BACTERIAL LIPOPOLYSACCHARIDE SIGNALING PATHWAYS IN MONONUCLEAR PHAGOCYTES INVOLVE PROTEIN AND LIPID KINASES

By

Patricia Herrera-Velit

B.Sc., Universidad Peruana Cayetano Heredia, Lima

M.Sc., Universidad Peruana Cayetano Heredia, Lima

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Microbiology and Immunology)

We accept this thesis as conforming to the required standards

THE UNIVERSITY OF BRITISH COLUMBIA

October 1997

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of MICROBIOLOGY AND IMMUNOLOGY

The University of British Columbia
Vancouver, Canada

Date DECEMBER 4 1997

DE-6 (2/88)

I. ABSTRACT

Mononuclear phagocyte activation enhances the functional properties of these cells, and endotoxin (bacterial lipopolysaccharide) is one of the most potent agonists known to cause an activated state. This model was used to investigate signaling pathways that regulate macrophage activation. Exposure of mononuclear phagocytes to lipopolysaccharide resulted in rapid and dose-dependent increases in tyrosine phosphorylation of a set of proteins. This was due, at least in part, to increased tyrosine kinase activity. In particular, compared to untreated cells, lipopolysaccharide-treated monocytes showed increased activity of the Src-family tyrosine kinase p*53/56*^{lyn}. In addition, both tyrosine kinase activity, as well as tyrosine phosphorylated proteins without endogenous kinase activity, associated with the lipopolysaccharide receptor CD14 in an inducible manner.

Lipopolysaccharide treatment of monocytes also activated the lipid kinase, phosphatidylinositol 3-kinase in a dose and time dependent manner. Activation of this enzyme resulted in increased levels of D3-phosphorylated phosphoinositides and this response was mediated through CD14. Activation of phosphatidylinositol 3-kinase also resulted in its association with activated p*53/56*^{lyn} without leading to a detectable change in the tyrosine phosphorylation state of the p85 regulatory subunit of the enzyme.

Increased protein kinase activity is not restricted to tyrosine kinases, as lipopolysaccharide also activated serine/threonine kinases. Anion exchange chromatography of endotoxin-treated mononuclear phagocytes showed increased myelin basic protein-kinase activity eluting as two major peaks. Based on immunoreactivity and substrate preference, the earliest eluting peak, peak "one" was identified as p42/p44 mitogen-activated protein kinases.

Tyrosine phosphorylation of both p42 and p44 mitogen-activated protein kinases increased in response to lipopolysaccharide. The second peak of myelin basic protein-kinase activity, peak "two", was identified as protein kinase C-ζ, an atypical isoform of protein kinase C. This conclusion is based on immunoreactivity, substrate preference, and cofactor independency of the enzyme.

The cellular mechanisms regulating the activity of protein kinase C-ζ were investigated. Protein kinase C-ζ activation by lipopolysaccharide occurred downstream of phosphatidylinositol 3-kinase. This conclusion is based on two findings: (i) phosphatidylinositol 3-kinase inhibitors blocked activation of protein kinase C-ζ, and (ii) transfection of cells with a dominant negative mutant of phosphatidylinositol 3-kinase impaired activation of protein kinase C-ζ by lipopolysaccharide.

Functional responses of mononuclear phagocytes dependent on signaling pathways involving phosphatidylinositol 3-kinase were examined. Phosphatidylinositol 3-kinase inhibitors abrogated lipopolysaccharide-induced adherence, but not adherence induced in response to phorbol 12-myristate 13-acetate in the monocytic cell line THP-1. In contrast, induction of transcription of cytokine genes by lipopolysaccharide was independent of phosphatidylinositol 3-kinase.

In conclusion, examination of lipopolysaccharide signaling in mononuclear phagocytes showed that endotoxin activates both protein and lipid kinases including p53/56^{lyn}, p42 and p44 isoforms of mitogen activated protein kinase and phosphatidylinositol 3-kinase through a CD-14 dependent mechanism. Activation of PKC-ζ is phosphatidylinositol 3-kinase dependent and signaling through this lipid kinase regulates monocyte adherence, but not cytokine production induced by lipopolysaccharide. Given the requirement that monocytes must adhere

to endothelium, to other leukocytes and to extracellular matrix proteins in order to mediate functional responses, it may be possible to modify macrophage responses to lipopolysaccharide and perhaps other agonists by targeting phosphatidylinositol 3-kinase.

II. TABLE OF CONTENTS

VIII. MATERIALS AND METHODS

III. LIST OF TABLES

IV. LIST OF FIGURES

V. LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CAK	Ceramide-activated protein kinase
CPAE	Cow pulmonary artherial cell
ECL	Enhanced chemoluminiscence
EDTA	Ethylendiaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetracetic acid
ELAM-1	Endothelial-leukocyte adhesion molecule-1
FCS	Fetal calf serum
FPLC	Fast performance liquid chromatography
G-CSF	Granulocyte-colony stimulating factor
GDP	Guanine 5′-diphosphate
GM-CSG	Granulocyte/macrophage-colony stimulating factor
GPI	Glycosylphosphatidylinositol
HBSS	Hank's balanced salt solution
HPLC	High pressure liquid chromatography
HRPO	Horse radish peroxidase
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercelullar cell adhesion molecule-1
IL	Interleukin
KDO	2-keto-3-dexyoctonic acid
LAM	Lipoarabinomannan

LBP	LPS binding protein
LPS	Lipopolysaccharide, endotoxin
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
mCD14	Membrane CD14
MBP	Myelin basic protein
MOPS	3,-[N-morpholino]propanesulfonic acid
Mϕs	Mononuclear phagocytes
NHS	Normal human serum
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PBMϕs	Peripheral blood mononuclear cells
PEI	Polyethylenimine cellulose
PIPES	Piperazine-N,N'-bis[2-ethanesulfonic acid]
PI 3-kinase	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear lymphocytes
PMSF	Phenylmethylsulfonyl fluoride
PNH	Paroxysmal nocturnal hemaglobinuria
PS	L–α-phosphatidyl-L-serine

PtdIns	L-α-phosphatidylinositol
PtdIns3P	L-α-phosphatidylinositol 3-phosphate
PtdIns4P	L-α-phosphatidylinositol 4-phosphate
PtdIns3,4P_2	L-α-phosphatidylinositol 3,4-bisphosphate
PtdIns4,5P_2	L-α-phosphatidylinositol 4,5-bisphosphate
PtdIns3,4,5P_3	L-α-phosphatidylinositol 3,4,5-trisphosphate
PTK	Protein tyrosine kinase
R-LPS	Rough LPS
RT	Room temperature
sCD14	Soluble CD14
S-LPS	Smooth LPS
TGF-β	Transforming growth factor-β
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-α

VI. ACKNOWLEDGMENTS

I would like to give innumerable thanks to Dr. Neil Reiner for all the advice he has given me along this way and to the proficient use of red pen he has practiced in my writing. Special thanks to the people in the Biochemistry Laboratory from Universidad Peruana Cayetano Heredia, Lima and to my family -especially my mother- for their infinite patience, and to all the people in Neil's lab, in particular to Keith Knutson and Zakaria Hmama for their help. I would also like to acknowledge scholarship support from the Canadian International Development Agency.

VII. INTRODUCTION/LITERATURE REVIEW

MONONUCLEAR PHAGOCYTES

Mononuclear phagocytes (Mϕs) are important components of both innate resistance and adaptive immunity. After extensive development in the bone marrow, relatively immature monocytes enter the circulation and migrate to tissue sites where they mature and differentiate into macrophages (1). Whether circulating or in the tissues, Mϕs are subject to a large variety of signals, both stimulatory and inhibitory in nature. This is especially true when homeostasis is altered, as occurs during tissue injury, inflammation and sepsis. Under these conditions, many of the innate properties of macrophages change, including their morphology and metabolism (2). These changes are associated with the induced expression of various gene products that enable the cells to perform functions otherwise not achieved by resting cells. This process of macrophage activation results in the appearance of an "immunocompetent" cell. Activated cells show enhanced responses to lymphokines and become highly secretory cells, with their secreted products exerting a wide variety of effects on many cells types (3). These products include lipid-derived inflammatory mediators such as platelet-activating factor (PAF) and prostaglandins, as well as chemokines that recruit other inflammatory cells to the site of injury, reactive oxygen species involved in microbial killing, cytokines such as tumor necrosis factor (TNF-α) with tumoricidal and microbicidal activities, and growth factors such as fibroblast growth factor and transforming growth factor-β (TGF–β) that promote repair of injured tissues. Mϕs are essential not only for natural immunity, but they also have evolved to play critical roles in acquired immunity. They are efficient in presenting foreign antigens to T cells , they bind

and phagocytose particles opsonized with complement, IgG, or other opsonins, and they respond to cytokines secreted by T lymphocytes and other cells (4-6). Activated Mϕs play a central role, therefore, in the host response to external stimuli.

LIPOPOLYSACCHARIDE (LPS)

Amongst the most potent stimuli known to lead to Mϕ activation are bacterial products. The classical and most thoroughly studied microbial product known to activate Mϕs is endotoxin or lipopolysaccharide (LPS), derived from cell walls of gram negative bacteria. Qualitatively, LPS is able to elicit many, but not all of the responses usually attributed to activated Mϕs (7,8). As has already been mentioned, this may be necessary as part of a proper protective innate host response against gram negative infection. However, uncontrolled Mϕ activation can be deleterious to the host and many of the clinical manifestations of systemic bacterial infections such as fever, hypotension, and metabolic dearrangements can be attributed, at least in part, to the effects of LPS on Mϕs (9).

Numerous studies have been performed to delineate the structural components of LPS (10-13). The minimal structure of endotoxin consists of a lipid component termed lipid A and a core hydrophilic polysaccharide region formed minimally by at least one or two, 2-keto-3-deoxyoctonic acid (KDO) residues or KDO derivatives (Figure 1). The composition of the hydrophilic region is usually considerably more complex, however, and is highly variable. In *Enterobacteriacea*, for example, it is formed by a heteropolysaccharide consisting of a core oligosaccharide and an O-specific chain (smooth- or S-LPS). The structure of the core region may be subdivided further into outer and inner components, the latter being formed by KDO and heptose residues (11), and the former consisting of the common hexoses D-glucose, D-

galactose and N-acetyl-D-glucosamine. Besides providing an attachment site for O-antigen, the function of the outer core is not known.

The O-specific chain is a polymer of oligosaccharides, containing between two and eight sugar monomers. Further complexity is added by the fact that the composition of the repeating units in the O-side chain differs from strain to strain within a serotype and exhibits large structural variability. This region also functions as an important surface antigen and determines the serological specificity of the LPS variant and of the bacteria containing it. The synthesis of the O-specific chain is determined by a cluster of genes termed rfb. A second type of LPS, the rough or R-type, lacks the O-specific chain, and was first identified in enterobacterial mutants with a defect in the rfb locus. Mutants are able to grow and multiply *in vitro* indicating that the O-specific chain is not necessary for bacterial viability.

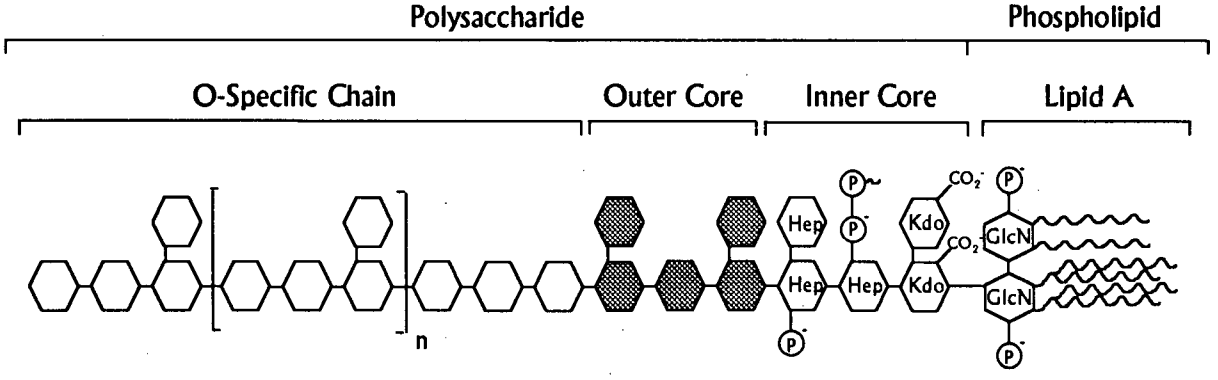


Figure 1. Chemical structure of lipopolysaccharide. From Holst, O. *et al.* (13).

Lipid A is a phosphoglycolipid that, together with the KDO-containing inner core, is the most conserved structure of LPS. It contains a β(1-6) residue-linked disaccharide with one glycosidic and one non-glycosidic phosphoryl group and medium to long chained fatty acids

linked by amide and ester bonds (10-13). Despite being highly conserved, variations in its structure exist. These derive from the type of hexoseamine residues present, the degree of phosphorylation, and the nature and length of the acyl groups (11). Most of the effects of endotoxins on animals or on cultured cells are attributable to lipid A, and certain chemical modifications of the constituents of lipid A result in a biologically inactive endotoxin. Lipid A expressing full endotoxic activity requires two hexosamine residues, two phosphoryl groups, and six fatty acids with defined lengths and distinct positions.

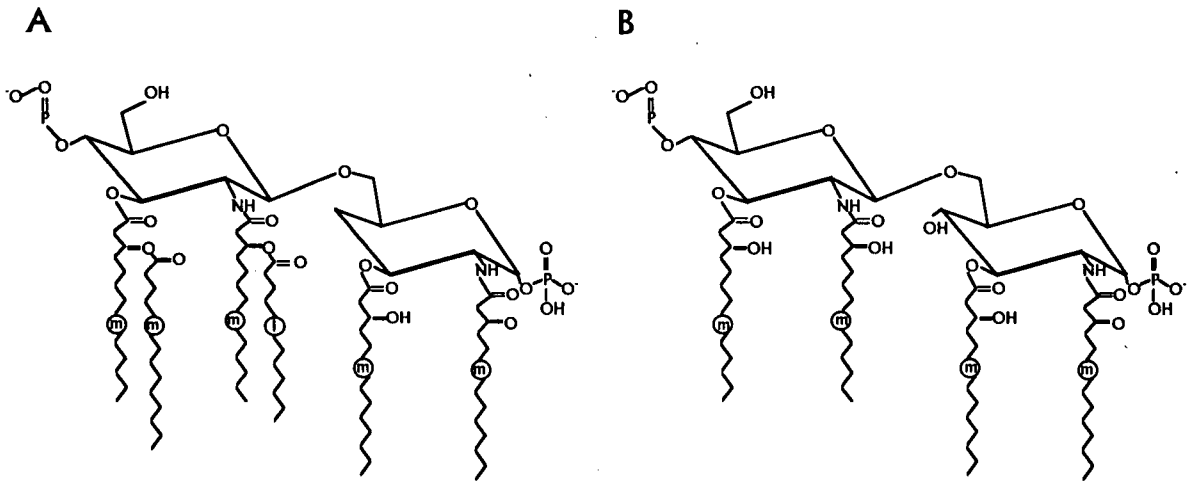


Figure 2. Chemical structure of lipid A (A) and the antagonistic lipid A partial structure, lipid IV_A (B). m= 14C-acyl group, l= 12C-acyl group. From Holst, O. *et al.* (13).

Cells affected by LPS include not only those of the monocyte/macrophages lineage, but also endothelial cells, smooth muscle cells, granulocytes and megakaryocytes. Brief exposure to endotoxin or lipid A results in the production of bioactive lipids, reactive oxygen intermediates and cytokines including TNF-α, interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8)

and interleukin 10 (IL-10), in addition to a multitude of other mediators (3). These responses are observed in murine, rabbit and human Mϕs of either alveolar, peritoneal or blood origin (8).

LPS RECEPTORS AND LPS BINDING PROTEINS

Cell-associated LPS binding proteins

In the presence of serum, Mϕs responses to LPS (or to lipid A) occur at concentrations in the picogram to nanogram/ml range. This sensitivity involves an LPS receptor at the cell surface, as well as a serum factor that facilitates LPS binding (14,15). CD14, a myeloid differentiation protein present mainly on peripheral blood monocytes and macrophages, as well as on neutrophils, has been shown to function as an LPS receptor. CD14 is anchored to the cell membrane by a glycosylphosphatidylinositol (GPI) tail (16) and is also found in serum in a soluble form. It appears that the GPI anchor may not be required for all the downstream events brought about by LPS binding. This conclusion is based on the finding that LPS is able to induce identical cellular responses in the 70Z/3 B lymphoma cell line, stably expressing CD14, irrespective of whether it is expressed as an integral membrane protein or if it is anchored to the membrane by a GPI tail (17).

Several experimental observations indicate the existence of other LPS cell surface receptors in Mϕs in addition to CD14. For example, antibodies against CD14 are not able to block completely responses to LPS when the latter is used in concentrations ≥ 100 ng/ml. Similarly, promonocytic cell lines such as U937 and THP-1 express very low levels of CD14, but are still able to respond to LPS in concentrations ≥ 100 ng/ml. Likewise, cell types that do not express CD14 such as, 70Z/3, respond to concentrations of LPS in excess of 100 ng/ml

(18). These findings indicate that, both in CD14 negative as well as in CD14 positive cells, there appears to be other cell surface proteins that can bind LPS and transmit signals, albeit at concentrations higher than those required for signaling through CD14. The function of CD14 as an LPS receptor in Mϕs and polymorphonuclear phagocytes (PMNs) will be discussed in more detail below.

Using a photoactivatable, radioiodinated LPS, Halling *et al.* (19) described the presence of several proteins in human blood cells, including Mϕs, that are able to bind LPS in a specific manner under serum free conditions. A 73 kDa LPS-binding protein was noted to be present in the different cell types examined in this study. The question of whether this protein is at the cell surface or intracellular was not addressed in this work. In the same study, when lymphocytic cells were examined, several additional lower molecular weight LPS-binding proteins were detected, the most abundant having approximate subunit sizes of 50, 31 and 18 kDa. These secondary proteins were also present in the adherent monocytic preparations, although they were less prominent. Surprisingly, even though several other LPS-binding proteins were reported, none had a size in the range of 55 kDa, potentially corresponding to CD14. Lei and Morrison (20) described a similar LPS binding protein of 80-kDa in B and T lymphocytes and in murine macrophage membranes with specificity for the lipid A region of LPS. Approximately 5,000-10,000 molecules of this putative LPS receptor were detected on these cells indicating that it appeared to be less abundant than CD14 [approximately $1x10^5$ copies/monocyte (21)]. Chen and colleagues reported that addition of purified mAbs specific for the 80-kDa protein to bone marrow-derived macrophages from LPS-responsive C3H/HeN mice, led to activation of these cells for tumoricidal activity towards mastocytoma cells *in vitro* (22). In contrast, when these antibodies were used with Mϕs derived from LPS unresponsive

C3H/He] mice, no tumoricidal activity was observed, even though there was equivalent binding of mAb to cells from these two strains. Using affinity-purified rabbit antibodies with specificity for the-80 kDa protein, and a variety of techniques to detect this protein at the surface of thioglycollate-elicited peritoneal macrophages, Perera *et al.* (23) were also unable to detect any difference in the surface expression of this putative 80-kDa LPS-receptor from cells derived from C3H/HeH and C3H/He] mice. Taken together, these results indicate that LPS hyporesponsiveness in macrophages from C3H/He] mice is not due to lack of this ~ 80-kDa LPS-binding protein, but to events distal to LPS binding.

Although some of the data regarding this 70-80-kDa LPS binding protein suggest that it may be a functional receptor, recent findings have called this into question. Dziarski (24) reported in 1994, that a 70-kDa protein present on the surface of lymphocytes and macrophages, capable of binding LPS, was in reality, cell bound albumin. Species-specific anti-bovine, anti-human, and anti-mouse albumin antibodies recognized the 70-kDa protein on mouse and human cells according to the species of albumin that was present in the culture medium or in the serum *in vivo*, but not according to the species of the cells. Anti-albumin antibodies immunoprecipitated a radiolabeled 70-kDa protein from ^{125}I-ASD-LPS cross-linked THP-1 cells grown in the presence of FCS. Furthermore, soluble albumin *per se* was also found to bind radiolabeled-LPS *in vitro*. Lastly, this "LPS-binding protein' was shown to originate from the cell culture medium or from the serum *in vivo,* but was not produced by the cells. Thus, these results indicate that, this 70-80-kDa putative LPS receptor may be an artifact.

In addition to the proteins described above, some of which may function as specific receptors for LPS or lipid A, there are other macrophage receptors with well defined ligands that have also been shown to bind LPS. Among these, the scavenger receptor has been

reported to bind lipid A. For example, subsequent to binding to the scavenger receptor, lipid IV_A, a bioactive precursor of lipid A, is metabolized to a less active form by the murine macrophage-like RAW 264.7 cells (25). Both binding and deactivation of lipid was shown to be mediated by the macrophage scavenger receptor. It seems unlikely, however, that this protein is involved as a functional LPS receptor in mononuclear phagocytes, since its natural ligand, acetylated LDL, is unable to block induction of TNF-α production by endotoxin. Similarly, Golenbock *et al.* (26) found that transfection of CHO cells with CD14 confers responsiveness to LPS as assessed by arachidonate release. In contrast, transfection with the macrophage scavenger receptor failed to produce the same effect. Thus, while not functioning as an LPS "signaling" receptor, the macrophage scavenger receptor may play an important role in clearance and detoxification of LPS *in vivo.*

Members of the CD18 or β2 complex of leukocyte integrins (CD11a-c/CD18), are known to participate in numerous cell-cell and cell-substrate interactions. In addition to functioning as adhesion molecules, β2 integrins on monocytes, macrophages and polymorphonuclear leukocytes (PMN) have also been shown to bind LPS (27). Using antibodies against this complex, Wright and coworkers (28) were able to block the binding of particulate LPS to Mϕs when it was presented on the surface of bacteria or as LPS-coated erythrocytes. However, the importance of this complex in Mϕ functional response to LPS is unclear for several reasons. First, antibodies to CD18 do not prevent LPS-induced synthesis of TNF-α by whole human blood (29). Second, antibodies against CD11a, CD11b or CD18 do not block the LPS-induced secretion of IL-1β and TNF-α by normal human monocytes (30). Finally, Mϕs from patients genetically deficient in CD18 show *in vitro* responses to LPS that are indistinguishable from those observed using cells from normal volunteers (31).

Recombinant human CD14 expressed at the surface of Chinese hamster ovary (CHO) fibroblasts interacts rapidly with LPS in the presence of serum or LBP, being almost complete within 5 min and reaching maximal binding by 10 min (32). If LPS and LBP are preincubated before being presented to the cells, binding occurs in a similar manner either at 10 ° or 20 °C. This suggests that this process does not require extensive mobility of CD14 within the cell membrane. The molar ratio of LPS bound to surface CD14 varies from 8:1 to 20:1. One explanation for this high stoichiometry is that LPS is present, not as a monomer, but as an aggregate of 8 to 20-mers and binds to CD14 as such. Another explanation is that CD14 has multiple LPS binding sites. In this respect, it has been found that there are ten leucine-rich repeats within CD14 that may be LPS binding motifs (33). If each leucine repeat has the capacity to bind one or two LPS molecules, this would allow for the observed stoichiometry.

Several human, promonocytic cell lines have also been used to study the effects of LPS. THP-1, U937 and HL-60 are amongst the cell lines used most commonly. Although CD14 can be detected in some of these cell lines (21,34) the level of expression of CD14 is minimal, and these cells respond poorly to low concentrations of LPS in terms of IL-1 and TNF-α production. Treatment of immature THP-1 cells with vitamin D_3 induces the expression of CD14 in a time and dose dependent manner. After 48-72 h of treatment with 100 nM of D_3, maximal expression of CD14 is observed (35-fold increase over baseline), whereas expression of other surface markers, such as CD18 increase less substantially (35). As THP-1 cells differentiate in the presence of D_3, they become increasingly responsive to LPS and LPS-LBP complexes. This phenomenon is likely explained by the interaction of the LPS-LBP complex with CD14 now present at the cell surface in greater numbers. Induction of competence for LPS cell activation is also seen in cells that are stably transfected with CD14.

CD14 transfected murine B-lymphoma cells respond to 1000-fold lower LPS concentrations than do parental CD14-negative cells (18,36,37). Similarly, CHO fibroblasts, a CD14 negative, LPS-unresponsive cell line, also become responsive to LPS when transfected with CD14 (26).

CD14 present at the surface of neutrophils appears to be indistinguishable from Mϕ-CD14. As is the case for monocytes, it is also anchored to the membrane of PMNs by a GPI-tail and is shed into the culture medium. Mϕs and PMNs derived from patients with paroxysmal-nocturnal hemoglobinuria (PHN), a disease characterized by a defect in the GPI-anchoring mechanism of proteins, show decreased levels of mCD14 (16,38,39). *In vitro*, cells from patients with PHN exhibit diminished functional responses to LPS, thus further substantiating the critical role of CD14 in LPS signaling.

The precise role of membrane CD14 in LPS signaling is a subject of intense interest. Some insight into this question was provided by Kuhns *et al.* (40) who reported the case of a 15-year old patient with recurrent bacterial infections who showed hyporesponsiveness to LPS, both *in vivo* and *in vitro*. The neutrophils and monocytes from this patient showed normal levels of various surface markers, including CD14, and the binding of LPS to CD14 was not different from that observed using cells from normal donors. *In vitro* incubation of Mϕs with LPS and IL-1 failed to induce the production of either TNF-α or granulocyte colony stimulating factor (G-CSF). LPS priming of neutrophils for N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced superoxide production was also deficient, while the cells responded to TNF-α- and PAF-priming for fMLP-induced superoxide production. The defect in this patient's cells, appears to localize to an early upstream event in the signal-transduction pathway activated by

LPS and IL-1. Thus, although required for binding of LPS to Mϕs, the presence of CD14 *per se* is not sufficient to confer functional responsiveness.

Soluble LPS binding proteins

A major advance in the understanding of LPS signaling was the discovery of LPS binding protein (LBP), a plasma protein capable of binding LPS specifically and which is required for ligand binding to CD14 (41,42). LBP is present in human serum at concentrations of ≤ 5 μg/ml, and increases more than ten-fold after induction of an acute phase response, a phenomenon that develops rapidly after an infection where the concentration of a number of serum proteins increases dramatically. LBP has a predicted size of 50 kDa and is produced in the liver. Before secretion, LBP is glycosylated and circulates as a 60 kDa glycoprotein with a binding site for lipid A, the active component of LPS (43). The binding of LPS to LBP appears to be monomeric, with a dissociation constant of 10^{-9} M (43). The region of LBP between amino acids 7 and 10 is necessary for signaling, but not for LPS binding, as constructs lacking this region, although capable of binding LPS, are unable to mediate LPS-dependent up-regulation of IL-6 in U373 cells, integrin activation in neutrophils, or NF-κB activation in U937 cells (44). The N-terminal 197 residues of the protein are involved in LPS binding (45). In the presence of LBP, rabbit peritoneal exudate Mϕs respond to LPS at < 1 ng/ml by secreting TNF-α. Under these conditions, cells also show more rapid induction of cytokine mRNA, higher steady-state mRNA levels and increased mRNA stability for TNF-α when compared to serum-free conditions (46,47). Similar effects of LBP are observed in the responses of rabbit and human alveolar Mϕs to LPS (48). The role of LBP is believed to be to bind endotoxin in blood and to transfer it to CD14 at the cell surface. Thus, by making LPS

more readily available to the cell, LBP lowers the threshold concentration of LPS required to activate CD14-positive cells (49).

Wright and coworkers (50) described a distinct plasma constituent named septin, also able to bind to LPS. Septin activity is present at very high levels in plasma and is thought to be formed by at least two protein species requiring proteolytic degradation of one of the components for LPS opsonic activity. The biological activity of septin appears to be similar to that of LBP and a model has been proposed in which, like LBP, septin mediates binding of LPS to CD14 in Mϕs and PMNs. According to this model, the concentration of LBP in serum under basal conditions -in the absence of an acute phase response- is too low to mediate efficient transfer of LPS to CD14. This model proposes that under normal conditions, septin and not LBP is the principal means for opsonizing LPS for recognition by CD14.

As discussed above, over 90% of the total pool of CD14 detectable in the circulation is accounted for by soluble CD14 (sCD14) with the remainder being associated with Mϕs or PMNs. Soluble CD14 is estimated to have a concentration in plasma of approximately 5 μg/ml (51). There appears to be two sources for sCD14: (i) mCD14 is shed from the cell surface by protease digestion, and (ii) before export to the cell surface, some of the intracellular pool of CD14 escapes the GPI-anchoring process and is secreted. The latter mechanism is supported by the finding that part of the sCD14 shed from cells spontaneously at 37 °C lacks ethanolamine present in the GPI-tail of normal mCD14 (16). Additional evidence that this form of sCD14, present in the culture supernatant of $CD14^+$ cells as well as in various body fluids, is not derived from mCD14, is its molecular size of 56 kDa. In contrast, sCD14 generated from proteolytic release of mCD14 has a molecular mass of 48 kDa. Proteins reserved for GPI anchoring have a C-terminal hydrophobic region of about 30 amino acids that

serve as a signal peptide for an enzyme responsible for GPI tailing. Normally, this peptide is replaced by the GPI anchor. Escape from this process, results in an unprocessed C-terminus and direct secretion. As demonstrated by Bufler *et al.* (52), the larger (56 kDa) form of sCD14 is not truncated at the C-terminus implying that the protein escapes the GPI anchor attachment and is directly secreted.

The surprising finding that some CD14-negative cells are able to respond to LPS suggested the possibility that other LPS receptors besides CD14 might be present in these cells, or that sCD14 present in blood might be involved in mediating these responses. In the presence, but not in the absence of normal human serum (NHS), CD14-negative human umbilical vein endothelial cells (HUVEC) respond to LPS by producing increased levels of PAF (53), TGF-β and intercellular cell adhesion molecule 1 (ICAM-1) (54), and activation of the transcription factor NF-κB (55). Frey and coworkers (56) demonstrated that in the presence of NHS, HUVEC respond to LPS in the range of 1-10 ng/ml with increased production of endothelial-leukocyte adhesion molecule 1 (ELAM-1) after a 4 h treatment, and U373 cells produce IL-6 after 24 h of incubation with 0.1-100 μg/ml LPS. CD14-negative pulmonary arterial endothelial bovine cells (CPAE) also respond to LPS in the presence of NHS resulting in enhanced cell permeability and cell death. None of these effects of LPS in these two cell types are observed if serum is omitted or if it has been immunodepleted of sCD14 (56). If sCD14 acts in a manner similar to LBP, i.e. simply as a carrier for LPS, it would be expected that LBP could substitute for sCD14 in this system. However, the involvement of LBP in this system seems negligible as it is not able to support the responses brought about by the presence of sCD14. In addition, Frey and coworkers found that IL-6 production by CD14-negative U373 cells in the presence of NHS is also blocked by preincubation with anti-CD14 antibodies, but

not by control anti-CD18 antibodies. Haziot *et al.* (54) noted that LPS is able to bind to sCD14 directly, and that under serum-free conditions, 5-50 ng/ml of LPS elicits ICAM-1 expression in human endothelial cells if sCD14 is present. However, working with lower concentrations of LPS (5 ng/ml), it was found that sCD14-stimulated activation is enhanced in the presence of LBP. These results suggest that sCD14 alone is sufficient for opsonization and presentation of LPS to CD14-negative cells. As a corollary to this, for cells devoid of mCD14, the lack of surface anchor does not hinder the capacity of sCD14 to bring LPS to the cell surface in a manner that elicits functional responses. This implies that sCD14 may bind to additional proteins on the cell resulting in transduction of a signal. In this respect, Vita *et al.* reported specific and saturable binding of sCD14 in a manner indicative of a receptor-ligand interaction, consistent with the presence of a cell surface binding structure for complexes of LPS and sCD14 (57).

The observed effects of sCD14 on CD14-positive cells have been somewhat inconsistent. Human Mϕs show increased synthesis of PAF when stimulated with LPS (1 ng/ml) in the presence of LBP. However, when sCD14 is added to LPS-LBP, a significant reduction of this response is observed (53). A similar effect is observed in peripheral blood Mϕs, where increased secretion of TNF-α in response to LPS in the presence of LBP is blocked by sCD14 in a dose dependent manner (58). In the murine model of endotoxic shock, the use of recombinant sCD14 has proven beneficial as it is able to protect animals from LPS-induced mortality (58). These results indicate that the predominant effect of sCD14 on CD14 positive cells is inhibitory in respect to LPS action. By contrast, freshly isolated PMNs show LPS-induced adherence even when sCD14 and LBP are present simultaneously (59). It has also been found (49) that the sensitivity of human Mϕs (IL-6 production) and neutrophils (adherence) to

complexes of LPS-LBP is the same as for complexes of LPS-sCD14. Thus, depending upon the experimental conditions, the effects of sCD14 on CD14+ cells have been observed to be either inhibitory or indifferent when LBP is also present, and in fact, sCD14 can substitute for LBP in some systems.

Several models can be proposed to explain how LPS might induce a signal through binding to CD14. In the first model, when LPS or LPS-LBP bind to mCD14, the receptor directly initiates a signaling cascade leading to activation of the cells. The fact that anti-CD14 antibodies mimic some LPS-elicited responses such as induction of homotypic adhesion (60), indicates that occupancy of CD14 alone might be sufficient to elicit a response. CD14, however, does not need to be the signal transducer *per se*. A protein analogous to gp130 in the IL-6 receptor complex could be active in the LPS system and be responsible for transducing the signal into the cell after LPS binds to CD14. A second model may be envisioned in which CD14 brings LPS to the membrane, acting like a shuttle, delivering it to a transmembrane co-receptor which is responsible for propagation of the signal.

CD14 may also function as a "pattern recognition" receptor with the capacity to bind structural components common to multiple microbial ligands, but without providing by itself ligand-specific responses. With respect to ligand binding, CD14 appears not to discriminate between stimulatory molecules, and those that are antagonistic to the effects of LPS. For instance, CD14 is capable of binding to lipid A, to soluble peptidoglycan from gram positive bacteria (61), or to lipoarabinomannan from *Mycobacteria* (62), as well as to antagonists, such as lipid IV_A (63), deacylated LPS (64,65), or LPS from *Rhodobacter spheroides* (66,67). Despite binding to CD14, Mϕ responses to these various ligands are disparate. These findings

point to the presence of an as yet unidentified element, distinct from CD14 that is responsible for signal propagation in response to LPS which imparts functional specificity.

LPS-INDUCED CELL SIGNALING

Protein Tyrosine Kinases

The functional consequences of Mϕ activation by LPS have been studied extensively and are well described [reviewed in (8,9,13)]. However, the details of the intracellular events that take place after engagement of LPS at the cell surface are only now beginning to be elucidated. Ligand-induced protein tyrosine phosphorylation is a rapid and common event mediating subsequent intracellular responses through diverse receptors in many cell types (68). Increased protein tyrosine phosphorylation in response to LPS is also observed in murine and human Mϕs (69-71), and in B cells transfected with CD14 (36,37). In the presence of sCD14, bovine and human endothelial cells also respond to LPS with transiently increased tyrosine phosphorylation (72).

Tyrosine phosphorylation appears to be important for downstream events taking place after LPS stimulation. Treatment with the protein tyrosine kinase (PTK) inhibitor herbimycin prevents LPS-induced release of eicosanoid mediators from the murine Mϕ cell line, RAW 264.7 (69). Herbimycin also blocks LPS-induced tumoricidal activity (73,74) and nitric oxide synthase activity of peritoneal Mϕs (21). Protein tyrosine kinase inhibitors of the tyrphostin family have also been observed to protect mice against LPS-induced lethal toxicity (75). Inhibition of tyrosine phosphorylation by these various compounds appears to correlate with their capacity to block LPS-induced production of TNF-α and nitric oxide by Mϕs *in vitro*, as well as LPS-induced production of TNF-α *in vivo*.

The importance of tyrosine kinase activity for LPS-elicited responses of Mϕs contrasts with findings in CHO cells transfected with CD14. CHO cells expressing CD14 show Mϕ-like responsiveness to LPS as treatment with endotoxin results in the production of arachidonate in a dose and time dependent manner similar to that observed in Mϕs (26). However, in contrast to Mϕs, no increased tyrosine phosphorylation is observed in CD14 positive CHO cells after LPS treatment. Furthermore, in CD14-transfected CHO cells, translocation of NF-κB by LPS, usually considered equivalent to its activation, is PTK-independent. This conclusion is based upon the finding that LPS-induced translocation of NF-κB is not blocked by pretreatment with either herbimycin A or genistein (76). These findings suggest that tyrosine phosphorylation is not always an essential event in eliciting functional responses to LPS. NF-κB had been thought to play a central role in the transcription of the IL-1β gene. However, in contrast to the finding on translocation of NF-κB in CHO cells, LPS-induced expression of IL-1β in Mϕs, is attenuated by PTK inhibitors. These apparently conflicting results, can be explained nevertheless, by the fact that even though treatment with tyrosine kinase inhibitors does not block NF-κB translocation, its ability to induce transcription is impaired under these conditions, indicating that translocation to the nucleus *per se* might not be sufficient to induce transcription (77).

At least some of the tyrosine kinases that become activated in LPS-treated Mϕs belong to the Src family of protein tyrosine kinases. In addition, treatment of macrophages with either LPS or LPS and interferon-gamma (IFN-γ) has been shown to increase steady-state levels of p*58/64*^{hck} and p*53/56*^{lyn} (78,79). Stefanova *et al.* (80) demonstrated that in human Mϕs, LPS treatment results in the transient activation of p*53/56*^{lyn}, p*58/64*^{hck}, and p*59*^{fgr}. In this system, despite its lack of a transmembrane domain, CD14 was shown to associate with p*53/56*^{lyn} in LPS-treated cells. In this regard, CD14 is similar to other GPI-linked molecules

such as decay accelerating factor, CD59, CD55, and CD48 which have also been reported to associate with Src tyrosine kinases in agonist-treated cells (81-83). Despite the unambiguous evidence that LPS brings about activation of Src kinases in Mϕs, it is not clear to what extent this is mechanistically important. Thus, Meng and Lowell (84) recently showed that peritoneal and bone marrow-derived Mϕs from *hck-/-fgr-/-lyn-/-*triple knock-out mice have no major defects in LPS-induced activation in terms of nitrite production and cytokine secretion. Similarly, it was found that the tumoricidal capacities of bone marrow-derived Mϕs from these animals were appropriately enhanced after LPS stimulation *in vivo*. In the case of peritoneal Mϕs, induction of tumoricidal activity was only partially impaired. These results seem to indicate that despite the biochemical evidence that Hck, Fgr and Lyn are activated by LPS treatment of Mϕs, cells devoid of these proteins and expressing no other detectable Src-family kinases, appear to have normal functional responses to LPS. It is important to note, however, that the responses measured by Meng and Lowell were induced after prolonged exposure to IFN-γ and LPS. Under similar conditions, Ziegler and coworkers (78) reported that exposure to LPS for 6-24 h resulted in increased expression of Hck in human macrophages and this was potentiated by priming of cells with IFN-γ. Therefore, the possibility exists that incubation of macrophages from knockout mice for prolonged periods with LPS and IFN-γ may induce the *de novo* expression of protein kinases -including Src-family kinases- that are not abundant under basal conditions. These newly induced enzymes may be able to compensate for the absence of Lyn, Fgr, and Hck.

Phosphatidylinositol 3-kinase

Non-receptor tyrosine kinases have been shown to associate not only with cell surface receptors, but also with cytosolic proteins. In this regard, the p85/p110 lipid kinase, phosphatidylinositol 3-kinase (PI 3-kinase) has been found to associate with tyrosine kinases and with other tyrosine phosphorylated proteins in response to a variety of signals including those mediated by growth factors, cytokines and G proteins (85-87). Prior to the studies reported in this thesis, no relationship between LPS signaling and PI 3-kinase had been established. However, since PI 3-kinase is known to be activated in association with tyrosine kinases -some of which are known to be regulated by LPS-, we postulated that PI 3-kinase may be involved in LPS cell activation.

PI 3-kinase phosphorylates the hydroxyl group at position 3 (D3) on the inositol ring of phosphoinositides (88). The most thoroughly studied of the PI 3-kinase family is a heterodimeric form comprised of a catalytic subunit (either p110α or p110β) and a regulatory subunit (p85α or p85β). The interactions of p85/p110 PI 3-kinase with other tyrosine phosphorylated signaling molecules appear to be mediated by SH2 domains in p85 and phosphotyrosine residues in the interacting proteins. A second mechanism for this association involves SH3 domains in p85 and proline-rich regions in the associated proteins (89,90), or vice versa (91). These interactions lead to the activation of the p110 catalytic subunit of PI 3-kinase resulting in increased levels of D-3-phosphorylated metabolites of phosphatidylinositol. Another PI 3-kinase enzyme, p110γ, present in neutrophils and U937 cells, is specifically activated by G protein $\beta\gamma$ subunits (92). This PI 3-kinase does not interact with any of the p85 subunits described thus far, and possesses a pleckstrin homology domain near its amino terminus (93). A third PI 3-kinase family member has recently been described by Stephens

and coworkers. This Gβγ-activated PI 3-kinase is a heterodimer comprised of a 120 kDa protein highly related to p110γ, and a p101 subunit that is not substantially related to protein in current protein sequence databases (94). The tight association existing between these subunits seems to be the mechanism for activation of the enzyme, as it leads to an amplified effect of Gβγ on the PI 3-kinase activity of p120.

The roles played by PI 3-kinase metabolites in cell regulation are not completely clear. Levels of these metabolites, especially PtdIns3,4P_2 and PtdIns3,4,5P_3 (Figure 3), in resting cells are negligible. Cell activation by various agonists, including membrane Ig cross-linking in B lymphocytes (95,96), IL-1 treatment of fibroblasts (97), and fMLP activation of neutrophils (88), leads to transient increases in their concentration. This supports a role for these polyphosphoinositides as second messengers. In contrast to the activity displayed by the enzyme *in vitro*, where PtdIns is the preferred substrate resulting in the formation of PtdIns3P, levels of this metabolite change minimally *in vivo* in response to agonist treatment (85). The D-3 phosphorylated phosphoinositides are not substrates for phospholipases, thus they are not sources of either inositol phosphates, or diacylglycerols. D3-phosphorylated phosphoinositides have been reported to bind to SH2 domains of Src and the p85 subunit of PI 3-kinase, to PH domains of Akt/PKB and to the non-receptor, Bruton's tyrosine kinase and to other proteins with novel phosphotyrosine binding domains [reviewed in (98)].

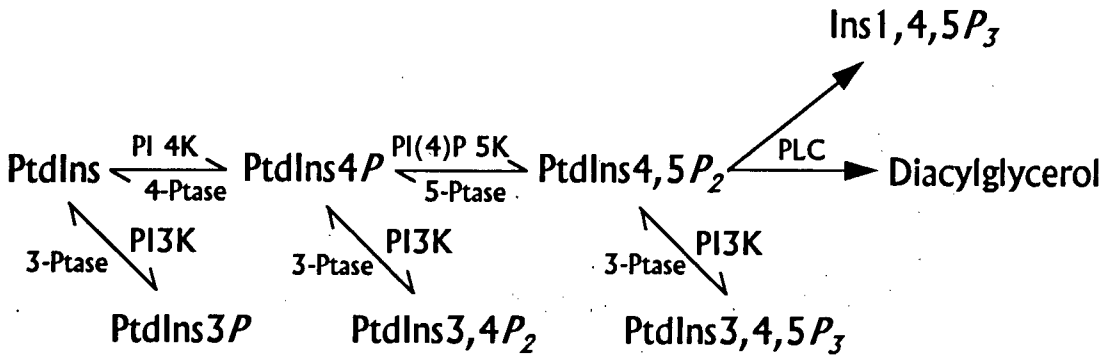


Figure 3. Metabolism of phosphoinositides. Generation of different inositol-derived metabolites and the enzymes involved. K= kinase; PI/PtdIns = phosphatidylinositol; PLC = phospholipase C; Ptase = phosphatase.

Several downstream signaling elements appear to be regulated by PI 3-kinase. This lipid kinase, for instance, appears to mediate activation of $p70^{s6k}$, as treatment of cells with the PI 3-kinase inhibitor, wortmannin, prevents activation of $p70^{s6k}$ (99) and partially inhibits activation of p42 and p44 MAP kinases by growth hormone (100), as well as platelet activating factor-induced MAPK activation in neutrophils (101). Similarly, activation $p70^{s6k}$ through the platelet-derived growth factor (PDGF) receptor seems to be mediated by PI 3-kinase, as activation of $p70^{s6k}$ is reduced either in PDGF-treated cells expressing a receptor unable to bind PI 3-kinase, or in normal cells after treatment with wortmannin (102). The protooncogene product, protein kinase B (Akt/PKB) is also activated by growth factors operating through PI 3-kinase. It has been shown that activation of PKB is inhibited by wortmannin and by coexpression of a dominant negative mutant of PI 3-kinase (103). In addition, Akt-1/PKB is able to bind to PtdIns3,4,5P_3 *in vitro*, but this binding does not affect

the enzymatic activity of the protein (104). These findings suggest that some other protein or factor may be required for activation of Akt/PKB *in vivo*, but that binding to phosphoinositides might recruit PKB to the membrane where it is activated in the context of other elements. Thus, PI 3-kinase, or its metabolites, may act as priming agents, in a manner analogous to the mechanism by which Ras is thought to prime c-Raf for activation. The involvement of PI 3-kinase in the activation of Akt/PKB is reinforced by findings that Akt can be directly regulated by phosphatidylinositol 3,4-bisphosphate both *in vivo* and *in vitro* (105). The PH domain present in the amino terminal region of PKB is known to bind to D3 inositol phospholipids *in vitro* (106), and PKB mutated at the PH domain is activated neither by PI 3-kinase *in vivo* nor by PtdIns3,4P_2 *in vitro* (105). Other proteins with PH domains such as cytohesin and "general receptors for phosphoinositides" (GRPs), have also been shown to bind PtdIns3,4,5P_3 in vitro (107). A PtdIns3,4P_2-dependent protein kinase-1 (PDK1) is also directly activated by both PtdIns3,4P_2 and PtdIns3,4,5P_3, and interestingly, has been found to phosphorylate and activate Akt *in vitro* (108). Other potential targets of a PI 3-kinase cascade include some members of the protein kinase C family (PKC). Certain PKC isoforms have been reported to be activated by PI 3-kinase metabolites (109) and this aspect is discussed below.

The functional alterations mediated by PI 3-kinase are only now being elucidated. PI 3-kinase has been implicated in protein sorting, as the sequence of the catalytic subunit p110, shows homology with another lipid kinase Vps34p, a yeast enzyme implicated in sorting of proteins to vacuoles (86,110). In neutrophils, functional responses to chemotactic peptides have been linked to activation of PI 3-kinase. Thus, the PI 3-kinase inhibitors wortmannin and LY294002 have been shown to block B cell proliferative responses (111), fMLP-induced superoxide production (112,113), and neutrophils treated with wortmannin develop

oscillatory changes in F-actin content (114). In contrast to observations in neutrophils where PI 3-kinase does not appear to be involved in cytoskeletal rearrangements (113,114), treatment of T cells with wortmannin leads to collapse of the actin architecture (115).

Protein kinase C and LPS cell signaling

An important, ubiquitous family of protein kinases also known to be involved in diverse signaling pathways in mammalian cells is protein kinase C (PKC). PKC participates in cellular differentiation, maturation and proliferation. This family of enzymes includes multiple isoforms with different biochemical characteristics and distinct patterns of tissue distribution. Eleven isoforms of PKC have been identified in mammalian tissues thus far, and they have been divided into three subfamilies (116): classical or conventional PKCs (cPKC), new or novel PKCs (nPKC) and atypical PKCs (aPKC). cPKC isoforms (α, βI, βII, γ) are activated by diacylglycerol (DAG) and Ca^{+2}. Tumor-promoting phorbol esters act as analogues of the physiological activator DAG, and activate some PKC isoforms *in vitro*. The nPKC isoforms (δ, ε, η, θ) do not require Ca^{2+} and exhibit enzymatic activity in the presence of phosphatidylserine (PS) and DAG or phorbol esters, whereas the activities of the members of the third PKC subfamily, aPKC (ζ, λ/ι) are not affected by either DAG, Ca^{+2} or phorbol esters.

Regulation of atypical PKC subfamily members is noteworthy for marked differences from that of other PKC isoforms. Phorbol esters have been shown to have profound effects on several members of the PKC family. Brief exposure of cells to phorbol 12-myristate, 13-acetate (PMA), for instance, results in translocation of some PKC isoforms to the membrane resulting in enhanced enzymatic activity. As well, prolonged exposure of cells to this tumor promoting agent results in reduced expression and downregulation of certain PKC isoforms [reviewed in

(117)]. In contrast, phorbol ester treatment of cells does not translocate PKC-ζ to the membrane (118), does not bind to it (119), and fails to alter its expression (120). The primary structure of aPKC family members explains, at least in part, their atypical biochemical characteristics (Figure 3). For example, atypical PKCs have only the first of two cysteine-rich zinc-finger motifs, common to all other isoforms of PKC. These cysteine-rich motifs have been reported to function in the phospholipid-dependent binding of tumor-promoting phorbol esters or DAG, thus explaining the insensitivity of the aPKC isoforms to PMA. Likewise, the C2 domain present in cPKC members confers calcium sensitivity upon conventional isoforms of the enzyme. Both nPKCs and aPKC lack a C2 domain and hence are insensitive to Ca^{2+} (Figure 4) (121,122).

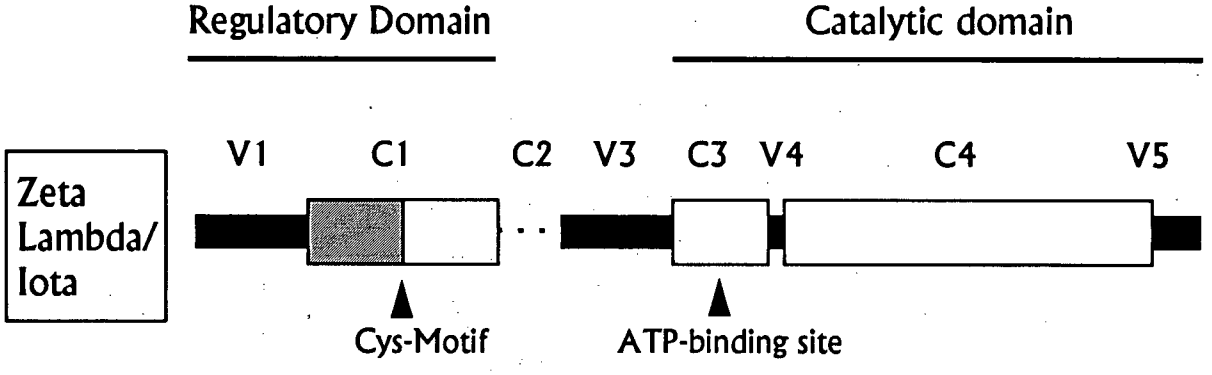


Figure 4. Structure of the atypical PKC isoforms. From Zhou G. *et al.* (122).

The regulation of aPKC family members *in vivo*, especially of PKC-ζ, is not yet fully understood, but the substrate preferences of these enzymes and the effects of different cofactors that modulate their activities *in vitro* have been examined (123). As stated above, in

contrast to other PKC isoforms, PKC-ζ expression is not downregulated by chronic exposure to phorbol esters. Depending on the assay conditions, the cell type, or whether it is a recombinant protein, wide variations in the biochemical characteristics of PKC-ζ have been reported. Several investigators have shown the activation of PKC-ζ by PI 3-kinase metabolites. In particular, PtdIns3,4P_2 and PtdIns3,4,5P_3 have been shown to stimulate the activities of PKC-ζ and PKC-ι (109,124).

The role of PKC in LPS signaling has been somewhat controversial. Findings supporting the participation of PKC in LPS signaling include experiments in which pretreatment of J774 cells with the PKC inhibitor H-7, abrogates LPS-stimulated tumoricidal activity (125). Similarly, it has also been shown that LPS transiently activates a PKC in human monocytes and treatment of these cells with H-7 completely blocks TNF-α and IL-1β secretion in response to LPS (126). Intracellular levels of the endogenous PKC activator DAG, were not, however, significantly modified by endotoxin in this study, indicating activation of a DAG-independent PKC isoform. The identity of the specific isoform(s) involved was not determined.

Translocation of PKC has frequently been associated with its activation, and this phenomenon has been reported for different effectors, especially for phorbol esters. Thioglycolate-elicited murine peritoneal Mϕs show LPS-induced translocation of PKC-β to the cytoskeleton. This effect is observed in LPS-responsive C3H/HeN mice, but not in LPS-hyporesponsive C3H/HeJ mice. Nevertheless, cells from the latter animals show PKC translocation in response to PMA (127). Results with murine macrophages, however, have not been consistent in respect to PKC activation. For instance, Hamilton and coworkers were unable to show LPS stimulation of Ca^{+2}/DAG/PS-dependent PKC in murine peritoneal Mϕs (128).

Numerous PKC-isoforms have been found to be expressed in primary Mϕs and in Mϕ cell lines. For instance, thioglycolate-elicited peritoneal Mϕs from C3H/HeN (LPS-sensitive), and C3H/HeJ (LPS-hyporesponsive) mice express the conventional PKC-β isoform, as well as two members of the nPKC family, PKC-δ and PKC-ε. PKC-ζ, as well as PKCβ–II and PKC-ε were observed to be expressed in the murine Mϕ cell line J774 (129), and PKC-β, and PKC-α have been detected in human monocytes by Western blot analysis (130). The human promonocytic cell line U937 expresses, both PKC-βI and PKC-βII, as well as PKC-ζ (131).

The participation of PKC-ζ in downstream events such as activation of NF-κB has been demonstrated in several different systems. For example, activation of NF-κB is observed in *Xenopus laevis* oocytes after insulin treatment or following microinjection of PC-phospholipase C (PC-PLC). This effect is abrogated when cells are microinjected with peptide Z, a specific PKC-ζ inhibitor based on the pseudosubstrate region of the enzyme (132). Likewise, overexpression of PKC-ζ in NIH-3T3 fibroblasts is sufficient to stimulate a persistent translocation of functionally active NF-κB. Transfection of a kinase-defective, dominant negative mutant of PKC-ζ dramatically inhibits κB-dependent transactivation of a reporter gene (133) as well as activation of NF-κB by sphingomyelinase in NIH-3T3 cells (134). As discussed previously, LPS brings about the activation of NF-κB in Mϕs and this suggests the possibility that PKC-ζ may play a role in the induction of functional responses elicited by LPS.

Ceramide-activated protein kinase

Ceramide-activated protein kinase (CAK) is a 97 kDa membrane-associated Ser/Thr protein kinase. The enzyme appears to be identical to kinase suppressor of Ras previously detected in *Caenorhabditis elegans* and *Drosophila* (135). CAK is activated *in vitro* by

ceramide, but not by other lipids such as diacylglycerol or arachidonic acid, and is able to phosphorylate Raf-1 very efficiently *in vitro* (136). LPS has also been shown to activate CAK in HL-60 cells and in human neutrophils in a CD14-dependent manner. Activation of this enzyme by LPS, in contrast to its activation by IL-1 or TNF, appears to occur independent of the hydrolysis of sphingomyelin and the generation of ceramide (34). Rather, LPS appears to act by mimicking the second messenger function of ceramide in these cells. Not surprisingly then, the LPS and ceramide signaling pathways appear to be regulated by some common elements. Macrophages from C3H/OuJ mice not only fail to respond to LPS, but also do not respond to ceramide analogues that enhance expression of LPS-inducible genes in LPS sensitive mice (137). These findings suggests the possibility that CAK may be involved in LPS stimulus-response coupling.

Mitogen-activated protein kinases, p21^{ras} and other signaling proteins

An additional signaling pathway potentially activated by LPS is one involving activation of mitogen activated protein kinases (MAPK). Indeed, human and murine Mϕs exposed to LPS, show increased tyrosine phosphorylation of a number of proteins, including the 42 and 44 kDa isoforms of MAP kinase (MAPK2 and MAPK-1, respectively, also known as ERK2 and ERK1) (70). Dong *et al.*, demonstrated that cells from the LPS-responsive strain C3H/HeN show increased tyrosine phosphorylation of numerous proteins including three with subunit sizes of 35, 41 and 45 kDa. Based upon immune complex kinase assays, the 41 and 45 kDa proteins appear to be respectively, MAPK2 and MAPK1 (138). In contrast, activation of MAPK was not observed in cells from LPS-unresponsive C3H/HeJ mice. In human neutrophils, in addition to the activation of MAPK1 and MAPK2, exposure to LPS results in tyrosine

phosphorylation and activation of p38 MAPK. This occurs in a dose-dependent manner through CD14 (139). Cloning and characterization of p38 has confirmed it to be member of the MAPK family (140). Increased tyrosine phosphorylation of p38 is also observed in response to LPS in 70Z/3 cells transfected with human CD14, and in peritoneal exudate Mϕs from LPS-responsive mice (C3HeB/FeJ). On the other hand, cells derived from LPS-unresponsive C3H/HeJ mice do not respond to LPS with increased phosphorylation of p38. On the other hand, exposure of these cells to hyperosmolarity does activate p38. Because Mϕs from hyporesponsive mice express normal levels of CD14, these results indicates that the defect in cells from these mice is localized to a functional element located distal to the LPS receptor and proximal to p38/p42/p44 MAPKs and CAK (140).

Activation of MAP kinases by growth factors and mitogens has been shown to involve a series of phosphorylating enzymes. One of the earliest events observed after engagement of certain cell surface receptors is the activation of p21^{ras} and with it, activation of a protein kinase cascade consisting of the Raf-1 kinase, the MAP kinase activator, MAP/ERK kinase (MEK), and ultimately MAP-kinases [reviewed in (141,142)]. The involvement of p21^{ras} in the activation of MAPK by LPS in Mϕs is as yet not clear. The murine macrophage cell line BAC-1.2F5, when stimulated with LPS shows increased MAP kinase activity in a Raf-dependent manner. This is also independent of increased p21^{ras} activity (143), whereas in the same system, activation of MAP kinase via colony stimulating factor 1 (CSF-1) involves activation of both Raf-1 kinase and p21^{ras}. The different signaling pathways recruited by these two agonists may underlie the contrasting responses elicited by these compounds in Mϕs. Thus, CSF-1 stimulates proliferation of Mϕs, while LPS inhibits cell growth and stimulates differentiation and activation. In contrast to these findings, Geng *et al.* (144) reported that LPS stimulation of

human Mϕs results in activation of p*53/56*^{lyn} and this is associated with tyrosine phosphorylation of Vav, and activation of p21^{ras}. The product of the protooncogene *vav* is a guanine nucleotide exchange factor for Ras, specifically expressed in cells of lymphoid lineage that becomes tyrosine phosphorylated upon cell activation. Nevertheless, these findings remain to be confirmed since in other studies, LPS did not activate Ras or Vav in human monocytes (Herrera-Velit P. and N. Reiner, unpublished, see below).

Thesis Rationale and Objectives

General Objective

Activation of Mϕs in response to extracellular signals is essential to induce immunological competence and maximal effector functions. While there is an extensive body of knowledge about the functional changes in Mϕs after activation, less known is about the signaling pathways used to bring about these changes. The activation process is known to involve a series of intracellular events including activation of protein kinases, gene transcription and new protein synthesis. To understand cell regulation leading to Mϕ activation, it is important to define the signaling elements involved in the process. As LPS is one of the most potent agonists of Mϕs, the studies described below examined signal transduction events taking place in response to LPS and their potential relationship to Mϕ activation.

Hypotheses and Specific Objectives

- Hypothesis 1. LPS-mediated cell signaling in human Mϕs involves activation of tyrosine kinases signaling through the LPS receptor, CD14.

Rationale: Increased protein tyrosine phosphorylation and activation of tyrosine kinases is a common phenomenon resulting from engagement of cell surface receptors with different agonists. LPS is a potent effector of Mϕ responses and tyrosine phosphorylation of different cellular proteins in response to LPS has been reported for Mϕs of different origins. This signal may originate at the Mϕ membrane through CD14, a monocyte differentiation, cell surface molecule described as an endotoxin receptor.

- Hypothesis 2. LPS brings about the activation of PI 3-kinase leading to increased levels of D3-phosphorylated phosphoinositides.

Rationale: Both receptor and non-receptor tyrosine kinases have been shown to associate with the lipid kinase, PI 3-kinase, leading to its activation. PI 3-kinase phosphorylates position D3 of inositol phospholipids resulting in a dramatic and transient increases in the intracellular levels of these novel second messengers. This lipid kinase associates with different proteins, including tyrosine kinases, and activation of PI 3-kinase results from this association. LPS has been found to activate several protein tyrosine kinases in myeloid cells, thus indicating the possible stimulation of PI 3-kinase through a pathway involving activated tyrosine kinases.

- Hypothesis 3. LPS treatment of Mϕs involves the activation of p21^{ras} and the Ras guanine nucleotide exchanger Vav.

Rationale: Activation of different enzymes, including MAPK, PKC and PI 3-kinase has been reported to be linked to activation of the protooncogene product p21^{ras}. Activation of Ras may occur through Vav, a guanine nucleotide exchanger for Ras present in several lymphocytic cells. Activation of Mϕs and neutrophils by different agonists, including LPS, has

been shown to result in activation of MAPK and some isoforms of PKC. This indicates that some LPS responses may be Ras-dependent.

- Hypothesis 4. LPS activation of PI 3-kinase, leading to increased levels of D3-phosphorylated inositol phospholipids, results in activation of PKC-ζ, an atypical isoform of PKC.

Rationale: The activation of PI 3-kinase by different agonists results in increased levels of D3 phosphoinositides. These metabolites have been shown to activate atypical isoforms of PKC-ζ *in vitro*. LPS-treatment of Mϕs results in activation of a PKC isoform independent of calcium and DAG for its activity. These features are characteristic of members of the aPKC subfamily, including PKC-ζ.

- Hypothesis 5. LPS activation of Mϕ-PI 3-kinase regulates monocyte adherence and cytokine induction.

Rationale: LPS treatment of Mϕs results in increased production of different cytokines, and enhanced cellular adhesion. LPS activates PI 3-kinase in Mϕs, and in T cells and neutrophils this lipid kinase has been linked to changes in F-actin content and to cytoskeletal rearrangements. These findings suggest the possibility that PI 3-kinase may regulate monocyte adherence and cytokine production in response to LPS.

VIII. MATERIALS AND METHODS

A. MATERIALS

1. Antibodies:

Horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG was from BioRad laboratories (Mississauga, Ontario, Canada). Upstate Biotechnologies (Lake Placid, NY) were the suppliers of: mouse monoclonals anti-phosphotyrosine (4G10, #05-321), and anti- PI 3-kinase, N-SH2, antibodies (UB93-3), and rabbit polyclonal antibodies against-MAP kinase (anti-ERK-1-CT), pan PKC, human lyn kinase (p53/56^{lyn}), PI 3-kinase, and PKC-ζ. A second polyclonal anti-PKC-ζ (C-20), anti-p70 S6 kinase (C-18), mouse monoclonal anti-PKC (MC5), and goat polyclonal anti-PI 3-kinase p110α (C-17) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GIBCO (Burlington, Ontario, Canada) was supplier of a third rabbit polyclonal anti-PKC-ζ. Goat anti mouse HRPO IgG was purchased from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). Mouse monoclonal anti-MAP kinase (#03-6600 recognizing both ERK1 and ERK2) was from Zymed Laboratories Inc. (San Francisco, CA). Anti-CD14 monoclonal antibody 3C10 was a generous gift from Dr. Wesley C. Van Voorhis (University of Washington, Seattle, WA), rat monoclonal anti-p21^{ras} antibody Y13-259 was a gift from Dr. Vince Duronio (University of British Columbia, Vancouver, B.C.), and rabbit polyclonal antibody against p97^{vav} was a gift from Dr. Amnon Altman (Division of Cell Biology, La Jolla Institute of Allergy and Immunology, La Jolla, CA).

2. Reagents

The following reagents were from Sigma Chemical Company (St. Louis, MO): agarose, ammonium acetate, aprotinin, benzamidine, boric acid, bovine serum albumin (BSA), bromophenol blue, dimethyldichlorosiline, cAMP-dependent protein kinase peptide inhibitor (TTYADFIASGRTGRRNAIHD), ethylendiaminetetraacetic acid, disodium salt, dihydrate (EDTA), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) Ficoll-Paque, β-glycerophosphate, herbimycin A, histone III-S, isoamyl alcohol, lauryl sulfate, sodium salt (SDS), leupeptin, mineral oil, 3-[N-Morpholino]propanesulfonic acid (MOPS), myelin basic protein from bovine brain (MBP), pepstatin A, phenylmethylsulfonyl fluoride (PMSF), L-α-phosphatidylinositol (PtdIns), L-α-phosphatidylinositol 4,5-bisphosphate (PtdIns4,5,P_2), L-α-phosphatidyl-L-serine (PS), piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES), phorbol 12-myristate 13-acetate (PMA), protamine sulfate, protein A Sepharose, RNase A, sodium chloride, soybean trypsin inhibitor, Trizma Base, Trizma hydrochloride, Tris-saturated phenol, xylene cyanol, and wortmannin. Reagents for polyacrylamide gel electrophoresis, protein A-agarose and D*c* protein assay kit were from BioRad Laboratories. Peptide ε and MAPK peptide substrate (APRTPGGRR) were from Upstate Biotechnology. Rainbow molecular weight markers, reagents and film for enhanced chemiluminiscence (ECL), $^{32}PO_4^{3-}$, 5'[α-^{32}P]UTP, triethylammonium salt (3000 Ci/mmol), [^{3}H]PtdIns4*P* and [^{3}H]PtdIns4,5P_2 were purchased from Amersham International (Oakville, Ontario, Canada). ATP, CTP, GTP, and UTP (all 10 mM), and 100 mM dithiothreitol (DTT), nuclease-free water, 5X transcription buffer, bacteriophage T7 RNA polymerase, recombinant RNasin RQ1, RNase-free DNase, and proteinase K were from Promega Corporation (Madison, WI). Yeast tRNA, formamide, RNase T1, TRIzol, LipofectAMINE were from Gibco BRL. Human AB+ serum was provided by the

Canadian Red Cross (Vancouver, BC). RPMI 1640, phosphate-free RPMI 1640, Iscove's methylcellulose, penicillin/streptomycin and Hank's Balance Salt Solution (HBSS) were from Stem Cell Technologies (Vancouver, British Columbia, Canada). Lipopolysaccharide (*E. coli.* 0127:B8) was from Difco Laboratories (Detroit, MI). Microcystin-LR, LY294002, and α,25-dihydroxy-vitamin D_3 were from Calbiochem (San Diego, CA). [γ-^{32}P]ATP (sp. act. 3000 Ci/mmol) was obtained from Dupont (Wilmington, DE). Mono Q HR5/5 columns and protein G-Sepharose were from Pharmacia Biotech Inc. (Piscataway, NJ). Unless specified otherwise, all reagents were of the highest quality available

The human monocytic cell lines U937 and THP-1 were from the American Type Culture Collection (Rockville, MD). THP-1 cells stably transfected with CD14 (THP1-WT) were provided by Dr. R. J. Ulevitch (Scripps Research Institute, La Jolla, CA).

B. METHODS

1. Isolation of monocytes and cell culture.

White cell enriched fractions of peripheral blood were obtained from normal human volunteers by the Cell Separator Unit of the Vancouver Hospital and Health Sciences Center (Vancouver, British Columbia) and fractionated by centrifugation (800 x *g* for 15 min) over Ficoll Hypaque as previously described (145). After three washes in HBSS, the cells were resuspended in RPMI 1640 with 10% human AB$^+$ serum at 10^7 viable cells per ml, dispensed into 150 cm^2 cell culture flasks at a density of 1.5 x 10^8 cells per flask, and incubated at 37 °C in a humidified atmosphere (5% CO_2, 95% air [vol/vol]) for 45 min. Non-adherent cells were removed by vigorous washing with divalent cation-free HBSS (37 °C) and the flasks were replenished with RPMI (without serum). The monolayers were allowed to equilibrate for 1 h

before further treatment. Adherent monolayers prepared in this manner were 89% $\pm$ 5% monocytes by morphologic and phagocytic criteria as determined by microscopic examination of preparations stained with Diff-Quik (CanLab, Vancouver, British Columbia). Cells were then either left untreated (controls) or incubated with different concentrations of LPS solubilized in RPMI + 10% human serum (final serum concentration 0.1%). Incubations were terminated by rinsing flasks with ice-cold phosphate buffered saline (PBS: 1.0 mM NaH_2PO_4, 8.1 mM $NaHPO_4$, 154 mM NaCl, pH 7.4) and monolayers were immediately frozen under liquid nitrogen and stored at -70 °C before further analysis.

Cell lines were cultured in RPMI supplemented with 10% heat inactivated fetal calf serum (FCS) and incubated in a humidified atmosphere (5% CO_2, 95% air [vol/vol]). THP-1-WT cells were maintained in a similar manner except that the medium was supplemented with G418 (350 μg/ml) for selection of positive transfectants. Unless otherwise stated, 12 to 15 h prior to incubation with LPS, cells were rendered quiescent in RPMI without FCS at a concentration of 5 x 10^5 cells/ml. Following stimulation with LPS, cells were lysed immediately and the detergent soluble material was frozen at -70 °C until further analysis.

2. Preparation of cell lysates

a. High speed supernatants

Monolayers were rapidly thawed by the addition of ice-cold extraction buffer (pH 7.5), containing 20 mM Hepes, 12.5 mM β-glycerophosphate, 5 mM EDTA, 2 mM EGTA, 1 mM Na_3VO_4, 0.2 mM PMSF, 2.5 mM benzamidine, 5 mM 2-mercaptoethanol, 100 nM microcystin, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 2 μg/ml leupeptin. Cells were dislodged and transferred to prechilled, Dounce glass homogenizers and disrupted by 20 strokes

on ice. After disruption, extracts were centrifuged at 100,000 x *g* for 60 min at 4 °C and high speed supernatants were assayed directly for protein kinase activity.

b. Lysates for anion exchange chromatography

Extracts were prepared by lysing cells on ice (20 min) in fast performance liquid chromatography (FPLC) extraction buffer (1% Nonidet P-40, 12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 2 mM EGTA, 1.0 mM Na_3VO_4, 1 mM PMSF, 100 nM microcystin, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). Lysates were centrifuged at 16,000 x *g* to remove insoluble material and were passed through a 0.2 μm filter prior to chromatography.

c. Lysates for analysis of phosphotyrosine content

Cells lysates for phosphotyrosine analysis, and for anti-Lyn, anti-Vav, anti-CD14, and anti-PI 3 kinase immunoprecipitates were prepared by addition of cold phosphotyrosine (PY) lysis buffer for 15-30 min at 4 °C (1% Triton X-100, 20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF, 100 nM microcystin, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin; 1 ml per flask, for approximately 4 x 10^6 cells in suspension). Detergent-insoluble material was removed by centrifugation for 15 min at 16,000 *x g* in the cold.

3. Ras immunoprecipitation

Adherent monocytes were incubated in phosphate-free RPMI for 1 h and labeled with ^{32}[P]orthophosphate for 2 h (2 mCi/150 cm^2 flask). Following stimulation, medium was removed and cells were rinsed in cold PBS. To increase labeling of cells, adherent monocytes

were removed from tissue culture flasks by incubation with 5 ml of cold HBSS (divalent cation-free) and gentle scraping. Cells were resuspended in Pi-free RPMI, and cell viability was assessed by trypan blue dye exclusion (~90% viable cells). After 1 h, cells were centrifuged and resuspended in RPMI + 0.5 mCi/ml ^{32}P-orthophosphate and incubated for 3 additional h. Cells were then solubilized in ice cold buffer consisting of 50 mM Hepes (pH 7.4), 1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 2 μg/ml leupeptin, 1 μM pepstatin, 10 μg/ml soybean trypsin inhibitor and 40 μg/ml PMSF and centrifuged in a microfuge for 1 min. Supernatants were transferred to new tubes and were immediately made up to 500 mM NaCl, 0.5% sodium deoxycholate, and 0.05% SDS. Protein A-Sepharose beads were precoated with 0.1% BSA, rabbit anti-rat IgG antiserum (1 h) and anti-p21^{ras} antibody (Y13-259; 1-2 h) and added to the lysates. After incubation for 45-60 min, the beads were washed 4 times with wash buffer (50 mM Hepes, pH 7.4, 0.1% Triton X-100, 500 mM NaCl, 0.005 % SDS, 5 mM $MgCl_2$) and resuspended in elution buffer (2 mM EDTA, 2 mM DTT, 0.5 mM GTP and 0.5 mM GDP). Samples were incubated at 67 °C for 20 min after which they were centrifuged and 10 μl of supernatant were loaded into polyethylenimine (PEI) cellulose plates with fluorescent indicator. Samples were developed in 1.0 M KH_2PO_4, pH 3.4 and radioactive spots detected on X-ray film were compared with unlabeled standards (GDP and GTP) detected using a UV lamp.

4. Anion-exchange chromatography

Detergent extracts were loaded onto a Mono Q FPLC column pre-equilibrated in buffer A (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 2 mM EGTA, and 0.5 mM Na_3VO_4). Proteins were resolved with a 20 ml linear gradient of 0-0.8 M NaCl in buffer A

at a flow rate of 0.5 ml/min. Fractions of 0.25 ml were collected and aliquots were assayed for protein kinase activity or immunoreactivity as described below. No significant MBP phosphorylating activity could be detected either in the column flow-through or wash fractions prior to initiation of the salt gradient.

5. Immunoadsorption of kinase activity

Cell lysates (1 mg) prepared in FPLC-extraction buffer from LPS-treated U937 cells, were incubated with either 7.5 μg of two commercially available rabbit anti-protein kinase C-ζ antibodies (Santa Cruz Biotechnology and Gibco) or with 7.5 μg of monoclonal anti-PKC antibody (recognizing α, β and γ isoforms) for 2-3 h at 4 °C. Immune complexes were then incubated for 1 h with either protein A-agarose or protein G-Sepharose. Solid phase complexes were washed 2 times with FPLC extraction buffer by centrifugation at 14,000 x *g* for 1 min and the supernatant fraction was subjected to a second immunoprecipitation with the same antibodies. Immunoadsorbed supernatants were fractionated by Mono Q chromatography as described above and fractions were analyzed by immunoblotting and protein kinase assays.

6. Western blotting

Samples were subjected to SDS-PAGE and separated proteins were transferred to nitrocellulose membranes. Membranes were blocked overnight at 4 °C, with 3% BSA dissolved in TBT-T (20 mM Tris, pH 7.4, 137 mM NaCl, 0.1% Tween 20) and immunoblotting was carried out by incubating the membranes for 2 h at room temperature (RT) with different monoclonal antibodies or with polyclonal rabbit antibodies raised against proteins of interest as described in figure legends. Immunoreactive bands were then detected

by incubation (1 h at RT) with horseradish peroxidase (HRPO) conjugates of either goat-anti-mouse or goat-anti-rabbit (1/5,000 dilution) antibodies and enhanced chemiluminescence (ECL) reagents (Amersham). When appropriate, after developing, antibodies were removed from membranes by incubating in stripping buffer containing 62.5 mM Tris, pH 6.7, 100 mM 2-mercaptoethanol and 2% SDS at 50 °C for 30 min. Membranes were then blocked and reprobed with antibodies of interest as just described.

7. Immunoprecipitation

Cell lysates (~500 μg protein) were incubated with either a monoclonal antibody specific for PI 3-kinase, rabbit anti-Lyn antiserum or irrelevant control antibodies (normal mouse serum and normal rabbit serum, respectively) overnight at 4 °C. Immune complexes were collected by incubation with protein A-agarose for 1 h and washed 5 times with lysis buffer. Samples were resuspended in Laemmli sample buffer (125 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 2% 2-mercaptoethanol plus bromophenol blue) and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting.

8. In-situ renaturation kinase assay

Whole cell lysates prepared in PY lysis buffer were used for detection of protein tyrosine kinases in an in situ renaturation kinase assay with a polydispersed tyrosine kinase substrate, [poly(Glu,Tyr) 30 to 94 kDa)] as described by Durocher *et al.* (146). Briefly, samples were mixed with substrate and separated by PAGE. After electrophoresis, SDS was removed from gels, proteins were denatured in guanidine hydrochloride, and renatured by incubating in buffer containing 0.04% Tween and 10% sucrose (four washes for a total of 19 h). Gels were

equilibrated in 10 mM Hepes pH 7.4 plus 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 5 mM $MnCl_2$ and 0.1 mM Na_3VO_4 and kinase assays were performed in the same buffer with the addition of 100 μCi [γ -^{32}P]ATP. Gels were soaked in 1 M KOH for 1 h at 56 °C to eliminate signals from non-tyrosine kinase enzymes and after staining and drying, phosphorylated bands were detected by autoradiography.

9. Immune complex kinase assay

Immune complexes precipitated with anti-Lyn antiserum, monoclonal anti-CD14 antibodies or appropriate controls plus protein A-agarose, were washed three times with lysis buffer and four times with kinase buffer (20 mM Hepes, pH 7.4, 10 mM $MgCl_2$). Washed immune complexes were suspended in 40 μl kinase assay buffer (20 mM Hepes, pH 7.4, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 10 μM ATP, 10 μCi [γ-^{32}P]ATP) and incubated at RT for 15 min. Reactions were stopped by adding 40 μl of 2X sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 2% 2-mercaptoethanol with bromophenol blue). Samples were boiled for 10 min and subjected to SDS-10% PAGE. Gels were dried and exposed to X-ray film at -70 °C.

10. PKC-ζ assays

Quiescent THP-1 cells were incubated with 1 μg/ml of LPS for different times or left untreated. When the effects of PI 3-kinase inhibitors were analyzed, cells were incubated with either 32 μM LY294002 or 100 nM wortmannin prior to LPS treatment for 10 min unless otherwise stated. Cells were immediately lysed for 30 min at 4 °C in lysis buffer (1% Triton X-100, 50 mM HEPES, pH 7.4, 150 mM NaCl with protease and phosphatase inhibitors at

the same concentrations as described for PY lysis buffer). Lysates were precleared with protein A-Sepharose and PKC-ζ was immunoprecipitated with rabbit polyclonal anti-PKC-ζ (5 μg per sample, Upstate Biotechnology). Kinase activity was measured in immunoprecipitates using MBP as substrate as previously described (115). Quantitation of kinase activity was done by excision of the band corresponding to MBP and scintillation counting. Incorporation of radioactivity into MBP in the absence of lysate was used as background and was subtracted from radioactivity present in the immunoprecipitates.

11. Protein kinase assays

Aliquots of either high speed supernatants or fractions from Mono Q chromatography (5 μl) were assayed for MBP phosphotransferase activity as described (147). When substrate preference of FPLC fractions was analyzed, substrates were used as indicated in the corresponding figures. Substrate phosphorylation was carried out in a final volume of 25 μl of kinase assay buffer (pH 7.5) containing 12.5 mM MOPS (pH 7.5), 12.5 mM β-glycerophosphate, 2 mM EGTA, 0.5 mM Na_3VO_4, 2 mM DTT, 10 mM $MgCl_2$, MBP (0.5 mg/ml), 75 ng/ml cAMP dependent protein kinase inhibitor peptide and [γ-^{32}P]ATP (50 μM). Reactions were allowed to continue for 10 min at 30 ^{o}C, unless otherwise stated, and were terminated by spotting 22 μl of the assay mixture onto Whatman P-81 phosphocellulose filter squares (1.5 x 1.5 cm^2). Filters were washed six times with ice-cold 175 mM phosphoric acid over a period of 60 min and incorporation of radioactivity was determined by liquid scintillation counting.

12. In vitro PI 3-kinase assay

Aliquots of cell lysates normalized for protein content (300-500 μg protein) were incubated overnight at 4 °C with either antibody (mAb) to PI 3-kinase, anti-Lyn antiserum or with appropriate control antibodies. Immune complexes were adsorbed onto protein A-agarose for 1 h. When 4G10 was used, it was first incubated with protein A-agarose for 1 h and cell lysates were then added for 2-4 h. Immunoprecipitates were washed twice with lysis buffer and three times with 10 mM Tris-HCl, pH 7.4. PI 3-kinase activity was measured as described (148). Briefly, immunoprecipitates were incubated with 10 μg of sonicated PtdIns and 10 μCi of [γ-^{32}P]ATP in the presence of adenosine in a final volume of 40 μl. Reactions were carried out for 15 min at room temperature and stopped by the addition of 0.1 ml of 1 N HCl and 0.2 ml of chloroform:methanol (1:1, v/v). Lipids were separated on oxalate-treated silica TLC plates using a solvent system of chloroform: methanol: water: 28% ammonia (45:35:7.5:2.5, v/v/v/v). Plates were exposed to X-ray film at -70 °C. Incorporation of radioactivity into lipids was quantitated by excising the corresponding portions of the TLC plate followed by liquid scintillation counting.

13. Cell labeling and extraction of inositol phospholipids

After adherence of monocytes to culture flasks, cells were incubated for 30 min at 4 °C with cold HBSS, without Ca^{+2}, or Mg^{+2} and removed gently with a cell scraper. Monocytes were then pelleted by centrifugation and resuspended in phosphate-free RPMI in sterile polypropylene tubes. Cells were incubated at a density of 2-4 x 10^6/ml for 1 h at 37 °C and labeled with 0.5 mCi/ml $^{32}PO_4^{3-}$ for an additional 2 h. Samples containing equal numbers of cells were treated or not with LPS for the indicated periods of time, and incubations

were terminated by the addition of 3.75 volumes of methanol:chloroform (2:1, v/v). Extraction of lipids was performed as described (95). Phases were separated by the addition of 1.25 volumes each of 2.4 N HCl and chloroform, and the lower organic phase was recovered after centrifugation. The upper phase was reextracted with 1 volume of chloroform:methanol (2:1, v/v). The combined lower phases were washed twice with 1 volume of methanol:0.1 M EDTA in water (1:0.9, v/v) and then dried under liquid nitrogen, resuspended in chloroform and loaded into oxalate-treated TLC plates for separation using a solvent system of n-propanol:2M acetic acid (13.7:7, v/v). Plates were exposed to X-ray film at -70 °C and spots were excised and counted as described above.

14. HPLC analysis of deacylated lipids

U937 cells cultured in RPMI plus 10% FCS were incubated in P_i-free RPMI (without serum) and labeled for 3 h with 0.5 mCi/ml $^{32}PO_4^{3-}$ as done for normal human Mϕs. Cells were recovered by centrifugation and resuspended at 4 x 10^7/ml before treatment with LPS (100 ng/ml) for either 5 or 10 min. Lipids were extracted as described above. Dried lipids were deacylated by adding 1.8 ml methylamine solution (methanol/25% methylamine/n-butanol, 45.7:42.8,11.4, v/v/v) for 50 min at 53 °C (148). Samples were dried *in vacuo*, resuspended in water, and extracted three times with equal volumes of n-butanol:light petroleum ether:ethyl formate (20:4:1, v/v/v). The aqueous phase was dried again, resuspended in water and stored at -70 °C until analyzed by HPLC on a Partisil SAX ion exchange column. Column runs were calibrated by coinjecting ADP and ATP as internal standards. Samples were separated with a 60 min linear gradient of 0 to 0.25 M ammonium phosphate, pH 3.8 followed by a 50 min gradient of 0.25 to 1 M ammonium phosphate, pH

3.8. Fractions (0.5 ml) were collected, mixed with scintillation fluid (Ecolite+, ICN) and quantitated by liquid scintillation counting. The elution times of deacylated PtdIns3*P*, PtdIns3,4P_2 and PtdIns3,4,5P_3 were determined by preparing these metabolites in an *in vitro* kinase assay with anti-PI 3-kinase immunoprecipitates using PtdIns, PtdIns4P and PtdIns4,5P_2 as substrates. The elution times of deacylated PtdIns4*P* and PtdIns4,5P_2 were determined using [^{3}H]PtdIns4P and [^{3}H]PtdIns4,5P_2.

15. Transfection of U937 cells

pSRα-based mammalian expression plasmids, containing the entire coding regions of either wild-type bovine p85α or mutant bovine p85α (Δp85α) were kindly provided by Masato Kasuga (Kobe University School of Medicine, Kobe, Japan) (Figure 5). The mutant has a deletion of 35 amino acids from residues 479-513 of bovine p85α and the insertion of two other amino acids (Ser-Arg) in the deleted position. This alteration prevents the association of mutant p85α with the p110 catalytic subunit. However, mutant p85α is able to compete with native p85 for binding to essential signaling proteins and behaves as a dominant negative mutant (149).

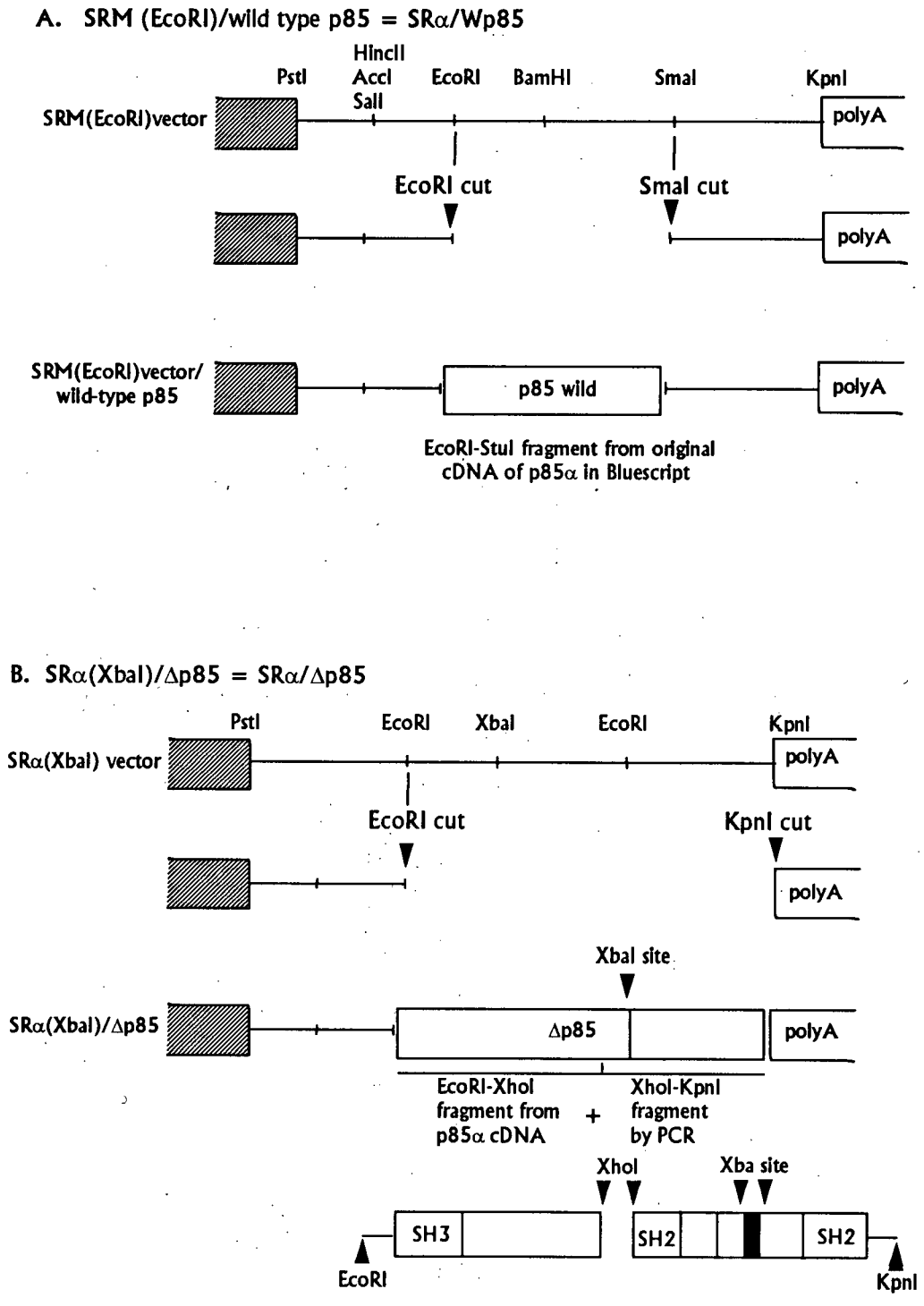


Figure 5. Cloning of wild-type bovine p85α (A) and dominant negative mutant Δp85 (B).

U937 cells were grown to a density of 4-8 x 10^5 cells/ml in RPMI-1640 media supplemented with 10% heat-inactivated FCS. Cells were washed and resuspended in 800 µl of antibiotic-free RPMI (without FCS). Transfection was done using LipofectAMINE according to the protocol supplied by the manufacturer. pSRα plasmids containing either wild-type or mutant p85α were cotransfected along with pMC1neo-polyA, a plasmid encoding resistance to the antibiotic, G418 sulfate. DNA/liposome complexes were added to the cells followed by a 5 h incubation after which the cultures were supplemented with RPMI containing 10% FCS, penicillin, and gentamicin. Expression of foreign DNA was allowed to proceed for 2 days followed by the addition of 350 µg/ml of G418. After 4 days in G418, the cells were suspended in Iscove's methylcellulose supplemented with 2-mercaptoethanol, FCS, BSA, G418 and glutamine. The cells were incubated at 37 °C for ten days and colonies were picked and resuspended in 400 µl of RPMI + 10 % FCS supplemented with G418. Thereafter, cells were maintained in medium containing 350 µg/ml G418.

16. RNase Protection Assay

Cells were incubated with LPS (0.1 or 1 µg/ml) for 2 h and RNA was extracted from cells using Trizol according to the manufacturer's instruction. Equal amounts of RNA (5-10 µg) were subjected to an RNase protection assay as described by Hobbs *et al.* (150). One cytokine-specific probe template set, HL-14, was assembled from EcoR1-linearized and purified subclones. The template set synthesized a group of ^{32}P-labeled riboprobes specific for several cytokines (Table I). Radiolabeled anti-sense RNA probes were prepared and hybridized in excess with test RNA dissolved in hybridization buffer (80% formamide, 1 mM EDTA, 400 mM NaCl, 40 mM PIPES, pH 6.7) for 12-15 h at 56 °C. Single stranded unhybridized RNA

was digested with RNase A and RNase T1. After treatment with a mixture of proteinase K (0.5 mg/ml) and yeast tRNA (100 mg/ml), RNA duplexes were isolated , dissolved in 80% formamide and dyes, and resolved in 5% acrylamide/8 M urea sequencing gels. Dried gels were placed on X-ray film and developed at -70 °C.

Table 1. HL-14 Template Set

Cytokine	Protected mRNA
IL-6	320 bases
IL-10	294
IL-1α	269
TNF-β	220
GM-CSF	208
TGF-β	170
IL-1β	149
TNF-α	124
L32	76

GeneBank Accession Number for nucleotide sequence for protected RNA is described in reference (150).

17. LPS induced adherence of Mϕs

Control protein (BSA) or fibronectin (20 mg/ml) dissolved in 50 mM bicarbonate buffer (pH 9.6) were added to 96 well cell culture dishes and incubated for 1 h at room temperature or overnight at 4 °C. Wells were blocked with 1% BSA in RPMI for 1 h at RT

and washed twice with RPMI. Cells were then added and incubated with the PI 3-kinase inhibitors, wortmannin or LY294002 or with anti-CD14 antibodies (10 μg/ml). Cells were stimulated with 1 μg/ml LPS overnight at 37 °C, washed and fixed with methanol/acetone (1:1 v/v) for 10 min at 4 °C. Fixative was removed and cells were stained for 10 min with a 0.05% solution of crystal violet dissolved in 20% ethanol. After washing and drying, remaining material was solubilized in methanol and adherence was measured as absorbance at 570 nm.

Alternatively, cells were added directly to uncoated wells, incubated with inhibitors or with anti-CD14 antibodies and then treated overnight with LPS as described above. After removing non-adherent cells, wells were observed at 200X magnification under an inverted microscope and the number or cells per field was taken as an estimate of adherence.

18. Protein determination

Protein concentrations of high speed supernatants were determined by Bradford method using Bio-Rad protein reagent and BSA as standard (151). Protein concentrations in detergent solubilized samples were determined by a modified Lowry method using D*c* protein assay kit and BSA as standard (152).

IX. RESULTS

A. TYROSINE PHOSPHORYLATION AND TYROSINE KINASE ACTIVITIES INDUCED BY LPS IN MΦS

1. LPS-induced tyrosine phosphorylation of Mϕ proteins

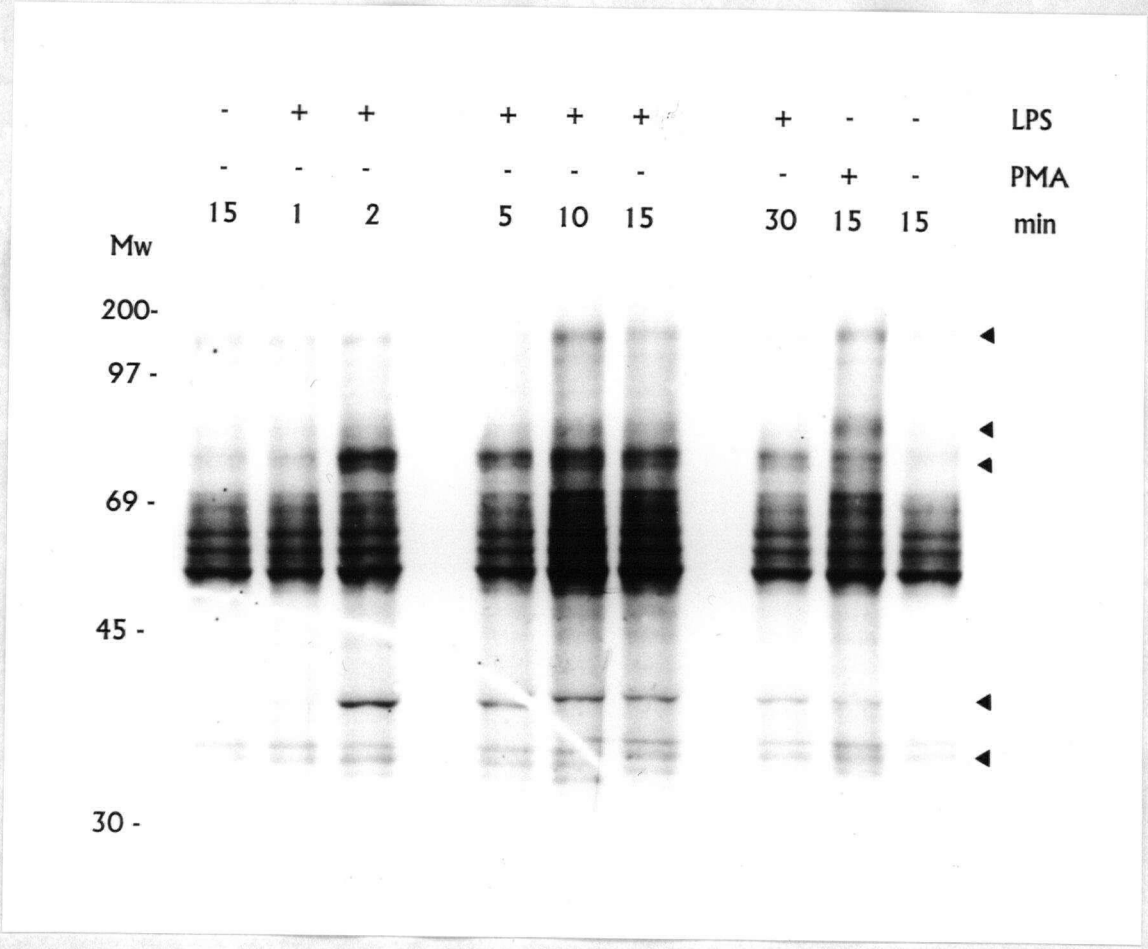
Treatment of peripheral blood Mϕs (PBMϕs) with 100 ng/ml of LPS led to a rapid increase in tyrosine phosphorylation of several proteins as observed by phosphotyrosine immunoblotting with 4G10 antibodies (Figure 6A). Responses were observed within 2 min of treatment and returned almost to basal levels by 30-40 min. The major tyrosine phosphorylated bands showed approximate subunit sizes of 35-38 kDa, 42 kDa, 77-79 kDa and 117-120 kDa. Enrichment of phosphotyrosine containing proteins by immunoprecipitation with 4G10 allowed the detection of a similar pattern of tyrosine phosphorylation (e.g. 117-120 kDa band, bottom arrow head in Figure 6B is also observed in Figure 6A), and also of additional bands, including a protein of approximately 155 kDa (Figure 6B, top arrow head;). For comparison, parallel cell cultures were treated with 100 nM PMA. Although some proteins were phosphorylated in response to both agonists, the presence of phosphotyrosine-containing proteins unique to each agonist was detected (i.e. a 105 kDa band observed after PMA treatment, Figure 6B).

2. LPS induced tyrosine kinase activity

The observed tyrosine phosphorylation of the proteins in whole cell lysates could be a consequence of autophosphorylation of tyrosine kinases occurring after cell activation. The proteins undergoing tyrosine phosphorylation could also be substrates of tyrosine kinases that become activated in response to LPS. To examine directly for the presence of activated

tyrosine kinases, a renaturation kinase assay was performed using whole cell lysates from control and LPS stimulated PBMϕs. This assay takes advantage of the fact that some protein kinases regain their enzymatic activity after PAGE and renaturation. By renaturing the enzyme in the polyacrylamide gel an approximate estimate of the molecular mass of the protein can be obtained. As seen in Figure 7, PBMϕs show at least three bands with renaturable tyrosine kinase activities with apparent subunit sizes of 47-49, 53-55 and 60-65 kDa (arrow heads). The activities of these proteins are increased in LPS treated cells as early as 2 min and return to near basal levels after 30 min. Prolonged exposure of gels showed two additional bands of approximately 75 and 79 kDa (n=2).

A



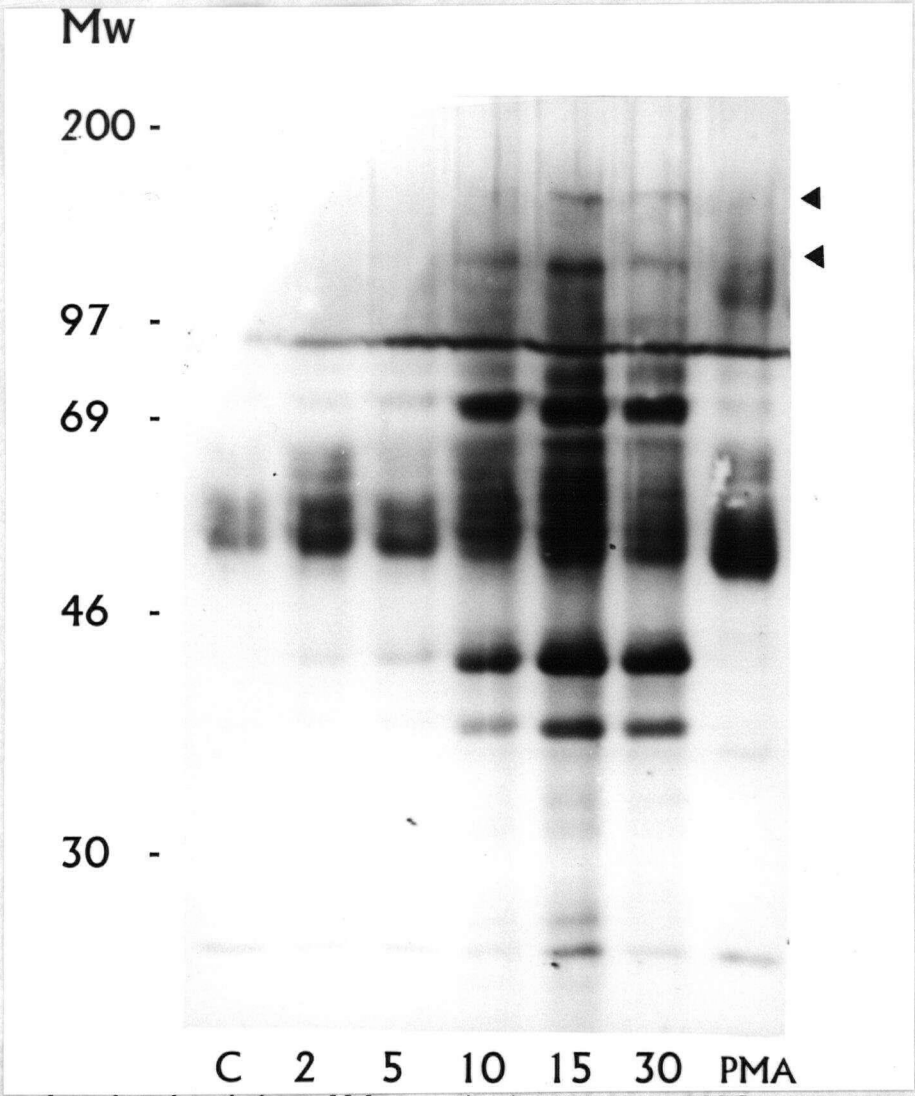


Figure 6. Tyrosine phosphorylation of Mϕ proteins in response to LPS. Adherent Mϕs were incubated for the indicated times with LPS (100 ng/ml dissolved in RPMI + 10% HS, final HS concentration = 0.1%), 100 nM PMA (15 min), or medium alone. Reactions were stopped by rinsing the flasks with cold PBS, cells were snap frozen, and kept at -70 °C until processing. Lysates were prepared as described in Materials and Methods and (A) 50 μg protein/lane were loaded onto a 10% polyacrylamide gel. Alternatively, (B) 250 μg of lysates from control or LPS treated cells were immunoprecipitated with anti-phosphotyrosine antibodies and immune complexes were electrophoresed. After electrophoresis proteins were transferred to nitrocellulose membranes and blocked overnight at 4 °C with 3% BSA in TBST. Blots were incubated with 4G10 (10 μg/ml) for 2 h, followed by goat anti-mouse HRPO-conjugated secondary antibody and developed by ECL according to the manufacturer's instructions. The migration of molecular mass marker proteins are shown in kDa.

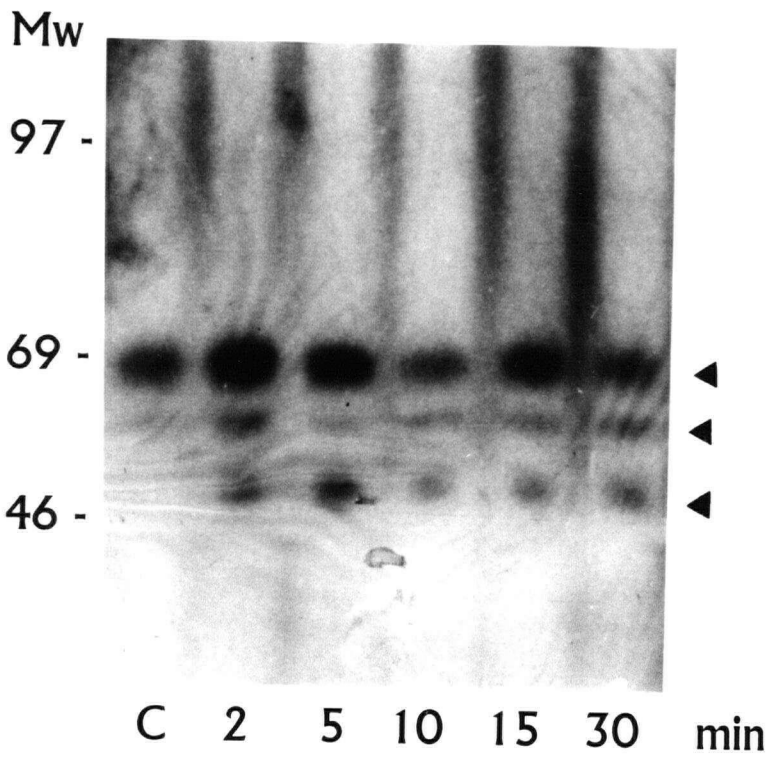


Figure 7. Activation of protein tyrosine kinases in Mϕs in response to LPS. Peripheral blood Mϕs were left untreated for 10 min (C) or were stimulated with 100 ng/ml LPS and reactions were stopped after the indicated times. Lysates were boiled together with Poly(Glu,Tyr) and electrophoresed in a 10% polyacrylamide gel. Proteins were renatured in the gel and enzyme activity was detected by incorporation of [γ-^{32}P]ATP as described in Materials and Methods. Gels were stained and radioactive bands were detected by exposure to X-ray film at -70 °C (n=3). The migration of molecular mass marker proteins are shown in kDa.

3. Association of CD14 with tyrosine kinases and phosphotyrosine proteins in response to LPS

As many receptors are known to associate with tyrosine kinases after binding of their ligands, the association of CD14 with tyrosine kinases was investigated. *In vitro* kinase assays using CD14 immune complexes and performed in the absence of exogenous substrate showed the presence of several protein kinases in the range of 55-66 kDa. Enzymatic activities were increased in lysates from LPS stimulated cells, and although the time course varied from donor to donor, increased activities were observed as early as 2 min, peaked by 5-10 min and decreased by 30-40 min of LPS treatment (Figure 8). CD14 associated not only with tyrosine kinases, but also with other tyrosine phosphorylated proteins lacking kinase activity. For example, 4G10 immunoblotting of CD14 immunoprecipitates showed a tyrosine phosphorylated band of 170-180 kDa (Figure 9) in lysates from LPS-treated cells, whereas no immunoreactivity was observed in immunoprecipitates prepared using a control antibody. Tyrosine phosphorylation of this protein was increased in response to LPS in a time dependent manner, was maximal at 2-5 min and returned nearly to basal levels after 20 min of treatment.

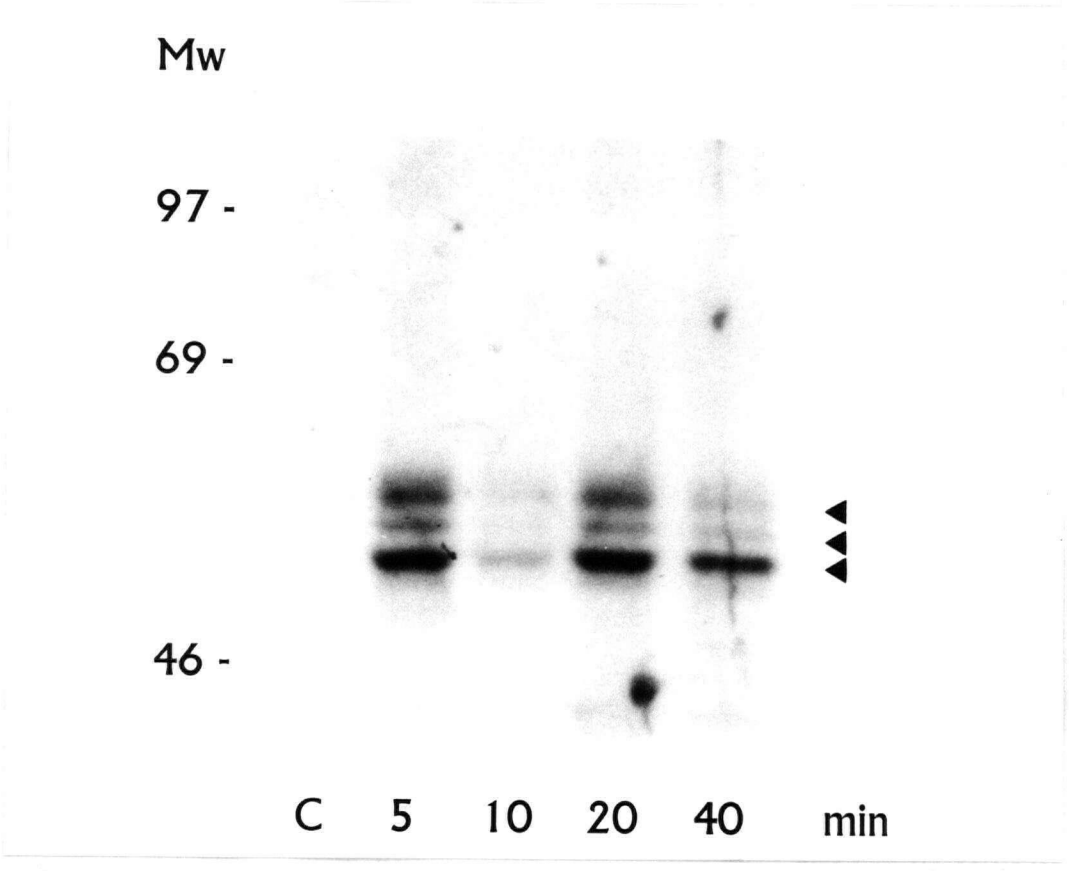


Figure 8. LPS induces the association of CD14 with tyrosine kinases. Peripheral blood Mϕs were left untreated for 10 min (C) or treated with 100 ng/ml LPS for the indicated times and lysed as described above. Equal amounts of lysates (750-1000 μg/ml) were immunoprecipitated with either anti-CD14 monoclonal antibodies or an isotype matched control overnight at 4 ^{o}C. After washing with lysis buffer, immunoprecipitated material was subjected to an *in vitro* kinase assay without exogenous substrate in the presence of [γ-^{32}P]ATP as described in Materials and Methods. After 15 min, reactions were stopped by boiling for 10 min with Laemmli buffer, and samples were electrophoresed in a 7.5% polyacrylamide gel. Gels were stained, dried and exposed to X-ray film for detection of radioactive proteins. Decreased intensity of bands in the lane corresponding to 10 min are a result of underloading of the lane and not to decreased activity, as other experiments showed kinase activities comparable to or higher than 5 min of LPS treatment. Shown is one of four similar experiments with cells from different donors. Migration of molecular mass marker proteins are shown in kDa.

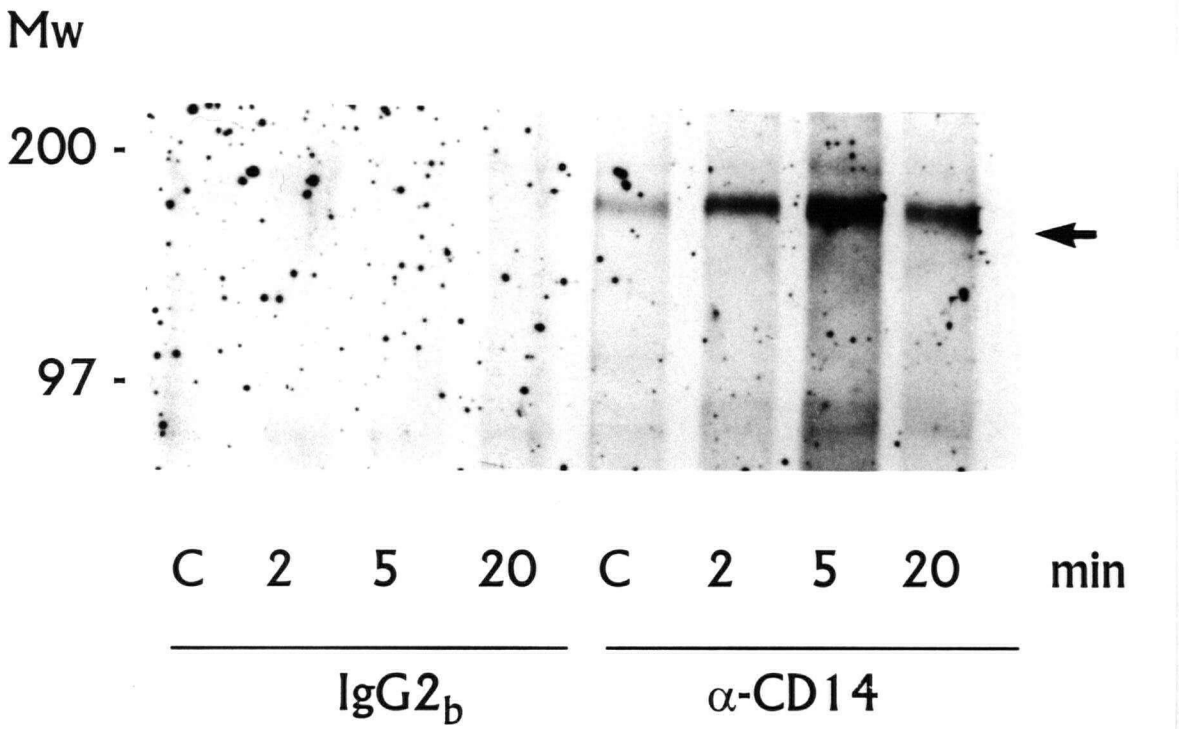


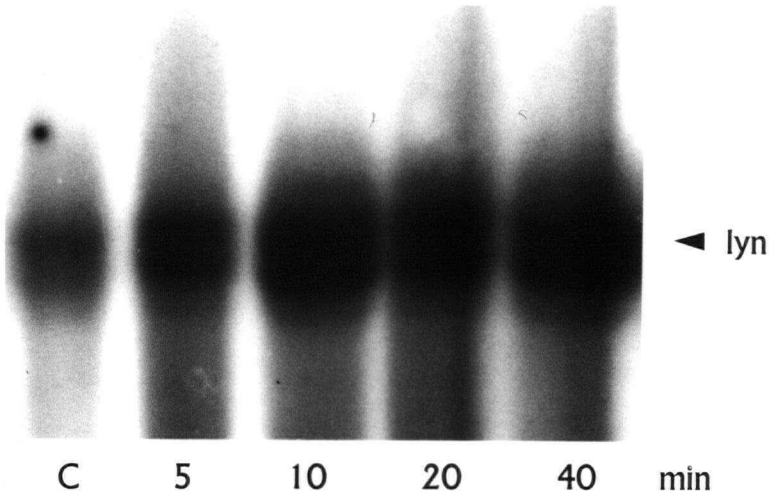
Figure 9. LPS induces association of phosphotyrosine containing proteins with CD14. After stimulation of PBMϕs with medium alone for 5 min (C) or with LPS for the indicated times, cells were lysed and equal amounts of protein were immunoprecipitated with anti-CD14 as described for Figure 8. Immunoprecipitated material was electrophoresed in a 7.5% polyacrylamide gel and proteins were transferred to nitrocellulose membranes for detection of tyrosine phosphorylated bands with 4G10 as described for Figure 6 (n=3). Migration of the molecular mass marker proteins are shown in kDa.

4. LPS treatment activates p53/56^{lyn} in Mϕs

Treatment of PBMϕs with LPS has been shown to bring about the activation of several Src-family tyrosine kinases including p*53/56^{lyn}*, p*58^{fgr}* and p*59^{hck}* (78-80). These responses are CD14-dependent. In the case of p*53/56^{lyn}*, this involves physical association of the kinase with CD14 (80). When p*53/56^{lyn}* immunoprecipitates were examined for kinase activity in an immune complex kinase assay, the enzyme was found to be more active in LPS treated samples when compared to control PBMϕs (Figure 10A, B).

This effect of LPS on p*53/56^{lyn}* activity was also examined in U937 cells. Whether left undifferentiated (Figure 11A) or differentiated with vitamin D_3 for 72 h (Figure 11B) this cell line showed increased activity of p*53/56^{lyn}* after 10 min treatment with 0.1 or 1 μg/ml LPS. As expected, p*53/56^{lyn}* activity in immunocomplexes was markedly reduced if cells were preincubated for 4 h with the tyrosine kinase inhibitor, herbimycin A (Herb) (5 μg/ml).

A



B

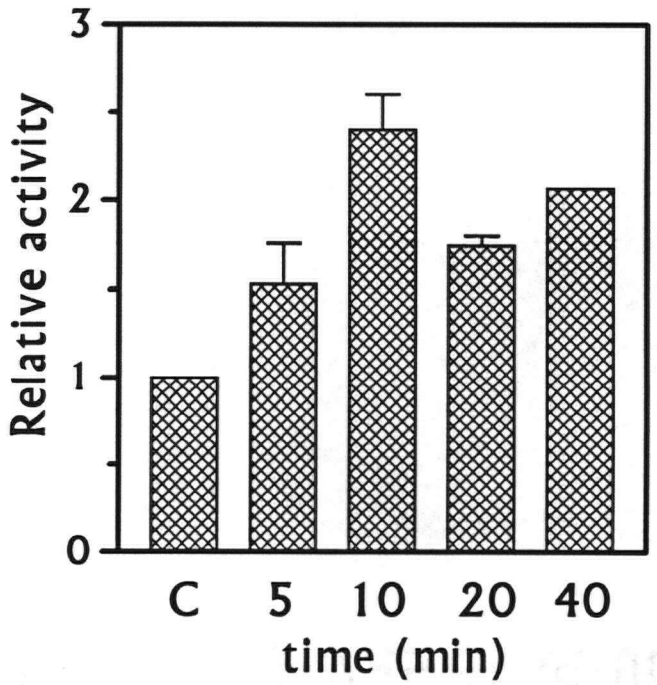


Figure 10. Activation of p*53/56*^{lyn} in PBMϕs treated with LPS. (A) Cells were left untreated ("C") or treated with 100 ng/ml LPS for the indicated times (min). Cell extracts were prepared and equal amounts of protein (~500 mg) were used for immunoprecipitation overnight with anti-lyn antibodies (or normal rabbit serum as control, not shown) as described under Materials and Methods. Immune complex assays were performed in the absence of exogenous substrate and proteins separated by SDS-PAGE as described for Figure 8. (B) After exposure of gels to X-ray film, radioactive bands were excised from the gel and quantified by liquid scintillation counting. Activity is expressed as a ratio with respect to control untreated cells (n=3, except for t=40 min where only one determination was done).

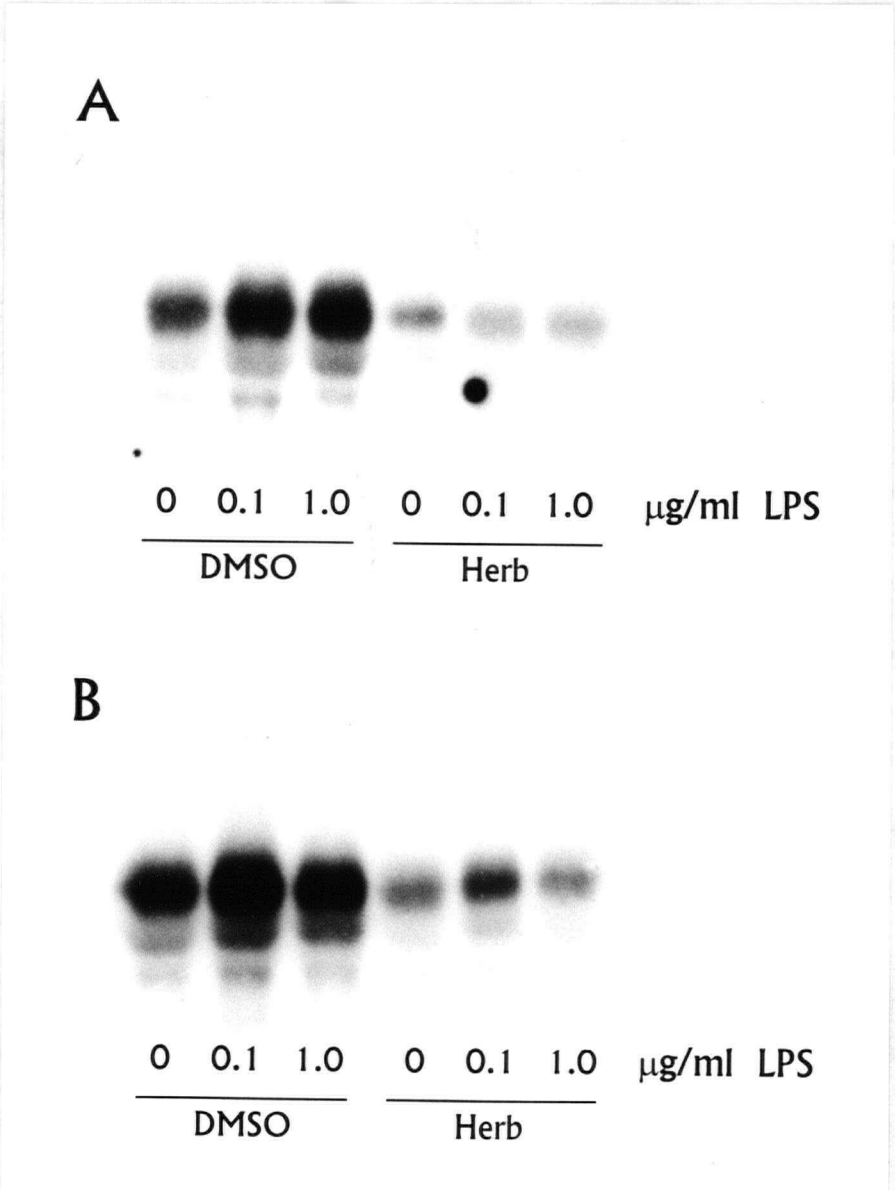


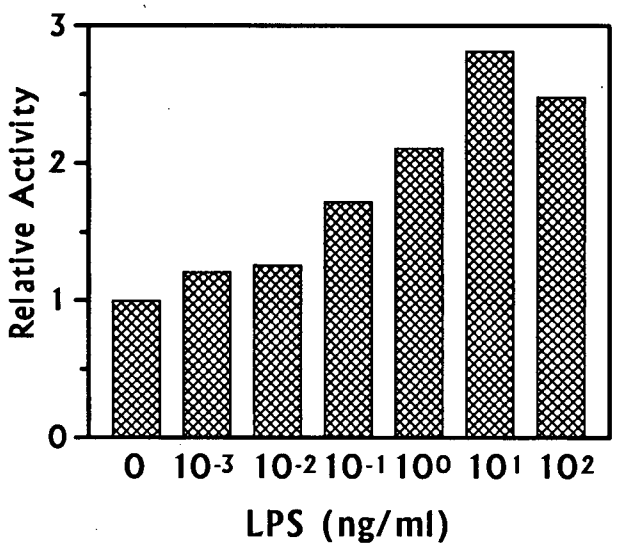
Figure 11. LPS-dependent activation of p*53/56*^{lyn} in undifferentiated and D_3-differentiated U937 cells. U937 cells were either left undifferentiated (A) in normal culture medium as described in Materials and Methods, or were treated with 100 nM 1,25-dihydroxy vitamin D (D_3) for 72 h (B). Twelve to 15 h before treatment with LPS, cells were left in RPMI (no FCS) and incubated with 5 μg/ml herbimycin A or vehicle alone (DMSO) for 4 h. Cells were then stimulated for 10 min with the indicated amount of LPS or left untreated. Immunoprecipitates were prepared with anti-Lyn antibodies and immunocomplex kinase assays were performed as for Figure 10.

B. SERINE/THREONINE KINASES ACTIVATED IN MΦS IN RESPONSE TO LPS

1. LPS induces time- and concentration-dependent increases in phosphotransferase activity towards myelin basic protein

Cytosolic preparations from cells stimulated with LPS for 30 min showed a dose-dependent increase of MBP-phosphotransferase activity. The stimulatory effects of LPS were first apparent with concentrations as low as 10 pg/ml, and maximal with 10-100 ng/ml (Figure 12A). LPS-elicited increases of MBP kinase activity were dependent on the incubation time. Increased enzymatic activity was first detected at 10 min of LPS treatment (100 ng/ml) and peaked by 20-30 min of stimulation (Figure 12B). By 40-60 min of LPS treatment, MBP kinase activity returned to a level similar to untreated, control cells. In four different experiments, activation of 2-5 fold over basal levels was observed with 100 ng/ml LPS.

A


Relative Activity
0
1
2
3
0
10-3
10-2
10-1
100
101
102
LPS (ng/ml)

B

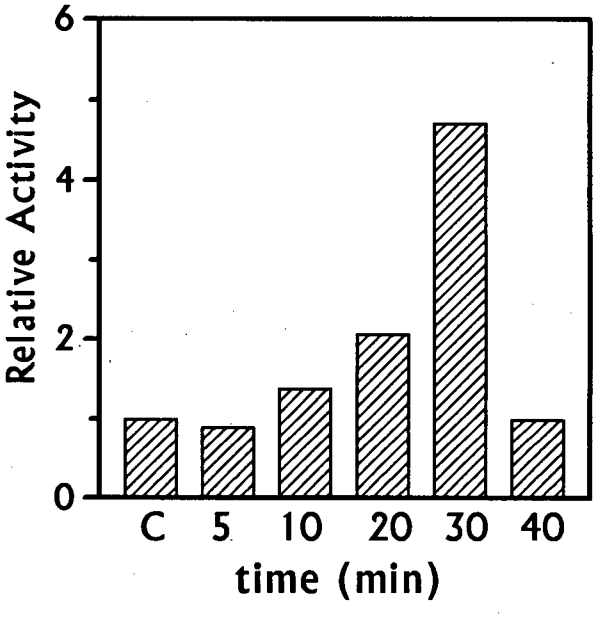

Relative Activity
0
2
4
6
C
5
10
20
30
40
time (min)

Figure 12. LPS treatment of Mϕs leads to increased myelin basic protein phosphorylating activity in a dose and time dependent manner. (A) PBMϕs were stimulated with different concentrations of LPS for 30 min, or (B) with 100 ng/ml LPS for the indicated times. High speed supernatants were prepared and protein kinase assays were performed using MBP as substrate as described in Materials and Methods. After 10 min the reactions were stopped by spotting the samples on phosphocellulose filter paper. Filters were washed five times in 0.85% (v/v) o-phosphoric acid and incorporated radioactivity was measured by scintillation counting. Activity is expressed as a ratio with respect to control untreated cells; shown is one of three experiments with similar results.

2. Activation of p42/p44 mitogen activated protein kinase by LPS in PBMϕs

Following exposure of cells to LPS (10 or 100 ng/ml) for 30 min two distinct peaks of LPS-activated MBP phosphotransferase activity were resolved by chromatography of cell lysates on Mono Q (Figure 13A). In a series of experiments with cells from different donors, both peaks of activity were consistently observed. A third peak of MBP phosphotransferase activity eluting later in the gradient was also present, but this was not consistently observed in preparations from different donors.

Fractions from Mono Q peaks one and two were subjected to SDS/PAGE on 10% gels and transferred to nitrocellulose for immunoblotting. Using rabbit polyclonal antibodies that recognized both ERK-1 and ERK-2 members of the MAP kinase family, two proteins with approximate subunit sizes of M_r 42,000 and M_r 44,000 were detected (denoted by the two arrowheads in Figure 13B, D, E) in fractions under peak one (the earliest eluting peak) from both control (Figure 13B) and LPS-treated cells (Figure 13D). In contrast, no immunoreactive MAP kinase protein was detected in fractions comprising either Mono Q peak two or three (data not shown). The anomalous appearance of p44 MAP kinase in fraction 33 (the distorted bands in Figures 13B and 13E) may have been due to the presence of a large amount of nonspecific protein of similar size (detected by staining with amido black, data not shown) that eluted in this fraction and caused increased migration of p44 MAP kinase. This band could also be accounted for by the 38 kDa protein kinase Hog that is known to become tyrosine phosphorylated in neutrophils in response to LPS and is weakly recognized by erk1-CT antibodies (139).

Membranes that had been immunoblotted with anti-MAP kinase antibodies were stripped and re-probed with the anti-phosphotyrosine monoclonal antibody 4G10. Two

proteins also of subunit sizes M_r 42,000 and 44,000, that coincided precisely with the p42 and p44 isoforms of MAP kinase were shown to become tyrosine phosphorylated within activated peak one fractions from LPS-treated cells (denoted by the two arrowheads in Figure 13E, fractions 30 and 31). No evidence for tyrosine phosphorylation of these proteins was found in the corresponding Mono Q fractions prepared from extracts of untreated monocytes (Figure 13C). Other protein species showed increased tyrosine phosphorylation in fractions from extracts of LPS-treated cells, but not in extracts from control cells (data not shown). Although the identities of the latter proteins have not been determined, their presence is consistent with the observation that treatment of monocytes with LPS was observed to provoke increased tyrosine phosphorylation of multiple proteins as detected in whole cell immunoblots (Figure 6).

3. Analysis of protein kinase activity using a MAP kinase-specific peptide substrate

To characterize further the nature of the kinase activities present in Mono Q fractions from LPS-treated cells, *in vitro* kinase assays were carried out in parallel using either MBP or a MAP kinase-specific peptide substrate APRTPGGRR (147,153). As shown in Figure 14A, LPS-activated phosphotransferase activity towards MBP was detected in Mono Q fractions from both, peaks one and two. In contrast, only fractions from Mono Q peak one demonstrated stimulated activity towards the MAP kinase-specific peptide (Figure 14B).

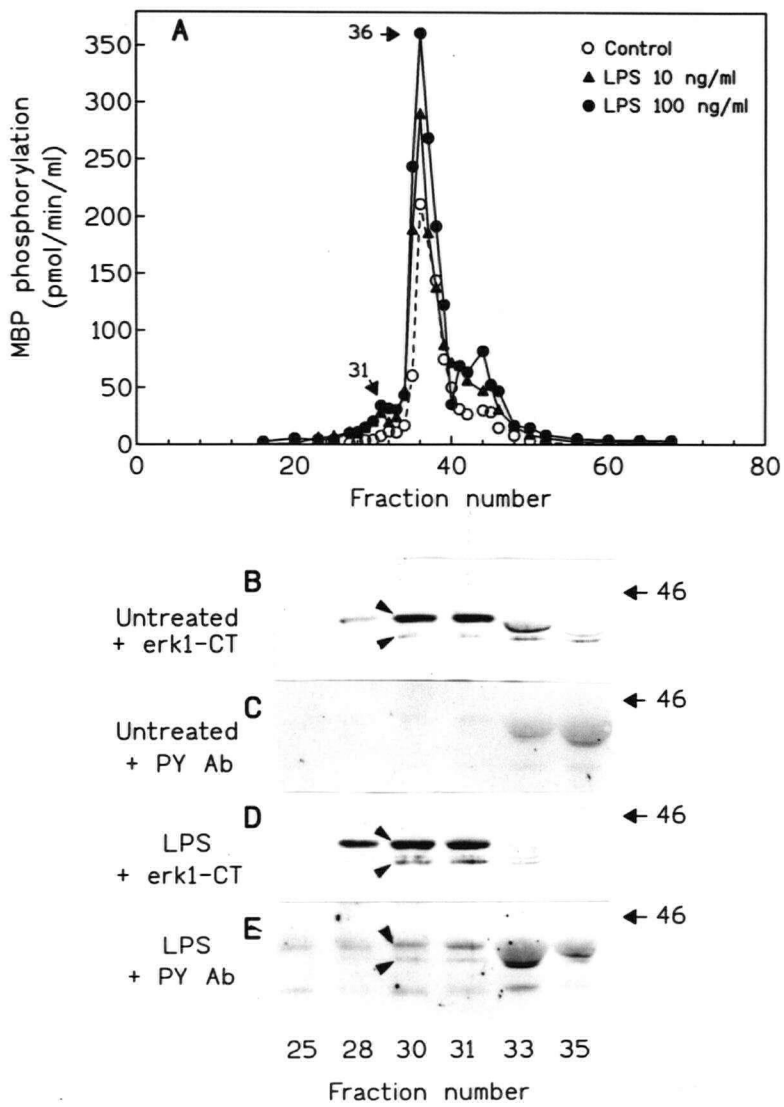

A
36
31
Control
LPS 10 ng/ml
LPS 100 ng/ml
MBP phosphorylation (pmol/min/ml)
Fraction number
B
Untreated + erk1-CT
C
Untreated + PY Ab
D
LPS + erk1-CT
E
LPS + PY Ab
46
25 28 30 31 33 35
Fraction number

Figure 13. Anion exchange chromatography of PBMϕs shows several peaks of MBP-phosphorylating activity stimulated by LPS. After 30 min of incubation with LPS (10 or 100 ng/ml) or vehicle (RPMI + 10% HS), detergent lysates were prepared and fractionated by anion exchange chromatography on a Mono Q column. Proteins were separated by a 20 ml linear gradient of 0-800 mM NaCl in buffer A (12.5 mM β-glycerophosphate, 12.5 mM MOPS, 2 mM EGTA, 0.5 mM ortho-vanadate). Fractions of 0.25 ml were collected and (A) aliquots were analyzed for MBP-kinase activity as described under Materials and Methods using MBP as substrate. Aliquots (75-100 μl) of fractions from control (B, C) or LPS treated samples (D, E) were analyzed for the presence of p42/p44 mitogen activated protein kinases by immunoblotting with erk1-CT (B, D), an antibody recognizing both isoforms of the enzyme. After stripping the blots of erk1-CT antibodies, the membranes were reprobed with 4G10 (C, E) for the detection of tyrosine phosphorylated proteins as for Figure 6. Arrowheads shown in B, D and E correspond to positions of p44 and p42 MAP kinases.

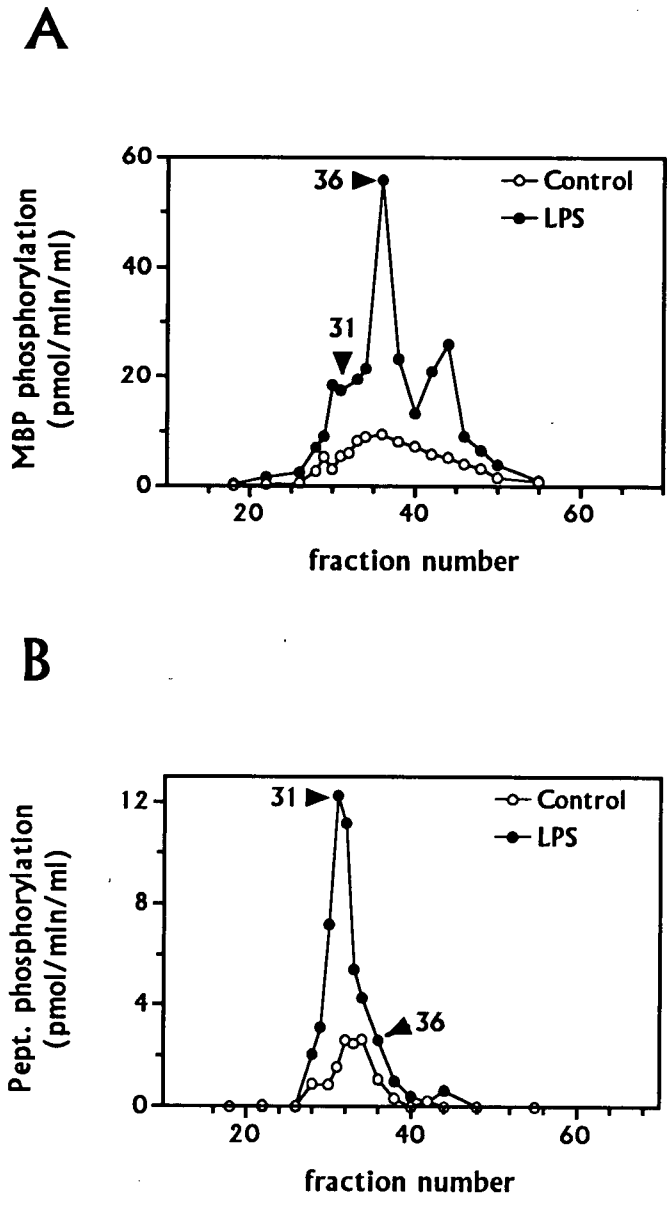


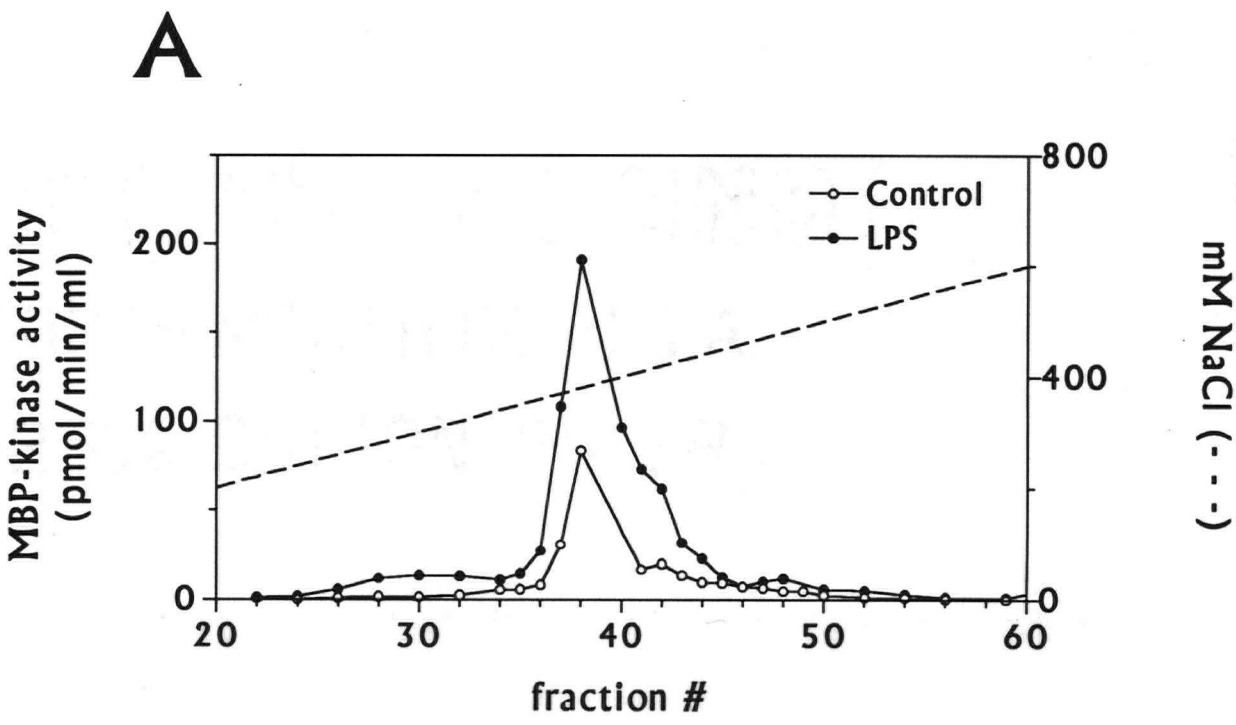
Figure 14. MBP and synthetic MAP kinase peptide substrate reveal distinct substrate specificities of peak one and peak two of MBP kinase activity. After lysis and FPLC fractionation, samples from control (untreated) or LPS (100 ng/ml, 30 min) treated PBMϕs were assayed for kinase activity using (A) myelin basic protein as described for Figure 12, or (B) the synthetic peptide (APRTPGGRR) as phosphate acceptors. Mono Q peak one was comprised of fractions 27-34 and peak two of fractions 35-42. For ease of reference between A and B, fractions 31 (Peak one) and 36 (Peak two) are labeled in both panels (arrows) (n=2).

4. Lipopolysaccharide-induced activation of protein kinase C-ζ in monocytes

Fractions from Mono Q peaks one and two were also subjected to immunoblotting with anti-pan PKC antibodies. A prominent immunoreactive band with an approximate size of M_r 81,000 was detected in peak two fractions from Mono Q that contained LPS-stimulated MBP phosphotransferase activity (data not shown). A corresponding protein, with an identical elution profile of subunit size M_r 80,000 was also detected in peak two fractions prepared from extracts of untreated cells. In contrast no immunoreactive PKC was detected in Mono Q peak one fractions obtained from LPS-treated cells. Based upon size, this protein was believed likely to represent a PKC and indicated the possibility that the LPS-activated MBP kinase activity detected in peak two may be due to PKC.

Compared to lysates prepared from untreated cells, the mean increase in activity of the major MBP-phosphotransferase activity peak was 2.5 ± 0.7 (mean ± S.E.M., n=6) fold. Since this activity (Figure 15A) was detected in the absence of calcium, phospholipids, or diacylglycerol, this indicated that this activity was a lipid-independent isoform of PKC. The possible presence of PKC-ζ was analyzed by immunoblotting fractions with an isoform-specific antibody. Immunoreactivity for PKC-ζ was observed in fractions corresponding to the peak of kinase activity (Figure 15B). The antibody recognized a protein of ~M_r 80,000-85,000 which was sometimes resolved into a doublet or triplet of closely migrating proteins. The specificity of antibody reactivity with this M_r ~80,000 protein complex was confirmed by peptide competition. Thus, as shown in Figure 15B, an excess of the PKC-ζ peptide used as immunogen to raise the antibody specifically abrogated recognition of these bands, whereas non-specific reactivity with other proteins was unaffected.

The effects of LPS on PKC-ζ were also examined in two human, promonocytic cell lines, THP-1 and U937. After Mono Q chromatography the MBP kinase activities exhibited similar elution profiles than that observed in peripheral blood monocytes, and immunoreactivity for PKC-ζ corresponded with peak 2 of activity (Figure 16). Immunoprecipitation of PKC-ζ from THP-1 cells showed increased activity towards MBP in the lysates from LPS treated samples (1 μg/ml) as compared to untreated cells (Figure 16C). LPS treatment resulted in a transient increase in kinase activity which was maximal by 10 min.


A
MBP-kinase activity
(pmol/min/ml)
200
100
0
Control
LPS
800
400
0
mM NaCl (- - -)
20
30
40
50
60
fraction #

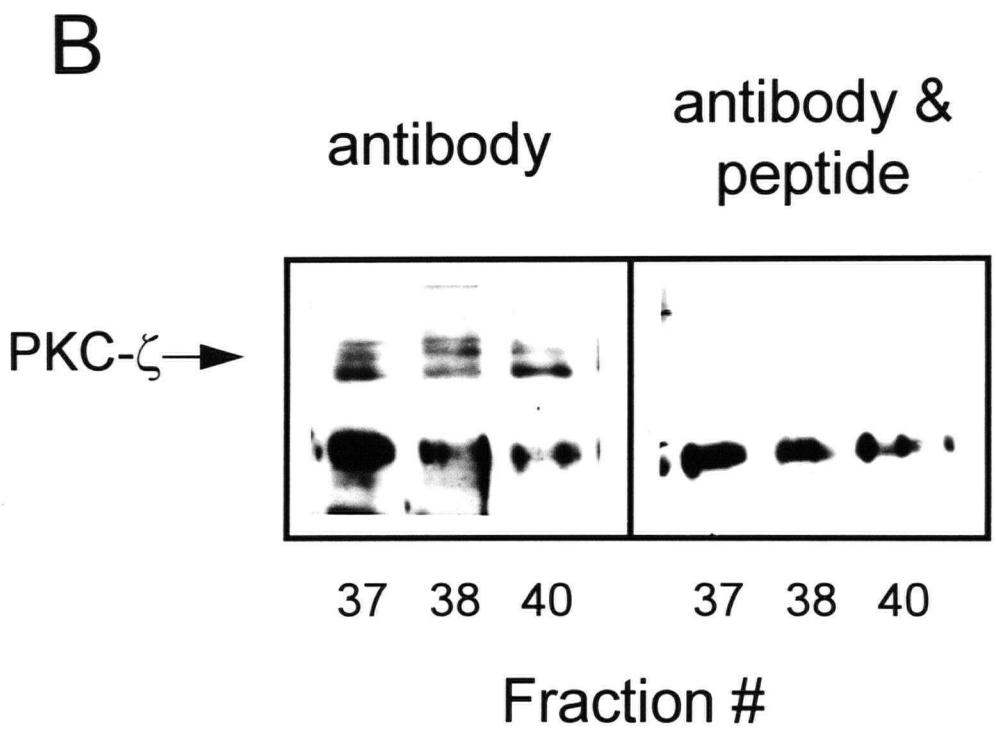
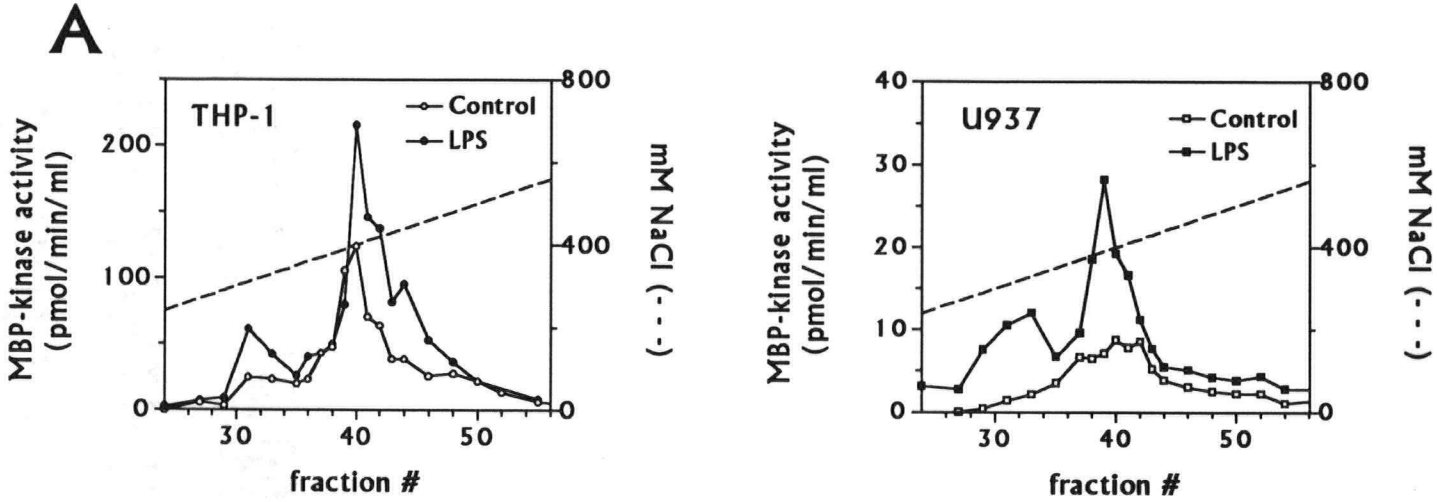
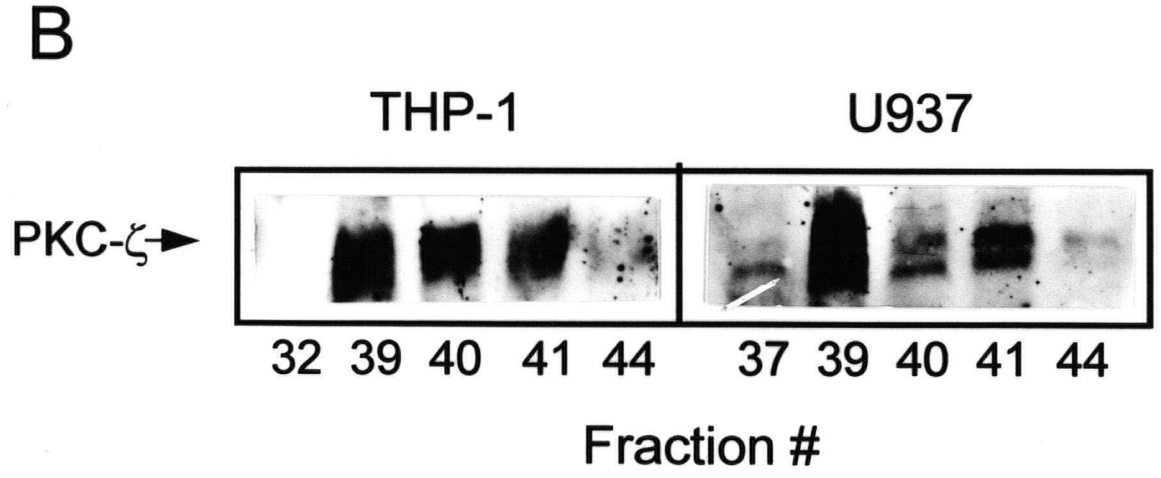

B
antibody
antibody &
peptide
PKC-ζ
37 38 40
37 38 40
Fraction #

Figure 15. LPS induces activation of PKC-ζ. (A) Human monocytes were incubated with LPS (100 ng/ml) or medium alone (final serum concentration, 0.1%) for 15 min. Cells were then and fractionated by Mono Q anion exchange chromatography and fractions were assayed for MBP kinase activity as described in Materials and Methods and in Figure 13. The data shown are from one experiment and are representative of results obtained in >7 independent experiments. (B) Detection of PKC-ζ immunoreactivity in fractions corresponding to the major peak eluting from the column at 390-420 mM NaCl. Aliquots of the fractions (125 μl) were analyzed for the presence of PKC-ζ by immunoblotting with anti-PKC-ζ antibody. Specific recognition of the Mr 80,000-85,000 protein by anti-PKC-ζ antibody was determined by immunoblotting in the absence or presence of peptide used to raise the antibody. Bands that were eliminated by preadsorbing the antibody with the competing peptide were deemed specific. The results shown are from one of three similar experiments.


A
THP-1
Control
LPS
MBP-kinase activity (pmol/min/ml)
mM NaCl (- - -)
fraction #
U937
Control
LPS
MBP-kinase activity (pmol/min/ml)
mM NaCl (- - -)
fraction #


B
THP-1
U937
PKC-ζ
32 39 40 41 44
37 39 40 41 44
Fraction #

C

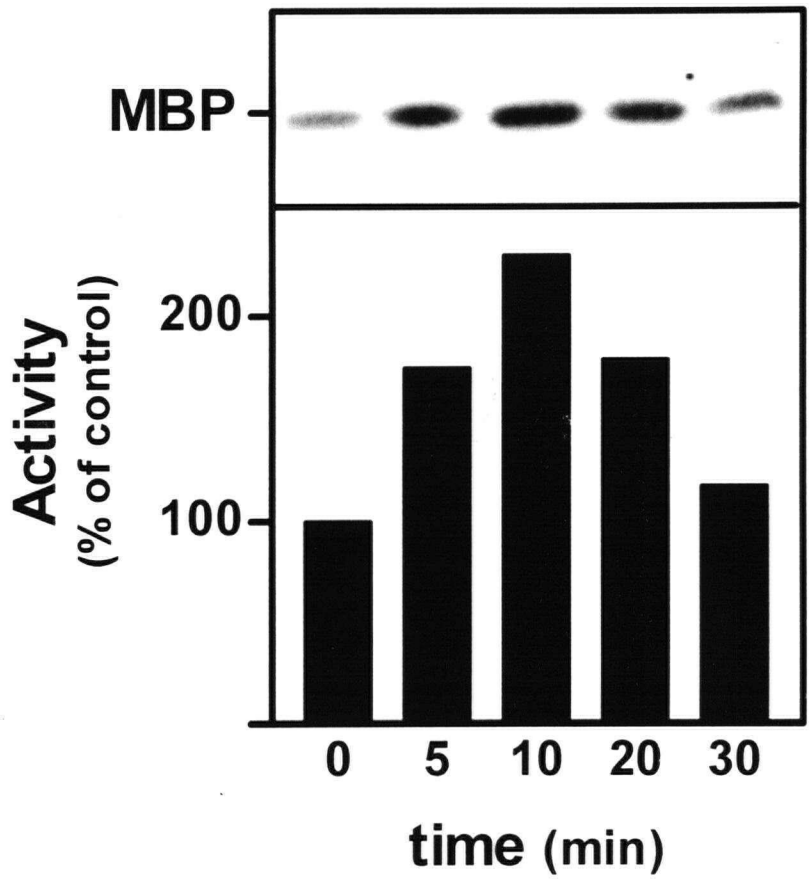
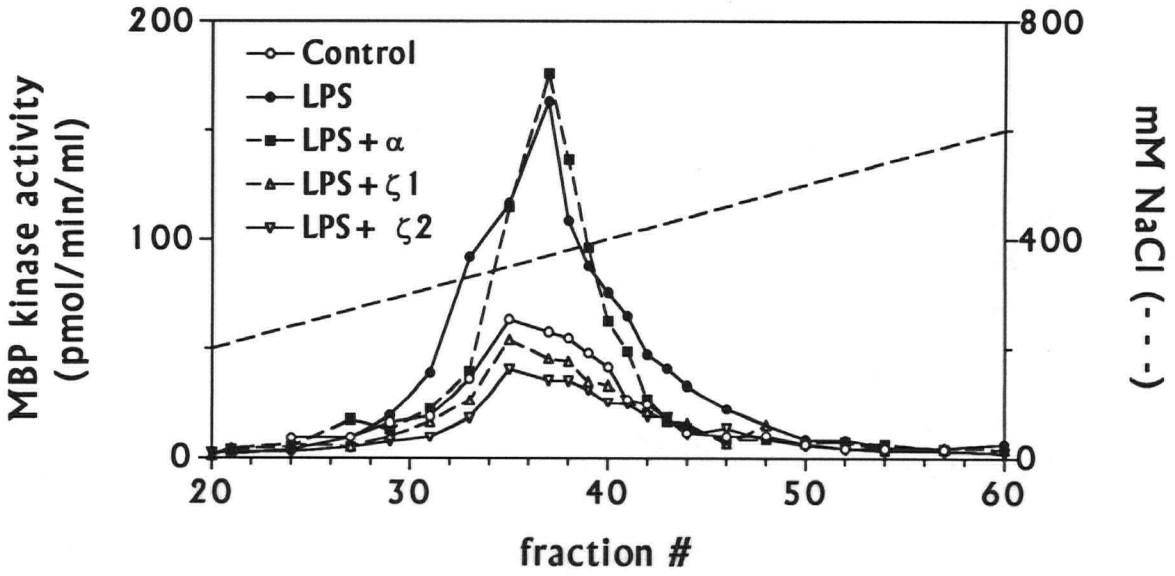


Figure 16. LPS induces activation of PKC-ζ activity in promonocytic cell lines. THP-1 or U937 cells were rendered quiescent in serum-free RPMI for 12 to 15 h and subsequently stimulated (15 min) with 1 μg/ml of LPS or medium as described in Materials and Methods. (A) Cells were lysed and subjected to Mono Q chromatography. MBP kinase activity in aliquots (5 μl) of each fraction from each cell line was determined as described in the legend to Figure 10. (B) Detection of PKC-ζ immunoreactivity in fractions corresponding to the major peaks was done as described in the legend to Figure 14 (n=3). (C) THP-1 cells were treated with LPS for the indicated times and PKC-ζ was immunoprecipitated from cell lysates with polyclonal anti-PKC-ζ antibodies (Upstate Biotechnology). *In vitro* kinase activities in the immunoprecipitates were measured using MBP as substrate.

5. Immunoadsorption of PKC-ζ activity

Immunodepletion was used to examine further whether the peak of MBP kinase activity was due to PKC–ζ. Prior to fractionation on Mono Q, lysates prepared from LPS-treated U937 cells were either untreated or immunoadsorbed with either of two anti-PKC-ζ antibodies. Anti-PKC antibodies able to recognize α, β, and γ PKC isoforms were used as a control for specificity. As shown in Figure 17A, immunoadsorption of lysates with anti-PKC-ζ antibodies resulted in a significant reduction in peak activity when compared to fractions from either unadsorbed lysates or to lysates immunoadsorbed with control anti-PKC antibodies. In fact, peak MBP kinase activity was reduced by treatment with anti-PKC-ζ to a level essentially equivalent to that observed in fractions from control (non-LPS-treated) cells. Immunoadsorption of MBP kinase activity also resulted in removal of PKC-ζ immunoreactivity from Mono Q fractions (Figure 17B) and this was observed with anti-PKC-ζ antibodies from two different sources. When either anti-cPKC (left panel, Figure 17B) or anti PKC-ζ antibodies were used for immunodepletion, a non-specific high molecular weight band was immunoprecipitated.

A


200
100
0
800
400
0
Control
LPS
LPS + α
LPS + ζ1
LPS + ζ2
MBP kinase activity (pmol/min/ml)
mM NaCl (- - -)
20
30
40
50
60
fraction #

B

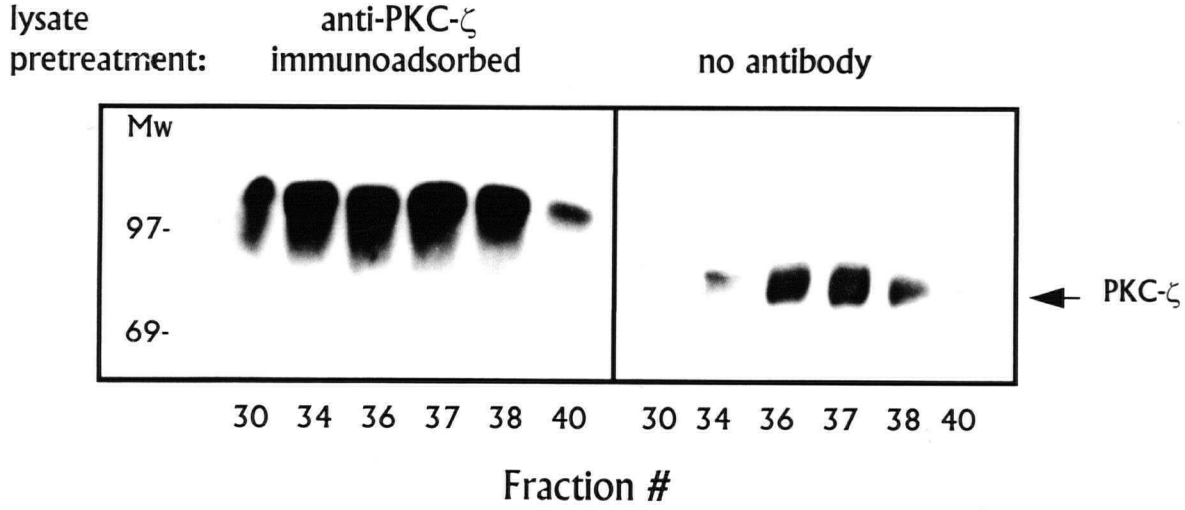

lysate pretreatment:
anti-PKC-ζ immunoadsorbed
no antibody
Mw
97-
69-
PKC-ζ
30 34 36 37 38 40
30 34 36 37 38 40
Fraction #

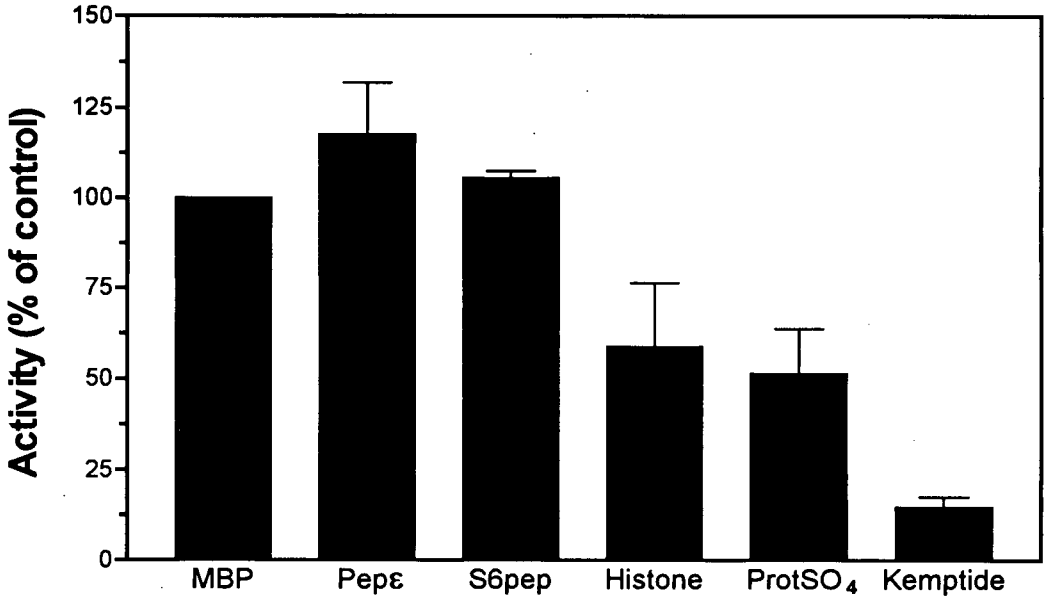
Figure 17. Immunoadsorption of the major peak of MBP kinase activity with anti-PKC-ζ antibodies. U937 cells were either stimulated with 1 μg/ml of LPS or medium alone (final serum concentration, 0.1%) and detergent-soluble lysates were prepared for immunoadsorption as described in Material and Methods. Lysates from medium control and LPS-stimulated cells were either untreated or subjected to immunoadsorption with either rabbit anti-PKC-ζ antibodies (ζ1 and ζ2, Gibco or Santa Cruz) or anti-cPKC antibodies (specificity control). (A) One mg of lysate from each sample was then fractionated by Mono Q chromatography and MBP kinase activities were measured as described in Materials and Methods. The data shown are from one of three independent experiments that gave similar results. (B) Aliquots of fractions from LPS-treated cells analyzed by immunoblotting with anti-PKC-ζ to confirm depletion of PKC-ζ.

6. Biochemical characterization of PKC-ζ

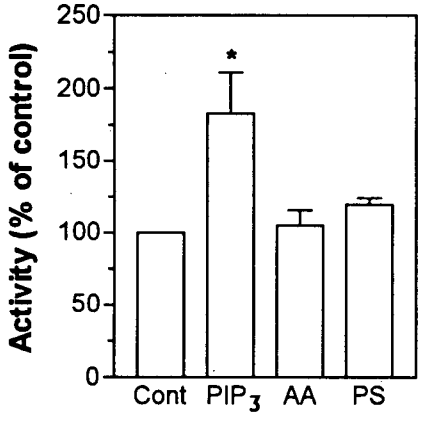
Since PKC-ζ is known to behave differently from other PKC family members, experiments were done to biochemically characterize the putative PKC-ζ in Mono Q peak two. Aliquots of Mono Q fractions containing PKC-ζ were analyzed for activity using multiple substrates as shown in Figure 18A. Of the different substrates tested, the kinase phosphorylated MBP (0.2 mg/ml), peptide ε (84 μM), and S6 peptide (0.1 mM) with similar efficiencies. In comparison, activities towards kemptide (0.2 mg/ml), histone (0.2 mg/ml), and protamine sulfate (0.2 mg/ml) were lower. This profile of substrate preferences is consistent with previous reports for PKC-ζ (109,118).

Cofactor requirements for PKC-ζ have also been found to differ from those of other PKC isoforms (109,154). Figure 18B shows the activity of the kinase toward MBP in the presence or absence of various PKC activators and cofactors. PtdIns3,4,5P_3, an activator of PKC-ζ, enhanced the activity of the kinase in Mono Q fractions prepared from control cells (Figure 18B), but not in those prepared from LPS-treated cells. Unlike PtdIns3,4,5P_3, neither arachidonic acid (alone or with diacylglycerol) nor phosphatidylserine significantly enhanced the activity of the kinase when compared to the activity detected in the absence of added lipids (Figure 18B). PMA, a known activator of several PKC isoforms other than PKC-ζ was also tested in the presence of PS and Ca^{2+}. The addition of PS, Ca^{2+}, and PMA together, resulted in an approximate 35% increase in activity when compared to activity in the absence of cofactors (Figure 18C). In contrast, fractions corresponding to cPKC (i.e. 15-16) showed a robust activation in response to the combination of PMA, PS, and Ca^{2+}.

A


Activity (% of control)
150
125
100
75
50
25
0
MBP
Pepε
S6pep
Histone
ProtSO4
Kemptide

B


Activity (% of control)
250
200
150
100
50
0
*
Cont
PIP3
AA
PS

C

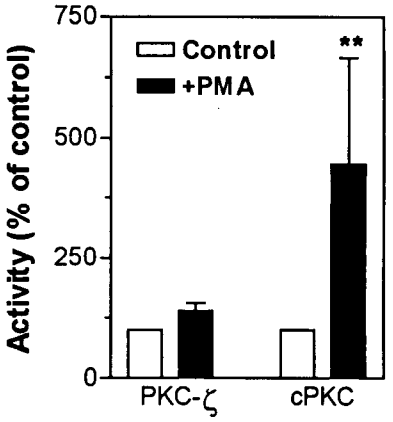

Activity (% of control)
750
500
250
0
Control
+PMA
**
PKC-ζ
cPKC

Figure 18. Substrate preference and cofactor dependency of monocyte PKC-ζ. Detergent lysates of human monocytes were prepared and fractionated by Mono Q chromatography. (A) Kinase activities were determined in peak 2 fractions (5 μl) using various substrates in the absence of cofactors. MBP, protamine sulfate, histone, and kemptide were used at 0.2 mg/ml. Peptide ε was used at 84 μM and S6 peptide at 100 μM. Activity is referred to MBP (100 5). (B) Kinase activity was measured in the absence (Cont) or presence of various cofactors (PtdIns3,4,5P_3, 0.1 μM; AA, 33 μM; PS, 12.5 μM) using MBP as substrate. (C) Peak kinase activity was determined in the fractions corresponding to both PKC-ζ and cPKC (fractions 15-16) in the presence of 50 nM PMA, 100 μM free Ca^{2+}, and 25 μM PS or absence of cofactors (Control). Results are expressed as percent of activity measured in the absence of any factors which was taken as 100%. The values shown are the mean ± s.e.m. of 3-4 independent experiments. *p=0.04 (control vs. PtdInsP_3); **p=0.04 (control vs. PMA), both by Student's t test.

C. PHOSPHATIDYLINOSITOL 3-KINASE ACTIVATION IN LPS-TREATED MΦS

1. Detection of PtdIns3,4,5P$_3$ in vivo in LPS-treated cells

Activation of PI 3-kinase results in rapid and transient increases in the production of D3-phosphorylated phosphoinositides. The effects of LPS treatment on the levels of these metabolites were examined in peripheral blood Mφs as well as in the monocytic cell line U937. Cells were labeled with ^{32}P-orthophosphate and were then incubated in the presence or absence of LPS. Phospholipids were extracted and analyzed by thin layer chromatography. As shown in Figure 19, when compared to control cells, the incorporation of ^{32}P into PtdIns3,4,5P$_3$ was increased in LPS treated monocytes. This effect was relatively rapid and transient with maximal accumulation of PtdIns3,4,5P$_3$ observed between 10 min and 15 min. Using cells from different donors, the magnitude of this increase ranged between 2- to 11-fold relative to control cells.

2. HPLC analysis of PI 3-kinase metabolites in U937 cells

When normal human Mφs were labeled with ^{32}P-orthophosphate and lipids were extracted for HPLC analysis it was found that the incorporation of radioactive material into the least abundant inositol phospholipids was not high enough to detect in this system (data not shown). This appeared to be due to relatively low specific activity of the monocyte ATP pool. In contrast, the ATP pool in the human promonocytic cell line U937 labeled to higher specific activity and was used, therefore, to study the effects of LPS on PI 3-kinase metabolites *in vivo*. As shown in Figure 20 and Table 2, treatment of cells with 100 ng/ml LPS brought about increased levels of the PI 3-kinase metabolites PtdIns3,4P$_2$, and PtdIns3,4,5P$_3$. LPS treatment also resulted in increased levels of PtdIns4P, and PtdIns4,5P$_2$. These effects were observed as early as 5 min after addition of LPS and were persistent through 10 min (data not shown) of treatment.

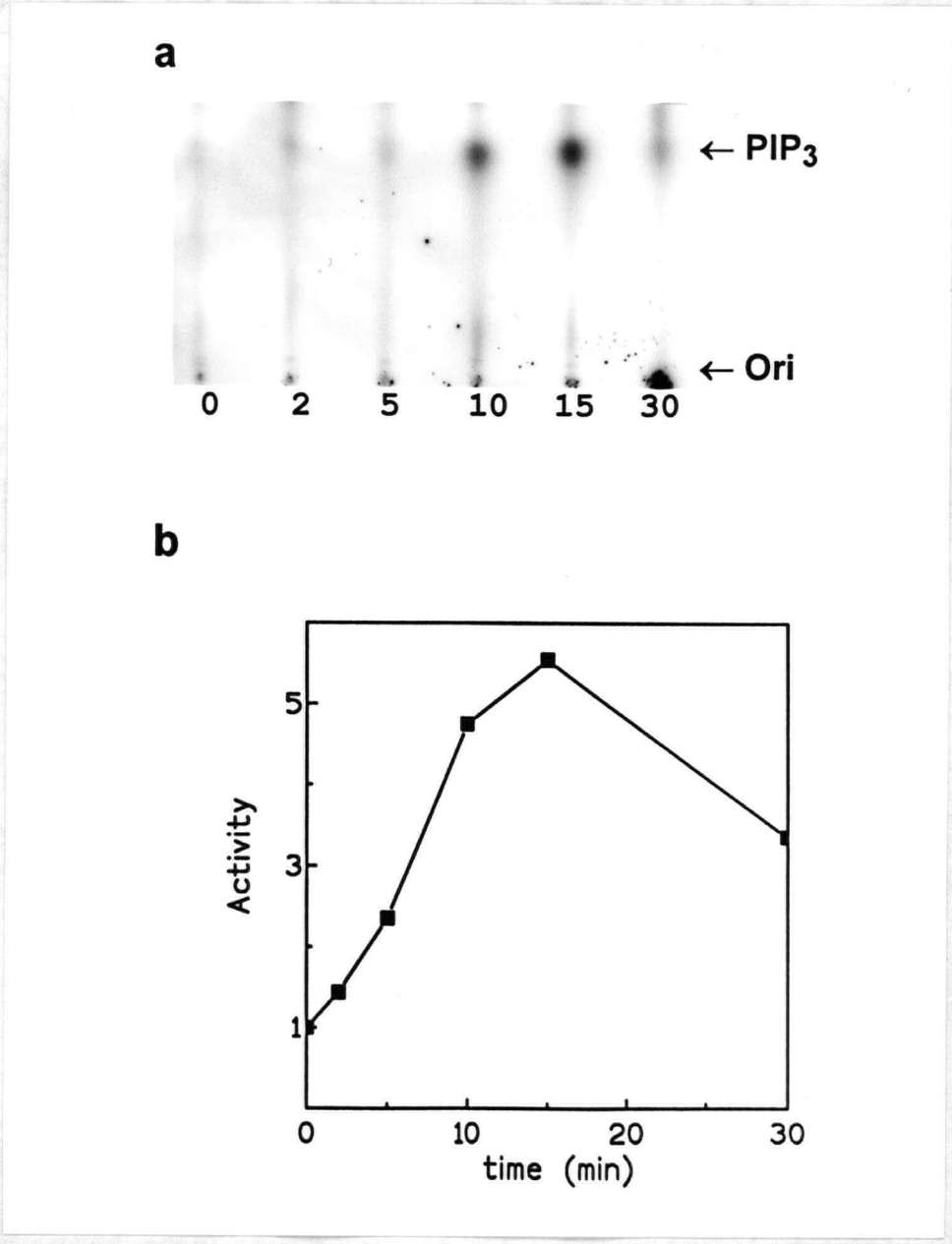


Figure 19. PtdIns3,4,5P_3 levels in Mϕs after LPS treatment. (a) Human Mϕs were labeled with [$^{32}P_i$] for 2 h before stimulation with LPS (100 ng/ml) for the indicated periods. Lipids were extracted and separated by TLC using a solvent system of n-propanol:2 M acetic acid (13.7:7, v/v) as described in Materials and Methods. (b) Spots corresponding to PtdIns3,4,5P_3 were excised and analyzed by liquid scintillation counting. The data shown are from one of three independent experiments.

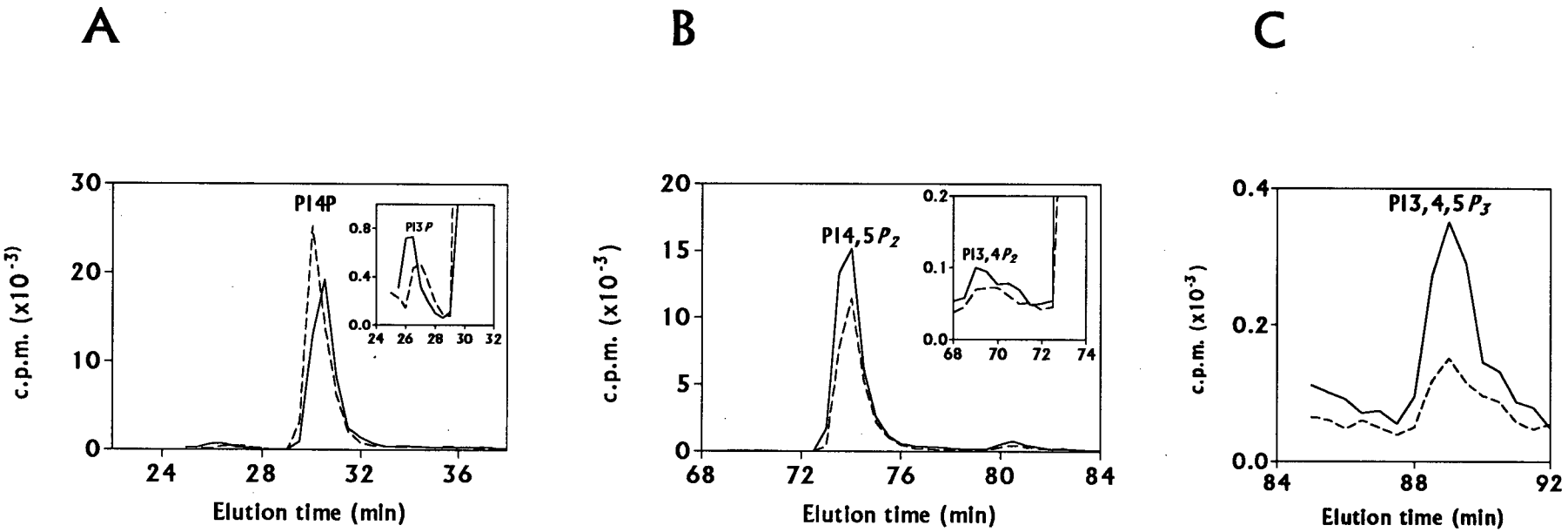


Figure 20. HPLC analysis of deacylated inositol phospholipids from U937 cells. [$^{32}P_i$]-labeled lipids were extracted from control (dashed lines), and LPS (100 ng/ml) treated cells (5 min, solid lines), deacylated and applied to a Partisil 10 SAX column. The deacylated products were identified by comparing their elution times with those of different standards as described in Materials and Methods. Elution profiles of monophosphorylated (A), bisphosphorylated (B), and trisphosphorylated phosphoinositides (C).

Table 2. Effects of LPS on inositol phospholipids in U937 cells. [^{32}P]-labeled phosphoinositides were extracted from control cells and cells treated with LPS for 5 min (100 ng/ml) as described under Materials and Methods and Figure 20. The data shown are the ratios of values observed in LPS-treated versus control, untreated cells (mean ± S.E.M., n=3).

	LPS 5 min
PtdIns3P *	1.24 ± 0.07
PtdIns4P	1.16 ± 0.1
PtdIns3,4P_2	1.73 ± 0.32
PtdIns4,5P_2	1.39 ± 0.16
PtdIns3,4,5P_3**	2.02 ± 0.19

*p = 0.018 vs. control; **p = 0.014 vs. control

3. Wortmannin inhibits the generation of PtdIns3,4,5 P_3 in vivo

Increased accumulation of PtdIns3,4,5P_3 in LPS-treated cells was likely related to increased specific activity of PI 3-kinase *in vivo* and this was supported by experiments (described below) in which PI 3-kinase activity was examined directly *in vitro* in cell lysates. Nevertheless, LPS-induced inhibition of a phosphatase that acts on PtdIns3,4,5P_3 could also have contributed to increased detection of this metabolite. To examine this possibility, $^{32}P_i$-labeled cells were pretreated for 10 min with 100 nM wortmannin, a concentration considered

to have relative specificity for PI 3-kinase (114). Cells were then either left untreated or incubated with LPS. As shown in Figure 21, pretreatment of monocytes with wortmannin completely abrogated the LPS-induced increase in PtdIns3,4,5P_3. Taken together, these findings provide direct evidence for activation of PI 3-kinase *in vivo* by LPS.

4. LPS-induced activation of PI 3-kinase is CD14-dependent

Many functional responses elicited by LPS in Mϕs are mediated through the cell surface receptor CD14 (29) and antibodies to CD14 are able to block a variety of LPS-induced responses (80). Incubation of $^{32}P_i$ labeled cells with 3C10 (10 μg/ml for 30 min prior to LPS treatment), a monoclonal antibody that blocks CD14, completely abrogated the LPS-induced increase in PtdIns3,4,5P_3 (Figure 22, lane 4). This effect was specific to CD14, since the expected increase in PtdIns3,4,5P_3 in LPS-treated cells was not prevented by pretreatment with an irrelevant isotype (IgG_{2b}) matched monoclonal antibody, OKT4 (Figure 22, lane 6).

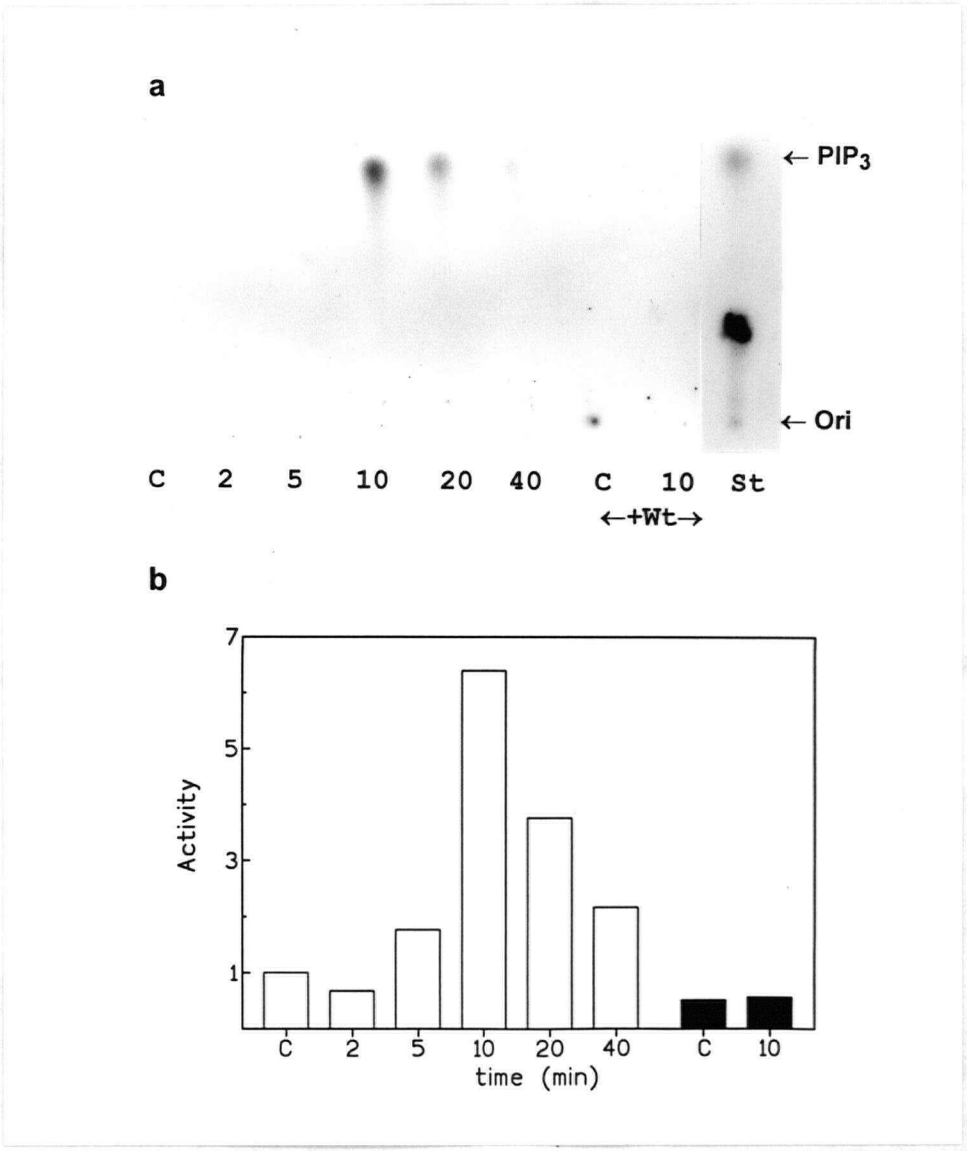


Figure 21. Effects of wortmannin on PtdIns3,4,5P_3 levels. Mϕs were labeled with [$^{32}P_i$] for 2 h as described and treated with 100 nM wortmannin or vehicle (Me_2SO) for 10 min. Cells were then treated with 100 ng/ml LPS for the indicated times. (a) Lipids were extracted and analyzed by TLC as indicated in Figure 18. The position of PtdIns3,4,5P_3 prepared *in vitro* and separated under the same conditions is shown (St). The signal in this lane migrating between the origin and PIP_3 corresponds to [γ-^{32}P]ATP from the *in vitro* reaction. (b) Spots corresponding to PtdIns3,4,5P_3 were excised and quantified by scintillation counting. Activity is expressed as a ratio compared to control samples. Data shown are from one of three independent experiments.

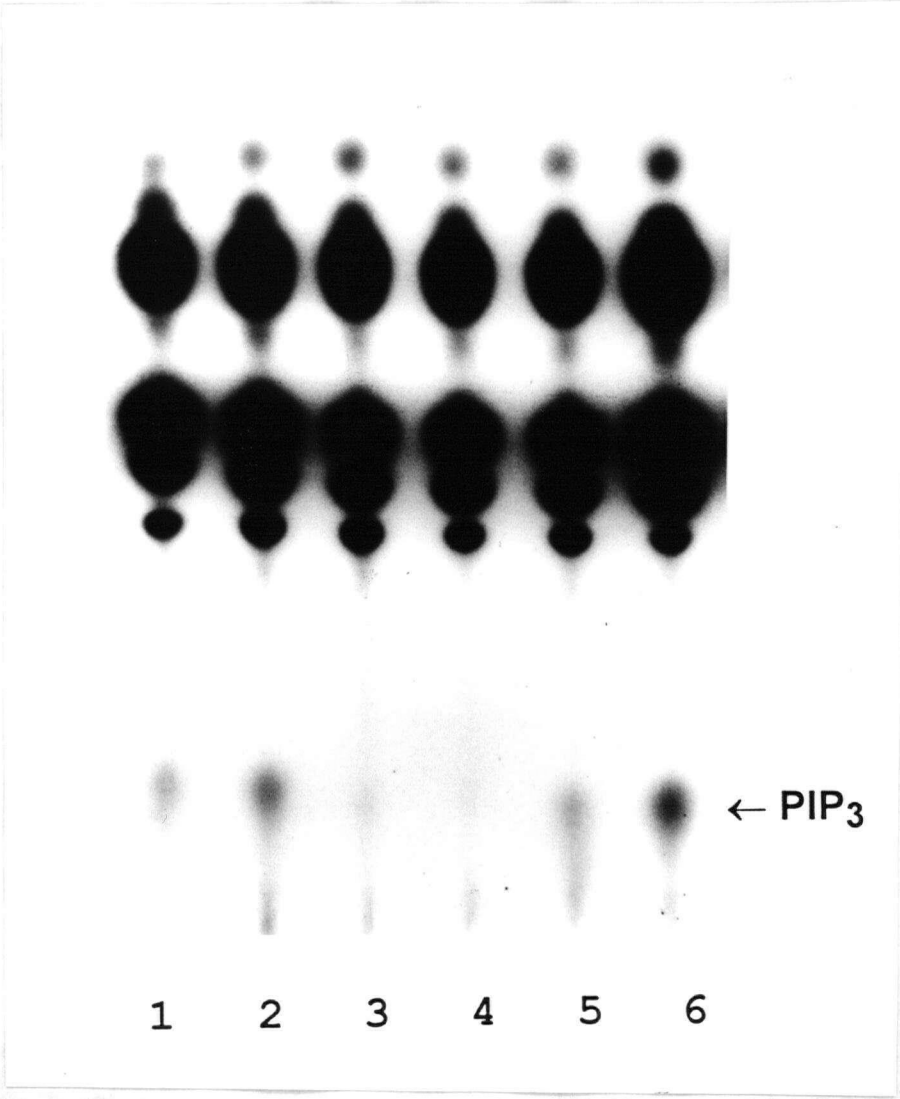


Figure 22. CD14-dependence of enhanced PtdIns3,4,5P_3 levels in LPS-treated human monocytes. Mϕs were labeled with [$^{32}P_i$] for 2 h and left untreated (lanes 1 and 2), or were incubated with 10 μg/ml 3C10 (lanes 3 and 4) or an irrelevant isotype matched control antibody (OKT4, IgG_{2b}) (lanes 5 and 6) for 30 min before treatment with 100 ng/ml LPS (lanes 2, 4, and 6) or vehicle (lanes 1, 3, and 5) for 10 min. Lipids were extracted and analyzed as described above (n=3).

5. Kinetic analysis of PI 3-kinase activity in LPS-treated monocytes

Treatment of monocytes with LPS brought about a substantial increase in PI 3-kinase activity assayed *in vitro* (Figure 23). Cell lysates were immunoprecipitated with a monoclonal antibody specific for PI 3-kinase and enzyme activity was measured in an *in vitro* kinase assay using PI as substrate. Increased PI 3-kinase activity in samples from LPS treated cells was detected as early as 2 min and was consistently maximal after 10 min of treatment (range, 2.5-5.7 fold greater than control samples using cells from five different donors). PI 3-kinase activity in LPS-treated cells returned to near basal levels by 40 min.

6. LPS dose-response analysis for activation of monocyte PI 3-kinase

Activation of monocyte PI 3-kinase was dose dependent and was detected with LPS concentrations as low as 10 pg/ml. Maximal activation was achieved at 1-10 ng/ml of LPS (Figure 24A). Immunoprecipitation of PI 3-kinase from cell lysates yielded comparable amounts of the M_r 85,000 regulatory subunit from both control and LPS-treated cells as detected by immunoblotting with a polyclonal antibody against p85 (Figure 24B). This result indicates that increased enzyme activity detected in samples from LPS-treated cells was not an artifact related to preferential immunoprecipitation of PI 3-kinase protein from these samples. Moreover, this finding provides direct evidence that LPS treatment brings about an increase in the specific activity of monocyte PI 3-kinase.

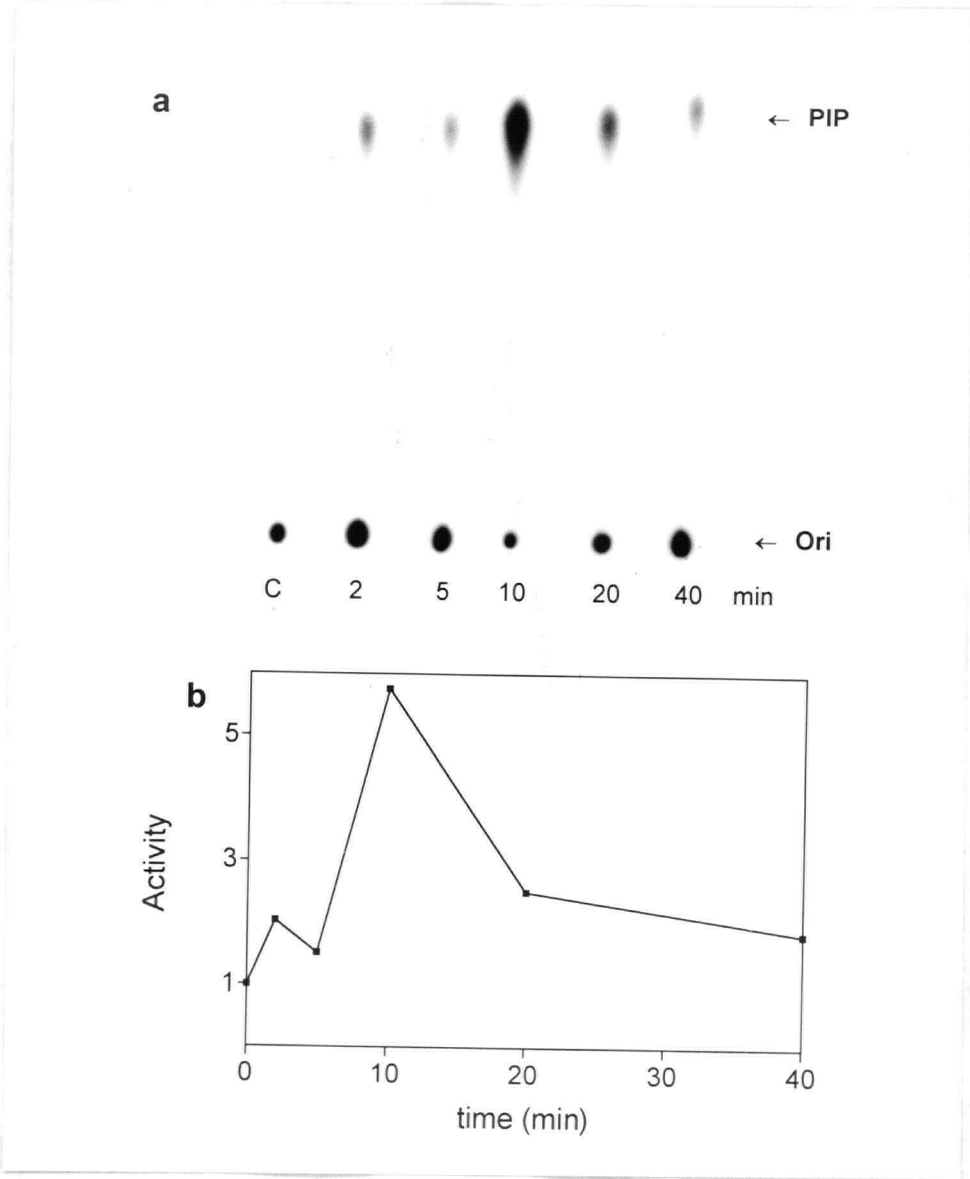


Figure 23. Time course of PI 3-kinase activity in LPS-treated cells. Monocytes were incubated for the indicated times with LPS (100 ng/ml). Equivalent amounts of cell lysates were immunoprecipitated with monoclonal anti-PI 3-kinase antibodies. (a) Immune complexes were assayed for PI 3-kinase activity by measuring the incorporation of [γ-^{32}P]ATP into PtdIns. The position of the migration of PtdInsP (PIP) and the origin of chromatography are indicated. (b) Spots corresponding to PtdInsP were excised and analyzed by liquid scintillation counting and the values obtained are expressed relative to control (480 cpm). Shown is one of five similar experiments.

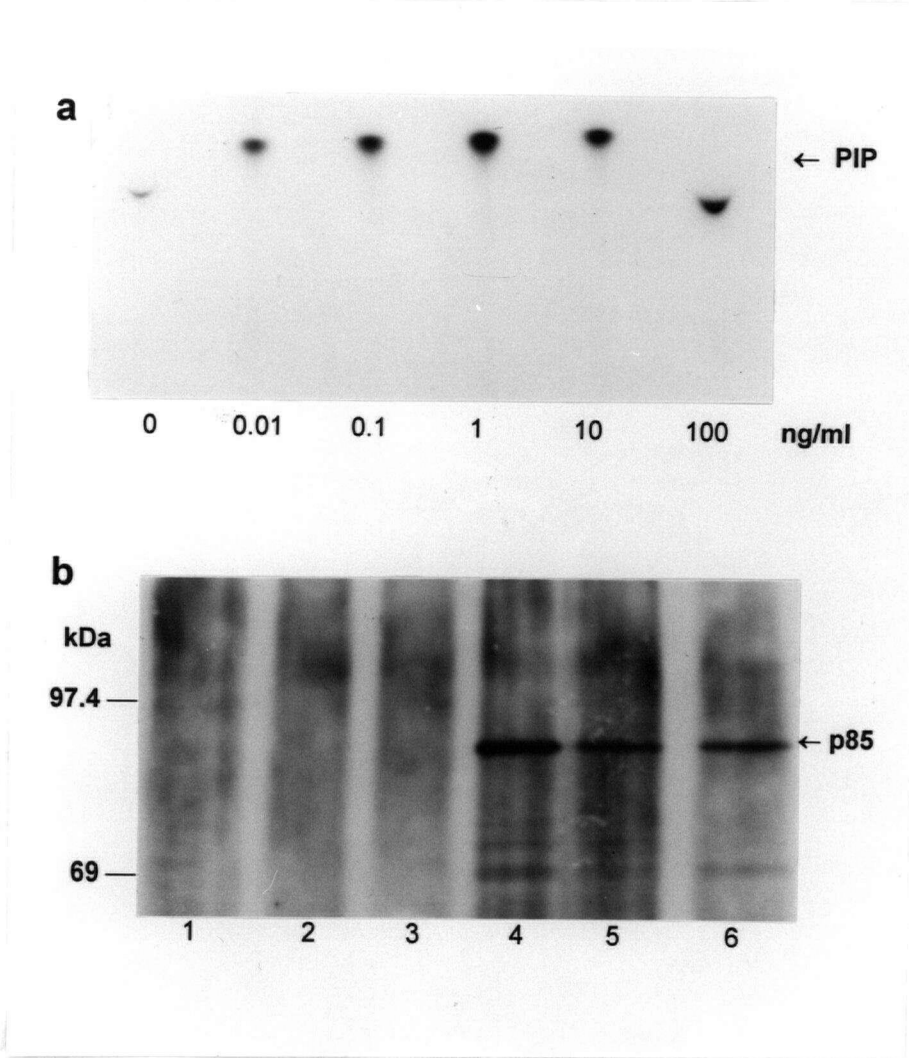


Figure 24. Dose dependence of PI 3-kinase activation in response to LPS. (a) Monocytes were incubated for 10 min with the indicated concentrations of LPS and assayed for PI 3-kinase activity as in Figure 23. (b) Lysates corresponding to control (lanes 1 and 4), or LPS treated cells (1 ng/ml LPS: lanes 2 and 5; 100 ng/ml: lanes 3 and 6) were immunoprecipitated with normal mouse serum (lanes 1-3) or monoclonal anti PI 3-kinase antibody (lanes 4-6). Immunoprecipitated material was separated by polyacrylamide gel electrophoresis and immunoblotted with rabbit anti-PI 3-kinase. The positions of p85 and molecular weight markers are indicated. Data are representative of three experiments.

7. LPS treatment activates p53/56^{lyn} and promotes its transient association with PI 3-kinase

As reported above, LPS brings about the activation of several tyrosine kinases, among them p53/56^{lyn}. It has been observed that PI 3-kinase may associate with and be activated by a variety of both receptor and non-receptor tyrosine kinases (86). Taken together, these findings indicated the possibility that activation of PI 3-kinase in LPS-treated Mϕs might involve its association with activated p53/56^{lyn}. In fact, when lysates from anti-lyn immunoprecipitates were examined in the *in vitro* PI 3-kinase assay, increased enzymatic activity was detected in samples prepared from LPS-treated cells (Figure 25). The association between p53/56^{lyn} and PI 3-kinase exhibited a time course similar to that of PI 3-kinase activation *per se* (e.g. see Figure 23). Maximal activity was observed after 5-10 min of LPS treatment and by 40 min evidence of association was no longer present. When anti-lyn immunoprecipitates were subjected to immunoblotting using an anti-PI 3-kinase rabbit serum a band corresponding to the M_r 85,000 regulatory subunit was not detected (data not shown). This indicated that the amount of enzyme that associated with p53/56^{lyn} was only a small percentage of the total PI 3-kinase present in the cell and this was below the sensitivity of the detection system. As discussed above, when p53/56^{lyn} immunoprecipitates were examined for Lyn phosphotransferase activity in an immune complex kinase assay, the enzyme was found to be more active in LPS treated samples when compared to control samples (Figures 10 and 11). The kinetics of Lyn activation showed that this response was maximal at 10 min and this coincided with its maximal association with PI 3-kinase and with PI 3-kinase activation *per se* (Figure 25). Taken together, these results indicate that LPS is able to bring about the

coordinate activation of p53/56^{lyn} and PI 3-kinase and that these enzymes become transiently associated for a period of time that corresponds to the duration of PI 3-kinase activation.

Further evidence to suggest that activation of PI 3-kinase is dependent upon its association with activated p53/56^{lyn} is provided by the finding that inhibition of Lyn activity with the tyrosine kinase inhibitor herbimycin A abrogates activation of PI 3-kinase (Figure 26). Addition of herbimycin to PI 3-kinase immunoprecipitates prepared from LPS-treated cells immediately before assay did not affect the lipid-kinase activity, indicating that the effect of herbimycin on intact cells was unlikely related to a direct effect on PI 3-kinase.

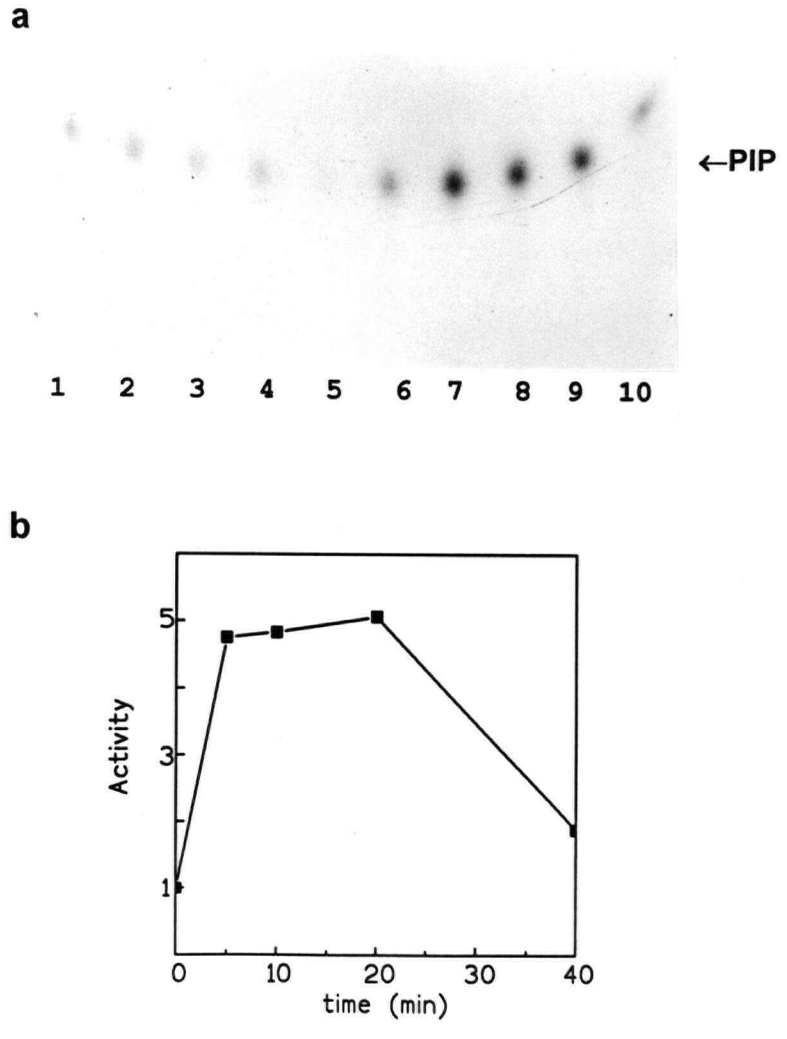


Figure 25. Association of p53/56^{lyn} with PI 3-kinase. Cells were either left untreated (lanes 1 and 6) or treated with 100 ng/ml LPS (lanes 2-5, 7-10) for 5 (lanes 2 and 7), 10 (lanes 3 and 8), 20 (lanes 3 and 9) or 40 min (lanes 5 and 10). (a) Cell lysates were prepared as described and equivalent amounts of protein were used for immunoprecipitation with normal rabbit serum (lanes 1-5) or Lyn antiserum (lanes 6-10). Immunoprecipitates were assayed for PI 3-kinase activity using PtdIns as substrate as described. (b) The autoradiogram was analyzed by densitometry, and the background signals from lanes 1-5 were subtracted individually from the corresponding signals in lanes 6-10. The difference between lane 6 and lane 1 was given an activity value of 1, and the other activity values shown reflect the effects of LPS on PI 3-kinase associated with p*53/56*^{lyn}. Shown is one of three experiments with similar results.

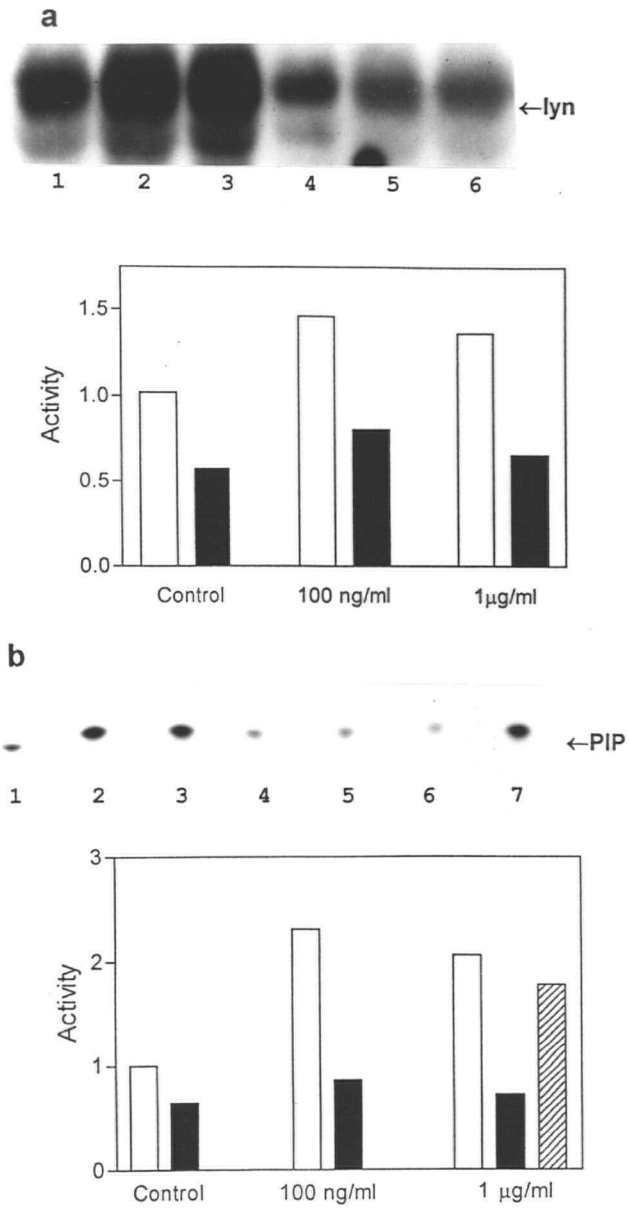

a
←lyn
1
2
3
4
5
6
Activity
1.5
1.0
0.5
0.0
Control
100 ng/ml
1μg/ml
b
←PIP
1
2
3
4
5
6
7
Activity
3
2
1
0
Control
100 ng/ml
1 μg/ml

Figure 26. Inhibition of PI 3-kinase activity by herbimycin A. U937 cells were pretreated with 5 μg/ml of herbimycin (lanes 4-6; solid bars) or with a similar volume of Me_2SO (lanes 1-3, empty bars) for 4 h before any stimulation. Cells were then left untreated (lanes 1 and 4) or were stimulated with either 100 ng/ml (lanes 2 and 5), or 1 μg/ml (lanes 3, 6 and 7) of LPS for 5 min. Cell extracts were prepared and equivalent amounts of protein were used for (a) immunoprecipitation with anti-Lyn antibodies. Immune-complex kinase assays were performed as described in Figure 10. Bands corresponding to lyn were excised, radioactivity was measured by scintillation counting and activity was expressed as compared to control cells. (b) Parallel immunoprecipitations with monoclonal anti-PI 3-kinase antibodies were performed and PI 3-kinase activity was detected as described in Figure 23. Spots corresponding to PtdInsP were excised and analyzed by liquid scintillation counting and expressed relative to control.

8. Tyrosine phosphorylation state of the M_r 85,000 subunit of PI 3-kinase

The M_r 85,000 regulatory subunit of PI 3-kinase is known to become phosphorylated by a variety of tyrosine kinases *in vitro*. Furthermore, PI 3-kinase may be detected in anti-phosphotyrosine immunoprecipitates prepared from different cell types following agonist treatment. Whereas this implicated tyrosine phosphorylation of p85 in enzyme activation, the latter does not strictly correlate with higher levels of tyrosine phosphorylation of the regulatory subunit. To examine whether p85 becomes phosphorylated on tyrosine in response to LPS in Mϕs, anti-PI 3-kinase immunoprecipitates were reprobed with 4G10. No detectable tyrosine phosphorylation of p85 was observed in either control or treated samples (data not shown). This negative result might be explained by the fact that the amount of tyrosine phosphorylated p85 brought down with the anti-PI 3-kinase antibody represented only a minor fraction of the total enzyme in the immunoprecipitates. To maximize the potential for detecting tyrosine phosphorylation of p85, cell lysates were immunoprecipitated with 4G10. As shown in Figure 27a, treatment of monocytes with LPS brought about an increase in the amount of PI 3-kinase activity that could be immunoprecipitated with anti-phosphotyrosine antibody 4G10. When parallel 4G10 immunoprecipitates were immunoblotted with 4G10, several proteins exhibited enhanced phosphorylation in response to LPS (Figure 27b). When this blot was stripped and reprobed with a polyclonal antibody specific for p85 it was found that p85 did not comigrate with any of the tyrosine phosphorylated bands previously detected (Figure 27c). This results indicates that tyrosine phosphorylation of the regulatory subunit of PI 3-kinase is not necessary for its activation in LPS-treated monocytes.

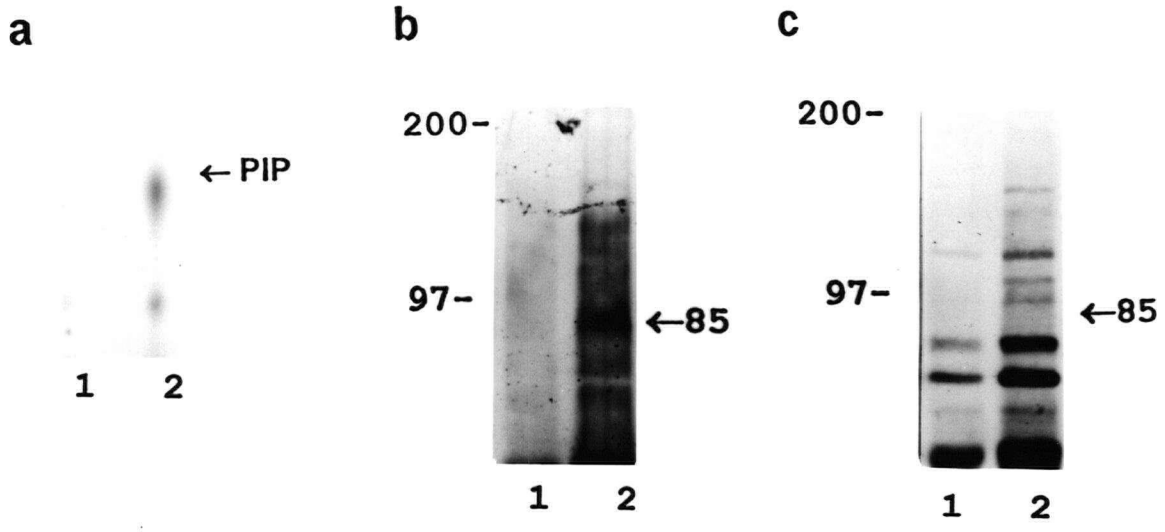


Figure 27. PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates. Human Mϕs were either left untreated (1) or treated with 100 ng/ml LPS for 10 min (2). Cell lysates were prepared and immunoprecipitated with 4G10. (a) Immune complexes were either assayed for PI 3-kinase activity as described previously or were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with (b) polyclonal anti-PI 3-kinase or (c) 4G10. The positions of PtdIns*P*, molecular weight markers and p85 are indicated (n=2).

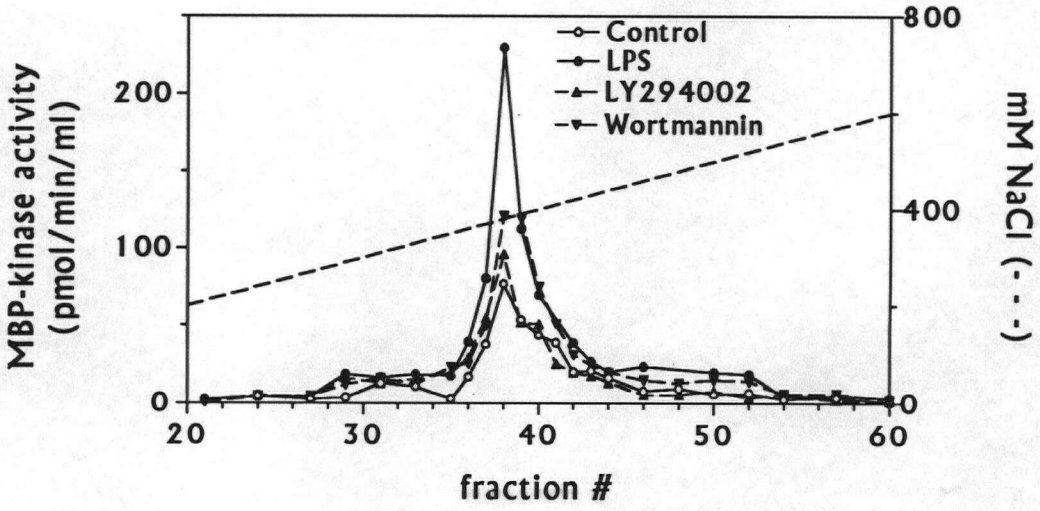
9. Activation of PKC-ζ is phosphatidylinositol 3-kinase-dependent

Recent evidence has implicated a role for PI 3-kinase metabolites in a signaling cascade leading to activation of PKC (124). To examine the potential involvement of PI 3-kinase in LPS-induced activation of PKC-ζ, cells were incubated with the PI 3-kinase inhibitors wortmannin or LY294002, prior to the addition of LPS. As shown in Figure 28A, when used at concentrations known to be relatively selective for inhibition of PI 3-kinase, both inhibitors markedly attenuated activation of PKC-ζ induced by LPS in peripheral blood monocytes. This effect was also observed in PKC-ζ immunoprecipitated from THP-1 cells. Preincubation of the cells with 32 μM LY294002 or 100 nM wortmannin for 30 min resulted in the abrogation of LPS-stimulated PKC-ζ activity (Figure 28B).

The requirement for PI 3-kinase for activation of PKC-ζ was also examined in cells transfected with a dominant negative mutant of p85 (Δp85). The expression of p85 in the different populations of U937 cells was examined (Figure 29). Lysates from non-transfected U937 cells (lane 1), and cells transfected with either Δp85 (lane 2), or with wild-type p85 (lane 3) were separated by PAGE and immunoblotted with anti-p85 antibodies. The results indicate that equivalent amount of p85 are expressed in these cell populations.

Stable transfection with Δp85 resulted in a significant reduction in both basal and LPS-stimulated PI 3-kinase activity (Figures 30a, b). In contrast, cells transfected with wild-type p85 showed the expected increase in PI 3-kinase activity in response to LPS stimulation.
cells and PKC-ζ immunoreactivity correlated with the peak kinase activity.

A


Control
LPS
LY294002
Wortmannin
MBP-kinase activity
(pmol/min/ml)
mM NaCl (- - -)
fraction #
0
100
200
400
800
20
30
40
50
60

B

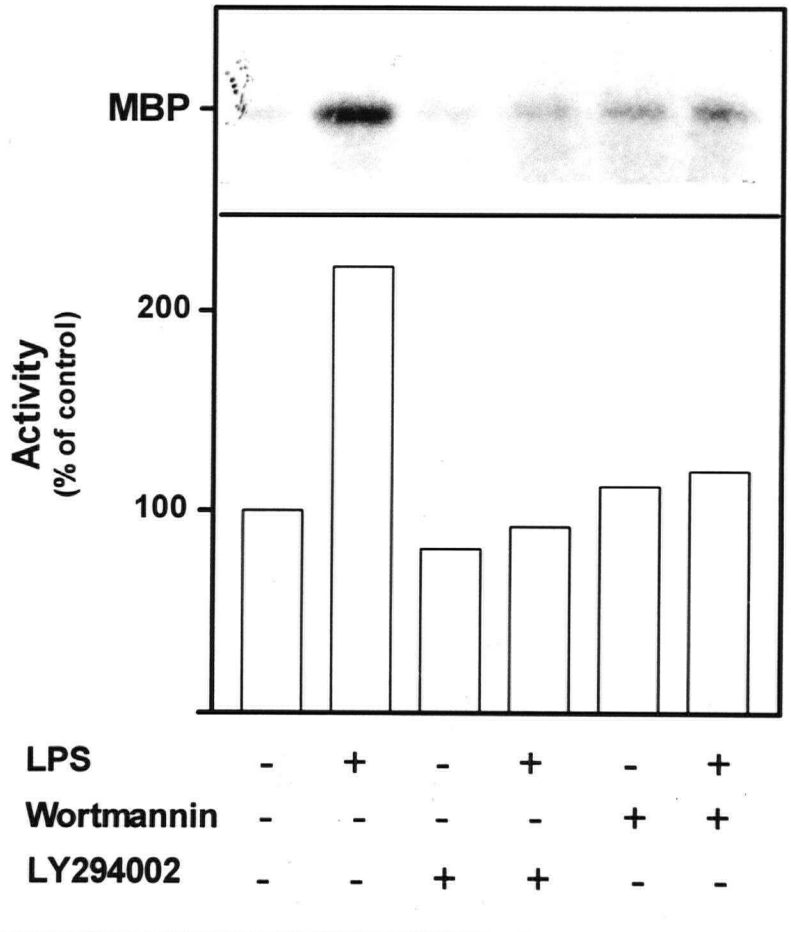

MBP
Activity
(% of control)
100
200
LPS - + - + - +
Wortmannin - - - - + +
LY294002 - - + + - -

Figure 28. Abrogation of LPS-induced activation of PKC-ζ by the PI 3-kinase inhibitors, wortmannin or LY294002. (A) Human monocytes were preincubated with 50 nM wortmannin (n=4), 16 μM LY294002 (n=2), or vehicle for 15 min. Cells were then treated with 100 ng/ml of LPS or medium alone for an additional 15 min. Detergent lysates were prepared and fractionated by Mono Q chromatography. MBP kinase activities were determined in fractions (5 μl) from each cell preparation as described in Figure 11. (B) THP-1 cells were preincubated with either 100 nM wortmannin, 32 μM LY294002 or vehicle for 20 min prior to addition of LPS (1 μg/ml, 10 min). Lysates were immunoprecipitated with anti-PKC-ζ antibodies and MBP-kinase activity was measured in the immunoprecipitates as described in the legend to Figure 15C (n=2).

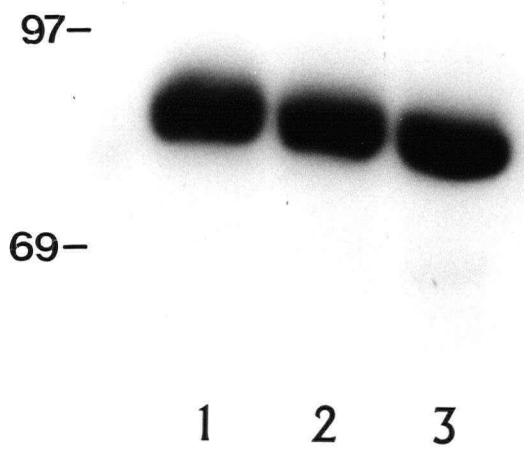


Figure 29. Expression of p85 in U937 cells. Fifty micrograms of whole cell lysates from untransfected (1), Δp85-transfected (2) and Wp85-transfected (3) U937 cells were electrophoresed and immunoblotted with polyclonal antibodies against the p85 subunit of PI 3-kinase. Migration of molecular mass marker proteins are shown in kDa.

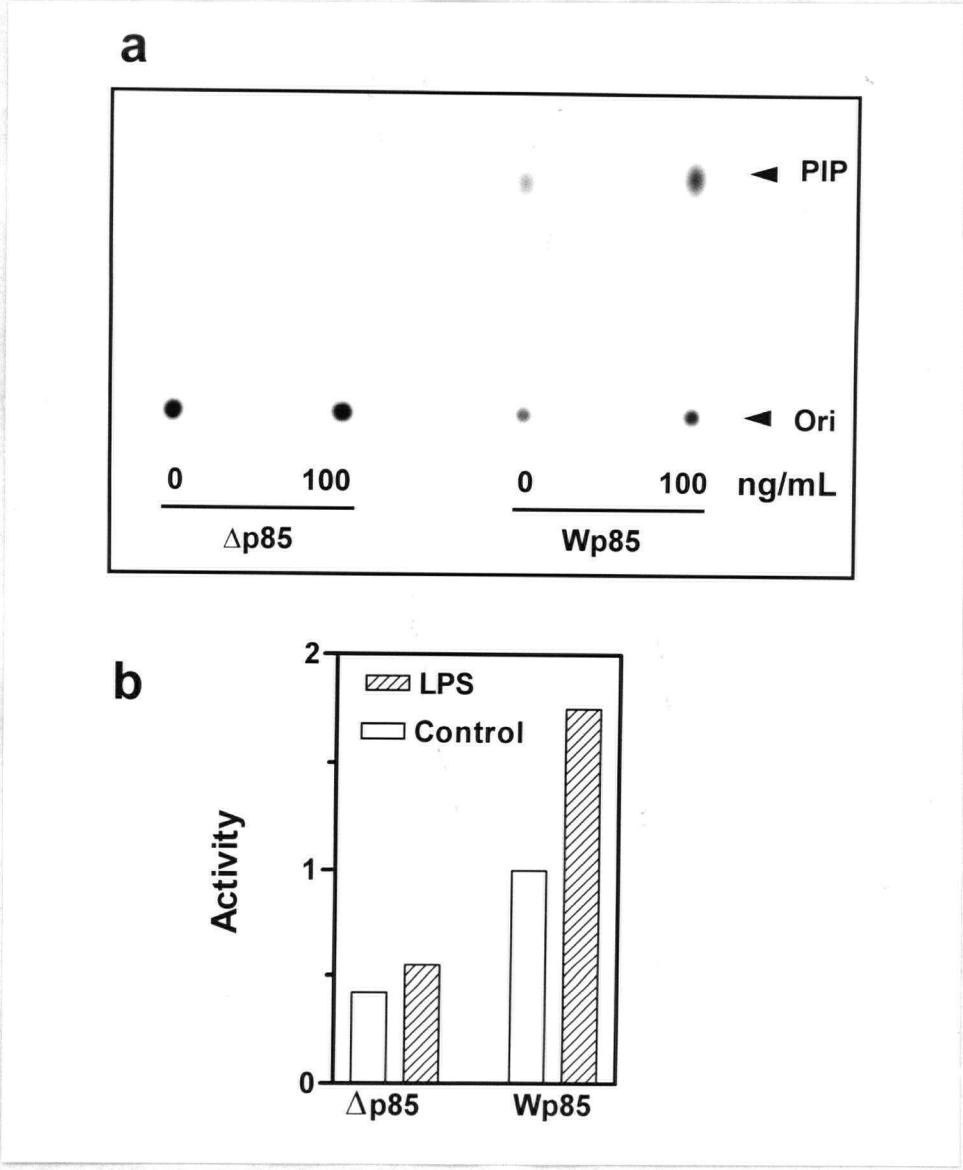
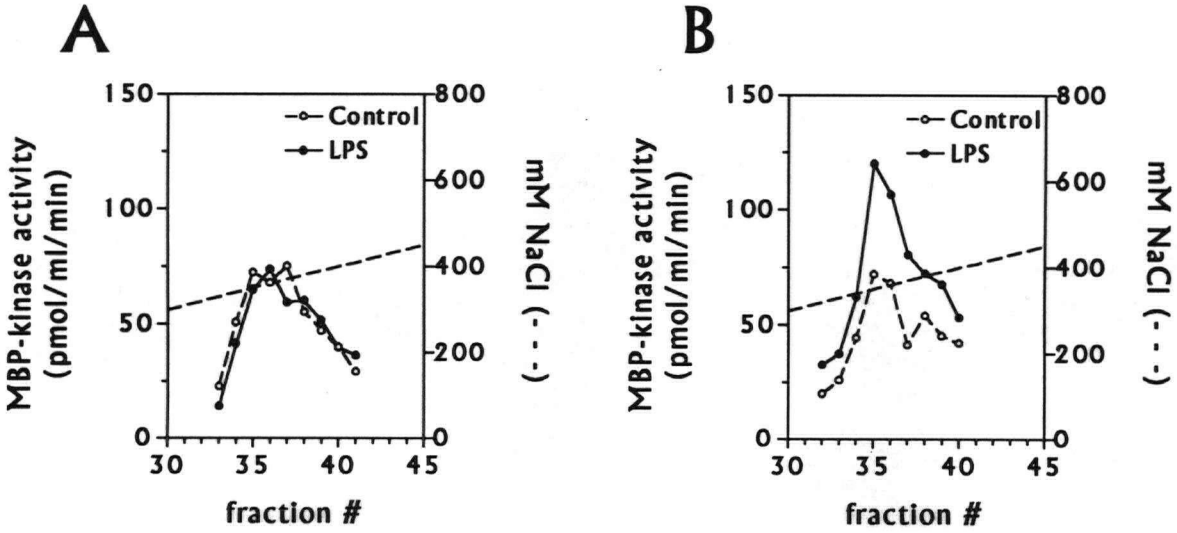


Figure 30. PI 3-kinase activity in U937 cells transfected with either wild-type, bovine p85α or dominant negative mutant, Δp85α. Cells were stimulated with 100 ng/ml LPS or medium alone followed by detergent lysis. (A) Lysates were immunoprecipitated with anti-PI 3-kinase antibody and phosphatidylinositol kinase activity was assayed as described in Materials and Methods. Radioactivity observed at the origin reflects residual, water-soluble ^{32}P-labeled material in the samples, the amount of which was not relevant to the results. (B) Spots corresponding to PtdInsP (PIP) were excised and analyzed by scintillation counting. Activity is expressed as compared to control (untreated) cells transfected with wild-type p85α (Wp85). Results presented are from one of two independent experiments with similar results.

To assess the effects of Δp85 on activation of PKC-ζ by LPS, lysates of transfected cells were analyzed using Mono Q chromatography. As shown in Figure 31A, activation of PKC-ζ by LPS was abrogated in cells transfected with Δp85 while cells expressing wild-type p85 showed activation of PKC-ζ in response to LPS (Figure 31B). Figure 31C demonstrates that PKC-ζ was expressed in Δp85 transfected; no significant difference in the relative abundance of PKC-ζ in two additional experiments was observed.


A
B
Control
LPS
MBP-kinase activity (pmol/ml/min)
mM NaCl (- - -)
fraction #
150
100
50
0
800
600
400
200
0
30
35
40
45

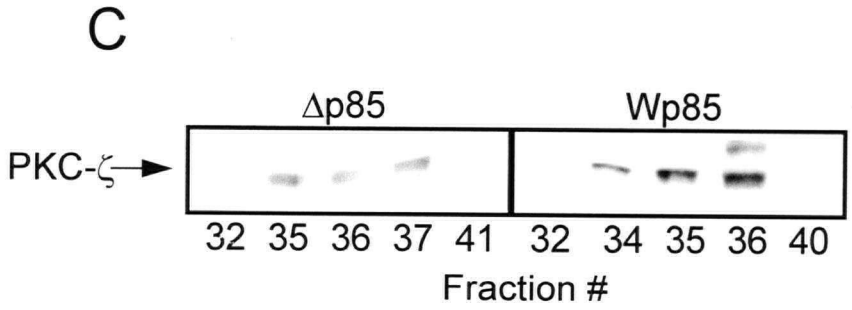

C
Δp85
Wp85
PKC-ζ
32 35 36 37 41 32 34 35 36 40
Fraction #

Figure 31. Attenuation of LPS-induced activation of PKC-ζ by a dominant negative mutant (Δp85) of the p85 subunit of PI 3-kinase. Quiescent, transfected U937 cells were treated either with LPS (1 μg/ml, 15 min) or medium alone and lysed in FPLC extraction buffer. Detergent lysates were fractionated by Mono Q chromatography as described in Materials and Methods. Aliquots (5 μl) of each fraction from cells transfected with Δp85 (A) or with wild-type p85 (Wp85) (B) were assayed for MBP kinase activity in triplicate. The standard deviation of each data point was <20% of the mean. (C) Aliquots of fractions from LPS-treated cells were analyzed by immunoblotting with anti-PKC-ζ antibodies. The results shown are from one of four experiments with similar results.

D. Effects of LPS on p21*ras* and the Vav protooncogene

p21*ras* (Ras) is a small G-protein which has been found to be positioned upstream of MAPK as well as PI 3-kinase in some systems (141,155,156). The protein is present in cells coupled to either GDP or GTP. Growth factor stimulation results in an increase in GTP-Ras with a corresponding decrease in GDP-Ras and this is usually expressed as a change in the ratio of GTP-Ras to GDP-Ras. When PBMϕs were examined, no consistent results were obtained to indicate that Ras activation was induced by LPS (data not shown).

The guanine nucleotide status of p21*ras* is controlled by exchange proteins. The product of the protooncogene *vav* has been reported to fulfill this function in cells of lymphocytic origin. In response to agonists, Vav is rapidly phosphorylated on tyrosine which activates the guanine nucleotide exchange activity of the protein. As shown in Figure 32A, when anti-Vav immunoprecipitates were electrophoresed and blotted, two immunoreactive bands of 90 and 104 kDa were observed with anti-Vav antibodies (arrow heads). When the blots were stripped and reprobed with 4G10 (Figure 32B), only a band comigrating with the fastest migrating band in the anti-Vav blot was detected. However, treatment with LPS did not bring about detectable increase in tyrosine phosphorylation of this protein. Taken together, these results indicate that p21*ras* and Vav are not involved in LPS signaling in human Mϕs.

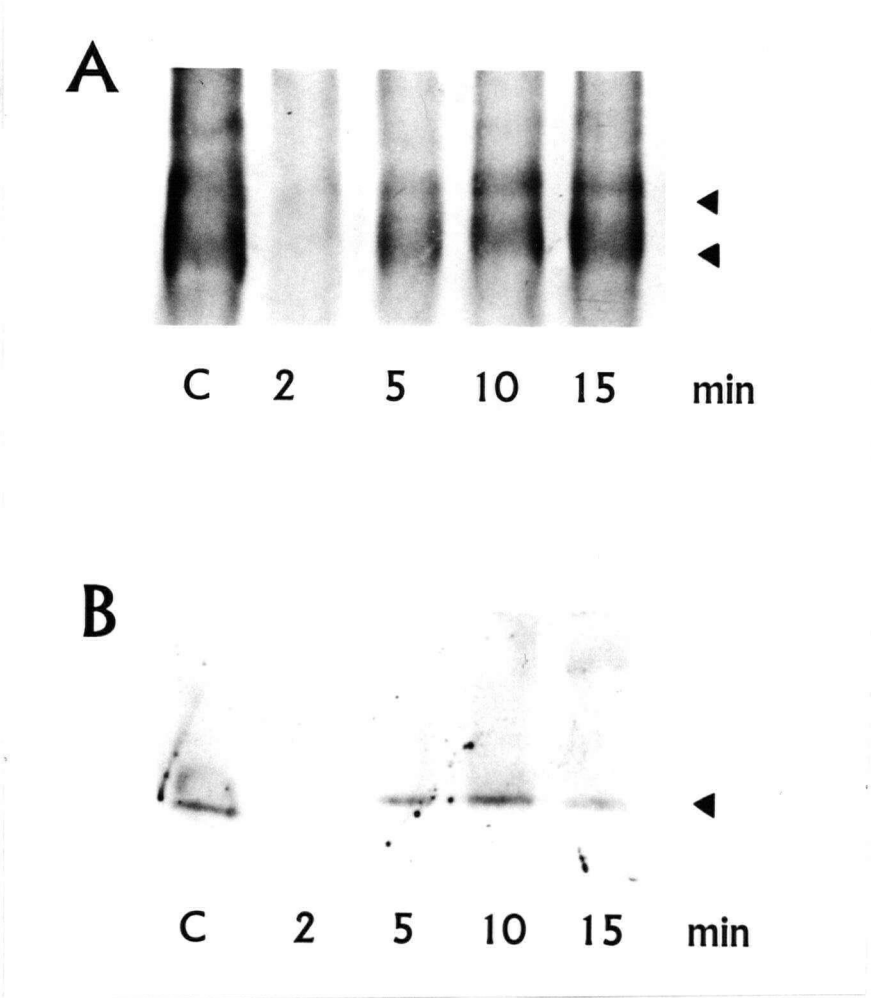


Figure 32. Tyrosine phosphorylation of p97^{vav} in PBMϕs. PBMϕs were treated with 100 ng/ml of LPS for the indicated times, or were left untreated ("C"). Whole cell lysates were prepared and Vav was immunoprecipitated using Vav-antiserum. Immune complexes were separated in a 7.5% polyacrylamide gel and immunoblotted with anti-Vav antiserum (A). The arrows indicate bands of 95 and 98 kDa recognized by the anti-vav antibodies. After stripping of antibodies, the blots were re-probed with 4G10 for the detection of phosphotyrosine residues (B). The immunoreactive band denoted by the arrow in panel B, comigrates with the lower band in panel A.

E. PI 3-KINASE AND LPS-ELICITED FUNCTIONAL RESPONSES

1. Effects of PI 3-kinase inhibitors on LPS-induced enhancement of cytokine mRNA levels

As reviewed above, the effect of LPS on Mϕs are pleiotropic. Amongst the most profound responses induced by LPS is the induction of cytokine production (3,8). To examine whether cytokine induction by LPS requires activation of PI 3-kinase, a set of templates able to recognize mRNA for eight different monokines was used (Table 1). Treatment of THP-1-WT (CD14 positive) cells with either 100 ng/ml or 1 μg/ml LPS for 3 h resulted in increased levels of mRNA for IL-1β, and TNF-α (Figure 31). Prolonged exposure of the gels allowed detection of enhanced mRNA levels for IL-1α as well. Pretreatment of cells with PI 3-kinase inhibitors, either wortmannin (100 nM), or LY294002 (32 μM), had no detectable effects on induction of cytokine mRNA levels by LPS. Treatment of undifferentiated U937 cells with LPS did not result in increased mRNA levels of any of the cytokines present in the H-14 set (data not shown). Thus, it was not possible to examine the effects of transfection of U937 cells with Δp85 on cytokine mRNA production.

2. PI 3-kinase and LPS-induced adhesion of Mϕs

The ability of Mϕs to adhere to substrates and to other cells is important for many of their functional activities. THP-1-WT cells expressing CD14, were used to study the role of PI 3-kinase in LPS-induced adherence. Overnight treatment of THP-1-WT cells with 1 μg/ml LPS resulted in increased adherence of cells to uncoated microtitre wells as compared to cells incubated with medium alone (34B vs. 34A). This effect was dependent on the LPS receptor, CD14, as pretreatment with 10 μg/ml of anti-CD14 markedly reduced the number of cells attached to the wells (Figure 34C and D). Treatment with 100 nM wortmannin (Figure 34E)

or 16 μM LY294002 (Figure 34F) for 30 min prior to LPS stimulation resulted in a marked reduction in LPS-induced adherence as compared to control, LPS-treated cells (Figure 34B). Quantitative results from this experiment are shown in Figure 34G .

A parallel adhesion assay was performed using fibronectin as substrate and adherence was quantitated spectrophotometrically based upon retention of crystal violet dye. As seen in Figure 35, overnight treatment of THP-1 cells with LPS resulted in increased adherence of the cells to fibronectin. As described for adherence of cells directly to plastic, treatment of cells with the PI 3-kinase inhibitors resulted in a marked reduction of LPS-induced adherence of cells to fibronectin-coated wells (Figures 34 and 35). In contrast, adherence induced in response to PMA was unaffected by the presence of wortmannin.

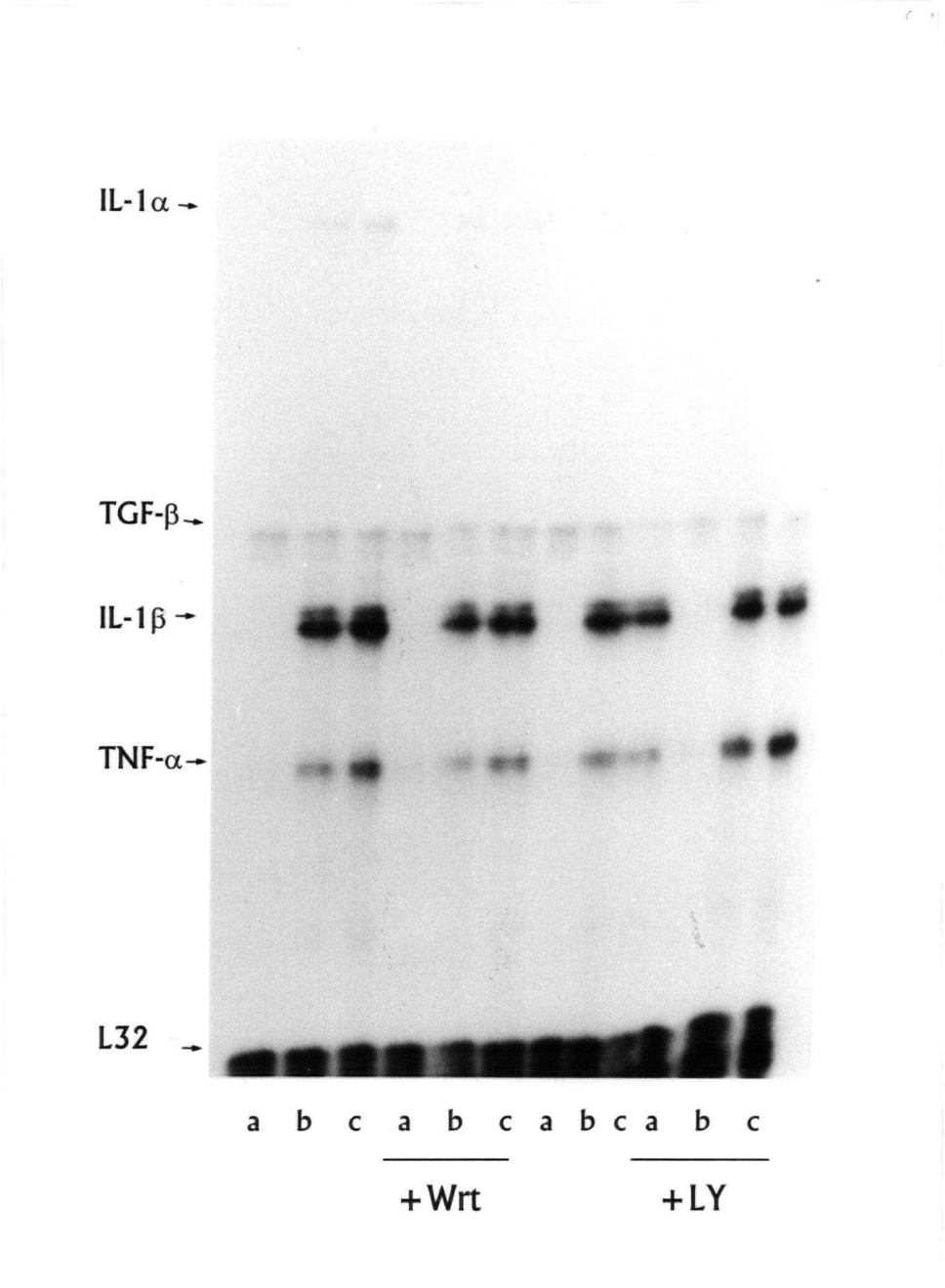
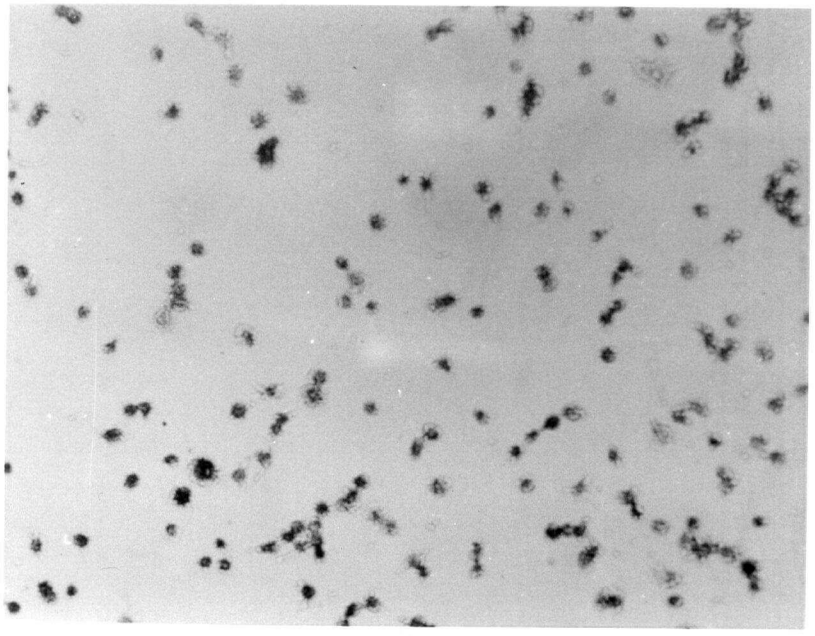
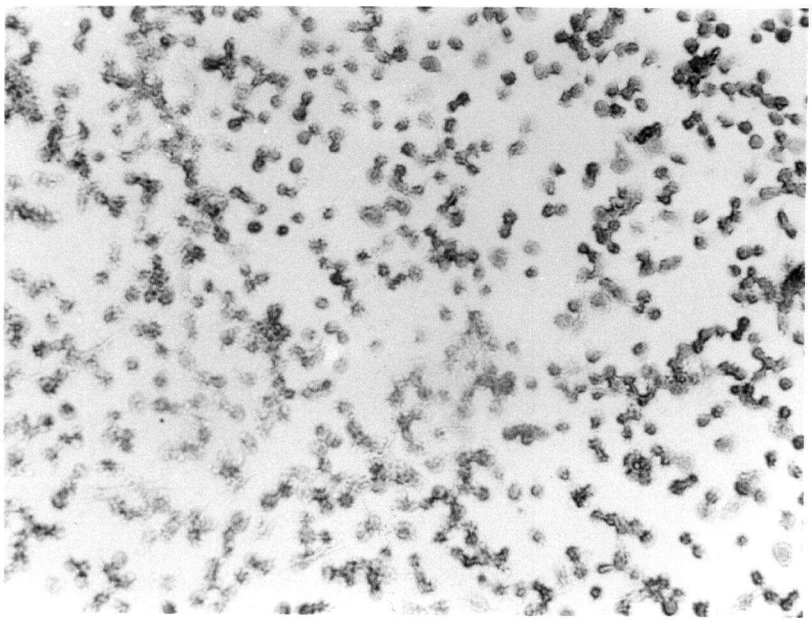


Figure 33. Effect of PI 3-kinase inhibitors on mRNA levels of different cytokines. Quiescent THP-1-WT cells were incubated with vehicle alone or with 100 nM wortmannin (+Wrt) or 32 μM LY294002 (+Ly) for 30 min, and then were either left untreated (a), or treated with 0.1 (b) or 1 μg/ml of LPS (c). Total RNA was extracted and ^{32}P-labeled cytokine RNA probes were used to detect specific, endogenous mRNAs in a ribonuclease protection assay as described under Materials and Methods.

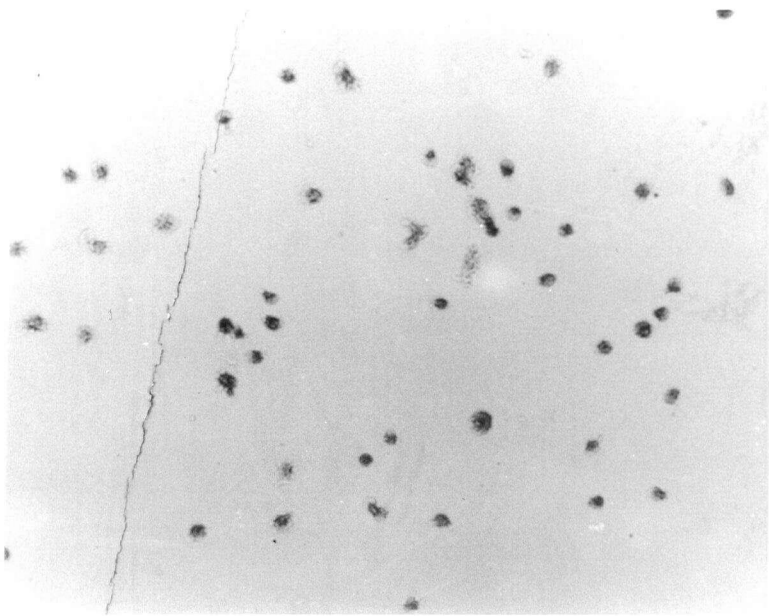
A



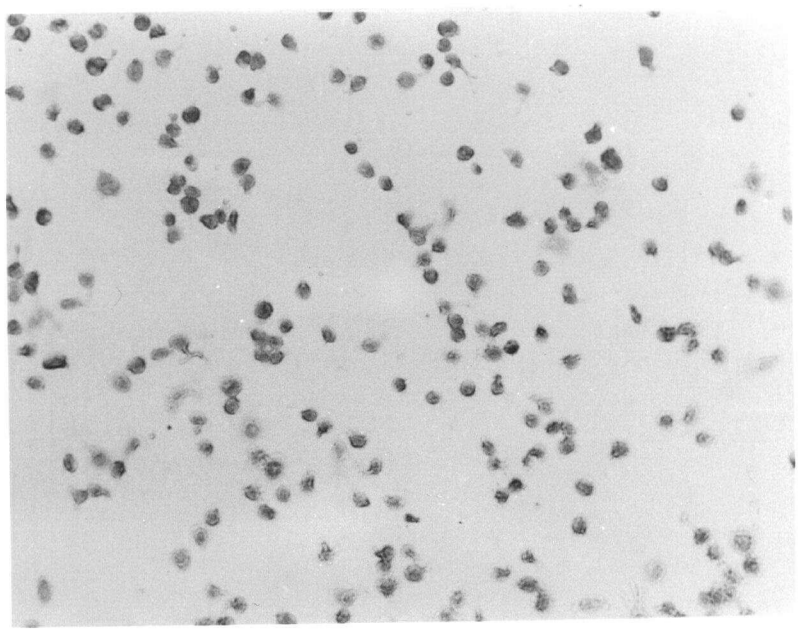
B



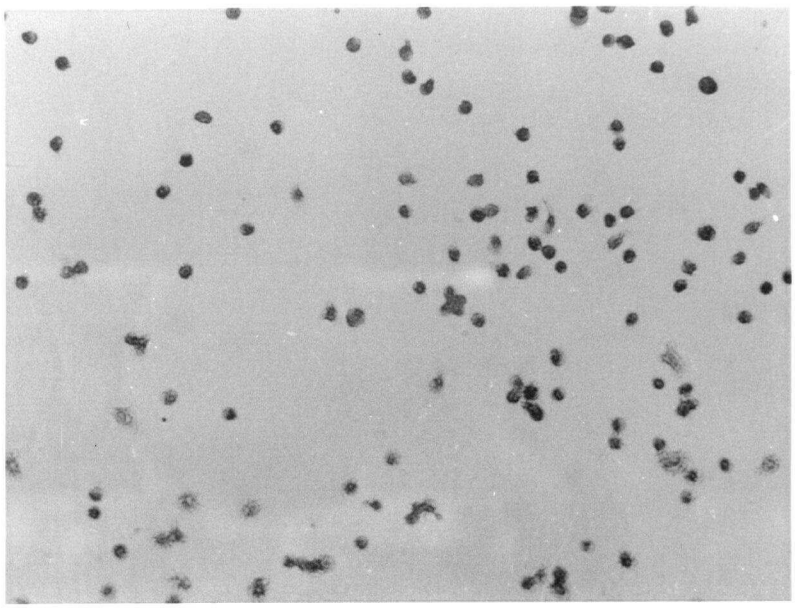
C



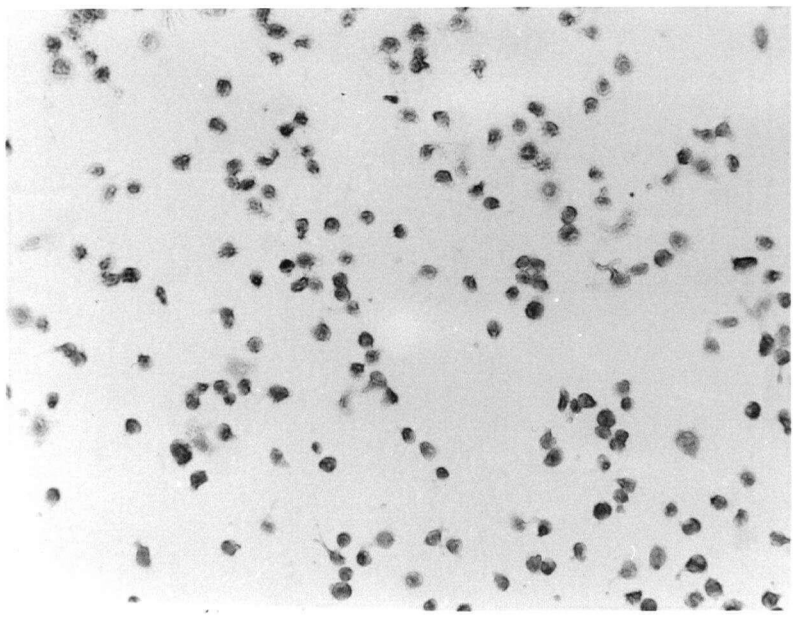
D



E



F



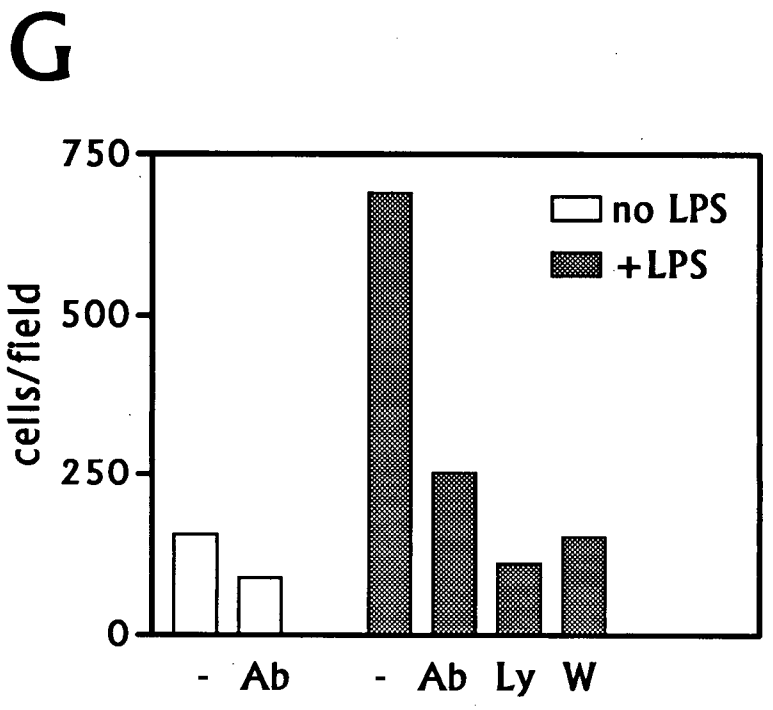


Figure 34. LPS-induced adherence of THP-1-WT cells is PI 3-kinase dependent. (A) THP-1-WT cells were incubated with vehicle (A, B, C), with 10 μg/ml anti-CD14 antibodies (C, D), with 100 nM wortmannin (E), or with 16 μM LY294002 (F). After 30 min, medium (A, C) or 1 μg/ml LPS (B, D, E, F) were added to the wells and incubated overnight. Non-adherent cells were removed by rinsing with HBSS and the wells were observed using an inverted microscope at 200X magnification (n=2). (G) Adherent cells per field (minimum of 3 fields counted at 200X magnification) were counted for each of the treatments described in A-F (Ab = anti-CD14, Ly = LY294002, W = wortmannin).

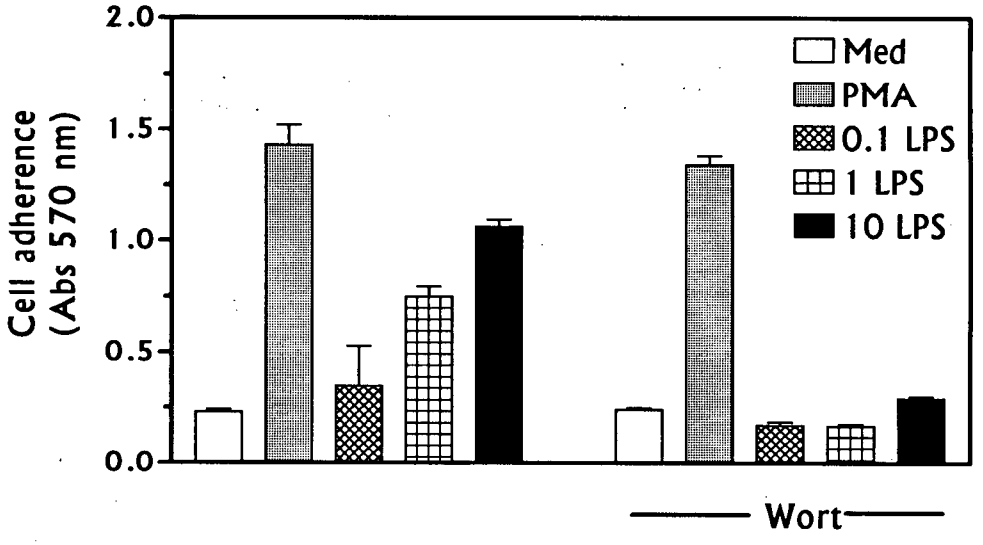


Figure 35. THP1-WT cells were added to wells coated with fibronectin. Cells were then incubated either with vehicle or with wortmannin prior to addition of either 50 nM PMA or the indicated concentrations (μg/ml) of LPS overnight. Adherent cells were quantified spectrophotometrically (570 nm) after staining with crystal violet as described in Materials and Methods (n=2).

X. DISCUSSION

Mononuclear phagocytes undergo many physiological and functional changes in response to LPS, these responses being initiated through CD14, a LPS cell surface receptor. The importance of this receptor in Mϕ activation is manifest by the findings that overexpression of CD14 leads to hypersensitivity to endotoxic shock in mice (157) and Mϕs derived from CD14-knockout mice do not express TNF-α, IL-1β, and interferon-inducible protein-10 genes after exposure to low concentrations of LPS (158).

One of the most rapid events reported after exposure of Mϕs to endotoxin is the increased tyrosine phosphorylation of proteins (69,70). These changes are apparently initiated through CD14, as antibodies against CD14 are able to block LPS-induced protein tyrosine phosphorylation (71). Working with freshly isolated peripheral blood Mϕs, we have been able to demonstrate the increased tyrosine phosphorylation of a range of proteins after exposure to LPS (Figure 6). Similar effects were observed using the monocytic cell lines THP-1 and U937 (data not shown). These responses are rapid, with increased tyrosine phosphorylation being apparent as early as 2 min and returning to near basal levels by 30-40 min. Increased protein tyrosine phosphorylation is elicited in peripheral blood Mϕs with concentrations of LPS in the picogram/ml range reaching a plateau at 10 to 100 ng/ml LPS (data not shown). These changes are due, at least in part, to increased tyrosine kinase activity in LPS-treated cells. This conclusion is based in part upon renaturation kinase assays, in which it was shown that renaturable tyrosine kinases become activated in response to LPS (Figure 7). The proteins with the highest activity observed in whole cell lysates appeared as three bands in the 49-70 kDa range. In addition, when p*53/56*^{lyn} was immunoprecipitated from equal amounts of whole cell

lysates, increased *in vitro* kinase activity was found in peripheral blood Mϕs, as well as in U937 cells (Figures 8 and 9) after treatment with LPS. Stefanová and coworkers have also reported that LPS-activated peripheral blood Mϕs have increased protein tyrosine kinase activity. Specifically they observed activation of p*58/64^{hck}*, p*53/56^{lyn}*, and p*69$^{c\text{-}fgr}$* (80). In a similar manner, Beaty and workers (159) have shown increased Hck and Lyn kinase activity after LPS treatment of human monocytes. The involvement of these tyrosine kinases in downstream events is reinforced in this study by the fact that tyrosine kinase inhibitors are able to block the LPS-induced TNF-α and IL-6 production.

Increased protein tyrosine kinase activity was also observed to be associated with CD14 in LPS-treated cells (Figure 8). Although the tyrosine kinases associating with CD14 were not specifically identified, the apparent molecular weights of these enzymes were in the range of the Src-kinases. These observations could be related to increased amounts of enzymes becoming associated with CD14 in response to LPS. On the other hand, the amounts of tyrosine kinases associated with CD14 could be the same in control and LPS treated monocytes, but the increased kinase activity observed could result from enhanced specific activities of the enzymes in LPS-treated samples. Stefanová and coworkers also demonstrated that p*53/56^{lyn}*, but not p*58/64^{hck}* or p*69$^{c\text{-}fgr}$*, associates with CD14 (80). The amounts of p*53/56^{lyn}* associated with CD14 in both resting and LPS-stimulated cells was similar, but Lyn activity was increased in LPS-treated samples. This favors the argument that LPS induces changes in the specific activities of the Src kinases. Taken together, these findings indicate that p*53/56^{lyn}* constitutively associates with CD14 and its activity is modified by LPS.

Lyn and other Src kinases have been shown to play an important role in the immune response. Mice homozygous for a disruption at the Lyn locus show defects in B cell and mast

cell functions implying an obligatory role for Lyn in cell signaling (160). In addition, macrophages from doubly homozygous $hck^{-/-}$-$fgr^{-/-}$ mice, show impaired phagocytosis and increased susceptibility to infection with *Listeria monocytogenes*, indicating that either *hck, fgr* or both are required for effective host defense (161). Despite these observations, the importance of Lyn and other Src kinases in Mϕ signaling has been questioned recently. Thus, Mϕs derived from mice deficient in Lyn, Hck, and Fgr, do respond to LPS if they are incubated with LPS and IFN-γ for 6-48 h (84) suggesting that these kinases may be dispensable for LPS responses. However, these cells were not examined for their functional responses to LPS after short term exposure (e.g., <2 h). This is an important caveat, since while Mϕs derived from Src-triple knockout mice expressed neither mRNA nor proteins corresponding to these enzymes under basal conditions, it is possible that prolonged exposure to LPS and IFN-γ may have led to the induction of other tyrosine kinases, including other members of the Src family. This possibility is supported by studies referred to above (78) in which prolonged incubation of Mϕs with LPS and IFN-γ resulted in induction of the Src kinases, Hck and Lyn. Moreover, in the Lyn^{-}-Hck^{-}-Fgr^{-}-cells, treatment with the general tyrosine kinase inhibitor, herbimycin, inhibited nitrite production induced by LPS and IFN-γ indicating continued dependency upon tyrosine kinases for signaling. the possibility remains that Lyn, Hck and Fgr may be required for rapid responses to LPS, but that with longer term exposure to LPS and IFN-γ, their absence may be compensated for by the induction of other tyrosine kinases.

The association of tyrosine kinases with GPI-linked molecules to is not limited to CD14, as several GPI-anchored proteins have been shown to associate with tyrosine kinases of the Src-family (83). The function of GPI anchors in proteins is still speculative. In the case of integral membrane proteins, the interaction of their cytoplasmic domains with cytoskeletal components

has the potential to influence their mobility. However, no such constraints apply to GPI-anchored proteins which results in their having increased mobility in the plane of the membrane. This feature may be important for their interaction with other transmembrane proteins with the capacity to transduce signals into the cell. In this regard, we observed the association of CD14 with a tyrosine phosphorylated protein of approximately 170 kDa (PY170) in phosphotyrosine immunoblots of CD14 immunoprecipitates from LPS-treated cells (Figure 9). It is unlikely that this protein is a protein tyrosine kinase *per se*, since no protein of similar size was observed either when CD14 immune complexes were subjected to *in vitro* kinase assays (Figure 8), or when whole cell lysates were analyzed by renaturation kinases assays (Figure 7). The identity of this protein has not been established. However, among the potential candidates, and because of similarity in molecular mass, we examined the possibility of it being the insulin receptor substrate-1 (IRS-1). IRS-1 is a protein without tyrosine kinase activity that becomes tyrosine phosphorylated when insulin binds to its receptor (162). IRS-1 is able to bind both to the insulin receptor and to intracellular proteins, thereby serving to propagate extracellular signals inside the cell. No immunoreactivity was observed when CD14-immunoprecipitates, positive for PY170, were examined with antibodies raised against IRS-1 (data not shown). Nevertheless, the distinct possibility exists that PY170 is involved in relaying signals inside the cell after LPS binds to CD14.

In murine Mϕs, amongst the most prominently tyrosine phosphorylated bands appearing after LPS treatment are a series of 40-45 kDa proteins. These have been demonstrated to be the p42 and p44 isoforms of MAP kinase (69). In human blood Mϕs, we have consistently observed increased tyrosine phosphorylation of proteins with apparent molecular masses in the 40-45 kDa range. This led us to investigate the possibility that some of these may be members

of the MAPK family. As shown in Figure 13A, anion exchange chromatography of lysates from LPS treated peripheral blood Mϕs, showed two peaks of increased MBP-kinase activity. Immunoblotting with anti-erk1 antibodies demonstrated the presence of p42 and p44 MAPKs and tyrosine phosphorylation of these proteins was markedly enhanced in LPS treated cells. Further evidence to support the conclusion that Mono Q peak one corresponded to p42/44 MAPKs was obtained based upon substrate specificity of the two peaks of MBP-kinase activity detected. Only Mono Q peak 1 was able to efficiently phosphorylate a peptide substrate specific for members of the MAPK family. As seen in Figure 14B, peak two showed almost negligible activity towards this substrate even though it was more efficient than peak one in phosphorylating MBP (Figure 14A). These findings support the conclusion that LPS treatment of human, peripheral blood mononuclear cells brings about the activation of two members of the MAPK family, MAPK1 and MAPK2. The importance of MAPK in Mϕ cell signaling is strengthened by the finding that MAPKs become activated in Mϕs, not only in response to LPS (163,164), but also in response to GM-CSF (163) and engagement of the TNF receptor (165). Furthermore, other cell types have also been shown to respond to LPS with activation of MAPK, including endothelial cells (72) and neutrophils (139).

In addition to p42 and p44 MAPKs, a p38 MAPK isoform is also activated in response to LPS in Mϕs, neutrophils (139) and in 70Z/3 cells transfected with CD14 (140). The activation of this isoform in our system cannot be excluded as antibodies specific for p38 MAPK were not used to probe Mono Q fractions. It is interesting to note, however, that a band of approximately 38-40 kDa appeared to be tyrosine phosphorylated and showed weak immunoreactivity with erk-CT antibodies (Figure 13). The importance of this enzyme in Mϕ

signal transduction is indicated by the finding that a family of cytokine-suppressive anti-inflammatory drugs are potent and selective inhibitors of this enzyme (166).

Work done in this laboratory (167) and presented here (Figures 13A, 15A, 16A, and 17A), indicated that the second and later eluting Mono Q peak (peak two) of LPS-activated MBP kinase activity might be accounted for by an apparently calcium-independent isoform of PKC. The evidence supporting this includes: (i) reactivity of peak fractions with anti-pan PKC antibodies in immunoblots of a protein with an apparent subunit M_r of approximately 80,000-81,000, (ii) immunoprecipitation of peak two MBP kinase activity with anti-pan PKC antibodies, (iii) sensitivity of this kinase to inhibition by a PKC inhibitor peptide, and (iv) detection of stimulated activity in the presence of 2 mM EGTA (free calcium below 10^{-8} M) and in the absence of diacylglycerol and phosphatidylserine. The identification and mechanism of activation of this PKC is discussed below.

The findings that Src-family tyrosine kinases are activated in LPS-treated Mϕs raised the question of potential downstream effectors and one possibility is the lipid kinase, PI 3-kinase. Concentrations of PI 3-kinase metabolites, such as PtdIns3,4,5P_3, are low or undetectable in resting cells and increase rapidly and transiently in response to various agonists. In this regard, activation of both receptor and non-receptor, cytosolic tyrosine kinases (168,169), including p53/56^{lyn} (91,170,171) have been linked to increases in PI 3-kinase metabolites. The finding that p53/56^{lyn} is activated in Mϕs incubated with LPS, therefore, supported the possibility that PI 3-kinase is a downstream effector in this system.

In fact, the results of the present study show that PI 3-kinase is activated in LPS-treated human Mϕs. This conclusion is based both upon the detection of elevated levels of PI 3-kinase metabolites *in vivo* in LPS-treated peripheral blood Mϕs and U937 cells, as well as increased

enzymatic activity assayed *in vitro* in PI 3-kinase immunoprecipitates. Detailed analysis of phosphoinositide levels done using LPS-treated U937 cells revealed that both PtdIns3P and PtdIns3,4,5P_3 are increased over basal levels (Figure 20 and Table 2). No significant changes were observed in the other phosphoinositides analyzed. Increased levels of these metabolites could be a consequence of either higher PI 3-kinase activity or decreased activity of phosphomonoesterases resulting in the accumulation of these phospholipids. Increased specific activity of PI 3-kinase observed in immunoprecipitates from LPS-treated cells as compared to control cells (Figures 23 and 24) is consistent with the first possibility. This conclusion is also supported by the finding that LPS-induced increases in levels of PtdIns3,4,5P_3 were completely abrogated when cells were pre-incubated with the PI 3-kinase inhibitor wortmannin (Figure 21).

Many responses of Mϕs to physiological concentrations of LPS are believed to involve the cell surface molecule CD14 (14). In the present study, PI 3-kinase activation in peripheral blood Mϕs was observed using LPS concentrations in the range of 10-100 picogram/ml. Thus, the concentrations of LPS that bring about activation of PI 3-kinase are similar to those required to induce Mϕs functional responses through CD14. Moreover, incubation of cells with antibody 3C10, specific for CD14 and which neutralizes Mϕ responses to LPS, abrogated LPS-induced-increases in PtdIns3,4,5P_3 levels (Figure 22), indicating that activation of PI 3-kinase by LPS is CD14-dependent.

In other systems, such as antigen receptor cross-linking in B cells, PI 3-kinase is known to interact physically with tyrosine phosphorylated p53/56^{lyn} and this is associated with increased lipid-kinase activity (170). Thus, the finding that p53/56^{lyn} is activated in LPS-treated Mϕs is of particular interest and the results presented in Figure 24 identify LPS signaling

in Mϕs as another system in which PI 3-kinase and p53/56^{lyn} transiently associate leading to increased lipid kinase activity. Furthermore, treatment of cells with the PTK inhibitor herbimycin A abrogates the LPS-induced increase in PI 3-kinase activity (Figure 26). This effect was not a result of a direct inhibition of PI 3-kinase, since *in vitro* incubation of herbimycin A with PI 3-kinase, immunoprecipitated from LPS-treated cells, did not affect lipid kinase activity (Figure 26c). The association of p85 with other tyrosine phosphorylated proteins is not excluded, however. As reported by Stefanová and coworkers (80) LPS brings about the activation of Lyn, Hck, and Fgr in Mϕs. The PI 3-kinase activity observed in phosphotyrosine immunoprecipitates could be the results of the association of p85 with any, or all, of these tyrosine kinases, as well as with any protein that became their target after activation by LPS.

Following agonist treatment, PI 3-kinase is known to associate not only with tyrosine kinases, but also with other tyrosine phosphorylated proteins. Reports in the literature show that, in some cases, these associations appear to be mediated through SH2 domains in the p85 regulatory subunit of the enzyme. For example, phosphotyrosine containing peptides derived from insulin receptor substrate-1 (89,172), polyoma virus middle t antigen and platelet derived growth factor receptor (173) activate PI 3-kinase from 3T3-L1 adipocytes or from CHO cells *in vitro*. Other mechanisms, either alternative or complementary, that mediate these protein-protein interactions appear to involve SH3 domains from either p59^{fyn} or p53/56^{lyn} binding to proline rich regions in p85. This binding also appears to upregulate PI 3-kinase activity (91). Thus, it is possible that either or both of these interactions with p53/56^{lyn} may account for activation of PI 3-kinase in LPS treated Mϕs.

Three potential tyrosine phosphorylation sites have been mapped in the regulatory subunit of PI 3-kinase and phosphorylation of one or more of these tyrosine residues has been proposed as an alternative mechanism for enzyme activation. In contrast, there are multiple reports of activation of PI 3-kinase in the absence of changes in tyrosine phosphorylation of p85 [reviewed in (86)]. The results presented in this thesis indicate that there is no apparent change in tyrosine phosphorylation of p85 in LPS-treated cells (Figure 27). Failure to detect a change in tyrosine phosphorylation of p85 could be explained by the fact that increased PI 3-kinase activity in the anti-p85 immunoprecipitates resulted from only a minor fraction of the enzyme being tyrosine phosphorylated. This might have been below the sensitivity of the detection system used thereby contributing to a false negative result. To maximize the chance of detecting tyrosine phosphorylated p85, 4G10 immunoprecipitates were prepared from LPS-treated cells to provide material enriched in phosphotyrosine containing proteins. Even under these conditions, p85 was not observed to comigrate with any of the tyrosine phosphorylated proteins detected in parallel Western blots prepared with anti-phosphotyrosine antibody (Figure 24b, c). Taken together, these results indicate that tyrosine phosphorylation of the regulatory subunit is not necessary for activation of the enzyme in response to LPS. Although p85 was not observed to be tyrosine phosphorylated in 4G10 immunoprecipitates from LPS-treated cells, relative to control cells these samples contained more PI 3-kinase protein (Figure 27b). This presumably indicates that PI 3-kinase associates with phosphotyrosine containing proteins (p53/56^{lyn} at least and perhaps others) preferentially in LPS-treated cells. As discussed above, these protein-protein interactions likely contribute to enzyme activation.

In *Schizosaccharomyces pombe*, Ras has been shown to be involved in regulating PI 3-kinase (174). In addition, Ras has been reported to be activated in LPS treated human

monocytes (144). However, we have been unable to demonstrate that LPS activation of Mϕs promotes increased GTP loading of Ras (data not shown). Similarly, work done by Büscher and coworkers in LPS-treated (1 μg/ml) BAC-1,2F5 macrophages showed stimulation of MAP kinase occurring independently of Ras activation (143). Guanine nucleotide exchangers such as Vav, are responsible for the appearance of GTP-Ras in activated T cells (175). This 97 kDa protein is a common target of protein tyrosine kinases (176,177), and in T cells Gulbins and coworkers reported that tyrosine phosphorylation of Vav correlates with increased nucleotide exchange activity (175). When Vav immunoprecipitates were analyzed for tyrosine phosphorylation by immunoblotting, we were unable to detect any difference between the control and LPS treated cells (Figure 32B). Taken together, these results indicate that Ras activation may not be involved in the LPS-dependent activation of PI 3-kinase.

The mechanisms for regulation of p85/p110 in mammalian cells are diverse. For instance, the fMLP receptor in neutrophils is known to be coupled to heterotrimeric G-proteins, and this chemoattractant peptide causes rapid activation of p85/p110 PI 3-kinase in neutrophils (87,88,101,113). Furthermore, Matsuo *et al.* (178) demonstrated that concanavalin A treatment of THP-1 cells results in PI 3-kinase activation through dual signaling pathways: one G-protein-coupled and the second phosphotyrosine-related. In addition to p85/p110 PI 3-kinase, another PI 3-kinase family member consisting of a single polypeptide chain, p100γ, has been described. This isoform is present in the human leukemia cell lines U937 and K562 (93) and becomes activated by heterotrimeric, G protein-linked receptors. Thus, while it appears that activation of PI 3-kinase by LPS in human monocytes occurs independent of Ras, based upon inhibitor studies (Figure 26), it appears to be regulated by tyrosine-kinase dependent mechanisms and the role of G proteins remains to be examined.

Increased activity of PI 3-kinase in human monocytes incubated with LPS results in elevated levels of PtdIns3,4,5P_3 as well as PtdIns3,4P_2 in U937 cells (Figure 20). The exact roles for these and other metabolites of PI 3-kinase are still not completely clear. Transient increases in the concentrations of these metabolites in response to activation by different agonists, however, does implicate their function as second messengers. Several PH-domain containing proteins have the ability to bind to D3-phosphorylated phosphoinositides [reviewed in (98)]. Among these, Akt/PKB has been reported to bind PtdIns(3,4)P_2 resulting in activation of the enzyme (105). Furthermore, treatment of cells with the PI 3-kinase inhibitor wortmannin results in inhibition of PKB activity, suggesting that activation of PKB is an event occurring downstream of PI 3-kinase (103). MAP kinase is another potential target of the action of PI 3-kinase. Treatment of COS-7 and CHO cells with wortmannin or LY294002, or overexpression of a dominant negative mutant of PI 3-kinase results in attenuation of MAP kinase activation through G proteins (179).

Other potential targets of activated PI 3-kinase are members of the PKC family. PKC-δ has been found to associate with PI 3-kinase following cytokine stimulation (180). It has also been reported that the inositol phospholipids PtdIns3,4P_2 and PtdIns3,4,5P_3 have the capacity to activate calcium-independent isoforms of PKC *in vitro* (109,124). As discussed above, the work reported here indicates that LPS treatment of human monocytes results in the activation of a form of PKC that is active in the absence of calcium and lipid co-factors. The activation of this form of PKC was not limited to monocytes from peripheral blood, as it was also observed in the monocytic cells lines THP-1 and U937 (Figures 16A, 16C and 17A). Detection of activity in the absence of cofactors is consistent with PKC-ζ, and immunoblotting of fractions from Mono Q peak two indicated the presence of PKC-ζ in these samples (Figures 15B , 16B

and 17B). PKC–ζ activity immunoprecipitated from LPS-stimulated THP-1 cells also showed increased *in vitro* kinase activity as compared with the activity present in immunoprecipitates from control, untreated cells (Figure 15C). Furthermore, LPS-enhanced MBP kinase activity could be removed by immunoadsorption with anti-PKC–ζ (Figure 17). Prior analyzes of PKC expression in blood monocytes, U937 cells, and HL-60 cells indicated the presence of PKC-α, -βI, -βII, -ε, –ζ and θ isoforms (118,130,131,181). In U937 cells, PKC-α, -β, and -ε elute from Mono Q at or below 320 mM NaCl, while PKC–ζ elutes at ~460 mM NaCl (118). The somewhat earlier elution of PKC–ζ (370-410 mM NaCl) observed in the present study most likely reflects procedural differences related to detergent solubilization and variations in elution buffers.

The evidence presented indicating that PKC-ζ is activated by LPS is of potential interest since previous studies concerned with the role of PKC in LPS cell signaling in Mϕs have been inconsistent. For example, it has been reported that treatment of human monocytes with LPS brings about translocation of PKC-α and PKC-β isoforms to the membrane fraction (182). In contrast, incubation of murine macrophages with LPS was not observed to activate PKC in the presence of calcium, phosphatidylserine and diacylglycerol (128). Studies from this laboratory also found no evidence for LPS-induced translocation of Ca/phospholipid dependent-PKC enzymatic activity to the membrane fraction of human monocytes (P. Herrera, R. Brownsey and N. Reiner, unpublished data).

Two of the three anti-PKC–ζ antibodies used in this study were raised against a peptide (SEFEGFEYINPLLLSAEESV) corresponding to amino acids 573-592 present in the COOH-terminus of the kinase. This exact sequence is not found in any of the other known PKC isoforms (183). The closely related PKC-ι contains a similar COOH-terminal sequence

(SEFEGFEYINPLLMSAEECV) differing at only two amino acids and is detected with antibodies to the COOH-terminus of PKC–ζ. However, it is unlikely that the major immunoreactive band detected in this study was PKC-ι, since this isoform is known to migrate at 65 kDa (184), consistently lower than the ~80-85 kDa protein observed in this study. The third antibody used (Upstate Biotechnology) is reported not to cross-react with PKC-α, PKC-ι or PKC-λ. It has also been reported that antibodies directed against the COOH-terminus of PKC–ζ react with a Ca^{2+} and phorbol ester-sensitive PKC isoforms (185). However, the kinase detected and characterized in this thesis was only weakly activated by a combination of PMA, Ca^{2+}, and PS and had sustained activity in the absence of exogenous lipids (Figure 18). These findings preclude the notion that it may be a member of either the cPKC or nPKC subfamilies and indicates strongly that it is PKC-ζ.

PKC isoforms have been observed to display different substrate specificity profiles and to some extent the results obtained are influenced by the specific assay conditions (118,154,186). In the present study, it was found that MBP, peptide ε, and S6 peptide were equally efficient substrates for monocyte PKC–ζ. In contrast, protamine sulfate, histone, and kemptide were relatively poor substrates (Figure 18). These findings are consistent with previous studies in which PKC–ζ phosphorylated MBP and peptide substrates with equal efficiency (109), but showed lower activity against histone and kemptide (118,123). While the data contrast somewhat with other results indicating that peptide ε may be a more efficient substrate for PKC-ζ than MBP (123,154), these comparisons are complicated by species differences as well as different assay conditions.

Activator and cofactor requirements for PKC isoforms have served as the basis for a general classification scheme for these kinases. Members of the cPKC and nPKC subgroups

have generally been found to require the presence of PS and diacylglycerol for activation and exhibit little activity in the absence of these factors (187,188). The cPKC members also require the presence of Ca^{2+}. In contrast, human PKC–ζ exhibits activator- and cofactor-independent activity which is not increased by the addition of PS (118). The biochemical characteristics of LPS-activated monocyte PKC–ζ reported in this paper (Figure 18) are consistent with previous studies. The findings presented contrast somewhat, however, with studies of PKC–ζ in other species. For example, rat and bovine homologues display activator- and cofactor-independent activity which is significantly enhanced by the addition of PS [reviewed in (122)].

The mechanisms leading to the activation of PKC–ζ in cells in response to external stimuli are not fully understood. As discussed above, the involvement of PI 3-kinase in the regulation of nPKC and aPKC isoforms has been implicated by studies showing a stimulatory effect of the PI 3-kinase products PtdIns3,4P_2 and PIP_3 *in vitro* (109,124). Mizukami and coworkers found an *in vivo* correlation between activation of PKC-ζ and PI 3-kinase. These authors reported that after ischemia of cardiac tissue, PKC-ζ is translocated to the nucleus and treatment with wortmannin prevents this translocation in a dose dependent manner (189). In addition, the PI 3-kinase products PtdIns3,4P_2 and PtdIns3,4,5P_3 show a stimulatory effect on the *in vitro* activity of aPKCs (109,124). The role of PI 3-kinase in LPS-induced activation of monocyte PKC–ζ was examined in this thesis using two different approaches. The first approach involved the use of two structurally unrelated PI 3-kinase inhibitors. LPS-induced activation of PKC–ζ was abrogated by both wortmannin and LY294002 (Figure 28A and 28B). The effects of wortmannin are considered to be relatively specific for PI 3-kinase at concentrations similar to those used in this study (50-100 nM). However the compound has

been shown to inhibit phospholipase A_2 with an IC_{50} similar to that previously reported for PI 3-kinase (190). The structurally unrelated compound, LY294002, has been shown to have inhibitory effects on PI 3-kinase by a different mechanism (191). Moreover, LY294002 shows no inhibitory effects on other lipid kinases or on several protein kinases, including PKC and MAP kinase (191). The findings that both compounds exhibit inhibitory effects on LPS-induced activation of PKC-ζ, therefore, support the argument that PI 3-kinase is involved in the regulation of PKC-ζ in this system.

The second approach to examine the role of PI 3-kinase in regulating PKC-ζ activation involved experiments in which the effects of transfection with a dominant negative mutant (DNM) of PI 3-kinase (Δp85) on PKC-ζ activity was analyzed. As described in the Materials and Methods section, the mutant Δp85 subunit lacks a region of the protein involved in binding to the catalytic subunit of the enzyme. However, Δp85 retains both SH2 domains and the SH3 domain of the wild type enzyme, allowing it to interact with other signaling molecules, for example tyrosine kinases and possibly others. Thus, coexpression of Δp85 with native enzyme results in competition between the proteins for binding to critical signaling molecules. Consequently, the native p110 catalytic subunit fails to interact with regulatory proteins and impaired PI 3-kinase signaling occurs.

When the activity of PI 3-kinase was examined in U937 cells that had been transfected with either Δp85 or bovine wild type p85 (Wp85), it was observed that cells transfected with the dominant negative mutant protein showed decreased in vitro activity as compared to Wp85-transfected U937 cells (Figure 30). Furthermore, treatment of cells with LPS failed to increased the enzymatic activity of PI 3-kinase, whereas Wp85-transfectants showed LPS-dependent responses similar to those observed in non-transfected cells. Immunoblotting of

whole cell lysates from non-transfected, Δp85- and Wp85-transfected U937 cells showed similar levels of expression of p85 in the three cell types. The fact that no overexpression of p85 was detected, could be explained by the low levels of expression that U937 cells show when transfected with foreign DNA (192). These results indicate that although there is no apparent overexpression of p85 in either of the two transfectants, expression of native p85 in the DNM is not impaired, and lower PI 3-kinase activity is not a result of diminished amount of enzyme present in the cell.

Expression of Δp85 in U937 cells impaired LPS-induced activation of PKC–ζ (Figure 31B) providing additional support for the argument that PI 3-kinase is an upstream activator of PKC-ζ. An important question arising from these observations is how PKC–ζ maintains its activation following exposure to PtdIns3,4,5P_3 produced by activated PI 3-kinase. One possibility is that PtdIns3,4,5P_3 induces a change in the phosphorylation state of the PKC–ζ which sustains its activity until it becomes dephosphorylated by a cellular phosphatase. Changes in PKC-ζ could also involve association with other proteins resulting in enhancement of its activity.

The importance of these signaling events in regulating the functions of Mϕs as immunologically competent cells is an issue of extreme interest. As discussed earlier, LPS is capable of eliciting a wide variety of responses in these cells, including the production and secretion of many growth factors and cytokines (193,194). We explored the hypothesis that PI 3-kinase may be important for the induction of cytokines production in human Mϕs. Using RNase protection assays to measure mRNA levels for different cytokines in U937 cells, we were unable to detect significant increases in any of eight different cytokines. Thus it was not possible to examine the effects of transfection of Δp85 in the LPS-elicited responses of U937

cells. THP-WT cells, however, showed increased mRNA levels for IL-1α, IL-1β and TNF-α in response to LPS. Treatment of THP-1-WT cells with the PI 3-kinase inhibitors LY294002 and wortmannin, while inhibiting PI 3-kinase and PKC-ζ activities, failed to inhibit LPS-induced increases of cytokine mRNA (Figure 33). These findings indicate that PI 3-kinase is not involved in signaling events leading to increased cytokine production by Mϕs. We cannot exclude completely the possibility that PI 3-kinase is involved in regulating cytokine production. Even though mRNA levels for these cytokines are not impaired after treatment with PI 3-kinase inhibitors, the expression or secretion of these proteins could be affected, consistent with post-transcriptional regulation.

In comparison to resting cells, activated Mϕs show increased adhesive properties which are vital for the many of their functions, including tumoricidal activity. As PI 3-kinase appears to be involved in cytoskeletal rearrangements in other cells from the immune system (115), we examined the effects of PI 3-kinase on the adhesive capacity of Mϕs. CD14-positive THP-1-WT cells growing in suspension showed increased adhesiveness to culture dishes (Figure 34) or fibronectin (Figure 35) when treated with LPS. After pretreatment with either wortmannin or LY294002, they showed marked reductions in LPS-induced adherence. PMA is an agent commonly used for differentiation of U937 and THP-1 cells and it causes the cells to become tightly adherent to plastic surfaces. When THP-1 cells were incubated with the PI 3-kinase inhibitors before the addition of PMA, no effect on PMA-induced adherence was observed. These results indicate that in THP-1 cells, the effects of the PI 3-kinase inhibitors on the adhesive properties of the cells are selective and likely related to inhibition of PI 3-kinase activation in response to LPS. Furthermore, the data show that LPS-induced, but not PMA-

induced cell adherence appears to be PI 3-kinase dependent. These findings are consistent with the observation that PMA does not activate PI 3-kinase in Mϕs (data not shown). These studies concerned with adherence were carried out using a human monocytic cell line. Experiments to determine whether LPS-induced adherence is similarly regulated in peripheral blood monocytes are in progress. However, the fact that many of the signaling events reported in this thesis were comparable in THP-1, U937 cells, and in peripheral blood monocytes, suggests that changes in monocyte adherence in response to LPS are likely to be PI 3-kinase dependent in the latter cells.

Several questions emanating from the studies reported in this thesis warrant further investigation. For example, the involvement of PKC-ζ in LPS-induce, macrophage adherence remains to be elucidated. Work done by Derman and coworkers showed that PtdIns3,4,5P_3 increases the motility of fibroblasts and suggested the involvement of a calcium-independent isoform of PKC in this process (195). The use of specific inhibitors of PKC-ζ, or transfection of cells with a dominant negative mutant of the enzyme could help establish the exact role PKC-ζ plays in the downstream events initiated by LPS activation. In addition, both tyrosine kinase- as well as G protein-dependent pathways for the activation of PI 3-kinase have been demonstrated (85-87). Whether one or both of these is involved in LPS cell signaling is an important question. Notably, G proteins have been shown to participate in LPS-induced responses in a macrophage cell line (196).

In conclusion, this thesis examined LPS signaling in Mϕs and the relationship of activated pathways to functional responses. The results show that LPS activates both protein and lipid kinases including p*56^{lyn}*, p42 and p44 isoforms of MAP kinase, PKC-ζ and PI 3-kinase through

CD14-dependent mechanisms. Activation of PKC-ζ was demonstrated to be PI 3-kinase dependent and signaling through this lipid kinase was shown to regulate monocyte adherence induced in response to LPS, but not PMA. In contrast to LPS-induced adherence, induction of transcription of cytokine genes in response to LPS did not appear to be dependent on PI 3-kinase.

XI. REFERENCES

1. Lydyard, P. and Grossi, C. Cells involved in the immune response. In: *Immunology*, edited by Roitt, I., Brostoff, J. and Male, D. London: Mosby, 1996, p. 2.2-2.18.

2. Gordon S. 1995. The macrophage. *BioEssays 17*: 977-986.

3. Nathan C. F. 1987. Secretory products of macrophages. *Journal of Clinical Investigation 79*: 319-326.

4. Unanue E. R. and Allen P. M. 1987. The basis for the immunoregulatory role of macrophages and other accessory cells. *Science 236*: 551-558.

5. Adams, D. O. and Hamilton, T. A. Phagocytic cells: cytotoxic activities of macrophages. In: *Inflammation: basic principles and clinical correlates*, edited by Gallin, J. I., Goldstein, I. M. and Snyderman, R. New York: Raven Press, Ltd. 1988, p. 471-492.

6. Wright S. D., Ramos R. A., Hermanowski-Vosatka A., Rockwell P., and Detmers P. A. 1991. Activation of the adhesive capacity of CR3 on neutrophils by endotoxin: Dependence on lipopolysaccharide binding protein and CD14. *J. Exp. Med. 173*: 1281-1286.

7. Raetz C. R. H., Ulevitch R. J., Wright S. D., Sibley C. H., Ding A., and Nathan C. F. 1991. Gram-negative endotoxin: An extraordinary lipid with profound effects on eukaryotic signal transduction. *FASEB J. 5*: 2652-2660.

8. Watson R. W. G., Redmond H. P., and Bouchier-Hayes D. 1994. Role of endotoxin in mononuclear phagocyte-mediated inflammatory responses. *J. Leukocyte Biol. 56*: 95-103.

9. Glauser M. P., Zanetti G., Baumgartner J.-D., and Cohen J. 1991. Septic shock: pathogenesis. *Lancet 338*: 732-736.

10. Raetz C. R. H. 1990. Biochemistry of endotoxins. *Annu. Rev. Biochem. 59*: 129-170.

11. Rietschel E. T., Kirikae T., Schade F. U. *et al.* 1994. Bacterial endotoxin: Molecular relationships of structure to activity and function. *FASEB J. 8*: 217-225.

12. Sweet M. J. and Hume D. A. 1996. Endotoxin signal transduction in macrophages. *J. Leukocyte Biol. 60*: 8-26.

13. Holst O., Ulmer A. J., Brade H., Flad H.-D., and Rietschel E. T. 1996. Biochemistry and cell biology of bacterial endotoxins. *FEMS Immunology and Medical Microbiolgy 16*: 83-104.

14. Ulevitch R. J. 1993. Recognition of bacterial endotoxins by receptor-dependent mechanisms. *Adv. Immunol. 53*: 267-289.

15. Kielian T. L. and Blecha L. 1995. CD14 and other recognition molecules for lipopolysaccharide: a review. *Immunopharmacology 29*: 187-205.

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

17. Lee J.-D., Kravchenko V., Kirkland T.N. *et al.* 1993. Glycosyl-phosphatidylinositol-anchored or integral membrane forms of CD14 mediate identical cellular responses to endotoxin. *Proc. Natl. Acad. Sci. USA 90*: 930-9934.

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

19. Halling J. L., Hamill D. R., Lei M.-G., and Morrison D. C. 1992. Identification and characterization of lipopolysaccharide-binding proteins on human peripheral blood cell populations. *Infect. Immun. 60*: 845-852.

20. Lei M.-G. and Morrison D. C. 1988. Specific endotoxic lipopolysaccharide-binding proteins on murine splenocytes. *J. Immunol. 141*: 996-1005.

21. Antal-Szalmas P., Van Strijp J. A. G., Weersink A. J. L., Verhoef J., and Van Kessel K. P. M. 1997. Quantitatiion of surface CD14 on human monocytes and neutrophils. *J. Leuk. Biol. 61*: 721-728.

22. Chen T.-Y., Bright S. W., Pace J. L., Russell S. W., and Morrison D. C. 1990. Induction of macrophage-mediated tumor cytotoxicity by a hamster monoclonal antibody with specificity for lipopolysaccharide receptor. *J. Immunol. 145*: 8-12.

23. Perera P. Y., Chen T.-Y., Morrison D. C., and Vogel S. N. 1992. Detection and analysis of the 80-kd lipopolysaccharide receptor in macrophages derived from Lps^n and Lps^d mice. *J. Leukocyte Biol. 51*: 501-506.

24. Dziarski R. 1994. Cell-bound albumin is the 70-kDa peptidoglycan-, lipopolysaccharide-, and lipoteichoic acid-binding protein on lymphocytes and macrophages. *J. Biol. Chem. 269*: 20431-20436.

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

26. Golenbock D. T., Liu Y., Millham F. H., Freeman M. W., and Zoeller R. A. 1993. Surface expression of human CD14 in Chinese hamster ovary fibroblasts imparts macrophage-like responsiveness to bacterial endotoxin. *J. Biol. Chem. 268*: 22055-22059.

27. Ingalls R. R. and Golenbock D. T. 1995. CD11c/CD18, a transmembrane signaling receptor for lipopolysaccharide. *J. Exp. Med. 181*: 1473-1479.

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

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

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

31. Wright S. D., Detmers P. A., Aida Y. *et al.* 1990. CD18-deficient cells respond to lipopolysaccharide in vitro. *J. Immunol. 144*: 2566-2571.

32. Kirkland T. N., Finley F., Leturcq D *et al.* 1993. Analysis of lipopolysaccharide binding by CD14. *J. Biol. Chem. 268*: 24818-24823.

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

34. Joseph C. K., Wright S. D., Bornmann W. G. *et al.* 1994. Bacterial lipopolysaccharide has structural similarity to ceramide and stimulates ceramide-activated protein kinase in myeloid cells. *J. Biol. Chem. 269*: 17606-17610.

35. Martin T. R., Mongovin S. M., Tobias P. S. *et al.* 1994. The CD14 differentiation antigen mediates the development of endotoxin responsiveness during differentiation of mononuclear phagocytes. *J. Leukocyte Biol. 56*: 1-9.

36. LeGrand C. B. and Thieringer R. 1994. CD14-dependent induction of protein tyrosine phosphorylation by lipopolysaccharide in murine B-lymphoma cells. *Biochim. Biophys. Acta Mol. Cell Res. 1223*: 36-46.

37. Han J., Lee J.-D., Tobias P. S., and Ulevitch R. J. 1993. Endotoxin induces rapid protein tyrosine phosphorylation in 70Z/3 cells expressing CD14. *J. Biol. Chem. 268*: 25009-25014.

38. Haziot A., Tsuberi B.-Z., and Goyert S. M. 1993. Neutrophil CD14: biochemical properties and role in the secretion of tumor necrosis factor-α in response to lipopolysaccharide. *J. Immunol. 150*: 5556-5565.

39. Schubert J., Ostendorf T., and Schmidt R. E. 1994. Biology of GPI anchors and pathogenesis of paroxysmal nocturnal hemoglobinuria. *Immunol. Today 15*: 299-301.

40. Kuhns D. B., Priel D. A. L., and Gallin J. I. 1997. Endotoxin and IL-1 hyporesponsiveness in a patient with recurrent bacterial infections. *J. Immunol. 158*: 3959-3964.

41. Tobias P. S., Soldau K., and Ulevitch R. J. 1986. Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. *J. Exp. Med. 164*: 777-793.

42. Schumann R. R., Leong S. R., Flaggs G. W. *et al.* 1990. Structure and function of lipopolysaccharide binding protein. *Science 249*: 1429-1431.

43. Tobias P. S., Soldau K., and Ulevitch R. J. 1989. Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. *J. Biol. Chem. 264*: 10867-10871.

44. Juan T. S.-C., Hailman E., Kelley M. J., Wright S. D., and Lichenstein H. S. 1995. Identification of a domain in soluble CD14 essential for lipopolysaccharide (LPS) signaling but not LPS binding. *J. Biol. Chem. 270*: 17237-17242.

45. Theofan G., Horwitz A. H., Williams R. E. *et al.* 1994. An amino-terminal fragment of human lipopolysaccharide-binding protein retains lipid A binding but not CD14-stimulatory activity. *J. Immunol. 152*: 3623-3629.

46. Mathison J. C., Tobias P. S., Wolfson E., and Ulevitch R. J. 1992. Plasma lipopolysaccharide (LPS)-binding protein: A key component in macrophage recognition of Gram-negative LPS. *J. Immunol. 149*: 200-206.

47. Betz Corradin S., Mauël J., Gallay P., Heumann D., Ulevitch R. J., and Tobias P. S. 1992. Enhancement of murine macrophage binding of and response to bacterial lipopolysaccharide (LPS) by LPS-binding protein. *J. Leukocyte Biol. 52*: 363-368.

48. Martin T. R., Mathison J. C., Tobias P. S. *et al.* 1992. Lipopolysaccharide binding protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide. Implications for cytokine production in normal and injured lungs. *J. Clin. Invest. 90*: 2209-2219.

49. Hailman E., Vasselon T., Kelley M. *et al.* 1996. Stimulation of macrophages and neutrophils by complexes of lipopolysaccharideand soluble CD14. *J. Immunol. 156*: 4384-4390.

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

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

52. Bufler P., Stiegler G., Schuchmann M. *et al.* 1995. Soluble lipopolysaccharide receptor (CD14) is release via two different mechanisms from human monocytes and CD14 transfectants. *Eur. J. Immunol. 25*: 604-610.

53. Camussi G., Mariano F., Biancone L. *et al.* 1995. Lipopolysaccharide binding protein and CD14 modulate the synthesis of platelet-activating factor by human monocytes and mesangial and endothelial cells stimulated with lipopolysaccharide. *J. Immunol. 155*: 316-324.

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

55. Read M. A., Cordle S. R., Veach R. A., Carlisle C. D., and Hawiger J. 1993. Cell-free pool of CD14 mediates activation of transcription factor NF-κB by lipopolysaccharide in human endothelial cells. *Proc. Natl. Acad. Sci. USA 90*: 9887-9891.

56. Frey E. A., Miller D. S., Jahr T. G. *et al.* 1992. Soluble CD14 participates in the response of cells to lipopolysaccharide. *J. Exp. Med. 176*: 1665-1671.

57. Vita N., Lefort S., Sozzani P. *et al.* 1997. Detection and biochemical characteristics of the receptor for complexes of soluble CD14 and bacterial lipopolysaccharide. *J. Immunol. 158*: 3457-3462.

58. Haziot A., Rong G. W., Lin X.-Y., Silver J., and Goyert S. M. 1995. Recombinant soluble CD14 prevents mortality in mice treated with endotoxin (lipopolysaccharide). *J. Immunol. 154*: 6529-6532.

59. Juan T. S.-C., Kelley M.J., Johnson D. A. *et al.* 1995. Soluble CD14 truncated at amino acid 152 binds lipopolysaccharide (LPS) and enables cellular response to LPS. *J. Biol. Chem. 270*: 1382-1387.

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

61. Weidemann B., Brade H., Rietschel E. T. *et al.* 1994. Soluble peptidoglycan-induced monokine production can be blocked by anti-CD14 monoclonal antibodies and by lipid A partial structures. *Infect. Immun. 62*: 4709-4715.

62. Savedra R., Jr., Delude R. L., Ingalls R. R., Fenton M. J., and Golenbock D. T. 1996. Mycobacterial lipoarabinomannan recognition requires a receptor that shares components of the endotoxin signaling system. *J. Immunol. 157*: 2549-2554.

63. Delude R. L., Savedra R., Jr., Zhao H. L. *et al.* 1995. CD14 enhances cellular responses to endotoxin without imparting ligand-specific recognition. *Proc. Natl. Acad. Sci. USA 92*: 9288-9292.

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

65. Wright S. D. 1995. CD14 and innate recognition of bacteria. *J. Immunol. 155*: 6-8.

66. Golenbock D. T., Hampton R. Y., Qureshi N., Takayama K., and Raetz C. R. H. 1991. Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. *J. Biol. Chem. 266*: 19490-19498.

67. Aida Y., Kusumoto K., Nakatomi K., Takada H., Pabst M. J., and Maeda K. 1995. An analogue of lipid A and LPS from *Rhodobacter sphaeroides* inhibits neutrophil responses to LPS by blocking receptor recognition of LPS and by depleting LPS-binding protein in plasma. *J. Leuk. Biol. 58*: 675-682.

68. Fant W. J., Johnson .D. E, and Williams L. T. 1993. Signalling by receptor tyrosine kinases. *Annu. Rev. Biochem. 62*: 453-481.

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

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

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

72. Arditi M., Zhou J., Torres M., Durden D. L., Stins M., and Kim K. S. 1995. Lipopolysaccharide stimulates the tyrosine phosphorylation of mitogen-activated protein kinases p44, p42 and p41 in vascular endothelial cells in a soluble CD14-dependent manner. Role of protein tyrosine phosphorylation in lipopolysaccharide-induced stimulation of endothelial cells. *J. Immunol. 155*: 3994-4003.

73. Dong Z., O'Brian C. A., and Fidler I. J. 1993. Activation of tumoricidal properties in macrophages by lipopolysaccharide requires protein-tyrosine kinase activity. *J. Leukocyte Biol. 53*: 53-60.

74. Dong Z., Qi X., Xie K., and Fidler I. J. 1993. Protein tyrosine kinase inhibitors decrease induction of nitric oxide synthase activity in lipopolysaccharide-responsive and lipopolysaccharide-nonresponsive murine macrophages. *J. Immunol. 151*: 2717-2724.

75. Novogrodsky A., Vanichkin A., Patya M., Gazit A., Osherov N., and Levitzki A. 1994. Prevention of lipopolysaccharide-induced lethal toxicity by tyrosine kinase inhibitors. *Science 264*: 1319-1322.

76. Delude R. L., Fenton M. J., Savedra R., Jr. *et al.* 1994. CD14-mediated translocation of nuclear factor-κB induced by lipopolysaccharide does not require tyrosine kinase activity. *J. Biol. Chem. 269*: 22253-22260.

77. Yoza B. K., Hu J. Y. Q., and McCall C. E. 1996. Protein-tyrosine kinase activation is required for lipopolysaccharide induction of interleukin 1β and NFκB activation, but not NFκB nuclear translocation. *J. Biol. Chem. 271*: 18306-18309.

78. Ziegler S. F., Wilson C. B., and Perlmutter R. M. 1988. Augmented expression of a myeloid-specific protein tyrosine kinase gene *(hck)* after macrophage activation. *J. Exp. Med.* 1801-1810.

79. Boulet I., Ralph S., Stanley E. *et al.* 1992. Lipopolysaccharide- and interferon-γ-induced expression of *hck* and *lyn* tyrosine kinases in murine bone marrow-derived macrophages. *Oncogene 7*: 703-710.

80. Stefanová I., Corcoran M. L., Horak E. M., Wahl L. M., Bolen J. B., and Horak I. D. 1993. Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase $p53/56^{lyn}$. *J. Biol. Chem. 268*: 20725-20728.

81. Shenoy-Scaria A. M., Kwong J., Fujita T., Olszowy M. W., Shaw A. S., and Lublin D. M. 1992. Signal transduction through decay-accelerating factor: Interaction of glycosyl-phosphatidylinositol anchor and protein tyrosine kinases $p56^{lck}$ and $p59^{fyn\ 1}$. *J. Immunol. 149*: 3535-3541.

82. Shenoy-Scaria A. M., Timson Gauen L. K., Kwong J., Shaw A. S., and Lublin D. M. 1993. Palmitylation of an amino-terminal cysteine motif of protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ mediates interaction with glycosyl-phosphatidylinositol-anchored proteins. *Mol. Cell. Biol. 13*: 6385-6392.

83. Stefanová I., Horejsí V., Ansotegui I. J., Knapp W., and Stockinger H. 1991. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science 254*: 1016-1019.

84. Meng F. Y. and Lowell C. A. 1997. Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. *J. Exp. Med. 185*: 1661-1670.

85. Stephens L. R., Jackson T. R., and Hawkins P. T. 1993. Agonist-stimulated synthesis of phosphatidylinositol(3,4, 5)-trisphosphate: A new intracellular signalling system. *Biochim. Biophys. Acta Mol. Cell Res. 1179*: 27-75.

86. Fry M. J. 1994. Structure, regulation and function of phosphoinositide 3-kinases. *Biochim. Biophys. Acta Mol. Basis Dis. 1226*: 237-268.

87. Stephens L., Jackson T., and Hawkins P. T. 1993. Synthesis of phosphatidylinositol 3,4,5-trisphosphate in permeabilized neutrophils regulated by receptors and G-proteins. *J. Biol. Chem. 268*: 17162-17172.

88. Stephens L. R., Hughes K. T., and Irvine R. F. 1991. Pathway of phosphatidylinositol(3,4,5)-trisphosphate synthesis in activated neutrophils. *Nature 351*: 33-39.

89. Herbst J. J., Andrews G., Contillo L. *et al.* 1994. Potent activation of phosphatidylinositol 3'-kinase by simple phosphotyrosine peptides derived from insulin receptor substrate 1 containing two YMXM motifs for binding SH2 domains. *Biochemistry 33*: 9376-9381.

90. Mak P., He Z. Q., and Kurosaki T. 1996. Identification of amino acid residues required for a specific interaction between Src-tyrosine kinase and proline-rich region of phosphatidylinositol-3' kinase. *FEBS Lett. 397*: 183-185.

91. Pleiman C. M., Hertz W. M., and Cambier J. C. 1994. Activation of phosphatidylinositol-3' kinase by Src-family kinase SH3 binding to the p85 subunit. *Science 263*: 1609-1612.

92. Stephens L., Smrcka A., Cooke F. T., Jackson T. R., Sternweis P. C., and Hawkins P. T. 1994. A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein $\beta\gamma$ subunits. *Cell 77*: 83-93.

93. Stoyanov B., Volinia S., Hanck T. *et al.* 1995. Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science 269*: 690-693.

94. Stephens L. R., Eguinoa A., Erdjument-Bromage H. *et al.* 1997. The $G\beta\gamma$ sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell 89*: 105-114.

95. Gold M. R. and Aebersold R. 1994. Both phosphatidylinositol 3-kinase and phosphatidylinositol 4-kinase products are increased by antigen receptor signaling in B cells. *J. Immunol. 152*: 42-50.

96. Gold M. R., Chan, V., Turck C. W., and DeFranco A. L. 1992. Membrane Ig cross-linking regulates phosphatidylinositol 3-kinase in B lymphocytes. *J. Immunol. 148*: 2012-2022.

97. Ballou L. R., Barker S. C., Postlethwaite A. E., and Kang A. H. 1991. Interleukin 1 stimulates phosphatidylinositol kinase activity in human fibroblasts. *J. Clin. Invest. 87*: 299-304.

98. Toker A. and Cantley L. G. 1997. Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature 387*: 673-676.

99. Monfar M., Lemon K. P., Grammer T. C. *et al.* 1995. Activation of pp70/85 S6 kinases in interleukin-2-responsive lymphoid cells is mediated by phosphatidylinositol 3-kinase and inhibited by cyclic AMP. *Mol. Cell. Biol. 15*: 326-337.

100. Kilgour E., Gout I., and Anderson N. G. 1996. Requirement for phosphoinositide 3-OH kinase in growth hormone signalling to the mitogen-activated protein kinase and $p70^{s6k}$ pathways. *Biochem. J. 315*: 517-522.

101. Ferby I. M., Waga I., Sakanaka C., Kume K., and Shimizu T. 1994. Wortmannin inhibits mitogen-activated protein kinase activation induced by platelet-activating factor in guinea pig neutrophils. *J. Biol. Chem. 269*: 30485-30488.

102. Chung J., Grammer T. C., Lemon K. P., Kazlauskas A., and Blenis J. 1994. PDGF- and insulin-dependent $pp70^{S6k}$ activation mediated by phosphatidylinositol-3-OH kinase. *Nature 370*: 71-75.

103. Burgering B. M. T. and Coffer P.J. 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature 376*: 599-602.

104. James S.R., Downes C. P., Gigg R., Grove S. J. A, Holmes A. B., and Alessi D. R. 1996. Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. *Biochem. J. 315*: 709-713.

105. Franke T. F., Kaplan D. R., Cantley L. C., and Toker A. 1997. Direct regulation of the *Akt* proto-oncogene product by phosphatidylinositol-3-4-bisphosphate. *Science 275*: 665-668.

106. DownwarD. J. 1995. Signal transduction: A target for PI(3) kinase. *Nature 376*: 553-554.

107. Klarlund J. K., Guilherme A., Holik J. J., Virbasius J. V., Chawla A., and Czech M. P. 1997. Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing pleckstrin and Sec7 homology domains. *Science 275*: 1927-1930.

108. Alessi D. R., James S. R., Downes C. P. *et al.* 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates PKB-α. *Curr. Biol. 7*: 261-269.

109. Toker A., Meyer M., Reddy K. K. *et al.* 1994. Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3. *J. Biol. Chem. 269*: 32358-32367.

110. Schu P. V., Takegawa K., Fry M. J., Stack J. H., Waterfield M. D., and Emr S. D. 1993. Phosphatidylinositol 3-kinase encoded by yeast *VPS*34 gene essential for protein sorting. *Science 260*: 88-91.

111. Aagaard-Tillery K. M. and Jelinek D. F. 1996. Phosphatidylinositol 3-kinase activation in normal human B lymphocytes. *J. Immunol. 156*: 4543-4554.

112. Okada T., Sakuma L., Fukui Y., Hazeki O., and Ui M. 1994. Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. *J. Biol. Chem. 269*: 3563-3567.

113. Vlahos C. J., Matter W. F., Brown R. F. *et al.* 1995. Investigation of neutrophil signal transduction using a specific inhibitor of phosphatidylinositol 3-kinase. *J. Immunol. 154*: 2413-2422.

114. Arcaro A. and Wymann M. P. 1994. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem. J. 296*: 297-301.

115. Gómez J., García A., Borlado L. R. *et al.* 1997. IL-2 signaling controls actin organization through Rho-like protein family, phosphatidylinositol 3-kinase, and protein kinase C-ζ. *J. Immunol. 158*: 1516-1522.

116. Hug H. and Sarre T. F. 1993. Protein kinase C isoenzymes: Divergence in signal transduction. *Biochem. J. 291*: 329-343.

117. Stabel S. and Parker P. J. 1991. Protein kinase C. *Pharmacol. Ther. 51*: 71-95.

118. Ways D. K., Cook P. P., Webster C., and Parker P. J. 1992. Effect of phorbol esters on protein kinase C-ζ. *J. Biol. Chem. 267*: 4799-4805.

119. Goodnight J., Kazanietz M. G., Blumberg P. M., Mushinski J. F., and Mischak H. 1992. The cDNA sequence, expression pattern and protein characteristics of mouse protein kinase C-ζ. *Gene 122*: 305-311.

120. Liyanage M., Frith D., Livneh E., and Stabel S. 1992. Protein kinase C group B members PKC-δ, -ϵ, -ζ and PKC-Λ(eta). Comparison of properties of recombinant proteins *in vitro* and *in vivo*. *Biochem. J. 283*: 781-787.

121. Nishizuka Y. 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science 258*: 607-614.

122. Zhou G., Wooten M. W., and Coleman E. S. 1994. Regulation of atypical ζ-protein kinase C in cellular signaling. *Exp. Cell Res. 214*: 1-11.

123. Kazanietz M. G., Areces L. B., Bahador A. *et al.* 1993. Characterization of ligand and substrate specificity for the calcium-dependent and calcium-independent protein kinase C isozymes. *Mol. Pharmacol. 44*: 298-307.

124. Nakanishi H., Brewer K. A., and Exton J. H. 1993. Activation of the ζ isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem. 268*: 13-16.

125. Novotney M., Chang Z., Uchiyama H., and Suzuki T. 1991. Protein kinase C in tumoricidal activation of mouse macrophage cell lines. *Biochemistry 30*: 5597-5604.

126. Shapira L., Takashiba S., Champagne C., Amar S., and Van Dyke T. E. 1994. Involvement of protein kinase C and protein tyrosine kinase in lipopolysaccharide-induced TNF-α and IL-1β production by human monocytes. *J. Immunol. 153*: 1818-1824.

127. Shinji H., Akagawa K. S., and Yoshida T. 1994. LPS induces selective translocation of protein kinase C-β in LPS-responsive mouse macrophages, but not in LPS-nonresponsive mouse macrophages. *J. Immunol. 153*: 5760-5771.

128. Hamilton T. A., Becton D. L., Somers S. D., Gray P. W., and Adams D. O. 1985. Interferon-γ modulates protein kinase C activity in murine peritoneal macrophages. *J. Biol. Chem. 260*: 1378-1381.

129. Fujihara M., Connolly N., Ito N., and Suzuki T. 1994. Properties of protein kinase C isoforms (βII, ∈, and ζ) in a macrophage cell line (J744) and their roles in LPS-induced nitric oxide production. *J. Immunol. 152*: 1898-1906.

130. Chang Z.-L. and Beezhold D H. 1993. Protein kinase C activation in human monocytes: Regulation of PKC isoforms. *Immunology 80*: 360-366.

131. Kiley S. C.and Parker P. J. 1995. Differential localization of protein kinase C isozymes in U937 cells: Evidence for distinct isozyme functions during monocyte differentiation. *J. Cell Sci. 108*: 1003-1016.

132. Dominguez I., Sanz L., Arenzana-Seisdedos F., Diaz-Meco M. T., Virelizier J.-L., and Moscat J. 1993. Inhibition of protein kinase C ζ subspecies blocks the activation of an NF-κB-like activity in *Xenopus laevis* oocytes. *Mol. Cell. Biol. 13*: 1290-1295.

133. Diaz-Meco M. T., Berra E., Municio M. M. *et al.* 1993. A dominant negative protein kinase C ζ subspecies blocks NF-κB activation. *Mol. Cell. Biol. 13*: 4770-4775.

134. Lozano J. , Berra E., Municio M. M. *et al.* 1994. Protein kinase C ζ isoform is critical for κB-dependent promoter activation by sphingomyelinase. *J. Biol. Chem. 269*: 19200-19202.

135. Therrien M., Chang H. C., Solomon N. M., Karim F. D., Wasserman D. A., and Rubin G. M. 1995. KSR, a novel protein kinase required for RAS signal transduction. *Cell 83*: 879-888.

136. Zhang Y., Yao ., Delikat S. *et al.* 1997. Kinase suppressor of Ras is ceramide-activated protein kinase. *Cell 89*: 63-72.

137. Barber S. A., Perera P.-Y., and Vogel S. N. 1995. Defective ceramide response in C3H/HeJ (*Lps*^{d}) macrophages. *J. Immunol. 155*: 2303-2305.

138. Dong Z., Qi X., and Fidler I. J. 1993. Tyrosine phosphorylation of mitogen-activated protein kinases is necessary for activation of murine macrophages by natural and synthetic bacterial products. *J. Exp. Med. 177*: 1071-1077.

139. Nick J. A., Avdi N. J., Gerwins P., Johnson G. L., and Worthen G. S. 1996. Activation of a p38 mitogen-activated protein kinase in human neutrophils by lipopolysaccharide. *J. Immunol. 156*: 4867-4875.

140. Han J., Lee J.-D., Bibbs L., and Ulevitch R. J. 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science 265*: 808-811.

141. Blenis J. 1993. Signal transduction via the MAP kinases: Proceed at your own RSK. *Proc. Natl. Acad. Sci. USA 90*: 5889-5892.

142. Matsuda S., Gotoh Y., and Nishida E. 1994. Signaling pathways mediated by the mitogen-activated protein (MAP) kinase kinase/MAP kinase cascade. *J. Leukocyte Biol. 56*: 548-553.

143. Büscher D., Hipskind R. A., Krautwald S., Reimann T., and Baccarini M. 1995. Ras-dependent and -independent pathways target the mitogen-activated protein kinase network in macrophages. *Mol. Cell. Biol. 15*: 466-475.

144. Geng Y., Gulbins E., Altman A., and Lotz M. 1994. Monocyte deactivation by interleukin 10 via inhibition of tyrosine kinase activity and the Ras signaling pathway. *Proc. Natl. Acad. Sci. USA 91*: 8602-8606.

145. Olivier M., Baimbridge K. G., and Reiner N. E. 1992. Stimulus-response coupling in monocytes infected with leishmania: attenuation of calcium transients is related to

defective agonist-induced accumulation of inositol phosphates. *J. Immunol. 148*: 1188-1196.

146. Durocher Y., Chapdelaine A., and Chevalier S. 1992. Identification of cytosolic protein tyrosine kinases of human prostate by renaturation after SDS/PAGE. *Biochem. J. 284*: 653-658.

147. Sanghera J. S., Aebersold R., Morrison H. D., Bures E. J., and Pelech S. L. 1990. Identification of the sites in myelin basic protein that are phosphorylated by meiosis-activated protein kinase p44. *FEBS Lett. 273*: 223-226.

148. Gold M. R., Duronio V., Saxena S. P., Schrader J. W., and Aebersold R. 1994. Multiple cytokines activate phosphatidylinositol 3-kinase in hemopoietic cells. Association of the enzyme with various tyrosine-phosphorylated proteins. *J. Biol. Chem. 269*: 5403-5412.

149. Hara K., Yonezawa K., Sakaue H. *et al.* 1994. 1-Phosphatidylinositol 3-kinase activity is required for insulin stimulated glucose transport but not for RAS activation in CHO cells. *Proc. Natl. Acad. Sci. USA 91*: 7415-7419.

150. Hobbs M. V., Weigle W. O., Noonan D. J. *et al.* 1993. Patterns of cytokine gene expression by $CD4^+$ T cells from young and old mice. *J. Immunol. 150*: 3602-3614.

151. Bradford M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*: 248-254.

152. Peterson G. L. 1979. Review of the folin phenol protein quantitation method of Lowry, Rosebrough, Farr, and Randall. *Anal. Biochem. 100*: 201-220.

153. Clark-Lewis ., Sanghera J. S., and Pelech S. L. 1991. Definition of a consensus sequence for peptide substrate recognition by $p44^{mpk}$, the meiosis-activated myelin basic protein kinase. *J. Biol. Chem. 266*: 15180-15184.

154. Nakanishi H. and Exton J. H. 1992. Purification and characterization of the ζ isoform of protein kinase C from bovine kidney. *J. Biol. Chem. 267*: 16347-16354.

155. Kodaki T., Woscholski R., Hallberg B., Rodriguez-Viciana P., Downward J., and Parker P. J. 1994. The activation of phosphatidylinositol 3-kinase by Ras. *Curr. Biol. 4*: 798-806.

156. Rodriguez-Viciana P., Warne P. H., Dhand R. *et al.* 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature 370*: 527-532.

157. Ferrero E., Jiao D., Tsuberi B.-Z. *et al.* 1993. Transgenic mice expressing human CD14 are hypersensitive to lipopolysaccharide. *Proc. Natl. Acad. Sci. 90*: 2380-2384.

158. Perera P. Y., Vogel S. N., Detore G. R., Haziot A., and Goyert S. M. 1997. CD14-dependent and CD14-independent signaling pathways in murine macrophages from normal and CD14 knockout mice stimulated with lipopolysaccharide or taxol. *J. Immunol. 158*: 4422-4429.

159. Beaty C. D., Franklin T. L., Uehara Y., and Wilson C. B. 1994. Lipopolysaccharide-induced cytokine production in human monocytes: Role of tyrosine phosphorylation in transmembrane signal transduction. *Eur. J. Immunol. 24*: 1278-1284.

160. Hibbs M. L., Tarlinton D. M., Armes J. *et al.* 1995. Multiple defects in the immune system of *Lyn*-deficient mice, culminating in autoimmune disease. *Cell 83*: 301-311.

161. Lowell C. A., Soriano P., and Varmus H. E. 1994. Functional overlap in the *src* gene family: Inactivation of *hck* and *fgr* impairs natural immunity. *Genes Dev. 8*: 387-398.

162. Wang L.-M., Myers M. G., Jr., Sun X.-J., Aaronson S. A., White M., and Pierce J. H. 1993. IRS-1: Essential for insulin- and IL-4-stimulated mitogenesis in hematopoietic cells. *Science 261*: 1591-1594.

163. Frendl, G. and Guan, R. J. Mitogen-activated protein (MAP) kinase is a common element of multiple macrophage activation pathways: activation of MAP kinase by lipopolysaccharide, granulocyte-macrophage colony-stimulating factor, and other macrophage-activating factors. In: *Immunotherapy of Infections*, edited by Masihi, K.N. N.Y./Basel/H.K. Marcel Dekker Inc. 1994, p. 421-453.

164. Sanghera J. S., Weinstein S. L., Aluwalia M., Girn J., and Pelech S. L. 1996. Activation of multiple proline-directed kinases by bacterial lipopolysaccharide in murine macrophages. *J. Immunol. 156*: 4457-4465.

165. Winston B. W. and Riches D. W. H. 1997. Activation of p42*mapk/erk2* following engagement of tumor necrosis factor receptor CD120a (p55) in mouse macrophages. *J. Immunol. 155*: 1525-1533.

166. Lee J. C. and Young P. R. 1996. Role of CSBP/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. *J. Leukocyte Biol. 59*: 152-157.

167. Liu M. K., Herrera-Velit P., Brownsey R. W., and Reiner N. E. 1994. CD14-dependent activation of protein kinase C and mitogen-activated protein kinases (p42 and p44) in human monocytes treated with bacterial lipopolysaccharide. *J. Immunol. 153*: 2642-2652.

168. Whitman M., Kaplan D. R., Schaffhausen B., Cantley L., and Roberts T. M. 1985. Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. *Nature 315*: 239-242.

169. Taichman R., Merida I., Torigoe T., Gaulton G. N., and Reed J. C. 1993. Evidence that protein tyrosine kinase p56-*lck* regulates the activity of phosphatidylinositol-3'-kinase in interleukin-2-dependent T-cells. *J. Biol. Chem. 268*: 20031-20036.

170. Yamanashi Y., Fukui Y., Wongsasant B. *et al.* 1992. Activation of Src-like protein-tyrosine kinase Lyn and its association with phosphatidylinositol 3-kinase upon B-cell antigen receptor-mediated signaling. *Proc. Natl. Acad. Sci. USA 89*: 1118-1122.

171. Ren C. L., Morio T., Fu S. M., and Geha R. S. 1994. Signal transduction via CD40 involves activation of *lyn* kinase and phosphatidylinositol-3-kinase, and phosphorylation of phospholipase Cγ2. *J. Exp. Med. 179*: 673-680.

172. Rordorf-Nikolic T., Van Horn D. J., Chen D., White M. F., and Backer J. M. 1995. Regulation of phosphatidylinositol 3'-kinase by tyrosyl phosphoproteins. Full activation requires occupancy of both SH2 domains in the 85-kDa regulatory subunit. *J. Biol. Chem. 270*: 3662-3666.

173. Carpenter C. L., Auger K. R, Chanudhuri M. *et al.* 1993. Phosphoinositide 3-kinase is activated by phosphopeptides that bind to the SH2 domains of the 85-kDa subunit. *J. Biol. Chem. 268*: 9478-9483.

174. Van der Geer P., Hunter T., and Lindberg R. A. 1994. Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu. Rev. Cell Biol. 10*: 251-337.

175. Gulbins E., Coggeshall K. M., Baier G., Katzav S., Burn P., and Altman A. 1993. Tyrosine kinase-stimulated guanine nucleotide exchange activity of Vav in T cell activation. *Science 260*: 822-825.

176. Bustelo X. R., Ledbetter J. A., and Barbacid M. 1992. Product of *vav* proto-oncogene defines a new class of tyrosine protein kinase substrates. *Nature 356*: 68-71.

177. Bustelo X. R. and Barbacid M. 1992. Tyrosine phosphorylation of the *vav* proto-oncogene product in activated B cells. *Science 256*: 1196-1199.

178. Matsuo T., Hazeki K., Hazeki O., Katada T., and Ui M. 1996. Activation of phosphatidylinositol 3-kinase by concanavalin A through dual signalling pathways, G-protein-coupled and phosphotyrosine-related, and an essential role of the G-protein-coupled signals for the lectin-induced respiratory burst in human monocytic THP-1 cells. *Biochem. J. 315*: 505-512.

179. Hawes B. E., Luttrell L. M., Van Biesen T., and Lefkowitz R. J. 1996. Phosphatidylinositol 3-kinase is an early intermediate in the Gβγ-mediated mitogen-activated protein kinase signaling pathway. *J. Biol. Chem. 271*: 12133-12136.

180. Ettinger S. L., Lauener R. W., and Duronio V. 1996. Protein kinase C δ specifically associates with phosphatidylinositol 3-kinase following cytokine stimulation. *J. Biol. Chem. 271*: 14514-14518.

181. Baier G., Telford D., Giampa L. *et al.* 1993. Molecular cloning and characterization of PKCθ, a novel member of the protein kinase C (PKC) gene family expressed predominantly in hematopoietic cells. *J. Biol. Chem. 268*: 4997-5004.

182. Bakouche O., Moreau J. L., and Lachman L. B. 1992. Secretion of IL-1: Role of protein kinase C. *J. Immunol. 148*: 84-91.

183. Nishida E. and Gotoh Y. 1993. The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci. 18*: 128-131.

184. Selbie L. A., Schmitz-Peiffer C., Sheng Y., and Biden T. J. 1993. Molecular cloning and characterization of PKCι, an atypical isoform of protein kinase C derived from insulin-secreting cells. *J. Biol. Chem. 268*: 24296-24302.

185. Batlle E., Fabre M., and De Herreros A. G. 1994. Antipeptide antibodies directed against the C-terminus of protein kinase Cζ (PKCζ) react with a Ca^{2+}- and TPA-sensitive PKC in HT-29 human intestinal epithelial cells. *FEBS Lett. 344*: 161-165.

186. Kochs G., Hummel R., Meyer D., Hug H., Marmé D., and Sarre T. F. 1993. Activation and substrate specificity of the human protein kinase C α and ζ isoenzymes. *Eur. J. Biochem. 216*: 597-606.

187. Shinomura T., Asaoka Y., Oka M., Yoshida K., and Nishizuka Y. 1991. Synergistic action of diacylglycerol and unsaturated fatty acids for protein kinase C activation: its possible implications. *Proc. Natl. Acad. Sci. USA 88*: 5149-5153.

188. Newton A. C. 1995. Protein kinase C: Structure, function, and regulation. *J. Biol. Chem. 270*: 28495-28498.

189. Mizukami Y., Hirata T., and Yoshida K. 1997. Nuclear translocation of PKCζ during ischemia and its inhibition by wortmannin, an inhibitor of phosphatidylinositol 3-kinase. *FEBS Lett. 401*: 247-251.

190. Cross M. J., Stewart A., Hodgkin M. N., Kerr D. J., and Wakelam M. J. O. 1995. Wortmannin and its structural analogue demethoxyviridin inhibit stimulated phospholipase A_2 activity in Swiss 3T3 cells - Wortmannin is not a specific inhibitor of phosphatidylinositol 3-kinase. *J. Biol. Chem. 270*: 25352-25355.

191. Vlahos C. J., Matter W. F., Hui K. Y., and Brown R. F. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem. 269*: 5241-5248.

192. Cotten, M., Wagner, E. and Birnstiel, M. L. Receptor-mediated transport of DNA into eukaryotic cells. In: *Methods in Enzymology*, edited by Wu, R. New York: Academic Press, 1993, p. 618-635.

193. Gallay P., Jongeneel C. V., Barras C. *et al.* 1993. Short time exposure to lipopolysaccharide is sufficient to activate human monocytes. *J. Immunol. 150*: 5086-5093.

194. Gessani S., Testa U., Varano B. *et al.* 1993. Enhanced production of LPS-induced cytokines during differentiation of human monocytes to macrophages: Role of LPS receptors. *J. Immunol. 151*: 3758-3766.

195. Derman M. P., Toker A., Hartwig J. H. *et al.* 1997. The lipid products of phosphoinositide 3-kinase increase cell motility through protein kinase C. *J. Biol. Chem. 272*: 6465-6470.

196. Jakway J. P. and DeFranco A. L. 1986. Pertussis toxin inhibition of B cell and macrophage responses to bacterial lipopolysaccharide. *Science 234*: 743-746.