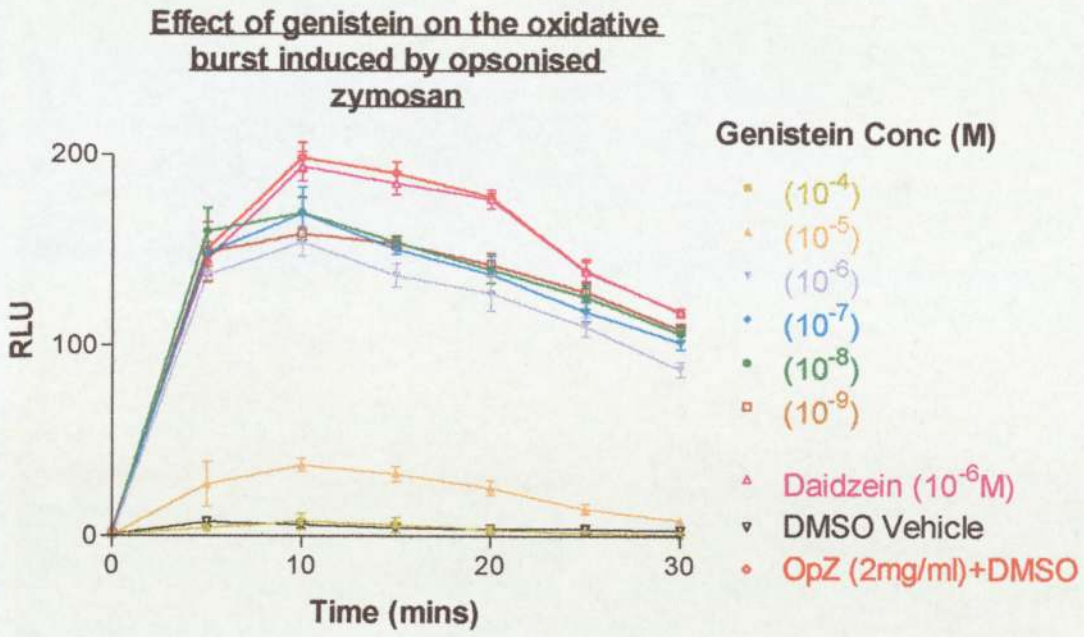
Peritoneal Mϕ's were incubated with opsonised zymosan (2mg/ml), which was freshly prepared or stored for various periods at 4^0C prior to use (Fig 3.7a). Values of the peak RLU response at 5 minutes after opsonised zymosan application at various times after preparation can be seen in (Fig 3.7b).

4. Signal Transduction

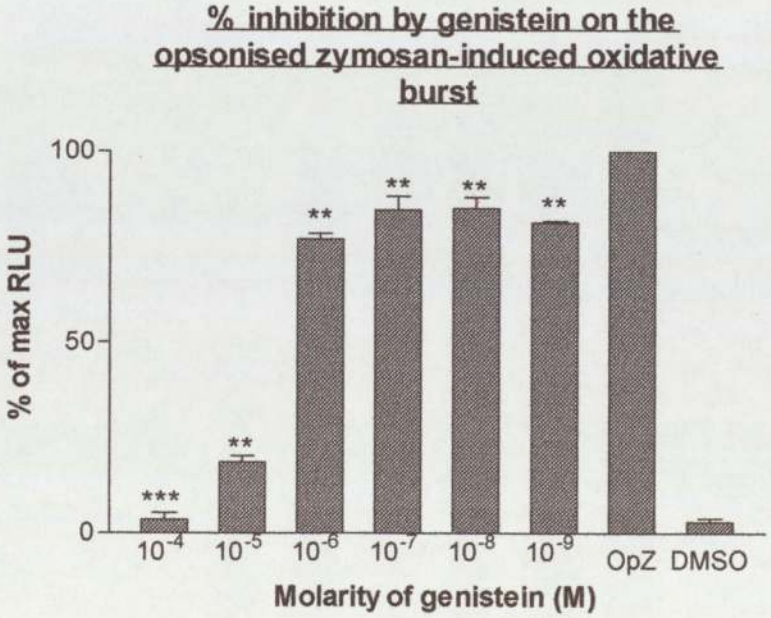
Experiments designed to elucidate the signal transduction pathways, which elicit the respiratory burst of murine peritoneal macrophages, are described in this chapter. The interaction or cross talk between different surface receptors and their associated second messengers is also considered.

As previously mentioned, Fcγ receptors initiate phagocytosis via their phosphorylated ITAM domains and tyrosine kinases are responsible for this (Aderem et al 1999). To investigate the role of PTK's in the respiratory burst triggered by zymosan or opsonised zymosan, the effect of the potent PTK inhibitor genistein was examined. Phagocytosis of these particles is coupled to the mannose and the C3b complement receptors respectively. Mouse peritoneal Mϕ were pre-treated with genistein for 30 minutes and then challenged with OpZ. There was a significant dose-dependent inhibition of reactive oxygen intermediate (ROI) production with almost total suppression at10^{-4}M to 19% inhibition at 10^{-9}M (Fig 4.0a and 4.0b). The inactive analogue daidzein did not exhibit any effects at any concentration (Fig 4.1). Genistein caused similar inhibitions when zymosan alone was used to elicit an oxidative burst. Thus tyrosine phosphorylation seems to be implicated in the oxidative burst triggered by ligand binding by all three receptor types. It is known that protein tyrosine phosphorylation is controlled by the balance between the activities of PTK's and of protein tyrosine phosphatases. Vanadate ions inhibit protein tyrosine phosphatases and should therefore potentiate the respiratory burst (Green et al 1994). Peritoneal Mϕ's were premixed with either 10^{-6}M or 10^{-7}M vanadate for 30,60 and 120 minutes and then challenged with OpZ. For all pre-incubation times there was a significant augmentation of the response to OpZ at 10^{-6}M vanadate (Fig's 4.2a-4.2c). The maximum potentiation was seen with 120-minute incubation where a 19% increase was noted. At 10^{-7}M the values were similar to those obtained with OpZ alone. This reinforces the idea that PTK's are required for the initiation of the respiratory burst, because de-phosphorylation would be curtailed by the vanadate leading to a greater or more protracted increase in tyrosine-phosphorylated proteins.

(Fig 4.0a)

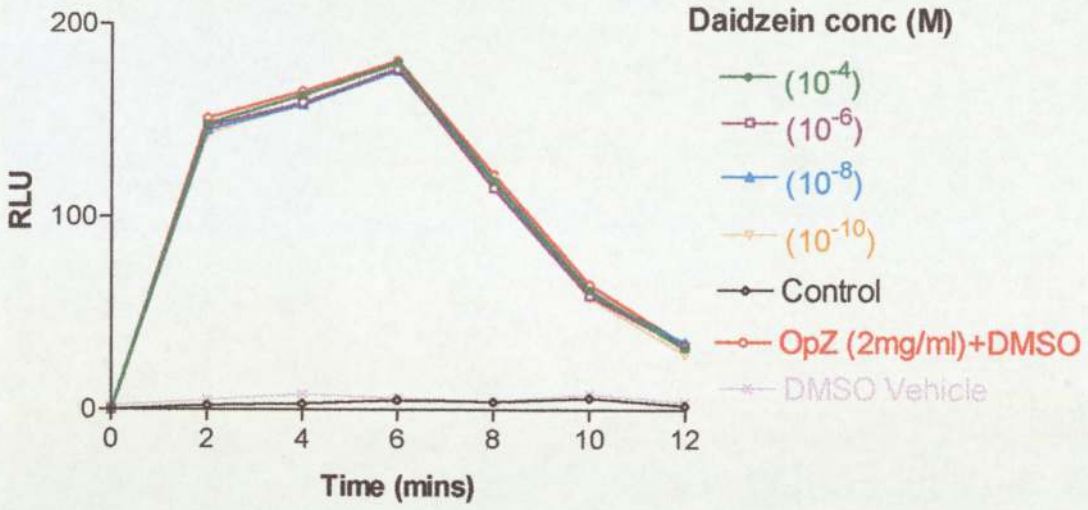


(Fig 4.0b)



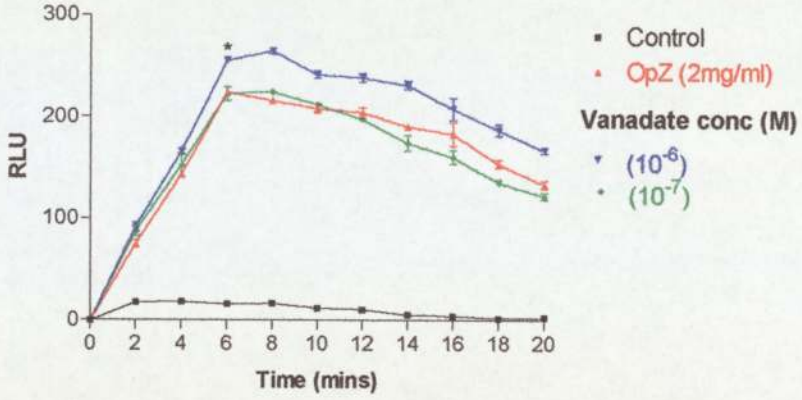
(Fig 4.1)

Effect of daidzein on the opsonised zymosan enhanced respiratory burst

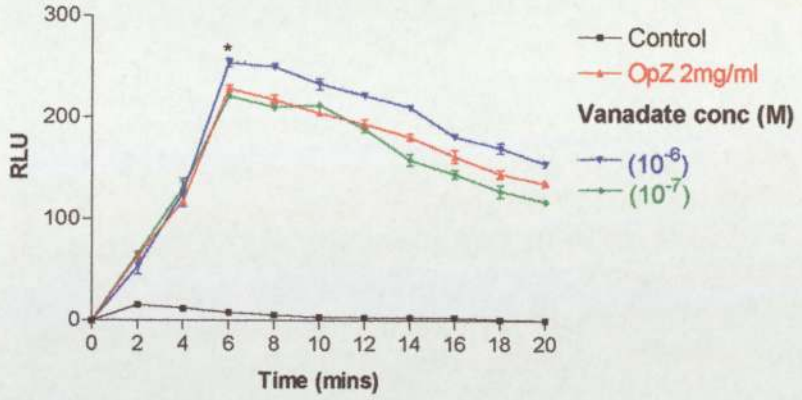


$1x10^5$ peritoneal Mϕ's/cuvette were pre-incubated for 30 minutes with the protein tyrosine kinase inhibitor genistein or its inactive analogue daidzein (Fig's 4.0a and 4.1). The respiratory burst was measured after the addition of OpZ (2mg/ml). The addition of OpZ and DMSO to Mϕ's alone was used as an activated positive control. Genistein was initially dissolved in DMSO, and Mϕ's were incubated with this solvent at a concentration of no greater than 0.01% so exposure would not be toxic (DMSO vehicle). Values represent mean +/- SEM derived from 4 independent experiments using cells from different mice. Genistein significantly reduced the opsonised zymosan-induced oxidative burst at a range of concentrations (Fig 4.0b). ($p<0.05$)* ($p<0.01$)** ($p<0.001$)***.

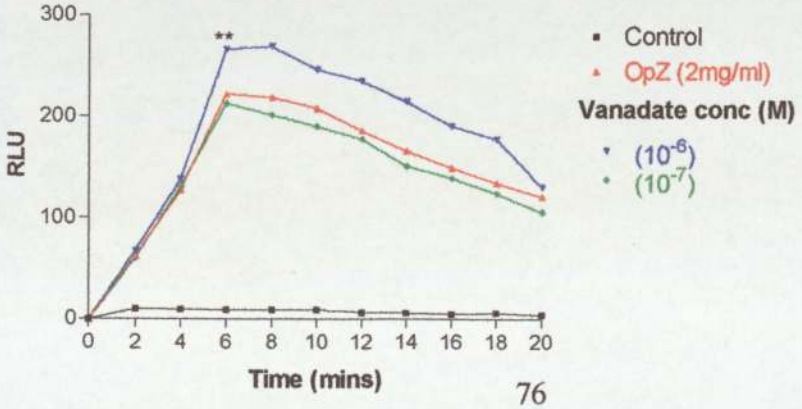
(Fig 4.2a) Opsonised zymosan enhanced respiratory burst of peritoneal MΦ after 30 minute pre-incubation with vanadate



(Fig 4.2b) Opsonised zymosan enhanced respiratory burst of peritoneal MΦ after 60 minute pre-incubation with vanadate



(Fig 4.2c) Opsonised zymosan enhanced respiratory burst of peritoneal MΦ after 120 minute pre-incubation with vanadate



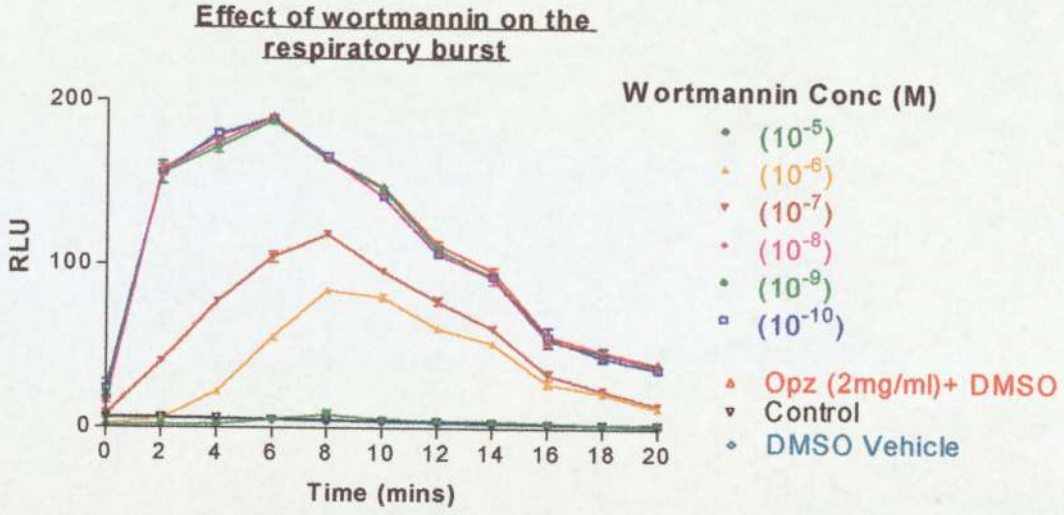
$1x10^5$ Mϕ's/cuvette were pre-incubated for 30, (a) 60, (b) or 120 (c) minutes with the protein tyrosine phosphatase inhibitor vanadate. Respiratory burst activity was initiated by the addition of OpZ (2mg/ml). The addition of OpZ (2mg/ml) to Mϕ's alone was used as a positive control. Values represent mean +/- SEM derived from 3 independent experiments using cells from different mice. Vanadate at 10^{-6}M showed a significant augmentation of the oxidative burst ($p<0.05$)* ($p<0.01$)**.

Recent evidence suggests that PI-3kinase participates in the signalling cascade of phagocytic receptors and is activated by many tyrosine kinase-linked receptors that trigger cytoskeletal re-arrangement during phagocytosis (Aderem et al 1999).

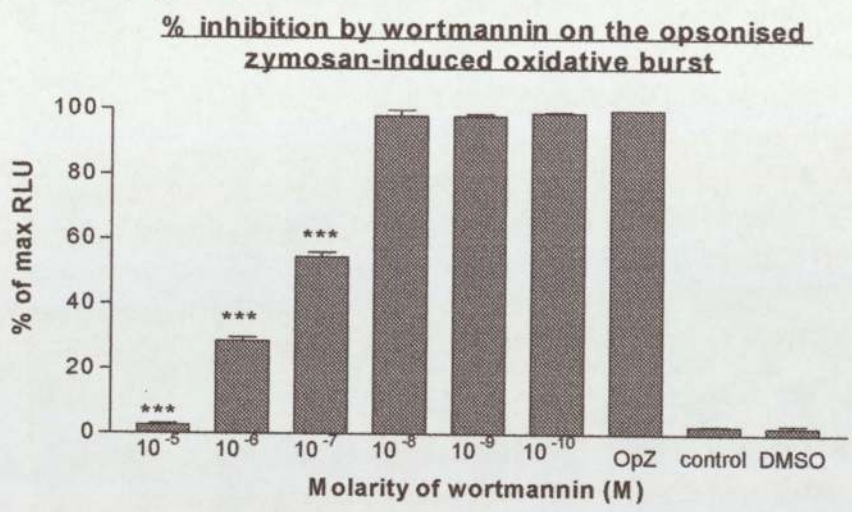
To study the possible involvement of PI-3 kinase on the oxidative burst, the potent and irreversible inhibitor wortmannin was utilised (Park et al 1997). Mϕ's were pre-treated with wortmannin (10^{-5} M to 10^{-10}M) for 30 minutes and then challenged with OpZ. A dose-dependent inhibition of the oxidative burst was observed from 10^{-5} M to 10^{-7}M. Lower concentrations had no effect (Fig 4.3a).

At 10^{-6}M and 10^{-7}M the burst was inhibited by 72% and 38% respectively with almost total suppression being seen at 10^{-5}M (Fig 4.3b). This would seem to indicate that the blocking of the phosphorylation of specific phosphoinositides at the 3-OH position of the inositol ring causes a reduction in the oxidative burst. This clearly emphasises the importance of PI-3 kinase in this signalling pathway.

(Fig 4.3a)



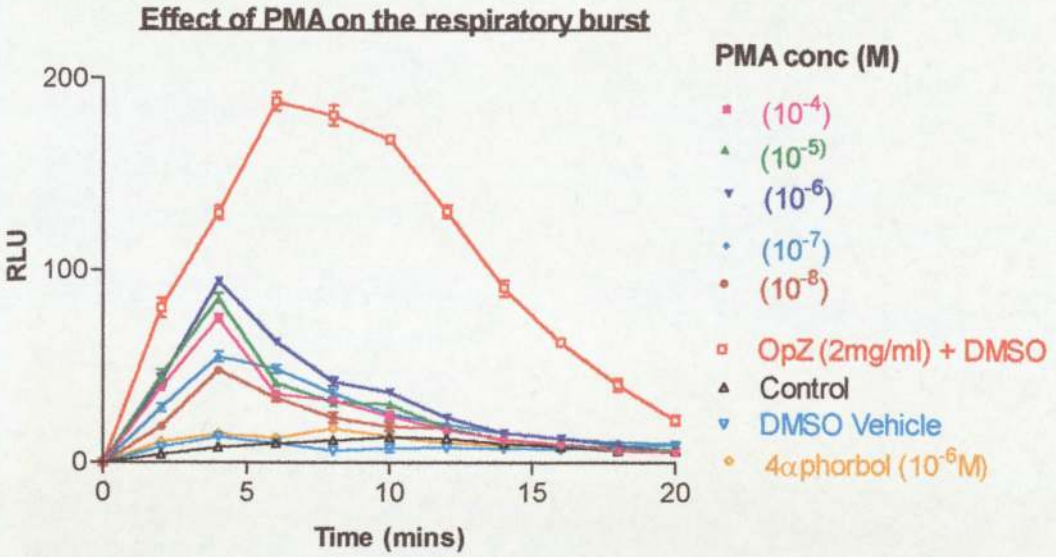
(Fig 4.3b)



$1x10^5$ Mϕ's/cuvette were pre-incubated for 30 minutes with the PI-3 kinase inhibitor wortmannin. The respiratory burst was measured after the addition of OpZ (2mg/ml). A positive control consisted of Mϕ's and OpZ + DMSO. Wortmannin was initially dissolved in DMSO and Mϕ's were incubated with DMSO of < 0.01% as a control (DMSO vehicle). DMSO at this concentration and below is non-toxic to cells. Values represent the mean +/- SEM from 3 independent experiments using cells from different mice. Wortmannin significantly reduced the opsonised zymosan-induced oxidative burst ($p<0.001$)***.

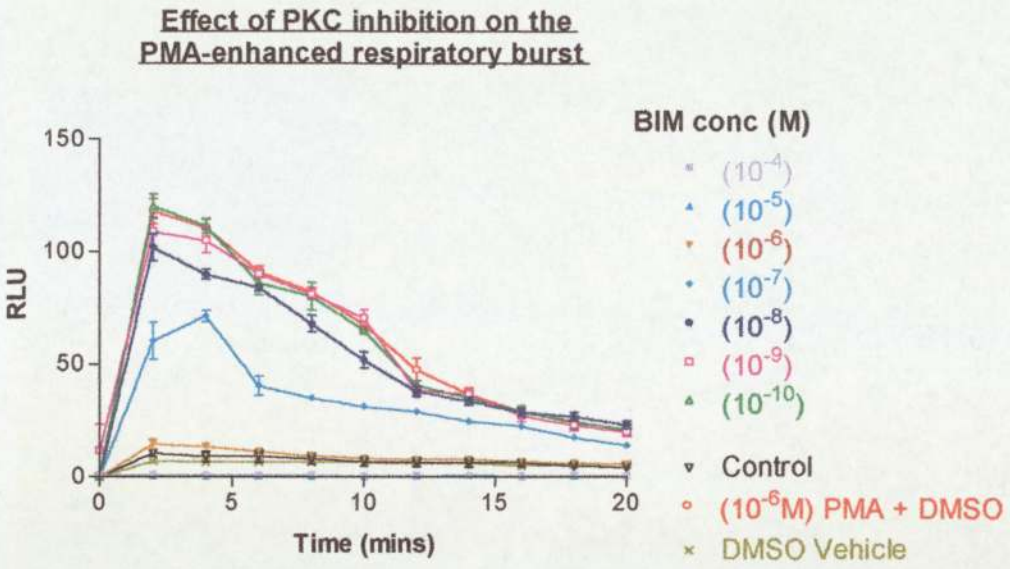
A known potent agonist of PKC, the phorbol ester PMA was used to see if it augmented the respiratory burst. PMA did indeed stimulate an oxidative burst with maximum activity observed at 10^{-6}M. Both higher and lower concentrations than this were less effective. Compared to the response to opsonised zymosan the burst occurred slightly more rapidly and was of shorter duration (Fig 4.4). The maximum PMA-induced response was only about half that induced by opsonised zymosan. An analogue of PMA 4α- phorbol 12,13 didecanoate, which does not activate protein kinase C, had no stimulatory effect on reactive oxygen intermediate generation at a concentration of 10^{-6}M (Fig 4.4). Pre-treatment of Mϕ's with a specific inhibitor of PKC, bisindolyl maleimide (BIM) for 30 minutes caused marked reduction in the oxidative burst elicited by both PMA and opsonised zymosan.

(Fig 4.4)

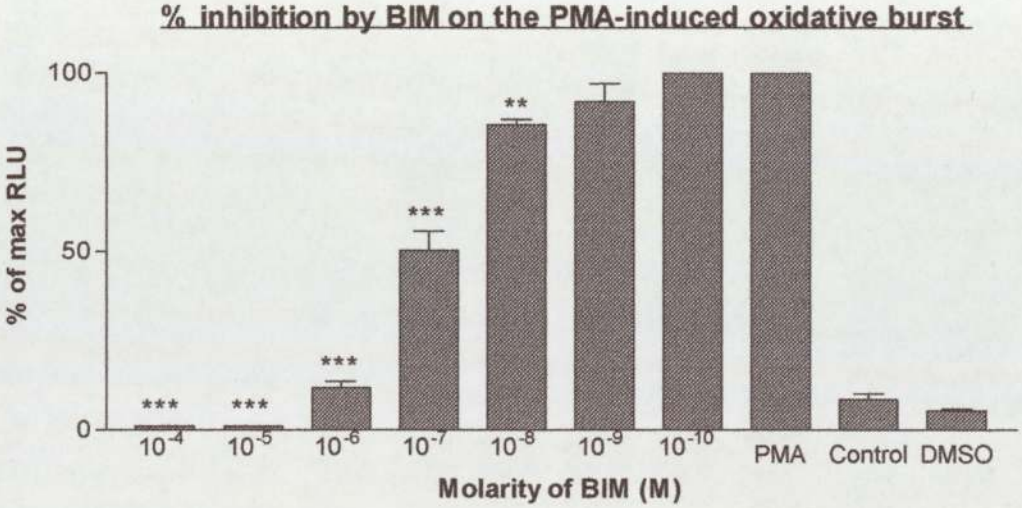


An activator of PKC, PMA was added to $1x10^5$ Mϕ's/cuvette and the respiratory burst was recorded every 2 minutes for 20 minutes. The inactive analogue 4α-phorbol 12,13 didecanoate was used with Mϕ's as a negative control. PMA was initially dissolved in DMSO so Mϕ's were incubated with this solvent with < 0.01% DMSO (vehicle). OpZ (2mg/ml) + DMSO was used as a comparable assay. Values represent mean +/- SEM derived from 3 independent experiments using cells from different mice.

(Fig 4.5a)

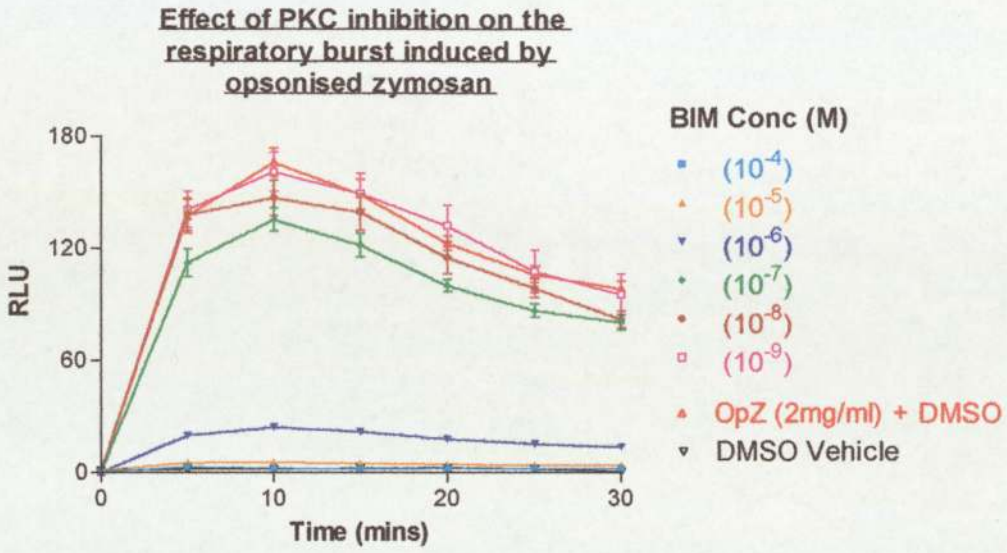


(Fig 4.5b)

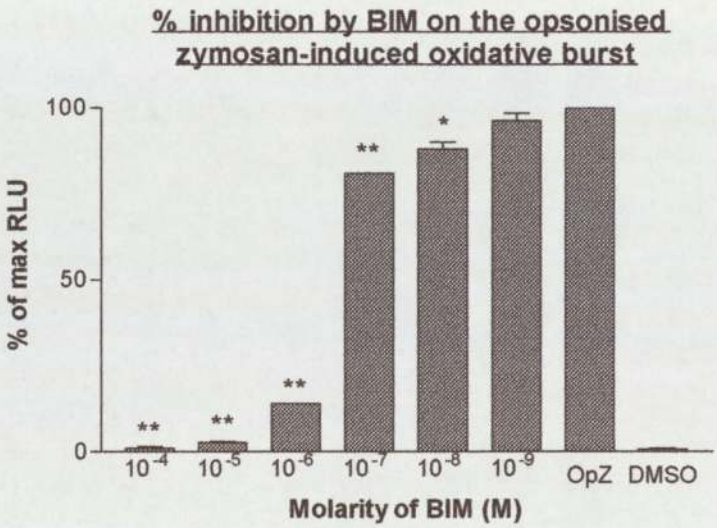


$1x10^5$ Mϕ's/cuvette were pre-incubated with the inhibitor of PKC (BIM) for 30 minutes and the respiratory burst in response to the subsequent addition of 10^{-6}M PMA recorded. Values represent mean +/- SEM derived from 3 independent experiments using cells from different mice. BIM significantly abrogated the PMA-induced oxidative burst ($p<0.01$)** ($p<0.001$)***.

(Fig 4.6a)



(Fig 4.6b)



$1x10^5$ Mϕ's/cuvette were pre-incubated for 30 minutes with BIM and the respiratory burst was recorded after the addition of OpZ (2mg/ml). A positive control of Mϕ's, OpZ and DMSO without BIM was utilised. Values represent mean +/- SEM derived from 3 independent experiments using cells from different mice. BIM significantly reduced the opsonised zymosan-induced respiratory burst ($p<0.05$)* ($p<0.01$)**.

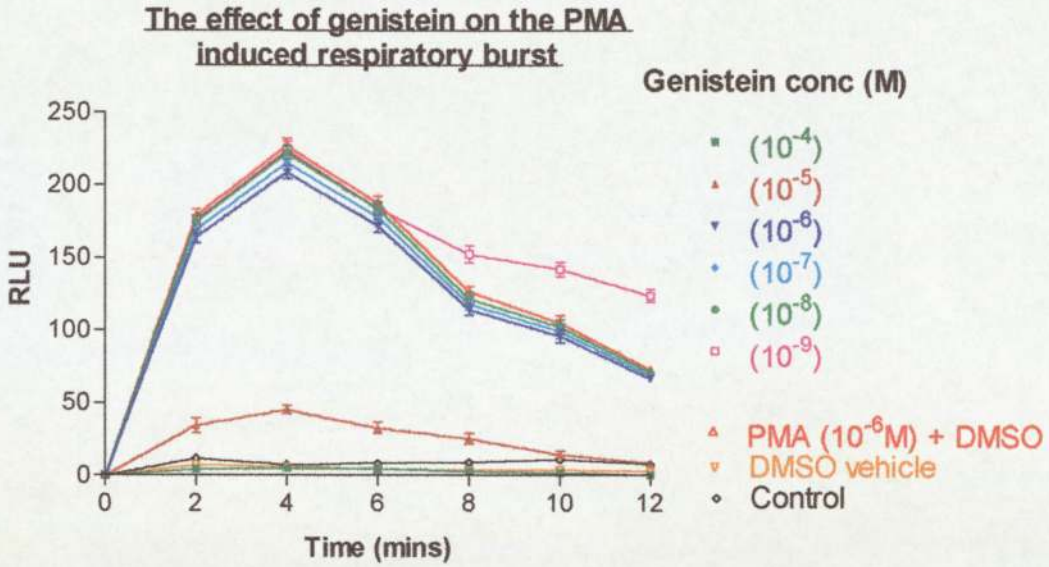
Thus PTK, PI-3 kinase and PKC are all involved in the signalling pathways responsible for the respiratory burst. If these enzymes are activated in a sequential manner then inhibition of PTK and PI-3 kinase should not block a PMA-induced respiratory burst.

When peritoneal Mϕ's were pre-treated with genistein at concentrations from 10^{-4}M to10^{-9}M for 30 minutes and then challenged with PMA at 10^{-6}M, 10^{-4}M and 10^{-5}M genistein still inhibited but only ~ 10% inhibition at 10^{-6}M and no inhibition at 10^{-7}M.

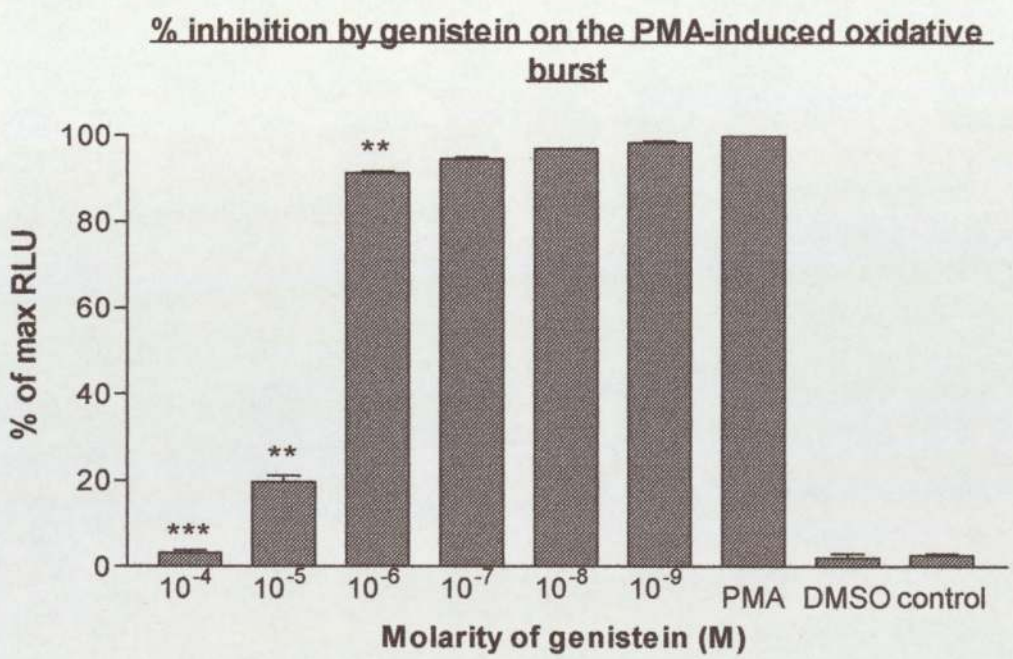
Compared to the effects of these two concentrations on the opsonised zymosan response (Fig 4.0) where 20-25% inhibition was noted provides evidence that genistein has a greater effect on the OZ response than it does on the PMA response.

Surprisingly vanadate which inhibits protein tyrosine phosphatase activity did, albeit very modestly, augment the effect of the PKC activator PMA which would promote protein phosphorylation on serine and threonine residues. Conceivably therefore concomitant tyrosine, threonine and serine phosphorylation is required for maximum NADPH oxidase activation. To investigate whether PI-3 kinase activation was required before or after these phosphorylations the effect of wortmannin on a PMA-elicited oxidative burst was examined. After a 30 minute preincubation with 10^{-7}M wortmannin the response to PMA was inhibited by about 45% (Fig 4.9b). Thus inositol sugars phosphorylated in position 3 of the inositol ring are required at the same time as, or subsequent to, PKC activation for full activation of the oxidative burst.

(Fig 4.7a)

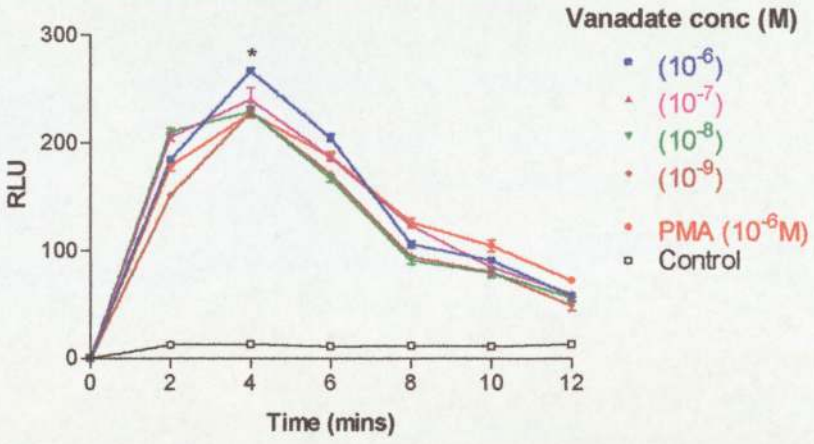


(Fig 4.7b)

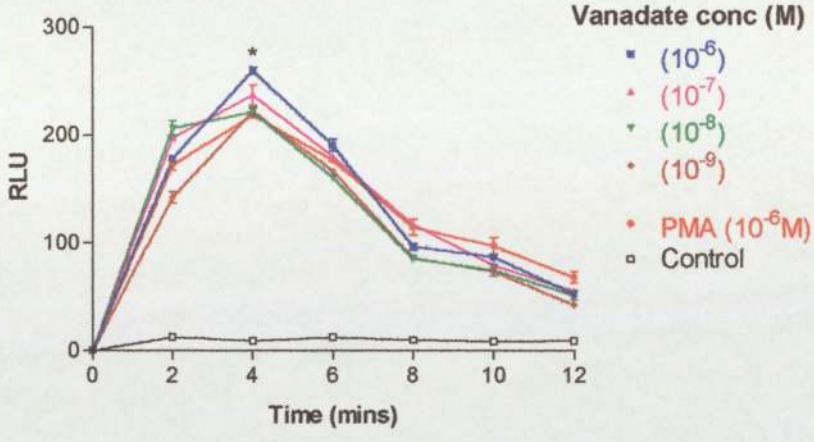


$1x10^5$ MΦ's/cuvette were pre-incubated with genistein for 30 minutes and then activated with PMA at (10^{-6}M). MΦ's were also activated with PMA, which had not been pre-incubated with genistein and this was used as a positive control. Values represent the mean +/- SEM derived from 3 independent experiments using cells from different mice. Genistein inhibited the PMA-induced oxidative burst significantly at concentrations to 10^{-6}M ($p<0.01$)** ($p<0.001$)***.

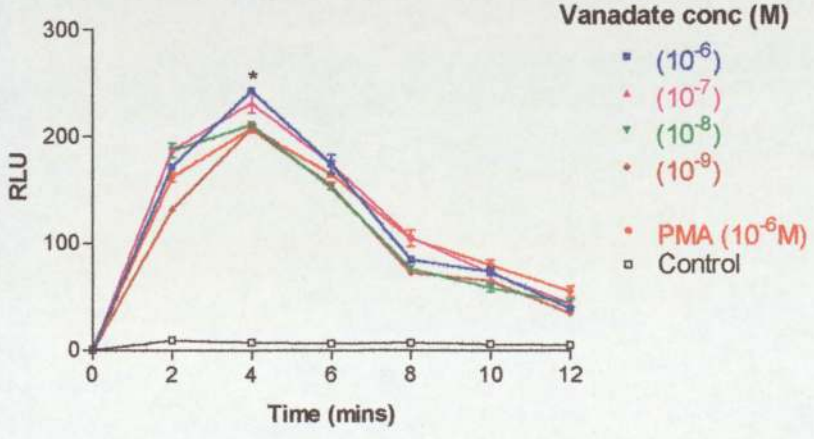
(Fig 4.8a) Effect on the PMA enhanced respiratory burst after 30 minute pre-incubation with vanadate



(Fig 4.8b) Effect on the PMA enhanced respiratory burst after 60 minute pre-incubation with vanadate

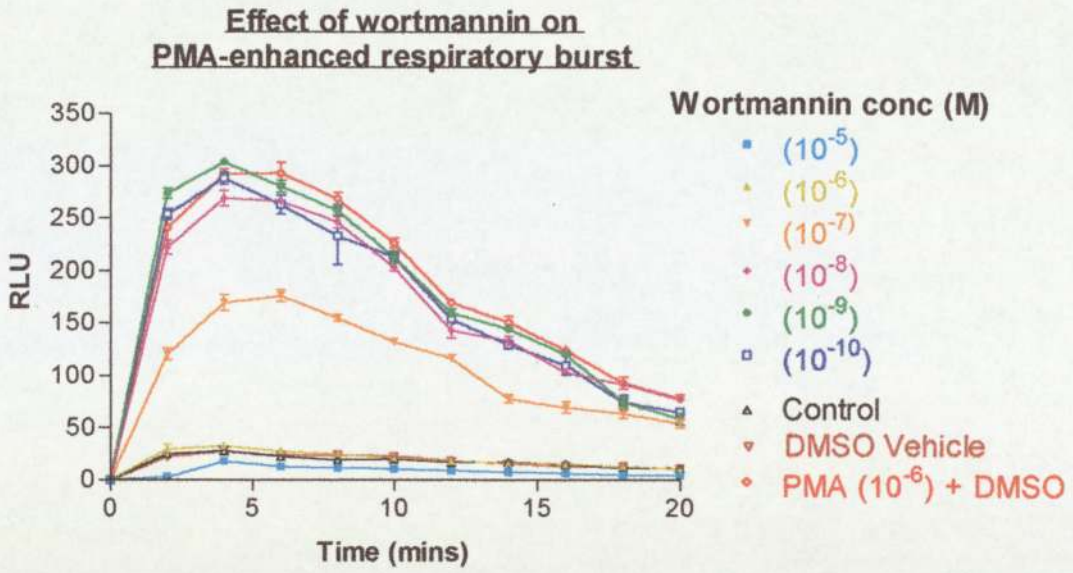


(Fig 4.8c) Effect on the PMA enhanced respiratory burst after 120 minute pre-incubation with vanadate

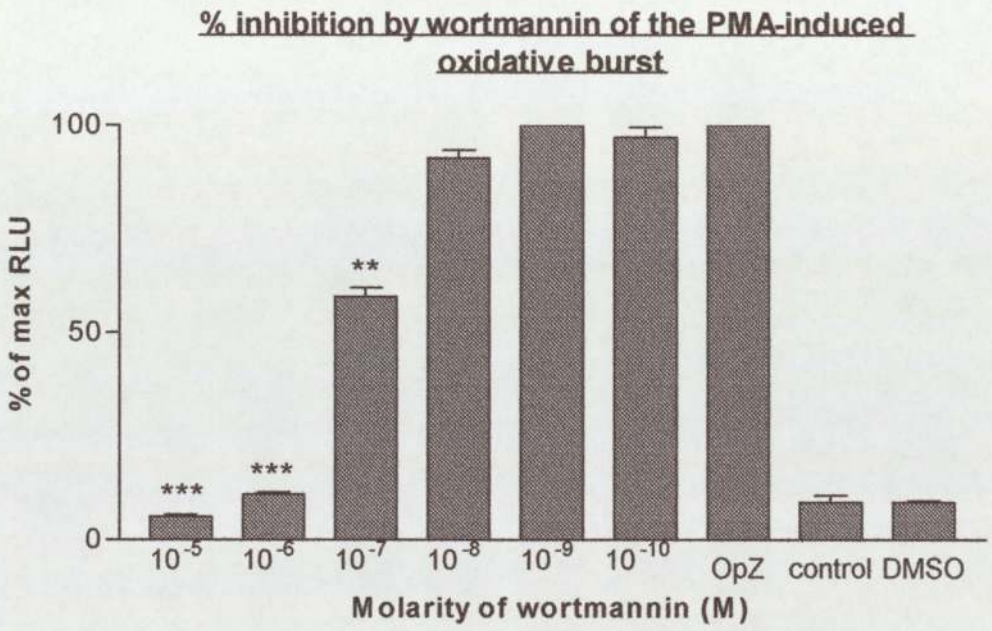


$1x10^5$ MΦ's/cuvette were pre-treated with the tyrosine phosphatase inhibitor vanadate for 30, 60 and 120 minutes respectively (fig's 4.8a to 4.8c). The MΦ's were then activated with PMA (10^{-6}M) and the respiratory burst measured every 2 minutes for 12 minutes. MΦ's untreated with vanadate and activated with PMA were used as a positive control. Values represent the mean +/- SEM derived from 3 independent experiments using cells from different mice. Vanadate significantly augmented the maximum PMA-induced respiratory burst at 10^{-6}M ($p<0.05$)*.

(Fig 4.9a)



(Fig 4.9b)

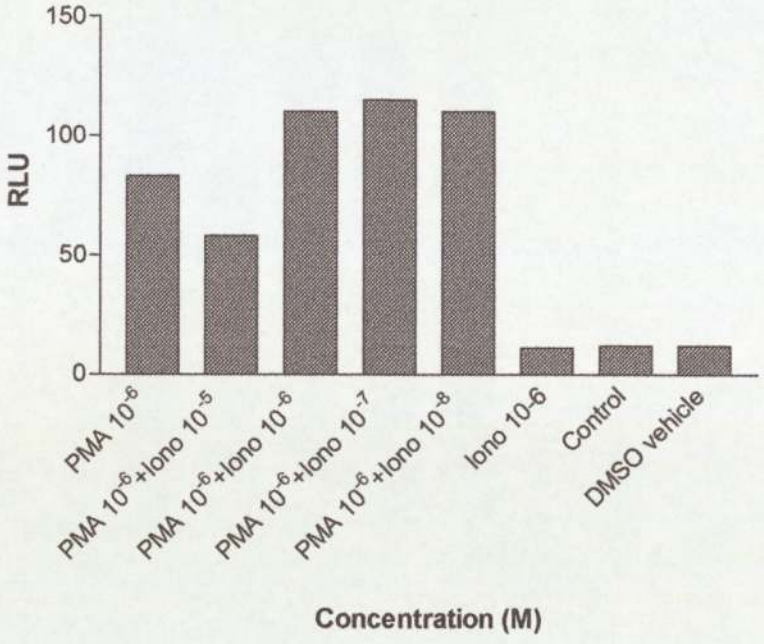


$1x10^5$ MΦ's/cuvette were pre-incubated with the PI-3 kinase inhibitor wortmannin for 30 minutes and then activated with PMA (10^{-6}M). Activated MΦ's containing <0.01% DMSO and untreated with wortmannin were used as a positive control. Values represent the mean +/- SEM from 3 independent experiments using cells from different mice. Wortmannin significantly inhibited the PMA-induced oxidative burst down to 10^{-7}M ($p<0.01$)** ($p<0.001$)***.

Although direct PKC activation with PMA could generate an oxidative burst the magnitude was only 50% of that elicited by OZ (see fig 4.4). This also suggests that additional factors acting at the same time or subsequent to PKC are required for full NADPH oxidase activity. One such factor could be an increase in intracellular Ca^{2+} which would accompany the natural activation of PKC via the products of PLC activation i.e. diacylglycerol and IP_3. When we attempted to mimic this condition by simultaneous application of PMA and A23187 a calcium ionophore, 10^{-6} and 10^{-7}M ionomycin enhanced the PMA-induced burst by about 30% whereas these concentrations of ionophore on their own were ineffectual (Fig 4.10). Thus a simultaneous rise in intracellular calcium and PKC activation seems to be required for optimum NADPH oxidase activation. An experiment was also undertaken to deplete Ca^{2+} from the assay medium using the Ca^{2+} chelating EGTA. Peritoneal Mϕ's were added to pre-mixed aliquots of EGTA and medium such that the final concentration of chelator ranged from 0.25mM to 2.5mM and their response to challenge with OpZ was recorded. As the [EGTA] increased, the OpZ enhanced response declined (Fig 4.11). In further experiments when Mϕ's were treated with 2.5 or 5.0mM EGTA the oxidative burst was inhibited by about 70%. Progressive Ca^{2+} restoration largely re-established the normal responsiveness (Figs 4.12a and 4.12b).

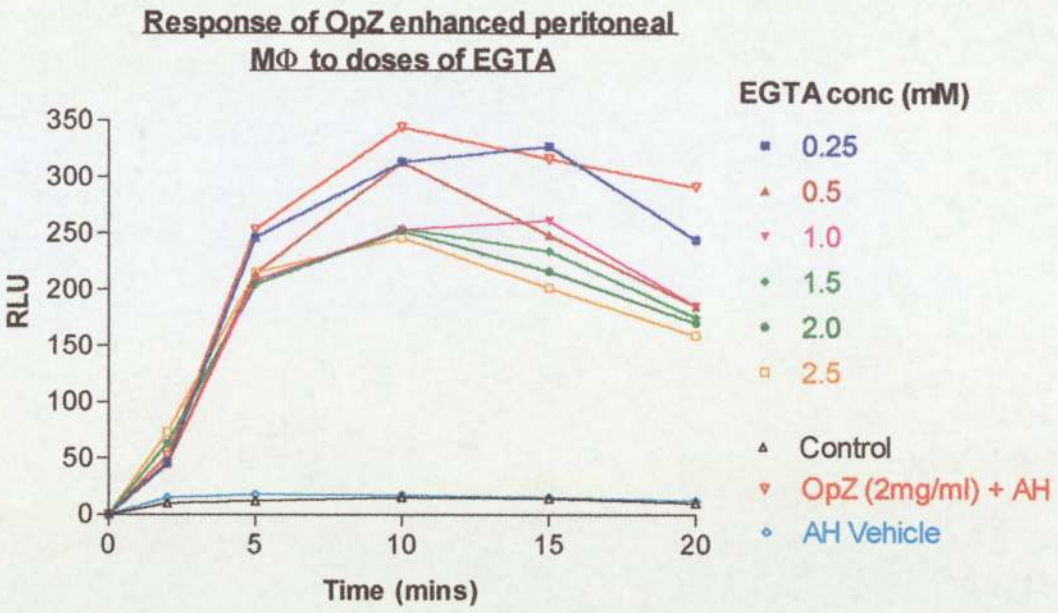
(Fig 4.10)

The effect of PMA used in conjunction with ionomycin on the respiratory burst



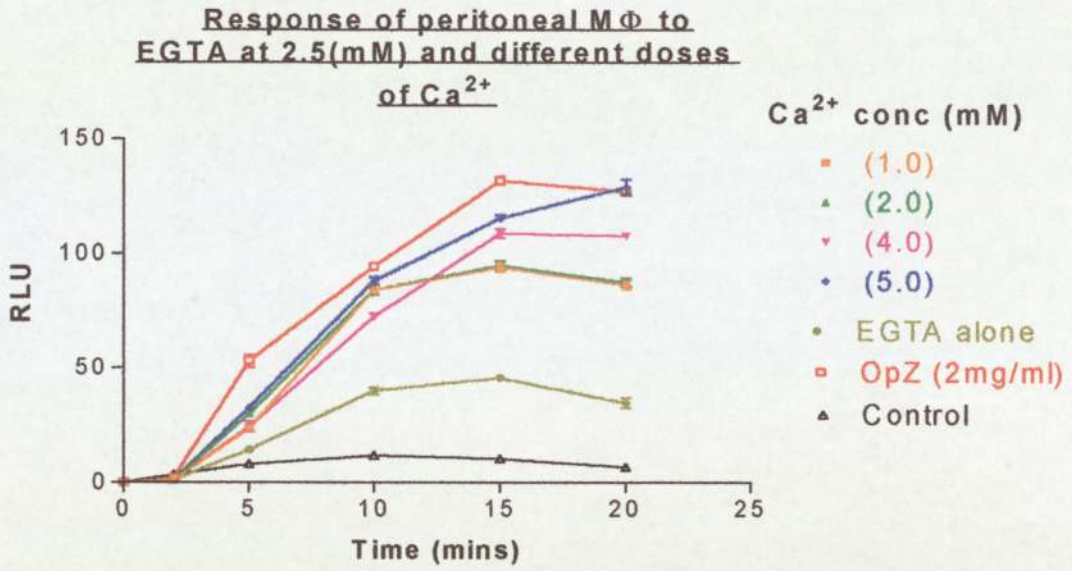
$1x10^5$ MΦ's/cuvette were pre-incubated for 30 minutes with the calcium ionophore (ionomycin) and the respiratory burst was recorded after the addition of PMA (10^{-6}M) Mϕ's untreated with ionomycin but activated with PMA were used as a positive control. MΦ's were also pre-treated with ionomycin without activation with PMA to elucidate if there was any reaction to the ionophore. Ionomycin had been previously dissolved in DMSO, so MΦ's were incubated with DMSO at a concentration < 0.01% to ensure there was no causative effect on the response. Values are representative of a single experiment. The PMA + 10^{-5}M ionomycin combination seemed to impair the oxidative burst; presumably there is an optimum intracellular Ca^{2+} concentration for NADPH oxidase activity.

(Fig 4.11)

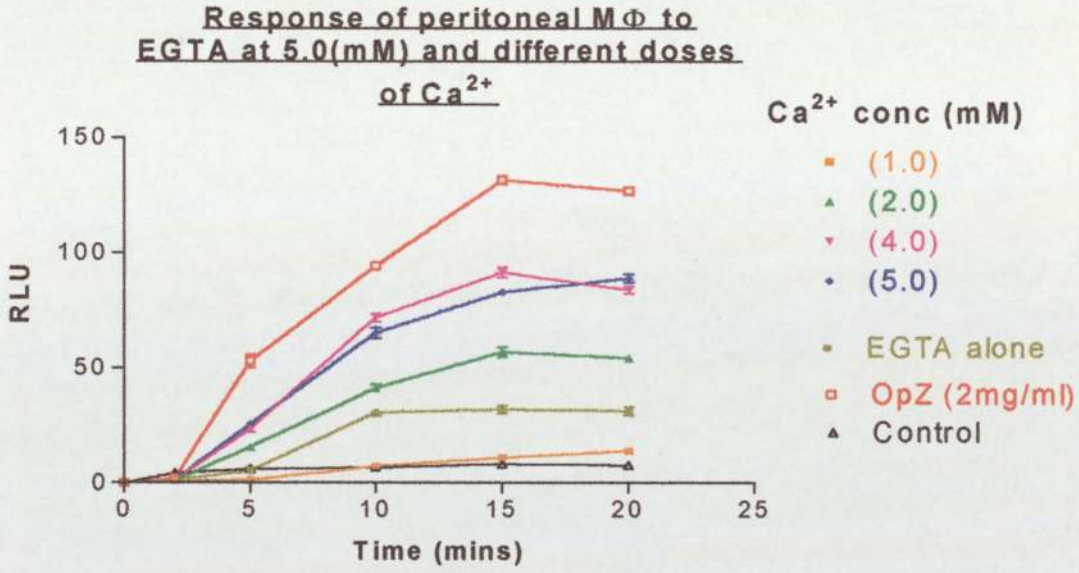


$1x10^5$ MΦ's/cuvette were pre-incubated for 30 minutes with medium that had been mixed with different concentrations of the Ca^{2+} chelator EGTA. MΦ's incubated with normal medium were used as a positive control. After activation by OpZ (2mg/ml) the respiratory burst was recorded. Because the EGTA was initially dissolved in ammonium hydroxide (AH), cells were incubated with ammonium hydroxide at a dose equivalent to that present with the highest concentration of EGTA. This established that EGTA and not ammonium hydroxide is responsible for any inhibitory effect. Values are representative of a single observation using cells extracted from the same mouse. EGTA (2.5mM) which should chelate all the extracellular Ca^{2+} reduced the oxidative burst from ~ 340 RLU to 240 RLU. Thus the response was reduced but not abolished.

(Fig 4.12a)

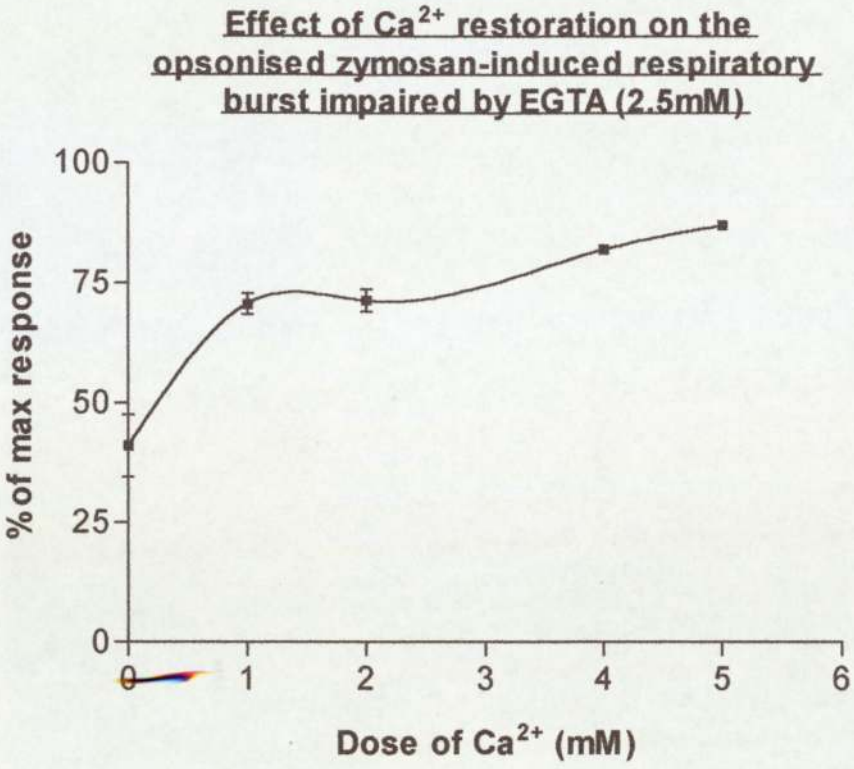


(Fig 4.12b)

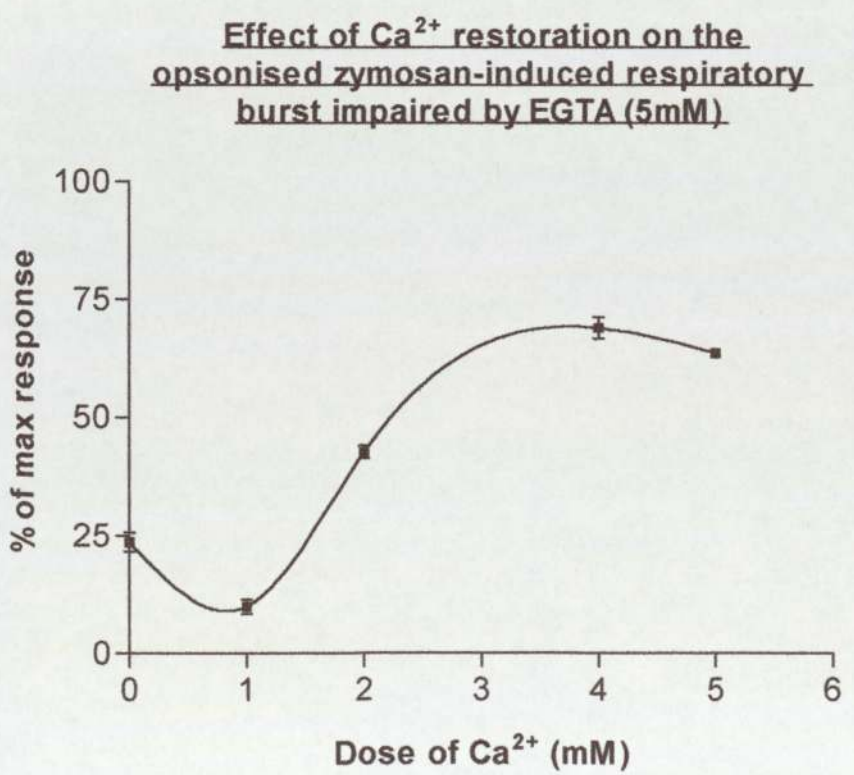


$1x10^5$ MΦ's/cuvette were pre-incubated with medium for 30 minutes that had been treated with EGTA at 2.5mM and 5.0mM and replenished with Ca^{2+} from 1.0mM to 5.0mM (fig's 4.12a and 4.12b respectively). After the addition of OpZ (2mg/ml) the respiratory burst was recorded at 2, 5, 10, 15, and 20 minutes. MΦ's activated in normal medium acted as the positive control. The values represent the mean +/- SEM derived from 3 independent experiments using cells from different mice. Fig's 4.12c and 4.12d show the response of the MΦ's at 15 minutes after replenishment with the stated doses of Ca^{2+} (see over).

(Fig 4.12c)



(Fig 4.12d)



Influx of extracellular Ca^{2+} is clearly important for genesis of the complete oxidative burst in response to opsonised zymosan. It is not clear which second messenger system induces this influx nor do we know the type of channel involved. Verapamil is an inhibitor of voltage gated calcium channels and peritoneal Mϕ's were pre-treated for 30 minutes with verapamil at concentrations from 10^{-4}M to 10^{-8}M and then challenged with OpZ. Verapamil appeared to reduce the respiratory burst by approximately 35% at 10^{-4}M (Fig 4.13). Although this concentration is rather high, the evidence would be consistent with a triggered opening of voltage-operated channels.

This view is supported by experiments using amiloride a Na transport inhibitor; a sodium influx might be instrumental in altering the transmembrane potential and therefore opening the voltage Ca^{2+} channels. Amiloride at 10^{-4}M and 10^{-5}M significantly impaired the respiratory burst to opsonised zymosan (Fig 4.14). It is known that verapamil can also elevate c-AMP levels within the cell (Bianca et al 1988) and that this may also act to dampen the respiratory burst. To test this possibility peritoneal Mϕ's were pre-treated for 30 minutes with forskolin) an activator of adenylate cyclase and then challenged with OpZ. The Mϕ response was again reduced in a dose dependent manner (Fig 4.15). This seems to suggest that a pathway involving c-AMP and protein kinase A, may serve to down-regulate the respiratory burst. This idea was reinforced when peritoneal Mϕ's were incubated with the c-AMP analogue (dibutyryl c-AMP). A significant abrogation of the oxidative burst was again observed (Fig 4.16). It is not clear whether the c-AMP, PKA pathway impairs the rise in intracellular Ca^{2+} or acts in some other way. In all the experiments in which an influx of extracellular Ca^{2+} was prevented, the oxidative burst in response to OpZ was always reduced but never abolished. It may be that mobilisation of Ca^{2+} from intracellular stores is able to support a partial but incomplete generation of reactive oxygen intermediates.

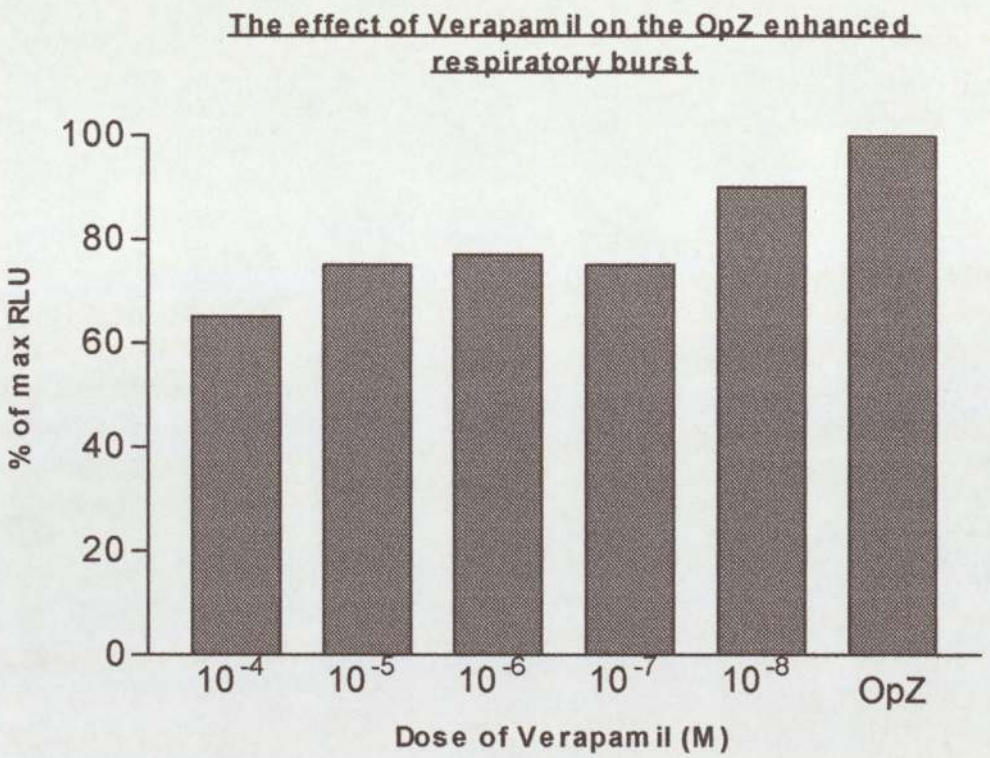
Whether Ca^{2+} directly affects the NADPH oxidase complex or perhaps does so via calmodulin activation is not certain. However, when peritoneal Mϕ's were pre-treated for 30 minutes with the calmodulin antagonist W7 at a concentration range of 10^{-4}M to 10^{-8}M and then challenged with OpZ, an inhibition of the response was again noted in a dose dependent manner (Fig 4.17) with the % inhibition ranging from 13% at 10^{-8}M to 74% at 10^{-4}M.

To establish whether Mϕ's release measurable amounts of Ca^{2+} from their intracellular stores, thapsigargin was employed. This material inhibits the Ca^{2+}-ATPases in the endoplasmic reticulum thus blocking replenishment of the stores by by cytosolic Ca^{2+}, which should consequently increase. It can be seen from (Fig 4.18) that 10 minutes after the addition of thapsigargin there was a uniform increase in $[Ca^{2+}]i$ in most cells with the exception of cells 7 and 8 (regions 7 + 8). Intracellular Ca^{2+} levels returned to basal levels by 30 minutes. In a separate experiment, Ca^{2+} transients were measured after the application of platelet activating factor (PAF). Upon application of 1μM PAF, cell 4 exhibited a sharp increase in $[Ca^{2+}]i$ reaching a peak in about 10 seconds and returning to basal levels after approximately 3 minutes (Fig 4.19). Cell 9 showed a much-reduced response to this concentration of PAF with cell 1 possibly giving a delayed response. When 10μM PAF was applied, no observable response was noted, which may be due to the Mϕ's being desensitised by the previous PAF application. The subsequent addition of 2μM thapsigargin produced a uniform increase in $[Ca^{2+}]i$ in all Mϕ's providing evidence that there was still Ca^{2+} present in the intracellular stores.

1μM PMA, which promotes an oxidative burst in the Mϕ's (Fig 4.4) showed an undulating response in cell 5 only (Fig 4.20). When 0.2mg/ml oz was introduced into the perfusion system, their appeared to be a reduction in response to below basal levels in all cells. This was attributed to the fact that the particulate nature of the opsonised zymosan prevented any Ca^{2+}-induced fluorescence reaching the photomultiplier tube. As the opsonised zymosan was washed away by perfusion, a slow recovery was noted. 1μM PAF application produced transient increases in $[Ca^{2+}]i$ in all cells, with varying intensities, except in cell 1. In a similar experiment,

levels in all cells. This was attributed to the fact that the particulate nature of the opsonised zymosan prevented any Ca^{2+}-induced fluorescence reaching the photomultiplier tube. As the opsonised zymosan was washed away by perfusion, a slow recovery was noted. 1μM PAF application produced transient increases in $[Ca^{2+}]i$ in all cells, with varying intensities, except in cell 5. In a similar experiment, 1μM PMA gave no response, while opsonised zymosan again caused an artefactual decrease in the fluorescence signal (Fig 4.22). After application of 10μM PAF, $[Ca^{2+}]$ was elevated in all Mϕ's providing evidence that the Mϕ's were still responsive to external stimuli.

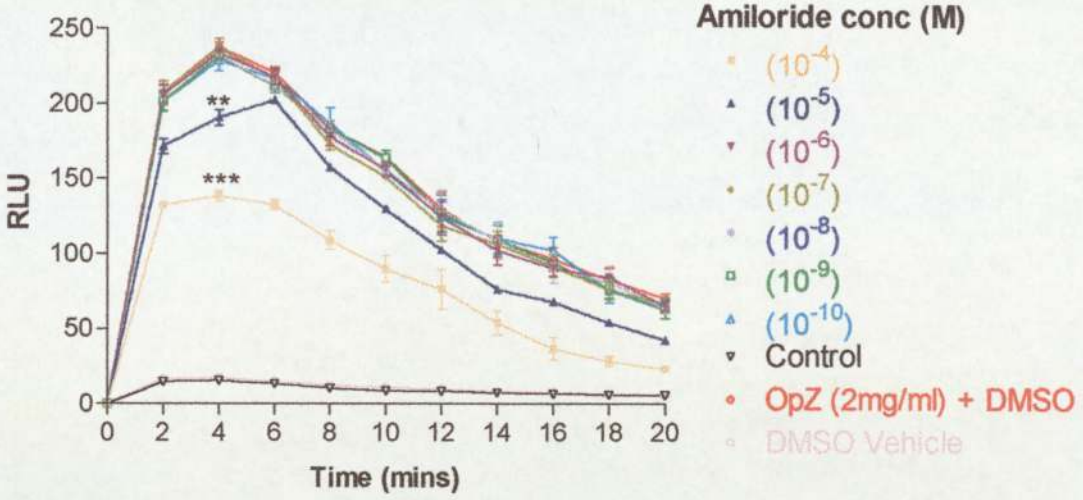
(Fig 4.13)



$1x10^5$ MΦ's/cuvette were pre-incubated with verapamil (a voltage gated Ca^{2+} channel blocker) for 30 minutes and activated with OpZ (2mg/ml). Readings were taken every 2 minutes for 20 minutes. MΦ's untreated with verapamil and activated with OpZ were used as a positive control. Values are depicted as the percentage of the maximum response of the positive control. Values are a representative of a single experiment.

(Fig 4.14)

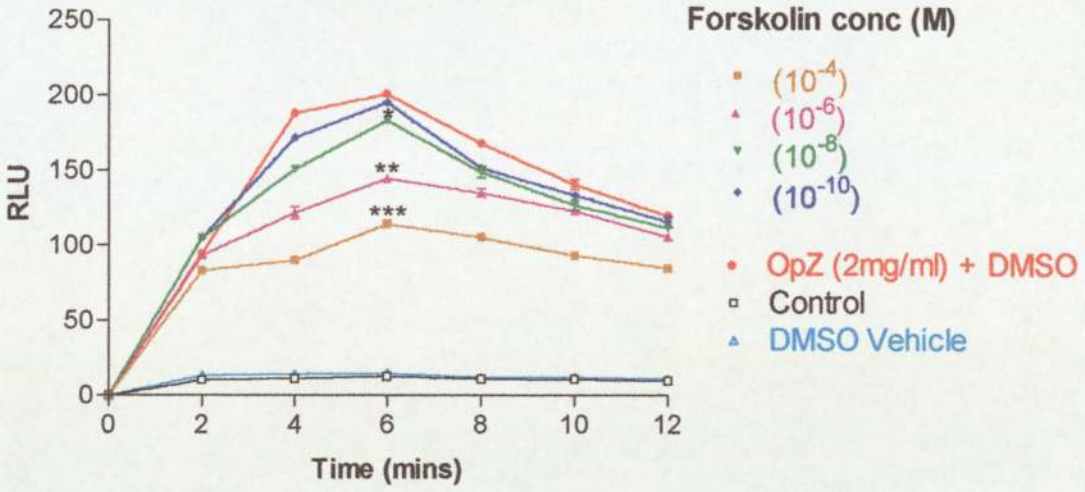
Effect of amiloride on the opsonised zymosan-induced respiratory burst



$1x10^5$ MΦ's/cuvette were pre-incubated with amiloride, (a Na^+/K^+ antiporter) for 30 minutes and then challenged with OpZ (2mg/ml). MΦ's untreated with amiloride and activated with OpZ were used as a positive control. MΦ's were again incubated with DMSO by itself (DMSO vehicle) at <0.01%. Values represent the mean +/SEM from 3 independent experiments. Amiloride exhibited an inhibition of the opsonised zymosan-induced respiratory burst at 10^{-4} and 10^{-5}M ($p<0.01$)** ($p<0.001$)***.

(Fig 4.15)

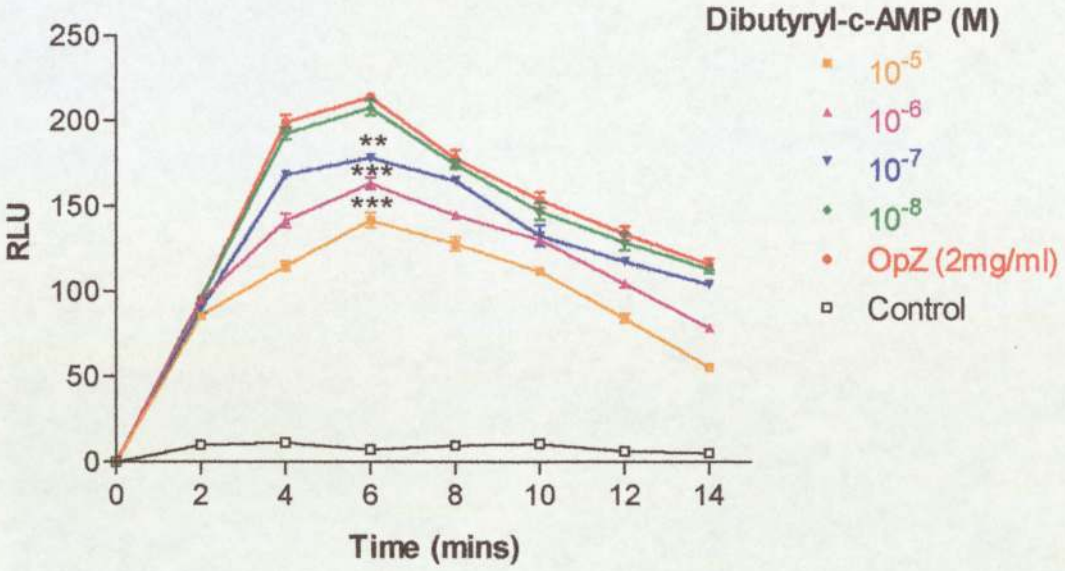
The effect of forskolin on the opsonised zymosan-induced oxidative burst



$1x10^5$ MΦ's/cuvette were pre-incubated with forskolin (an activator of adenylyl cyclase) for 30 minutes and the respiratory burst recorded after the addition of OpZ (2mg/ml). MΦ's untreated with forskolin and activated with OpZ were used as a positive control. MΦ's were also incubated with <0.01% DMSO because forskolin was initially dissolved in this solvent. DMSO was seen not to cause any debilitating effects on the response and had values similar to the control. All values represent the mean +/- SEM derived from 3 independent experiments using cells from a different mouse. Forskolin significantly abrogated the oxidative burst ($p<0.05$)* ($p<0.01$)** ($p<0.001$)***.

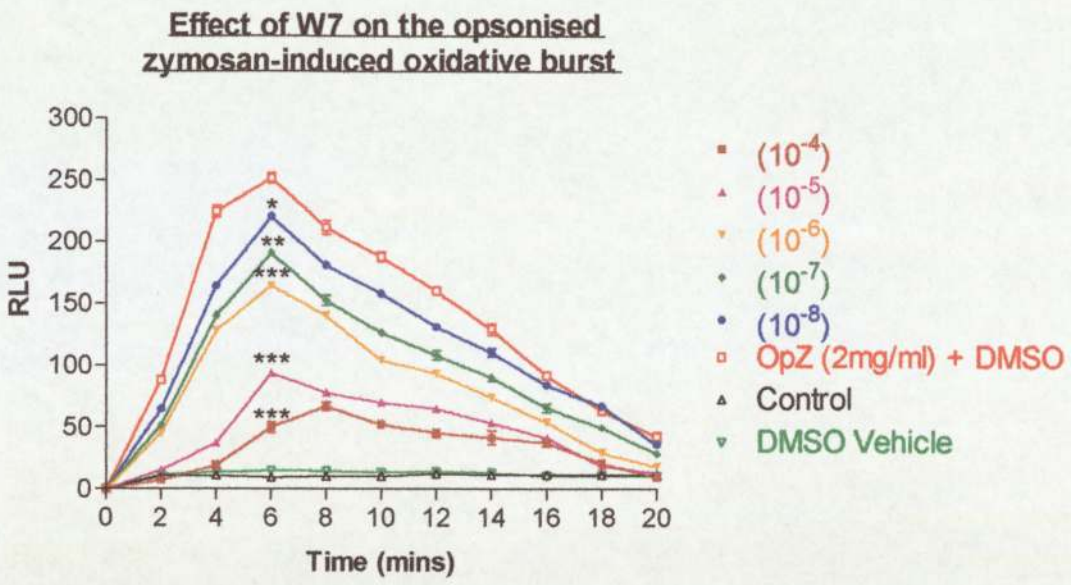
(Fig 4.16)

The effect of dibutyryl cyclic-AMP on the opsonised zymosan-induced oxidative burst



$1x10^5$ Mϕ's/cuvette were preincubated with dibutyryl cyclic-AMP (an analogue of cyclic AMP) for 60 minutes and the oxidative burst was recorded after the addition of opsonised zymosan (2mg/ml). Untreated Mϕ's challenged with opsonised zymosan (2mg/ml) were used as an activated positive control. All values represent the mean +/- SEM derived from 3 independent experiments using cell from a different mouse. Dibutyryl c-AMP significantly abrogated the opsonised zymosan-induced burst. ($p<0.05$)* ($p<0.01$)** ($p<0.001$)***.

(Fig 4.17)



$1x10^5$ MΦ's were pre-incubated for 30 minutes with doses of W7 (a Ca^{2+}/calmodulin inhibitor) and then activated with OpZ (2mg/ml). MΦ's untreated with W7 and activated with OpZ were used as the positive control. W7 had to be dissolved in DMSO and as a result of this MΦ's were incubated with <0.01% of this (DMSO vehicle). The DMSO alone caused no aberrations, indicating that inhibition was an effect of W7. Values represent the mean +/- SEM derived from 3 independent experiments using cells from different mice. A significant dose dependent inhibition of the opsonised zymosan-induced oxidative burst was observed ($p<0.05$)* ($p<0.01$)** ($p<0.001$)***.

Fig 4.18

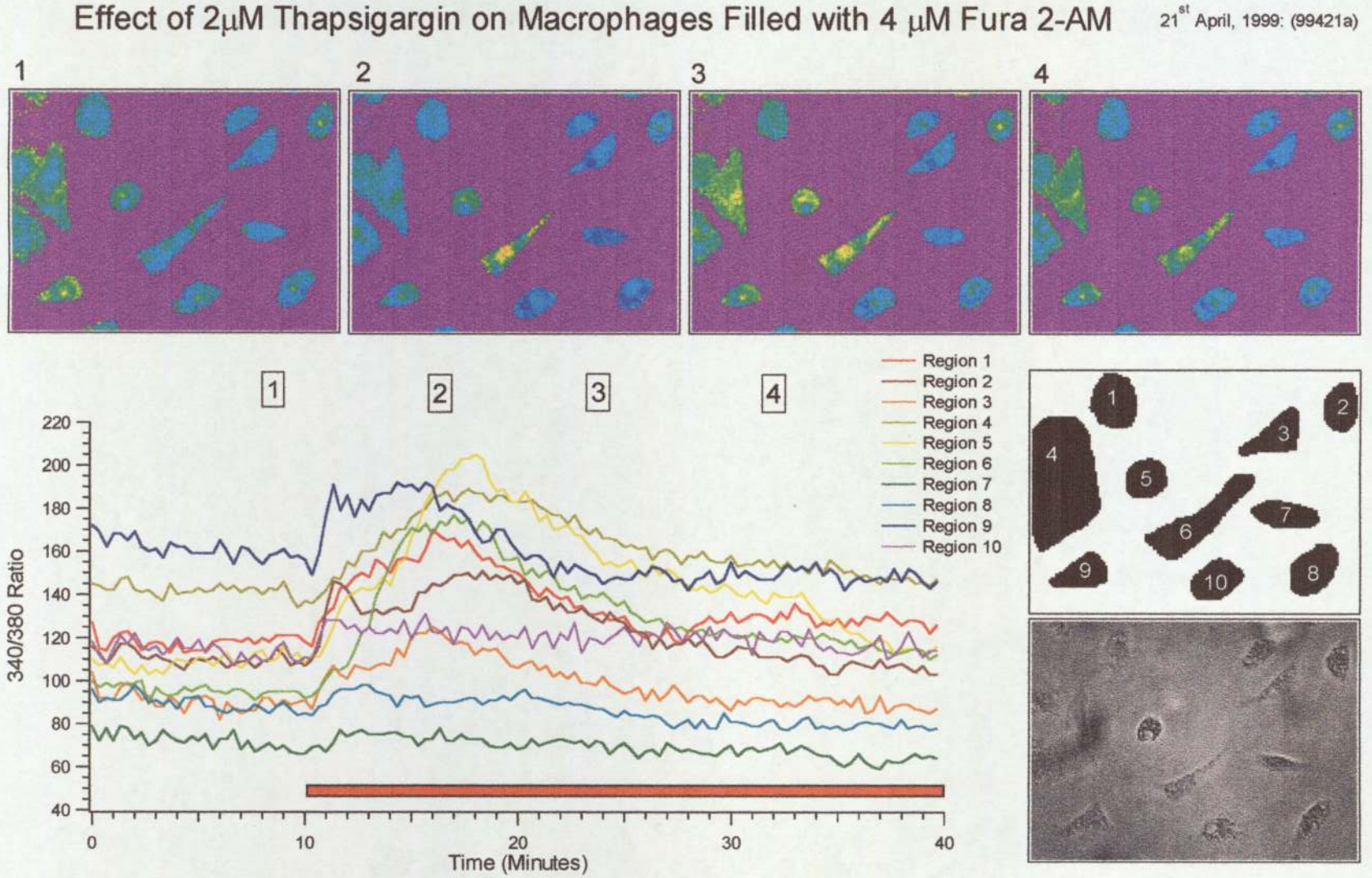

Effect of 2μM Thapsigargin on Macrophages Filled with 4 μM Fura 2-AM
21st April, 1999: (99421a)
1
2
3
4
Region 1
Region 2
Region 3
Region 4
Region 5
Region 6
Region 7
Region 8
Region 9
Region 10
340/380 Ratio
Time (Minutes)
220
200
180
160
140
120
100
80
60
40
0
10
20
30
40

Fig 4.19

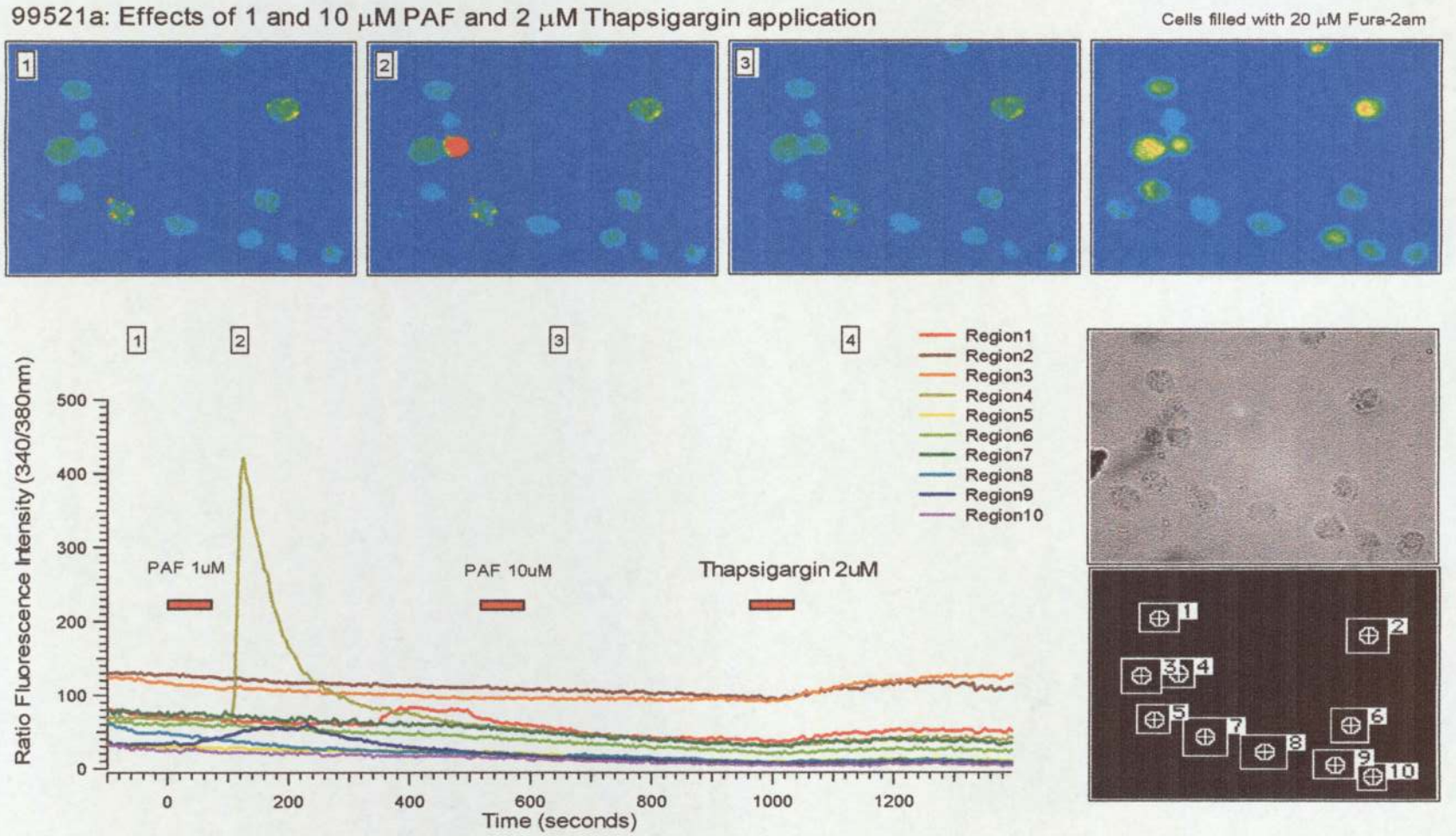

99521a: Effects of 1 and 10 μM PAF and 2 μM Thapsigargin application
Cells filled with 20 μM Fura-2am
1
2
3
4
Region1
Region2
Region3
Region4
Region5
Region6
Region7
Region8
Region9
Region10
PAF 1uM
PAF 10uM
Thapsigargin 2uM
Ratio Fluorescence Intensity (340/380nm)
Time (seconds)
0
100
200
300
400
500
0
200
400
600
800
1000
1200

Fig 4.20

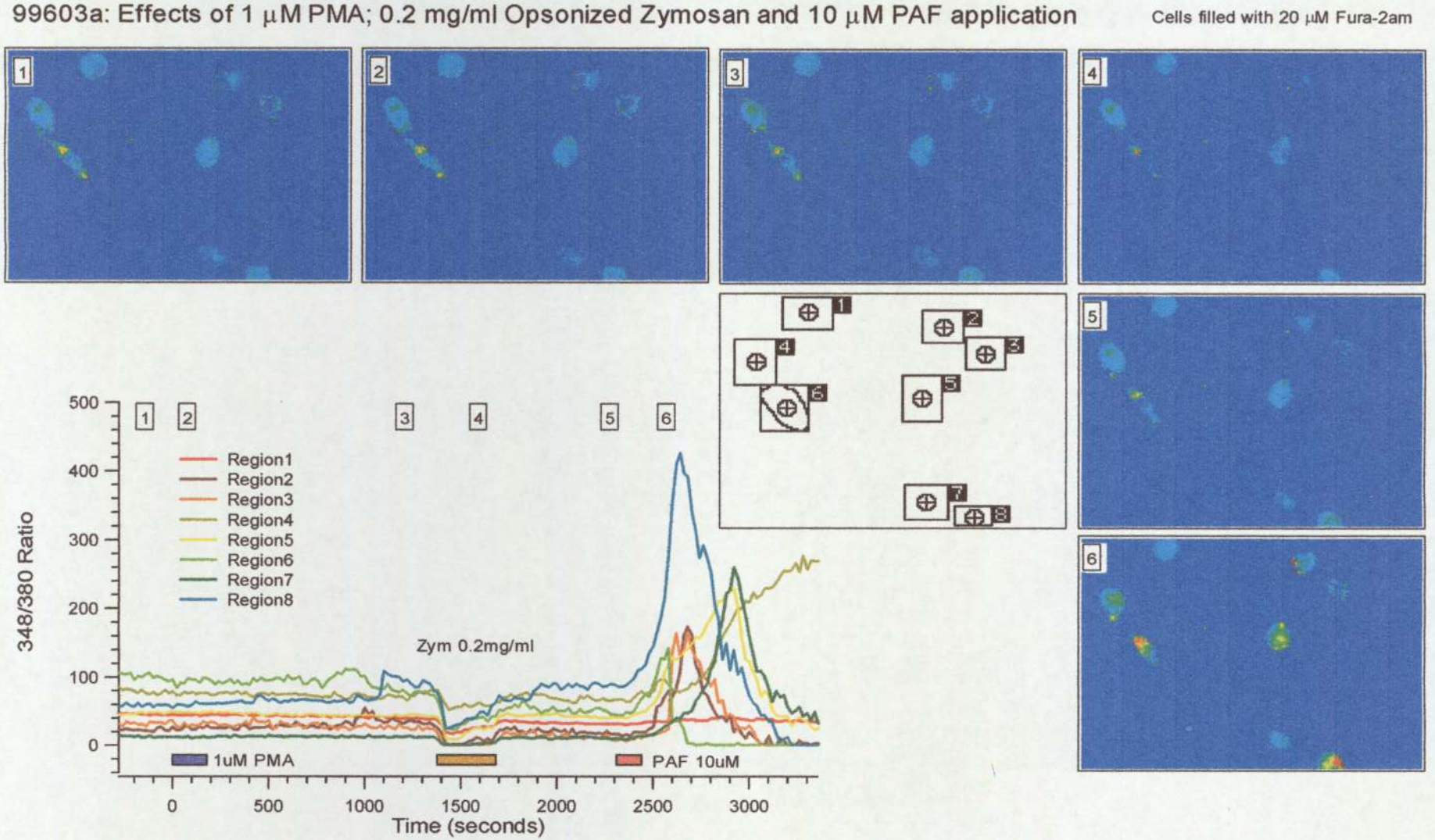
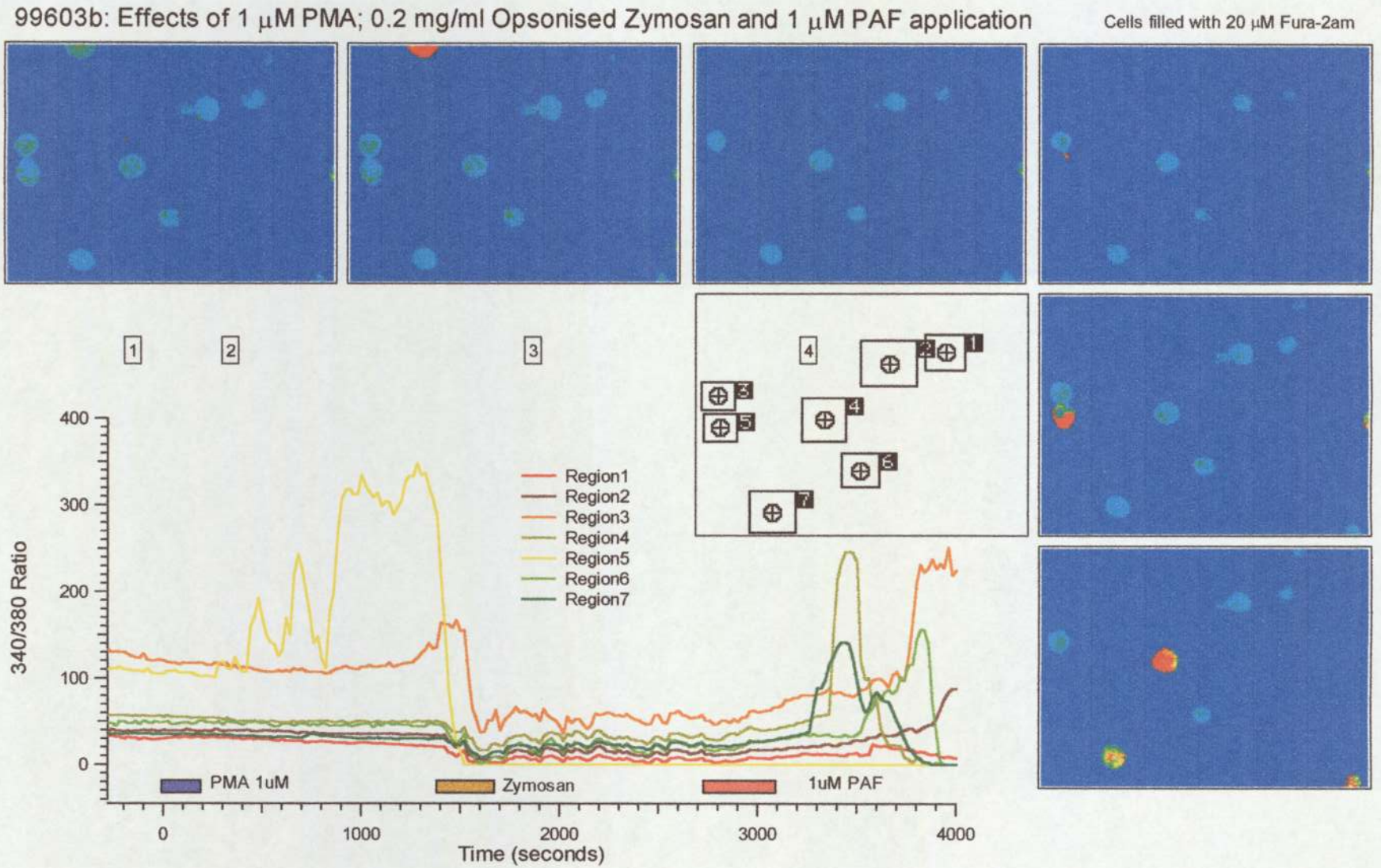

99603a: Effects of 1 μM PMA; 0.2 mg/ml Opsonized Zymosan and 10 μM PAF application
Cells filled with 20 μM Fura-2am
1
2
3
4
5
6
348/380 Ratio
Region1
Region2
Region3
Region4
Region5
Region6
Region7
Region8
Zym 0.2mg/ml
1uM PMA
PAF 10uM
Time (seconds)

Fig 4.21


99603b: Effects of 1 μM PMA; 0.2 mg/ml Opsonised Zymosan and 1 μM PAF application
Cells filled with 20 μM Fura-2am
1
2
3
4
Region1
Region2
Region3
Region4
Region5
Region6
Region7
340/380 Ratio
400
300
200
100
0
PMA 1uM
Zymosan
1uM PAF
0
1000
2000
3000
4000
Time (seconds)

5. Discussion

Having established the best technique for mouse peritoneal Mϕ isolation and harvesting and optimum concentrations of cells and indicator to monitor the generation of O_2^-, these studies investigated the signaling pathways responsible for the oxidative burst. The initial experiments were utilised to elucidate the best standard procedure. Zymosan particles (zymosan A) can induce chemiluminescence in murine Mϕ's (Sipka et al 1986) and this is achieved by the ligation of the sugar receptors on the Mϕ with the sugar residues on zymosan (Sung et al 1983). A binding site has been described on peritoneal Mϕ's that recognise glycoproteins having terminal mannose, n-acetylglucosamine or fucoidin residues (Adams et al 1984). The present study revealed that reactive oxygen species could be elicited by application of both zymosan and opsonised zymosan (OpZ). The magnitude of the oxidative burst elicited with OpZ was 40% greater than with zymosan alone (Fig 3.1) and this can be attributed to the capacity of zymosan to bind complement components and activate the Mϕ's through their C3b receptors enhancing the overall response. A dose-dependent response to both zymosan and OpZ was observed with OpZ producing a greater oxidative burst at all concentrations used (Fig's 3.2a and 3.2b). The optimum response reached a plateau in both instances at 2mg/ml. This may be due to receptor saturation and for the maximum response to occur most of the C3b or man/fuc/glunac receptors have to be ligated with OpZ or zymosan. Thus, increasing doses of zymosan correspond to increasing activity of the NADPH-oxidase. Various cytosolic factors ($p47^{phox}$ and $p67^{phox}$) have been found to be required for activating the membrane bound NADPH-oxidase and the corresponding production of O_2^- (Mizuno et al 1992). OpZ-treated HL-60 cells have been shown to phosphorylate $p47^{phox}$ (causing its translocation from the cytosol to the membrane probably through PKC activation). More significantly perhaps this treatment also causes rapid dephosphorylation of a 21K protein, which is associated with $p47^{phox}$ and $p67^{phox}$ (Suzuki et al 1995). This 21K protein is dephosphorylated via a phosphoserine phosphatase, which may work synergistically with the phosphorylated $p47^{phox}$ on the pathway for activation of the respiratory burst.

Two reports have demonstrated that tyrosine phosphorylation of specific proteins may play an important part in the activation of the respiratory burst (Berkow et al 1990) and zymosan certainly stimulates tyrosine kinase activity in murine Mϕ's (Green et al 1992). One of the most widely studied inhibitors of tyrosine kinase is genistein. This isoflavone was originally isolated from the fermentation broth of Pseudomonas and has been shown to abolish epidermal growth factor receptor autophosphorylation. The present studies demonstrated that genistein significantly inhibited the OpZ-induced oxidative burst (Fig's 4.0a and 4.0b), with an approximate Ec50 of 5μM, which is close to the expected value of 2.5μM. The inactive analogue daidzein, which lacks protein tyrosine kinase inhibiting activity, did not abrogate the opsonised-induced respiratory burst at any concentration (Fig 4.1). It is therefore reasonable to assume that tyrosine kinase plays a necessary part in the pathway for respiratory burst activity. To reinforce the involvement of tyrosine kinase in this process, sodium orthovanadate was utilised. Vanadate is known to induce phosphotyrosine accumulation and activate O_2 consumption in HL-60 cells (Trudel et al 1991). Vanadate in the pentavalent oxidation state has a host of biological activities with the most important being the analogous effect to phosphate in biological systems thus inhibiting the enzymes mediating phosphoryl transfer reactions such as phosphatases (Nechay 1984). Vanadate has also been shown to modulate with the surface expression and function of Fc2b receptors (Vaddi et al 1996) and superoxide generation (Cohen et al 1988).

Vanadate (10^{-6}M) significantly augmented the OpZ-induced respiratory burst (Fig's 4.2a-4.2c) presumably by inhibition of tyrosine phosphatases. Superoxide production by the action of NADPH oxidase triggers the formation of peroxyvanadyl [V(4+)-OOH] and vanadyl hydroperoxide [V(4+)-OO] and that one of these species is required for phosphotyrosine formation and the activation of the respiratory burst (Trudel et al 1991). Thus an amplification cycle seems to exist in cells treated with vanadate whereby trace amounts of superoxide initiate the formation of these peroxides of vanadate. The subsequent accumulation of critical tyrosine phosphorylated proteins then initiates the respiratory burst with the abundant production of more superoxide, which in turn could catalyse the formation of additional vanadate species magnifying the response. As vanadium derivatives are ubiquitous in animal tissues, they could become tyrosine phosphatase inhibitors

when they encounter reduced oxygen. A correlation therefore seems to exist between the induction of tyrosine phosphorylation and the activation of the respiratory burst.

Many tyrosine kinase type receptors have been shown to phosphorylate unique proteins, which are able to bind to PI-3 kinase after phosphorylation at their tyrosine residue (Fukui et al 1998). Although these phosphotyrosine-containing proteins have not been characterised well, these molecules can act as adaptor molecules for PI-3 kinase to be activated. As PI-3 kinase appears to play various important roles including signalling to the nucleus, vesicle transport and rearrangement of the cytoskeleton the potential involvement of this enzyme on the respiratory burst was investigated. Mϕ's were pretreated with wortmannin (a sterol fungal metabolite) and potent irreversible inhibitor of PI-3 kinase (Raeder et al 1999) A significant dose dependent inhibition of the opsonised zymosan-induced oxidative burst was observed, producing an observed Ec50 value of approximately 100nM, (Fig's 4.3a and 4.3b). This may be evidence that the PI-3 kinase lipid products phosphatidylinositol-(3)-phosphate (PtdIns (3) P), phosphatidylinositol-(3,4)-bisphosphate (PtdIns (3,4) P_2), phosphatidylinositol-(3,5)-bisphosphate (PtdIns (3,5) P_2) and phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns (3,4,5) P_3) may be important mediators for the signalling pathways for respiratory burst activity. The PI-3 kinase lipid products are not substrates for the phosphatidylinositol-specific PLC enzymes that cleave inositol phospholipids into membrane bound DAG and soluble inositol phosphates like inositol trisphosphate (IP_3) because the 3-PI's are converted by kinases and phosphatases that act on the inositol ring (Vanhaesebroeck et al 1999). This separates PI-3 kinase signal transduction from the classical PLC/PtdIns (4,5) P_2 pathway that leads to among others Ca^{2+} release. PI3 kinase is a heterodimer made up of an 110kda catalytic subunit and an adaptor/regulatory SH2 domain. The SH2 domains bind to phosphorylated tyrosine residues that are generated by activated tyrosine kinases in receptors and various adaptor proteins. This is believed to allow translocation of the cytosolic PI-3 kinase to the membrane where their lipid substrates and Ras reside. Inhibition of PI-3 kinase also blocks the actin reorganisation and chemotaxis stimulated by tyrosine kinases (Vanhaesebroeck et al 1999). This inhibition can be overcome by the introduction

of constitutively activated Rac suggesting that Rac acts downstream of PI-3 kinase in the signaling pathways in actin reorganisation and possibly in the induction of the oxidative burst.

A separate pathway that also plays an important part in the respiratory burst is the classical PLC/PtdIns (4,5) P_2 system that leads to the generation of DAG and IP3. DAG is known to activate PKC and this enzymes importance in the signal transduction pathway was investigated. The effects of DAG can be mimicked by phorbol esters such as PMA which is a potent tumour promoter and binds to PKC and activates it directly rather than being receptor mediated. PMA stimulated an oxidative burst more rapidly than opsonised zymosan but the response was only 50% of that induced by opsonised zymosan at a concentration of 10^{-6}M (Fig 4.4). The quicker response time of PMA may be attributed to its more direct action on PKC. It may be possible that PMA is acting as a substitute for DAG, which in turn is stimulating O_2^- production and this, has been demonstrated in murine peritoneal Mϕ's (Czerniecki et al 1989). This is supporting evidence that PMA is believed to involve the activation of PKC resulting in its translocation from the cytosol to a membrane-associated site. One of the most straightforward approaches to study the role played by PKC in cellular processes is to inhibit enzymatic activity of the kinase in intact cells. For this purpose permeable potent and selective compounds are required. Bisindolylmaleimide (BIM) (GF109203X) has been established as a potent and selective inhibitor of PKC (Toullec et al 1991) and as a consequence it was used to assess the role of PKC in the respiratory burst. Upon application of BIM a significant dose-dependent inhibition of the PMA-induced oxidative burst was observed at concentrations as low as 10^{-8}M (Fig 4.5b) Previous work in different cellular models established that BIM has an IC_{50} of 10nM (Toullec et al 1991) indicating in this study that the potency is lower at ~ 500nM. Pre-treatment of Mϕ's with BIM also significantly abrogated the OpZ-induced oxidative burst producing an Ec50 of approximately 200nM, suggesting that receptor binding may ultimately result in PKC activation as well as PTK activity (Fig 4.6). This is therefore evidence that PKC-dependent serine/threonine phosphorylation is involved in the signal pathways leading to the activation of NADPH oxidase. Recent cloning studies have revealed that the PKC gene family contains at least ten

different isozymes that can be divided into two or three major groups (Hong et al 1995). The first group consists of four conventional PKC's: α, βI, βII, and γ and these are activated by Ca^{2+}, phosphatidylserine and DAG or phorbol esters. The second group consists of four novel PKC's δ, ε, η(L) and θ which are activated by phosphatidylserine and DAG or phorbol ester but not Ca^{2+}. A third group consists of two atypical PKC's ζ and λ which only require phosphatidylserine for activation. Because the individual isozymes have different cofactor requirements, sub cellular distribution and substrate specificity, the profile of PKC isoforms expressed in a particular cell is likely to be an important determinant of that cell in response to a particular stimulus. The functional roles of the individual isozymes in general and especially in Mϕ's are still ill defined. It would have been helpful to investigate which of these isozymes is responsible for initiating the production of a respiratory burst but at the moment this is difficult due to the lack of specific activators and inhibitors (Nakanishi et al 1992, Ways et al 1992). It has been reported that by using specific antibodies mouse peritoneal Mϕ's express five PKC isozymes: α, β, δ, ε and ζ and that PKC α triggers arachidonic acid metabolism and eicosanoid synthesis when induced by zymosan (Huwiler et al 1993) and this may suggest a role for this isozyme in the formation of a respiratory burst. Other isozymes as yet unidentified may still be involved however. Although PMA is believed to act through PKC this agent has also been shown to stimulate tyrosine phosphorylation in a number of cell types (Force et al 1991, Munoz et al 1991, Weinstein et al 1991). Genistein inhibited the PMA-induced respiratory burst from 10^{-4}M to10^{-6}M giving an approximate Ec50 value of 5μM, with concentrations below this having no effect (Fig's 4.7a and 4.7b). It appears that the oxidative burst induced by opsonised zymosan was more sensitive to inhibition by genistein compared to that induced by PMA (see Fig 4.0). It is possible therefore that a cross talk may exist between the tyrosine kinase and the serine/threonine kinase pathways for the onset of the respiratory burst. The contention that both of these pathways may be synergistically important has been strongly supported by correlation of a net tyrosine phosphorylation and increased activity of the respiratory burst after stimulation with PMA (Green et al 1994). A connection between tyrosine phosphatase inhibition and PKC has also been shown in this study as peritoneal Mϕ's pretreated with vanadate and then induced with PMA showed an augmentation of the respiratory burst at a

concentration of 10^{-6}M vanadate (Fig's 4.8a to 4.8c). This corroborates well with the fact that PMA and vanadate used in synergy enhanced reactive oxygen species formation and protein tyrosine phosphorylation (Goldman et al 1994).

Novel PKC's have been identified as the targets of PI-3 kinase by products and this has been shown when (PtdIns-(3,4) P_2) or (PtdIns-(3,4,5) P_3) was necessary for the in vitro activation of purified PKC in in vitro experiments (Nakanishi et al 1993, Toker et al 1994). When Mϕ's were pretreated with wortmannin a significant inhibition of the PMA-induced oxidative burst was observed with an approximate Ec50 of 100nMbeing noted, (Fig 4.7). No significant inhibition of the response was seen at lower concentrations. Therefore, pathways from PI-3 kinase to PKC appear to be involved.

Phosphinositide-specific PLC is known to play a pivotal role in cells stimulated with several agonists (Berridge et al 1993). As previously discussed PLC generates two second messengers DAG and IP3. The IP3 mobilises Ca^{2+} from intracellular stores and the next avenue of investigation in this study was to elucidate the involvement of Ca^{2+} in the oxidative burst. To study the complex interplay between Ca^{2+} influx and Ca^{2+} mobilisation, various artificial means have become available. These include removal of extracellular Ca^{2+} or the use of Ca^{2+} channel blockers, inhibitors of Ca^{2+}-ATPase from the endoplasmic reticulum such as thapsigargin and of Ca^{2+} ionophores. The Ca^{2+} ionophore (A23187) was unable to induce a respiratory burst alone but was able to enhance the oxidative burst when used in conjunction with PMA (Fig 4.10) suggesting that PKC activation and rises in intracellular Ca^{2+} are involved. In order to support further the role of $Ca2^{+}$ in the signaling of the oxidative burst, extracellular Ca^{2+} stores were depleted by incubating the Mϕ's in a Ca^{2+} free medium containing EGTA. Progressive chelation of extracellular Ca^{2+} by increased concentrations of EGTA caused greater inhibition of the oz-induced respiratory burst (Fig 4.11). Complete inhibition was however never observed. When small aliquots of Ca^{2+} were added to replenish the medium a recovery of the oxidative burst was seen (Fig's 4.12a to 4.12c) reinforcing the importance of Ca^{2+} in this process. This is in reasonable agreement

with published data which has shown that incubation with murine peritoneal Mϕ's in a Ca^{2+} free, EGTA-containing medium slightly decreased but did not abolish the zymosan-stimulated inositol phosphate release (Moscat et al 1986). It could be interpreted that extracellular Ca^{2+} is necessary for the complete binding of zymosan to its receptor or for the adequate coupling of the components of the transduction system. So, full oxidative burst production relies on both the contribution from intracellular Ca^{2+} release and extracellular Ca^{2+} influx. Macrophages exposed to thapsigargin showed elevated cytosolic Ca^{2+} concentrations (Fig 4.18). This was probably due to the inhibition of Ca^{2+}-ATPases in the endoplasmic reticulum preventing sequestration of the Ca^{2+} into these stores. There is also evidence that thapsigargin may promote an influx of extracellular Ca^{2+} (Randriamampita et al 1990). The phospholipid mediator PAF is a known potent stimulant of Mϕ's and amongst one of its roles is to induce Ca^{2+} release from intracellular stores via the activation of PLC (Randriamampita et al 1991). A rapid and explosive release of Ca^{2+} was seen to be induced by PAF at 1 and 10μM (Figs 4.19-4.21). It has been demonstrated that IP3-sensitive Ca^{2+}-stores are essential during PAF stimulation, as this Ca^{2+} release could be mimicked by intracellular injection of IP3. PAF responses could also be blocked by heparin which is thought to mask/competitively inhibit the IP3 receptor (Randriamampita et al 1991). This suggests that IP3 is an important second messenger in the response of Mϕ's to PAF. When Mϕ's were also challenged with opsonised zymosan no Ca^{2+} transients could be measured probably because the particulate nature of the saccharomycetes interfered with any possible measurements of $[Ca^{2+}]i$ This was observed very quickly after the application of opsonised zymosan as the fluorescence intensity instantly declined to below basal levels (Figs 4.20-4.21). 1 μM PMA gave a response in only an individual cell (cell 5, Fig 4.21). Taken together, these results suggest that transient changes in $[Ca^{2+}]i$ occur in murine peritoneal Mϕ's upon stimulation with thapsigargin and the agonist PAF. It is uncertain however whether PMA evokes a definite response and any $[Ca^{2+}]i$ changes caused by the application of opsonised zymosan could not be measured due to its masking effect on the mode of measuring $[Ca^{2+}]i$ fluxes in this system. It is important to point out that not all the Mϕ's were excitable and this could be attributed to the cells being in different phases of differentiation. This is however a good experimental technique, as soluble activators produced a reasonable

response. These findings deserved to be extended perhaps by examining soluble immune complexes, which could bind to Fc and C3b receptors.

A still greater complexity of the mechanisms regulating intracellular Ca^{2+} stems from the existence of various PLC isoforms, which are all more or less sensitive to Ca^{2+} and which appear to be regulated by different mechanisms (Pinelli et al 1994) i.e. G-proteins for PLC-β, protein tyrosine kinases for PLC-γ and possibly Ca^{2+} for PLC-δ (Berridge et al 1993). Depending on the cell and on the agonist, elevation of intracellular Ca^{2+} can thus either result from PLC activation during its triggering or modulation. In addition, Ca^{2+} itself can also regulate the activity of various inositol phosphate-dependent or independent Ca^{2+} channels. This has led to various hypothetical models of Ca^{2+}-induced Ca^{2+} release (Berridge et al 1993). Verapamil an inhibitor of voltage gated Ca^{2+} channels has been shown to elevate c-AMP concentration in neutrophils and potentiate the c-AMP by exposure to FMLP and PMA (Della Bianca 1985). In this study it was revealed that verapamil abrogated the oxidative burst by a maximum of ~35% at 10^{-4}M. There could also be an inhibition of the Ca^{2+} activated phospholipid-dependent PKC. Intracellular Ca^{2+} increases could lead to the activation of other enzymes including calmodulin-dependent protein kinases and phospholipase A2. W7 is a calmodulin antagonist blocking Ca^{2+}/ calmodulin regulation of other response elements. Calmodulin functions as a multipurpose intracellular Ca^{2+} receptor mediating many Ca^{2+}-regulated processes and Ca^{2+} can induce calmodulin to bind to various target proteins in the cell and therefore alter their activity. W7 inhibited the opsonised zymosan-induced oxidative burst (Fig 4.17) indicating that Ca^{2+}/calmodulin regulated proteins play a part in this process such as Ca^{2+}/calmodulin-dependent protein kinases i.e. (Ca^{2+} kinase II).

A Na^{+}/Ca^{2+} exchange mechanism has been described in human neutrophil that constitutes the principal pathway for Ca^{2+} influx into resting cells (Simchowitz et al 1990). Amiloride an inhibitor of the Na^{+}/H^{+} antiporter system was used to pre-treat Mϕ's and was shown to inhibit the oxidative burst at concentrations of 10^{-4}M and 10^{-5}M (Fig 4.14). This suggests that this antiporter is a requirement for the respiratory burst activity. Amiloride and Na^{+} deprivation have been shown to

significantly inhibit both superoxide production and lysozyme release in neutrophils stimulated with opsonised zymosan (Wright et al 1986). It is therefore possible that a Na^+/H^+ antiporter similar to that found in neutrophils may be prevalent in murine peritoneal Mϕ's and linked to the activation of the respiratory burst. Cytoplasmic pH has been shown to be an important determinant of the activity of NADPH oxidase in phagocytic cells (Swallow et al 1990). It is possible that H^+ secretion during hexose monophosphate shunt activity is vital in order for a Na^+/H^+ exchange mechanism to occur.

The TK, PLC, PKC/Ca^{2+} axis is not the only signalling pathway in the Mϕ. Via a host of different receptors the adenylate cyclase system is likely also to affect Mϕ function. To ascertain whether the accumulation of cyclic AMP (cAMP) had an effect on the respiratory burst cells were treated with forskolin to directly stimulate adenylate cyclase. A significant reduction in the burst was observed using10^{-8}M forskolin (Fig 4.15). Cyclic-AMP mediates its second messenger function through the activation of protein kinase A (PKA) which is capable of phosphorylating PLC thus inhibiting its function (Snow et al 1994). This will then lead to the reduction in polyphosphoinositide turnover decreasing the amount of IP3 and DAG produced, ultimately causing a reduction in the oxidative burst. This experimental result indicates a possible role for c-AMP in regulating phosphoinositide turnover in the signalling pathways leading to the oxidative burst. This conclusion was reinforced when dibutyryl c-AMP (a more stable derivative of c-AMP) was used to pre-treat the Mϕ's. A similar dose-dependent inhibition to that observed with forskolin was seen (Fig 4.15). Over a number of years numerous investigators have demonstrated that activation of PKC by phorbol esters alters the c-AMP levels in various tissues and cultured cells. Indeed, PMA has been shown to significantly enhance forskolin-stimulated c-AMP accumulation in a cell model (Kawabe et al 1996) with staurosporine abolishing this enhancement indicating the positive role of PMA sensitive PKC in this reaction.

From these findings the signal transduction events during the process of phagocytosis leading to the stimulation of NADPH oxidase involves PLC activation when the complement and sugar receptors are ligated. Since genistein a PTK

inhibitor is seen to reduce opsonised zymosan-induced respiratory burst activity, then a PTK is thought to be one of the candidates involved. Tyrosine phosphatase involvement was also found to important, as vanadate augmented the oxidative burst. PLC then catalyses the hydrolysis of PIP2 to IP3 and DAG. DAG then causes the activation of PKC. The production of IP3 causes the mobilisation of intracellular Ca^{2+} stores and when IP3 is phosphorylated to IP4 (1,3,4,5 tetrakisphosphate), it may lead to the opening of Ca^{2+} channels in the membrane.

Verapamil, which inhibited the Ca^{2+} channels, caused a reduction in the respiratory burst, as did EGTA and this demonstrated the importance of Ca influx across the plasma membrane. The Ca^{2+} can bind calmodulin, and W7 abrogated this process indicating the importance of downstream calmodulin-dependent effectors, possibly like phosphodiesterases. Calmodulin can also stimulate PLA2 to enhance arachidonic acid production, which is then converted to PGE2 and leukotrienes, which activate adenylate cyclase. Adenylate cyclase causes the conversion of ATP to c-AMP, which subsequently stimulates PKA causing more protein phosphorylation. Increases in intracellular c-AMP however, profoundly inhibited the ability of the Mϕ's to produce an oxidative burst as demonstrated using forskolin and dibutyryl c-AMP. This suggests the importance of c-AMP as a second messenger in the oxidative burst by its action on PKA to suppress PLC activation and phosphorylation, dampening PIP3 and DAG turnover, creating a feedback inhibition.

PKC was also shown to be very important in the overall signalling pathways. The PKC activator PMA initiated an oxidative burst and BIM inhibited the production of reactive oxygen intermediates indicating that this enzyme also plays a major role in activating NADPH oxidase.

From these studies there is also compelling evidence that PI-3 kinase is also involved in the signalling pathway, as wortmannin significantly prevented the oxidative burst. It is uncertain however, whether these PI-3 kinase effects are occurring upstream or downstream of a tyrosine kinase or alternatively at both locations simultaneously. Inhibition by amiloride may indicate a constant

cytoplasmic pH level must be maintained for proper functioning of the respiratory burst. It is worth mentioning that there is some deviation in the experimental EC50 values of the inhibitors from cited estimates. Genistein exhibited EC50 values of 5μM (figs 4.0a and 4.7a), which compared favourably with the expected 2.5-10μM. However, BIM when used to block the effects of PMA and OPZ on PKC, produced EC50's of approximately 500nM and 200nM respectively (fig's 4.5a and 4.6a), compared to its quoted value of 10nM. Likewise, wortmannins EC50 values of the PMA and OPZ effects were 100nM (fig's 4.3a and 4.9a) respectively, in comparison to the stated EC50 of 5nM. The reduced efficacy of these inhibitors could be explained by the experimental assay conditions implemented. The specificity of the inhibitors could have been reduced due to changes in pH and ionic strength during the assay. It is also possible that there was association with other proteins and non-specific binding to the sample tubes during incubations. The EC50 values may also vary depending on what in vitro model was used. The experimental EC50's obtained during this analysis are within acceptable limits.
A summary diagram of the signalling pathways that are thought to be operative in this study can be seen in fig 5.0.

Subsequent experiments that might add to the findings of this study would certainly include the use of calmodulin antagonists and their roles in the transduction pathways. Measurement of intracellular Ca^{2+} would clearly be beneficial. A more detailed insight into the c-AMP system could also be achieved using phosphodiesterase inhibitors and beta agonists to examine in more detail the role of c-AMP in the regulation of the oxidative burst.

Mouse IgG coated latex beads or antibody coated sheep red blood cells could be used to study the Fcγ receptor-mediated signal transduction during phagocytosis. Monoclonal antibodies that bind to specific Fcγ receptors could distinguish which Fcγ receptors play the more important role.

Nitric oxide may be a potent regulator during the Mϕ respiratory burst and it would be interesting to elucidate if the induced expression of nitric oxide synthase has any

impact. Studies on the potential role of nitric oxide using antagonists (such as L-arginine analogues) or nitric oxide generating agents could be used.

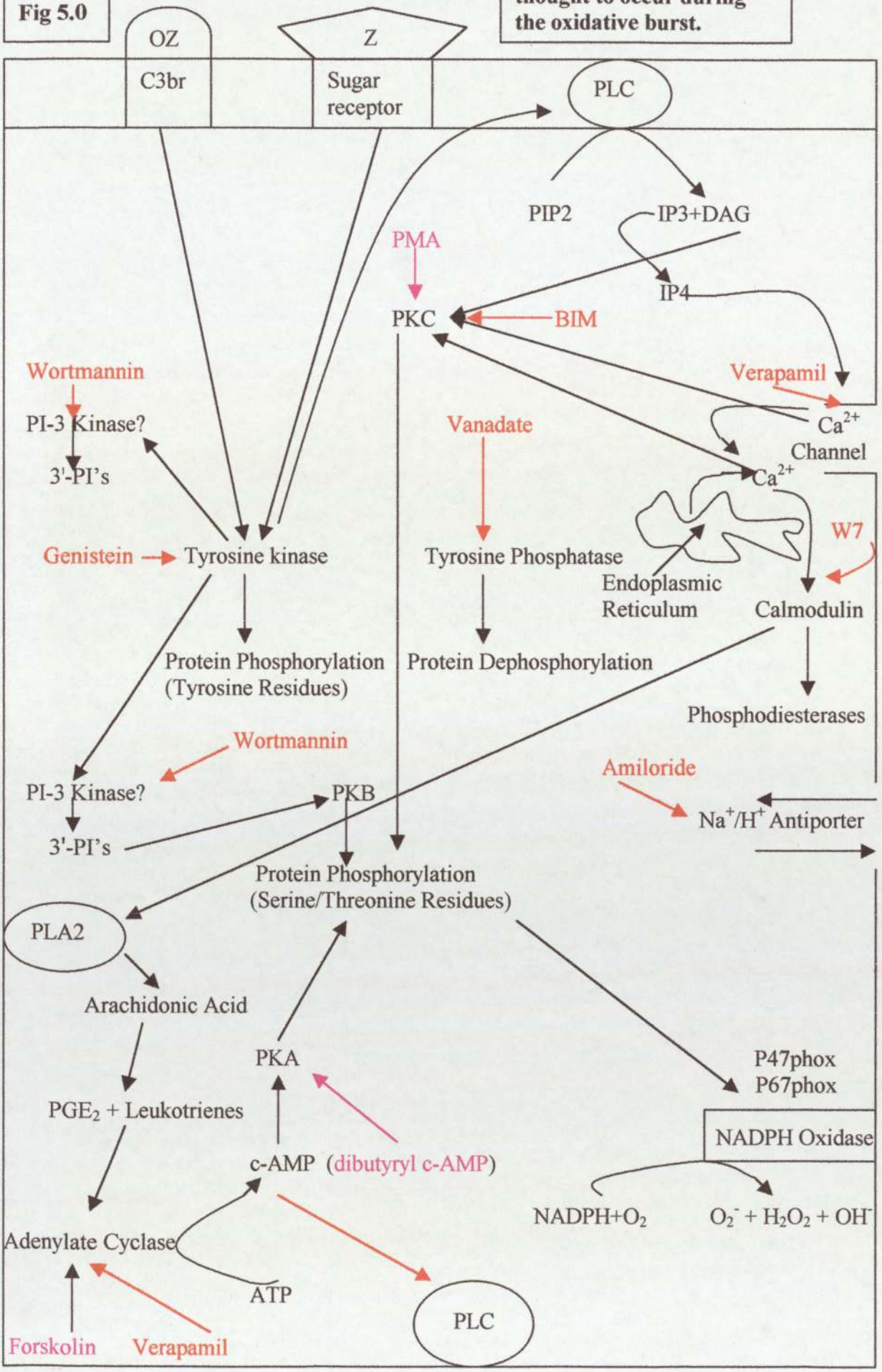
Neural and immune systems may have important interconnections during the oxidative burst, as it has been demonstrated that the neuropeptide calcitonin gene related peptide (CGRP) found in sensory neurones causes phagocytosis in cultured mouse peritoneal Mϕ's (Ichinose et al 1996). Studies could establish whether neural and endocrine mediators influence the generation of reactive oxygen intermediates. Certainly, macrophages are known to carry a multiplicity of receptors for various hormones and neuropeptides (Ichinose et al 1996).

The importance of G-protein coupled receptors in the system leading to NADPH oxidase activation could be investigated. For instance, the involvement of adrenergic receptors could be looked at using α and β antagonists.

If more specific inhibitors targeting the PKC's, PI-3 kinases and tyrosine kinases were available then a more detailed picture of the roles of isomers of these enzymes in the respiratory burst could be achieved. It can be seen therefore, that there are many more avenues of investigation that could be deployed in order to clarify the complexity of signal transduction during the respiratory burst.

Fig 5.0

Summary diagram of the signalling pathways thought to occur during the oxidative burst.



6. References

Abo, A., Pick, E., Hall, A., Torry, N., Teahan, C.G., and Segal, A.W., The small GTP binding protein p21rac1 is involved in activation of the NADPH oxidase. *Nature*, **353:** 668-670, 1991.

Adachi, Y., Ohno, N., and Yadomae, T., Inhibitory effect of β-glucans on zymosan-mediated hydrogen peroxide production by murine peritoneal macrophages in vitro. *Biology and Pharmaceutical Bulletin* **16:** 462-467, 1993.

Adams, D.O., and Hamilton, T.A., The cell biology of macrophage activation. *Annual Reviews of Immunology.* **2:** 283-318, 1984.

Aderem, A., and Underhill, M., Mechanisms of phagocytosis in macrophages. *Annual Review in Immunology.* **17:** 593-623, 1999.

Aderem, A.A., Wright, S.D., Silverstein, S.C., and Cohn, Z.A., Ligated complement receptors do not activate the arachidonic acid cascade in resident peritoneal macrophages. *Journal of Experimental Medicine*, **161:** 617-622, 1985.

Alford, B., Xue, Y., Thai, S-F., and Shackleford, R.E., Maleylated-BSA enhances production of nitric oxide from macrophages. *Biochemical and Biophysical Research Communications.* **245:** 185-189, 1998.

Allen, L-A.H., and Aderem, A., Molecular definition of distinct cytoskeletal structures involved in complement and Fc receptor-mediated phagocytosis in macrophages. *Journal of Experimental Medicine.* **184:** 627-637, 1996.

Allen, L-AH., and Aderem, A., A role for MARCKS, the α isozyme of protein kinase C and myosin I in zymosan phagocytosis in macrophages. *Journal of Experimental Medicine.* **182:** 829-840, 1995.

Allen, L-AH., and Aderem, A., Mechanisms of phagocytosis. *Current Opinions in Immunology.* **8:** 36-40, 1996.

Aparna, H.S., and Salimath, P.V., Galactose terminated oligosaccharides activate macrophage respiratory burst. *Nutrition Research,* **14:** 433-444, 1994.

Araki, N., Johnson, M.T., and Swanson, J.A., A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *The Journal of Cell Biology,* **135:** 1249-1260, 1996.

Babior, B.M., The respiratory burst of phagocytes. *Journal of Clinical Investigation.* **73:** 599-601, 1984.

Balsinde, J., Fernandez, B., and Diez, E., Regulation of arachidonic acid release in mouse peritoneal macrophages: The role of extracellular calcium and protein kinase C. *The Journal of Immunology,* **144:** 4298-4304, 1990.

Balsinde, J., Fernandez, B., Solis-Herruzo, J.A., and Diez, E., Pathways for arachidonic acid mobilisation in zymosan-stimulated mouse peritoneal macrophages. *Biochemica et Biophysica Acta*, **1136:** 75-82, 1992.

Barker, S.A., Caldwell, K.K., Hall, A., Martinez, A.M., Pfeiffer, J.R., Oliver, J.M., and Wilson, B.S., Wortmannin blocks lipid and protein kinase activities associated with PI-3-kinase and inhibits a subset of responses induced by FcγR1 cross linking. *Molec.Biol.Cell.* **6:** 1145-1158, 1995.

Beppu, M., Takahashi, T., Hayashi, T., and Kikugawa, K., Mechanism of macrophage recognition of SH-oxidised erythrocytes: recognition of glycophorin A on erythrocytes by a macrophage receptor for sialosaccharides. *Biochemica et Biophysica. Acta,* **1223:** 47-56, 1994.

Berken, A., and Benacerref, B., Properties of antibodies cytophilic for macrophages. *Journal of Experimental Medicine.* **123:** 119-144, 1966.

Berkow, R.L., Dobson, R.W., and Kraft, A.S., Human neutrophils contain distinct cytosolic and particulate tyrosine kinase activities: possible role of neutrophil activation. *Biochim.et Biophysica. Acta,* **997:** 292-301, 1989.

Berkow, R.L., and Dodson, R.W., Tyrosine-specific protein phosphorylation during activation of human neutrophils. *Blood,* **75:** 2445-2452, 1990.

Berridge, M.J., Inositol trisphosphate and calcium signalling. *Nature,* **361:** 315-325, 1993.

Blackwell, J.M., Ezekowitz, R.A.B., Roberts, M.B., Channon, J.Y., Sim, R.B., and Gordon, S., Macrophage, complement and lectin-like receptors bind leishmania in the absence of serum. *Journal of Experimental Medicine.* **162:** 324-331, 1985.

Bohnsack, J.F., Takahashi, T., and Brown, E.T., Interaction of cultured-derived macrophages with the fibroblast binding domain of fibronectin is a necessary but insufficient signal for fibronectin enhancement of CR1 mediated phagocytosis. *Journal of Immunology,* **136:** 3793-3798, 1986.

Bokoch, G.M., Regulation of the phagocyte respiratory burst by small GTP-binding proteins. *Trends in Cell Biology.* **5:** 109-113. 1995.

Breton, P., Petit, C., Bousser, M-T., Monsigny, M., Mayer, R., and Roche, A-C., Secretion of macrophage cytotoxic factors induced by new desmuramyl acylpseudopeptide analogs of muramyl dipeptide. *Oncology Research,* **4:** 103-107, 1992.

Byorkmann, P.J., Saper, M.H., Samaraoui, B., Bennet, W.S., Strominger, J.L., and Wiley, D.C., Structure of the human class 1 histocompatability antigen, HLA-AQ. *Nature.* **329:** 506-512, 1987.

Cohen, M.D., and Wei, C.I., Effects of ammonium metavanadate treatment upon macrophage glutathione redox cycle activity, superoxide production and intracellular glutathione status. *Journal of Leukocyte Biology,* **44:** 122-129, 1988.

Conde, M., Chiara, M.D., Pintado, E., and Sobrino, F., Modulation of phorbol ester-induced respiratory burst by vanadate, genistein, and phenylarsine oxide in mouse macrophages. *Free Radical Biology and Medicine,* **18:** 343-348, 1995.

Cox, D., Chang, P., Zhang, Q., Reddy, P.G., Bokoch, G.M., and Greenberg, S., Requirement for both Rac1 and Cdc42 in membrane ruffling and phagocytosis of leukocytes. *Journal of Experimental Medicine.* **186:** 1287-1294, 1997.

Cox, D., Tseng, C-C., Bjekic, G., and Greenberg, S., A requirement for phosphatidylinositol 3-kinase in pseudopod extension. *The Journal of Biological Chemistry,* **274:** 1240-1247, 1999.

Cross, C.E., Collins, H.L., and Bancroft, G.J., CR3 dependent phagocytosis by murine macrophages: different cytokines regulate ingestion of a defined CR3 ligand and complement-opsonized Cryptococcus neoformans. *Immunology,* **91:** 289-296, 1997.

Crowley, M.T., Costello, P.S., Fitzer-Attas, C.J., Turner, M., Meng, F., Lowell, C., Tybulewicz, V.L.J., and DeFranco, A.L., A critical role for syk in signal transduction and phagocytosis mediated by Fcγ receptors on macrophages. *Journal of Experimental Medicine,* **186:** 1027-1039, 1997.

Czerniecki, B.J., and Witz, G., Tumour promoters stimulate the formation of 1,2 diacylglycerol in murine peritoneal macrophages: a possible mechanism for stimulating superoxide anion radical production. *Cancer Letters*, **48:** 29-36, 1989.

Dana. R., Leto, T.L., Malech, H.L., and Levy, R., Essential requirement of cytosolic phospholipase A2 for activation of the phagocyte NADPH oxidase. *The Journal of Biological Chemistry.* **273:** 441-445, 1998.

De Camilli, P., Emr, S.D., McPherson, P.S., and Novick, P., Phosphoinositides as regulators of membrane traffic. *Science,* **271:** 1533-1539, 1994.

Della Bianca, V., Grzeskowiak, M., De Togni, P., Cassatella, M., and Rossi, F., Inhibition by verapamil of neutrophil responses to formyl-methionyl-leucyl-phenylalanine. and phorbol myristate acetate. Mechanisms involving Ca^{2+} changes, cyclic AMP and protein kinase C. *Biochemical et Biophysical Acta,* **845:** 223-236, 1985.

Derman, M.P., Toker, A., Hartwig, J.H., Spokes, K., Falck, J.R., Chen, C-S., Cantley, L.C., and Cantley, L.G., The lipid products of polyphosphoinositide 3-kinase increase cell motility through protein kinase C. *The Journal of Biological Chemistry.* **272:** 6465-6470, 1997.

De Lis, C., and Berzofsky, J., T cell antigenic sites tend to be amphipathic structures. *Proceedings of the National Academy of Sciences.* **82:** 7048-52, 1986.

Dewald, B., Thelen, M., and Baggiolini, M., Two transduction sequences necessary for activation by receptor agonists. *Journal of Cell Biology.* **263:** 16179-16184, 1988.

Diatchuk, V., Lotan, O., Koshkin, V., Wikstroem, P., and Pick, E., Inhibition of the NADPH oxidase activation by 4-(2-aminoethyl)-benzenesulfonyl fluoride and related compunds. *Journal of Biological Chemistry.* **272:** 13292-13301, 1997.

Dickmann, D., Abo, A., Johnston, C., Segal, A.W., and Hall, A., Interaction of rac with $p67^{phox}$ and regulation of phagocytic NADPH-oxidase activity. *Science,* **265:** 531-533, 1994.

Didichenko, S.A., Tilton, B., Hemmings, B.A., Ballmer-Hofer, K., and Thelen, M., Constitutive activation of protein kinase B and phosphorylation of P-47 phox by a membrane-targeted phosphoinositide 3-kinase. *Curr. Biol.* **6:** 1271-1278, 1996.

Dustin, M.L., Rothlein, R., Bhan, A.K., Dinarello, C.A., and Springer, T.R., Induction by interleukin-1 and interferon-γ. Tissue distribution, biochemistry and function of a natural adhesion molecule (ICAM -1). *Journal of Immunology.* **137:** 245-254, 1986.

Emilsson, A., and Sundler, R., Evidence for a catalytic role of phospholipase A in phorbol diester and zymosan-induced mobilisation of arachidonic acid in mouse peritoneal macrophages. *Biochemica et Biophysica Acta.* **876:** 533-542, 1986.

Exton, J.H., New developments in phospholipase D. *The Journal of Biological Chemistry.* **272:** 15579-15582, 1997.

Fernandez, B., and Balsinde, J., Calcium and G-protein-dependent activation of arachidonic acid release by concanavalin-A-stimulated mouse macrophages. *Biochimica et Biophysica Acta,* **1176:** 169-174, 1996.

Fernandez, B., and Balsinde, J., Receptor-mediated activation of arachidonic acid release in mouse peritoneal macrophages is linked to extracellular calcium influx. *Biochemical and Biophysical Research Communications,* **180:** 1036-1040, 1991.

Fernandez, B., Balboa, M.A., Solis-Herruzo, J.A., and Balsinde, J., Phosphatidate-induced arachidonic acid mobilisation in mouse peritoneal macrophages. *The Journal of Biological Chemistry,* **269:** 26711-26716, 1994.

Force, T., Kyriakis, J.M., Avruch, J., and Bonventre., Endothelin, vasopressin and angiotensin II enhance tyrosine phosphorylation by protein kinase C-dependent and independent pathways in glomerular mesangial cells. *Journal of Biological Chemistry,* **266:** 6650-6656, 1991.

Fukui, Y., Ihara, S., and Nagata, S., Downstream of phosphatidyl inositol-3-kinase, a multifunctional signaling molecule and its regulation in cell responses. *Journal of Biochemistry,* **124:** 1-7, 1998.

Furth, R.V., and Cohn, Z.A., The origin and kinetics of mononuclear phagocytes. *Journal of Experimental Medicine.* **128:** 415-435, 1968.

Giaimis, J., Lombard, Y., Fontenenau, P., Muller, CD., Levy, R., Makaya-Kumba, M., Lazdins, J., and Poindrin, P., Both mannose and beta-glucan receptors are involved in phagocytosis of unopsonised heat killed saccharomycetes cerevisiae by murine macrophages. *Journal of Leukocyte Biology,* **54:** 564-571, 1993.

Goldman, R., Ferber, E., Meller, R., and Zor, U., A role for reactive oxygen species in zymosan and β-glucan induced protein tyrosine phosphorylation and phospholipase A_2 activation in murine macrophages. *Biochemica et Biophysica Acta,* **1222:** 265-276, 1994.

Goldman, R., and Zor, U., Activation of macrophage PtdIns PLC by phorbol ester and vanadate: involvement of reactive oxygen species and tyrosine phosphorylation. *Biochemical and Biophysical Research Communications,* **199:** 334-338, 1994.

Green, S.P., and Phillips, W.A., Activation of the macrophage respiratory burst by phorbol myristate acetate: evidence for both tyrosine-kinase-dependent and independent pathways. *Biochemica et Biophysica Acta,* **1222:** 241-248, 1994.

Greenberg, S., Signal transduction in phagocytosis. *Trends in Cell Biology.* **5:** 93-99, 1995.

Grey, H.M., Sette, A., and Buus, S., How T cells see antigen. *Scientific American.* November 1989.

Griffin, F.M., Activation of macrophage C3 receptors by a unique lymphokine. *Lymphokines,* **14:** 431-441, 1987.

Griffin, F.M., and Silverstein, S.C., Segmental response of the macrophage plasma membrane to a phagocytic stimulus. *Journal of Experimental medicine.* **139:** 323-336, 1974.

Griffin, F.M., Griffin, J.A., Leider, J.E., and Silverstein, S.C., Study of the mechanisms of phagocytosis. Requirements for the circumferential attachment of peptide bond ligands to specific receptors on the macrophage plasma membrane. *Journal of Experimental Medicine.* **142:** 1263-1282, 1975.

Hackam, D.J., torstein, O.D., Schreiber, A., Zhang, W.J., and Grinstein, S., Rho is required for the initiation of calcium signalling in phagocytosis by Fcγ receptors in macrophages. *Journal of Experimental Medicine.* **186:** 955-966, 1997.

Hamilton, T.A., Molecular mechanisms of signal transduction in macrophages. *Immunology Today.* **8:** 151-158, 1987.

Hishikawa, T., Cheung, J.Y., Yelamarty, R.V., and Knutson, T.W., Calcium transients during Fc receptor-mediated and nonspecific phagocytosis by murine peritoneal macrophages. *The Journal of Cell Biology.* **115:** 59-66, 1991.

Holers, V.M., Kinoshita, T., and Molina, H., The evolution of mouse and human complement C3 binding proteins: divergence of form but conservation of function. *Immunology Today,* **13:** 231-236, 1992.

Hong, D.H., Huan, J., OU, B., Yeh, J., Saido, T.C., Cheeke, P.R., and Forsberg, N.E., Protein kinase C isoforms in muscle cells and their regulation by phorbol ester and calpain. *Biochimica et Biophysica Acta,* **1267:** 45-54, 1995.

Howard, A.D., and Erickson, K.L., Alteration of macrophage responsiveness to platelet-activating factor by interferon-γ and lipopolysaccharide. *Cellular Immunology.* **174:** 155-164, 1996.

Huwiler, A., and Pfeilschifter, J., A role for protein kinase C-α in zymosan-stimulated eicosanoid synthesis in mouse peritoneal macrophages. *European Journal of Biochemistry,* **217:** 69-75, 1993.

Huwiler, A., and Pfeilschifter, J., A role for protein kinase c-α in zymosan-stimulated eicosanoid synthesis in mouse peritoneal macrophages. *European Journal of Biochemistry.* **217:** 69-75, 1993.

Ichinose, M., and Sawada, M., Enhancement of phagocytosis by calcitonin gene related peptide (CGRP) in cultured mouse peritoneal macrophages. *Peptides.* **17:** 1405-1414, 1996.

Janeway,C.A., How the immune system recognises invaders. *Scientific American.***Sept 1993:** 73-80.

Johnson, W.J., Pizzo, S.V., Imber, M.J., and Adams, D.O., Receptors for maleylated proteins regulate secretion of neutral proteases by murine macrophages. *Science.* **218:** 574-576, 1982.

Johnston, P.A., Jansen, M.M., Somers, S.D., Adams, D.O., and Hamilton, T.A., Maleyl-Bsa and fucoid induce expression of an early set of proteins in murine peritoneal macrophages. *Journal of Immunology.* **138:** 1551-1558, 1987.

Jouvin, M-H, H., Adamczewsk, M., Numerof, R., Letourner, O., Valle, A., and Kinet, J-P., Differential control of the tyrosine kinases lyn and syk by two signalling chains of the high affinity immunoglobulin E receptor. *The Journal of Biological Chemistry*, **269:** 5918-5925, 1994.

Kan, V.L., and Bennet, J.E., Lectin-like attachment sites on murine pulmonary alveolar macrophages and Aspergillus fumigatus conidia. *Journal of Infectious Disease.* **158:** 407-414,1988.

Kawabe, J., Toshiaki, E., Yoshiyuki, T., Naoki, O., Schwenke, C., Duzic, E., and Ishikawa, Y., Regulation of type V adenylyl cyclase by PMA sensitive and insensitive protein kinase C isoenzymes in intact cells. *FEBS Letters,* **384:** 293-276, 1996.

Knoller, S., Shpungin, S., and Pick, E., The membrane-associated component of the amphiphile-activated, cytosol-dependent superoxide-forming NADPH oxidase of macrophages is identical to cytochrome b_{559}. *The Journal of Biological Chemistry.* **266:** 2795-2804, 1991.

Kraft, A.S., and Berkow, A.L., Tyrosine kinases and phosphotyrosine phosphatase activity in human promyelocytic leukamia cells and human polymorphonuclear leukocytes, *Blood.* **70:** 356-362, 1987.

Kuang, H.C., Borak, V., Laramee, G.R., and Casanellie, J.E., Protein tyrosine phosphorylation in rabbit peritoneal neutrophils. *Biochemical Journal,* **269:** 431-436, 1990.

Kuby, J., *Immunology 2nd Edition.* W.H.Freeman and Company. New York. 1994.

Kusner, D. J., Hall, C.F., and Schlesinger, L.S., Activation of phospholipase D is tightly coupled to the phagocytosis of Myobacterium tubercelosis or opsonised zymosan by human macrophages. *Journal of Experimental Medicine.* **184:** 585-595, 1996.

Kusner, D. H., Hall, C.F., and Jackson, S., Fc gamma receptor-mediated activation of phospholipase D regulates macrophage phagocytosis of IgG-opsonised particles. *Journal of Immunology.* **162:** 2266-2274, 1999.

Lennartz, M.R., Phospholipases and phagocytosis: the role of phospholipid-derived second messengers in phagocytosis. *The International Journal of Biochemistry and Cell Biology.* **31:** 415-430, 1999.

Lewis, C.E., and McGee, J.D., *The Macrophage* Oxford University Press. 1991.

Li, G., D'Souza-Schorey, C., Barbieri, A., Roberts, R.L., Klippel, A., Williams, L.T., and Stahl, P.D., Evidence for phosphatidylinositol 3-kinase as a regulator of endocytosis via activation of Rab5. *Proceedings of the National Academy of Sciences USA.* **92:** 10207-10211, 1995.

Lloret, S., and Moreno, J.J., Role of kinases and G-proteins on arachidonate release induced by zymosan in mouse peritoneal macrophages. *International Journal of Biochemistry and Cell Biology,* **28:** 465-472, 1996.

Lloret, S., and Moreno, J.J., Stimulation of arachidonic acid mobilisation by adherence of resident peritoneal macrophages to plastic substrate. *Comp. Biochem. Physiol.,* **113C** :403-408, 1996.

Loegering, D.J., and Schwacha, M.G., Macrophage hydrogen peroxide production and phagocytic function are decreased following phagocytosis mediated by Fc receptors but not complement receptors. *Biochemical and Biophysical Research Communications,* **180:** 268-272, 1991.

Lokesh, B.R., and Kinsella, J.E., Intracellular calcium does not appear to be essential for arachidonic acid release from stimulated macrophages as shown by studies with Quin-2. *Biochemica et Biophysica Acta.* **845:** 101-108, 1985.

Maldonado, G., Porras, F., Fernandez, L., Vazquez, L., and Zenteno, E., Effect of lectins on mouse peritoneal macrophage phagocytic activity. *Immunological Investigations,* **23:** 429-436, 1994.

Manser, E., Leung, T., Salihuddin, H., Zhao, Z.S., and Lim, L., A brain serine/threonine protein kinase activated by cdc42 and rac1. *Nature,* **367:** 40-46, 1994.

Marshall, M.S., Ras target proteins in eukaryotic cells. *Faseb Journal.* **9:** 1311-1318, 1995.

Martinez, J., and Moreno, J.J., Influence of superoxide radical and hydrogen peroxide on arachidonic acid mobilisation. *Archives of Biochemistry and Biophysics,* **336:** 191-198, 1996.

Masihi, K.N., Ozel, M., Lange, W., and Azuma, I., Lipophilic muramyl dipeptide – induced changes in electron microscopic morphology and phagocytic function of murine macrophages. *Journal of Biological Response Modifiers,* **5:**20-26, 1986.

McNeil, P.L., Swanson, J.A., Wright, S.A., Silverstein, S.C., and Taylor, D.L., Fc receptor-mediated phagocytosis occurs in macrophages without an increase in average $[Ca^{++}]i$. *The Journal of Cell Biology.* **102:** 1586-1592, 1986.

Meagher, L.C., Moonga, B.S., Haslett, C., Huang, C. L-H., and Zaidi, M., Single pulses of cytoplasmic calcium associated with phagocytosis of individual zymosan particles by macrophages. *Biochemical and Biophysical Research Communications.* **177:** *460-465,* 1991.

Meats, J.E., Steele, L., and Bowen, J.G., Identification of phospholipase D (PLD) activity in mouse peritoneal macrophages. *Agents and Actions,* **39:** C14-C16, 1993.

Meuret, G., and Hoffman, G., Monocyte kinetic studies in normal and disease states. *British Journal of Haematology.* **24:** 275-285, 1973.

Mizuno, T., Kaibuchi, K., Ando, S., Musha, T., Hiraoka, K., Takaishi, K., Asada, M., Nunoi, H., Matsuda, I., and Takei, M., Regulation of the superoxide-generating NADPH oxidase by a small GTP-binding protein and its stimulatory and inhibitory GDP/GTP exchange proteins. *Journal of Biological Chemistry,* **267:** 10215-10218, 1992.

Moscat, J., Aracil, M., Diez, E., Balsinde, J., Barrero, P.G., and Municio, A.M., Intracellular Ca^{2+} requirements for zymosan-stimulated phosphoinositide hydrolysis in mouse peritoneal macrophages. *Biochemical and Biophysical Research Communications,* **134:** 367-371, 1986.

Moscat, J., Herrero, C., Garcia-Batreno, P., and Municio, A.M, Zymosan induced release of inositol phosphates at resting cytosolic Ca^{2+} concentrations in macrophages. *Biochemical Journal,* **242:** 441-447, 1987.

Mosser, D.M., Receptors on phagocytic cells involved in microbial recognition. *Immunology Series,* **60:** 99-114, 1994.

Munoz, E., Zubiaga, A.M., and Huber, B.T., Tyrosine protein phosphorylation is required for protein kinase-mediated proliferation in T cells. *FEBS Letters,* **279:** 319-322, 1991.

Nagao, N., Akagawa, K.S., Okada, F., Harada, Y., Yagawa, K., Kato, K., and Tanigawa, T., Species dependency of in vitro macrophage activation by bacterial peptidoglycans. *Microbiol. Immunol,* **36:** 1155-1171, 1992.

Nagao, S., Akagawa, K.S., Yamada, K., Yagawa, K., Tokunada, T., and Kotani, S., Lack of response of murine peritoneal macrophages to in vitro activation by muramyl dipeptide. I. Macrophage activation by MDP is species dependent. *Microbiol. Immunol,* **34:** 323-335, 1990.

Nakanishi, H., and Exton, J.H., Purification and characterisation of the ζ isoform of protein kinase C from bovine kidney. *Journal of Biological Chemistry,* **267:** 16347-16354, 1992.

Nakanishi, H., Brewer, K.A., and Exton, J.H., Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5 trisphosphate. *Journal of Biological Chemistry,* **268:** 13-16, 1993.

Nakano, M., and Shinomiya, H., Molecular mechanisms of macrophage activation by LPS, 1990, In: cellular and molecular aspects of endotoxin reactions, Nowotny, A., Spitzer, J.J., and Ziegler, E.J., (eds). Elsevier, Amsterdam-New York, 205pp.

Nechay, B.R., Mechanisms of action of vanadium. *Annual Reviews of Pharmacology and Toxicology.* **24:** 501-524, 1984.

Ninomiya, N., Hazeki, K., Fukui, Y., Tsukasa, S., Okada, T., Hazeki, O., and Ui, Michio, Ui., Involvement of phosphatidylinositol 3-kinase in Fcγ receptor signaling. *The Journal of Biological Chemistry.* **269:** 22732-22737, 1994.

Ofek, J., and Sharon, N., Lectinophagocytosis: a molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. *Infection and Immunity.* **56:** 539-547, 1988.

Okazaki, M., Chiba, N., Adachi, Y., Ohno, N., and Yadomae, T., Signal transduction pathway on β-glucans-triggered hydrogen peroxide production by murine peritoneal macrophages. *Biol. Pharm. Bull,* **19:** 18-23, 1996.

Pabst, M.J., and Johnston, R.B., Increased production of superoxide anion by macrophages exposed in vitro to muramyl dipeptide or lipopolysaccharide. *Journal of Experimental Medicine,* **151:** 101-114, 1980.

Palmer, R.H., Dekker, L.V., Woscholski, R., Le Good, J.A., Gigg, R., and Parker, P.J., Activation of PRK1 by phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. A comparison with protein kinase C isotypes. *The Journal of Biological Chemistry.* **270:** 22412-22416, 1995.

Park, Y.C., Lee, C.H., Kang, H.S., Chung, H.T., and Kim, H.D., Wortmannin, a specific inhibitor of phosphatidylinositol-3-kinase, enhances LPS-induced NO production from murine peritoneal macrophages. *Biochemical and Biophysical Research Communications.* **240:** 692-696 (1997).

Paul, M.S., Aegerter, M., O'Brien, S.E., Kurtz, C.B., and Weis, J.H., The murine complement receptor gene family: Analysis of MCRY gene products and their homology to human CR1. *Journal of Immunology,* **142:** 582-589, 1989.

Pinelli, E., Cambon, C., Tronchere, H., Chap, H., Teissie, J., and Pipy, B., Ca^{2+} dependent activation of phospholipase C and D from mouse peritoneal macrophages by a selective trigger of Ca^{2+} influx, γ-hexachlorocyclohexane. *Biochemical and Biophysical Research Communications.* **189:** 699-705, 1994.

Polanski, M., Vermeulen, M.W., Wu, J., and Karnovsky, M.L., Muramyl dipeptide mimicry in the regulation of murine macrophage activation by serotonin. *International Journal of Immunopharmacology,* **17:** 225-232, 1995.

Prpic, V., Uhing, R.J., Weiel, J.E., Jakoi, L., Gawdi, G., Herman, B., and Adams, D.O., Biochemical and functional responses stimulated by platelet-activating factor in murine peritoneal macrophages. *The Journal of Cell Biology,* **107:** 363-372, 1988.

Raeder, E.M-B., Mansfield, P.J., Hinkovska-Galcheva, V., Shayman, J.A., and Boxer, L.A., Syk activation initiates downstream signaling events during human polymorphonuclear leukocyte phagocytosis. *Immunology,* **163:** 6785-6793, 1999.

Randriamampita, C., and Trautmann, A., Arachidonic acid activates Ca^{2+} extrusion in macrophages. *The Journal of Biological Chemistry,* **265:** 18059-18062, 1990.

Randriamampita, C., Bismuth, G., and Trautmann, T., Ca^{2+}-induced Ca^{2+}release amplifies Ca^{2+} response elicited by inositol trisphosphate in macrophages. *Cell Regulation,* **2:** 513-522, 1991.

Ravetch, J.V., Fc receptors. *Ann. Rev. Immunol,* **9:** 457-492, 1991.

Rothlein, R., Dustin, M.L., Marlin, S.D., and Springer, T.A., A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *Journal of Immunology.* **137:** 1270-1274, 1986.

Rotrosen, D., Yeung, C.L., Leto, T.L., Malech, H.L., Kwong, C.H., Cytochrome b558 the flavin binding component of the phagocyte NADPH oxidase. *Science,* **256:** 1459-1462, 1992.

Sampson, L.L., Heuser, J., and Brown, E.J., Cytokine regulation of complement receptor-mediated ingestion by mouse peritoneal macrophages: M.CSF and Il-4 activate phagocytosis by a common mechanism requiring autostimulation by β interferon. *Journal of Immunology,* **146:** 1005-1013, 1991.

Sanchez-Majorada, G., and Rosales, C., Signal transduction by immunoglobulin Fc receptors. *Journal of Leukocyte Biology.* **63:** 521-533, 1998.

Schreiber, R.D., Identification of γ-interferon as a murine macrophage activating factor for tumour cytotoxicity. *Curr. Topics. Immunobiol,* **13:** 171-198, 1984.

Segal, A.W., West, I., Wientjes, F.B., Nugert, J.H.A., Chavan, A.J., Haley, B., Garcia, R.C., Rosen, H., and Scrace, G., Cytochrome b-245 is a flavocytochrome containing FAD and the NADPH binding site of the microbial oxidase of phagocytes. *Biochemical Journal,* **284:** 781-758, 1992.

Sette, A., Prediction of major histocompatability complex binding regions of protein antigens by sequence pattern analysis. *Proceedings of the National Academy of Sciences., USA.* **86:** 3296-3300, 1989.

Sharma, P., Evans, A.T., Parker, P.J., and Evans, F.J., NADPH-oxidase activation by protein kinase C-isotypes. *Biochemical and Biophysical Research Communications,* **177:** 1033-1040, 1991.

Sharon, S., Carbohydrates as recognition determinants in phagocytosis and in lectin-mediated killing of target cells. *Biol.Cell,* **51:** 239-246, 1984.

Shinomiya, H., Hirata, H., and Nakano, M., Purification and chracterisation of the 65Kda protein phosphorylated in murine peritoneal macrophages by stimulation with bacterial lipopolysaccharide. *Journal of Immunology,* **146:** 3617-3625, 1991.

Silverman, D.H.S., Wu, H., and Karnovsky, M.L., Muramyl peptides and serotonin interact at specific binding sites on macrophages and enhance superoxide release. *Biochemical and Biophysical Research Communications,* **131:** 1160-1167, 1985.

Simchowitz, L., Foy, M.A., and Crague, E.J. Jnr., A role for Na^{+}/Ca^{2+} exchange in the generation of superoxide radicals by human neutrophils. *The Journal of Biological Chemistry,* **265:** 13449-13356, 1990.

Sipka, S., Abel, G., Csongor, P., Nyirkos, P., and Fachet, J., Effect of mannozym on the chemiluminescence of phagocytes. *Acta Microbiologica Hungarica.* **133:** 263-270, 1986.

Sodhi, A., and Geetha, B., Effect of cisplatin, lipopolysaccharide, muramyl dipeptide and recombinant interferon-gamma on murine macrophages in vitro. II. Production of H_2O_2, O_2^- and interleukin 1. *Nat. Immun. Cell. Growth. Regul,* **8:** 108-116, 1989.

Somers, S.D., Hamilton, T.A., and Adams, D.O., Maleylated bovine serum albumin triggers cytosolic function in selected populations of primed murine macrophages. *Journal of Immunology.* **139:** 1361-1368 1987.

Speert, D.P., Wright, S.D., Silverstein, S.D., Mah, B-A., Functional characterisation of macrophage receptors for in vitro phagocytosis of unopsonised Pseudomonas aeruginosa. *Journal of Clinical Investigation.* **82:** 872-879, 1988.

Stahl, P.D., and Ezekowitz, A.B., The mannose receptor is a pattern recognition receptor involved in host defense. *Current Opinion in Immunology.* **10:** 50-55, 1998.

Stites, D.P., and Terr, A.Z., *Basic and Clinical Immunology,* 7th edition. Norwalk Conn, Appleton and Lange., London. Prentice Hall.

Sung, S.S., Nelson, R.R., and Silverstein, S.C., Yeast mannans inhibit binding and phagocytosis of zymosan by mouse peritoneal macrophages. *Journal of Cell Biology.* **96:** 160-166, 1983.

Sung, S-S. J., Nelson, R.S., and Silverstein, S.C., Mouse peritoneal macrophages plated on mannan and horseradish peroxidase-coated substrates lose the ability to phagocytose by their Fc receptors. *The Journal of Immunology,* **134:** 3712-3717, 1985.

Suzuki, K., Yamaguchi, T., Oshizawa, T., Yamamoto, Y., Nishimaki-Mogami, T., Hayakawa, T and Takahashi, A., Okadaic acid induces both augmentation and inhibition of opsonised zymosan-stimulated superoxide production by differentiated HL-60 cells. Possible involvement of dephosphorylation of a cytosolic 21K protein in respiratory burst. *Biochemical et Biophysica Acta,* **1266:** 261-267, 1995.

Swallow, C.J., Grinstein, S., and Rotstein, O.D., Regulation of cytoplasmic pH in resident and activated peritoneal macrophages. *Biochemical et Biophysical Acta,* **1022:** 203-210, 1990.

Tanaka, A., Nagao, S., Imai, K., and Mori, R., Macrophage activation by muramyl dipeptide as measured by macrophage spreading and attachment. *Microbiol. Immunol,* **24:** 547-557, 1980.

Tarsi-Tsuk, D., and Levy, R., Stimulation of the respiratory burst in peripheral blood monocytes by lipoteichoic acid. The involvement of calcium ions and phospholipase A_2. *The Journal of Immunology.* **144:** 2665-2670, 1990.

Teslenko, V., Rogers, M., and Lefkowith, J.B., Macrophage arachidonate release via both the cytosolic Ca^{2+}-dependent and –independent phospholipases is necessary for cell spreading. *Biochimica et Biophysica Acta,* **1344:** 189-199, 1997.

Toker, A., Meyer, M., Reddy, K.K., Falck, J.R., Aneja, R., Aneja, S., Parra, A., Burns, D.J., Ballas, L.M., and Cantley, L.C., Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P-2 and PtdIns-3,4,5-P-3. *Journal of Biological Chemistry,* **269:** 32358-32367, 1994.

Toullec, D., Pinetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D., and Kirilovsky, J., The Bisindolylmaleimide GF109203X is a potent and selective inhibitor of protein kinase C. *Journal of Biological Chemistry,* **266:** 15771-15781, 1991.

Trudel, S., Paquet, M.R., and Grinstein, S., Mechanism of vanadate-induced activation of tyrosine phosphorylation and of the respiratory burst in HL-60 cells. Role of reduced oxygen metabolites. *Biochemical Journal.* **276:** 611-619, 1991.

Uhing, R.J., and Adams, D. O., Molecular events in the activation of murine macrophages. *Agents and Actions,* **26:** 9-14, 1989.

Unkeless, J.C., Scigliano, E., and Freedman, E.H., Structure and function of human and murine receptors for IgG. *Ann. Rev. Immunol,* **6:** 251-281, 1988.

Vaddi, K., and Wei, C-I., Modulation of macrophage activation by ammonium metavanadate. *Journal of Toxicology and Environmental Health,* **49:** 631-645, 1996.

Vanhaesebroeck, B., and Waterfield, M.A., Signaling by distinct classes of phosphoinositide 3-kinases. *Experimental Cell Research,* **253:** 239-254, 1999.

Waite, K.A., Wallin, R., Qualliotine-Mann, D., and McPhail, L,C., Phosphatidic acid-mediated phosphorylation of the NADPH oxidase component of p47-phox. Evidence that phosphatidic acid may activate a novel protein kinase. *The Journal of Biological Chemistry.* **272:** 15569-15578, 1997.

Ways, D.K., Cook, P.P., Webster, C., and Parker, P., Effect of phorbol esters on protein kinase C ζ. *Journal of Biological Chemistry,* **267:** 4799-4805, 1992.

Weinstein, S.L., Gold, M.R., and DeFranco, L.A., Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages. *Proceedings of the National Academy of Sciences USA,* **88:**4148-4158, 1991.

Werderlin, O., Mouritsen, S., Peterson, B.L., Sette, A., and Buus, S., Facts on the fragmentation on antigens in presenting cells, on the association of antigen fragments and speculation on the cell biology of antigen processing. *Immune Review.* **106:** 183-193, 1988.

Wientjes, F.B., and Segal, D.W., NADPH oxidase and the respiratory burst. *Seminars in Cell biology.* **6:** 357-365, 1995.

Wijkander, J., and Sundler, R., A role for protein kinase C-mediated phosphorylation in the mobilisation of arachidonic acid in mouse macrophages. *Biochemica et Biophysica Acta.* **1010:** 78-87, 1989.

Wright, J., Schwartz, J.H., Olson, R., Kosowsky, J.M., and Tauber, A.I., Proton secretion by the sodium/hydrogen ion antiporter in the human neutrophil. *Journal of Clinical Investigation,* **77:** 782-788, 1986.

Wright, S.D., and Jong, M.T.C., Adhesion promoting receptors on human macrophages recognise Eschericia coli by binding to lipopolysaccharide. *Journal of Experimental Medicine.* **164:** 1876-1878, 1986.

Wright, S.D., Multiple receptors for endotoxin. *Curr. Opin. Immunol,* **3:** 83-90, 1991.

Young, J. D-E., Ko, S-S., and Cohn, Z-A., The increase in intracellular free calcium associated with IgG2b/Fc receptor ligated interactions: role in phagocytosis. *Proceedings of the National Academy of Sciences.* **81:** 5430-5434, 1984.

Zimmerli, S., Majeed, M., Gustavsson, M., Stendahl, O., Sanan, D.A., and Ernst, J.D., Phagosome-lysosome fusion is a calcium-independent event in macrophages. *The Journal Of cell biology.* **132:** 49-61, 1996.

Zinchenko, V.P., Mysyakin, Y.B., Dolgachev, V.A., Dedkova, Y.N., Safronova, V.G., Gapeyev, A.B., Shebzukhov, V., and Vaisbud, M.Y., The action of structural analogues of the platelet-activating factor on the transmission of intracellular signals in mouse peritoneal neutrophils and macrophages of the P388D1 line. *Biophysics,* **42:** 1117-1125, 1997.