

CONTRIBUTION OF RECEPTORS TO
Neisseria meningitidis INTERACTIONS
WITH HUMAN MACROPHAGES

A thesis submitted for the degree of
Doctor of Philosophy

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**Contributions of receptors to *N. meningitidis* interactions with
human macrophages**

Abstract

N. meningitidis is a gram-negative human pathogen that invades human cells, evades immune responses and goes on to cause meningococcal disease, sepsis and death. Serogroups B and C are most prevalent in industrialised countries, with over 50% of cases due to serogroup B, which as yet, has no effective vaccine. The work described involved developing methods and adapting effective model systems used throughout this study, and examination of which possible opsonic receptors may be involved in bacterial interactions in the presence of serum. The study revealed the involvement of CD14 in meningococcal recognition was examined using a number of approaches, including glycosylphosphatidylinositol- (GPI) cleaving enzyme, phosphatidylinositol phospholipase C (PIPLC), appropriate anti-CD14 blocking antibody and CD14 transfectants. Class A scavenger receptor (SR-A) were also found to contribute significantly to meningococcal recognition by human macrophages. This observation was supported by the use of THP-1 monocytic cells; PMA-differentiation resulted in recovery of phagocytosis that could be inhibited with SR-A blocking reagent, Poly I. Immune serum-mediated interactions implicated CR3 and Fcγ receptor involvement as well as that of cholesterol-rich lipid rafts. I also examine the role of non-opsonic receptors by using the first viable LPS-deficient strain of *N. meningitidis*, and note that the *lpxA* mutant adheres to primary human macrophages more effectively than its parent strain, but internalisation, LAMP-1 incorporation and TNF production were all severely reduced.

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NUMBERING

AS ORIGINAL

Abbreviations

acLDL	acetylated low-density lipoprotein
ATCC	American Tissue Culture Collection
BHI	brain heart infusion broth
bp	base pairs
c.f.u	colony forming units
CD	cluster of differentiation
CR3	complement receptor 3
CSF	cerebrospinal fluid
dH ₂ O	distilled water
DiI	1,1' dioctadecyl-1-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DiI-acLDL	DiI-labelled acetylated low-density lipoprotein
DMEM	Dulbecco's modification of Eagle's Medium
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
FcR	Immunoglobulin Fc receptor
FCS	foetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
HIFCS	heat-inactivated foetal calf serum
hMSR-A	human macrophage scavenger receptor
IgG	gamma immunoglobulin
IL	interleukin
IFN- γ	gamma interferon
Kb	kilobase
kDa	kilodalton
LBP	lipid binding protein
LPS	lipopolysaccharide
LTA	lipoteichoic acid
mAb	monoclonal antibody
M-CSF	macrophage colony stimulating factor
MDM	monocyte-derived macrophages
MHC	major histocompatibility complex
MOI	multiplicity of infection
MR	mannose receptor
PAF-R	platelet-activating factor receptor
PAF-Ra	platelet-activating factor receptor antagonist
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear neutrophil

Poly C	polycytidylic acid
Poly I	polyinosinic acid
PRR	pattern recognition receptor
SEM	standard error of mean
SR	Scavenger receptor
SR-A	Class A scavenger receptor
dTHP-1	PMA-differentiated THP-1 cells
TLR	Toll-like receptor
TNF- α	tumour necrosis factor alpha
WT	wild-type

Chapter 1

Introduction

1.1 Innate Immunity

Innate immunity is essential for the recognition and destruction of pathogens in the absence of acquired immunity using a limited number of pattern recognition receptors, including phagocytic receptors. Mononuclear phagocytes, neutrophils, mast cells, complement system and anti-microbial peptides are all included in the armoury of innate immunity. Two hypotheses that complement one another are used to illustrate how the innate immune system responds to a specific antigenic attack, and describe how the body is able to distinguish between “self” and “non-self”.

Janeway (Janeway 1992) proposed that pattern recognition receptors (PRR) present on the surface of host cells were the mediators of this discriminatory function. They suggest that these receptors are germ-line encoded and able to recognise a range of conserved motifs which are prevalent on pathogens, but not host molecules. The targets for these receptors are referred to as pathogen-associated molecular patterns (PAMPs) and include components of yeast, such as mannans, components of bacteria including lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan and double-stranded RNA of viruses (Janeway 1992). Two types of PAMPs have been described, those that are cell-associated and soluble; the latter are considered to be components of the humoral immune system such as mannose binding lectin (MBL) (Medzhitov and Janeway 1997). Macrophages express a number of PRRs including CD14, complement receptor 3 (CR3), scavenger receptors (SR), mannose receptor (MR) and toll-like receptors (TLRs), which will be discussed in more detail later. Part of Janeway's hypothesis is that not only must PRRs be able to distinguish between non-self

and self, but they are also be able to recognise modified-self. Although Janeway's model of PRRs may require expansion as a number of the PRRs mentioned have endogenous and exogenous ligand and have be associated with homeostatic functions (Devitt, Moffat et al. 1998): as yet it is not known how these receptors discriminate between different ligands and carry out a suitable response. Matzinger developed this hypothesis further by suggesting the "danger theory" hypothesis (Matzinger 1998). She theorised that as well as monitoring the presence or absence of pathogens, the system may also monitor the damage by detecting molecules released by invading antigens or cells with defective innate cellular programming, and thus detect the "danger signals" released.

Her theory is supported by the identification of host-derived ligands for some PRR. Mammalian and bacterial heat-shock proteins are highly conserved and HSP60 can cause potent pro-inflammatory cytokine release in various mammalian cells as predicted by using this model (Chen, Syldath et al. 1999). Toll-like receptor 4 (TLR4) recognise some host-derived heat shock proteins such as the 60Da heat shock protein (HSP60) (Ukeki, Tabeta et al. 2002), and host response to them supports this model of innate immune function (Matzinger 1994; Kop and Medzhitov 1999).

1.2 Macrophage differentiation and ontogeny

Macrophages belong to the mononuclear-phagocyte system (MPS) (van Furth 1972) and originate in the bone marrow (BM) from stem cells (van Furth 1985). The sequence of differentiation is as follows; promonoblasts give rise to monoblasts, promonocytes, monocytes and mature macrophages (van Furth 1985). All the cell types are found in the bone marrow, and monocytes enter the circulation where they remain for 36-104h and from there, in response to environmental stimuli, they migrate into the tissue where maturation and differentiation occurs, giving rise to long-lived mature macrophages (van Furth 1985; Johnston Jr. 1988). As the body is constantly challenged with an enormous variety of exogenous material, it is essential that phagocytes, being amongst the body's first line of defence, are able to distinguish between cells or soluble molecules that are harmless or potentially dangerous ("non-infectious self and infectious non-self") and signal that danger is present (Janeway 1992).

These terminally differentiated macrophages are quite distinctive in their ability to perform a wide variety of functions. They play a central role in tissue remodelling for both development, repair and maintenance, are active in scavenging effete cells, as well as playing a role in regulating differentiation of other cell types e.g. phagocytosis, microbicidal and tumouricidal activity, antigen presentation. They can secrete a range of substances including enzymes, complement components, tissue clotting factors, growth factors and immunoregulating substances. Monocytes in circulation represent around 5% of the white blood cell count, and are heterogeneous with regard to cell density, size, morphology, cytochemistry, expression of cell surface receptors and cytokine secretion (Forster and Landy 1981; Figdor, te Velde et al. 1986). Most of this heterogeneity is produced as a result of differences in the maturational stage or activation state of the cells (Dransfield, Corcoran et al. 1988). It is unclear whether cells of the MPS all develop along a single pathway and it is suggested that macrophage heterogeneity may be based on the existence of

distinct precursor subsets in the BM. Despite their probable common BM progenitor, macrophages display considerable heterogeneity (Forster and Landy 1981; Walker, Warner et al. 1985). And unlike granulocytes, they differ considerably in phenotype and morphology depending on their location. Once in the tissue, monocytes differentiate into characteristically different tissue macrophages depending on tissue-specific stimuli present in the local microenvironment.

While the most common haemopoietic stem cell origin of macrophages is regulated by haemopoietic growth factors, including CSF and interleukin, macrophages in tissues have also been studied extensively for their self-renewal capacity *in situ* (Huang, Nocka et al. 1990). After leaving the bone marrow, the elements of most lineages of haematopoietic cells retain this attribute, including lymphocytes and mononuclear phagocytes. Proliferation of these cells is dependant on specific haematopoietic growth factors, such as GM-CSF.

1.2.1 Interactions between cytokines and macrophages

The two principle classes of professional phagocytic cells are monocyte-macrophages and polymorphonuclear leukocytes. Monocytes/macrophages are very versatile cells and play a central role in non-specific immunity through their ability to remove antigens by phagocytosis and intracellular killing. They are also involved in specific cell-mediated immunity by acting as antigen presenting cells (APC), as well as their ability to release a number of soluble mediators; cytokines. Cytokines are low molecular weight secreted proteins, usually between 15-25 kDa, and are mediators of inflammation, cell growth, immunity, differentiation and repair. Cytokines are able to act in a paracrine fashion in the local microenvironment, but also in an endocrine-like manner on distant organs. Most cytokines have a number of biological activities on different target cells,

and in addition to macrophages, a number of other cell types secrete them in response to different stimuli. So cytokines have a pleiotropic effect, in that they have multiple effects on the growth and differentiation of a number of cell types, and the expression of one cytokine is influenced by others, forming a cytokine network.

The table below lists some of the cytokines secreted by activated macrophages, and their properties:

Biological properties of some cytokines produced by activated macrophages

Cytokine	Biological properties
IL-1 α / β	Immunostimulatory molecule, mediator of shock and inflammation
IL-6	Induction of acute phase response, regulation of haematopoiesis and immune response
IL-8	Chemoattractant for granulocytes and lymphocytes
IL-10	Suppresses cytokine production (Th1 lymphocyte subset, monocytes) and monocyte/macrophage antigen-presenting function
TNF- α	Immunostimulatory molecule, mediator of shock and inflammation
M-CSF	Induction of monocyte migration, growth and differentiation factor, potentiates survival and effector function of mature monocytes/macrophages
GM-CSF	Induction of monocyte migration, growth and differentiation factor, potentiates survival and effector function of mature monocytes/macrophages
MCP	Monocyte chemoattractant protein
TGF- β	Acts on cell proliferation, fibrosis, healing angiogenesis, immune response, haematopoiesis, etc.

Adapted from (Stites, Terr et al. 1997)

1.3 Macrophage phagocytosis

Metchnikoff coined the term phagocytosis over a century ago and it is now defined as the ingestion of large particles ranging 0.5-5 μ m in diameter in an actin-based conserved mechanism. Phagocytosis is the primary defence of both innate and adaptive host defence against infectious agents as it allows the uptake and degradation of microbial pathogens, by participating in the immune and inflammatory responses.

The process of phagocytosis is comprised of several sequential and complex events that are initiated by the crucial step of recognition of the ligand destined for phagocytosis via specific receptors on the surface of phagocytic cells. The status of professional phagocytes is given to cells such as monocytes, macrophages, dendritic cells and neutrophils, whilst other cell types that display some of the attributes of professional phagocytes are referred to as semi-professional phagocytes (Rabinovitch 1995). The main differences between these types are their expression of a diverse array of dedicated phagocytic receptors designed to bind and internalise infectious agents, and the relative efficiency of particle phagocytosis.

Phagocytosis is a complex process that is initiated by ligand attachment to a specific phagocytic receptor; although binding to a receptor does not automatically prompt uptake (Aderem and Underhill 1999). Receptor clustering at the attachment site generates a phagocytic signal that in turn leads to local polymerisation of actin filaments directly beneath the phagocytic target, which pushes the membrane forward to form pseudopodia and form a phagocytic cup through which the particle is internalised. Actin polymerisation beneath the phagocytic target drives particle uptake and is a trademark of phagocytosis, but is shed rapidly after particle ingestion as the phagosome subsequently fuses with intracellular endocytic markers such as lysosomes leading to the breakdown of

the particle. Among the best-characterised phagocytic receptors are the opsonic receptors, Fc γ receptors (Fc γ R) and complement receptors (CR1, CR3 and CR4) that recognise IgG or complement bound particles in an opsonin-dependent manner (Aderem and Underhill 1999). Other opsonins include lipopolysaccharide binding protein (LBP), fibronectin, thrombospondin and collectins. Non-opsonic receptors such as CD14, scavenger receptor (SR) can bind particles directly and will be discussed in further detail.

1.3.1 Opsonophagocytosis

The word opsonin is derived from the Greek for “prepare for eating”. Opsonins are soluble mediators that promote the phagocytosis of bacteria and other antigens, by coating their surface, which has the effect of making the antigens bind more readily to the phagocytic cells, as they possess surface receptors for the opsonic proteins, thus allowing the target to undergo receptor-mediated engulfment (Stites, Terr et al. 1997).

The most effective antibody opsonins are from the IgG subclass, and their ability to opsonise is mediated through Fc receptors found on the surface of professional phagocytes. There are three distinct classes of receptors specific to the Fc portion of IgG, and they are collectively termed Fc γ receptors (Fc γ Rs). IgG1 and IgG3 bind with a high affinity to the Fc receptors (Fc γ RII and Fc γ RIII respectively), and so are the most effective opsonins. Complement receptors (CRs) recognise complement-opsonised particles and are present on macrophages and PMN's. C3 components can act as opsonins, specifically C3b and iC3b that bind to CR1 and CR3 respectively (Mosser 1994). Fc γ receptors and CR3 differ in a number of ways, for example Fc γ receptors are constitutively active for phagocytosis (Ravetch 1994), whereas CR3 requires additional activation to phagocytose particles (Wright, Craigmyle et al. 1983). Cytokines, such as IFN- γ ,

are able to up-regulate receptor expression in monocytes by as much as a 20-fold increase.

A number of precise regulatory signalling molecules linking ligated phagocytic receptors to actin polymerisation are involved in FcγR-mediated phagocytosis (type I phagocytosis), including Rho GTPases and members of Src family of tyrosine kinases Lyn, Hck, Fgr and Syk kinases (Greenberg, Chang et al. 1993; Greenberg, Chang et al. 1996). Many of these signalling cascades have yet to be completely understood, but it is clear that during particle uptake in mammalian professional phagocytes, Rho GTPases control the cytoskeletal rearrangements (Ridley and Hall 1994). These small GTPases control the formation of actin-rich filopodia and membrane ruffling, (Cox, Chang et al. 1997) as well as controlling different stages in FcR-mediated phagocytosis process, namely pseudopod emission for Cdc42 and phagocyte closure for Rac1. (Caron and Hall 1995; Massol, Montcourrier et al. 1998) IgG-opsonised particles require the presence of Cdc42 and Rac1 for F-actin polymerisation through the WASP (Wiskott-Aldrich Syndrome Protein) family of proteins that induce actin nucleation mediated by the Arp2/3 complex which translocates to phagocytic cup and co-localises with actin, (Machesky and Insall 1998) leading to F-actin rich lamellipodia extensions that engulf the particle. The central role of WASP in Fc-mediated phagocytosis is highlighted in WAS patients, who display an absence or reduced concentration of WASP protein in their leukocytes. This condition results in a marked reduction in the internalisation of IgG-opsonised particles (Lorenzi, Brickell et al. 2000). Vesicle fusion with the plasma membrane is controlled by regulatory proteins, such as phosphatidylinositol 3-kinase (PI3-K), an enzyme activated during phagocytosis (Cox, Tseng et al. 1999), localised to the phagocytic cup (Marshall, Booth et al. 2001), and involved in the supply of membrane required for the formation of phagosomes. Soon after phagocytosis is completed F-actin is quickly lost from the phagosomes.

Recruitment of the active forms of these proteins to the plasma membrane occurs via the rapamycin system, and has confirmed the differential roles of Cdc42 and Rac1 (Castellano, Montcourrier et al. 1999; Castellano, Montcourrier et al. 2000). In contrast, the actin polymerisation downstream of the integrin family member CR3 depends on the activity of RhoA, and neither Rac1 nor Cdc42 are necessary in this pathway. Complement factors bind particles through thiol ester bonds on free surface hydroxyl or α groups. CR3-mediated uptake (type II phagocytosis) leads to a transient accumulation underneath bound particles of F-actin and various cytoskeletal elements in small foci (Caron and Hall 1995; Allen and Aderem 1996); and particles sink straight into the cytoplasm of the cell without being engulfed through protruding pseudopodia as is the case for antibody-opsonised particles (Kaplan 1977). Although the presence of Arp2/3 complex has recently been demonstrated in the actin-rich foci (Mary, Caron et al. 2000), the upstream signalling of type II phagocytosis, linking CR3 activation to particle uptake is not known. Protein kinase C (PKC) involvement has been observed in both types of phagocytosis described, but unlike antibody-mediated phagocytosis protein tyrosine kinases are not thought to be involved in phagocytosis through complement receptors (Allen and Aderem 1996). Additionally type I phagocytosis usually triggers a pro-inflammatory response and respiratory burst that is not evident in type II phagocytosis. As actin dynamics are central to the process of phagocytosis, it is not surprising that several bacterial pathogens have evolved virulence mechanisms able to interfere with or promote uptake. What is interesting is that the regulatory switch of Rho GTPases is a common target, and emphasises the important role these signalling elements play in phagocytosis.

From the point of view of the microbe, phagocytosis can be seen as an opportunity for intracellular pathogens to enter immune cells without inciting an anti-microbial response by avoiding engulfment altogether or by blocking a phagocytic response once inside the cell. Bacterial pathogens have developed various strategies to avoid recognition of phagocytic receptors, *Neisseria*

meningitidis, *Klebsiella pneumoniae* and *Streptococcus pneumoniae* for example are all in possession of a polysaccharide capsule which can physically interfere with complement deposition and therefore impair opsonin-mediated phagocytosis. *Neisseria* can also alter the antigenicity of certain surface molecules. Other microbial pathogens, such as *Yersinia* species, *Salmonella* and *Pseudomonas aeruginosa*, are able to use specialised highly regulated secretion systems (type III secretion systems or TTSS) to interrupt the formation of cytoskeletal arrangements and/or phagocytic signalling pathways of opsonin-independent receptors (Simonet, Richard et al. 1990).

A normal functioning complement system, as well as the co-operation of specific antibodies, are essential in protection from invasive *N. meningitidis*, since intravascular clearance of gram-negative bacteria is mediated through the deposition of complement components: C3b for phagocytic clearance, the MAC or C5-C9 for lysis. The fact that immune lysis is the immunological barrier that restricts meningococci from colonising mucosal sites is most apparent in individuals who have genetic deficiencies in the terminal complement components required for immune lysis. These individuals are prone to recurrent neisserial infections, despite having acceptable levels of anti-meningococcal antibodies and having intact C3-dependent opsonophagocytic killing. Whereas other mucosal bacteria that can also cause disseminated disease in the absence of immune lysis do not disseminate in the sera of complement deficient people. There must therefore be something unique in the way that complement interacts with the surface of *Neisseria* (Brandtzaeg 1995).

The role of other serum opsonins are not explored in this manuscript, but it is important to note that collectins, which include mannose-binding lectin (MBL), lung surfactant proteins A and D, are thought to contribute to innate immune defence. MBL is distinguished by its ability to activate the complement system, and enhance host bactericidal responses by binding to the surface of a range of

organisms, including *Staphylococcus*, *Salmonella* and *N. meningitidis* (in the absence of sialylated lipooligosaccharides or LOS) (Jack, Klein et al. 2001).

The complexity, diversity and variety of phagocytic receptors and their signalling pathways are becoming more apparent as time goes on; as are the incredibly intricate methods that some bacterial pathogens have developed to avoid phagocytosis, as a means of intracellular survival. Non-opsonic phagocytic pathways have yet to be characterised in such detail; therefore *in vitro* studies such as this are important, as the role of opsonophagocytosis in the defence against meningococcal infection *in vivo* is largely unknown.

1.4 Mucosal immunity against *N. meningitidis*

The nasopharynx is the initial site of meningococcal colonisation and is presumed to be the primary site of invasion. Certain strains of *Neisseria meningitidis* are potential pathogens, and this appears to be conferred in part by their ability to adhere and invade mucosal surface structures (Townsend, Goodwin et al. 2002), and both survive and multiply after entering the bloodstream of certain individuals (Brandtzaeg 1995). The evolution of bacterial meningitis follows the general sequence of exposure to the pathogen, acquisition and then invasion; and invasion itself can be said to comprise three steps, mucosal penetration, invasion into the bloodstream and finally invasion of the meninges (Stephens and Farley 1991; Brandtzaeg 1995).

Specific surface structures are thought to be responsible for the pathogenic potential of a meningococcal strain, coupled with individual response to colonisation (Dehio, Gray-Owen et al. 2000), as it is the host's immunological response to these organelles that restricts the bacteria to mucosal sites of colonisation. It has also been hypothesised that if a strain makes more of these immunogenic organelles, there is a greater chance that it will colonise the mucosa without invading/killing the host. However, the low incidence of disease suggests that for most individuals natural immunity is successful in preventing meningococcal invasion. Studying disease in immunodeficient individuals has been extremely useful in highlighting the complexity of immune response to meningococci (Ross and Densen 1984).

There are a number of structures on the surface of the bacteria that are involved in host cell attachment; these include pili, capsule, and outer membrane proteins. Such attachment processes are important as they allow the bacteria to overcome repulsive charge, or the hydrophilic/phobic affects of different surfaces (Criado, Ferreira et al. 1985). Some of these structures can also undergo phase and/or antigenic variation, by DNA rearrangement, as a mechanism to evade specific

host defences (McLeod Griffis 1995). Acquisition of *N. meningitidis* in the nasopharynx and the severity of disease are affected by both host (Heist, Solis-Cohen et al. 1922) and environmental factors, such as age, antibody or complement deficiency, any existing bactericidal or opsonic activity, co-infection and any changes that damage the nasopharynx (Goldschneider, Gotschlich et al. 1969; Reller, MacGregor et al. 1973; Nicholson and Lepow 1979). Tobacco smoke also affects the surface charge of cells, and consequently the adhesion of potential pathogens associated with an increased risk of invasive disease (El Ahmer, Essery et al. 1999).

The carriage of *N. meningitidis* in adults is known to induce cross-protection between strains, and carriage of *N. lactamica*, a non-pathogenic species of *Neisseria* usually carried during early childhood, is associated with an increase in antibody titres to pathogenic meningococci strains. Over 90% of 12-18 year olds in a UK-based study displayed bactericidal activity against *N. lactamica*, that were consistent with high levels of carriage of the same organism or related/cross-reactive organisms throughout childhood (Zorgani, James et al. 1996). Carriage of non-pathogenic organisms that are capable of inducing immune responses are thought to be important for acquiring a natural immunity to pathogenic meningococci; and it may be these differences in carriage of cross-reacting non-pathogenic strains within a population may, in some part contribute to the worldwide variation of prominent serogroups and strains, and incidence of meningococcal disease (MCD). Relatively little is known about mucosal immunity to MCD, but in order for *Neisseria meningitidis* to cause disease it must both attack (colonise) and invade epithelial cells. So, in fact effective mucosal immunity really need only prevent invasion, not colonisation - but as yet we have no way of measuring mucosal immunity to epithelial cells, other than by noting the absence of disseminated disease.

1.5 Antigen recognition by macrophages

1.5.1 CD14

1.5.1.1 Structure and expression

CD14 was first described in 1982 during the First Leukocyte Typing Workshop (Bernard, Boumsell et al. 1984) and was later identified as a 55kD glycoprotein expressed on neutrophils, monocytes and macrophages (Todd, Bhan et al. 1984). Although it was not until 1990 that Wright and colleagues established the receptors main functional role, which was that of a high affinity LPS receptor that could induce macrophage activation and cytokine production in response to LPS (Wright, Ramos et al. 1990).

Human CD14 receptors are present on cells belonging to myeloid, erythroid, megakaryocytic and lymphoid lineages, and the gene consists of two exons that encode a 356 amino acid protein with multiple leucine-rich repeats. The mature protein occurs in two forms, as a membrane-bound glycosylphosphatidyl-inositol (GPI)-anchored protein (mCD14) (Haziot, Chen et al. 1988), and a soluble form (sCD14), which is present in normal human serum at a concentration of 2-6µg/ml (Bazil, Horejsi et al. 1986; Wright 1999). The presence of this anchor means that it is classified as belonging to the PI-anchored family of receptors (Haziot, Chen et al. 1988; Simmons, Tan et al. 1989). Macrophages express greater numbers of mCD14 than neutrophils, with approximately 10^5 receptors per cell compared to 3×10^3 receptors per cell respectively (Antal-Szalmas, Van Strijp et al. 1997). Two soluble forms of sCD14 are obtained in quite different ways; proteolytic cleavage of the cell membrane through a serine protease generates a 49kD form and a 55kD form of sCD14 can be liberated from its GPI-anchor (Bufler, Stiegler et al. 1995) by phospholipase D cleavage. Treatment of macrophages with phosphatidylinositol phospholipase C (PI-PLC), an enzyme

specific for the cleavage of all PI-anchored molecules, results in the removal of CD14 from the cell surface (Haziot, Chen et al. 1988).

Expression of mCD14 and release of sCD14 are subject to bacteria and cytokine-induced regulation, as both mCD14 and sCD14 expression are up regulated in the presence of bacterial ligands (Landmann, Ludwig et al. 1991; Watanabe, Takeshita et al. 1996). sCD14 can bind a wide range of amphipathic lipids, including bacterial proteins OSP-A, PI, PS, PC and Rhodamine-PE (Hailman, Lichenstein et al. 1994; Savedra, Delude et al. 1996; Yu, Hailman et al. 1997; Wang, Kitchens et al. 1998; Wooten, Morrison et al. 1998; Wang and Munford 1999; Wright 1999), and contributes to the transport of phospholipids in the blood by mediating the transport of bacterial LPS and phospholipid monomers both in and out of cell membranes (Sugiyama and Wright 2001).

1.5.1.2 Ligands and binding properties

CD14 is an important LPS receptor, and the majority of low-dose LPS effects in myeloid cells are attributed to CD14-dependant interactions (Wright, Ramos et al. 1990). Moreover, it is essential for TNF- α and IL-1 production (Couturier, Jahns et al. 1992; Martin, Mathison et al. 1992). CD14 can bind both soluble LPS and whole gram-negative organisms, which are subsequently internalised in a complement-independent manner, but the role of mCD14 in bacterial phagocytosis is not yet clear.

The binding of most ligands to CD14 evokes a pro-inflammatory response with a few notable exceptions including anionic phospholipids which are present on the surface of apoptotic cells (Savill 1998); thus ensuring that the surrounding tissue is protected from inflammatory injury (Savill 1998). Such a pro-inflammatory cytokine response in macrophages against apoptotic cells is inhibited by autocrine TGF β , PGE₂ and PAF release (Fadok, Bratton et al. 1998). Both mCD14 and sCD14 bind LPS (Gallay, Jongeneel et al. 1993; Tobias, Soldau et

al. 1995), and a number of studies to establish the precise region of the LPS binding site have been undertaken, but results have been variable and largely depend on the methods used. C-terminus amino acid residues after 152 are not thought to have a role in LPS binding and the promoting of cellular responses, so further work has concentrated on hydrophilic regions at the N-terminus. Several regions between residues 7-65 have been implicated, and deletion mutagenesis studies of sCD14 have identified residues 39-44 and 57-64 as important LPS binding domains (Juan, Hailman et al. 1995; McGinley, Narhi et al. 1995; Stelter, Bernheiden et al. 1997).

As well as LPS, as a PRR CD14 can also interact with a number of microbial products including muramyl dipeptide, polymannuronic acid, lipoarabinomannan and high doses of peptidoglycan, but it has yet to be established whether such ligands all bind to the same site on the receptor (Pugin, Heumann et al. 1994). CD14 is mobile in the plane of the membrane, and is quite distinct from other macrophage receptors. Cells that do not express CD14 (e.g. lymphocytes, NK cells, fibroblasts) still respond to high concentrations of LPS in a CD14-independent manner.

1.5.1.3 Function of CD14

Pattern recognition receptors on cells of the innate immune system include signalling receptors and phagocytic receptors, but CD14 lacks a transmembrane or cytoplasmic domain, so its role as a receptor capable of transducing signals into the cell is rather controversial. LPS interactions with macrophages via CD14 trigger several phosphorylation cascades, including NF κ B activation and MAP kinase pathways.

Due to its structure, CD14 is not able to function as signal transducing molecule and must therefore transmit appropriate signals into the cell by an alternate

method. A number of mechanisms have been proposed for CD14-mediated macrophage activation by LPS, and include:

- ◆ Studies using labelled anti-CD14 monoclonal antibodies have demonstrated that CD14 internalisation into the cell occurs through uncoated invaginations which is a known characteristic of receptor-mediated endocytosis which may serve to transduce a signal (Schutt, Ringel et al. 1988; Jonas, Schutt et al. 1990).
- ◆ TLRs and CD14 may co-operate to transduce signal into the cell (Shimazu, Akashi et al. 1999; Beutler 2000), Studies by Pridmore and Wylie *et al* also suggest that CD14 responsiveness to *N. meningitidis* LPS occurs in conjunction with TLR4 and its cofactor MD2 (Pridmore, Wylie et al. 2001).
- ◆ Physical and functional association of GPI-anchored receptors, including CD14, with β_2 integrins that can in turn act as a signalling conduit for CD14 (Petty and Todd 1996).
- ◆ Ceramide has structural similarity to LPS, so a suggestion is that if LPS binds to CD14, the LPS can be integrated into the phospholipid layer of the cell membrane and interact with ceramide-activated protein kinase (CAPK) in place of ceramide, thus leading to activation of the cell (Joseph, Wright et al. 1994). Later evidence calls into question whether this effect is specific to LPS, as both TNF- α and IL-1 can also activate CAPK. Perhaps then ceramide mimics LPS, rather than the converse as was first thought (Sweet and Hume 1996).
- ◆ Unknown co-receptors or accessory proteins involved in the binding of microbes and the subsequent activation of the cell.

The targeted deletion of the CD14 gene in mice confirmed the importance of CD14 in LPS responsiveness, as monocytes and macrophages were found to be profoundly hyporesponsive to LPS. Small amounts of TNF- α and trivial amounts of IL-1 β and IL-6 were produced in response to high doses of LPS (1-10 μ g/ml); this is 4-5 logs above threshold concentration for LPS responses. In

this manuscript the role of CD14 as a potential binding or signalling receptor for serogroup B *N. meningitidis* is explored.

1.5.1.4 Paroxysmal nocturnal haemoglobinuria (PNH)

PNH is a relatively rare acquired clonal disorder of the haemopoietic stem cell (HSC). The disease is characterised by the formation of defective granulocytes, lymphocytes, platelets and erythrocytes. Sufferers display a large number of membrane protein deficiencies, including CD14, CD59, CD58, decay accelerating factor (DAF), and acetylcholinesterase (AChE) and alkaline phosphates in erythrocytes and neutrophils respectively (Schubert, Uchiechowski et al. 1990; Bessler and Fehr 1991; Rotoli, Bessler et al. 1993). Current pathogenetic models predict that autoreactive T cells target normal (non-mutated) HSCs sparing mutated HSCs. In this context, PNH HSC cells expand and supply the periphery with mature blood cells which at various proportions lack surface expression of glycosylphosphatidylinositol (GPI)-linked proteins.

All the deficient membrane proteins possess a GPI-anchor, and PNH effects the formation of GPI-anchors (Rosse 1990; Rotoli, Bessler et al. 1993). The range of cells this disorder affects suggests that the somatic mutations have taken place in a totipotent cell, confirming that PNH is a stem cell disorder. The proportion of PNH cells in PNH patients who have a normal or reduced cell count can be up to 90% or even more. The genetic lesion is mutation(s) affecting the X-linked gene *PIG-A*. The *PIG-A* gene has been isolated and mapped to the X-chromosome on Xp21.3 (Takeda, Miyata et al. 1993; Bessler, Hillmen et al. 1994). *PIG-A* has been shown to correct the membrane abnormality of PNH cells, and further analysis of the gene has revealed mutations (often small insertion deletions or point mutations) in the PNH cell population, but not in the normal cell population of PNH sufferers (Bessler, Mason et al. 1994; Ware, Rosse et al. 1994; Nafa, Mason et al. 1995; Yamada, Miyata et al. 1995).

CD59, also referred to as membrane inhibitor of reactive lysis (MIRL), is important in preventing cellular death via the complement membrane attack complex (MAC), and cells lacking CD59 become highly susceptible to complement-mediated lysis. Consequently many patients with PNH tend to be anaemic, and have neutropenia or thrombocytopenia (or both) at some in the course of their disease. Inherited absence of PIG-A protein product produces a similar phenotype to PNH (Yamashina, Ueda et al. 1990).

1.5.2 Complement Receptor 3

Part of the complexity of phagocytosis is due to the wide variety of microbes that can interact with the cell via a diverse array of cellular receptors. A number of phagocytic receptors have dual functions, and are able to mediate opsonic and non-opsonic interactions, as well as bind and subsequently internalise invading microbes; complement receptor 3 (CR3) is an example of such a receptor. CR3 is widely held as being a crucial component of the innate immune system, and an essential LPS receptor. As a versatile adhesion and recognition receptor, CR3 mediates phagocytosis and has signalling capabilities via kinase cascades and cytoskeletal arrangements, as well as activating other important adhesion and defence receptors (Todds III 1996; Wagner, Hansch et al. 2001).

The integrin superfamily are a collection of transmembrane glycoproteins that can mediate both cell-substrate and cell-cell interactions, and CR3 shares many common features with other members; such as a propensity for clustering, associating with cytoskeletal and signalling complexes, activating signalling cascades and regulation of cell spreading and migration through Rho GTPases (Giancotti and Ruoslahti 1999). Integrins are present in a wide variety of species from sponges to mammals, and are therefore widely held as an important link between cells and the extracellular matrix (ECM).

1.5.2.1. Structure

CR3 is a heterodimeric type I transmembrane glycoprotein, comprising an α sub-unit (CD11a, b or c) and a non-covalently associated β sub-unit (CD18). CD18 is a 95kD polypeptide of which there is only one form that is shared by all the three β_2 -integrins. CD18 is encoded on chromosome 21 and is linked to one of the three CD11 subunits that are all encoded on chromosome 16. Electron microscopy studies suggest that structurally an integrin comprises a globular

“head” that is formed by the amino regions of the α and β chains, which is thought to be the ligand binding site. This is in turn attached to “legs” that are formed by the carboxyl regions of each chain. Disulphide bonds stabilise the complicated tertiary structure of the receptor, and form bridges between a number of cysteine residues that are highly conserved (Todds III 1996).

The four homologous heterodimers that comprise the β_2 -integrin family of receptors are: leukocyte-function associated molecule-1 (LFA-1, CD11a/CD18, CR1), complement receptor type 3 (CR3, CD11b/CD18, Mac-1, Mo-1), CR4 (CD11c/CD18) and the more obscure $\alpha_D\beta_2$ (CD11d/18). CR1, CR3 and CR4 are all expressed on macrophages and participate in the phagocytosis of complement opsonised particles, though CR3 is present in the greater numbers (Larson and Springer 1990; Springer 1995).

LFA-1 contains the 170kDa α -subunit CD11a (or α_L), is expressed on all leukocytes and mediates intercellular adhesion between leukocytes and other host cells via intercellular adhesion molecule (ICAM) –1, 2 and 3. The CR3 α -subunit CD11b (α_M) is 1136 residues long and encodes a 165kD polypeptide that is expressed on a range of leukocytes such as monocytes, macrophages, NK cells, granulocytes, but only a minor subset of B-cells (CD5+) and even less T-cells. Together CR3 and LFA-1 are essential in the process of diapedesis, where they act as cellular adhesion receptors that facilitate the migration of phagocytic cells from the blood vessels to sites of infection.

1.5.2.2 Ligands

A known PRR, CR3 receptors interact with an impressive array of ligands both protein and non-protein which bind to the two main binding regions present on the α -subunit. Bacterial LPS, as well as other diverse microbial conjugates, bind to CR3 both in the presence or absence of serum opsonins; and the ability of

CR3 to recognise a variety of endogenous and exogenous substances has raised a lot of interest in the receptor.

The I (inserted) domain (also known as A domain) is located at the N-terminus, and most, if not all, of the endogenous and microbial ligands bind via this specialised domain, including complement components C3b, iC3b, C3d and C3dg, coagulation proteins such as fibrinogen (Wright, Weitz et al. 1988) and factor X (Altieri and Edington 1988), Zymosan (Yalamanchili, Lu et al. 2000) and counter receptors ICAM-1 and 2 (Diamond, Staunton et al. 1990). ICAM-1 and iC3b bind to overlapping sites within the I-domain of CD11b. The I domain contains binding sites that are distinct and overlapping but not identical, and also has a distinctive metal ion-dependent adhesion site (MIDAS) (Diamond, Garcia-Aguilar et al. 1993).

The second main binding domain is located C-terminus to the I domain and is referred to as the polysaccharide lectin binding site. The lectin site is a divalent cation-independent domain that has a broad specificity for certain glucose- and mannose-containing polysaccharides (Thornton, Vetvicka et al. 1996). This lectin binding site is less well resolved, but is known to have a wide sugar specificity, able to bind both bacterial and yeast cell wall polysaccharide, which in turn triggers phagocytosis and extracellular cytotoxicity. CR3 expressing cells, blocking monoclonal antibodies and transfected cell lines have all been used in studies to identify the ligand specificity of this site (Shang and Issekutz 1998; Xia and Ross 1998). Microbial ligands include lipopolysaccharide (LPS), *Mycobacteria* polysaccharides, *K. pneumoniae* acylpolygalactoside (APG), *Leishmania* lipophosphoglycan (LPG), as well as soluble and particulate saccharides such as β -glucan and zymosan.

Suggestions that LPS and β -glucan binding may occur at distinct sites on the lectin binding site have been made and imply that either the lectin site is an individual binding site that can recognise different types of sugars, or a second

lectin site may exist. A broad range of micro-organisms have been shown to interact with leukocytes in a CR3-mediated manner, including *Mycobacterium tuberculosis* (Zimmerli, Edwards et al. 1996; Peyron, Bordier et al. 2000; Thorson, Doxsee et al. 2001), HIV (Bouhlal, Galon et al. 2001), *Cryptococcus neoformans* (Levitz, Tabuni et al. 1997), group B *Streptococci* (Goodrum, McCormick et al. 1994), *Listeria monocytogenes* (Drevets, Leenen et al. 1996), *Pseudomonas aeruginosa* (Heale, Pollard et al. 2001), *Escherichia coli* (Agramonte-Heva, Gonzalez-Arenas et al. 2002) and *Neisseria gonorrhoeae* (Edwards, Brown et al. 2002).

Signalling is quite complex for CR3 as it has two activation signalling processes that are quite different. High-affinity ligand binding results in an inside-out signalling state for CR3, and phagocytic activation of CR3 leads to outside-in signalling. LPS activation of macrophages induces a marked upregulation in CR3 expression and initiates inside-out signalling, which is essential for high-affinity binding of integrin ligands. This is an intrinsic property of integrin receptors where binding of ligands results in conformational changes in the external domain of the receptor; and results in allowing the ligand to gain better access to the binding pocket formed by the intertwined β and α chains. For example LPS binding to CR3 has the affect of revealing otherwise hidden, high-affinity binding sites for ICAM-1. Activated CR3 form a strong attachment with host endothelial cells that express ICAM-1, without eliciting a cytotoxic response. Another form of activated CR3 can recognise opsonin-coated bacteria and cell wall polysaccharide through the lectin-binding site, resulting in a strong cytotoxic response, which includes phagocytosis and cytokine production and secretion. It is clear that functionally both forms of the activated CR3 are quite different.

A number of studies have observed that CR3 can associate with several GPI-linked proteins, both physically and functionally (Petty and Todds III 1996). These proteins tend be heavily glycosylated and are thought to bind directly to

CR3 via the lectin site. GPI-linked receptors, such as CD14, CD16 (FcγRIIIB) and uPAR aggregate on cholesterol-rich regions, referred to as lipid rafts, present on cell surfaces, and this association may mediate the cross-talk of both receptors in response to LPS and result in effective signal transduction to the cell. Association of CR3 to GPI-anchored receptors is thought to result in their translocation to lipid rafts, at which point pathogenic microbes, such as *Mycobacteria* species are able to bind to and internalise through CR3 receptors without inciting a cytotoxic immune response (Peyron, Bordier et al. 2000).

Although CR3 is central to our innate immune response, it is interesting to note that a number of diverse microorganisms use CR3 as a safe portal of entry into the cell. *N. gonorrhoeae* for example uses CR3 as a primary receptor to infect and invade cervical epithelial cells, but whether gonococci interact with CR3 directly or if complement deposition is a specific requirement or whether these interactions take place via the I domain are all questions that have yet to be answered. Edwards *et al* (Edwards, Brown et al. 2001; Edwards, Brown et al. 2002) suggest that gonococci can bind to human cervical epithelium in an opsonin-dependent and –independent manner. The relationship between *N. meningitidis* and CR3-mediated entry has not been previously studied in detail, and will be examined as part of this study.

1.5.2.3 CR3 priming and activation

The functional status of inflammatory cells can be enhanced depending on specific conditions *in vivo* and *in vitro*. Priming is a phenomenon that is used by a number of cell types, including neutrophils, basophils, eosinophils as well as macrophages. Both human and murine CR3 can prime macrophages, neutrophils and NK cells for increased cytotoxicity or phagocytosis.

As well as an increase in function, priming is also associated with morphological, physical and phenotypic changes of the cell and provides the basis for the concept of cellular heterogeneity. Johnson *et al* (Johnston Jr. 1988) first introduced the concept of macrophage priming by observing an increase in oxidative burst after exposure to proteases *in vitro*. The local microenvironment of inflammatory cells accounts for their heterogeneity but the relationship between priming and other cell changes has yet to be elucidated.

Analysis of the response of human leukocytes to β -glucans has revealed that CR3 is primarily responsible for the high affinity binding of particulate (Ross, Cain et al. 1987) or soluble (Thornton, Vetvicka et al. 1996) β -glucans (Cain, Newman et al. 1987; Vetvicka, Thornton et al. 1996; Vetvicka, Thornton et al. 1997) that have highly variable structures and sizes and are present on a wide variety of fungi. For over 30 years β -glucans have been known to promote tumouricidal activity in mice; and recent *in vitro* studies revealed that β -glucans can activate these human and murine cells to kill sensitive tumour cells and potentiate T-cell responses to cellular antigens (Cook, Taylor et al. 1978; Amino, Noguchi et al. 1983; Seljelid, Bogwald et al. 1984; Sugawara, Lee et al. 1984; Morikawa, Noguchi et al. 1986; Sherwood, Williams et al. 1987).

High molecular weight soluble or particulate β -glucans have been shown to cross-link membrane CR3 and multiple cell surface CR3 molecules, resulting in the secretion of IL-1, TNF- α from macrophages (Doita, Rasmussen et al. 1991; Ohno, Saito et al. 1993; Ljungman, Leanderson et al. 1998), degranulation and respiratory burst of neutrophils. But small soluble β -glucans were shown to saturate the individual cell surface CR3 molecule (Thornton, Vetvicka et al. 1996) and generate a primed state of the receptor capable of mediating cytotoxicity of iC3b-opsonised tumour cells that otherwise would not trigger this CR3-dependent response (Vetvicka, Thornton et al. 1996; Vetvicka, Thornton et al. 1997). But interestingly β -glucan priming of CR3 does not lead to the presentation of a high-affinity epitope required for the efficient binding of

ligands, such as ICAM-1 (Vetvicka, Thornton et al. 1996); suggesting that CR3 has more than one primed or activation state. β -glucan tumouricidal activity by neutrophils, macrophages and NK cells require tumour cells to be opsonised with iC3b, therefore β -glucan therapy may be more effective when tumour cells generate a humoral response and are opsonised with antibody and C3. *In vitro* macrophage CR3 can remain primed for toxicity of iC3b-opsonised tumour cells hours after exposure, which may be useful in the study of β -glucan therapeutics in cancer research. The use of β -glucan as a tumouricidal agent has produced variable results in studies carried on cancer patients in Japan, but a clearer understanding of the mechanisms involved will allow their use to be more precise.

Small soluble β -glucans released from *S. cerevisiae* have been shown to be particularly effective in blocking macrophage phagocytosis of yeast (Goldman 1988; Goldman 1988) and Giamis *et al* observed that macrophage phagocytosis of non-opsonised heat-killed yeast was mediated by both β -glucans and mannose receptors (Giamis, Lombard et al. 1993). Seljelid *et al* produced a soluble β -glucan that activated murine macrophages for both anti-tumour and anti-bacterial activities (Seljelid, Bogwald et al. 1984; Seljelid 1986; Seljelid, Rasmussen et al. 1987; Doita, Rasmussen et al. 1991), and showed that the activity of these soluble β -glucans could not be blocked by mannose or mannan, demonstrating that the murine receptor for soluble β -glucan was distinct from the mannose receptor (Konopski, Smedsrod et al. 1994). The relationship between the CR3 activation states for high affinity adhesion versus cell-mediated cytotoxicity is unknown.

The important role of CR3 in human innate and microbial defences is highlighted in disorders associated with this receptor. Leukocyte adhesion deficiency (LAD) syndrome is a condition where the patient suffers recurrent life-threatening infections due to impaired transendothelial emigration, the result of impaired leukocyte adhesion and phagocytosis. This condition occurs because of

mutations in the β -subunit CD18, which affects the expression of all β_2 integrins. CR3 also has a role in apoptosis, as CR3-deficient mice display a significant delay in the apoptosis of extravasated neutrophils.

Some integrin ligands such as fibrinogen, vitronectin and fibronectin, contain an Arg-Gly-Asp (RGD) motif that is thought to be important for receptor binding. Small RGD-containing peptides can readily gain access to the ligand-binding region, and the presentation of this motif on a β -turn has been linked to the specificity of integrin receptors. Cyclic peptides containing the RGD motif are known inhibitors of integrin receptors, and RGD-based antagonists inhibit tumour-induced angiogenesis and tumour growth. Further investigation revealed a family of potent and selective RGD-based peptides – called the S787 class of integrin antagonists-specific for $\alpha_v\beta_3$ integrin, which have marked anti-tumour and anti-angiogenic properties in tumour models (Buerkle, Pahernik et al. 2002).

The involvement of CR3 and members of the integrin family that recognise the RGD motif in *N. meningitidis* will be examined in this study.

1.5.3 Mannose receptor

The macrophage mannose receptor (MR) is a 175kD type I transmembrane glycoprotein that mediates the endocytosis and phagocytosis of micro-organisms via carbohydrates expressed on their surface, such as fucose, mannose and D-N-acetyl-glucosamine (Stahl, Rodman et al. 1978). Macrophage MR activity was initially identified in the liver and on alveolar macrophages (Stahl, Rodman et al. 1978) and expression has since been shown on dendritic cells and most differentiated macrophages, including resident and elicited peritoneal macrophages (Stahl and Gordon 1982), monocyte-derived macrophages (Shepherd, Konish et al. 1985), bone marrow-derived macrophages (Shepherd, Campbell et al. 1982) and rat kupffer cells (Maynard and Baenziger 1987). Soluble receptor which maintains carbohydrate-binding function has also been detected in mouse serum (Martinez-Pomares, Mahoney et al. 1998).

Structurally the receptor is composed of a cytoplasmic and a transmembrane domain, eight carbohydrate recognition domains (CRDs), a fibronectin type II-like repeat and a cysteine-rich domain. Ligands bind to the cysteine-rich domain and the CRDs, particularly CRDs4-8 (Taylor, Bezouska et al. 1992; Martinez-Pomares, Kosco-Vilbois et al. 1996). MR are involved in the phagocytosis of a range of bacteria, yeast and protozoa, including *M. tuberculosis* (Schlesinger 1994), *K. pneumoniae* (Kabha, Nissimov et al. 1995), *P. aeruginosa* (Speert, Wong et al. 1997), *S. cerevisiae* (Giamis, Lombard et al. 1993), *C. albicans* (Ezekowitz, Sastry et al. 1990) and *Pneumocystis carinii* (Ezekowitz, Williams et al. 1991). Production of cytokines, TNF- α , IL-6, IL-1 and IL-12, following ligand binding has also been reported (Fraser and Ezekowitz 1998). It has been suggested that this receptor may be involved in antigen transport, by shuttling antigens from its cysteine-rich domain to lymphocytes present in the lymph nodes and spleen

As a PRR mannose receptors have a prominent role in host defence and molecular scavenging from plasma or extracellular medium. It can also recognise a variety of endogenous glycoproteins such as lysosomal glycosidases and proteases and tissue plasminogen activator that may associate the receptor in homeostasis (Pontow, Kery et al. 1992; Linehan, Martinez_Pomares et al. 2000).

1.5.4 Toll-like receptors

Toll-like receptors (TLRs) are members of the IL-1 receptor gene family that play a role in the host defence of organisms from *Drosophila* to plants. This high homology with *Drosophila* Toll receptors has led to them being named Toll-like receptors (Medzhitov, Preston Hurlburt et al. 1997; Kopp and Medzhitov 1999) of which there are ten members of this growing family of receptors (TLRs 1-10). Regarded as PRRs, numerous studies have shown these receptors to be involved in innate immune defence systems, and are particularly associated with CD14. As discussed above, CD14 is a GPI-linked outer membrane proteins that lacks transmembrane and cytoplasmic domains, and can bind LPS but have no signal transducing ability thus requiring co-receptor(s) to fulfil this function; of which TLRs are an example. Molecules involved in TLR signalling cascades include MyD88, which serves as an adapter molecule, and binds to IL-1 receptor-associated (serine/threonine) kinase (IRAK), which in turn undergoes autophosphorylation and dissociates from the complex. IRAK then interacts with TNF-receptor associated factor 6 (TRAF6) and results in the activation of NF κ B-inducing kinases (NIK) and I κ B kinases (IKK); and it is IKK that phosphorylates I κ B that results in NF κ B translocation to the nucleus where it is involved in activation of specific genes, inducing release of pro-inflammatory cytokines and immune activation (Kopp and Medzhitov 1999).

In flies, Toll is activated by a proteolytically cleaved form of a secreted ligand, known as Spätzle (Morisato and Anderson 1994), but mammalian TLRs are orphan molecules and it is not yet known whether they bind molecules directly or require activation from molecules similar to Spätzle; Smiley *et al* (Smiley, King *et al.* 2001) recently suggested that fibronectin might function as a Spätzle homologue in higher eukaryotes .

TLRs appear to provide protection against invading pathogens, such as gram-positive and gram-negative bacteria and yeast, of which TLR2 and TLR4 have been studied most, but the roles of these two TLRs in LPS responsiveness *in vivo* and *in vitro* has caused some controversy. Studies using TLR2 and TLR4 knockout mice to investigate LPS responsiveness have suggested that TLR2^{-/-} mice responded normally to *Salmonella* and *Escherichia* LPS but TLR4^{-/-} were unresponsive (Tapping, Akashi *et al.* 2000). MD2, a protein bound to TLR4 that facilitates LPS responsiveness, and TLR4 are thought to be in close proximity to CD14 (Jiang, Akashi *et al.* 2000). *E.coli* LPS, which depends on TLR4 for signalling, and *P. gingivalis* LPS, a TLR2 agonist, both bind to CD14 but on distinctive sites (Cunningham, J. *et al.* 1999). Recent work by Pridmore and Wylie *et al* (Pridmore, Wylie *et al.* 2001) using *N. meningitidis* observed that activation of signalling pathways occurred via TLR4/MD2 and CD14, but results using an LPS-deficient mutant showed that the organisms elicited biological responses via TLR2 and not TLR4/MD2, implying that other outer membrane components of the bacteria could induce signalling activity. Also TLR4-mediated NFκB activation by LPS is enhanced by sCD14 (Chow, Young *et al.* 1999). The role of CD14 in *Neisseria* recognition will be discussed in more detail.

1.6 Class A Scavenger receptor

1.6.1 Introduction

Scavenger receptors (SRs) were first discovered in 1979 when studies examining the mechanism by which cholesterol from lipoproteins (LDL) were able to accumulate in macrophages (Goldstein, Ho et al. 1979). Studies have shown that scavenger receptors are involved in cholesterol deposition on the arterial wall, as is the case in the formation of atherosclerotic plaques, as the excessive internalisation of modified LDL, via SR, converts macrophages to lipid-rich foam cells which form fatty streaks and atherosclerotic lesions (Brown and Goldstein 1983). Scavenger receptors are multidomained transmembrane glycoproteins that are defined by their ability to recognize and bind a broad range of polyanionic macromolecules, including modified (not native) LDLs. No single domain is common to all members of this family of receptors, but they share the ability to bind an extensive and varied range of ligands. Macrophages, dendritic cells, endothelial and smooth muscle cells have all been shown to express SRs. The receptor present on microglia also binds β -amyloid peptides and may cause the onset of Alzheimer's disease (Christie, Freeman et al. 1996; el_Khoury, Hickman et al. 1996). Human families have been identified in which some members display extensive collections of cholesterol under the skin, producing a yellow discolouration (xanthelasmas and planar xanthomata), and have a 4 to 7-fold increase in Class A Scavenger receptor (SR-A) expression.

Macrophage SR-A (MSR-A) play important roles in the non-inflammatory clearance and degradation of endotoxic lipids (Hampton, Golenbock et al. 1991), resulting in the non-inflammatory removal of LPS from the body (Haworth, Platt et al. 1997). Studies using SR-A^{-/-} mice have demonstrated that these animals become hypersensitive to endotoxin, and exhibit an increased susceptibility to

invasion by bacteria such as *Listeria monocytogenes* (Suzuki, Kurihara et al. 1997; Peiser, Gough et al. 2000; Thomas, Li et al. 2000).

As a multiligand receptor, MSR-A have been implicated in a number of macrophage-associated processes such as phagocytosis of microbes, apoptosis (Platt, Suzuki et al. 1996; Terpstra and van_Berkel 2000), clearance and detoxification of microbial products; as well as diseases such as arteriosclerosis, Alzheimer's disease and other CNS disorders (Kodama, Freeman et al. 1990; Matsumoto, Naito et al. 1990; Naito, Kodama et al. 1991; el_Khoury, Hickman et al. 1996; Paresce, Ghosh et al. 1996).

1.6.2 Structure and expression of SRA

Class A macrophage scavenger receptors (MSR-A) are homotrimeric multidomained molecules that comprise types I and II, a non-functional splice variant III, and a more distant receptor known as MARCO (Gough, Greaves et al. 1998). A single gene encodes types I and II receptors (SR-AI, SR-AII) but generates the two forms through alternative mRNA splicing (Freeman, Ashkenas et al. 1990; Kodama, Freeman et al. 1990).

Types I and II SR-A comprise six domains, of which I-V are shared by the two receptor types, whereas domain VI is restricted to the Type I receptor. Murine type I is 1457bp in length and encodes a protein of 454 amino acid residues, whereas the 4kb transcript of type II receptor cDNA encodes a 350 amino acid molecule. The two sequences diverge at residue 348, at which point the type II cDNA encodes 3 additional amino acids to complete the 6 amino acid C-terminal domain, and a long 3' UTR follows these 9 nucleotides.

The 6 domains that make up SR-A are as follows:

Domain I (residues 1-51 of murine sequence) comprises the SR-A cytoplasmic tail that contains the N-terminal part of the polypeptide, which orientates the protein as a type II transmembrane molecule. Typical motifs that are present in other endocytic receptors are notably absent from the cytoplasmic tail. Of the three conserved potential phosphorylation sites, two contain the consensus motif for protein kinase C and one for calmodulin-dependant protein kinase II. There is species divergence in the N-terminal region of the cytoplasmic tail, this implies that it does not play a critical role in receptor-mediated endocytosis. Domain II is the transmembrane domain (26-28 hydrophobic residues) and contains a conserved proline residue at position 67, which may affect the packing of the trimers in the membrane.

Domain III is the spacer domain and has 2 of the seven potential N-linked glycosylation sites. Domain IV forms a α -helical coiled coil structure on the basis of 23 heptad repeats, and has been strongly implicated in trimer assembly during SR-A biosynthesis (Ashkenas, Penman et al. 1993). Analyses of recombinant mutants of human SR-A have established that the seven-residue sequence Ile¹⁷³ – Ser¹⁷⁹ is essential for receptor oligomerisation, and the remaining five sites for N-linked glycosylation are located in this domain (Frank, Lustig et al. 2000). Intracellular dissociation of endocytosed ligands and recycling of the receptor are thought to be due to a pH-dependent conformational change within this domain.

Domain V, the collagenous domain, mediates the binding of ligands, including modified lipoprotein such as acetylated and oxidised LDL (ac-/ox-LDL) (Doi, Higashino et al. 1993). Both SR-AI and SR-AII express uninterrupted Gly-X-Y (where X or Y is any residue) tripeptide repeats - 23 (human) or 24 (bovine, murine, rabbit) - which form the collagenous triplex helix. The majority of these residues are believed to be neutral or positively charged at physiological pH with clusters of positively charged residues highly conserved across species (Ashkenas, Penman et al. 1993).

Within this collagenous domain is a cluster of five lysine residues, which are highly conserved among animal species. Binding studies on a series of point mutation and truncation receptors indicates this region is essential for mediating the specific binding and internalisation of chemically modified lipoproteins (acetylated and oxidized LDL) (Kodama, Freeman et al. 1990; Matsumoto, Naito et al. 1990; Rohrer, Freeman et al. 1990; Naito, Kodama et al. 1991).

These unpaired lysine residues at the outer edge of the helix would be available for intermolecular interactions with negatively charged ligands, such as Poly I and G, which can both block the ligand-binding site by forming quadruplexes on the surface of the receptor, with the stereospecific position of the phosphate groups together generate a negative charge that is complimentary to this binding site (Yamamoto, Nishimura et al. 1997). In addition to LDLs, other ligands include the lipid A domain of LPS, LTA, some sulphated polysaccharides (fucoidan, dextran sulphate) and some polynucleotides (Poly I and Poly G, but not Poly C or A) (Brown, Basu et al. 1980).

Studies using truncation and point mutation constructs have revealed that the carboxyl terminal 22 amino acids contain the site of acLDL recognition. Mutation of Lys³³⁷ of the bovine protein abolished all acLDL binding (Doi, Higashino et al. 1993). Although studies using the collagenous domain of the rabbit receptor indicated that residues distinct from the 22 amino acids also affect binding of modified lipoprotein (Andersson and Freeman 1998): the fact that these studies were performed at different temperatures (37°C and/or 4°C) may explain these differences. Ligand recognition appears to require complex conformational interactions independent of the terminal collagenous sequence, which may explain the failure of short peptide models to mimic the properties of the entire domain (Mielewczyk, Breslauer et al. 1996).

SR-A type II receptor is defined by Domain VI. SR-AII contains a truncated C-terminal domain of seventeen (human) or six (bovine and murine) residues. In

contrast, the type I receptor contains a hinge (VIa; 7 amino acids) and a cysteine rich domain (SRCR – VIb 101 amino acid residues) at the C-terminus. No clear role has been assigned to this domain and its function remains obscure, but as a structural motif it has been highly conserved across a wide range of species and is found in an increasing number of proteins, including other immune system proteins such as CD5 and CD6 (Freeman, Ashkenas et al. 1990). No NMR spectroscopy or crystallographic data are available for either Type I or II SR-A, but electron microscopy and biophysical methods have reported the presence of covalent structures that resemble those predicted from the primary amino acid sequence.

Domains IV and V are joined by a flexible hinge with a variable angle between the two. When most extended the receptor resembles the structure predicted from the primary sequence, but at physiological pH the structure is more compact with the collagen domain bent back onto the coiled coil. The functional significance of these alternative forms is unclear. A highly flexible hinge appears to join the fibrous domains which allow the receptors a fair degree of movement as the angle ranges between 0 - 180°.

1.6.3 Molecular Structure

The organisation of the genes for human and murine SR-A has been reported. Both are located on chromosome 8 and the coding regions are spread across 11 exons; the first 8 encode sequences common to both types I and II receptors. Type I is generated through splicing of exons 8 to 10 and 11, the latter encodes the SRCR domain. Type II results from splicing of exon 8 to 9, which includes the C-terminal structure. A third splice variant was reported by Gough and colleagues (Gough, Greaves et al. 1998) and arises from the splicing of exon 8 directly onto exon 11 which results in a novel shorter cysteine-rich region of 44 amino acids. When this form of SR-A was transfected into CHO cells, it was not

expressed at the cell surface and was unable to internalise acLDL but inhibited the binding of lipoproteins to SRA-I and SRA-II by acting as a dominant negative receptor.

1.6.4 Ligand-binding properties of SRA

The feature of SRA that almost certainly underlines its contributions to a multitude of biological processes is its broad, high-affinity ligand binding property. This feature is shared with members of other classes of SRs and sets SRA apart from the majority of membrane receptors that can bind only a single ligand. Hence SRs have been termed “molecular flypaper”. The range of ligands include non-physiological ones (e.g. acLDL), those derived from the environment (Silica particles, crocidolite asbestos) and those of microbial origin (LPS). Chemical modification that includes acetylation and oxidation convert LDL into a SRA ligand and abolish binding to the LDL receptor. A minimum modification of 15-20% of lysine residues of LDL is sufficient to facilitate binding to SRA. Another interesting and important feature of the binding properties of SRA is the phenomenon of “non-reciprocal cross-competition”. That is the binding of ligand A to the receptor can be totally competed out with an excess of ligand B, and the reverse is not true. An example is the relationship of acLDL and oxLDL with SR-A: as competition of acLDL for SRA by oxLDL is absolute, but when the situation is reversed, inhibition is only partial. This implies the existence of distinct, though probably overlapping, binding sites on SRA for the two modified lipoproteins. The list of known ligands is long (Platt and Gordon 1998; Gough and Gordon 2000), but as work in this area continues it becomes apparent that more are being found.

1.6.5 Expression of SR-A

The presence of SRA has been determined in many species, including murine (Hughes, Fraser et al. 1995) bovine (Naito, Kodama et al. 1991) and humans (Matsumoto, Naito et al. 1990) cells. Less information exists on the expression pattern of SRA-I/II, though co-expression was observed in atherosclerotic streaks, Kupffer cells and alveolar macrophages, but the specificity of antipeptide antibodies has been questioned (Honda, Akiyama et al. 1998). The murine monoclonal antibody 2F8 reacts to both receptors type I and II and staining is usually macrophage specific but is restricted to subpopulations in various organs; cellular expression may also be species-dependant (Matsumoto, Naito et al. 1990; Hughes, Fraser et al. 1995).

There have been a number of studies that have demonstrated the association of SR-A with disease, particularly atherogenesis where the presence of SRA on macrophage foam cells are characteristic of lipid accumulation in human lesions (Naito, Suzuki et al. 1992) and murine and rabbit models (De_Winther, Gijbels et al. 2000). This study implicates SRA in the phagocytosis of *N. meningitidis* by human macrophages.

1.6.6 Conservation of SR-A

Cloning of the cDNA encoding SR-A from four mammalian species revealed remarkable conservation of the species, with around 70% homology between amino acid sequences. Differences have been found in the properties of the receptor for each species, for example the Re form of LPS can inhibit high-affinity binding of acLDL to murine type I receptor, but had no such effect on the bovine receptor (Ashkenas, Penman et al. 1993); this may be due to the difference in sequence of particular Gly-X-Y triplets in the collagenous domain.

Due to our limited understanding of the evolution of SRA, conclusions of their evolutionary origin and relationships are unclear.

1.7 Membrane rafts

Membrane rafts or lipid rafts are distinct sub-microscopic microdomains characteristically rich in cholesterol and sphingolipids (sphingomyelin and glycosphingolipids) (Thompson and Tillack 1985) and are present on the plasma and endosomal membrane of eukaryotic cells (Brown and Rose 1992; Fridriksson, Shipkova et al. 1999). These lipid-enriched regions are too small to view using optical microscopes, as they appear to range from 10-300nm in size, and have certain characteristics that differentiate them from the surrounding environment, including their low buoyant density and resistance to solubilisation by non-ionic detergents, such as Triton X-100, at 4°C.

Detergent-insolubility is thought to be due to the tight packing of cholesterol with saturated fatty acid chains of sphingolipids, resulting in the formation of a liquid-ordered (l_o) phase at low temperatures (Ahmed, Brown et al. 1997). In contrast, the phospholipids in the surrounding cell membrane are detergent-soluble, as they lie in a more fluid, liquid-disordered state due to the presence of unsaturated, branched (kinked) fatty acid chains. It is these liquid-ordered regions present on the fluid lipid bilayers that are referred to as “lipid rafts” (Simons and Ikonen 1997). Based on biochemical characteristics, these distinctive raft microdomains are also referred to by a number of names including detergent-resistant membranes (DRMs), detergent-insoluble glycolipid-rich membranes and glycolipid-enriched membranes, but throughout this study the terms “lipid raft” or “membrane rafts” shall be used to refer to these structures.

It may be erroneous to assume cell membranes are merely composed of “raft” or “non-raft” environments. Recently rafts have been further sub-divided into different types based on their resistance to solubility in non-ionic detergents, (Roper, Corbeil et al. 2000), and this may be reliant, to some degree, on the

detergent used. Furthermore, chemical disruption of actin cytoskeleton in epithelial cells was required to isolate a subset of lipid rafts containing CD44, which would have otherwise been missed by sucrose density gradient, suggesting that two distinct types of lipid rafts are present, dependent on their association to actin cytoskeleton of the cell (Oliferenko, Paiha et al. 1999). CD44 is a transmembrane receptor associated with ordered microenvironments in a number of different cell types is linked to actin cytoskeleton.

In addition to lipids, it has been discovered that a number of molecules also cluster on lipid rafts. Yamada (Yamada 1955) assigned the name “caveolae intracellularis” to distinctive 50-70nm flask-shaped or omega-shaped invaginations present on the plasma membranes of a number of cell types, which have since been implicated in endocytosis and signal transduction. Caveolae are largely covered by a family of integral membrane proteins ranging in size from 21-25-kDa called caveolins, of which there are three main isoforms of caveolin, caveolin-1, -2 and -3 (Scherer, Tang et al. 1995). Caveolins play an essential role in the structural formation of caveolae, (Rothberg, Heuser et al. 1992; Fra, Williamson et al. 1995) and their discovery greatly aided research in this field, as caveolin expression was the first marker protein used to correlate the presence of caveolae on cells (Rothberg, Heuser et al. 1992; Fra, Williamson et al. 1995; Smart, Ying et al. 1996). An important point is that lipid rafts are not limited to caveolae, as rafts have been implicated in signal transduction processes on cells such as T cells and basophils, which notably lack distinct caveolae.

Many proteins found on membrane rafts of mammalian cells are linked to saturated acyl chains (found particularly in sphingolipids), as it is probable that they prefer a more ordered environment. Two ways in which proteins are linked to saturated acyl chains on mammalian cells are through acylation with myristate or palmitate (Brown and London 1997) or by attachment to a glycosylphosphatidylinositol (GPI) anchor (McConville and Ferguson 1993). Proteins tethered to the membrane by their GPI-anchor are characteristically

found in rafts (Brown and Rose 1992), and the hydrophobic interactions between long saturated fatty acids hold these structures together on the cell (Schroeder, London et al. 1994).

Cholesterol-depleting agents such as β -cyclodextrin (β CD), nystatin, filipin, lovastatin and cholesterol oxidase are all used to disrupt lipid rafts resulting in loss of distinct caveolae and causing GPI-linked proteins to disperse into the plasma membrane (Smart, Graf et al. 1999; Pralle, Keller et al. 2000). Use of cholesterol depleting agents to disrupt rafts is known to block a number of compartmentalised signalling pathways (Simons and Toomre 2000). Lipid rafts appear to have a variety of functions from membrane transport, transcytosis across epithelial monolayers to signal transduction and generating cell polarity.

In terms of cell polarity, the transition from resting cells to polarised cells able to migrate to sites of infection, is a complex series of reactions that requires a high level of co-ordination of signalling molecules to ensure that cellular attachment and cell membrane extension take place at the front of the cell (lamella) and detachment and membrane retraction at the back (uropod). Recent studies have found that the distribution of lipids on the plasma membrane are essential for this process, and treatment of motile cells with cholesterol-depleting agents results in the inhibition of cellular polarisation and migration. This suggests that the presence of lipids normally associated with raft formation, which in turn are essential in signalling networks, may be important sites for the signal transduction events associated with cell polarisation and motility. Recent work by Gómez-Moutón and colleagues found that different types of rafts were situated at opposite ends of polarised migrating T-cells, with ganglioside GM3-enriched rafts concentrated at the lamella and GM1-enriched rafts at the back of the cell (Gómez-Moutón, Abad et al. 2001).

Signal transducing family of proteins, Src-family kinases, are involved in the tyrosine phosphorylation of conserved sequences in the cytoplasmic domain of

cell surface receptors in a range of haematopoietic cells (Isakov 1997). Ligation or clustering of GPI-linked proteins can, in itself, induce transmembrane signal transduction which can be through Src-family kinase activation (Brown 1993; Zisch, D'Alessandri et al. 1995). As neither GPI-proteins nor Src-family kinases penetrate the cell membrane, precisely how these signals are transduced into the cell is unknown, but signal transducing Toll-like receptors have been associated with GPI-linked receptor. TNF receptor-1 are also able to translocate to these microdomains on binding TNF- α , where the receptor associates with signalling complexes such as Serine/Threonine kinases, to form signalling complexes, as disruption of lipid rafts results in the TNF- α transferring its signal from inducing NF κ B activation to upregulation of apoptotic signals (Legler, Micheau et al. 2003).

1.7.1 Rafts and disease

The study of lipid rafts, though limited by current techniques, is potentially a useful way of examining how microbes enter host cells and survive or how lipid rafts alert the host to the presence of pathogenic organisms. Signalling networks affect the growth, differentiation, motility and adhesion of cells, and so up-regulation of signalling can result in a number of diseases. Rafts play an important role in signalling pathways, and have been implicated in a number of diseases including prion diseases, Alzheimer's disease and cancer. In the case of Alzheimer's disease the production of β -amyloid plaques from precursor proteins has been shown to take place on lipid rafts, in a cholesterol-dependent manner (Ikezu, Trapp et al. 1998).

Evasions of cytotoxic immune responses are a common characteristic of intracellular pathogenic organisms, as their route of entry into immune cells will ultimately determine their survival. Lipid rafts do not make up a large proportion of the cell membrane, but the dense collection of signalling molecules

prevalent in these structures has meant that a range of microbial pathogens specifically target lipid rafts as a favourable or preferential point of entry into host cells. Rafts can be viewed as “safe” points of entry for some pathogens but they also have the potential to trigger cytotoxic responses in host cells. Studies using cholesterol-disrupting agents have been found to block the internalisation of organisms through rafts. Bacteria, prions, viruses and parasites have all been associated with using lipid rafts to gain entry into immune cells, and may aid the intracellular survival of these organisms

1.8 *Neisseria meningitidis*

1.8.1 Meningitis

Meningitis is inflammation of the meninges, which comprise the three membranes that enclose the brain and spinal cord. It may involve the dura and lead to pachymeningitis or the pia/arachnoid to cause leptomeningitis. The latter is more common, and is generally referred to as meningitis (Strausbaugh). The brain and spinal cord are relatively well protected from bacteria, but once the microbes have penetrated the host's defences the infection can spread rapidly via CSF pathways. So many microbes that ordinarily pose a threat elsewhere in the body can cause serious and fatal infections of the CNS (Strausbaugh; McLeod Griffis 1995).

Acute pyogenic meningitis is caused by infection of the sub-arachnoid space, the most common organisms being *N.meningitidis* (*Nm*), *S. pneumoniae* and *Haemophilus* – with *E.coli* being common in infants. Meningitis may also occur as a result of local spread from infection in the bones of the skull, or after compound fracture of the skull. A number of pathological processes can also result in the onset of meningitis including: cyst/haemorrhage rupture into the subarachnoid space and/or tumourous involvement of the meninges. The extension of inflammation from the bones in the skull almost always results in acute inflammation of the dura. Suppuration occurs between the bone and dura resulting in the formation of an extradural abscess. The pus can spread widely over the cerebral hemisphere and the infection may spread through the dura to form a sub-dural abscess (Strausbaugh; Stephens and Farley 1991). Diagnosis can often be established by examining the CSF, and principal findings in the CSF are inflammatory cells, proteinaceous exudates (and depending on the cause, reduction in glucose concentration) or viable microbes. Changes in CSF include it becoming turbid or becoming distinctly purulent, with a marked increase in the

number of neutrophils (Stephens and Farley 1991; Brandtzaeg 1995). Bacteria, fungi, viruses, protozoa and helminths can all cause this disease, and other more common bacteria that can cause meningitis include *Listeria monocytogenes*, *Streptococcus agalactiae*, aerobic gram-negative bacilli such as *E.coli*), *S. aureus* and *M. tuberculosis*.

1.8.2 *N. meningitidis*

The *Neisseria* species are non-motile, non-spore forming gram-negative diplococci that have a characteristic kidney/coffee bean shape. Size can vary from 0.6 - 1.5µm, this can depend on the species, the age of the culture and the source of the isolate. They also have specific growth requirements, as the optimum temperature is between 35 and 37°C. All species are aerobes and produce catalase (except *N. elongata*), and cytochrome oxidase (Pujol 1997). Only *N. gonorrhoeae* and *N. meningitidis* are primary pathogens that only infect humans. Although both *N. gonorrhoeae* and *N. meningitidis* can both be isolated from asymptomatic individuals, *N. gonorrhoeae* (*Ng*) is always considered to be a disease producer, whereas *N. meningitidis* (*Nm*) is commonly isolated from the throat or nasopharynx of healthy individuals as well as from patients with meningococcal disease (MCD) (Pujol 1997; Stites, Terr et al. 1997). Meningococci colonise the nasopharynx and gain entry into the bloodstream, where they cause meningococcaemia or fulminant septicaemia, and/or progress to the CSF to cause meningitis (McLeod Griffis 1995).

Humans are the major reservoir for meningococci, and transmission between individuals occurs as a result of direct contact with respiratory secretions or by aerosol droplets that are contaminated with the organism. Meningococci are susceptible to drying and do not survive long in airborne droplets; person-to-person transmission requires frequent or close contact. MCD is still a relatively rare disease between 1 and 12 cases of invasive MCD occur per 100,000 of

population in industrialised nations, each year with high rates of mortality, and morbidity, with a peak incidence of children between 6 months to 2 years of age (Riedo, Plikaytis et al. 1995). Up to approximately 10% of healthy individuals carry the organism in the nasopharynx at any one time, usually for between 6-9 months; but this rises significantly in confined populations such as army barracks. (Riordan, Cartwright et al. 1998)

N. meningitidis are subdivided into a number of serological groups depending on the chemical composition of capsular or cell wall antigens; such groups include A, B, C, D, X, Y, Z, W135. Worldwide five of these serogroups are responsible for causing the majority of MCD cases: A, B, C, W135 and Y. Serogroups B and C are most prevalent in industrialised countries, with over 50% of cases due to serogroup B, which as yet has no effective vaccine (Connolly and Noah 1999; Donaldson, Moores et al. 1999). Most epidemic and endemic cases of MCD are caused by strains of a limited number of genetically defined meningococcal clonal groups that are associated with high rates of disease once acquired. These include ET-35 (mostly serogroup B), ET-37 (mostly serogroup C, Y and W135), serogroup A clonal groups and the A4 cluster. Once in the population, these virulent clonal groups can both diversify and decay over time through immune selection, genetic recombination and mutation (Fell 1999), but the reduction of carriage rates and acquisition of carriage of circulating meningococcal clonal groups may be an important strategy in the development of meningococcal vaccines. Such genetic alterations are advantageous to the organisms, as the variation enables them to evade immune responses and go on to cause disease (Levin and Bull 1994; Zhu 2001).

1.8.3 Virulence determinants

Pathogenesis of meningococcal infection is as yet unclear, but evidence suggests that *N. meningitidis* has developed mechanisms to avoid recognition by the immune system, including the presence of structures on the surface of the cell. Virulence determinants whose presence is essential for successful meningococcal invasion include outer membrane proteins, pili, and capsular polysaccharide and LPS, which are discussed in more detail below.

1.8.3.1 Polysaccharide capsule

Most meningococci are surrounded by an acidic and hydrophilic polysaccharide capsule that provides the surface charge and humid environment necessary for the survival of the MC in aerosol droplets, and the structural differences among these capsules account for serogroup specificity. The sialylation of bacterial cell membrane glycoconjugates prevent the assembly of the membrane attack complex (MAC) on the bacteria, thus aborting complement-mediated lysis. Capsule also prevents C3b attachment to the surface of the bacteria during incubation in non-immune serum (Capel, Groeneboer et al. 1978), which may be an important virulence determinant of *N. meningitidis*, and one of the contributing factors for the predominance of serogroup B disease in industrialised countries.

The (α 2-8)-linked polysialic acid capsule of serogroup B *N. meningitidis* is a major virulence factor since only capsulate organisms are recovered from the blood and CSF, whereas both capsulate and acapsulate organisms were recovered from the nasopharynx (Read, Zimmerli et al. 1996). Serogroups B, C, Y and W all possess capsules that are polymers of sialic acid. As the meningococcal capsule is involved in adhesion to target cells, modifications to the capsule can affect the transmissibility of the organisms. Capsule switching is thought to be a result of allelic replacement of the *SiaD* gene through recombination and

transformation. *SiaD* is responsible for sialic acid capsule specificity. A recent example of this would be the outbreak of W135 MCD in returning Hajj pilgrims in 2000 (Popovic 2000; Taha 2000). The organisms isolated were all identical and belonged to the most common ST (sequence type) of ET-37, ST-11; the origin of the clone was unknown. Pilgrims were vaccinated against serogroup A and C, but the extremely high numbers of people congregating at the Hajj allowed the epidemic clonal expansion within the ET37 clonal complex. This outbreak may have been a result of a capsule switch from C to W135 or as a result of an old clone of ET-37. The extremely rapid spread of this new variant to both household and non-household members suggest a new member of a virulent clone (Taha 2000). Vaccination campaigns against serogroup A and C may give rise to specific immune responses against serogroup C, so W135 organisms may in fact represent genetic variants (capsule escape switch) of a virulent serogroup C ET37 clone. The horizontal exchange, DNA repeats and recombination of *Neisseria* allows genome shuffling within a population that leads to rapid phenotypic diversity or fitness, which may account for the apparent success of this human-based organism.

Studies using serogroup B *Nm.* have shown that the polysaccharide capsule non-specifically inhibits macrophage bactericidal activity against meningococci, and Read *et al* observed that capsule presence prevented bacterial binding to macrophages, and also appears to delay phagolysosome formation (Read, Zimmerli et al. 1996). Though the serum of adults carries low levels of anti-B-polysaccharide IgM, the antibodies are not particularly bactericidal and are of low avidity, so their presence probably does not contribute a great deal to the immunity against group B meningococci (Griffiss, Brandt et al. 1984). Serogroup B capsule is poorly immunogenic in humans, as human complement appears to direct its bactericidal abilities at non-capsulate antigens; but as the capsule is the antigen common to all group B meningococci work continues on developing a polysaccharide-based vaccine, as it is essentially the only antigenic structure shared by all serogroup B meningococci (Fusco, Michon et al. 1997).

1.8.3.2 *Lipopolysaccharide*

Lipopolysaccharide (LPS), or endotoxin, is present in the outer membrane of gram-negative bacteria and is a major inflammation-inducing component of the bacterial cells. An important characteristic is that unlike exotoxins, the toxic symptoms of LPS are not caused by its direct action on the phagocyte, but instead the macrophages respond to LPS by secreting cytokines such as TNF- α , IL-1, 8, 6, 10, IFN- α / β , TGF- β and CSFs, which in turn induce the inflammatory responses that may ultimately lead to the destruction of bacteria (Beaty, Franklin et al. 1994). Early studies showed that the LPS is a major toxic agent, and once it has entered the circulation, the range of inflammatory responses that it activates can ultimately lead to organ failure and death. High levels of endotoxin were found in lethal cases with irreversible septic shock and further studies confirmed that increased levels of circulatory endotoxin were associated with an increase in fatality rate (Brandtzaeg, Kierulf et al. 1989).

LPS can affect macrophage physiology in a number of ways, including provoking an increase in glucose, oxygen consumption, cell size and membrane ruffling. It also increases the synthesis of a number of cellular and secreted proteins/lipids, whilst others are reduced. The ability of macrophages to destroy bacteria and tumour cells is also enhanced, although their motility is diminished. Structurally meningococcal LPS is an amphipathic glycolipid, and is composed of a lipid A core structure that is embedded in the outer membrane of the bacterium, and covalently linked to the lipid A is a polysaccharide structure that extends out from the surface of the cell. The hydrophobic lipid A region is highly conserved within families of bacteria, and is referred to as the endotoxic centre. The segment of polysaccharide furthest away from the lipid A, known as the O-antigen, are variable, immunodominant and confer serological specificity to the bacteria. Between the O-antigen and the lipid A is the core region, and this is more conserved than the O-antigen. *Neisseria* LPS, often referred to as

lipooligosaccharide (LOS), is relatively small in size compared to *E.coli* or *Salmonella* LPS and lacks O-antigen altogether.

An important aspect of bacterial protection against immune responses is the sialylation of LOS, which can protect *Neisseria* from complement-mediated serum lysis and phagocytosis by neutrophils (Smith, Parsons et al. 1995; Ram, Mackinnon et al. 1999). Sialylation is catalysed by an α -2,3-sialyltransferase (Lst) using both endogenous and exogenous sources of 5'-cytidinemonophospho-N-acetylneuraminic acid (CMP-NANA) to monosialylate the terminal galactose of LOS (Mandrell, Lesse et al. 1990; Gilbert, Cunningham et al. 1997). The extent of serum resistance conferred by meningococcal LPS sialylation varies between different serogroups and at different stages of meningococcal disease pathogenesis (Kahler, Martin et al. 1998; Vogel, Claus et al. 1999).

Previous work has shown that free LPS binds poorly to macrophages, but found that LPS-plasma protein complexes bind efficiently via specific receptors. Such serum proteins include lipopolysaccharide binding protein (LBP), O-Ag specific antibodies, complement, HDL albumin and septin. LBP functions both as a bacterial opsonin and a cofactor that increases the efficiency of LPS stimulation of macrophages (Schumann, Leong et al. 1990). This 60kD glycoprotein is synthesized in hepatocytes as a 50kD single chain polypeptide, and binds specifically to the lipid A component of LPS. Even though LBP is an acute-phase reactant, there is enough present in normal plasma to function as an early defence mechanism during infection.

The binding of LPS/LBP complexes to CD14 receptors, which lack transmembrane or cytoplasmic regions, and are mobile in the plane of the membrane, results in cellular activation through CD14 or LPS neutralisation through high-density lipoproteins (HDL). Kinetics of these reactions have suggested that LPS/LBP complexes bind to CD14 before LPS is transferred to HDL; suggesting that cellular activation by immune cells occurs before the LPS

is neutralised as a mechanism of avoiding over stimulation of the immune system (Frey, Miller et al. 1992).

LPS is also a known ligand for a number of macrophage receptors, including CR3, Toll-like receptors and scavenger receptors. Type A scavenger receptors (SR-A) bind to a wide range of polyanionic ligands such the tetra-acyldisaccharide lipid A precursor lipid IV_A, modified lipoproteins and lipoteichoic acid of gram-positive bacteria. *In vivo* studies using mice lacking SR-A were found to be more susceptible to endotoxic shock and produced more TNF- α and IL-6 in response to LPS. TNF- α activity was also suppressed using antibodies, and this was found to reduce LPS-induced mortality in the mice. SR-A is therefore considered to have a protective role in host defences by scavenging LPS and reducing the release of pro-inflammatory cytokines (Haworth, Platt et al. 1997).

Nuclear factor- κ B (NF κ B) is a heterodimeric transcription factor formed by dimerization of proteins from the Rel family of proteins, and is thought to play a central role in immune and inflammatory responses by regulating genes that encode pro-inflammatory cytokines, adhesion molecules, growth factors, chemokines and inducible nitric oxide synthase (iNOS) (Hobbs and Moncada 1999; Karin and Ben-Neriah 2000). NF κ B comprises 50kD and 65kD structures bound to the cytosolic protein I κ B. Once the cell is activated I κ B is phosphorylated, NF κ B dissociates and moves to the nucleus, where it binds upstream of specific genes.

LPS alters the distribution of microfilaments and microtubules of macrophages, resulting in reduced phagocytic activity, suggesting that LPS may be suppressing phagocytosis via its effects on the cell cytoskeletal network. LPS suppression of phagocytosis is not mediated by the induction of cytokines, and was observed when LPS was administered *in vivo* (Wonduly, Ghaffar et al. 1996).

The development of the first viable LPS-deficient mutant of *N. meningitidis* may help in illustrating the role of LPS in meningococcal-cell interactions in more detail, as even though this mutant lacks LPS, the capsule and PorA expression appear to remain intact.

1.8.4 Possible mechanisms for meningeal invasion

Meningeal invasion usually takes place as a result of nasopharyngeal colonisation followed by bloodstream invasion, but exactly how these pathogenic microbes gain access from the bloodstream to the meninges and subarachnoid space is not known. The site of entry could involve the choroid plexus epithelium or the vascular endothelium.

It is unlikely that leukocytes are used to gain entry into the subarachnoid space, as a large number of bacteria may be present in the CSF before leukocyte migration commences. In order to cross the brain barriers *N. meningitidis* must cross a monolayer of endothelial or epithelial cell tight junctions, by either moving between the tight junctions (paracellular route) or by intracellular transport (transcellular route). Further understanding of these mechanisms might allow us to understand the cause for the neurotropism of meningeal pathogens, including *N. meningitidis*, and elucidate the role of microbial factors e.g. pili, capsule, OMP and LPS; as these factors may themselves mediate brain barrier opening or endothelial cell entry.

Unfortunately animal models that can adequately represent individual components of the blood brain barriers are not available, as a result of this indirect evidence is used to suggest that both *N. meningitidis* and *S. pneumoniae* pass through the monolayer by transcytosis. Evidence for this hypothesis comprises *in vivo* studies that have shown *N. meningitidis* localized inside endothelial cells of the brain of an infected patient, and *in vitro* meningococci

have been observed crossing the monolayer of tight junction-forming cells by transcytosis, without altering the organization of tight junctions. The implication being that once the organisms are internalized, they cross the cytoplasm and are exocytosed at the other side. *In vitro* studies using *S. pneumoniae* found that only organisms that express an adhesin, choline-binding protein, are internalized and transcytose through endothelial cells before exiting at the basolateral surface.

There are few bacterial pathogens able to invade the brain, due to the physiological barriers between the CNS and bloodstream. Two main structures also act as brain barriers, the blood brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB), make up one of the tightest barriers in the body. The exact site of entry into the CSF is unknown for most organisms, but studies using *N. meningitidis* have shown that they come into direct contact with the BBB, and judging by the frequency and efficiency that meningococci cross the blood brain barriers, meningococci appear to have developed highly elaborate methods to overcome these physical obstacles. A favoured hypothesis is that meningococcal structures primarily required to interact with mucosal cells, may not have meant to be of use in meningeal invasion, and may be an unlucky accident (Meyer 1999; Pollard and Frasch 2001).

1.9 Aims of thesis

The aim of this thesis was to investigate the contribution of a number of cell surface receptors, both opsonic and non-opsonic, expressed by monocytes and/or macrophages, and study roles these receptors may play in the adherence and uptake of serogroup B *N. meningitidis*, as well as events following ingestion. Model systems were established to examine the contribution of a number of pattern recognition receptors in these interactions, including CR3, CD14, SR-A, PAF receptor, MR.

Declaration

No portion of this work has been submitted in support of an application for another degree or qualification of this or any other university, except where due acknowledgment has been made in the text.

Chapter 2

Methods and Materials

2.1 Materials

2.1.1 General chemicals

Chemicals were of analytical grade and obtained from either BDH Merck (Dorset, UK) or Sigma (Dorset, UK).

2.1.2 Human Cell Lines

Cell lines were obtained from stocks kept at the University of Sheffield, and had been originally from ATCC (American Type Culture Collection) or ECACC (European Collection of Animal Cell Cultures). THP-1, human pre-monocytic leukaemia cells (Tsuchiya, 1980), HeLa (ECACC 85060701) cervical epithelial cell line and J774A.1 a macrophage-like mouse cell line were all used.

2.1.3 Cell Culture Reagents

RPMI 1640 and Dulbecco's modified Eagle media (DMEM) were obtained from GibcoBRL (Paisley, UK), foetal bovine (calf) serum (FCS) from Autogen Bioclear (Wiltshire, UK), L-glutamine and phorbol 12-myristate 13-acetate (PMA) was from Sigma. Heat-inactivated FCS was made by incubating FCS for 30 minutes at 56°C before cooling and centrifuging at 3000g to remove fibrin.

2.1.4 Other reagents

DiI-labelled acetylated LDL (DiI-acLDL) and DAPI (4',6-diamidino-2-phenylindole dihydrochloride) were obtained from Molecular Probes (Cambridge, UK). Platelet activating factor receptor antagonist (PAFRa) was from Calbiochem (UK). β -cyclodextrin, nystatin and phosphatidylinositol-specific phospholipase C (PIPLC), bovine- α -D-mannosylated-PITC-albumin (mBSA), RGDS and RGES containing peptides, poly-inosinic acid (poly I), -guanylinic (Poly G), -cytidylic (Poly C) and -adenylic acid (Poly A), saponin (Quillaja bark), trypan blue (0.4%), Lidocaine-HCl and Triton X-100 (t-octylphenoxyethoxyethanol) were all obtained from Sigma (Dorset, UK). Cytokine ELISA kit was from Pharmingen.

2.1.5 Solutions

Phosphate buffered saline (PBS) was obtained from Oxoid and tablets were made up according to manufacturers instructions and autoclaved prior to use. PBS: 137mM NaCl, 8.1mM Na₂HPO₄, 2.7mM HCl and 1.5mM KH₂PO₄ at pH 7.3. Distilled water (dH₂O) was purified using the Millipore-Ro6 plus system and autoclaved prior to use. Paraformaldehyde (pfa) was obtained from BDH Merck (Dorset, UK): 4% (w/v) pfa was made up in distilled water, buffered to pH 7.2 using 1N sodium hydroxide, and passed through a 0.22 μ m filter prior to use. To avoid using trypsin where possible, a detachment buffer used to remove adherent macrophages was made up 10mM EDTA and 10mM Lidocaine-HCl in PBS buffered to pH7.3 and passed through a 0.22 μ m filter.

2.1.6 Antibodies

The table below lists some of the antibodies used:

Antigen	Species	Clone	Conjugation	Source
Mouse IgG	sheep		FITC	Sigma (Dorset, UK)
Mouse IgG	sheep		Cy3	Sigma
Rabbit IgG	goat		FITC	Sigma
Human IgG (Fc specific)	goat		FITC	Sigma
Anti-human CD11b IgG1	mouse	2LMP19c		DAKO (Cambridgeshire, UK)
IgG1 (negative control)	mouse	DAK-G01		DAKO
Anti-human CD14 IgG2b	mouse	MY4		Beckman Coulter
Anti-human CD14 IgG2b	mouse	MY4	FITC	Beckman Coulter
IgG2b (negative control)	mouse	DAK-G09		DAKO
Anti-serogroup B <i>N.meningitidis</i>	rabbit			Difco (Detroit, USA)
Anti-human LAMP-1 IgG1	mouse	H4A3		

2.2 Mammalian cells

2.2.1 General growth conditions for mammalian cell culture

MDMs and cell lines were all maintained at 37°C in 5% CO₂ and a 95% humidity incubator. All growth culture media was supplemented with 10% heat-inactivated FCS (HIFCS); 2mM L-glutamine was also added to RPMI 1640. Primary macrophages were detached by 10min incubation in detachment buffer (see above) followed by vigorous pipetting (Rabinovitch and de Stefano 1976). HeLa cell lines were detached by incubating with 5mM EDTA and 0.125% (w/v) trypsin in PBS for 5 min at room temperature.

2.2.2 Isolation and culture of human monocytes from peripheral blood monocytes

Peripheral blood mononuclear cells (PBMC) were obtained from apparently healthy volunteers and isolated using Ficoll-Paque (Pharmacia, Amersham) by density sedimentation at 1500g for 20min at 21°C (Falcon 6/300). Cells were washed three times in PBS in order to remove platelets and red blood cells and resuspended in enriched RPMI 1640 culture media, and plated on glass coverslips in 24-well tissue culture plates (Costar) at a density of 1×10^6 cells/ml. Non-adherent cells, such as lymphocytes, were separated by allowing cells to adhere to tissue culture plates for one hour at 37°C and subsequently washing wells three times with warm culture media. Cells were cultivated for a further 24h at 37°C in enriched RPMI 1640 before washing and incubating in fresh media.

Monocytes differentiated into mature macrophages, gaining a distinctive flattened, adherent phenotype and becoming terminally differentiated after 6-7

days in culture: 8-12day old cells were used throughout this study. Previous studies have indicated this type of protocol isolates over 95% of the pre-macrophage population (Montaner, 1996). Isolation and culture of all blood monocytes used in this study were carried out by staff in the tissue culture laboratory, due to the high volume of cells required by researchers in the department.

2.2.3 Cell lines

THP-1 are a human pre-monocytic leukaemia cell line (Tsuchiya 1980) and are semi-adherent cell line. Cells were plated onto glass coverslips on 24-well tissue culture plates at a density of 1×10^6 cells/ml in enriched RPMI 1640 media and used the following day. Incubation of 1×10^5 cells/ml in the presence of 25nM PMA (0.05% DMSO) over 7 days, with media changed every 2 days, resulted in differentiation to adherent macrophage-like cells (Hassall 1992).

HeLa cells are a cervical epithelial cell line and J774A.1 cells, which are a macrophage-like mouse cell line derived from a tumour in female BALB/c mouse, were used. Both cell lines were grown and maintained in enriched DMEM. All cell lines were grown in 25ml tissue culture flasks (NUNC), and were routinely passaged between 1:5 and 1:10 every three days.

2.2.4 Transfection of HeLa cells

HeLa cells were passaged every 3days in DMEM supplemented with antibiotics. Cells were seeded down at 3.5×10^4 cells/ml in 24-well tissue culture plates for 24h before achieving ~80% confluency, and were underwent transient transfection using a Superfect kit (Qiagen) according to manufacturers instructions. Generation of HeLa cell CD14 transfectants has been previously

described (Pridmore, Wylie et al. 2001). Wild-type HeLa cells and HeLa cells transfected with the vector were used as controls. The cells were kept in DMEM and 10% heat-inactivated FBS and were propagated by seeding down 3.5×10^5 cells/ml in 24-well culture plates and passaged using EDTA treatment. Immediately before exposure to bacteria the confluent HeLa cells were washed 3 times and fresh culture media was added. Plasmids pCDM8-CD14 was a gift from Prof B. Seed (Molecular Biology, Harvard Medical School, Boston) and vector pcDNA3 was purchased from Invitrogen. (Simmons, Tan et al. 1989).

2.2.5 Selection of high levels of CD14 expression in HeLa cells

Transfected cells were incubated with FITC-conjugated anti-human anti-CD14 monoclonal antibody and analysed using a fluorescent microscope to ensure that a high proportion of cells expressed mCD14.

2.3 Bacteria

2.3.1 Bacterial strains

Neisseria used throughout study are listed in the table below:

Species	Strain or acquisition no.	Genotype	Serogroup	Immunotype
<i>N. meningitidis</i>	MC58 ₁		B	L3
	H44/76 ₂		B	L3
	H44/76 (pLAK33) ₂	<i>LpxA</i> mutant	B	-
	K454		B	L3
<i>N. lactamica</i>	10617		-	
<i>N. animalis</i>	10212 N462		-	

₁ MC58 was isolated from a clinical case in UK (Virji, Kaughty et al. 1991) and ₂ H44/76 was isolated in Norway 1976. (Holten 1979) ₂ *N. meningitidis* H44/76 and pLAK33 were obtained from L. Steeghs at Laboratory of Vaccine Research (Bilthoven, Netherlands).

2.3.2 Bacterial growth media

H44/76 and LPS-deficient mutant were grown in Muller-Hinton broth, and remaining *Neisseria* species were cultivated in Tryptone Soya broth, both made up to manufacturers instructions.

2.3.3 Growth and maintenance of bacteria

All *Neisseria* strains were maintained in liquid nitrogen, and once removed from storage were sub-cultured twice on chocolate or blood agar plates before transferring ~8 colonies to liquid growth media and grown to mid-log phase at 37°C, 5% CO₂ (v/v). Cultures required between 4 and 4.5h in this environment to reach optimum growth. Gentle shaking during incubation did not appear to have a detrimental affect on bacteria and resulted in significantly reduced incubation time, but both *N. lactamica* and LPS-deficient mutant pLAK33 did not respond well to this method. Bacterial growth was measured using optical density at 620nm (Jenway 6105 spectrophotometer) and viability was determined by Miles and Misra method of colony counting throughout experiments (Miles and Misra, 1938).

2.4 Methods

2.4.1 Serum extraction from LCCD patient

Following approval by the South Sheffield Research Ethics Committee, my supervisor (Prof. RC Read) would periodically draw 50ml aliquots of blood from a female patient with absolute C7-deficiency who presented to the Royal Hallamshire Hospital in 1997 with serogroup B meningococcal disease. Blood was centrifuged at 3000 x g for 10min, and serum was harvested and stored at -70°C in 0.5ml aliquots. In order to establish that serum contained C3 and IgG, MDM were incubated with *N. meningitidis* opsonised with serum (see above) and treated with anti-human C3 and/or FITC-conjugated goat anti-human IgG. To ensure no bacterial contamination was present, serum was cultured on blood agar plates

2.4.2 IgG adsorption

To remove IgG from serum, serum was passed through ImmunoPure plus Immobilised Protein A IgG purification kit (Pierce, IL, USA) according to manufacturer's instructions. Fluorescence for both IgG and C3 was observed in serum opsonised organisms; absence of IgG in IgG-depleted serum was also checked this way prior to use.

2.4.3 Cultivation and opsonisation of *N. meningitidis*

Bacterial strains were grown to mid-log phase in liquid culture media, and bacterial suspensions were washed by being centrifuged at 2000g for 5 min and resuspended in PBS; this was repeated three times. At this stage, non-opsonised bacteria were resuspended in RPMI 1640 and used to inoculate 24-well cell cultures; or alternatively bacteria were opsonised. Viability was measured by serial dilutional counting (Miles and Misra 1938). After a third wash bacteria were resuspended in RPMI 1640 and complement-deficient serum (immune serum or IgG deplete serum). Bacteria were placed on a roller at 37°C for 15min, washed twice using PBS, resuspended in RPMI 1640 enriched with 10% heat-inactivated FCS, and vortexed with glass beads to disrupt any clumped bacteria. Again, final concentration of viable bacteria was established by standard serial dilution technique.

2.4.4 Receptor inhibition of mammalian cells

All monocyte/macrophage populations were seeded into 24-well culture plates prior to bacterial inoculation. Cultured cells were washed three times using warmed culture media and incubated in the presence of inhibitor(s) for 15-30 min before infecting with prepared mid-log phase bacteria (as described above).

Viability was ensured using a trypan blue exclusion assay. Inhibitor(s) were used at the following concentrations and remained in culture media throughout experiments unless otherwise stated.

Table below shows inhibitory reagents used:

Reagent	Target	Concentration	Incubation time (min)	Source
Poly-I (& Poly C control)	Scavenger receptor	50 µg/ml	30	Sigma
MY4 (& isotype control IgG2b)	CD14	10 µg/ml	30	
Phosphatidylinositol phospholipase C enzyme	GPI-receptors	1 unit/ml	30	Sigma
2LMP19c (& isotype control IgG1)	CD11b/CD18	2.5 µg/ml	30	DAKO
mannosylated-BSA	Mannose receptor	0 – 100µg/ml	30	Sigma
PAF-Ra	PAF receptor	1 – 100nM	30	Calbiochem
RGDS-containing peptides (& control RGEs)	Integrins	50 µg/ml	30	Sigma
Cytochalasin D	Actin disruptor	3µM		Sigma
β-cyclodextrin	Lipid rafts	5 – 20 µM	15	Sigma
Nystatin	Lipid rafts	25 µg/ml	15	Sigma
DiI-acetylated LDL	Class A scavenger receptors	5 µg/ml	60	Molecular Probes

2.4.5 Analysis of bacterial association by mammalian cells

Association studies involved cooling cells (final density 5×10^7 cfu/ml unless otherwise stated) and culture media was replaced with 250 μ l of the prepared bacterial suspension (MOI of 30-50:1) and incubated for one hour at 4°C to allow bacteria to adhere to the surface of cells without being internalised. Supernate was aspirated, wells were washed three times to remove non-adherent cells and fixed with 4% paraformaldehyde for 15min. When necessary, the inoculum was adjusted so that the numbers of organisms that associated on the surface of the MDM were approximately equal, thus allowing direct comparisons to be made between intracellular behaviour of opsonised and non-opsonised meningococci.

2.4.6 Bacterial uptake by mammalian cells

Bacterial uptake was measured following by washing infected wells three times to remove non-adherent organisms following adherence at 4°C, and replacement with 250 μ l warm enriched media and incubated at 37°C before fixing at set time points. Internalisation was also measured by establishing suitable MOI for equal binding, and omitting the cold incubation step and incubating infected cells directly at 37°C. Wells were then fixed at different time points. Dil-acetylated LDL, a ligand of SR-A, was used to observe bacterial uptake. After incubation with bacteria, cells remain unfixed and are washed 3 times with warm culture media. Flasks/wells were incubated in the presence of 5 μ g/ml DilacLDL in enriched culture media and incubated for at least 1h at 37°C, 5% CO₂. For microscopic analysis cells were then treated with 4% pfa or further treatment took place in preparation of FACS analysis. Viability was measured using trypan blue exclusion.

2.4.7 Microscopic analysis

Paraformaldehyde fixed cells were washed three times with PBS. In order to distinguish between extracellular and intracellular bacteria, cells were incubated for 12 min with rabbit anti-group B meningococcal antibody diluted 1:50 in PBS. Wells were washed three times with PBS, and incubated for 12min in the presence of FITC-conjugated goat anti-rabbit IgG and goat serum, both diluted 1:20 in PBS. Cells were then counter-stained with 0.5 μ g/ml DAPI. Intracellular trafficking to terminal phagolysosomes/endosomes was observed by noting LAMP-1 co-localisation in cells. MDM were treated with the anti-LAMP-1 monoclonal antibody (H4A3) in the presence of saponin, before counterstaining with DAPI. Wells were washed once with PBS and three times with distilled water, air-dried and mounted onto glass slides in Vectashield (Vector Lab Inc, Burlingame, CA) mounting media with DAPI. Results were obtained by fluorescent microscopy as previously described. (Read, Zimmerli et al. 1996) Extracellular bacteria bound to the surface of cells were FITC-positive and DAPI-positive, whereas internalised bacteria were only DAPI-positive. Percentage internalisation was calculated by subtracting FITC-positive from FITC-negative (DAPI positive) bacteria.

2.4.8 Measurement of extracellular TNF- α

In order for sufficient quantities of TNF- α to be measured, the MDM were incubated with non-opsonised H44/76 for 2h at 37°C. The concentration of TNF- α from the supernatants were measured by ELISA, using a murine anti-human TNF- α Mab (PharMingen) for capture, a recombinant human TNF- α Mab (PharMingen) as the standard, and a biotinylated murine anti-human TNF- α Mab (PharMingen) as the detection antibody. The assay was performed according to the manufacturer's protocol. The lower limit of detection of the assay was 15 pg/ml of TNF- α .

2.4.9 Flow cytometry analysis

To prepare cells for flow cytometric analysis detachment buffer (10mM EDTA and 10mM Lidocaine-HCl in PBS) was used to lift cells tissue culture flasks. Standard RPMI 1640 was substituted with phenol red free RPMI 1640 (GibcoBRL) for all experiments involving flow cytometric analysis to minimize acquired autofluorescence. Culture media in wells was aspirated, and wells were washed 3 times with PBS before adding enough detachment buffer to cover cells (5ml) and incubate for 15min at 37°C, 5% CO₂. Cells were lifted by vigorously pipetting buffer over cells and transferring to centrifuge tubes; from this point cells were kept at 4°C to prevent the cells adhering to the inside surface of the plastic centrifuge tubes. Cells were harvested by centrifugation at 1500 x g for 5min and aspirated in 4% pfa, azide and PBS for 15min. Viability using trypan blue exclusion assay was carried out before fixation, and suspension was analysed for clumping using inverted light microscope. Fluorescence was analysed using FACScan (Beckton Dickinson) using the FL-1 or FL-2 photomultiplier as required, and results were analysed using CellQuest software. Results are representative of at least 3 independent experiments.

Statistics throughout this study are non-parametric and results were generally determined using Mann-Whitney and/or Kruskal-Wallis analysis.

Chapter 3

Development of a model of macrophage phagocytosis of *N.meningitidis*

3.1 Introduction

Despite their probable common bone marrow progenitor, macrophages display considerable heterogeneity (Forster and Landy 1981; Walker, Warner et al. 1985), and depending on their location, they can differ considerably in phenotype and morphology. Once in the tissue, monocytes differentiate into characteristically different tissue macrophages depending on tissue-specific stimuli present in the local microenvironment.

Numerous studies have shown that contact between phagocytes and bacterial pathogens result in the release of toxic compounds including reactive oxygen intermediates (ROI), and pro-inflammatory cytokines such as TNF- α , IL-1, IL-6 (Beaty, Franklin et al. 1994; Van Deuren, van der Ven-Jongekrijg et al. 1995; Astiz, Saha et al. 1996); however gram-positive and gram-negative cells are thought to induce monocytic cytokine production through different signal transducing pathways (Rabehi, Irinopoulou et al. 2001). These cytotoxic compounds are not only lethal for the microbe, but also for host cells, thus leading to the destructive affects that characterise inflammation (Perdomo, Cavillon et al. 1994). It is, therefore, the relationship between phagocyte and bacteria that lies at the very heart of the innate immune system.

The nasopharynx is the known site of meningococcal colonisation (Olcen, Kjellander et al. 1979), and is presumed to be the primary site of invasion; although relatively little is known about mucosal immunity to meningococcal disease, or precisely where within the nasopharynx meningococci colonise and

attack. In order for cells of *N. meningitidis* to cause disease they must both colonise and invade mucosal epithelial cells, where they come into direct contact with macrophages which make up a large proportion of host immune cells in the region. Experiments using human nasal explants have shown that macrophages phagocytose *N. meningitidis* on infection (Read, Fox et al. 1995; Read and Goodwin 2001), suggesting that macrophages are a central line of defence against meningococci. No previous work has been published on the close relationship between *N. meningitidis* and human macrophages in terms of specific receptors engaged by the bacteria, and subsequent intracellular trafficking.

Particular strains of *Neisseria* are potential pathogens, and this is exhibited in their ability to adhere to and invade mucosal surface structures, and both survive and multiply after entering the bloodstream of certain individuals. Therefore, in this chapter the direct interactions between macrophages and different *Neisseria* species were studied; specifically in terms of recognition, phagocytosis, internal trafficking and subsequent cytokine production in the absence of serum opsonins.

The role of serum opsonins in this relationship was also examined as the principal role that terminal pathways of the complement system play in meningococcal lysis are most apparent in individuals that have a late complement-component deficiency (LCCD). Meningococci are highly susceptible to complement-mediated lysis, therefore LCCD individuals have an almost 1000-fold higher risk of developing MCD than complement-sufficient individuals, as they lack one of the 'late' or 'terminal' components (from C5 to C9) and serum bactericidal function is severely impaired (Figueroa and Densen 1991; Platanov, Beloborodov et al. 1993; Esser 1994). In order to examine the role serum opsonins play in meningococcal processing by macrophages via specific opsonic receptors, *N. meningitidis* cells were opsonised with serum obtained from an LCCD patient who has a C7-deficiency, as the absence of the

late complement components in the serum prevented complement-mediated lysis of the meningococci.

Suitable cell-line models for the use of *N. meningitidis* studies are rather limited, as most phagocytic cell lines are derived from immature precursor cells of monocytes and permanent human mature cell lines do not exist. None the less I looked at the use of cell lines to model meningococcal interactions within this study. Previous studies have shown that the *in vitro* differentiation of a monocytic cell line such as THP-1 provides an appropriate model to study macrophage-pathogen interactions, as differentiation induces the cells to take on a more macrophage-like phenotype, resulting in the improved phagocytic capability of the cells compared to undifferentiated cells (Tsuchiya, Kobayashi et al. 1982), this was explored further in Chapter 4.

3.2 Results

To examine the relationship between *Neisseria* and macrophages an effective general method of measuring bacterial adherence and uptake by macrophages was required. Throughout all the experiments in this chapter a monolayer of 1×10^6 peripheral blood monocyte cells, obtained from apparently healthy volunteers, were grown in 24-well culture plates for 8-12 days at 37°C, (described in Chapter 2). At this point, the cells had matured and acquired the distinctive characteristics of macrophages. Each *Neisseria* strain was cultured and grown individually to mid-exponential phase at which point the bacteria were harvested and introduced to the monocyte-derived macrophages (MDM) at a concentration of 5×10^7 cfu/ml (MOI 50:1) unless otherwise stated.

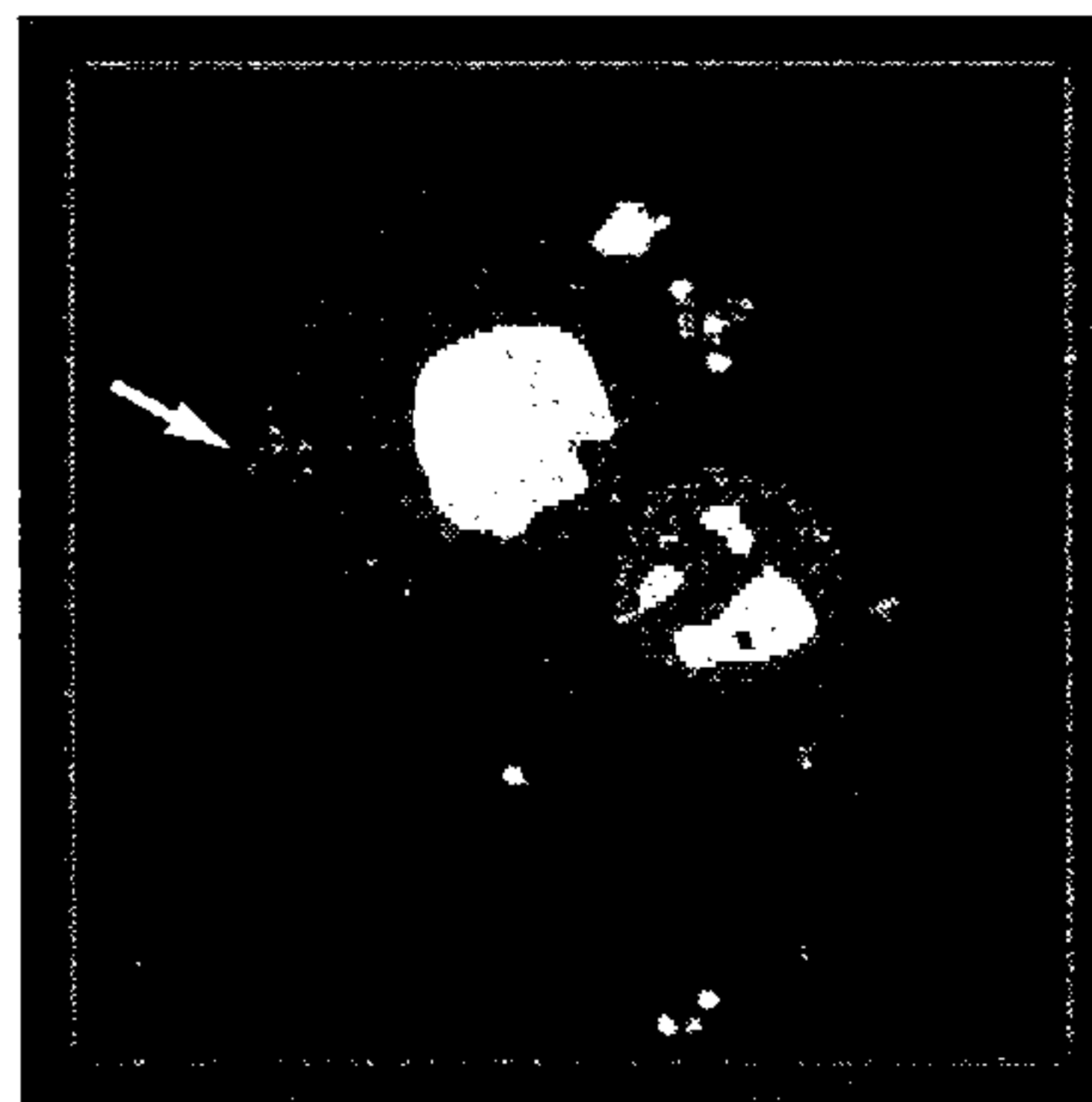
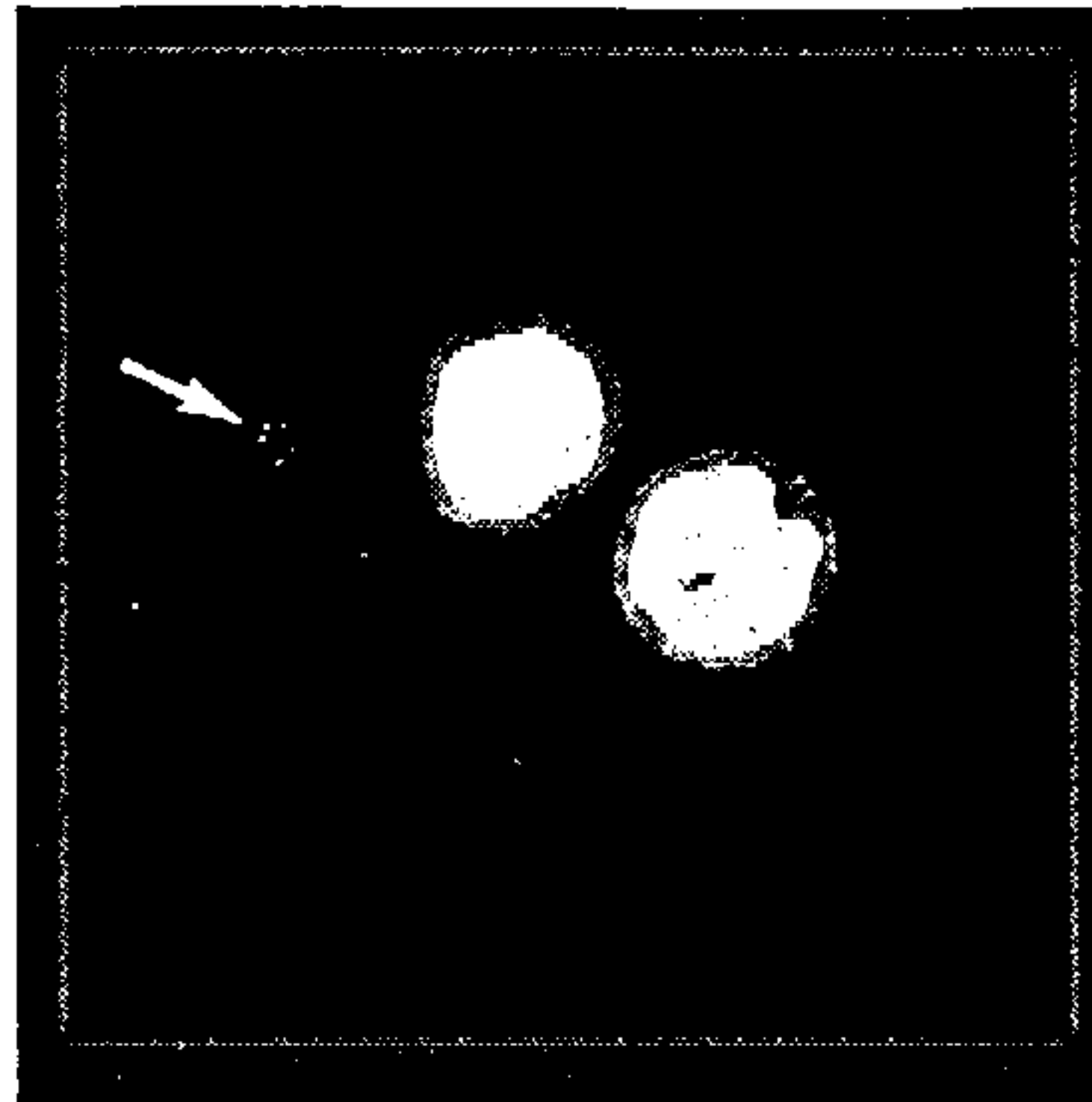
Experiments to examine binding of bacteria to the surface of macrophages were carried out at 4°C for one hour, and were based on previous established methods of studying bacterial adhesion, which had ascertained that phagocytes could survive the cooling/warming process involved allowing bacteria to bind at this temperature. Incubation at 4°C allows the macrophages to remain viable but deprives the cell membrane of its fluidity, and so the actin-based phagocytic machinery of the cell does not function. This permits the bacteria to interact with available surface receptors present on the macrophages without initiating phagocytosis, thus allowing the measurement of adherence independently of phagocytosis.

Tissue culture wells were fixed using 4% paraformaldehyde and layered with fluorescent antibodies (see Methods and Materials). Differential fluorescent microscopy identified extracellular organisms as being FITC positive (green), DAPI positive (blue), and internalised organisms as FITC negative, DAPI positive (see Figure 1). This method of staining was extremely effective in establishing how successfully the bacteria bound to these cells as well as

measure the proportion of adherent organisms that were subsequently internalised by the cells.

Figure 3.0

Indirect Immunofluorescent staining



Bacteria stained both FITC-positive (green) and DAPI-positive (blue) are extracellular
Bacteria stained DAPI-positive (blue) alone indicate internalisation, and internalised organisms surrounded by labelled LAMP-1 rings (red – see arrow) indicate trafficking to late endosome/phagosome.

3.2.1 *N. lactamica* associates more effectively than pathogenic *Neisseria* to human macrophages

Experiments were conducted to observe how effectively two strains of serogroup B *N. meningitidis* widely used in studies of pathogenesis (MC58 and K454) adhered to the surface of monocyte-derived macrophages (MDM) when compared with two contrasting *Neisseria* strains. The first was *Neisseria lactamica*, a human commensal that rarely causes disease. This organism is chiefly a commensal of children and is considered capable of generating an acquired cross-reactive immunity against meningococci. *Neisseria animalis* was the second species used and is a commensal of guinea pigs and has never been isolated from a human source. These organisms also differ in their outer membrane structure, and direct comparison between species would be an insightful way of noting if *N. meningitidis* behaves differently to these bacteria in relation to macrophages.

All organisms were grown to mid-log phase, used to infect human macrophages under non-opsonic conditions and incubated for one hour at 4°C; units were measured as mean numbers of bacteria per macrophage. Under each condition 100 macrophages from each slide were examined and the numbers of bacteria adherent to the surface of the cell and/or internalised were noted. It was possible to measure bacterial adherence to macrophage surfaces and compare species of *Neisseria*.

As shown in Fig. 3.1 although the binding of *Neisseria* species to the surface was broadly comparable, it is notable that *N. lactamica* exhibited quite exuberant adherence.

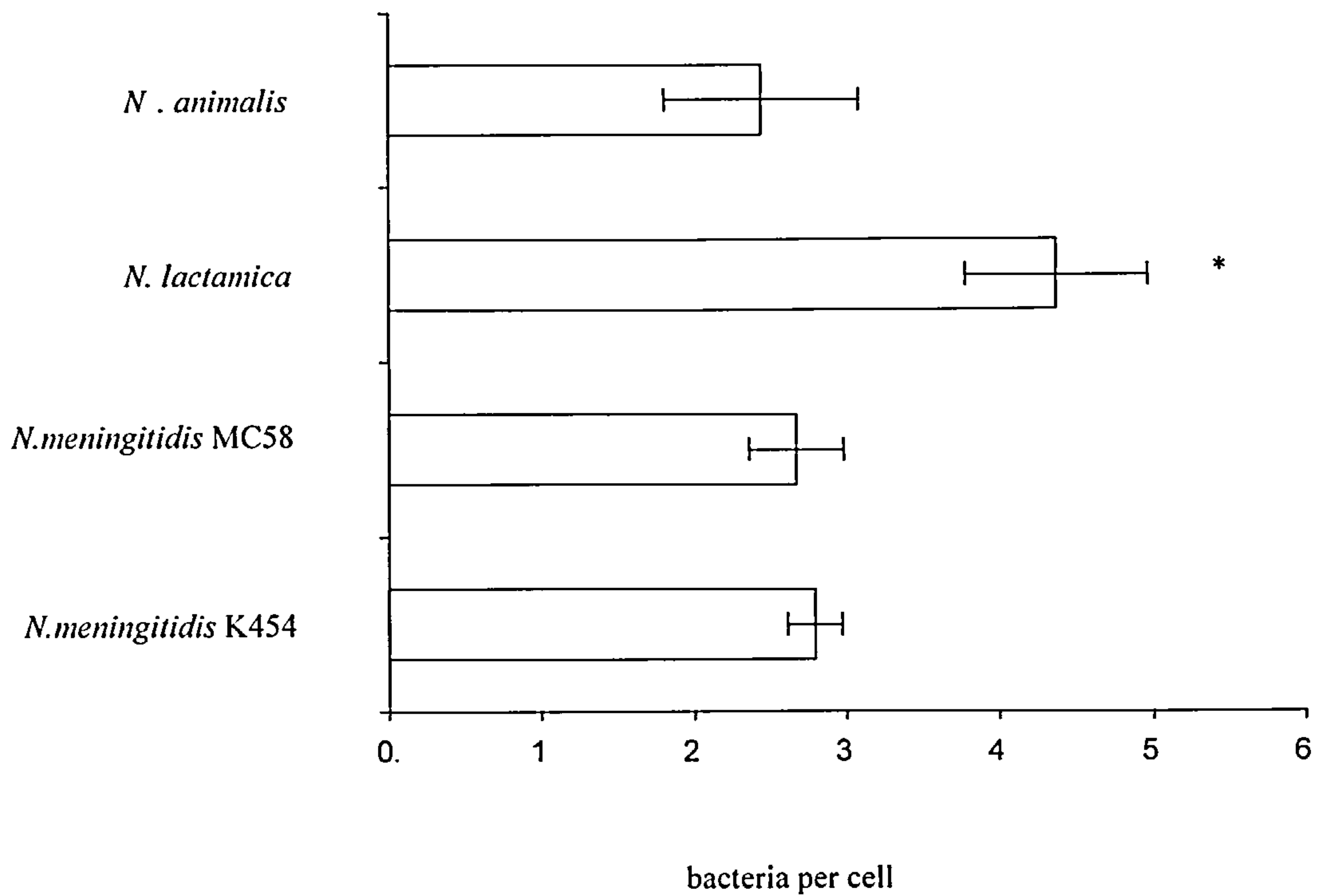


Figure 3.1

***N. lactamica* binds in greater numbers than *N. meningitidis* to MDM**

Adherence of *Neisseria* strains to MDM after 60min incubation at 4°C and MOI 50:1. Data represented as mean (±SEM); experiment performed using five donors each experiment done in triplicate. Statistical significance denoted using * $P < 0.05$; calculated using Mann-Whitney U test and comparing strains with *N. meningitidis*.

3.2.2 Uptake of *Neisseria* species

A methodological approach was designed to measure subsequent internalisation of species of *Neisseria*. Experiments were performed by allowing the bacteria to adhere to the surface of the macrophages, as described earlier, macrophages were then washed three times in warm culture medium in order to remove any non-adherent organisms present, fresh culture media replaced and cells were allowed to incubate at 37°C over 2h. Every 30min wells were fixed using 4% paraformaldehyde. Experiments were performed (data not shown) to find the suitable concentration at which *N. lactamica* adhered to macrophages in similar numbers to the other bacteria; attempts were made to increase the numbers of adherent *N. meningitidis* and *N. animalis* to levels comparable to *N. lactamica*, but I observed that under non-opsonic conditions *N. meningitidis* MC58 did not bind at more than 2-3 bacteria per cell up to an MOI 200:1. (data not shown).

The number of adherent bacteria per macrophage were subsequently equalised by reducing the MOI of *N. lactamica* from 50 to 20, thus allowing internalisation of all four *Neisseria* species to be directly compared. MDM were incubated at 4°C for one hour, before a further 2h incubation at 37°C, fixing wells every 30min, and internalisation at each time point was measured by differential fluorescent microscopy. Fig. 3.2 demonstrates that by 30min both *N. lactamica* and *N. animalis* were apparently internalised by MDM more effectively than the two *N. meningitidis* species. Interestingly here we observe that MDM internalised the only non-human pathogen, *N. animalis*, at significantly greater levels than the two human species. This suggests that in this study *N. animalis*, the non-human pathogen, may possess certain attributes that are more likely to alert the human macrophages to their presence. In contrast, the two pathogenic meningococci strains are found in humans, and are known to express virulence determinants that are thought to help successfully mask their presence from the hosts immune systems.

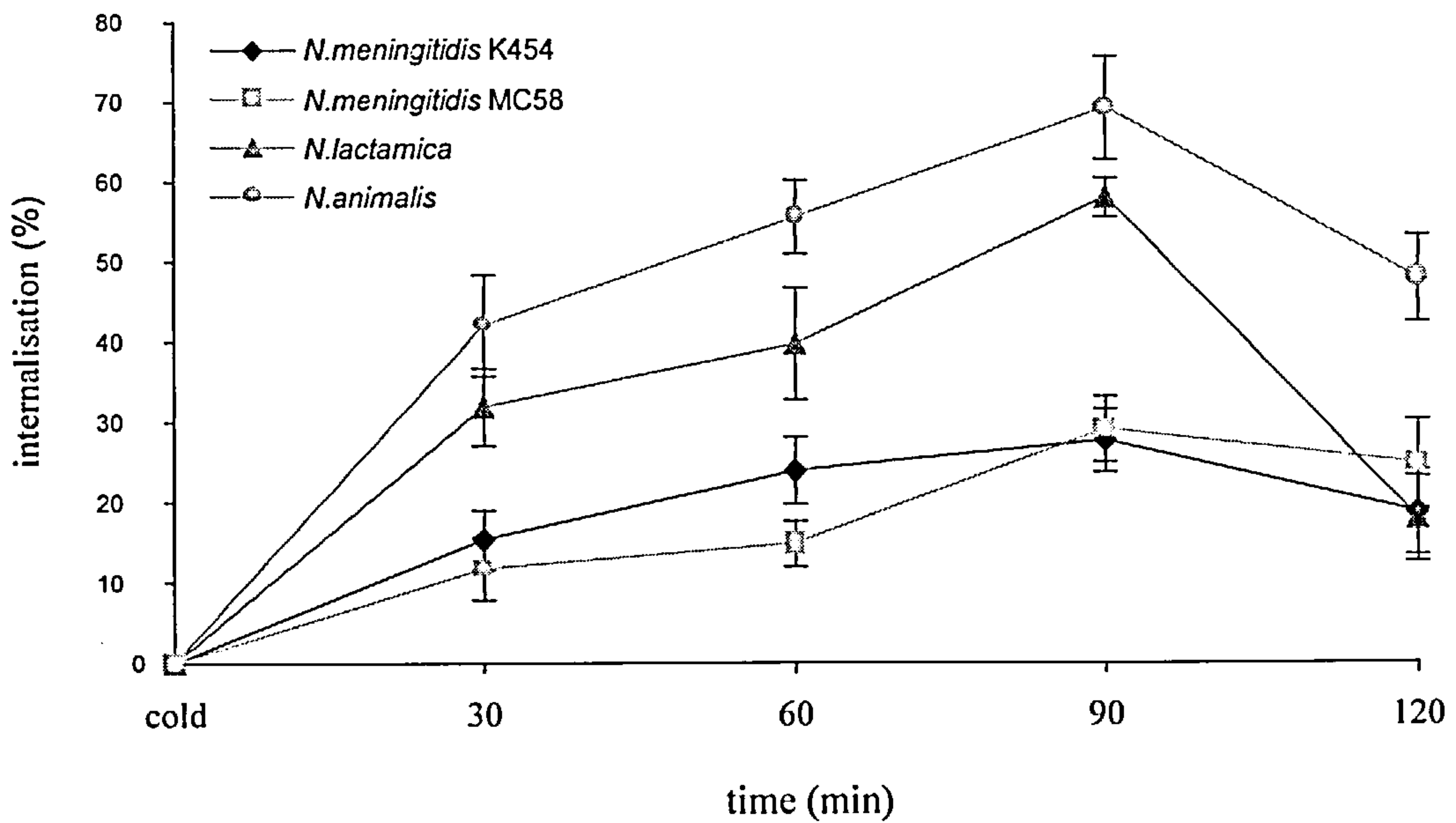


Figure 3.2

Non-pathogenic *Neisseria* strains ingested most effectively

Graph shows percentage of adherent *Neisseria* internalised by MDM. Concentration of *N. lactamica* was reduced to ensure adherence of *Neisseria* strains at 4°C was equal,, bacteria were then incubated for a further 120min at 37°C. Data represented as mean (\pm SEM); experiment performed using six donors each experiment done in triplicate.

3.2.3 Intracellular trafficking of *N. meningitidis*

Lysosome-associated membrane protein 1 (LAMP-1) is a member of a highly glycosylated protein group present in the lysosomal membranes of most cells. During phagocytosis, LAMP proteins are concentrated around the internalised particle and fluorescent antibodies raised against LAMP-1 proteins can be used to track bacteria within late endosomal/lysosomal compartments of macrophages. Serogroup B *N. meningitidis* are known to co-localise with LAMP in macrophages and are able to affect the bactericidal mechanism of the cell by reducing the rate of LAMP incorporation (Read, Zimmerli et al. 1996).

The fusion of lysosomes to phagosomes is the primary bactericidal mechanism of macrophages. In order to determine whether phagosome-lysosome fusion was occurring in MDM, the mouse mAb H4A3 that is directed against the late endosome-lysosomal marker glycoprotein LAMP-1, is used as a marker for fusion. The ability of *N. meningitidis* to prevent lysosomal association may play a role in the survival of *N. meningitidis* inside MDM cells. Intracellular bacteria can prevent association with lysosomes by preventing phagosome-lysosome fusion or by escaping the phagosome into the cytoplasm.

Lysosome-associated membrane protein 1 (LAMP-1) co-localisation of *Neisseria* is noted in Fig. 3.3, *N. lactamica* is taken into terminal phagolysosomes, and by 120 min the proportion of internalised *N. meningitidis* MC58, co-localising with LAMP-1 is significantly higher than the other *Neisseria* species. These two points show that *N. lactamica* appears to be both bound in greater numbers and internalised into terminal phagosomes more quickly than the other species.

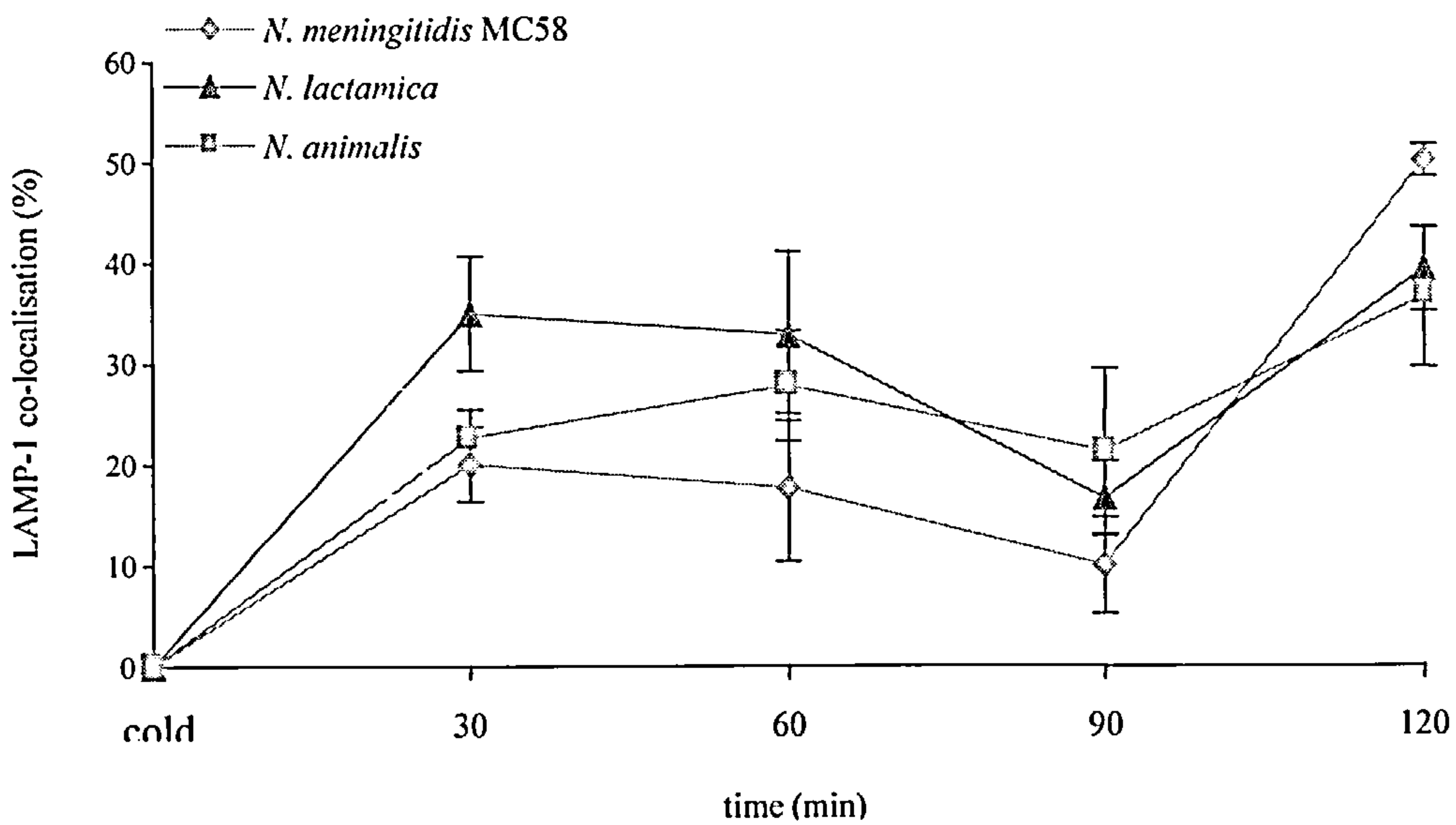


Figure 3.3

Intracellular trafficking of *Neisseria*

Graph shows percentage of ingested *Neisseria* (see Fig 3.2) taken into LAMP-1-labelled phagolysosome/endosome vacuoles by MDM. Concentration of *N. lactamica* reduced to ensure adherence of *Neisseria* strains at 4°C are equal, bacteria were then incubated for a further 120min incubation at 37 °C. Data represented as mean (\pm SEM); experiment performed using six donors each experiment done in triplicate.

3.2.4 IgG contributes to efficient binding of opsonised *N. meningitidis*

I was able to study both the adherence and the internalisation of *Neisseria* strains as two quite distinct individual processes by the use of the protocols discussed above; thus facilitating the study of possible receptor involvement in each of these interactions. Fig. 3.4A and 3.4B show findings obtained with the use of C7-deficient serum opsonised bacteria. *N. meningitidis* MC58 were cultured as described previously and opsonised for 15min at 37°C. Serum from the same source was also passed through a Protein A column in order to remove IgG antibodies, leaving IgG-depleted serum, which was also used to opsonise bacteria before inoculation onto MDM. Non-opsonised control organisms were opsonised in an equal concentration of PBS. Fluorescently labelled antibody staining revealed that this treatment resulted in opsonised bacteria that were not coated with human IgG, but complement opsonins were present (see Methods and Materials).

Interactions of serogroup B *N. meningitidis* MC58 with MDM were observed after opsonisation in order to assess whether bacteria were more readily bound and internalised when compared with control organisms. Fig. 3.4A demonstrated that when bacteria were opsonised and used to inoculate MDM in relatively equal numbers (MOI 50:1), immune serum opsonised bacteria appeared to bind in greater numbers per macrophage than IgG-deplete opsonised and non-opsonised MC58. This observation was obtained using six human donors, and implies that Fcγ receptors are involved in meningococcal adherence to macrophages as the absence of IgG clearly impairs bacterial association.

In order to observe any differences in internalisation of the opsonised organisms, the MOI was reduced from 50 to 10 thus equalising the mean numbers of adherent bacteria per cell after 1h at 4°C (Fig. 3.4A); thus allowing direct comparisons between the treatments used. Subsequent internalisation of organisms over 90min at 37°C were measured using fluorescent microscopy (Fig.

3.4B). By 60 min uptake of both groups of opsonised bacteria is four times higher than the non-opsonised control organisms, suggesting that as the absence of IgG has no effect on internalisation, non-FcγR appear to play a role in meningococcal uptake. Interestingly IgG-deplete opsonised bacteria were internalised by macrophage receptors as efficiently as immune serum opsonised bacteria over 90min. The role of the phagocytic complement receptors such as CR3 will be explored in Chapter 5.

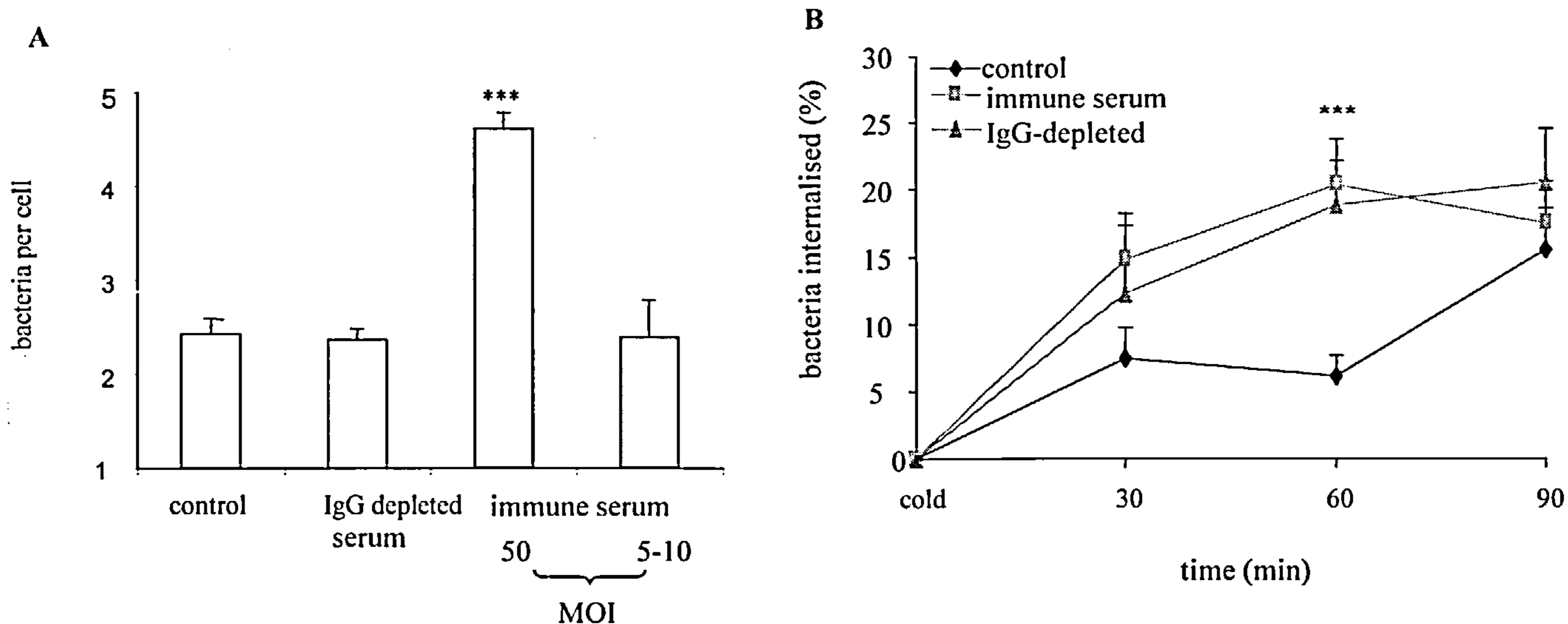


Figure 3.4

Immunoglobulin-mediated binding of meningococci

Adherence and internalisation of *N. meningitidis* MC58 by MDM under opsonic conditions (A) Ratio of bacteria per macrophage (1×10^6), cells fixed after 1h incubation at 4°C ; includes equalised results for immune serum opsonised MC58; bacterial concentration 5×10^7 cfu/ml (open bars), $5-10 \times 10^6$ cfu/ml (filled bar). (B) Percentage internalisation of adherent bacteria, using equalised data; (◆) non-opsonised PBS control, or opsonised using (◻) immune serum (▲) adsorbed serum (IgG deplete). Macrophages washed and subsequently incubated at 37°C , and at each time point monolayers were washed extensively and fixed. Presence or absence of IgG was confirmed using FITC-labelled anti-IgG antibodies (see Methods). Data are depicted as mean values (\pm standard error of mean) from six donors, with each experiment done in triplicate. Significance denoted by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using Mann Whitney test comparing opsonised bacteria with control organisms.

3.2.5 Primary human macrophages internalise *N. meningitidis* more effectively than cell lines

Throughout the study a number of different cell types are used, including MDM, THP-1 monocytic cell line, J774 murine macrophage-like cells. The expression of surface receptors on these cells determines how efficiently they bind and ingest bacteria such as *N. meningitidis*. Fig. 3.5 demonstrates that meningococcal adherence to cell lines is most effective in macrophage-like cells such as J774. Fig. 3.6 demonstrates that MDM appear to express more functional phagocytic receptors that can internalise *N. meningitidis* H44/76 successfully.

THP-1 cells are undifferentiated leukaemic cell lines whose growth is arrested at the precursor stage before they develop into mature macrophages, and the relative lack of phagocytic potency is a characteristic of immature cells. Differentiated THP-1 interactions with meningococci are explored further in in Chapter 4 and 6.

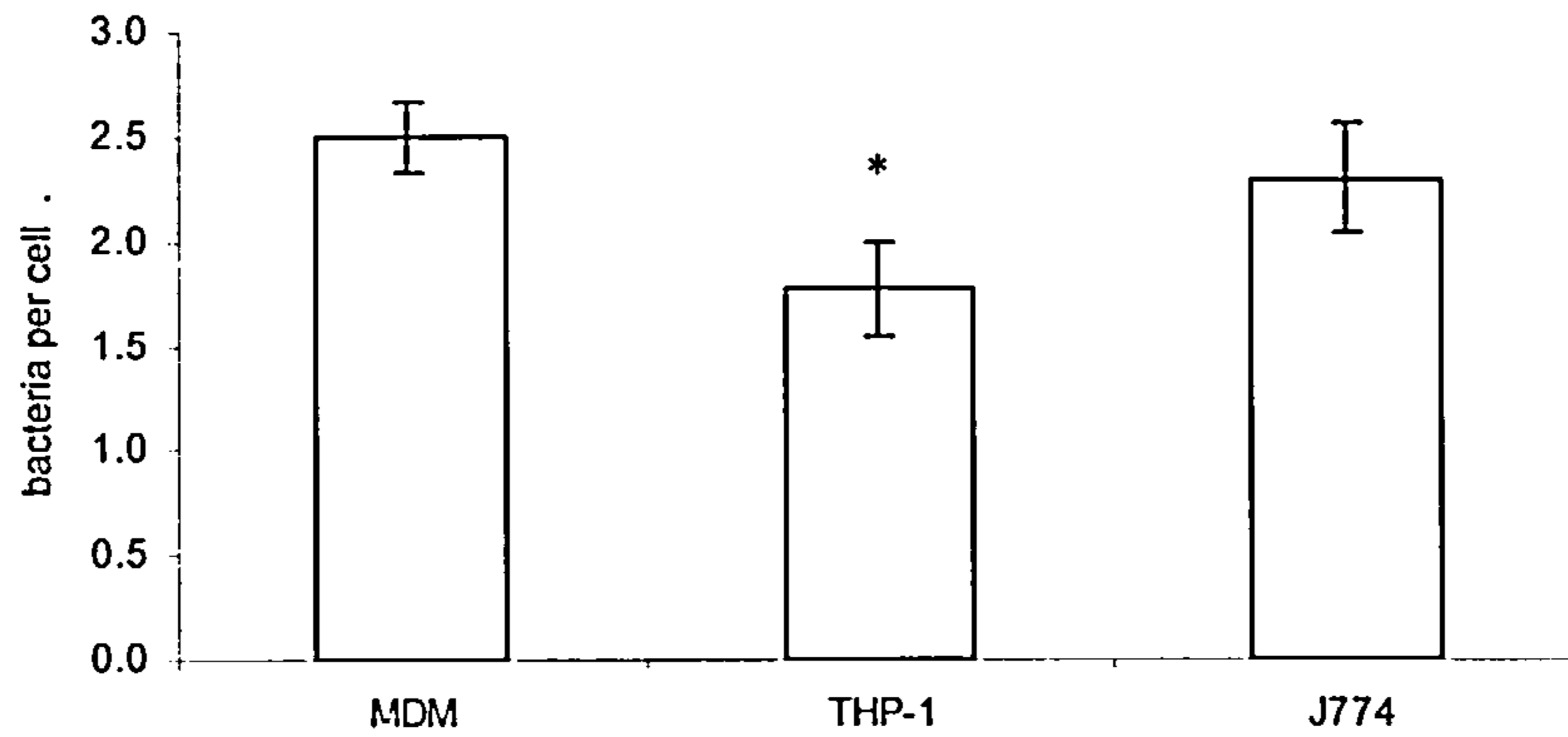


Figure 3.5

Cell lines models

Binding of *N. meningitidis* H44/76 to different cell types after 60min incubation at 4°C. Cells used were monocyte-derived macrophages (MDM), undifferentiated THP-1 cells (THP-1) and a murine macrophage cell line J774.A. Results based on four separate experiments, each repeated at least three times. Significance denoted by * $P < 0.05$ using Mann Whitney test comparing cell lines to primary human macrophages.

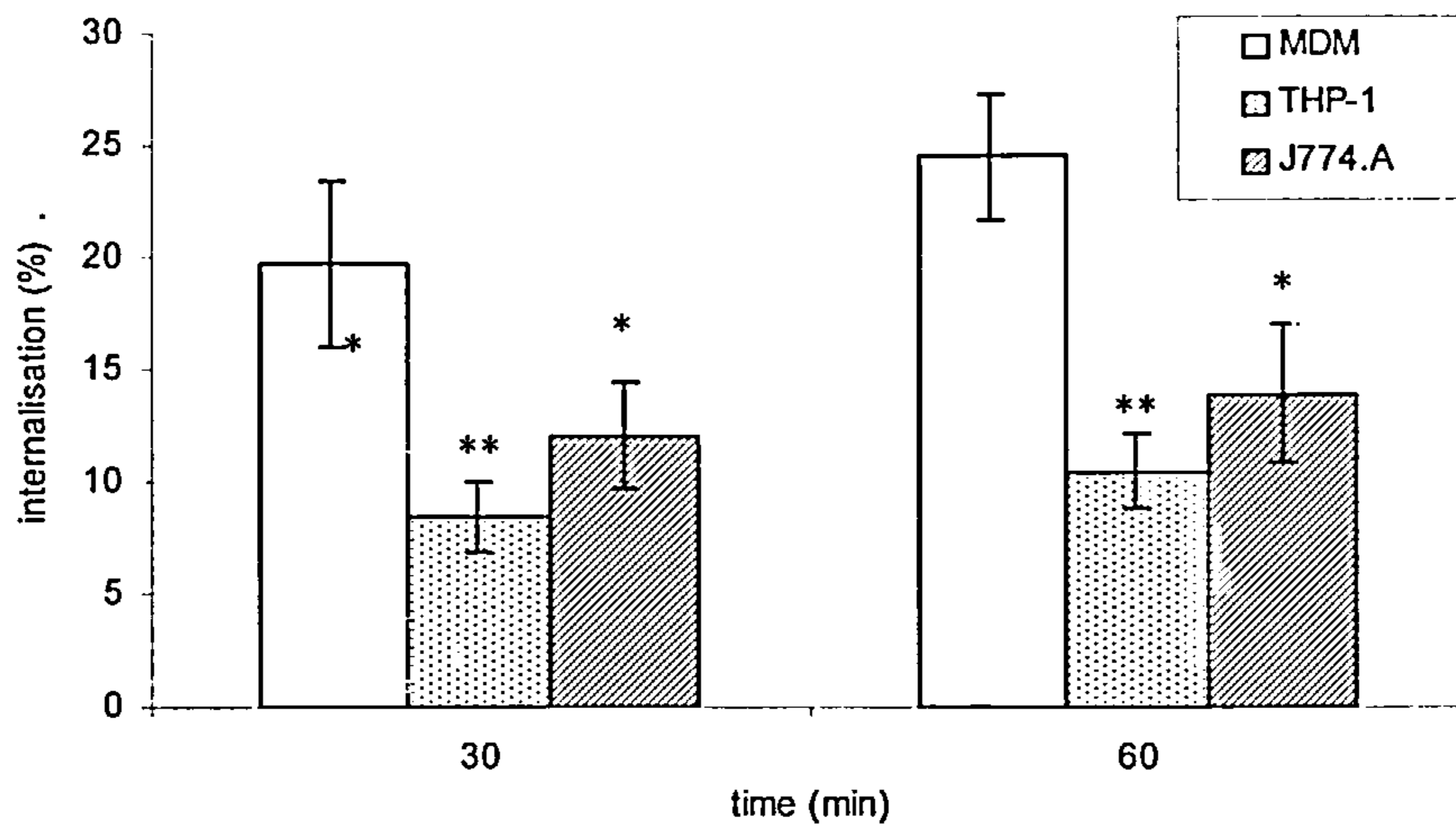


Figure 3.6

Uptake of bacteria by cell lines

Phagocytosis of *N. meningitidis* H44/76 to different cell types after 60min incubation at 37°C. Cell were monocyte-derived macrophages (MDM), undifferentiated THP-1 cells (THP-1) and a murine macrophage cell line J774.A. Results based on four separate experiments, each repeated at least three times. Significance denoted by * $P < 0.05$, ** $P < 0.01$ using Mann Whitney test comparing cell lines with MDM.

3.3 Summary and discussion

Macrophages are considered the first line of defence against invading *Neisseria* species in the upper respiratory tract, and it is therefore important to consider the question of how these distinctive organisms are able to overcome a number of innate defence systems present in their environment. This study examines the involvement of specific macrophage receptors in the recognition of *N.meningitidis*, and to this end, cell lines have proven to be a useful tool.

This chapter has described initial pilot work to design a robust assay of the interaction of human macrophages with pathogenic *Neisseria*. The process of phagocytosis is complex and often involves cross talk and synergy between receptors and signal transduction molecules. Macrophages express an array of surface receptors; multiple receptors can be involved in the processing of single bacteria cells. These events are initiated by the crucial step of ligand recognition via specific receptors.

In the absence of serum, *Neisseria* strains *N. lactamica* and *N. animalis*, were respectively bound and internalised by macrophages in significantly greater numbers than pathogenic strains of *N. meningitidis* using this assay. These findings suggest that *N. lactamica*, as the only non-pathogenic strain, is more readily recognised by the MDM as its outer membrane differs from that of *N. meningitidis* and *N. animalis* which might allow for greater affinity between cells. Although a relatively rare occurrence, the fact meningococci are able to avoid the gauntlet of innate immune responses, suggests that either host cells fail to recognise and destroy the invasive bacteria, or that virulence determinants on the outer surface of the microbes are successful in preventing direct recognition. The *N. meningitidis* strains used are capsulate and express both Opa and PilC, whereas *N. lactamica* lacks Opa expression and *N. animalis* expresses neither Opa nor PilC. Of all three *Neisseria* species examined, *N. animalis* was internalised most effectively by MDM, which is consistent with the fact that this

organism has never been isolated in humans, and is more usually found in mammals including guinea pigs. *N. lactamica* was found to bind most successfully to macrophages. LAMP incorporation was dependent on species, strain and time of incubation. A significant decrease in LAMP incorporation was observed by 90min (see Fig 3.3), even though internalisation had reached its peak, and this may occur because even though organisms are being ingested effectively it may be via different receptors or alternatively the bacteria may not all be viable and by 90min a proportion have died and are not therefore being picked up by the staining antibodies; or perhaps the bacteria are multiplying in the media during incubation and there is a limit to the number the macrophages can traffic into terminal phagolysosomes.

Present results indicate that different cell lines interact to varying degrees with *N. meningitidis* H44/76, and these interactions are likely to be dependent on receptor expression. The experiments demonstrated that cultured cell lines, such as THP-1 and J774, can express receptors able to interact directly with *N. meningitidis* and could be used as suitable models for the study of bacteria-macrophage interactions.

Once meningococci are in the bloodstream complement-mediated responses are the first line of defence, and are usually dependent on bacterial lysis by C5b to C9 complement components. If organisms can evade such an attack then they are able to proliferate relatively unhindered and reach concentrations that activate a more pronounced complement-mediated response that can lead to tissue damage by neutrophil activation. Therefore examination of opsonic receptor involvement in meningococcal recognition of macrophages was carried out, and experiments using this model implied that meningococcal interactions in the presence of immune serum were governed by different opsonic receptors. Adherence to macrophages appeared to be an FcγR-mediated process, whereas the data suggested that non-FcγR receptors contributed significantly to bacterial uptake (see Fig 3.4B). A similar pattern of opsonin-mediated bacterial recognition was

observed by Gordon and colleagues (Gordon, Irving et al. 2000) when similar experiments were performed using *S. pneumoniae* and alveolar macrophages. The role of CR3 will be examined further in Chapter 5. Mutant strains of *N. meningitidis* were used in the following chapter to examine the role of meningococcal lipopolysaccharide, a pathogen-associated molecular pattern, on immune activation.

Chapter 4

Influence of lipopolysaccharide on internalisation of *N. meningitidis* by human macrophages

4.1 Introduction

N. meningitidis is a host-adapted pathogen that possesses known conserved key surface structures, and has developed a high level of antigenic variability that is used as an effective strategy to evade immune surveillance by the host. Both capsular polysaccharide and lipopolysaccharide display this variability, and are essential virulence factors in meningococcal pathogenesis, as both structures have been shown to contribute to the bactericidal activity of human sera (Kahler and Stephens 1998; Tzeng and Stephens 2000). As an integral and essential component of the outer membrane of gram-negative bacteria, LPS can activate macrophages and trigger a number of pro-inflammatory cytotoxic responses. Lipopolysaccharide (LPS) is the major lipid component of the outer surface monolayer of the outer membrane, is highly conserved and present on the outer membrane of all gram-negative bacteria. Considerable work has been amassed on the role of LPS as a trigger for a range of pro-inflammatory responses in macrophages (Beaty, Franklin et al. 1994).

In the studies described in this chapter wild-type *N. meningitidis* H44/76 and an LPS-deficient isogenic mutant pLAK33 were used. LPS is responsible for the composition or asymmetry of the outer membrane of most gram-negative organisms, and is therefore an essential component of these bacteria. Steeghs and colleagues (Steeghs, den Hartog et al. 1998) developed the LPS-deficient mutant used in this study by the insertional inactivation of the *lpxA* gene that encodes UDP-GlcNAc acyltransferase that is required for the first step in lipid A

biosynthesis, leaving a viable organism completely deficient of LPS and yet in possession of a complete outer membrane.

Further study confirmed that LPS is not essential for the assembly of the outer membrane proteins such as porins, and to date the main difference between *N. meningitidis* pLAK33 and the wild-type strain *N. meningitidis* H44/76 are the phospholipid composition on both the inner and outer membrane. Evidence suggests that the missing LPS is replaced with short fatty acyl chain phospholipids, that have an increased saturation of the acyl chains, comparable to the fatty acyl chains of LPS that are also highly saturated. The presence of a α -2,8-polysialic acid capsule also appears to be essential for viability of the mutant strain (Steeghs, de Cock et al. 2001). Recent observation using a LPS-deficient serogroup C *N. meningitidis* strain observed differences in expression of pilus-associated characteristics, which have not been reported for the LPS-deficient pLAK33 strain used in this study, and this affected the organisms ability to enter human epithelial cells (Albiger, Johansson et al. 2003). Capsulate and piliated pathogenic meningococci are normally recovered from the CSF.

The LPS-deficient mutant strain, *N. meningitidis* pLAK33, is the first viable gram-negative bacteria that is completely deficient of LPS, and is therefore an extremely useful organism in the study of meningococci-host cell contact, and involvement of endotoxin in meningococci-macrophage interactions in terms of receptor interactions, phagocytosis, subsequent cytokine production, and intracellular trafficking.

The method of cellular differentiation employed is known to affect receptor expression, and a number of agents have been shown to induce differentiation in human leukaemia-derived cell lines, including vitamin analogues, cytokines and anti-leukaemic drugs such as phorbol esters, retinoic acid, bufalin, vitamin D₃, TNF- α , IFN γ and GM-CSF, all of which been reported to promote monocyte-

macrophage differentiation (Grilli, Chiu et al. 1993; Schwende, Fitzke et al. 1996). Treatment of THP-1 cells induces them to mature into macrophage-like cells, as cellular receptor expression is upregulated resulting in a larger number of receptors that can internalise meningococci. THP-1 cell differentiation increases cellular adherence, stop proliferating, increase in latex bead phagocytosis was observed and increased expression of macrophage receptors (Schwende, Fitzke et al. 1996). The differentiation process is a complex one and is controlled by the expression or activation of several transcription factors, and the actual events during this process of terminal differentiation of the cells that lead to improved bactericidal performance have yet to be fully elucidated (Valledor, Borrás et al. 1998). Differentiating agents are known to induce the appearance of NFκB in the cytoplasm and its translocation into the nucleus (Grilli, Chiu et al. 1993), and differentiation of THP-1 cells using phorbol myristate acetate (PMA) strongly activates protein kinase C (PKC). Using a PMA-induced cell differentiation model of this human monocytic cell line, we were able to study the effects of the differentiation process on the functional activity of the macrophages in relation to *N. meningitidis*.

4.2 Results

4.2.1 *lpxA* mutant exhibits increased adherence to THP-1 cells

Undifferentiated monocytic cell line, THP-1, cells were infected with equivalent numbers of mid-log phase *N. meningitidis* H44/76 and the isogenic strain *N. meningitidis* pLAK33, (MOI 50:1) and incubated for 1h at 4°C. A number of phagocytic receptors normally present in mature macrophages are noticeably absent on THP-1 cells, including scavenger receptors and mannose receptors, and though complement receptors and CD14 are present they are significantly reduced in number (Schwende, Fitzke et al. 1996). No difference was observed in either adherence or uptake of *N. meningitidis* H44/76 and isogenic mutant by THP-1 after 1.5h at 37°C. This sharply contrasts with the following data using MDM.

4.2.2 *lpxA* mutant exhibits increased adherence to human monocyte-derived macrophages (MDM)

The experiment described above was repeated substituting undifferentiated THP-1 cells for primary human macrophages, which were infected with *N. meningitidis* H44/76 and the isogenic strain *N. meningitidis* pLAK33, (MOI 50:1) and incubated for 1h at 4°C. Figure 4.2A shows that LPS-deficient *N. meningitidis* mutant associates with MDM in significantly greater numbers than the wild-type strain. Significant differences were observed in both adherence and uptake of parent and mutant strain., and the differences in outer membrane composition of the two strains (see discussion) may explain how LPS-deficient *N. meningitidis* pLAK33 can to adhere to the surface of MDM more effectively.

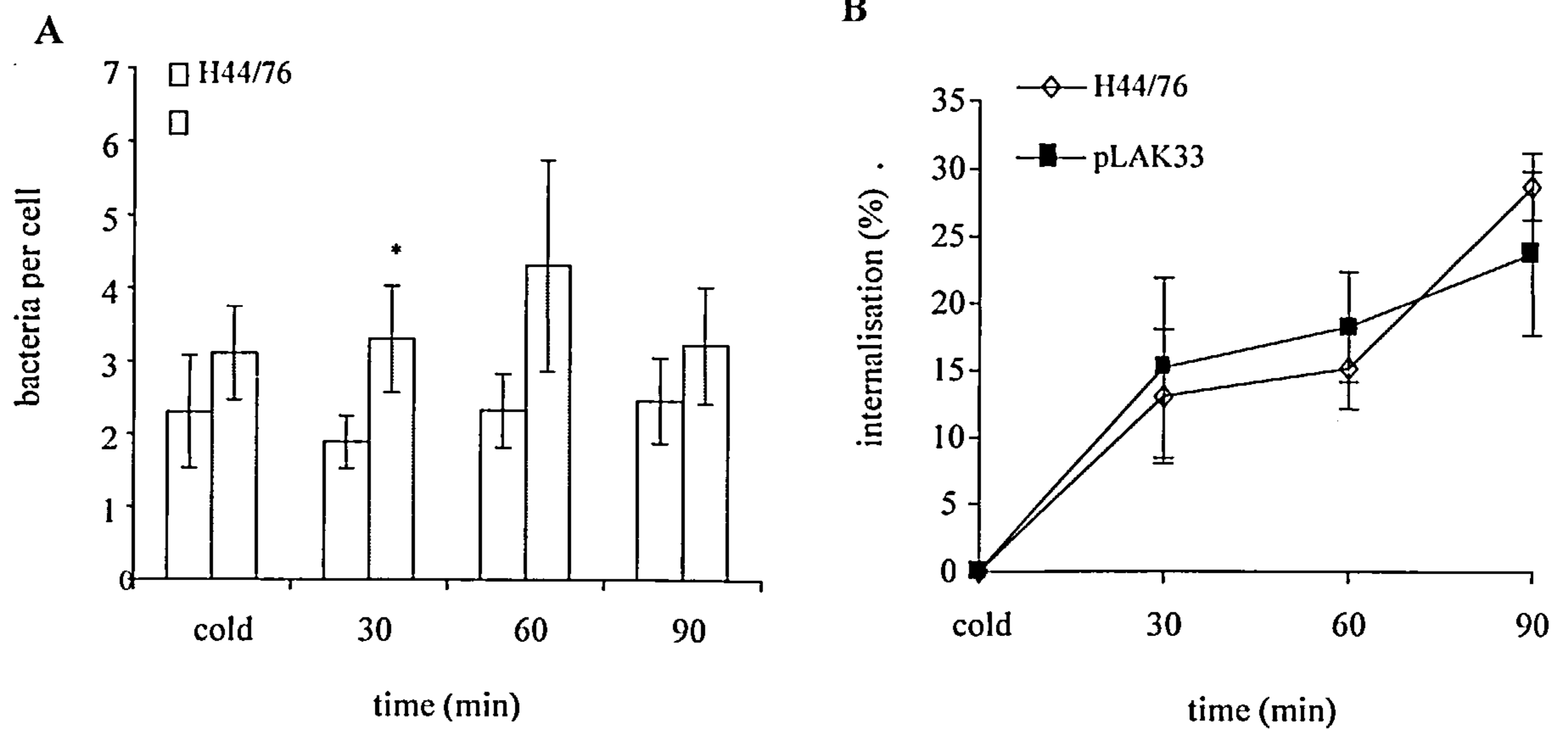


Figure 4.1

Ingestion of *lpxA* mutant by THP-1 cells is not impaired

Comparison of bacterial binding and internalisation of LPS-deficient mutant and wild type strain by undifferentiated THP-1 cells (10^6 cfu/ml).

(A) Adherence of LPS-deficient mutant increased after 30min incubation ($*P < 0.05$) (B) Percentage of bound organisms internalised. Results show no significant difference between phagocytosis of the two strains ($P > 0.05$). Significance calculated using Mann-Whitney U test at each time point. Experiments were performed eight times, each experiment repeated at least three times.

4.2.3 Internalisation of *lpxA* mutant bound to the surface of MDMs is impaired

N. meningitidis H44/76 and LPS-deficient mutant were used to infect MDM in the absence of serum. Macrophages were incubated at 4°C, before unattached bacteria were washed off and cells were incubated at 37°C for a further 1.5h. The complete absence of LPS in the *lpxA* mutant had a profound effect on the internalisation of mutant organisms once bound to MDM: as the LPS-deficient mutant bacteria were internalised 58% less effectively than *N. meningitidis* H44/76 parent strain by 30min (Fig. 4.2A).

LPS is an important ligand for a number of phagocytic receptors, and the marked decrease in the internalisation of associated LPS-deficient organisms compared to wild-type implies that LPS receptors play a pivotal role in meningococcal phagocytosis.

4.2.4 Impaired LAMP-1 acquisition by phagosomes containing *lpxA* mutant

Along with a marked decrease in internalisation of LPS-deficient mutants, subsequent maturation of phagosomes was also impaired in Figure 4.3. Extensive measurement of intracellular trafficking using a range of antibodies, such as Rab 5 and Rab 7 proved difficult using this organism, as it would require levels of bacterial internalisation of different strains to be equalised; this study has found macrophage internalisation of adherent meningococci or populations of macrophages engaged in meningococcal internalisation/binding in the absence of immune serum rarely exceeded 25%, therefore monitoring differential trafficking would be limited. Intracellular trafficking of meningococci was therefore restricted to measurement of LAMP-1 co-localisation as a marker of late endosome/phagosome fusion.

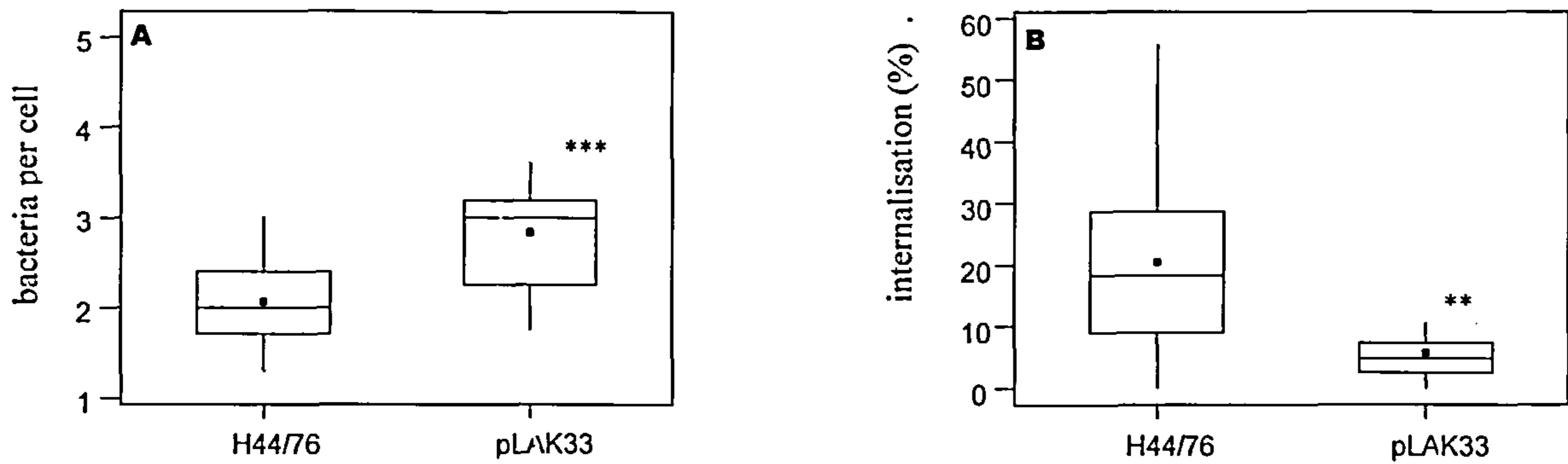


Figure 4.2

Contribution of LPS to interactions with primary human macrophages

LPS-deficient mutant pLAK33 and wild-type H44/76 were incubated with MDM (A) Adherence ratio per macrophage (B) Internalisation after 30min (as shown in Fig. 1) was reduced by 72.5% for the mutant when compared to the wild-type strain. Illustrating that LPS receptors appear to be important for meningococcal internalization. Boxes represent first and third quartiles, lines within each box are median values and black dots represent mean values. Results based on eight human donors, each experiment done in triplicate. Statistical significance denoted by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using Mann-Whitney U test

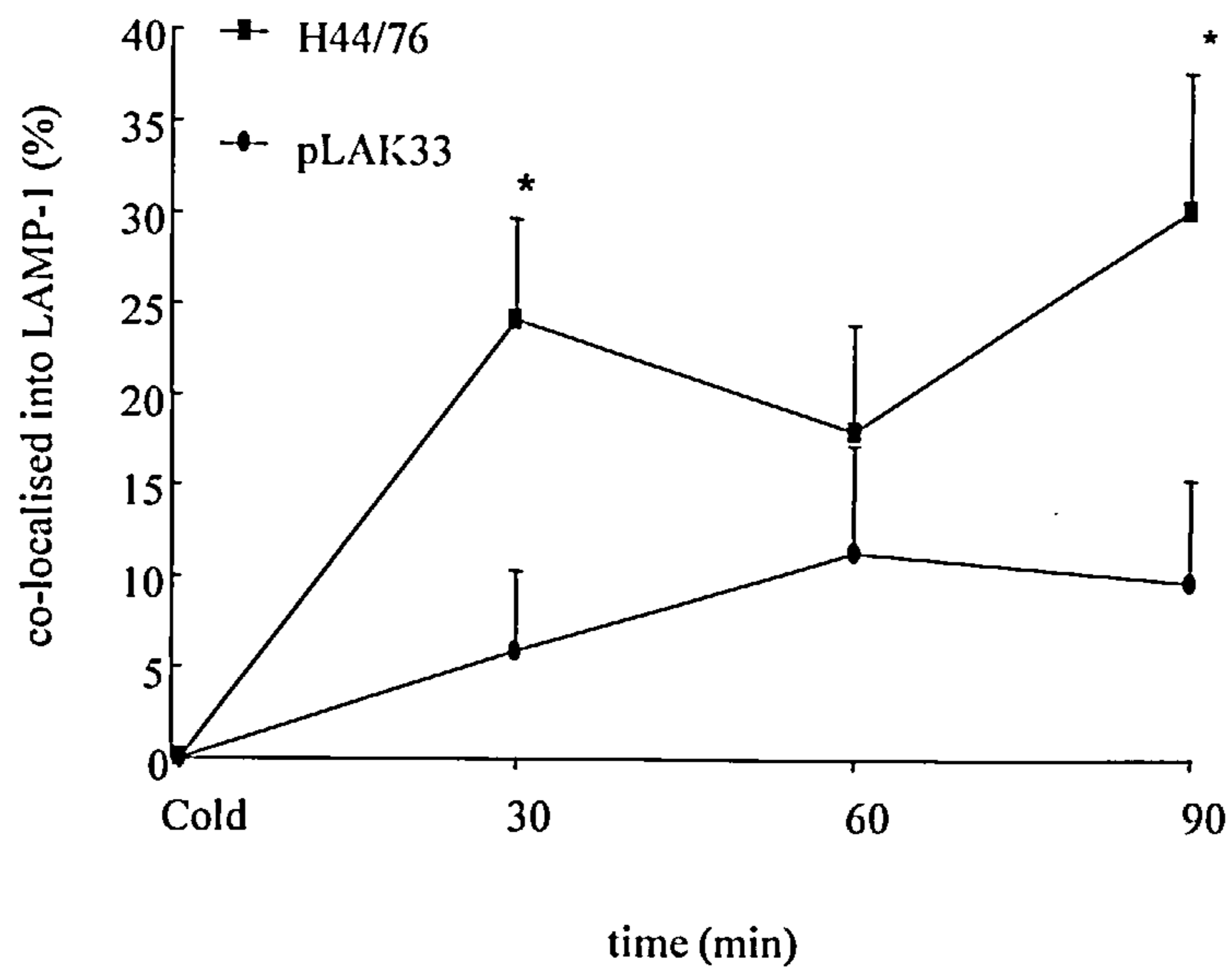


Figure 4.3

***lpxA* mutant taken into phagolysosomes less efficiently than wild type**

Graph shows percentage of ingested bacteria taken into LAMP-1-labelled phagolysosome/endosome vacuoles by MDM. Both H44/76 and pLAK33 were incubated at 4°C, before a further 90min incubation at 37 °C. Data represented as mean (\pm SEM); experiment performed using five donors each experiment done in triplicate. Statistical significance between two strains is denoted using * $P < 0.05$, calculated using Mann-Whitney U test.

4.2.5 Differentiation of THP-1 with phorbol myristate acetate (PMA) results in recovery of internalisation

THP-1 cells were incubated in the presence of PMA for 7 days at a final concentration of 25nM, with media changed every two days. Undifferentiated and differentiated THP-1 cells (dTHP-1) were incubated in the presence of *N. meningitidis* H44/76 for 1h at 37°C, 5% CO₂. Preliminary experiments comparing primary human MDM to differentiated (dTHP-1) and undifferentiated THP-1 reveals enhanced phagocytic activity of THP-1 cells following PMA-induced differentiation was detected (Fig. 4.5) and was accompanied by a marked increase in numbers of bacteria adhering to the surface of the cells (Fig. 4.4). Demonstrating that *in vitro*-differentiated monocytes, such as THP-1, can express receptors able to interact directly with *N. meningitidis* and could be used as suitable models for the study of bacteria-macrophage interactions, as PMA-differentiation appears to confer the same ability to recognise *N. meningitidis* as primary human cells (see Fig. 4.4).

Fig. 4.5 shows that levels of adherence, uptake and the proportion of cells directly interacting with bacteria at 37°C, all significantly increased following PMA treatment. Suggesting that receptors employed in meningococcal recognition are specific to mature macrophages, expression of which can be induced by PMA.

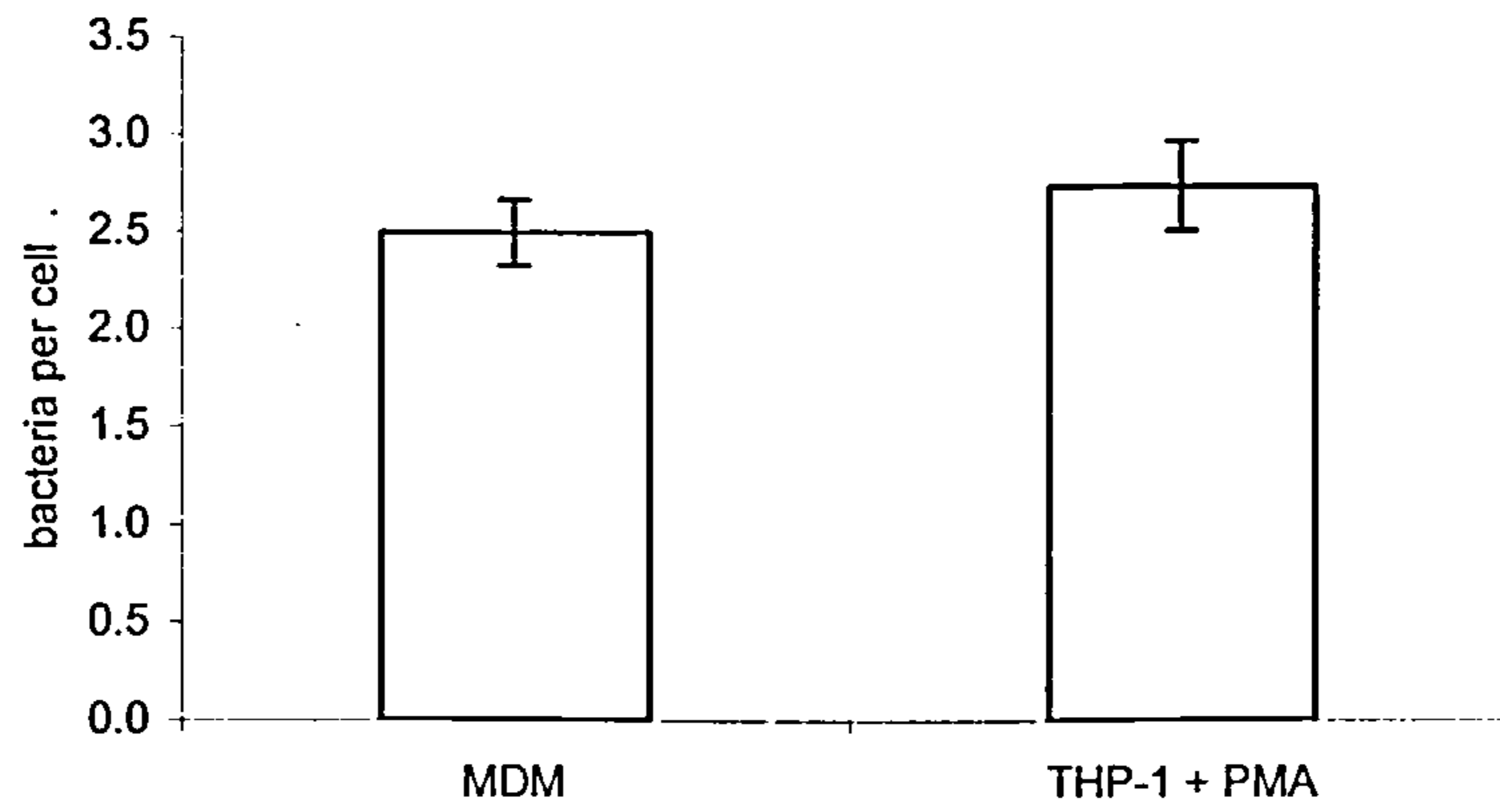


Figure 4.4

Monocyte differentiation enhances adherence

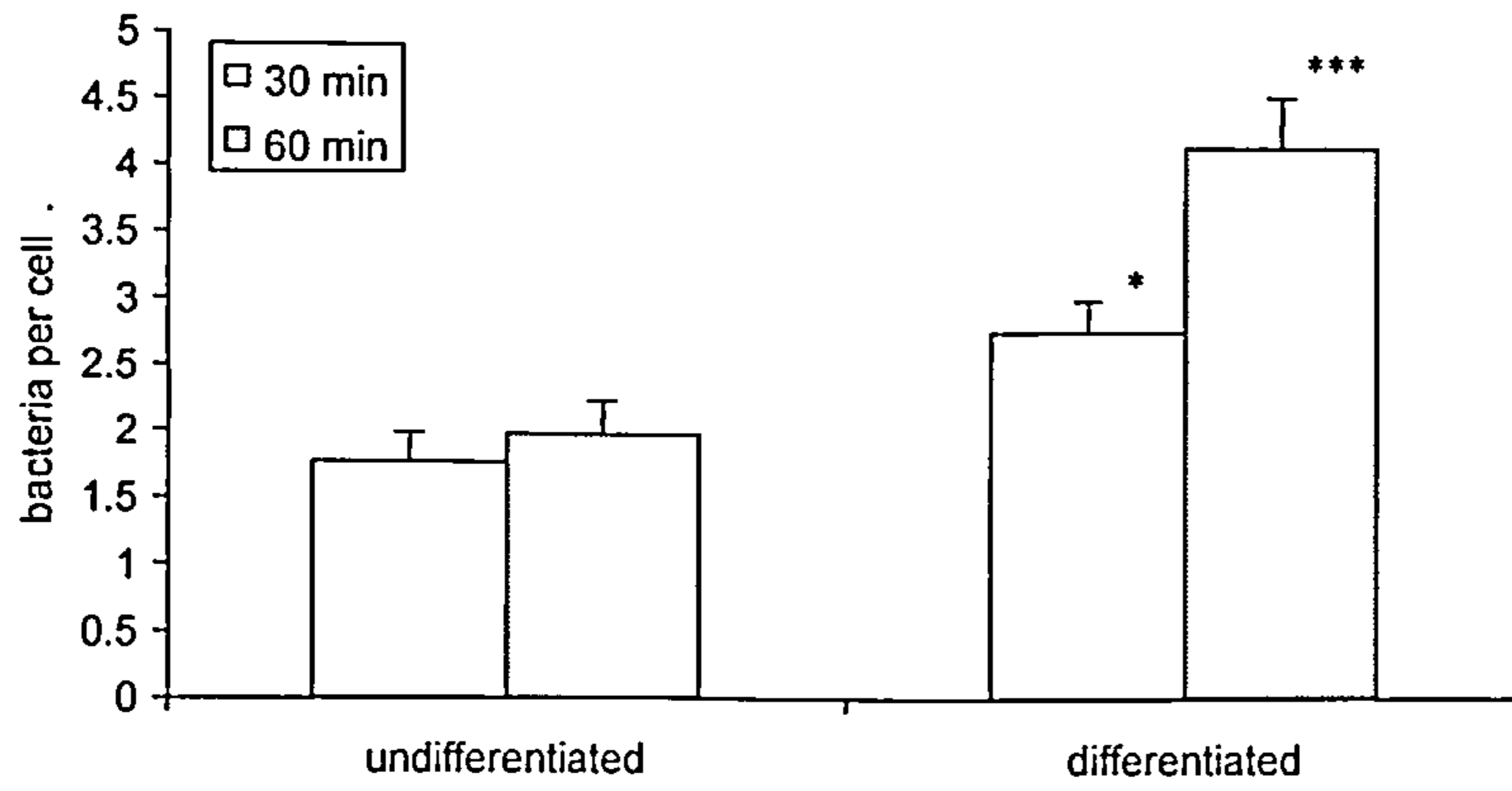
Comparison of PMA-differentiated and MDMs incubated with non-opsonised *N. meningitidis* H44/76 and incubated at 4°C for 60min. Results were based on at least four experiments, each experiment performed in triplicate. No significant difference observed using Mann-Whitney U test to compare each condition ($P>0.05$).

Figure 4.5

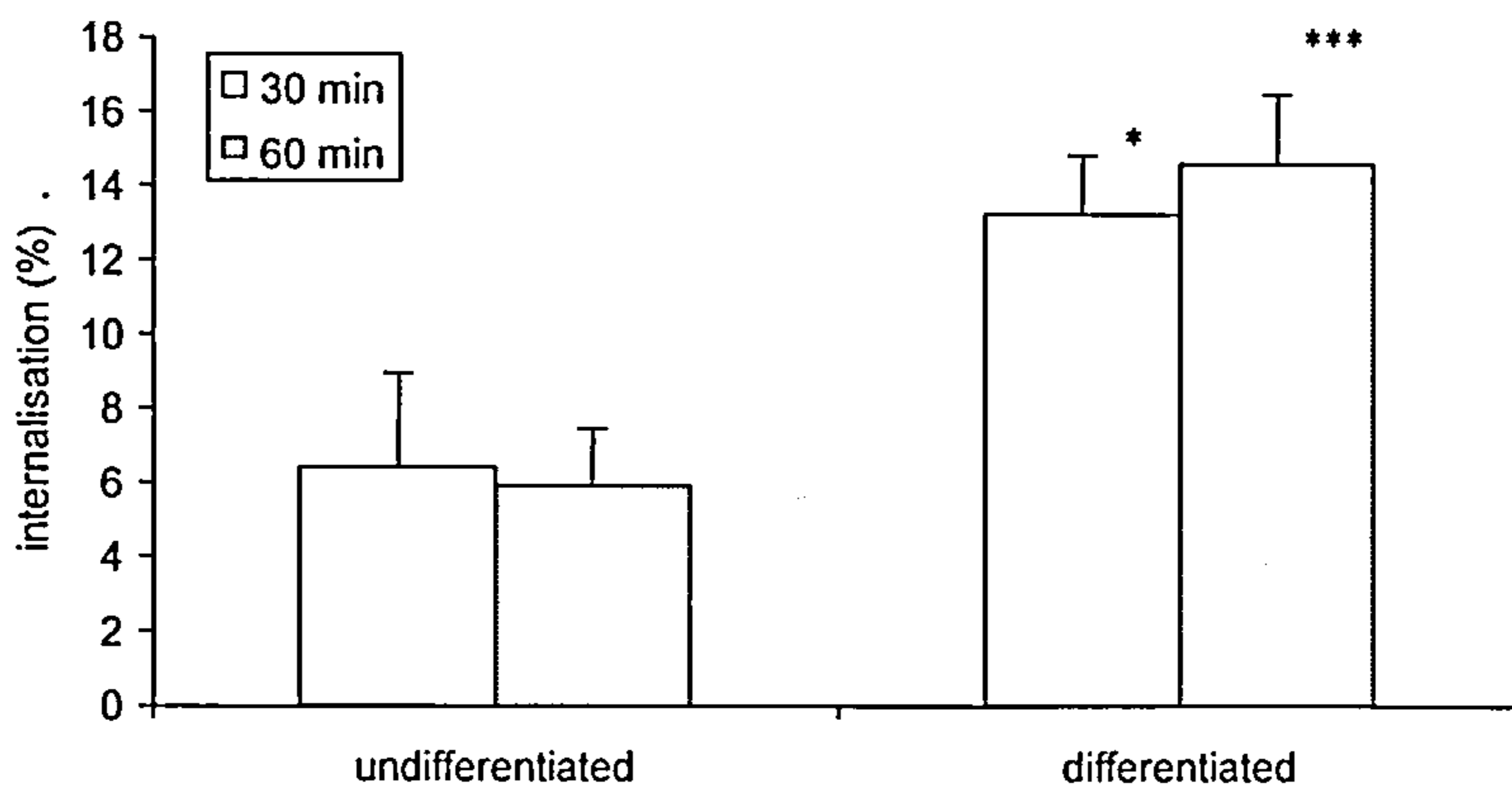
Monocyte differentiation enhances *N. meningitidis* recognition

Comparison of PMA-differentiated and undifferentiated THP-1 cells inoculated with non-opsonised *N. meningitidis* H44/76 and incubated at 37°C for 60min. (A) mean number of bacteria per cell (B) percentage of organisms ingested (C) percentage of cells interacting with bacteria. Results were based on at least four experiments, each experiment performed in triplicate. Statistical significance denoted by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using Mann-Whitney U test to compare dTHP-1 and THP-1 at each time point..

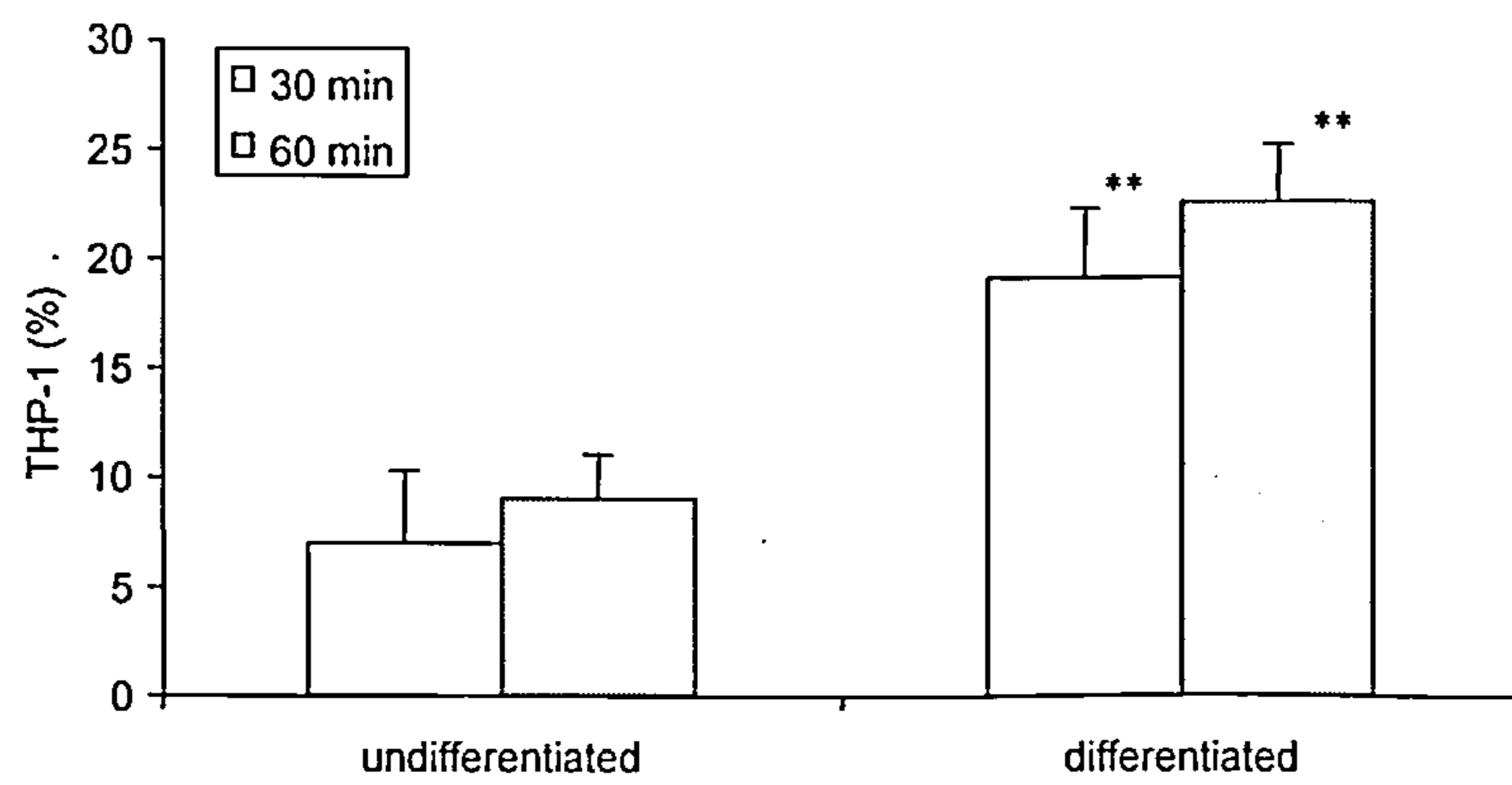
A



B



C



4.2.6 Absence of LPS reduces TNF- α production

Meningococcal disease (MCD) is characterised by high levels of circulating endotoxin which have a detrimental effect on the host, and trigger a range of inflammatory reactions that can ultimately lead to septic shock and death (Brandtzaeg 1996). These increased levels of circulatory LPS indicate severity of disease, and are closely associated with an increase in fatality rate (Brandtzaeg, Kierulf et al. 1989). The binding of LPS to specific surface receptors can induce macrophage activation, and result in a number of cellular changes, including secretion of pro-inflammatory cytokines such as TNF- α .

To examine the contribution of endotoxin on *N. meningitidis*-induced TNF- α secretion upon exposure to wild-type *N. meningitidis* H44/76 and LPS-deficient mutant pLAK33, MDM were incubated in the presence of the two strains for 2h at 37°C, 5% CO₂. The culture media was harvested and concentrations of TNF- α secreted measured using ELISA. Supernates were obtained from six human donors. Fig. 4.6 shows significant differences in levels of secreted TNF- α between parent and mutant *N. meningitidis* strains of H44/76. Results were expressed as proportion of the mean response to 3.3x10⁶cfu/ml doses of H44/76 and pLAK33.

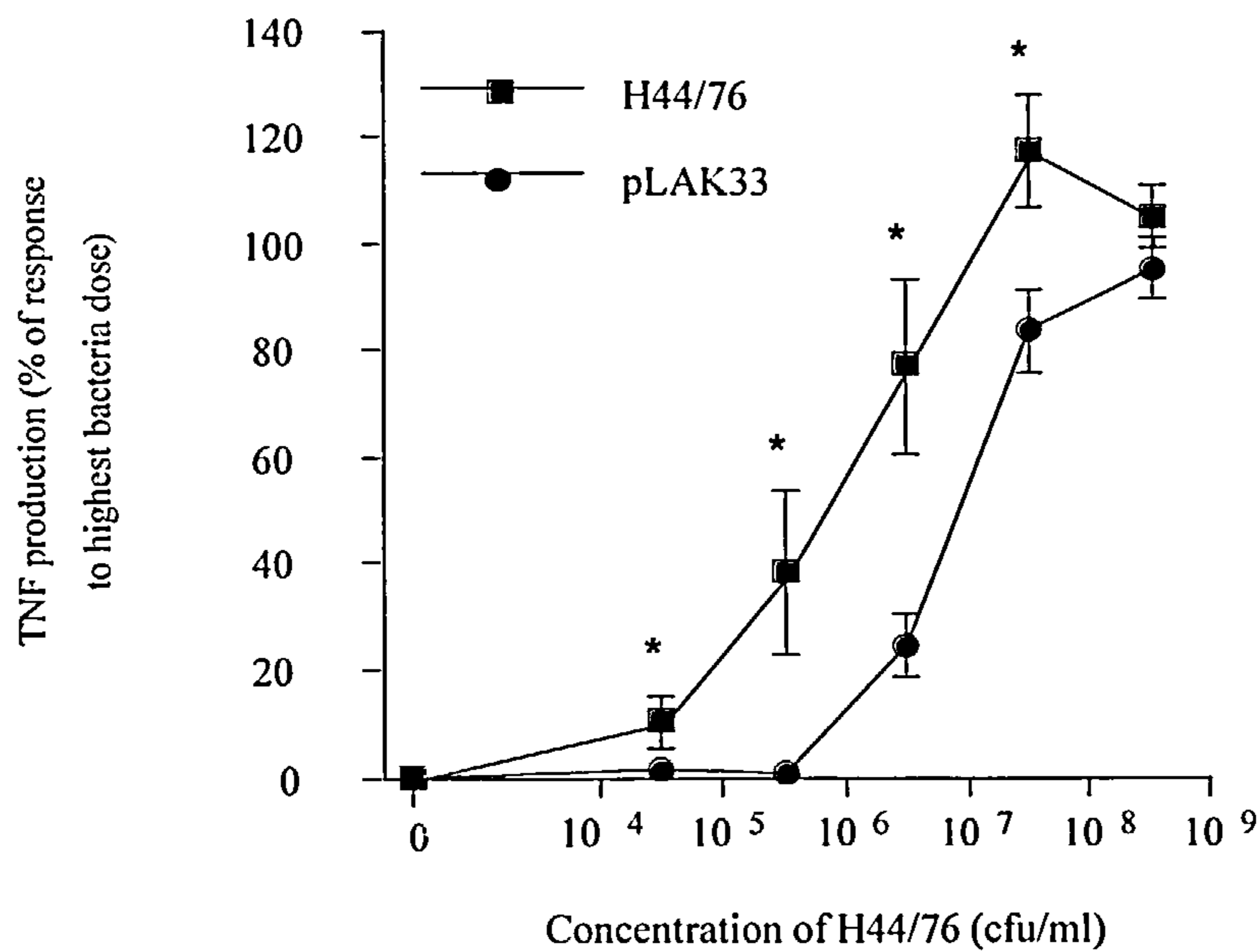


Figure 4.6

Absence of LPS impairs TNF- α production

Difference in TNF- α secretion after exposure to a range of concentrations of LPS-deficient mutant pLAK33 and wild-type H44/76 to MDM. Results based on six human donors and results expressed as a percentage of the mean TNF- α concentration induced in each preparation of MDM by 3.3×10^8 cfu/ml of H44/76 and pLAK33. Statistical significance denoted by * $P < 0.05$.

4.3 Summary and discussion

The examination of non-opsonic interactions of *N. meningitidis* is noteworthy, since once meningococci are in the nasopharynx they are likely to be exposed to resident macrophages and dendritic cells which are widely distributed extravascularly, where opsonins may be low or absent, and also in the liver, adrenal glands and choroid plexus they would be in direct contact with blood and CSF (Gordon, Lawson et al. 1992):

The use of the first viable *N. meningitidis* organism that completely lacks LPS allowed us to study how effectively whole organisms interact with, and are recognised by, host immune cells. These observations strongly suggest that LPS plays an important role in meningococcal recognition by macrophages during non-opsonic interactions. Internalisation, intracellular trafficking and cytokine production by human macrophages were all inhibited by the notable absence of LPS in the pLAK33 *lpxA* mutant compared with wild-type H44/76. Although TNF- α secretion following exposure to the LPS-deficient mutant was markedly reduced, it was not completely absent, implying that outer membrane components of gram-negative bacteria, other than LPS, may also trigger cytokine production and induce cellular activation.

Adherence by the LPS-deficient mutant was improved in the *lpxA* mutant strain. It is known that the outer membrane structure of the LPS-deficient mutant is quite different from that of the wild type, particularly with respect to phospholipid composition, as the length and the saturation of the fatty acyl chains of phosphatidylethanolamine (PE), and the relative proportions of other phospholipids are different. These differences could affect how successfully these organisms interact with the macrophages; for example in the mutant strain a greater proportion of more positively charged phospholipids, such as phosphatidylcholine (PC), are present which might affect bacterial adherence to

macrophages. The LPS-deficient mutant may engage with alternative receptors with a high affinity via its other outer membrane proteins, such as pili and opacity proteins, or perhaps the differences in the proportion of charged molecules present may enhance adherence to macrophages (see Fig. 4.2A).

The conspicuous difference between the ability of primary human macrophages and undifferentiated THP-1 to internalise these two strains of *N. meningitidis* H44/76 (see Figs. 4.1 and 4.2) may be due to these differences in outer membrane compositions of the neisserial strains used, but is more likely a result of the significant differences in receptor expression of MDM and undifferentiated THP-1 cells. Meningococcal recognition by THP-1 was enhanced considerably by PMA-differentiation (see Fig. 4.4 and 4.5), and previous studies have observed that PMA differentiation of THP-1 cells increases numbers of receptors expressed as well as diversity thus allowing us to conclude that one or more receptors expressed on mature macrophages are able to interact directly with meningococci.

The notable difference in LPS-mutant behaviour compared to H44/76 prompted further exploration into the role of LPS receptors as a possible route of entry into the macrophage was the central theme of this study; as the receptor(s) of choice for *N. meningitidis* by human macrophages under opsonic and non-opsonic conditions have yet to be reported. The following chapter examines the roles of two LPS receptors, CD14 and CR3, and the contribution of cholesterol-rich membrane rafts in meningococcal interactions with human macrophages.

Chapter 5

Roles of CD14, CR3 and membrane rafts in non-immune and opsonic internalisation of *N. meningitidis*

5.1 Introduction

The work described in this chapter examines how potential macrophage receptors utilised by *N. meningitidis* to gain entry into human macrophages under non-immune and opsonic conditions were explored. Evidence from Chapter 4 suggests that LPS is an important component of these interactions, attention was therefore directed at two important LPS receptors, CD14 and CR3.

CD14 is a GPI-anchored LPS receptor (Simmons, Tan et al. 1989) that can bind whole gram-negative bacteria as well as soluble LPS, and apart from a few notable exceptions, most CD14 ligands induce a pro-inflammatory response which serves to eliminate pathogens and their toxic compounds in a controlled manner. Uncontrolled stimulation could result in the life-threatening symptoms of septic shock. Interaction of LPS with monocytes/macrophages via specific membrane receptors (Bone, Grodzin et al. 1997) is the first step in the pathogenesis of endotoxic shock. Based on a number of *in vitro* studies, CD14 involvement in the phagocytosis of gram-negative bacteria via LPS has been observed (Grunwald, Fan et al. 1996), but its lack of transmembrane and cytoplasmic domains suggests that the organisms are internalised independently of CD14 (Kop and Medzhitov 1999), implicating one or more other phagocytic receptors such as TLR4, CR3 and as this study suggest SR-A. It is not known exactly how signals are transduced by CD14 over the plasma membrane, as GPI lacks the ability to transduce signals directly into the cells. Stimuli such as PMA, LPS or IFN γ induce the shedding of mCD14 (to 48kDa sCD14) from the surface of monocytes/macrophages; which may be mediated by the activation of

a membrane-associated serine protease. The role of CD14 in cell-cell interactions with monocytes has been reported, as the use of anti-CD14 mAb induces the monocyte-mediated activation of T-cells and ICAM-1-mediated attachment to monocytes (Lauener, Geha et al. 1990; Lue, Lauener et al. 1991), but no reports into the role of CD14 in the processing of *N.meningitidis* by macrophages have been published to date.

Phosphatidylinositol-specific phospholipase C (PIPLC) is an enzyme that specifically cleaves protein from the phosphatidylinositol component of the GPI anchor, and structural studies focusing on the glycan component that connects the protein to phosphatidylinositol molecule has shown that it has been conserved during evolution. Use of PIPLC is an effective method of removing GPI-linked proteins from the surface of macrophages or neutrophils. The GPI anchor may play a versatile role in the regulation of cell surface expression, and may offer a site for the degradation of proteins by specific endogenous phospholipases, resulting in the release of protein from the cell surface. Products of such degradation may then in turn have a role in cellular communication.

In this study MDM cells were treated with phosphatidylinositol phospholipase C (PIPLC), which cleaves the GPI-anchor of surface proteins, resulting in the complete removal of CD14 from the surface of the cell, leaving sCD14 molecules in the surrounding media which were subsequently removed during washing. In order to examine the relationship between mCD14 and *N. meningitidis* specifically, an anti-human CD14 blocking mAb (MY4) was also used in adherence and internalisation studies in different environments.

As human macrophages express a wide array of receptors capable of binding and internalising gram-negative bacteria, a model system was used to allow the study of human CD14 in isolation from other phagocytic receptors, by transfecting HeLa cells with CD14. HeLa cells are a human epithelial cell line that possess

limited phagocytic ability, but are able to successfully express transfected receptors and incite pro-inflammatory responses as a consequence. Transfected cells were therefore used to examine meningococcal association, and differential fluorescent microscopy was used to obtain these results. In this chapter, the results indicate show that serogroup B *N. meningitidis* adhere to non-immune macrophages almost exclusively through CD14 in the absence of serum.

A range of integrins also express RGD (Arg-Gly-Asp) sequences which function as cell adhesion sites for a number of endogenous molecules including fibronectin, vitronectin, fibrinogen, thrombospondin and laminin (Ruoslahti 1996). A number of microorganisms have been found to bind to these sequences as a means of gaining entry into the cell, for example *Bordetella pertussis* through its surface protein (Leininger, Roberts et al. 1991). *Yersinia pseudotuberculosis* produces an invasin to invade cells, and although this invasin does not contain RGD sequences, RGD peptides can inhibit invasin binding and bacterial entry (Van Nhieu and Isberg 1991). RGD peptides have also been successfully used to block entry of *N. meningitidis* into human endothelial cells (Virji, Makepeace et al. 1995), therefore these peptides were also used to examine whether meningococci interact with RGD-containing sites present on the surface of macrophages using this model.

Observations from Chapter 3 led us to conclude that non-Fcγ receptors are involved in the internalisation but not the binding of *N. meningitidis* in the presence of immune serum, we therefore also examined *Neisseria* interactions with CR3 in this chapter. This was a natural avenue to explore, as CR3 is considered a cornerstone of the innate immune system, and as a prominent phagocytic receptor that binds and internalises particles in both the presence and absence of opsonins, I wanted to observe the role, if any, this receptor played in neisserial interactions with human macrophages. A number of studies over the years have established that a range of saccharides, including bacterial LPS and NADG (Thornton, Vetvicka et al. 1996), bind to the polysaccharide lectin site of

CR3, situated C-terminus to the I-domain, but this has not been observed with *Neisseria* species.

Previous studies have shown that a number of different micro-organisms, such as *Mycobacteria* species and *Listeria monocytogenes*, use macrophage CR3 as a port of entry into leukocytes. *Listeria* are killed effectively, but in contrast *Mycobacteria* enter the cells without alerting normal immune responses (Stokes, Haidl et al. 1993; Drevets, Leenen et al. 1996; Zimmerli, Edwards et al. 1996; Le_Cabec, Cols et al. 2000). GPI-proteins aggregate on cholesterol-rich microdomains known as lipid rafts, and evidence has been found to suggest that in the binding and internalisation of certain pathogenic bacteria, such as *Mycobacteria kansasii*, CR3 are also mobilised to these lipid rafts. Here they interact both physically and functionally with CD14, which themselves form large non-covalently bound aggregates on specific plasma membrane microdomains known as caveolae, which are known to be sites on which GPI-linked receptors localise (Pierini and Maxfield 2001). Lipid raft disrupting agents, Nystatin and β -cyclodextrin, were used in this study in an attempt to identify if this is also the case in meningococcal processing.

In summary, this chapter examines the roles of CD14 and CR3; meningococcal-CR3 interactions are observed in a number of different ways; (a) Non-opsonic interactions with the lectin binding site of CD11b/18 were inhibited using N-acetyl D-glucosamine (NADG) (b) The I-domain (and/or lectin site) was blocked with anti-human CD11b mAb (2LMP19c) under both opsonic and non-opsonic conditions. (c) RGD-containing peptides were used to block integrin RGD binding sites on primary human macrophages and interactions with meningococci observed. The contribution of lipid-rich membrane rafts in macrophage interactions with *N. meningitidis* in the presence/absence of immune serum was also examined by using raft disrupting chemicals, β -Cyclodextrin and Nystatin.

5.2 Results

Part I: CD14

5.2.1.1 Human macrophages can capture *N. meningitidis* through GPI-anchored receptors

Activating agents, such as LPS, have the effect of inducing the translocation of intracellular pools of GPI-linked proteins to the cell surface, and therefore increase expression of CD14 receptors. Therefore, when internalisation studies at warm temperatures looking specifically at the role of CD14 were carried out, this effect was compensated for by ensuring the continuous presence of PIPLC enzyme or anti-CD14 antibody in the surrounding media. I had initially observed that following PIPLC treatment of human macrophages, the absence of the PIPLC enzyme during bacterial infection did not inhibit GPI protein function with respect to binding, as no difference in meningococcal binding was observed between enzyme-treated and control cells (data not shown). A difference was only observed if the enzyme was maintained in the media throughout.

For this adherence study MDM were incubated with 1 unit/ml PIPLC for 30min at 37°C, 5% CO₂ before cells were washed three times in warm culture media and infected with non-opsonised *N. meningitidis* H44/76 at MOI of 50:1 in the presence of enzyme for 1h at 4°C. Cells were fixed after incubation and analysed. Following incubation with PIPLC at 4°C a 25.1% reduction in the numbers of *N. meningitidis* H44/76 adherent per macrophage was noted. (Fig. 5.1A) GPI-receptor cleavage also had a marked effect on the percentage population of macrophages capable of binding *N. meningitidis* H44/76 after PIPLC treatment. Compared with untreated control cells, a 58.14% reduction was noted. (Fig. 5.1B)

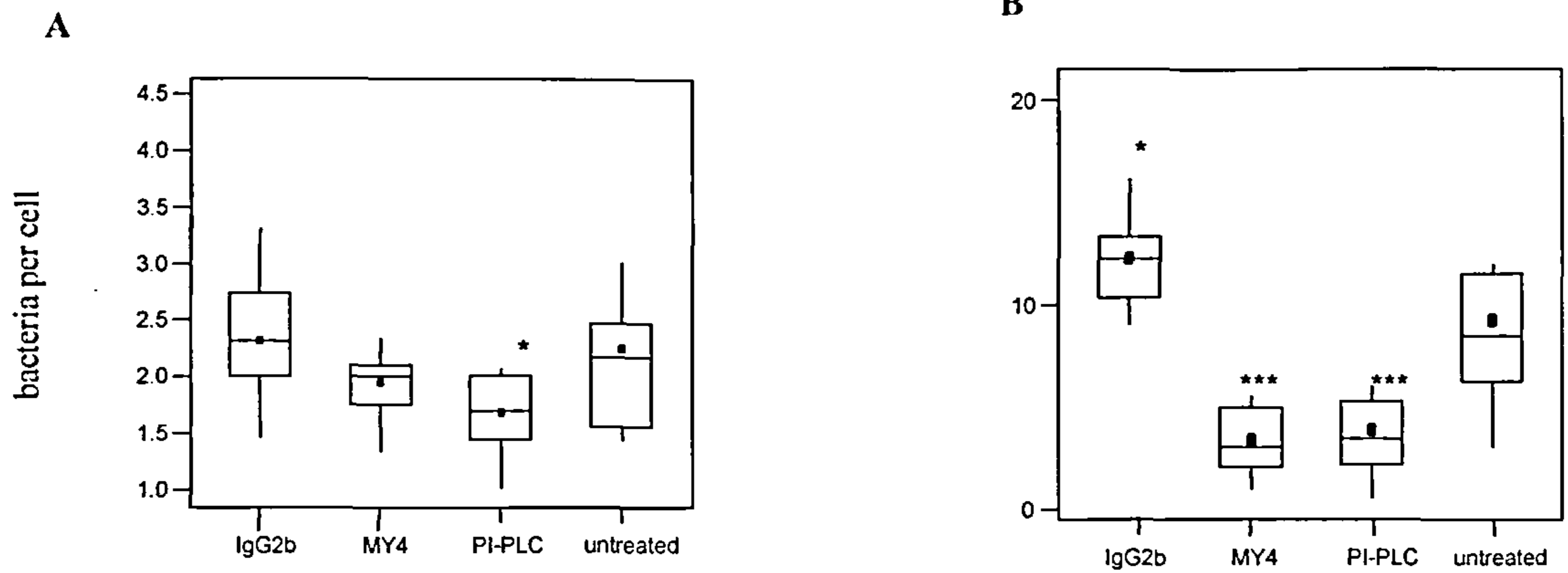


Figure 5.1

Inhibition of GPI-linked proteins

Comparison of H44/76 adherence to pre-treated MDM under non-opsonic conditions. MDM were treated with anti-CD14 mAb MY4 , control antibody IgG2b or 1 unit/ml phosphatidylinositol phospholipase C (PI-PLC) before exposure to bacteria for 1h at 4°C. Graphs represent (A) ratio of bacteria associated per macrophage, out of 200 (B) Percentage population of MDM with bacteria associated on surface. Data shown from four different donors, each experiment done in triplicate. Boxes represent first and third quartiles; lines within each box are median values; black dots represent mean values. Individual *P* values obtained using Mann-Whitney U test comparing antibody-treated cells with untreated control. Kruskal Wallis $P < 0.01$ for graph (B).

The conspicuous difference of meningococcal recognition at 4°C following PIPLC treatment may have been a temperature dependent response, therefore the experiment was repeated at 37°C, and bacteria were incubated for 30min and 60min before fixing and staining. The effects of PIPLC treatment followed a different pattern at 37°C, as the only change observed was the percentage of adherent bacteria internalised following 30min incubation fell dramatically by 57.7% (Fig. 5.2). Effects of PIPLC on bacterial adherence also appear to be temperature dependent, as no difference was observed at 37°C.

5.2.1.2 Blocking CD14 inhibits meningococcal adherence and internalisation by human macrophages

As a general GPI cleavage enzyme, the action of PIPLC was not specific to one surface protein expressed on macrophages, so in order to identify which possible receptors were involved in meningococcal recognition monoclonal antibodies were used. As a prominent LPS receptor present on human macrophages and known to contribute to bacterial recognition by immune cells, I wanted to ascertain whether CD14 contributed to the marked reduction in bacterial adherence and internalisation of serogroup B *N. meningitidis* to PIPLC-treated macrophages. In order to examine this phenomenon in greater detail, MDM were incubated in the presence of MY4, a mouse anti-human CD14 monoclonal antibody that blocks the LPS-binding site of CD14.

MDM were incubated in the presence of 5µg/ml MY4 mAb for 30min at 37°C, 5% CO₂, before wells were washed three times in warm culture media and exposed to *N. meningitidis* H44/76 in the absence of serum opsonins. Again, for all studies carried out at 37°C, macrophages were continuously incubated in the presence of MY4 in order to compensate for the translocation of intracellular

pools of CD14 to the cell surface. MY4 appeared to reduce macrophage recognition of meningococci at both 4°C and 37°C.

5.2.1.3 Effect of temperature on bacterial interactions

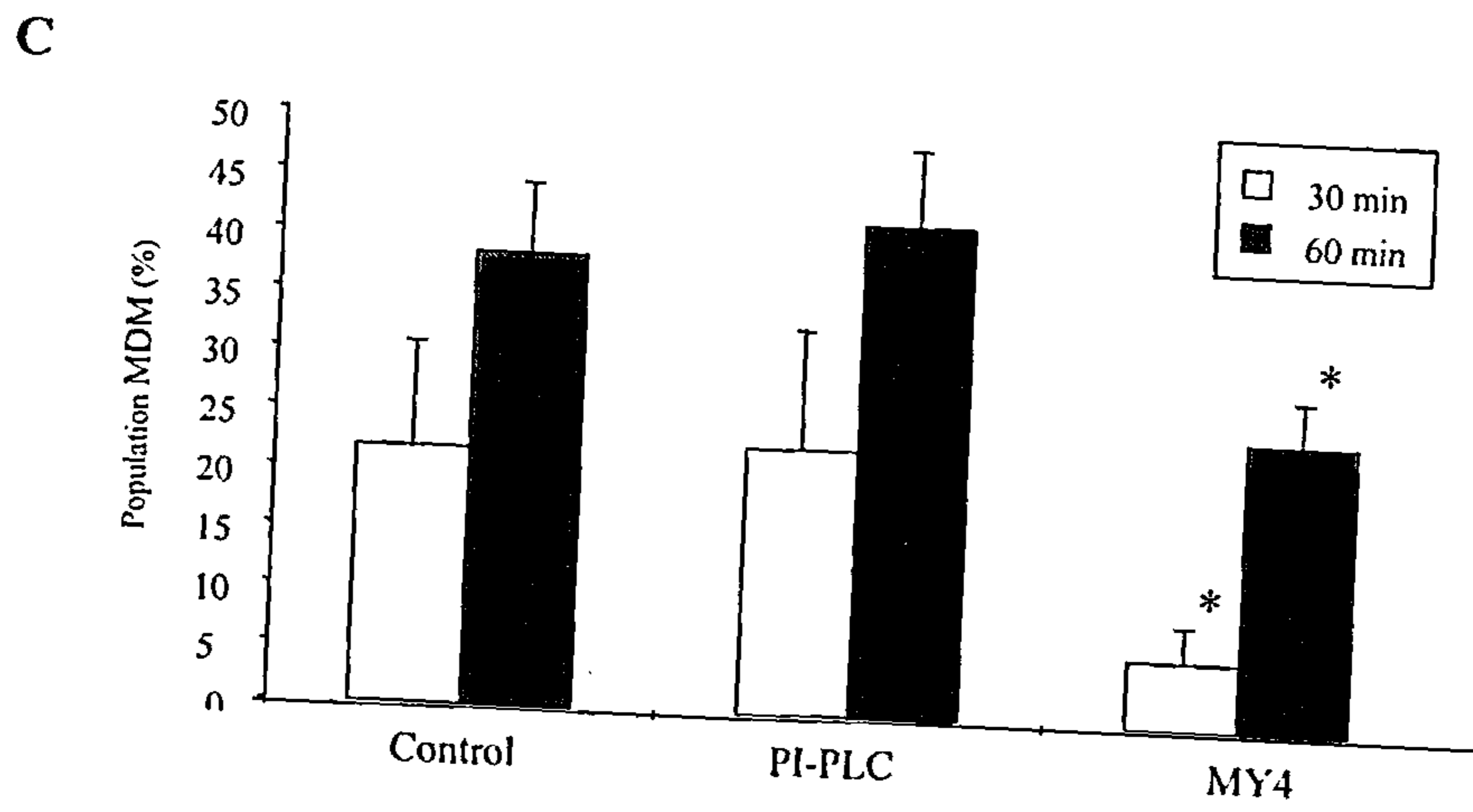
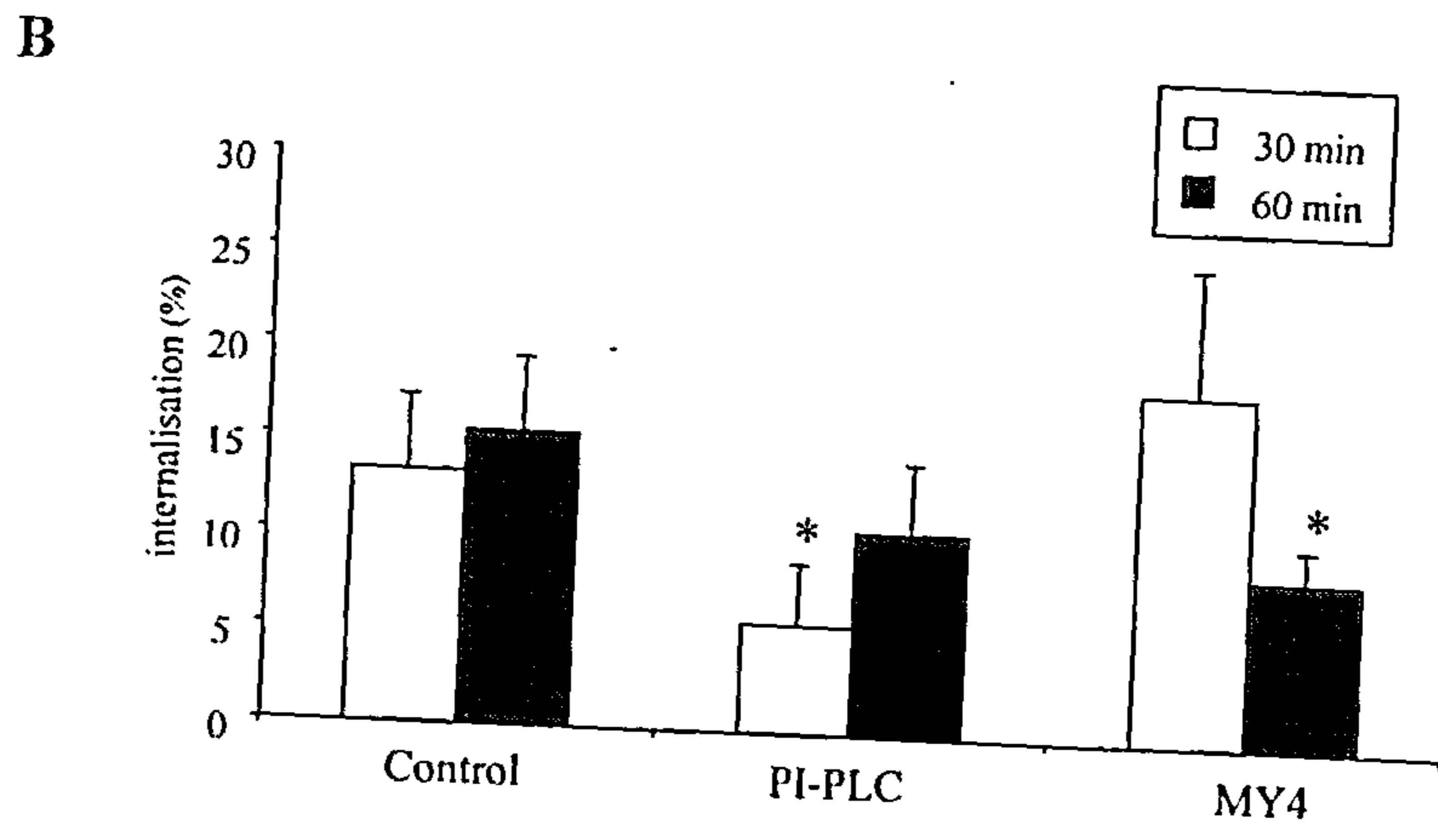
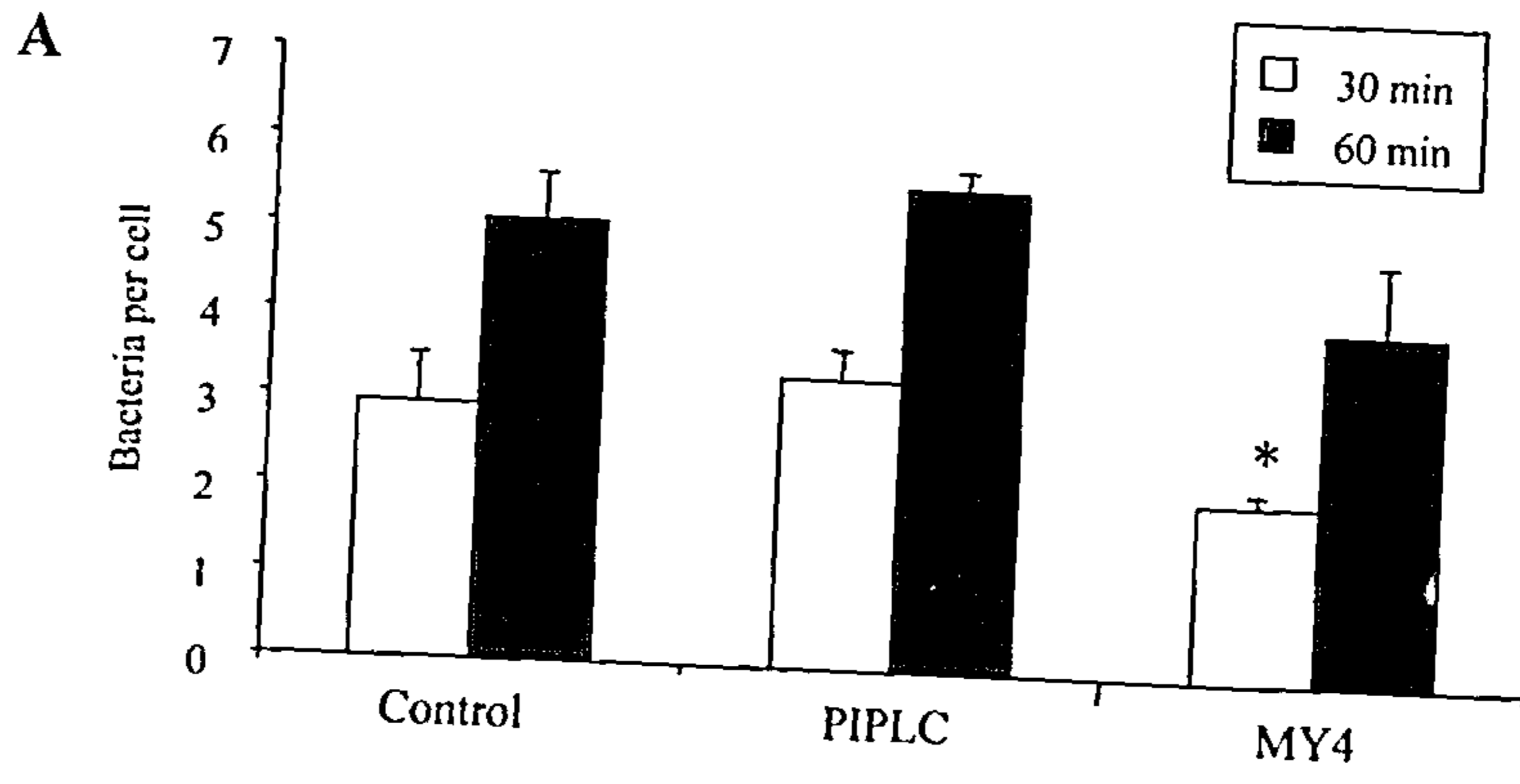
MDM were pre-incubated in the presence of 5µg/ml MY4 or same concentration of the isotype control antibody IgG2b before infecting with *N. meningitidis* H44/76. Bacterial adherence studies carried out at 4°C show that MY4 treatment clearly diminishes bacterial recognition when compared to both untreated control cells and isotype control antibody IgG2b (see Fig. 5.1). MY4 had a most significant effect on the population of macrophages with adherent bacteria on their surface, as a 72.36% reduction was observed in Figure 5.1B. This response to anti-CD14 mAb was even more marked than the more general PIPLC enzyme, suggesting that of the GPI-anchored receptors present on the surface on human macrophages, CD14 may play an important role in meningococcal recognition.

To study the effects of these treatments on bacterial internalisation, pre-treated MDM cells were incubated with bacteria at 37°C for 30min and 60min. Fig. 5.2A demonstrates numbers of adherent bacteria declined by 30.27%, although by 60min this had recovered to levels equivalent with control cells. MY4 appeared to have no significant effect on the internalisation of *N.meningitidis* H44/76 at 30min, but by 60min bacterial uptake had fallen by 43.05% (Fig. 5.2B). Following 30min incubation, fewer organisms are adherent to fewer macrophages (Fig. 5.2C) and the percentage of these bacteria internalised by MDM remains the same as the untreated control cells.

Figure 5.2

Absence of GPI-linked proteins and CD14 reduces bacterial uptake

Comparison of H44/76 adherence to pre-treated MDM under non-immune conditions. MDM were treated with anti-CD14 mAb MY4 (5µg/ml), or 1 unit/ml phosphatidylinositol phospholipase C (PI-PLC) before incubation with bacteria for 60min at 37°C. Graphs represent (A) ratio of bacteria associated per macrophage, from 200 cells counted (B) Internalisation (C) Percentage population of MDM with bacteria associated on surface. Data shown from four different donors, each experiment performed at least three times. Statistical significance denoted by * $P < 0.05$ using Mann Whitney U test comparing pre-treated cells with untreated control cells.



The population of macrophages with adherent or internalised organisms fell dramatically at both time points; 73.75% at 30min and 36.52% by 60 min.

5.2.1.4 Increased association of *N. meningitidis* to HeLa CD14-transfectants

HeLa cells do not possess the necessary machinery for efficient phagocytosis of microbes, and as a result are not considered to be “professional” phagocytes. Transfecting HeLa cells with human CD14 confers the ability of these cells to express mCD14, and this was confirmed by staining transfected cells with FITC-conjugated MY4. CD14-transfected cells and control cells were incubated for 1h at 4°C with *N. meningitidis* H44/76, and analysed for any changes in adherence alone. Control cells were HeLa cells which had been transfected with the plasmid vector which lacked CD14 cDNA sequence. This model was used as a means of establishing the influence of CD14 in the absence of a number of macrophage receptors.

Fig. 5.3 illustrates the difference in bacterial adherence between control cells (vector) and CD14-transfected cells. A 60.24% increase in adherence to transfected cells was observed. There was a 65.48% increase in the proportion of CD14-transfected HeLa cells with adherent meningococci present on the surface, and a 40.40% increase in total adherence (total bacteria/number of HeLa cells with adherent bacteria) (see Fig. 5.3B and Fig. 5.3C).

Other possible receptors utilised by *N. meningitidis* may exist on HeLa cells that recognise OMP structures, such as pili, but these results suggest that their effects appear to be less prominent, as the presence/absence of CD14 appears to have a profound effect on bacterial recognition.

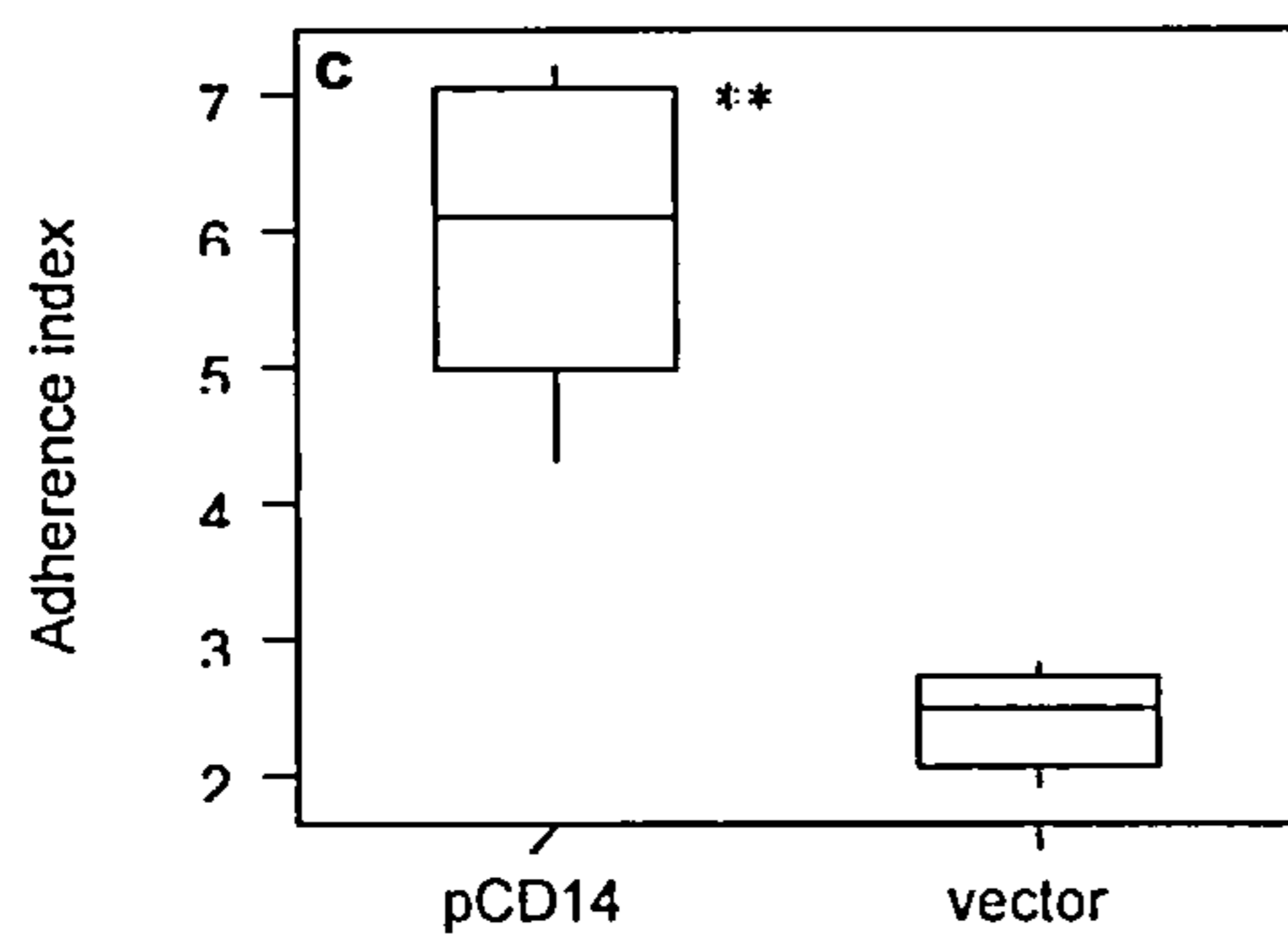
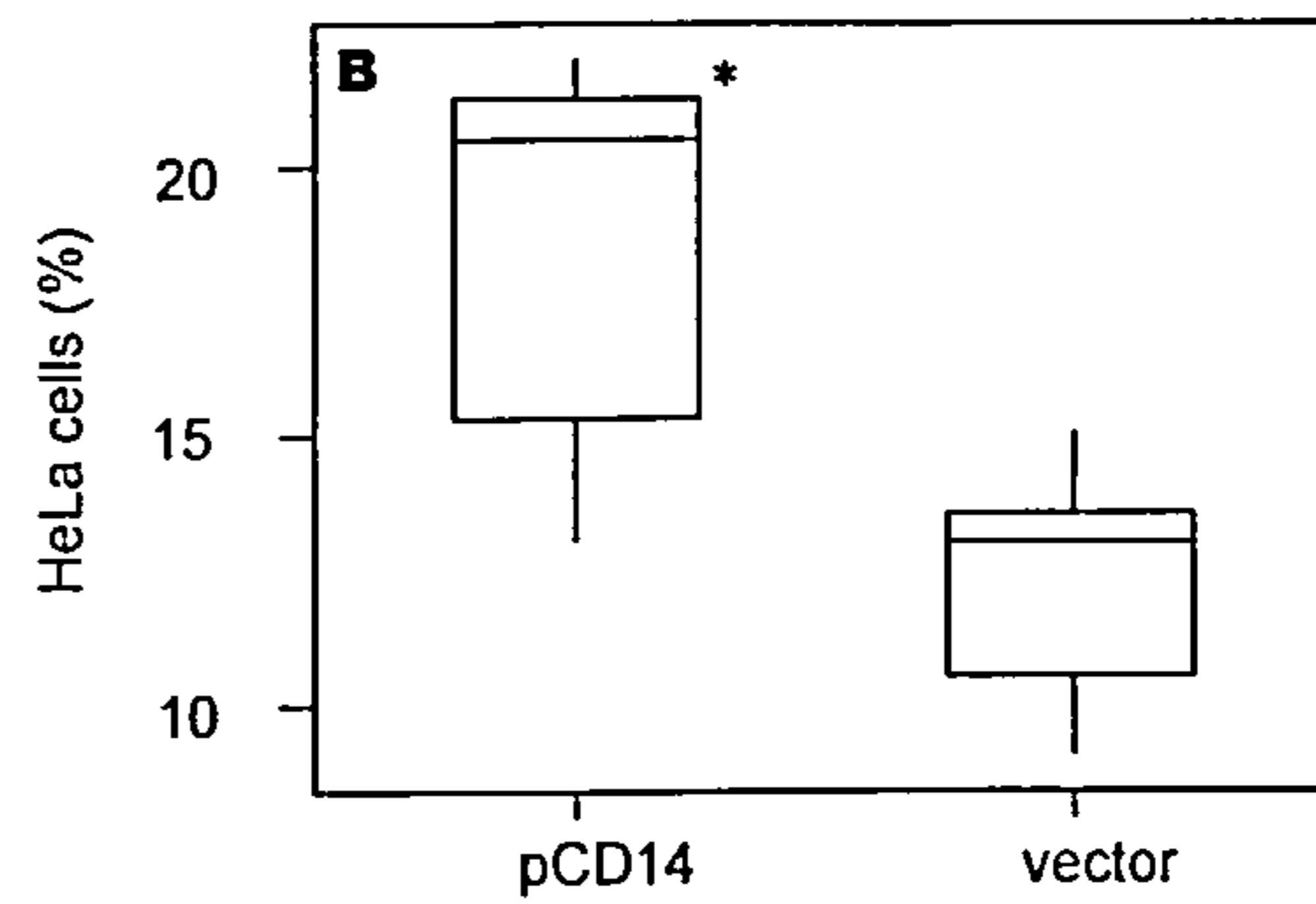
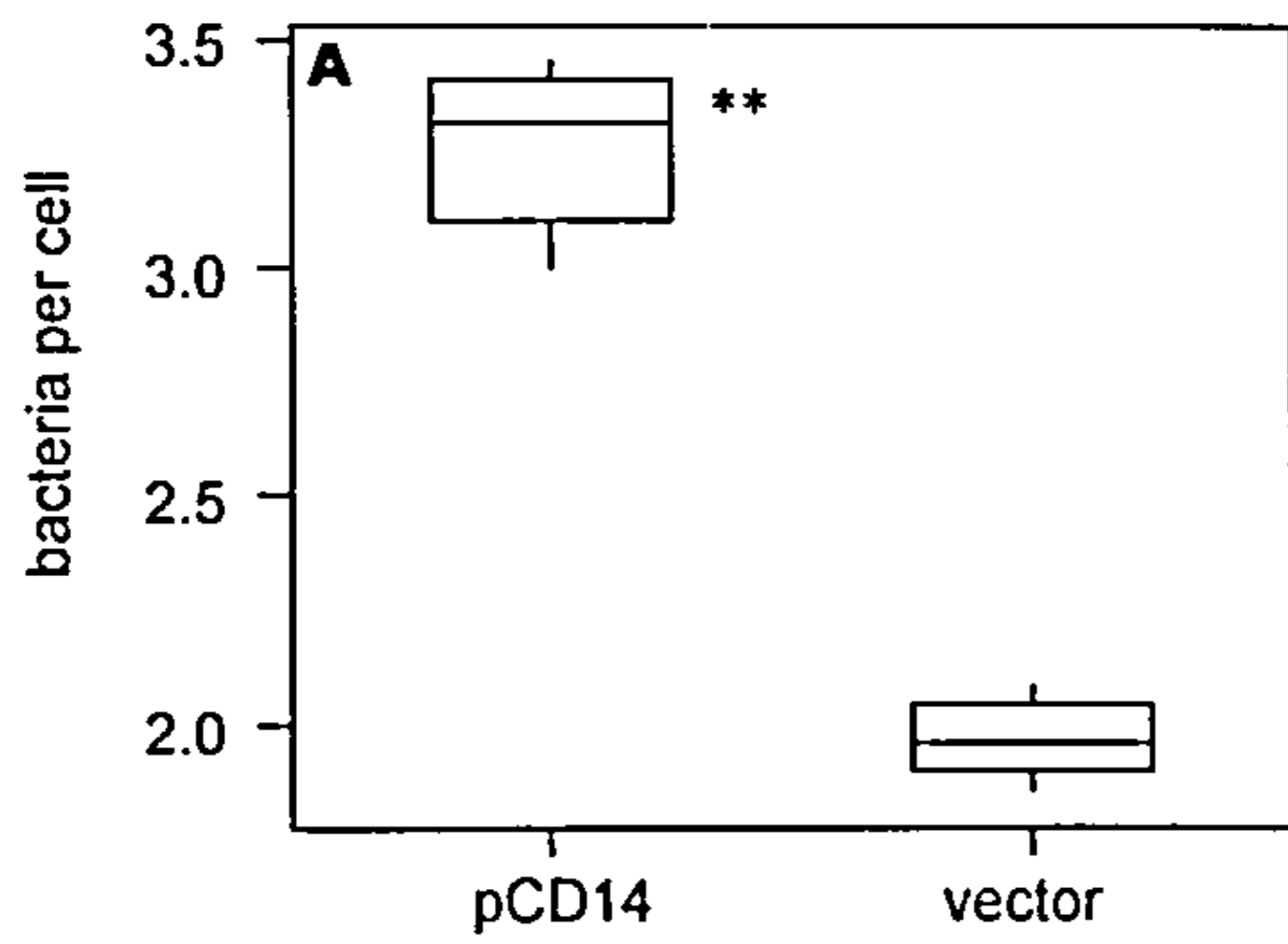


Figure 5.3

CD14-transfected HeLa cells

Absence of CD14 significantly impairs effective association of H44/76 to CD14-transfected HeLa cells under non-opsonic conditions. Box-plots show (A) ratio of bacteria associated per HeLa cell, out of 200 (B) Percentage HeLa cells with bacteria associated (C) adherence index (total MC/100). Data based on two experiments, each done in triplicate. Statistical significance denoted by * $P < 0.05$, ** $P < 0.01$ using Mann-Whitney U test.

Part II: Complement receptor 3 (CR3, CD11b/18)

5.2.2.1 Blockade of CD11b/CD18 has no effect on binding or internalisation of *N. meningitidis* in the absence of serum opsonins

Findings in Part I suggested that CD14 is an important recognition receptor for *N. meningitidis*, but as CD14 lacks transmembrane and cytoplasmic domains essential for bacterial phagocytosis, it was not considered to be a receptor directly involved in transduction of *Neisseria* internalisation. Therefore the role of complement receptor 3 (CR3 or CD11b/18) which does have phagocytic potential was examined. Two important binding sites, I-domain and lectin site, were inhibited using different reagents and effects on meningococcal processing observed.

2LMP19c is a murine anti-human mAb specific to an epitope found on the I-domain of CD11b subunit (Cywes, Godenir et al. 1996), a binding site of opsonic complement fragments such as C3b and iC3b. Studies carried out by Peyron and colleagues (Peyron, Bordier et al. 2000) also found that 2LMP19c mAb inhibited non-opsonic Mycobacterial internalisation by neutrophils, presumably by blocking the lectin site to which *Mycobacteria* are known to bind directly. A number of monoclonal antibodies known to be specific to the I-domain also block the lectin site. MDM were treated with 2LMP19c, before cells were exposed to *N. meningitidis* H44/76 in the presence/absence of serum opsonins.

Macrophages were incubated with 2.5µg/ml of 2LMP19c, isotype control IgG1, or culture media as a control for 30min at 37°C, cells were then washed thoroughly to remove excess reagent. Mid-log phase *N. meningitidis* H44/76 were used to infect cells at MOI of 50:1. Non-opsonised *N. meningitidis* H44/76 were incubated in the presence of antibody for all 37°C incubations. Fig. 5.4 shows 2LMP19c-treated cells incubated with bacteria for 1h at 4°C. No

significant difference between adherence of non-opsonised *N. meningitidis* H44/76 by pre-blocked MDM were observed (Fig 5.4); nor was any difference apparent at a range of concentrations (data not shown).

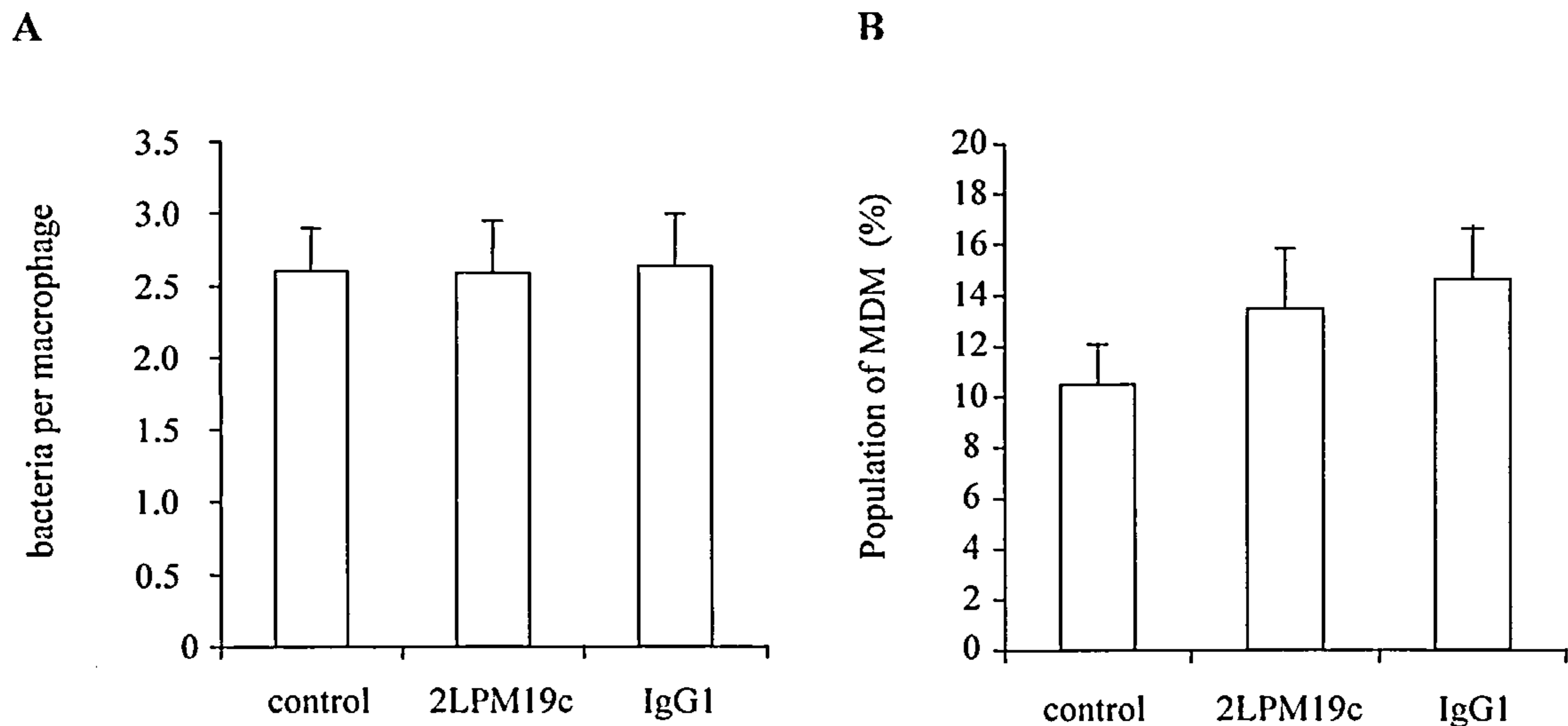


Figure 5.4

CD11b I-domain blockade

Anti-CD11b/18 mAb 2LMP19c was used to block macrophages before inoculating with non-opsonised H44/76, incubating for 60min at 4°C. (A) Bacteria associated per macrophage (B) Percentage of macrophages with adherent bacteria on surface of cell. Results based on macrophages from four different donors, with each experiment done in triplicate. No significant difference was observed between antibody-treated and control macrophages using Mann-Whitney U test. ($P>0.05$)

5.2.2.2 Macrophages do not internalise opsonised *N. meningitidis* via CR3

Previous observations in Chapter 1 indicated that non-Fcγ macrophage receptors were involved in the opsonin-dependant internalisation of *N. meningitidis*, therefore the I-domain of CD11b was blocked using 2LMP19c monoclonal antibody. The I-domain is a known binding site of complement fragments such as iC3b and C3b. Macrophages were incubated with bacteria at 4°C and 37°C to ascertain if a direct relationship between opsonised *N. meningitidis* H44/76 and macrophage CR3 exists.

Fig. 5.5 shows that no reduction was observed in serum opsonised bacterial adherence or internalisation of 2LMP19c-treated cells when compared with untreated control cells ($P>0.05$). The only significant effect of anti-CD11b mAb under opsonic conditions occurred after 30min incubation at 37°C, when the percentage of macrophages with adherent or internalised bacteria (ie. DAPI positive organisms) notably fell by 32.98%. Although by 60min MDM appeared to return to levels comparable to untreated control levels.

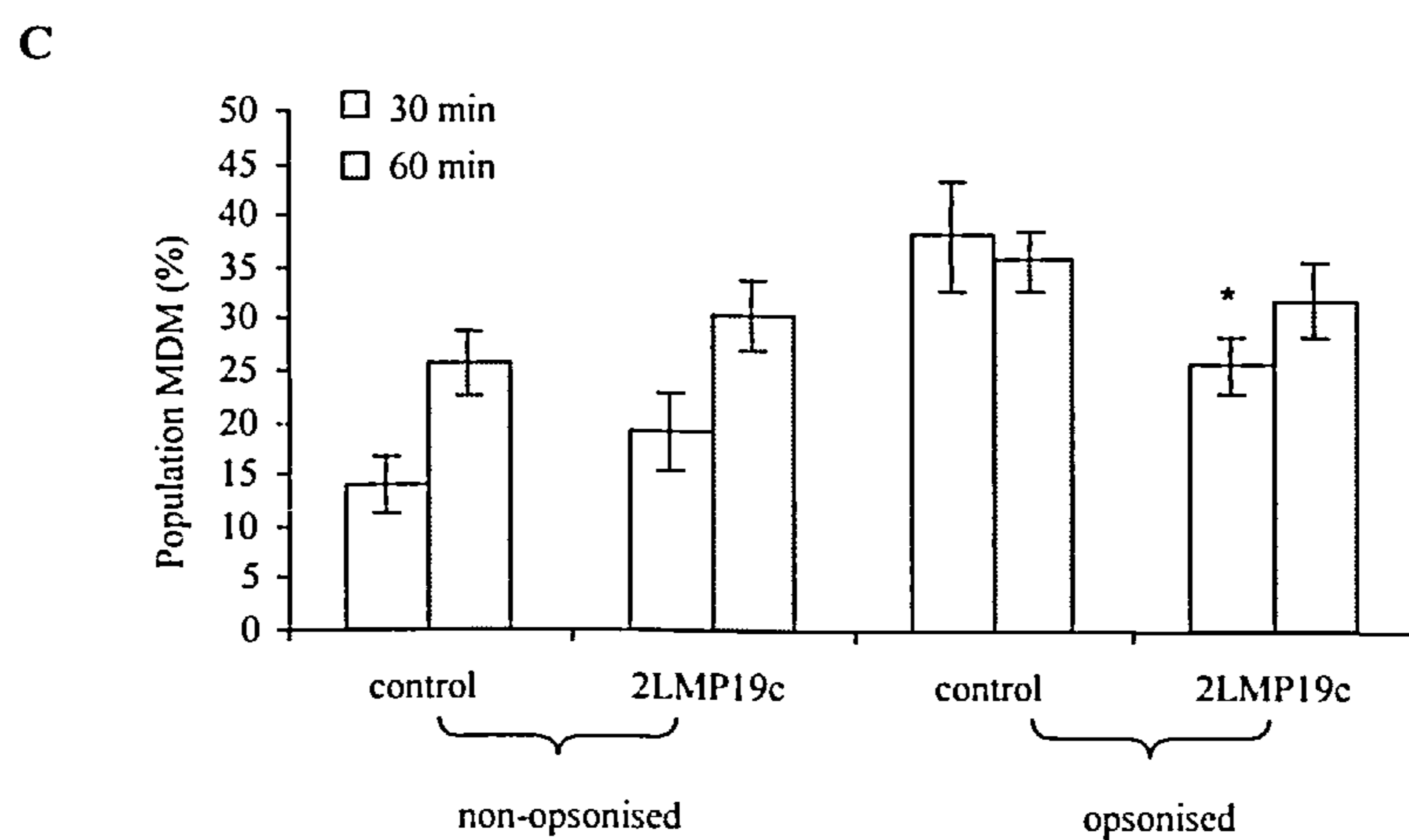
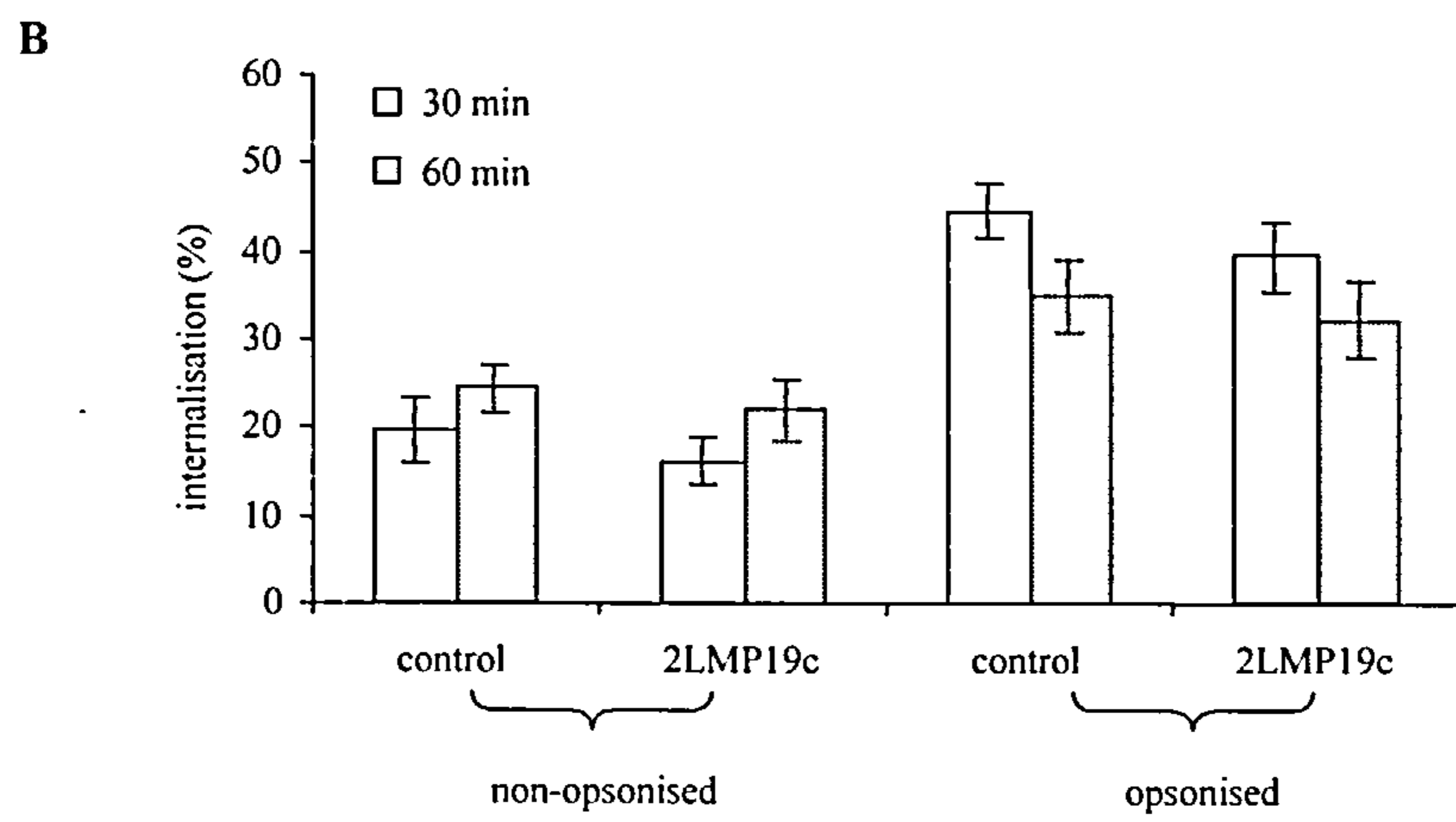
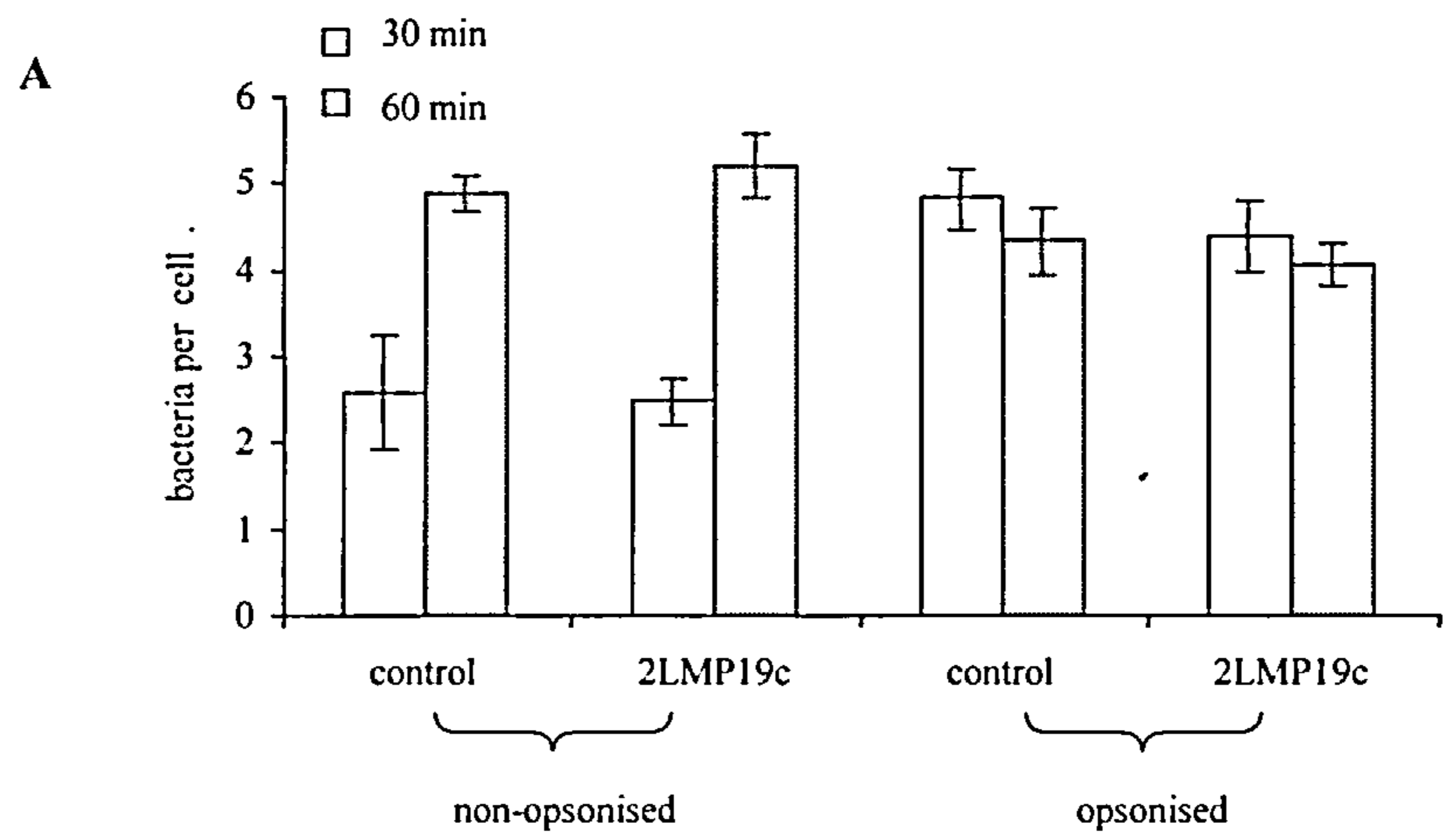
5.2.2.3 *N*-acetyl-D-glucosamine (NADG) treatment of lectin site of macrophage CR3

The lectin-binding site of CD11b is divalent cation independent and can be blocked by competing certain polysaccharides, including NADG, glucose and mannose all of which can bind to the lectin site (Thornton, Vetvicka et al. 1996). A number of integrins and GPI-anchored receptors associate with one another, as

Figure 5.5

CD11b I-domain blockade in presence of immune serum

Bacterial uptake examined by treating MDM with anti-CD11b mAb 2LMP19c for 15min at 37°C before inoculating with *N. meningitidis* H44/76 in the presence/absence of serum opsonins for 60 min at 37C. Control MDM were not treated with antibody. Graphs represent (A) bacteria per cell (B) Percentage of bound organisms internalised (C) Percentage population of macrophages interacting with bacteria. Data obtained from three donors with each experiment performed at least three times. Values represented as mean (\pm standard error of mean). Statistical significance calculated by Mann Whitney U test and denoted by * $P < 0.05$.



GPI proteins, such as CD14, tend to be heavily glycosylated and previous studies using neutrophils have found NADG disrupts these interactions by competing for the binding site, and consequently preventing bacterial interactions on this region of the receptor (Zhou, van der Winkel et al. 1993; Galon, Gauchat et al. 1996). Work using NADG to block interactions between *Mycobacteria* strains and neutrophils found that the reagent appeared to disrupt the formation of CD14-CR3 complexes and therefore inhibit bacterial association to the neutrophils (Peyron, Bordier et al. 2000). Such complexes are also known to form on macrophages, but no evidence of meningococcal involvement with CD14-CR3 has been published. Therefore, part of the non-opsonic studies of CR3 in this chapter involved the use of NADG at varying concentrations over time. MDM were incubated in the presence of NADG for 15min before infecting with *N. meningitidis* H44/76. Experiments were performed using at least three donors and each experiment repeated in triplicate. Fig. 5.6 shows that no difference was observed in the binding or internalisation of *N.meningitidis* H44/76 to control cells or NADG-treated cells under non-opsonic conditions.

5.2.2.4 Inhibition of RGD-integrins does not influence interaction of *N. meningitidis* with macrophages

Human MDM were treated with RGDS-containing peptides for 30min at 37°C prior to infection with *N. meningitidis* H44/76 at MOI of 50. MDM were incubated with bacteria at 4°C (Fig. 5.7) and 37°C (data not shown) for 1h and no significant difference was found then compared with control cells (RGES-treated and untreated MDM).

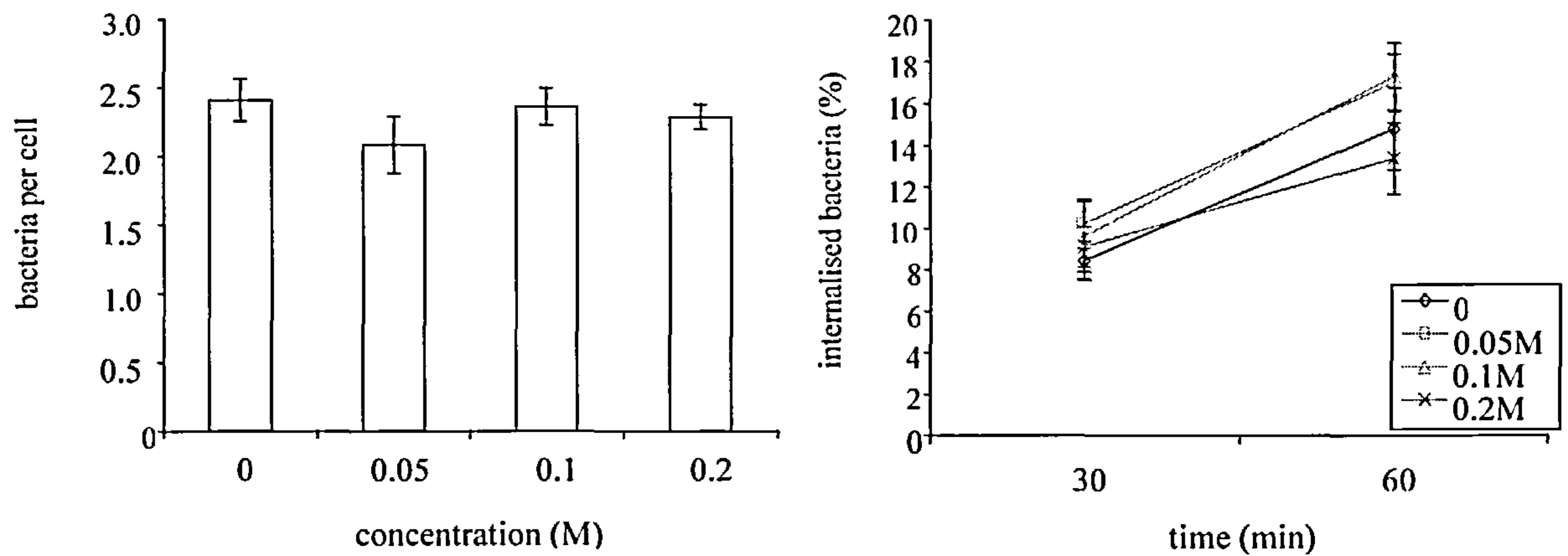


Figure 5.6

Polysaccharide binding site blockade using NADG

MDM incubated with a range of concentrations of N-acetyl D-glucosamine (NADG) before infecting with non-opsonised *N.meningitidis* H44/76 and incubating for 60min at 4C or 37°C. Graphs show (A) Numbers of adherent bacteria per cell after incubation at 4°C (B) Internalisation following 37 °C incubation. Graphs Data obtained from three donors and each experiment repeated at least three times. No significant difference in binding or uptake of meningococci using Mann-Whitney U test ($P>0.05$).

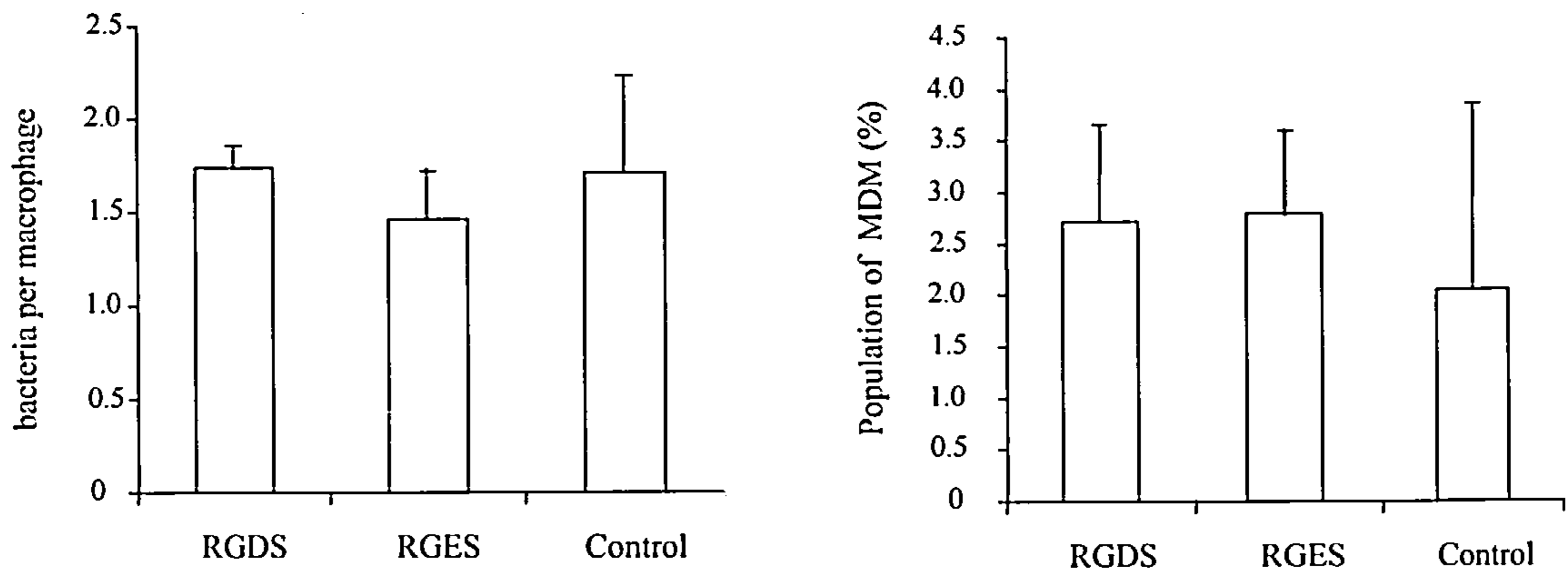


Figure 5.7

Inhibition of RGD-containing binding sites

Integrin binding sites were blocked using RGDS-containing peptides (RGES used as control) before exposure to H44/76 in the absence of immune serum. (A) bacterial association (B) Percentage of macrophages with bacteria associated. Each value is mean (\pm standard error of mean) from four donors, each experiment done in triplicate. No difference shown under these conditions using Mann-Whitney U test ($P > 0.05$).

Part III: Lipid rafts

5.2.3 Role of macrophage lipid rafts in trafficking of *N. meningitidis*

5.2.3.1 Cholesterol depletion of macrophages has no effect on bacterial phagocytosis

Cholesterol-rich lipid rafts are used as a portal of entry by a number of organisms, and studies have attributed this trend to the high density of molecules, such as GPI-proteins, caveolin and heterotrimeric G-proteins, that tend to cluster at these sites (Brown and London 1998).

Integrins are not only in high concentration, but are also in close proximity to signalling molecules on these specialised regions of the cell. Lipid rafts are areas on the plasma membrane that have more highly ordered lipids and are rich in sphingolipids and cholesterol. Chemical disruption of these rafts may give some insight into the preferred sites of entry of *N. meningitidis*, as we have observed in previous chapters that both CD14 and CR3 are involved, in varying degrees, with macrophage recognition of meningococci. The only opsonic receptor so far reported to cluster on lipid rafts is CR3, therefore another way of studying the possible relationship between CD14 and CR3 involved treating MDM with a cholesterol-scavenging antibiotic, nystatin, and cholesterol-depleting agent, β -cyclodextrin, both of which disrupt lipid rafts structure which is dependent on the presence of cholesterol.

Cells were treated with 25 μ g/ml of Nystatin or β -cyclodextrin for 15 min at 37°C before incubating with *N. meningitidis* H44/76 and analysing by fluorescent microscopy or flow cytometry. Both opsonised and non-opsonised organisms were studied. The observations in the studies described in this chapter using an

anti-CR3 monoclonal antibody suggested that neither opsonised nor non-opsonised meningococci were internalised via CR3

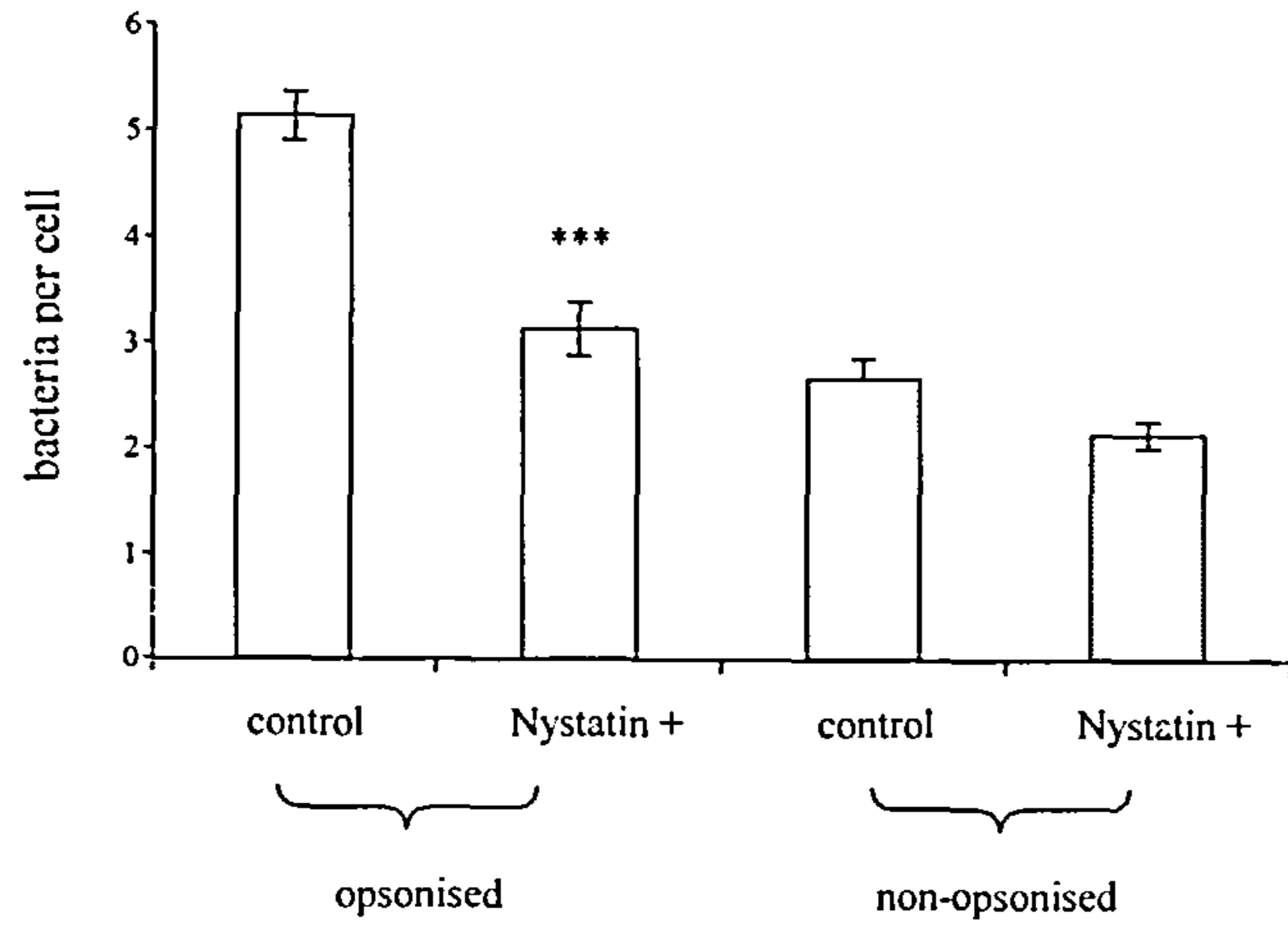
Interestingly Fig. 5.8 demonstrates that following Nystatin treatment of MDM, opsonised meningococci adhered 38.91% less efficiently than untreated control cells ($P < 0.001$), and the population of phagocytic macrophages increased by 22.67% ($P < 0.05$). Internalisation of organisms remained the same between untreated control and cholesterol-depleted cells to macrophages at 37°C. No differences were observed in the adherence or internalisation of non-opsonised meningococci by Nystatin-treated cells. This suggests that receptors that recognise opsonised *N. meningitidis* H44/76 may also be found in these lipid-rich domains. The numbers of organisms internalised have not been affected by treatment of the cells with Nystatin, but there appears to be an increase in the number of macrophages with less meningococci associated on their surface. It would seem that cholesterol-depletion increases the number of opsonic receptors available for binding meningococci, but fewer organisms actually bind to each macrophage (see discussion).

Figure 5.8

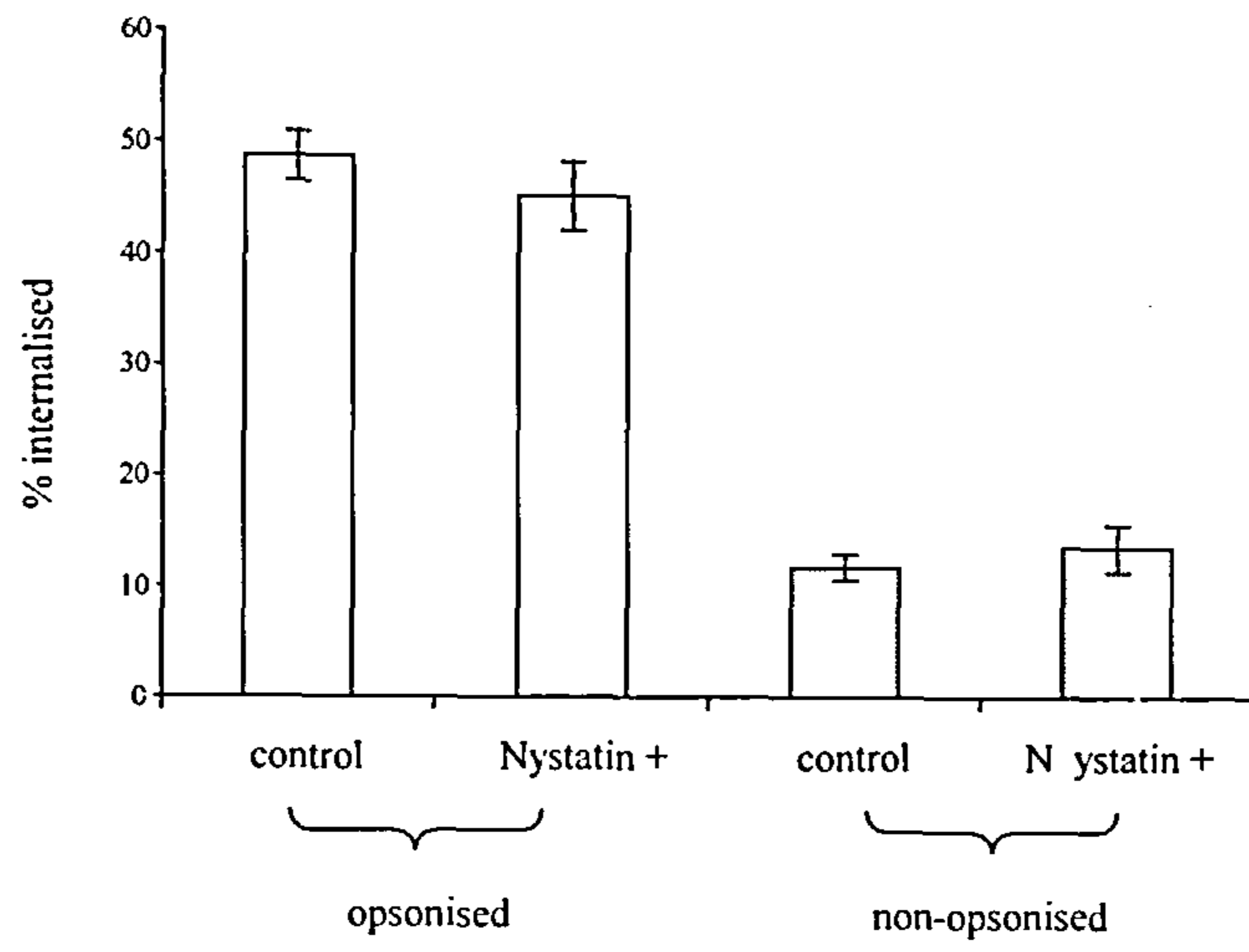
Chemical disruption of lipid rafts using Nystatin

Bacterial uptake examined by treating MDM with cholesterol-depleting agent Nystatin for 15min at 37°C before inoculating with *N. meningitidis* H44/76 in the presence/absence of serum opsonins at 37°C. Control MDM were not treated with Nystatin. Graphs represent (A) bacteria per cell (B) Percentage of bound organisms internalised (C) Percentage population of macrophages interacting with bacteria. Data obtained from four donors with each experiment performed at least three times. Values represented as mean (\pm standard error of mean). Statistical significance calculated by Mann Whitney U test and denoted by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when comparing Nystatin-treated cells with control cells under each condition.

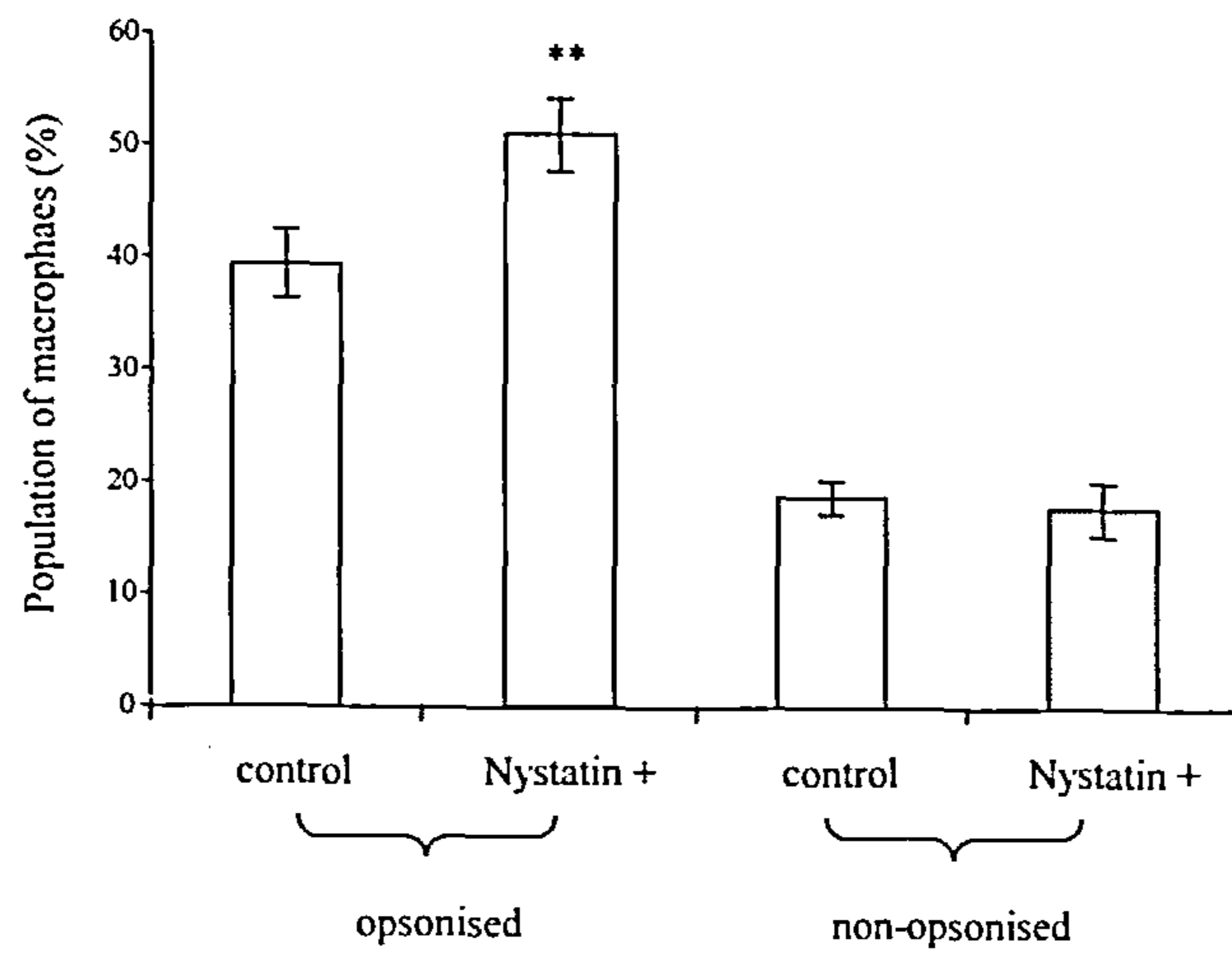
A



B



C



5.3 Summary and discussion

Lipid rafts comprise dynamic assemblies of cholesterol and sphingolipids in plasma and endosomal membrane of eukaryotic cells (Simons and Ikonen 1997; Brown and London 1998; Brown and London 1998). Rafts were originally reported to explain the lipid-based sorting properties of cell membranes, and were therefore implicated in cell membrane cholesterol-based micro-heterogeneity (Simmons and van Meer 1988). GPI-anchored receptors present on the surface of macrophages and neutrophils have been found to aggregate on distinct cholesterol-enriched lipid rafts, which appear to have a number of functions including signal transduction, cholesterol trafficking, endocytosis and transcytosis across epithelial cells (Anderson 1998; Okamoto, Schlegel et al. 1998). CR3 and GPI-anchored proteins, including CD14, were examined together in this chapter as previous studies have observed that CD14 aggregate on lipid rafts and on activation CR3 also appear to translocate to lipid rafts (Petty and Todd 1996).

Rafts are of current interest as they are unique micro-environments enriched with caveolae, signal transducing molecules and appear to be used by pathogenic organisms as a site of entry into cells. Organisms include *C. jejuni*, *L. pneumophila* (Watarai, Derre et al. 2001), *Plasmodium falciparum*, as well as LPS and *Mycobacterium bovis* BCG (Gatfield and Pieters 2000) where cholesterol aggregates at the site of bacterial entry and cholesterol depletion inhibits *M. bovis* uptake. Selective lipid recruitment has also been observed during HIV-1 or influenza virus budding from mammalian cells (Scheiffele, Rietveld et al. 1999; Ono and Freed 2001).

Nystatin or β CD treatment depletes cholesterol from membranes causing degradation of lipid rafts, dissociation of proteins attached and disappearance of caveolae from rafts. Nystatin treatment of macrophages before exposure to non-opsonised meningococci had no significant affect on macrophage-neisserial

interactions, but in the presence of immune serum bacterial adherence was impaired, suggesting that the presence of membrane rafts does appear to contribute significantly to the recognition of opsonised *N. meningitidis* H44/76 by macrophages. Cholesterol-depletion increases the number of opsonic receptors available for binding meningococci, but fewer organisms actually bind to each macrophage. This may be due to Nystatin treatment resulting in release of receptors from these rafts, and their dispersal on the macrophage surface, making more available.

Importance of these specialised microdomains is highlighted by the role of lipid rafts in signal transduction (Anderson 1998; Okamoto, Schlegel et al. 1998), and cross-linking of signalling receptors increases their affinity for rafts. A new microenvironment results from the partitioning of receptors into lipid rafts, where their state of phosphorylation can be adjusted by local kinases and phosphatases. Such compartmentalised signalling has been observed when antibodies or other ligands cross-link GPI-anchored proteins present in lipid rafts. Clustering of several lipid rafts may result in amplifying signal, and consequently affect signal transduction. Transmembrane and GPI-linked proteins present on rafts are solubilised and vesicles of rafts are released from the cell. The problem with the use of cholesterol-depleting or -scavenging reagents that disrupt the native structure of lipid rafts is that they are fairly crude tools as they are not specifically targeted to cholesterol present in lipid rafts alone, but will also deplete cholesterol from non-raft membrane. Results are also dependent on different modes of action, i.e. Cholesterol binding agents compared with those that inhibit cholesterol synthesis by the cell. Much more information on the properties is needed to understand the roles of lipid rafts in different cells. Little is known about the size, heterogeneity, dynamics or composition of lipid rafts, but it is apparent that a number of different types of lipid rafts can exist on a single cell distinguished by their lipid and protein compositions. Separation of lipid rafts from cell membrane can be done by density gradient ultracentrifugation of detergent-solubilised membranes, and will produce a mixture of

different rafts that cannot be differentiated from one another; and it is likely that heavier rafts with higher lipid content will evade detection using these methods.

PIPLC enzyme was used to cleave GPI-anchored proteins from the surface of the cell, consequently bacterial internalisation and recognition by macrophages was markedly impaired. As a prominent GPI-anchored receptor that recognises gram-negative bacteria, CD14 receptors were then blocked using a monoclonal antibody (MY4), which again resulted in a significant decrease in meningococcal interactions with macrophages. As PIPLC and MY4 treatments reduce the availability of mCD14 receptors to meningococci, the contribution of alternative macrophage receptors able to associate and internalise *N. meningitidis* H44/76 may become more prominent as neither treatment resulted in complete abolition of adherence or phagocytosis. These observations may be due to these alternative receptors binding *N.meningitidis*, but not internalising them effectively. CD14-independent uptake therefore appears to be less efficient than CD14-dependent uptake. This suggests that GPI-anchored receptors, and specifically CD14, have a role in trafficking of *N. meningitidis* H44/76 by human macrophages. This was subsequently confirmed using CD14-transfected HeLa cells, and I found that bacterial adherence was enhanced in the presence of CD14. HeLa tranfects were a useful tool in attempting to isolate CD14 from a number of other macrophage receptors, and support the hypothesis that this receptor plays a significant role in non-opsonic meningococcal recognition. Previous studies have suggested that the effect of anti-CD14 monoclonal antibodies, such as MY4, may in fact be a result of the antibody binding directly to the lectin binding site of CR3, thus inhibiting binding of CD14 or bacteria to CR3, rather than blocking CD14 directly.

NADG has been used in previous studies to prevent CR3-GPI protein interactions or microbial binding to CR3 by occupying the lectin site, but this reagent appeared to have no significant effect on meningococcal recognition by macrophages, and may be due to mBSA binding to CR3 lectin site or

alternatively other serum components may can bind NADG. The anti-CD11b monoclonal antibody 2LMP19c is directed specifically at an epitope of the I-domain, which is a known binding region of complement components such as C3b and iC3b. MDM cells underwent treatment with this antibody but in the presence and absence of serum opsonins, no effect on adherence or phagocytosis of meningococci was apparent: except the observation that the percentage population of cells able to interact with serum opsonised organisms declined. This decrease at 30min following anti-I domain treatment was quickly recovered by 60min, and may have been due to an up-regulation in receptor expression by 60min, or perhaps alternative receptors playing a more prominent role in the absence of CD11b. It was also important to perform similar experiments in the absence of serum, as a number of monoclonal antibodies also directed specifically at the I-domain have been found to block lectin site activity, despite the fact that this binding site is located at the C-terminus; these include LM2/1, OKM1 and M1/70 (Xia and Ross 1998; Xia, Vetvicka et al. 1999). Even though this antibody was bought in good faith, it may be that 2LMP19c was not very effective due to a problem with antibody production by the company, consequently it is possible that CR3 receptors may not have all been blocked.

As neither CD14 nor CR3 appear to internalise *N. meningitidis*, alternative phagocytic receptors were examined in the following chapter, namely Class A scavenger receptors, mannose receptor and PAF receptors.

Chapter 6

Non-opsonic receptor recognition of *N. meningitidis*

Part I: Class A Scavenger receptor

6.1.1 Introduction

Work published by Hampton and colleagues (Hampton, Golenbock et al. 1991) first implicated the Class A scavenger receptor in lipid A binding, and it is now considered a prominent receptor on human macrophages that is able to directly bind an extensive range of ligands. Although the cytoplasmic tail of SR-A contains potential protein kinase C (PKC)-interaction sites, it lacks any known motifs associated with phagocytosis and yet a number of both gram-negative and gram-positive bacteria are internalised via SR-A, including *E. coli*, *L. monocytogenes*, *S. pyogenes*, *S. aureus* and *M. tuberculosis* (Suzuki, Kurihara et al. 1997; Peiser, Gough et al. 2000; Thomas, Li et al. 2000).

Direct interaction with LPS normally results in macrophage activation, and the initiation of a range of intracellular signalling events and cellular responses including production of nitric oxide and pro-inflammatory cytokines such as TNF- α and IL-6. A significant amount of evidence suggests that scavenger receptors play a role in the down-regulation of these inflammatory responses (van_Lenten and Fogelman 1992; Yang, Galeano et al. 1996; Matsuno, Aramaki et al. 1997), and the suppression of cellular responses that traditionally accompany macrophage activation, including transcriptional activation of NF κ B, fluxes in intracellular Ca²⁺ concentrations and hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP2) (Schackelford, Misra et al. 1995).

The presence of SR-A on the surface of macrophages has therefore been associated with the protection of the host against endotoxic shock (Haworth, Platt et al. 1997).

The use of SR-A^{-/-} mice, rat anti-murine monoclonal antibody (2F8) and transfection using both murine SR-A isoforms (types I and II) has been the source of most microbe-SR-A relationship studies to date. Peiser and colleagues recently observed that murine macrophages recognise a range of gram-negative bacteria including *N. meningitidis* via SR-A, but to date little evidence of meningococcal processing through SR-A in human cells has been observed. The lack of available blocking antibodies to human SR-A meant that an alternative reagent was sought. Polyinosinic acid (Poly I), a known SR-A ligand, binds to a lysine cluster present on the collagenous domain which acts as an LPS binding site (Yamamoto, Nishimura et al. 1997) and is effective in blocking ligand binding to this site. In order to establish that Poly I does indeed block the SRA ligand-binding site, I conducted a series of flow cytometry experiments using DiI-labelled acLDL, a fluorescently-labelled modified low-density lipoprotein that is bound and endocytosed specifically through SR-A. No distinction between the two SR-A isoforms can be made using DiI-labelled acLDL .

As discussed in previous chapters, differentiation of THP-1 using the phorbol ester, PMA (phorbol myristate acetate) provokes the maturation of the cell into a more macrophage-like cell and induces the expression of a range of surface membrane receptors associated with terminally differentiated cells, including SR-A. PMA-treated THP-1 cells were the source of the first cDNA clones for human SR-AI and SR-AII (Matsumoto, Naito et al. 1990).

Following on from Chapter 4, this chapter continues the search for possible non-opsonic macrophage receptors utilized by serogroup B *N. meningitidis*. No previous studies have demonstrated that human macrophages phagocytose serogroup B *N. meningitidis* through SR-A. Previous results reported in this

thesis indicate that the individual blockade of a number of CR3 binding sites did not impair non-opsonic meningococcal uptake; and CD14 is involved in meningococcal uptake (see Chapter 5). I therefore incubated *N. meningitidis* H44/76 with Poly I-treated cells in order to establish whether SR-A plays any role in the adherence and phagocytosis of the organisms in human macrophages.

Individually inhibiting binding to specific receptors offered some interesting results, in particular with respect to CD14 and SRA involvement. Complimentary experiments were also carried out, where blocking reagents and antibodies were used to simultaneously block two or more receptors in order to give more insight into which non-blocked receptor(s) the organisms might utilise in the absence of others. CD14, SRA and CR3 were all blocked. Two of these receptors appear to have a role in meningococcal trafficking in this study, and CR3, as a phagocytic receptor favoured by a number of pathogenic microbes, has been observed here to be involved in non-immunoglobulin-based interactions.

6.1.2 Results

6.1.2.1 Poly I blocks SR-A ligand uptake

1×10^6 MDM were cultured in 24-well tissue culture plates as described earlier, and incubated in the presence of 50 μ g/ml Poly I for 30min at 37°C. Wells were then washed three times and media replaced with RPMI 1640, 2mM glutamine, 10% HI FCS (heat-inactivated foetal calf serum) and 50 μ g/ml Poly I, to which DiI-labelled acLDL (DiI-acLDL) was added to the Poly I treated cells. Cells were incubated at 37°C, 5% CO₂ for 2h and prepared for flow cytometry analysis (see Methods). MDM were checked for viability using a trypan blue exclusion assay. This assay demonstrated that Poly I was an effective SR-A blocking agent, as DiI-acLDL uptake of MDM was almost completely abolished following Poly I treatment, and the non-SRA ligand control reagent Poly C had no such affect (Fig. 6.1). This assay did not distinguish between binding and uptake of acLDL, but was used as a general marker of Poly I effectiveness.

6.1.2.2 *Neisseria meningitidis* do not bind to human macrophages via Scavenger Receptor A

Role of scavenger receptors in the direct binding of *N. meningitidis* H44/76 to the surface of MDM cells under non-opsonic conditions was examined. Poly I-treated MDM were incubated with *N. meningitidis* H44/76 for 1h at 4°C, and Fig. 6.2A demonstrates that no difference was observed in the number of bacteria adherent to each cell or in the percentage of macrophages with bacteria associated (Fig. 6.2C).

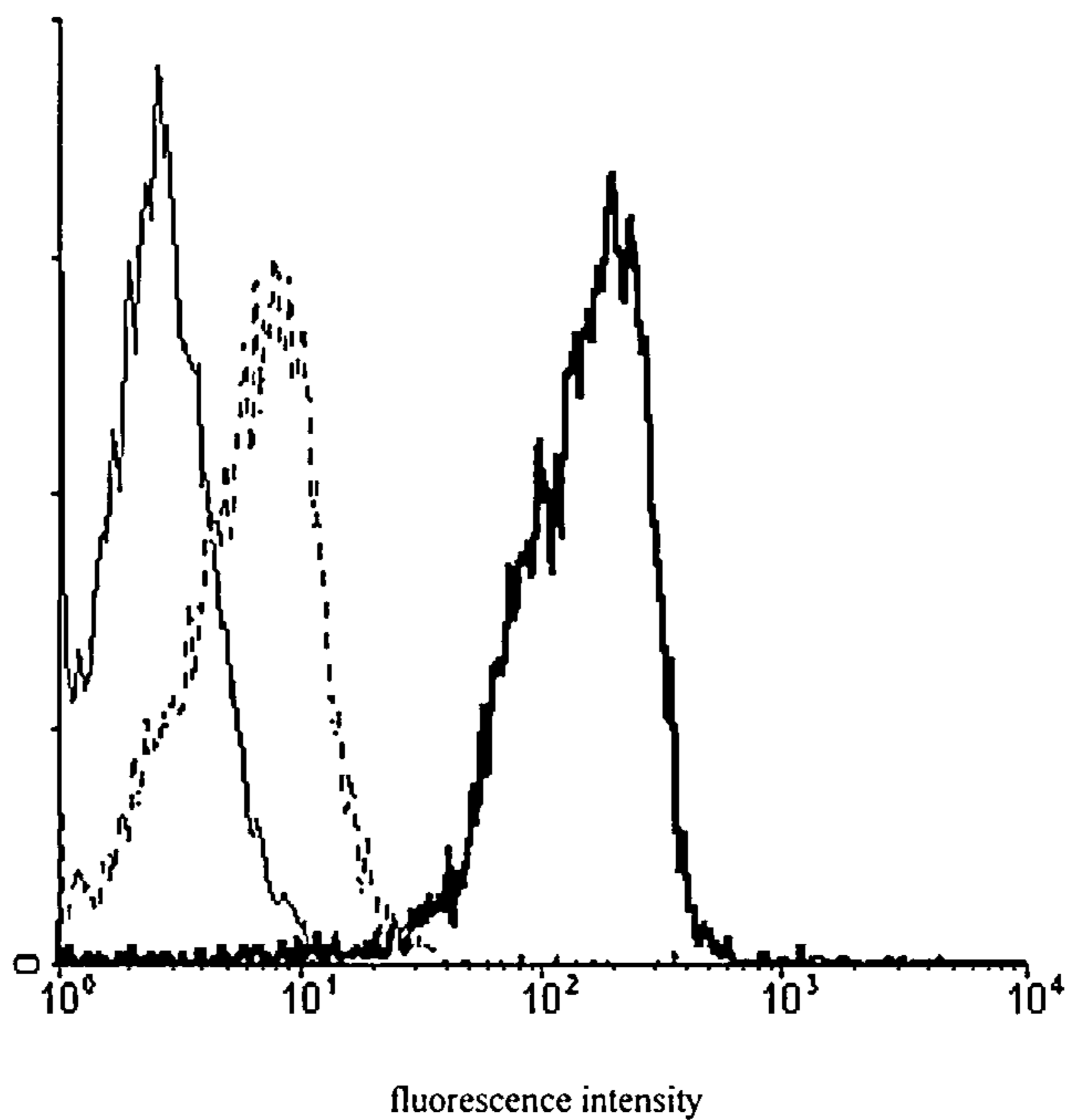


Figure 6.1

Poly I inhibition of acLDL uptake

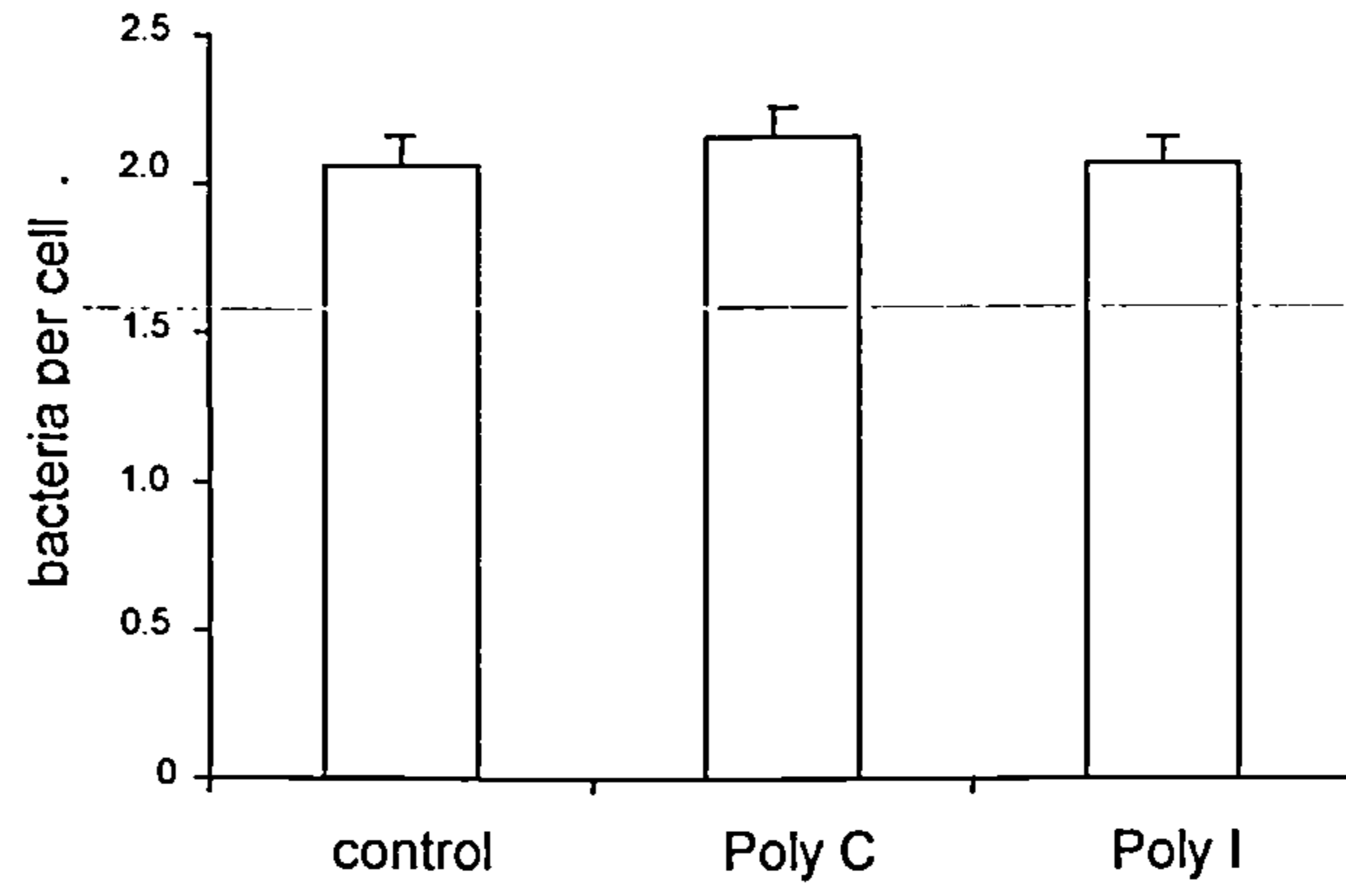
MDM were incubated with Poly I before incubating with DiI-labelled acLDL (5ug/ml) for 120min at 37°C, before FACS analysis. Filled line represents DiI-acLDL fluorescence of control cells that were not treated with Poly I, dark line represents control DiI-acLDL fluorescence and unfilled line indicates DiI-acLDL fluorescence by Poly I-treated MDM. Experiments was performed in three human donors and repeated three times. Figure represents one experiment done in triplicate

Figure 6.2

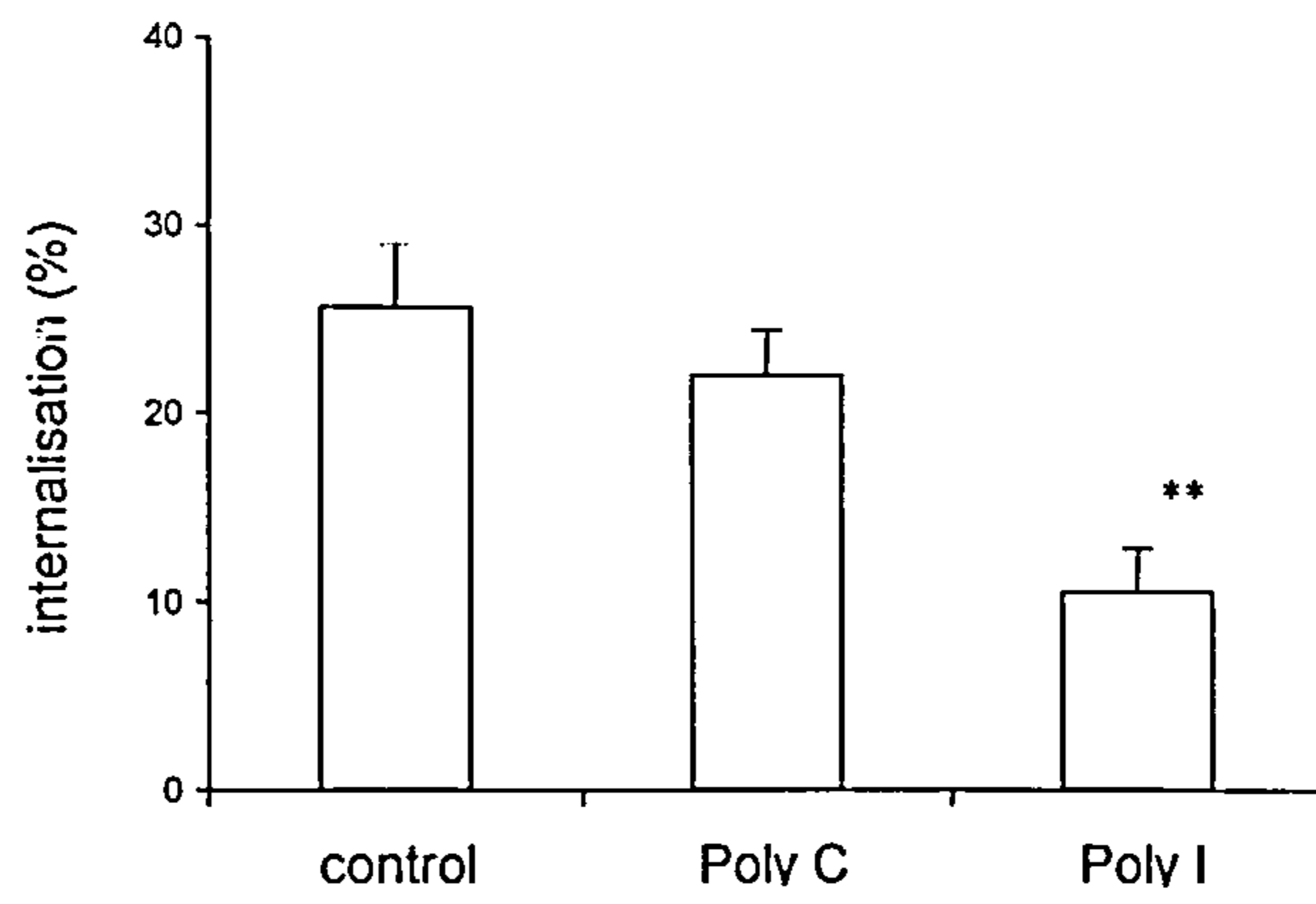
Phagocytosis inhibited by scavenger receptor blockade

MDM were pre-treated with Poly I and inoculated with *N. meningitidis* H44/76 strain (control cells were untreated MDM) and incubating at 4°C for 60min. Unbound organisms were washed off and cells underwent further incubation for 30min at 37°C. (A) Ratio of adherent bacteria per macrophage after cold incubation (B) Population of MDM with bacteria bound or internalised after both cold and 37°C incubation (C) Percentage of bacteria internalised by MDM after incubation 4C and 30min at 37C. Results from six donors, values represent mean (\pm standard mean of error) and significance is denoted by * $P < 0.05$, ** $P < 0.01$ calculated by Mann Whitney U test.

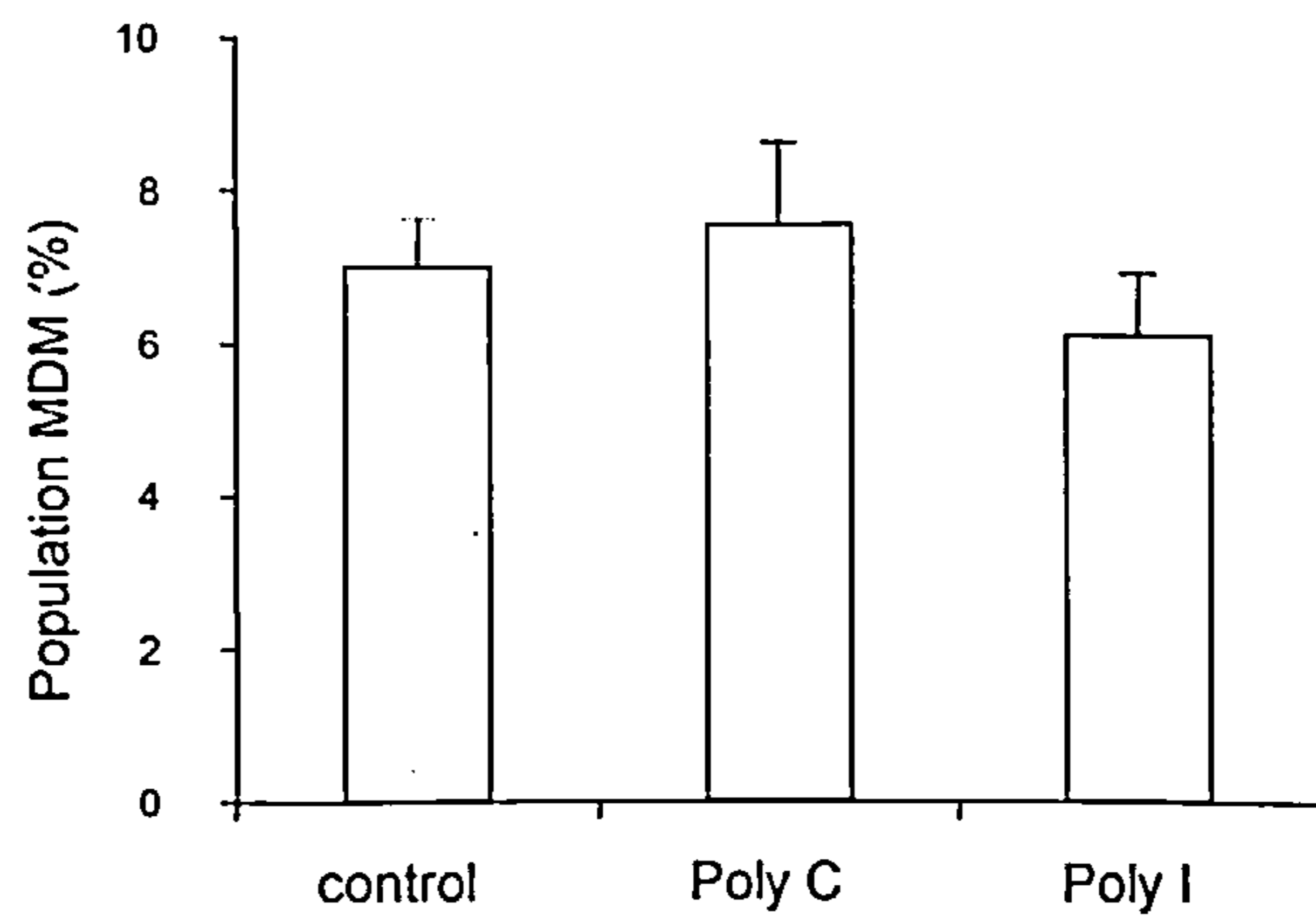
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6.1.2.3 Class A Scavenger Receptor enhances phagocytosis of *Neisseria meningitidis*

MDM were incubated with 50µg/ml Poly I or non-SR-A ligand Poly C or untreated cells for 30 minutes at 37°C before inoculating with *N. meningitidis* H44/76 for one hour at 4°C to ensure binding was equal. Wells were then washed of any unbound organisms and incubated at 37°C for a further 30 minutes, and analysed using fluorescent microscopy and 200 cells were counted per well. The internalisation of *N. meningitidis* H44/76 by human MDM in the presence of the scavenger receptor inhibitor Poly I was reduced by 58% (P<0.01) when compared to untreated control cells(Fig 6.2B).

6.1.2.4 Poly I influences uptake when cold step is omitted

In order to establish whether the effect of Poly I, as described in 6.1.2.3, was temperature dependent, meningococcal response to Poly I-treated MDM were performed at 37°C, without the 4°C step. After 30min incubation with *N. meningitidis* H44/76 no difference in adherence was observed. By 60min there was a significant drop in the association, internalisation and recognition of meningococci by Poly I-blocked macrophages (Fig. 6.3).

6.1.2.5 PMA-differentiated THP-1 internalised *N. meningitidis* more effectively

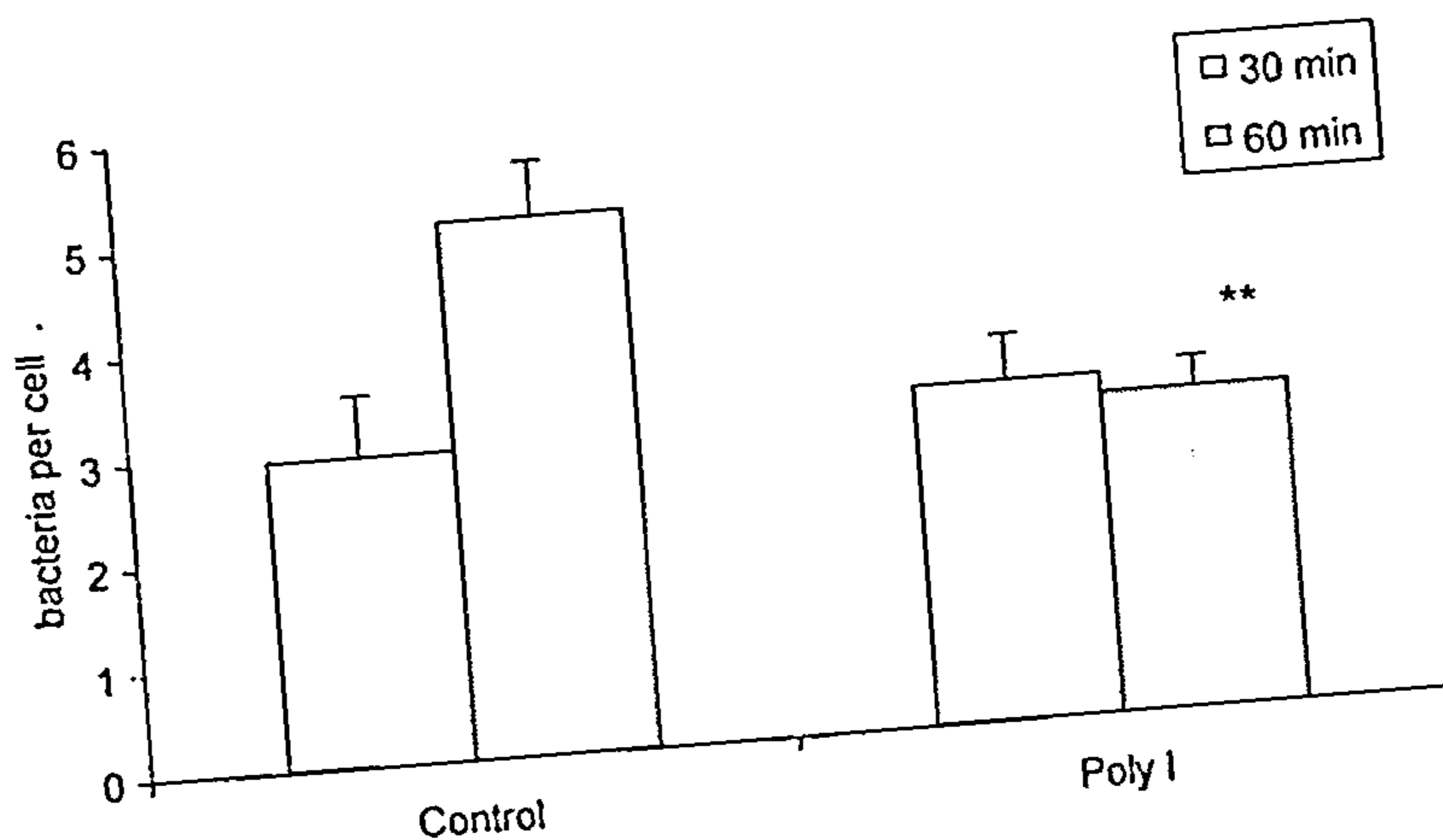
Previous results (see Chapter 3) have shown that undifferentiated THP-1 cells do not internalise *N. meningitidis* as effectively as human macrophages, which may, in part, be due to the absence of SR-A. As human SR-A monoclonal antibodies were not available, the THP-1 monocytic cells were incubated with PMA in order

Figure 6.3

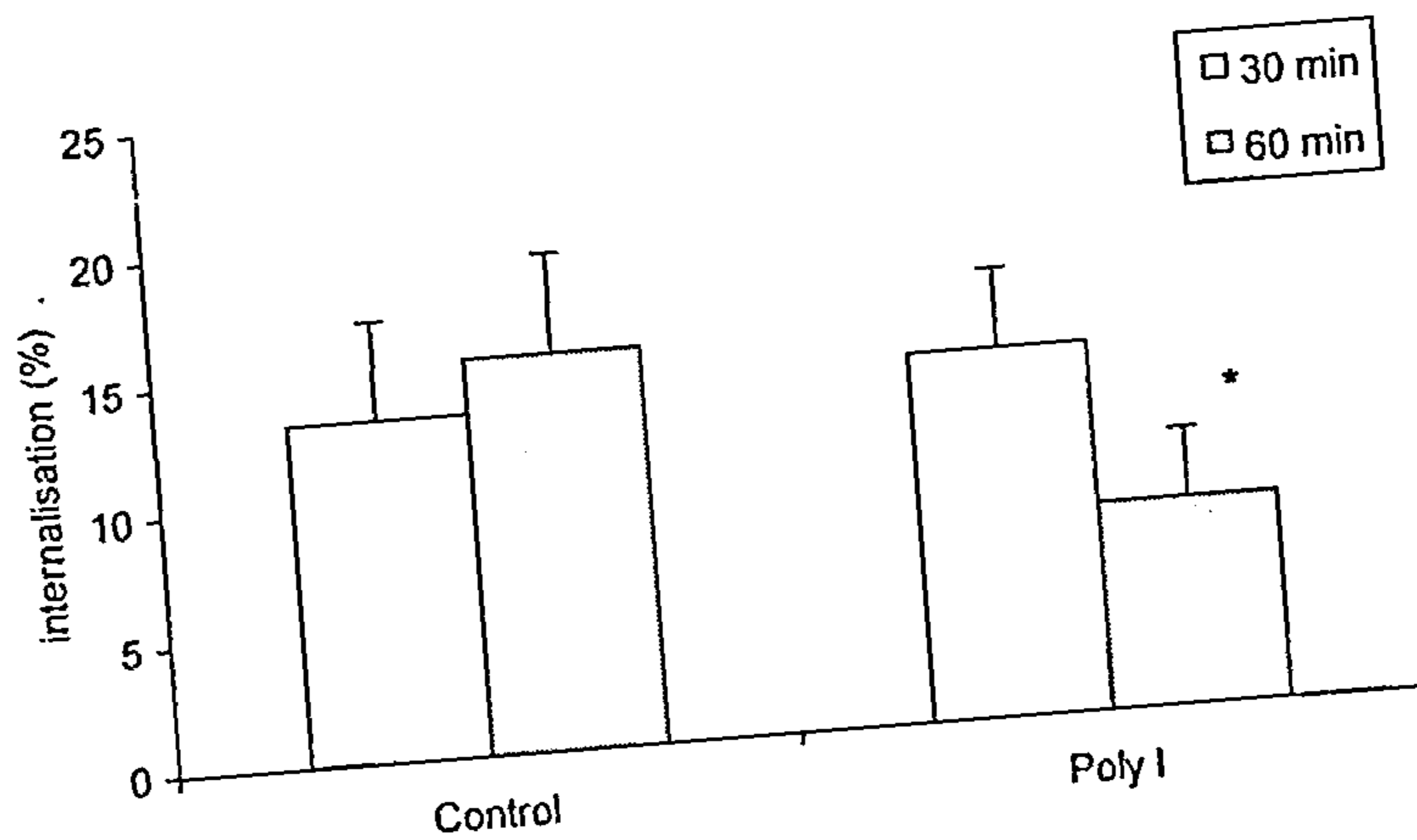
Class A Scavenger receptors and MDM

Monocyte-derived macrophage cells were treated with Poly I before incubating for 60min at 37°C with *N. meningitidis* H44/76, in the presence of Poly I. Graphs represent (A) Ratio of bacteria per cell (B) Percentage of phagocytosis (C) Population of cells interacting with bacteria. Results were obtained from four separate donors, repeated at least three times. Values represent mean (\pm standard error of mean). Statistical significance indicated by * $P < 0.05$, ** $P < 0.01$ using Mann Whitney U test comparing Poly I-treated cells with control cells at each time point.

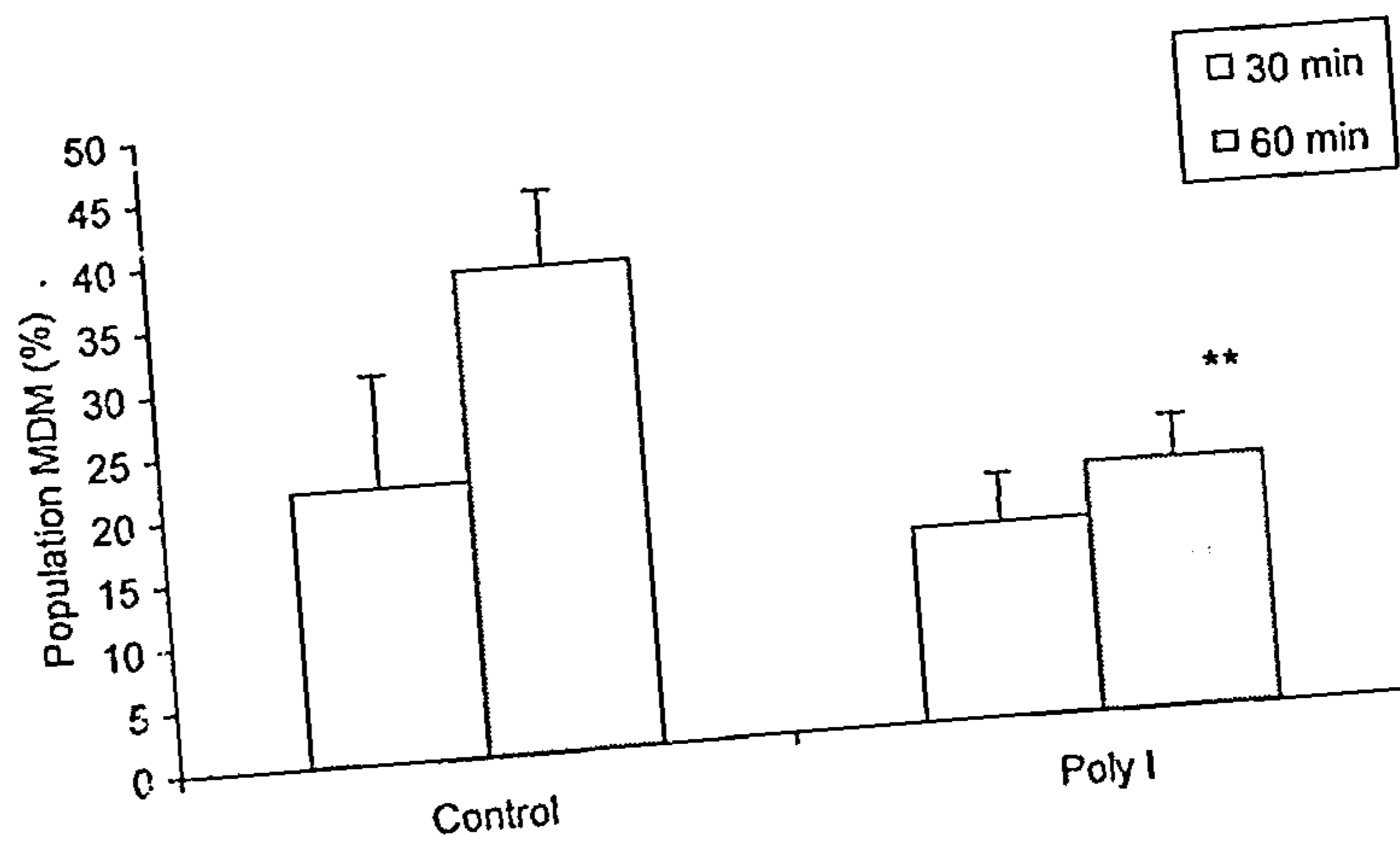
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to up-regulate SR expression. dTHP-1 cells were then treated with/without Poly I and inoculated with *N. meningitidis* H44/76 in order to examine whether dTHP-1 cells expressed functional macrophage SR-A capable of internalising bacteria. This was tested by analysing whether treatment with Poly I would impair meningococcal uptake by dTHP-1, as was observed using primary human macrophages.

Comparisons of Fig. 6.3 and Fig. 6.4 shows that PMA stimulation and therefore up-regulation in receptor expression, conferred in monocytes the capacity to internalise *N. meningitidis* more effectively than undifferentiated THP-1 cells, to levels quite comparable to MDM cells; and Poly I treatment of these differentiated cells resulted in a 31.4% loss in meningococcal adherence and 24.2% decrease in bacterial internalisation after 60 min incubation at 37°C. These findings imply SR-A does contribute to the non-opsonic phagocytosis of *N. meningitidis* H44/76.

6.1.2.6 Population of phagocytic cells interacting with bacteria in the absence of SRA remains the same irrespective of Poly I treatment

The marked reduction in bacterial internalisation due to SR-A blockade appeared to have no significant effect on the population of MDM or indeed dTHP-1 binding and internalising organisms (Fig. 6.3C and 6.4C). Hence the same mean number of bacteria are binding to the same mean number of macrophages, but in the presence of Poly I internalisation is reduced by over half; although not completely abolished. Therefore, SR-A are likely to be key receptors involved in the non-opsonic internalisation of *N. meningitidis*. As phagocytosis is not completely abolished following Poly I treatment, this suggests that in the presence of Poly I (and absence of available SR-A) meningococci uptake occurs through, as yet unknown, alternative macrophage receptors. Experiments

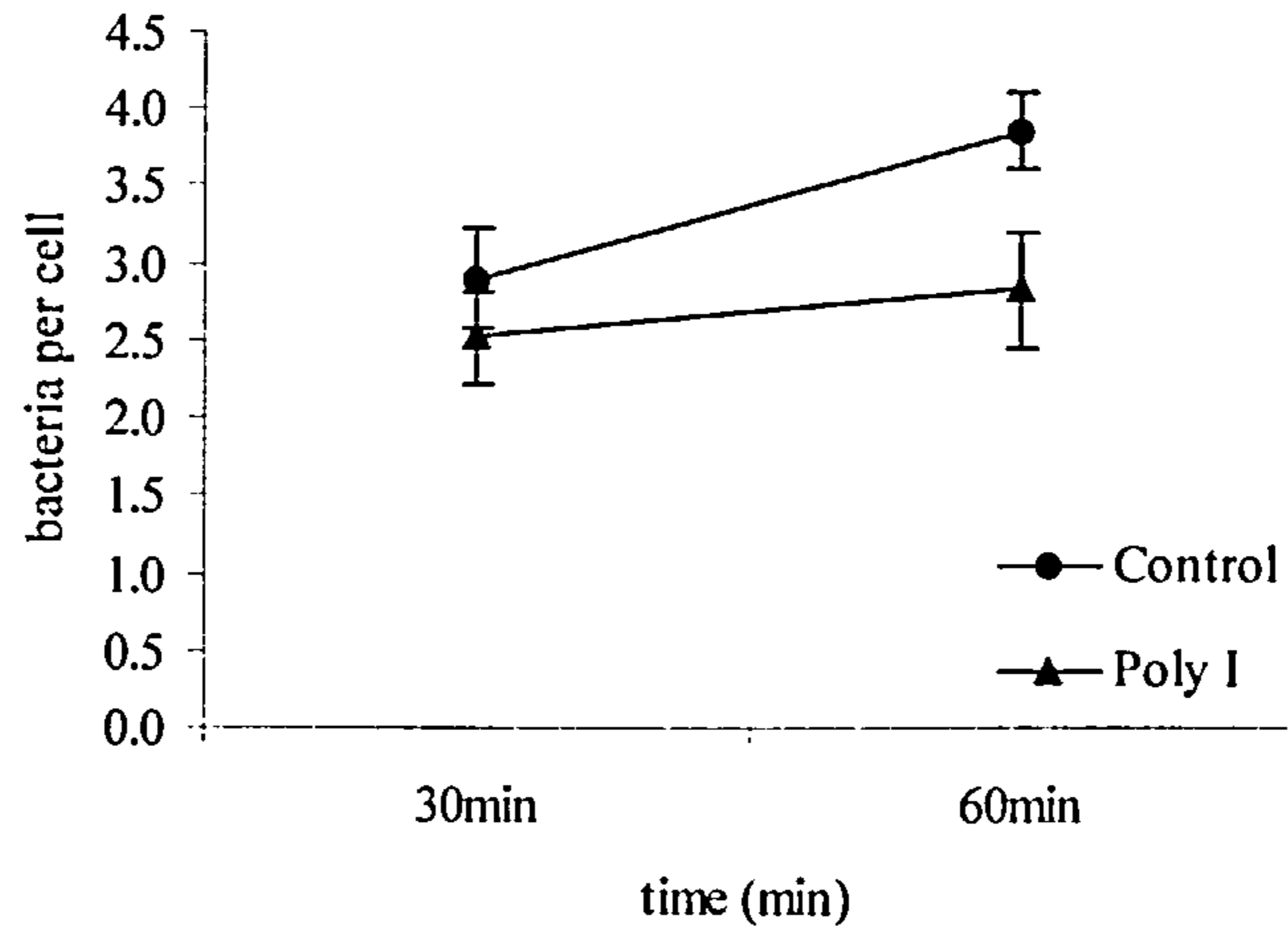
transfecting cell lines with human SR-A clones were not possible, as the relevant clones were not available.

Figure 6.4

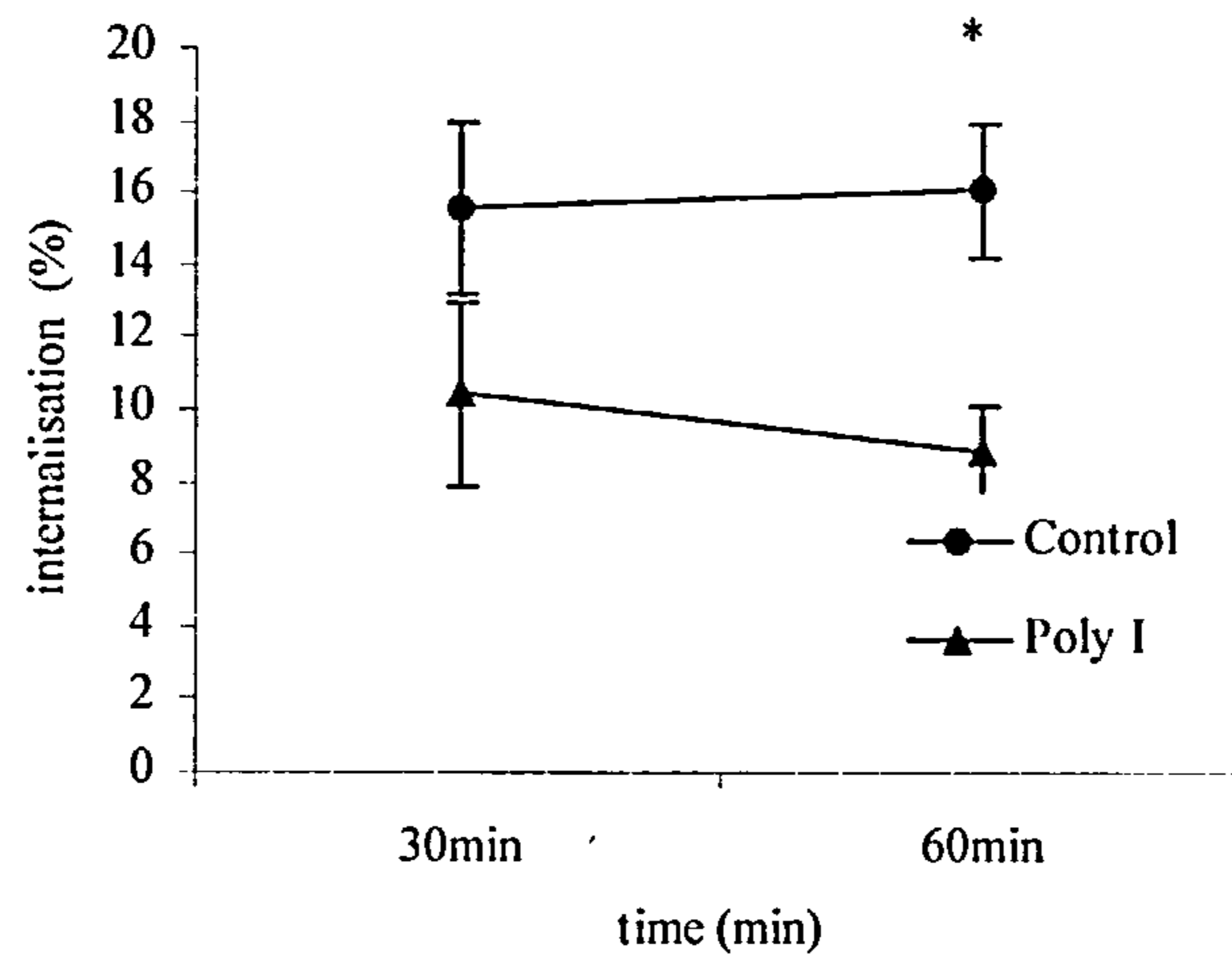
SRA-mediated uptake in dTHP-1 inhibited by Poly I

PMA-differentiated THP-1 (dTHP-1) cells treated with Poly I before incubating for 60min at 37°C with *N. meningitidis* H44/76, in the presence of Poly I. Graphs represent (A) Ratio of bacteria per cell (B) Percentage of phagocytosis (C) Population of cells interacting with bacteria. Results were obtained from four separate experiments, repeated at least three times. Values represent mean (\pm standard error of mean). Statistical significance indicated by * $P < 0.05$, ** $P < 0.01$ using Mann Whitney U test.

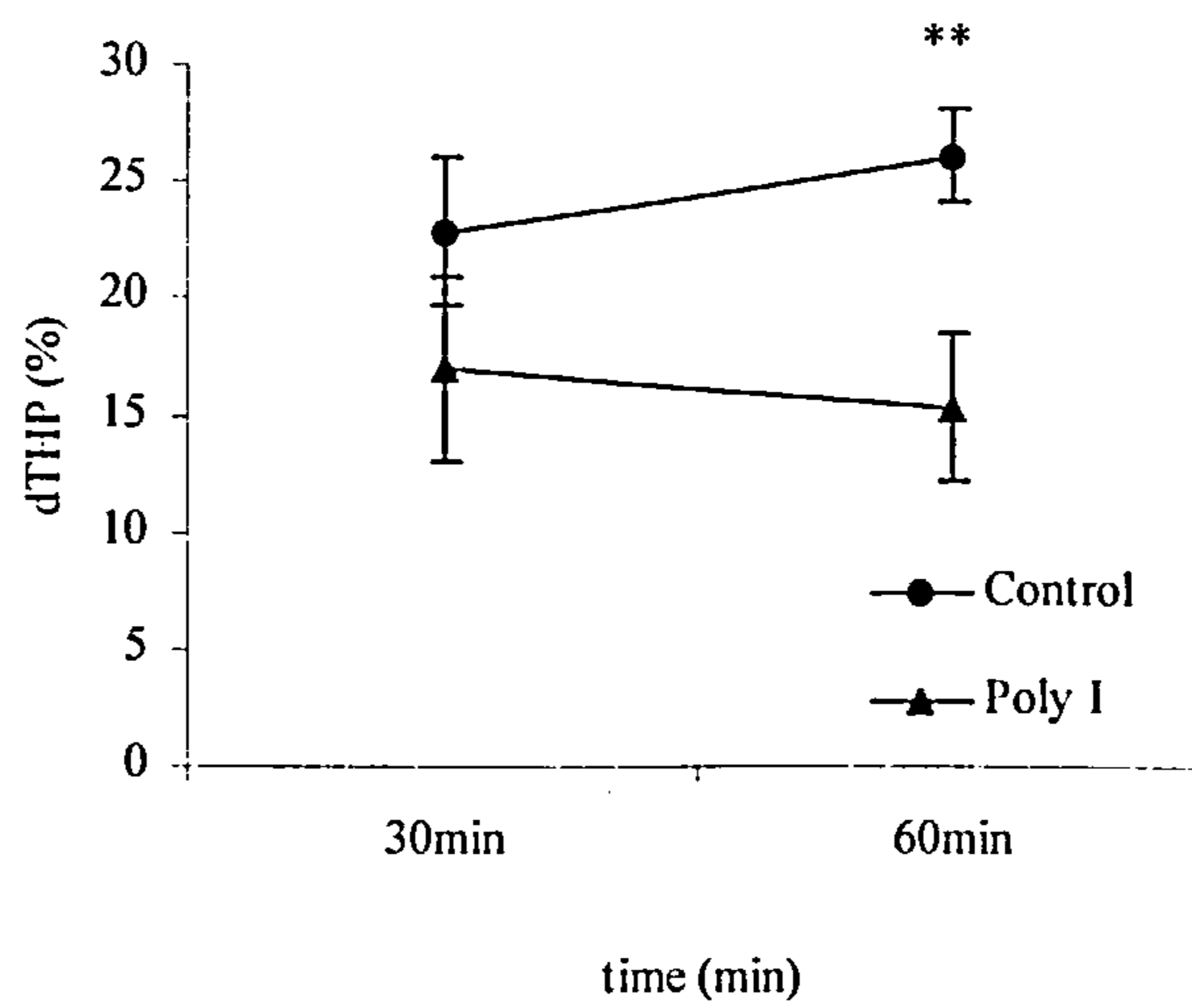
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6.1.2.7 *N. meningitidis* is internalised in an actin-dependent manner

To ensure that meningococcal uptake by MDM was an actin-dependent process, MDM were treated with Cytochalasin D for 30min before infecting cells with *N. meningitidis* H44/76 for 60min at 37°C. Figure 6.5 demonstrates that phagocytosis was reduced by over 70%.

6.1.2.8 Blockade of phagocytosis by Poly I is dose-dependent

MDM were incubated with Poly I at a range of concentrations (0-500µg/ml) before inoculating with *N. meningitidis* H44/76, incubating for 1h at 4°C and 30min at 37°C. Percentage of adherent bacteria internalised is demonstrated in Figure 6.6. By 25µg/ml Poly I-treatment of MDMs inhibited internalisation of meningococci to the point where maximal numbers of SR-As appear to have been blocked, and from 50 - 200µg/ml this appears to be reversed which may be due to alternative phagocytic receptor(s) playing more prominent roles in the absence of SR-A.

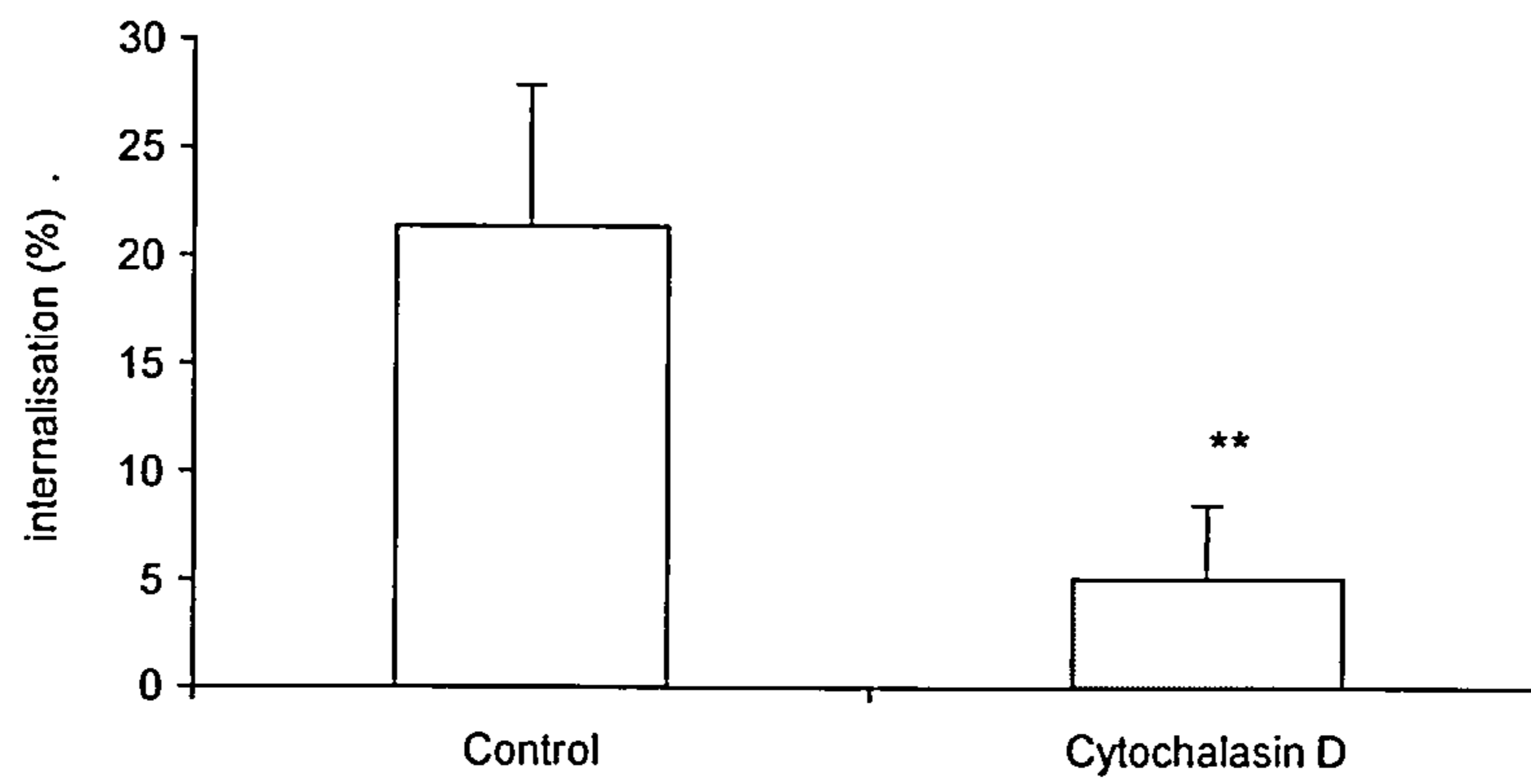


Figure 6.5

Disruption of actin development inhibits phagocytosis

MDM were incubated in the presence of 3 μ M Cytochalasin D for 30min before exposure to bacteria. Graph shows results from two experiments, based on mean values (\pm SEM). Statistical significance indicated by * $P < 0.05$ using Mann Whitney U test.

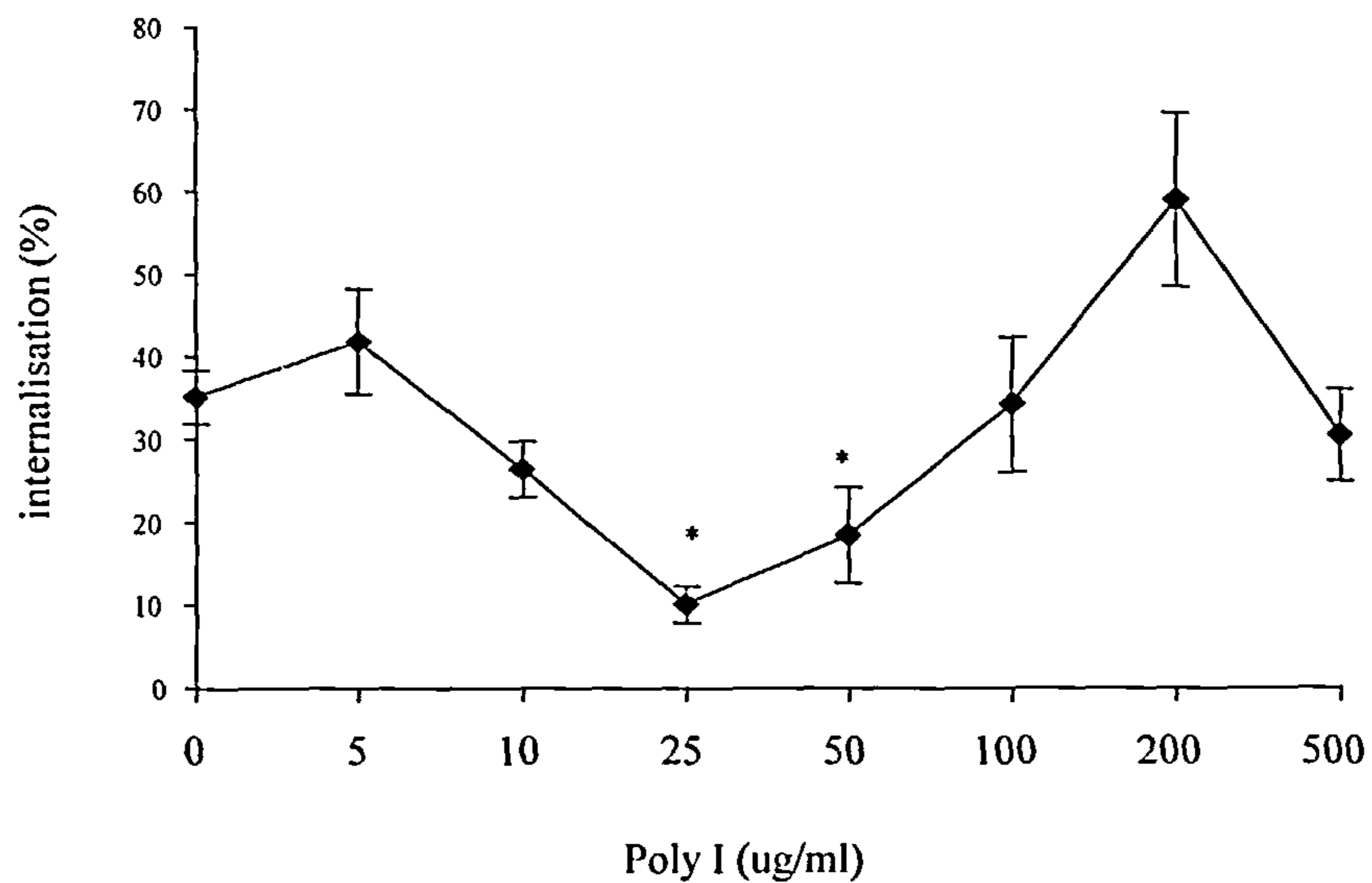


Figure 6.6

Effect of Poly I

Range of Poly I concentrations used to block SR-A, and internalisation measured. MDM were incubated with *N.meningitidis* H44/76 (MOI 50:1) in the absence of serum for 60min at 4°C followed by 30min at 37°C. Observations were based on three human donors, each experiment repeated at least three times. Values represent mean (\pm standard error of mean) with statistical significance calculated using Mann Whitney U test to compare each concentration against untreated control cells.

6.1.2.9 Pro-inflammatory cytokine secretion not affected by SR blockade

MDM were pre-incubated with Poly I to investigate whether the internalisation of *N. meningitidis* H44/76 stimulates a pro-inflammatory cytokine response normally associated with LPS: macrophage interactions. MDM were incubated with *Neisseria meningitidis* for 2h in the presence or absence of Poly I, after which supernates from each well were collected and frozen for further analysis to detect the levels of TNF- α . Enzyme-linked immunosorbent assays (ELISA) were carried out on the supernatants. Figure 6. shows that TNF- α secretion was not significantly altered in the presence/absence of increasing doses of Poly I up to 50 μ g/ml. However Poly I at 100 μ g/ml did exhibit an attenuating effect.

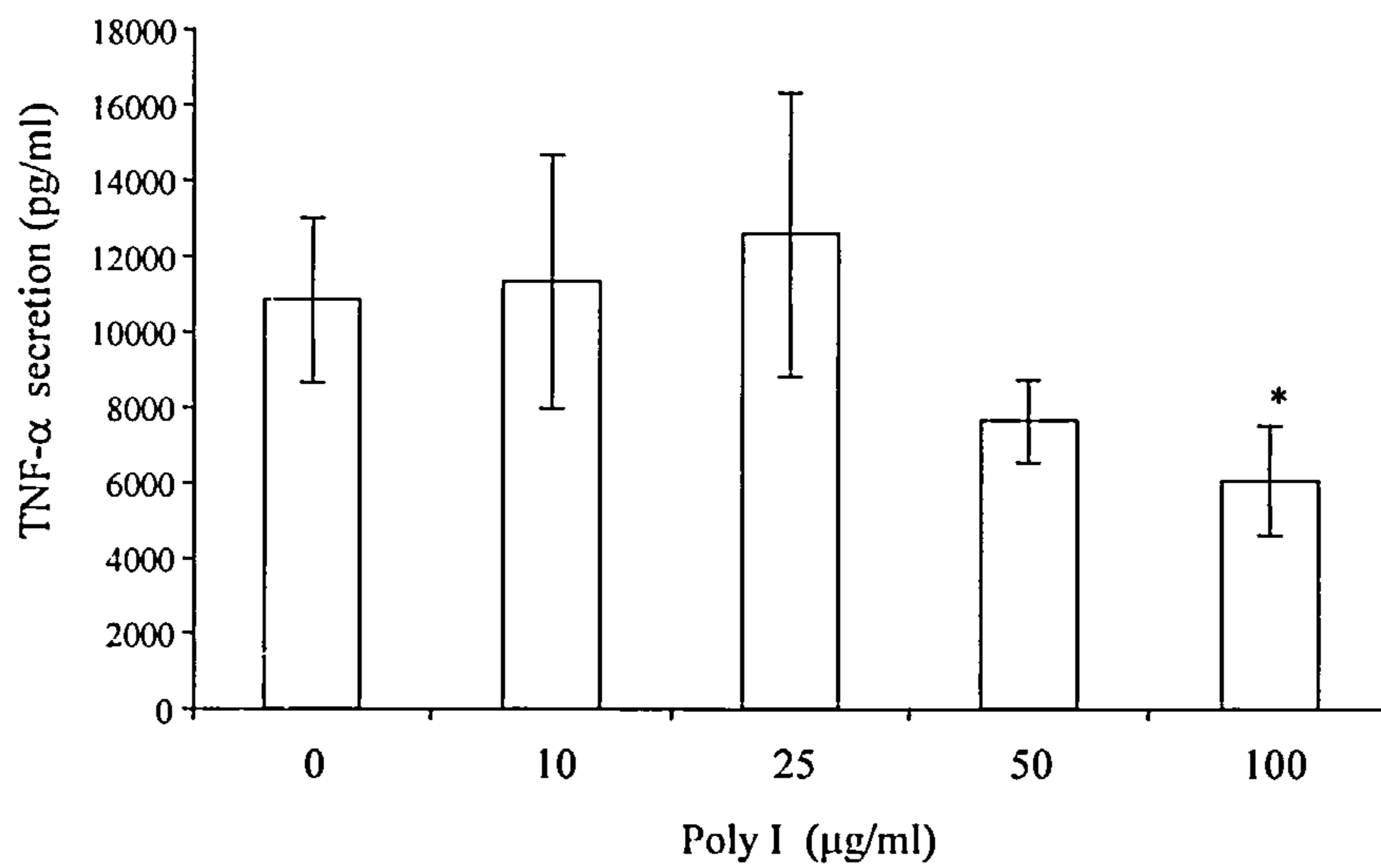


Figure 6.7

TNF-α secretion following scavenger receptor blockade

MDM were treated with a range of concentrations of Poly I before incubation with *N.meningitidis* H44/76 for 120 min, and levels of TNF- α secreted into the surrounding media were measured. Observations were based on four human donors, each experiment repeated at least six times. Values represent mean (\pm standard error of mean) and significance denoted by * $P < 0.05$ using Mann Whitney U test.

6.1.2.10 Examination of the effect of multiple receptor blockade on bacterial phagocytosis

To examine if three of the macrophage receptors investigated individually in this study, namely CD14, SRA and CR3, showed any differences in bacterial internalisation when more than one receptor was absent, combinations of the three blocking reagents/antibodies were used at same concentration as before (see Chapter 5); anti-CD14 mAb ([MY4], 5µg/ml), anti-CD11b mAb ([2LMP19c], 2µg/ml) and SR blocking agent ([Poly I], 50µg/ml). Combinations of reagents were incubated with MDM simultaneously for 30min, before washing three times and infecting with *N. meningitidis* H44/76 (MOI 50:1) and culture media containing blocking agents. Cells were then incubated for 60min before fixing, staining and microscopic analysis.

Fig. 6.8A shows that after 60min incubation at 37°C the absence of CD14, through MY4 treatment, significantly impairs bacterial recognition by macrophages. This reduction is apparent in the total number of bacteria binding, the ratio of bacteria to each macrophage (23% reduction) and the proportion of macrophages interacting with bacteria. Moreover, this observation is repeated when CD14 receptors are blocked in combination with either CR3 or SRA, where compared to control macrophages association is reduced by 18.3% and 23.7% respectively. These findings suggest again that macrophage CD14 receptors are involved in bacterial capture at physiological temperature.

Fig. 6.8B represents the differences in adherent meningococci phagocytosed by MDM. Only macrophages treated with Poly I and anti-CD14 reagents, both individually and together, showed a significant difference in bacterial internalisation; although not when co-incubated with anti-CR3 antibody, even though this combination of blocks significantly reduces adherence. Compared to control cells, anti-SRA treatment alone reduced internalisation by 26.5% and anti-CD14/anti-SRA resulted in a 25% decrease. Similar patterns were observed

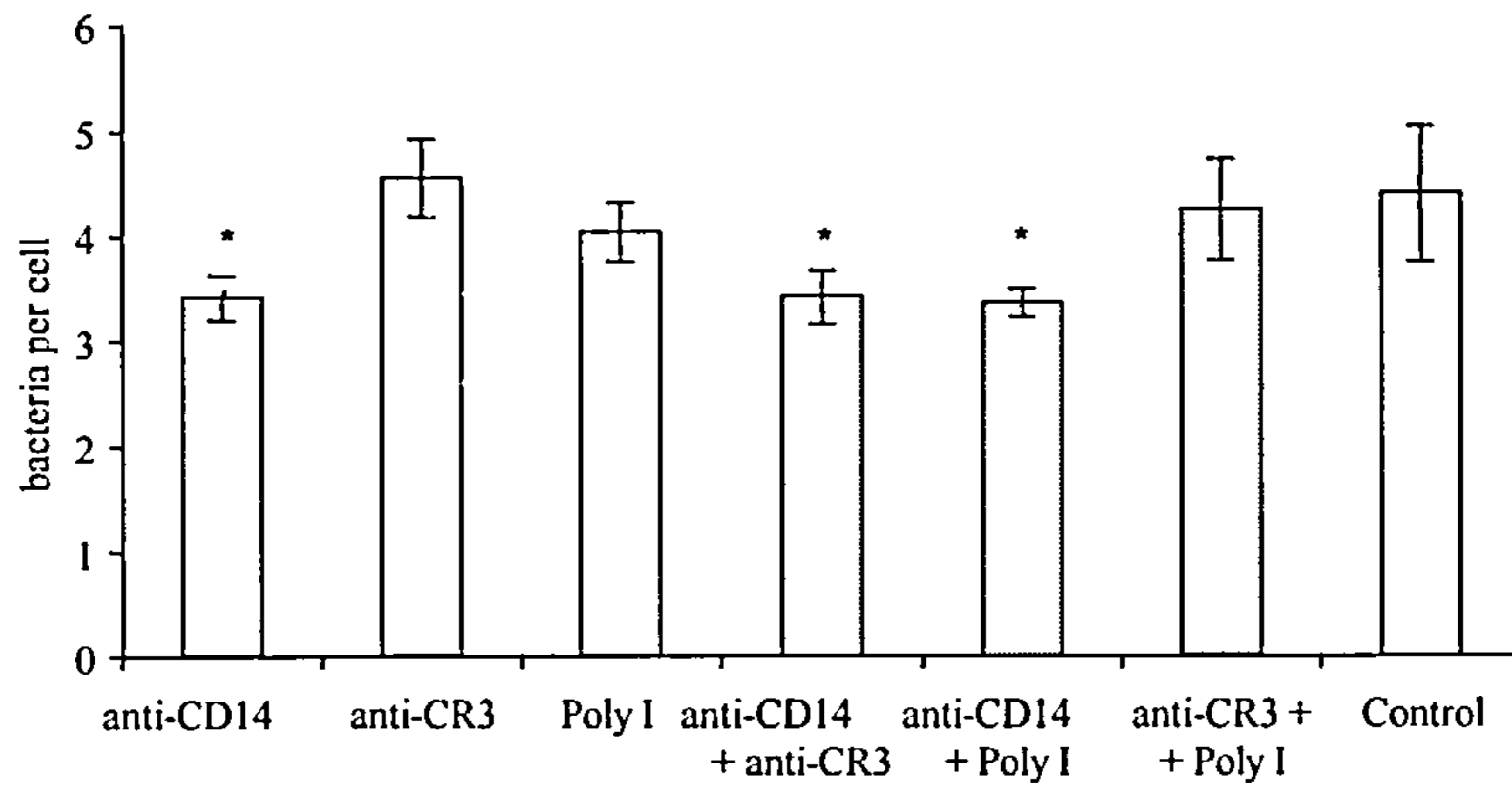
in number of organisms able to bind, and proportion of macrophages binding and internalising the bacteria. These findings suggests that CD14 and SRA both contribute significantly to meningococcal uptake by macrophages, and may work in concert. Similar patterns were observed in Figure 6.8C and D.

Figure 6.8

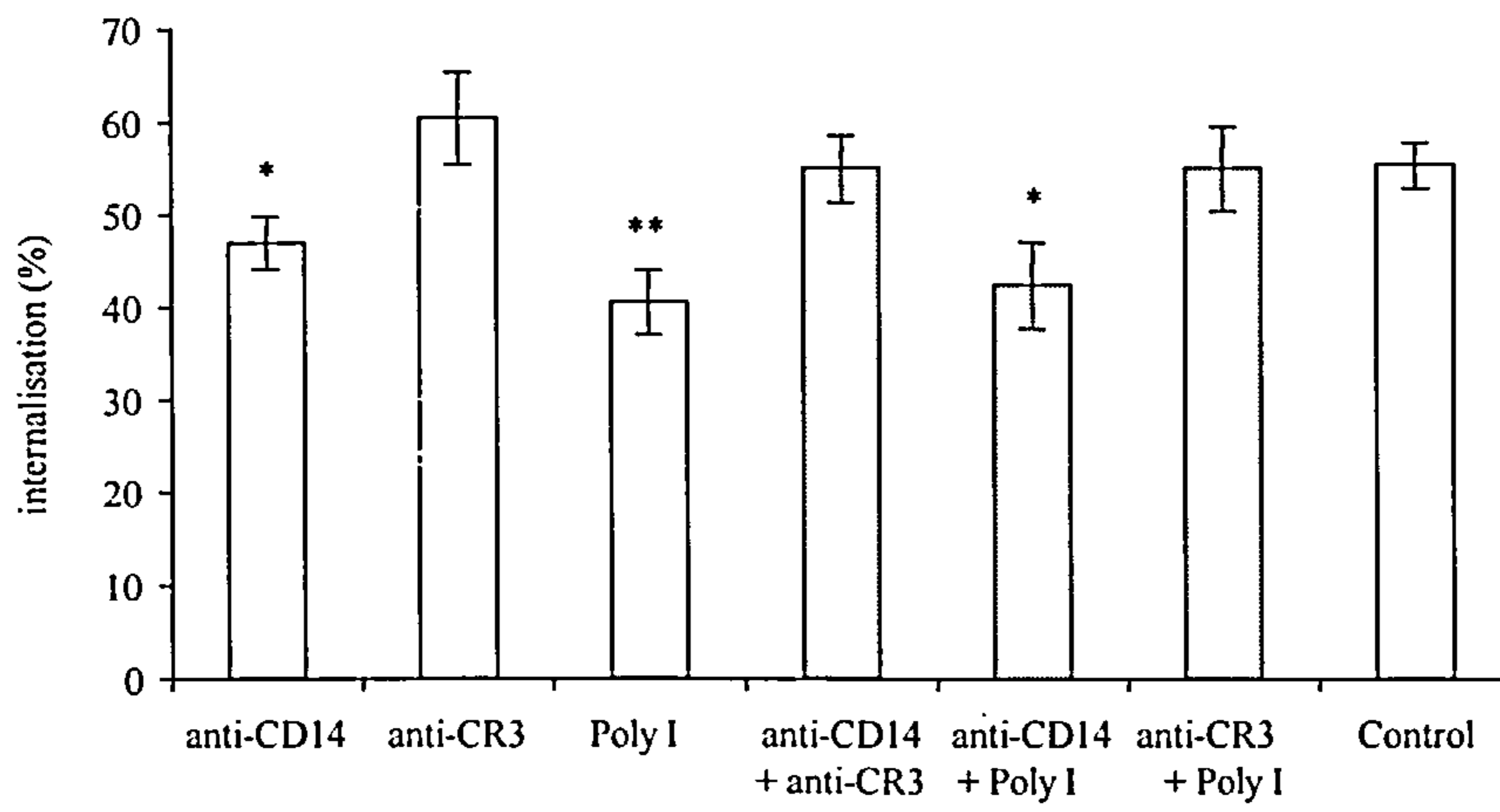
Multiple receptor blockade affects bacterial phagocytosis

MDM were simultaneously exposed to combinations of blocking antibody/reagents before infecting macrophages with *N. meningitidis* H44/76 and incubating for 60min at 37°C. Anti-CR3 mAb is 2LMP19c, anti-CD14 mAb is MY4 and anti-SRA is Poly I. Graphs show (A) mean numbers of bacteria bound to each macrophage (B) mean percentage of total organisms internalised and (C) mean percentage population of macrophages interacting with bacteria and (D) total number of bacteria bound/ingested by 100 macrophages (filled bars) in the absence of serum., with bars representing mean (\pm standard error of mean). Results are based on three donors, each experiment repeated three times. Statistical significance is denoted by * $P < 0.05$, ** $P < 0.01$ using Mann Whitney U test comparing treated cells with control.

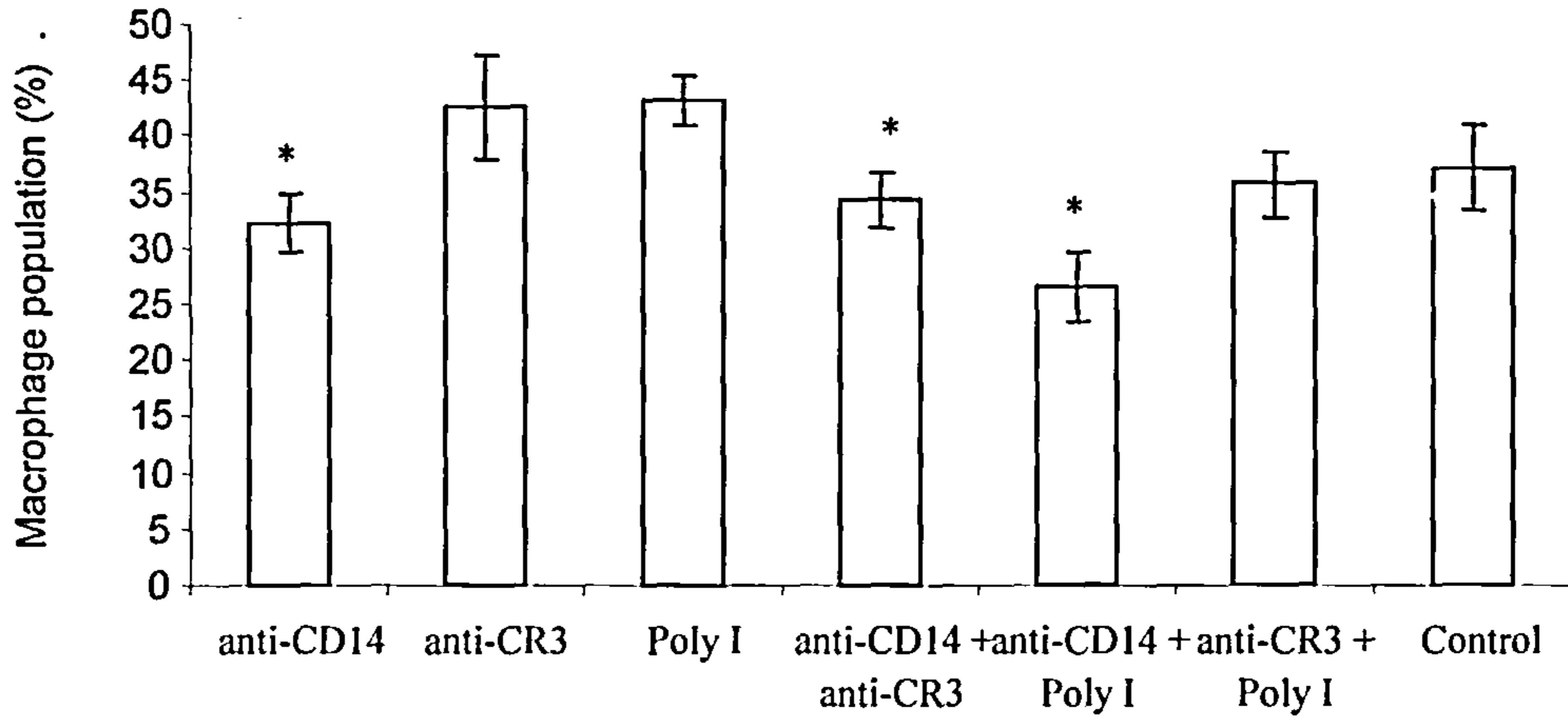
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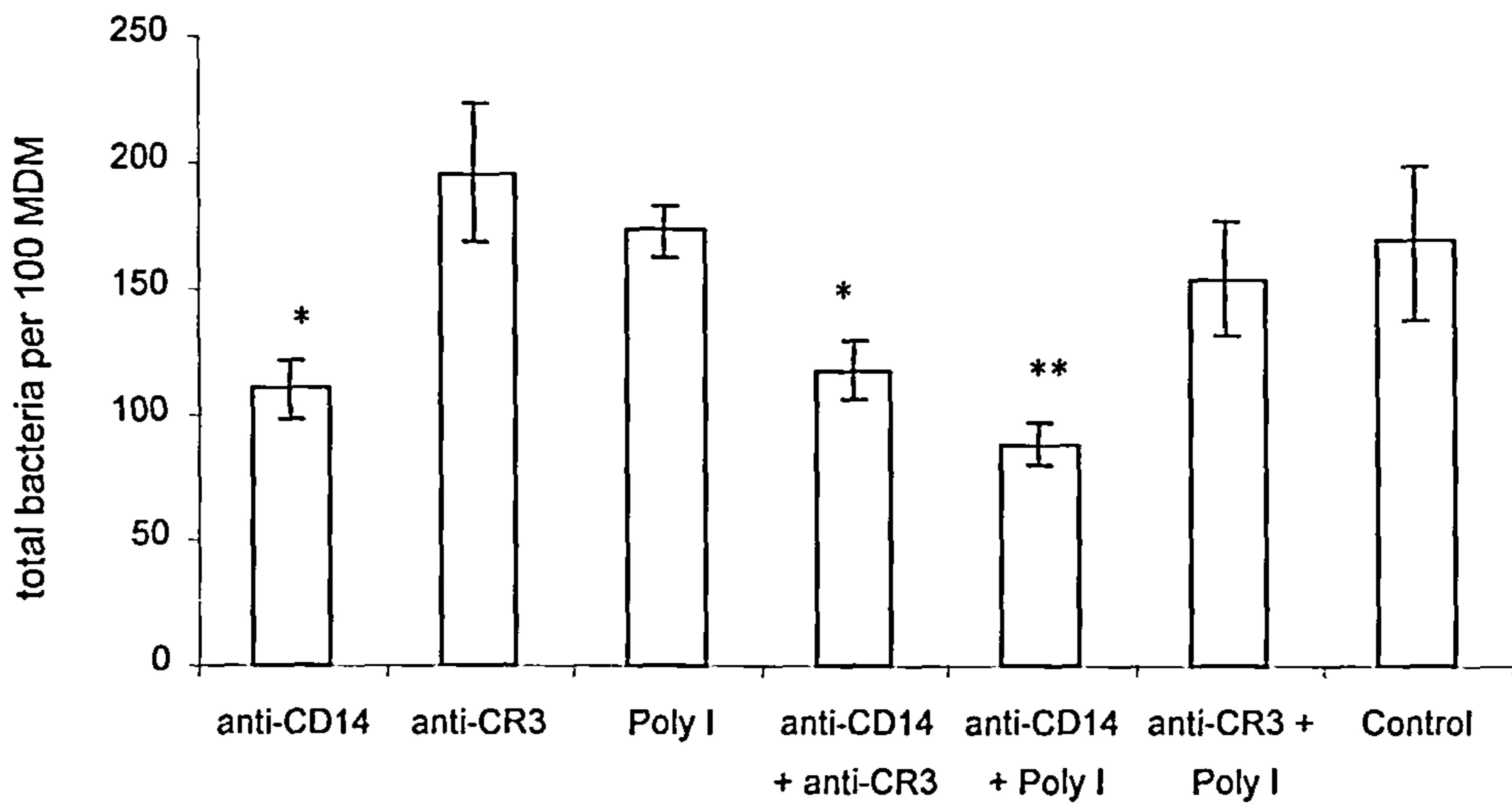
B



C



D



6.1.2.11 Cholesterol depletion of macrophages does not inhibit SR-A ligand uptake

β CD is a carbohydrate that contains a cholesterol-binding pocket that depletes cholesterol, whereas nystatin sequesters cholesterol within the membranes. β CD, extracts cholesterol from outside the rafts (not within) without binding to or inserting itself into the membrane. If CD14 and SRA do work in concert to internalise *N. meningitidis* they would need to be in close proximity, therefore as previous studies (see Chapter 1) have identified GPI-linked proteins such as CD14 tend to cluster on these lipid-rich microdomains disruption of these rafts might affect bacterial uptake. MDM were therefore treated separately with two different cholesterol disrupting agents used previously, Nystatin (data not shown) and β -Cyclodextrin (β CD). Following raft disruption cells using a range of concentrations of β CD for 15min at 37°C, cells were then incubated with a known SRA ligand, DiI-acLDL, for 2h before looking for any changes in SR-A ligand recognition by the macrophages; and these initial experiments show that no differences were observed Fig 6.9.

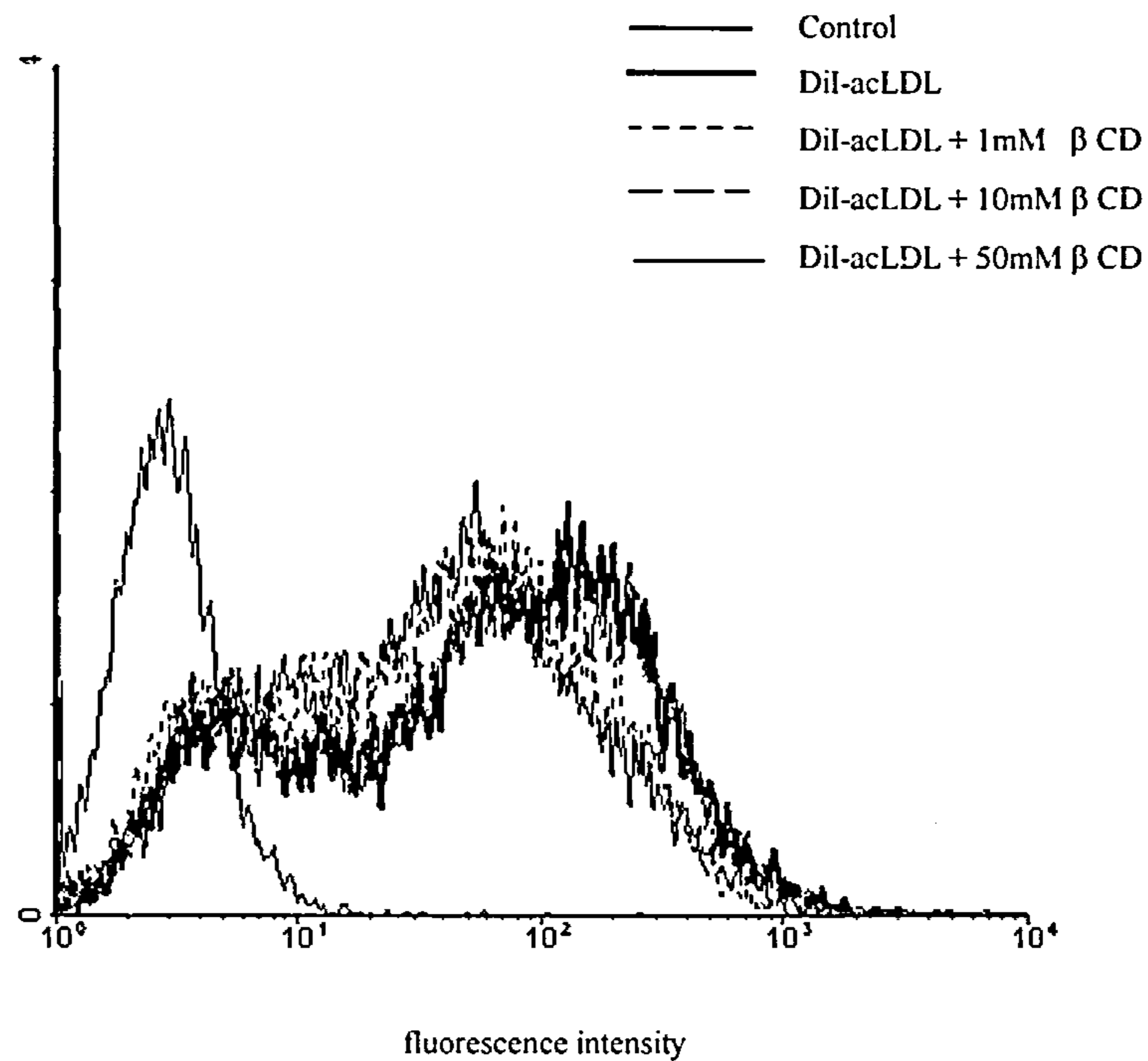


Figure 6.9

β -Cyclodextrin treatment of lipid rafts

DiIacLDL uptake examined by treating MDM with a range of concentrations (1, 10 and 50mM) of a cholesterol-depleting agent, β -Cyclodextrin, for 15min at 37°C before incubating with DiIacLDL for 2h. Control MDM were not treated with β -Cyclodextrin. Figure shows one experiment representative of three, each experiment performed at least three times.

Part II: Mannose receptor and PAF receptor

6.2.1 Introduction

In previous chapters the possible roles of a number of macrophage receptors in non-immune opsonic phagocytosis were examined, and several lines of evidence have led us to suggest that CD14 and scavenger receptors are likely to play a role in the recognition of *N. meningitidis*. In this chapter more receptors that could contribute to these processes are examined, specifically mannose receptor (MR) and platelet-activating factor receptor (PAF-R).

Platelet-activating factor is an ether-linked acetylphosphorylcholine molecule, that functions through G-protein linked receptors as an inflammatory signal mediator (Snyder 1990). PAF, along with other phosphorylcholine-linked molecules, binds to the PAF receptor which is a non-opsonic receptor present on epithelial cells, endothelial cells and macrophages. Previous research has shown that strains of non-typeable *H. influenzae* invade human epithelial cells via a number of pathways, including PAF receptor-mediated uptake (Swords, Buscher et al. 2000). *S. pneumoniae* also invade host cells through phosphorylcholine interactions with PAF receptors, as PAF receptor expression increases in inflamed airways, of individuals with asthma or chronic bronchitis (Cundell, Gerard et al. 1995). As a result of direct recognition through PAF receptors, pneumococci can invade host cells and subsequently migrate into the subarachnoid space by altering the permeability of the blood brain barrier (Cabellos, MacIntyre et al. 1998; Ring, Weiser et al. 1998). As no previous studies have implicated PAF-R in playing a role in meningococcal interactions here an effective PAF receptor antagonist was used to investigate whether the blockade of PAF-R would affect meningococcal entry into human macrophages.

The mannose receptor (MR) is the second receptor examined in this chapter. MR is an integral membrane protein and a member of the lectin family of receptors that are expressed on tissue macrophages, but not circulating monocytes. Ligation of pathogens or mannose-rich glycoconjugates to the receptor results in the phagocytosis of the ligand accompanied by a pro-inflammatory response. A non-specific receptor involved in host defence, MR has been associated with uptake of yeasts (*C. albicans*, *S. cerevisiae*) (Ezekowitz, Sastry et al. 1990; Giamis, Lombard et al. 1993), parasites (*P. carinii*) (Ezekowitz, Williams et al. 1991) and bacteria (*E. coli*) (Lefkowitz, Lincoln et al. 1997). A number of studies have observed that the phagocytosis of these particles can be inhibited with the treatment of mannose, yeast mannans and mannose-terminal glycoconjugates; therefore mannosylated BSA (mBSA) was used to act as a competitive ligand to the mannose receptor binding site, and observe the affect on meningococcal recognition.

6.2.2 Results

6.2.2.1 Mannose receptors do not appear to have a role in meningococcal interaction *in vitro*

Mannose receptors were blocked with mannosylated-BSA (mBSA; α -D-mannosylated albumin) in order to prevent bacterial interactions via the receptors known ligand-binding site. Macrophages were incubated with a range of concentrations of 0-100 μ g/ml, before infecting with *N. meningitidis* H44/76. Fig. 6.10 shows adherence measured after 60min incubation with bacteria at 4°C, and no difference was observed.

Internalisation was then measured over 60min at 37°C, mBSA was used at a concentration of 50 μ g/ml (see Fig6.11). When compared to untreated control cells, no significant difference in binding or internalisation of *N. meningitidis* H44/76 by mBSA-treated cells was observed. Experiments carried out using the same range of concentrations of mBSA (0-100 μ g/ml) did not have any effect on bacterial ingestion at 37°C (data not shown).

6.2.2.2 Involvement of platelet-activating receptor (PAF-R) in recognition of *N. meningitidis*

PAF receptors were blocked using the PAF receptor antagonist, PAF-16, (1-0-hexadecyl-2-acetyl-*sn*-glycero-3-phospho-(N,N,N-trimethyl)hexanolamine) which is an analogue of PAF that can also inhibits phospholipid turnover and secretion, as well as PAF-induced platelet aggregation. MDM were incubated in final concentrations of PAF-16 that ranged from 1-100nM final concentration, before cells were infected with *N. meningitidis* H44/76 and incubated at 4°C for an hour (see Fig. 6.12) or 37°C for up to an hour (data not shown). No difference

was observed between pre-blocked and control cells at 1 and 10nM, but a marked decrease in bacterial recognition was observed at 100nM PAF-16. Cundell *et al* (Cundell, Gerard et al. 1995) observed a difference in *Streptococcus pneumoniae* recognition following receptor inhibition with this reagent. I observed that this high concentration of reagent appeared to have a detrimental affect on primary human macrophages, in terms of morphology the cells had reduced in size and taken on a different appearance, even though trypan blue exclusion indicated that these cells were still viable. Therefore even though the data indicate a significant difference at this concentration further experiments alternative inhibitors should be sought to gain a clearer perspective.

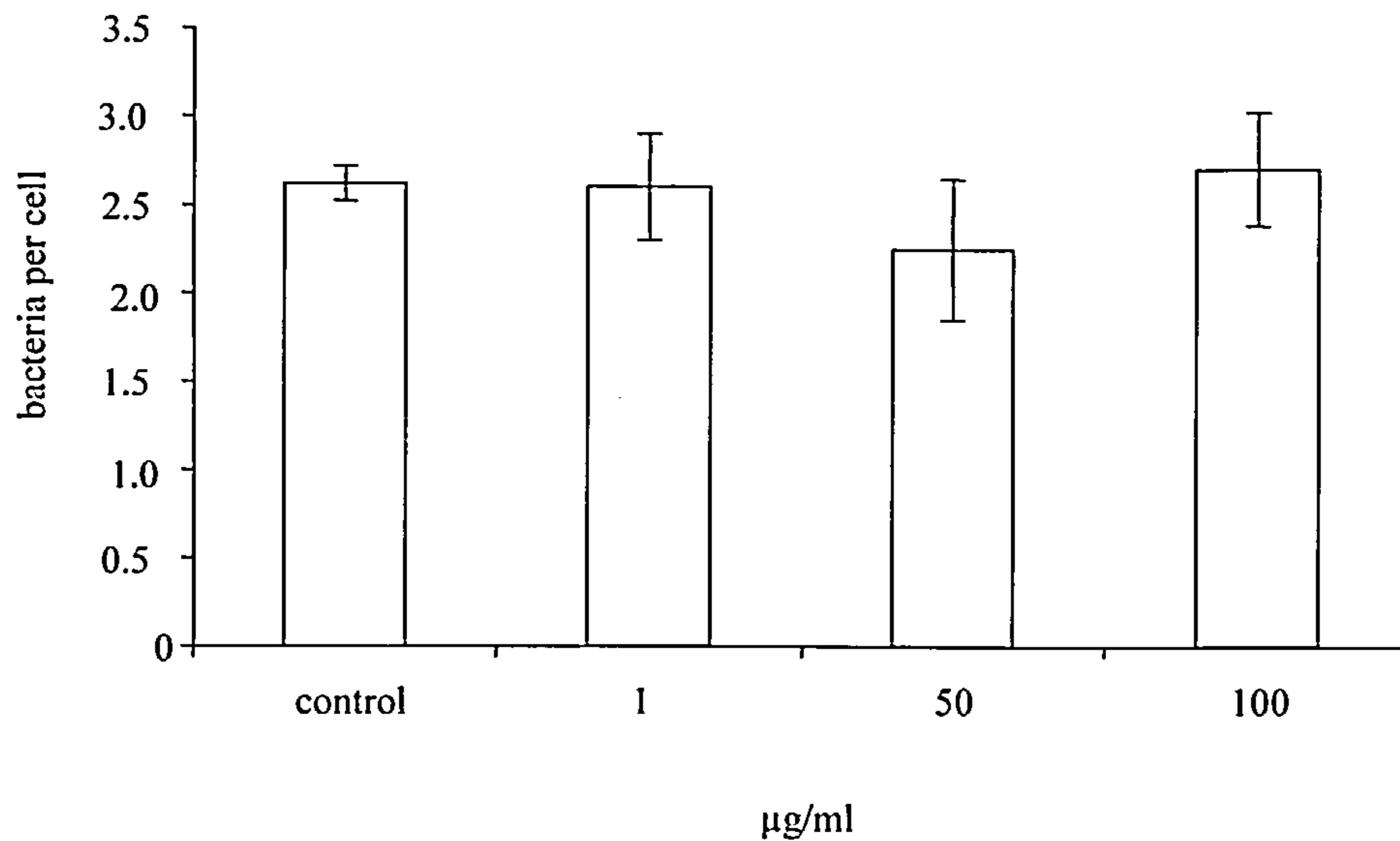


Figure 6.10

Bacterial adherence following mannose receptor blockade

MDM were exposed to different concentrations of mannosylated bovine serum albumin (mBSA) before infecting macrophages with *N. meningitidis* H44/76 and incubating for 60min at 4°C. Graphs shows numbers of bacteria bound to each macrophage, with bars representing mean (\pm standard error of mean). Results are based on four donors, each experiment repeated at least three times. No difference in bacterial adherence was observed ($P>0.05$).

Figure 6.11

Mannose receptor blockade

MDM were exposed to 50µg/ml mannosylated bovine serum albumin (mBSA) before infecting macrophages with *N. meningitidis* H44/76 and incubating for 60min at 37°C. Graphs shows numbers of bacteria bound to each macrophage, with bars representing mean (\pm standard error of mean). Results are based on four donors, each experiment repeated at least three times. No difference in bacterial adherence was observed ($P>0.05$).

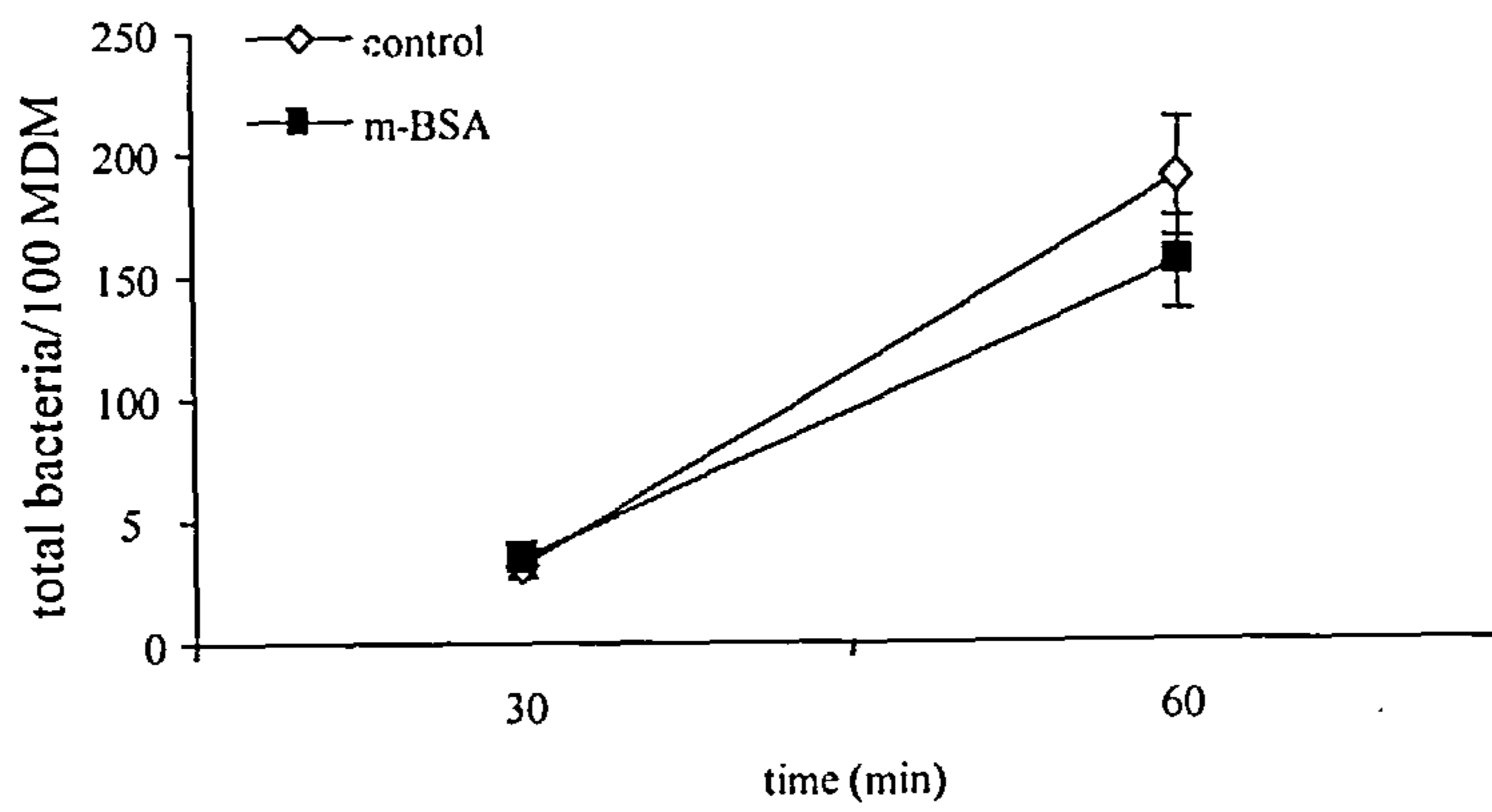
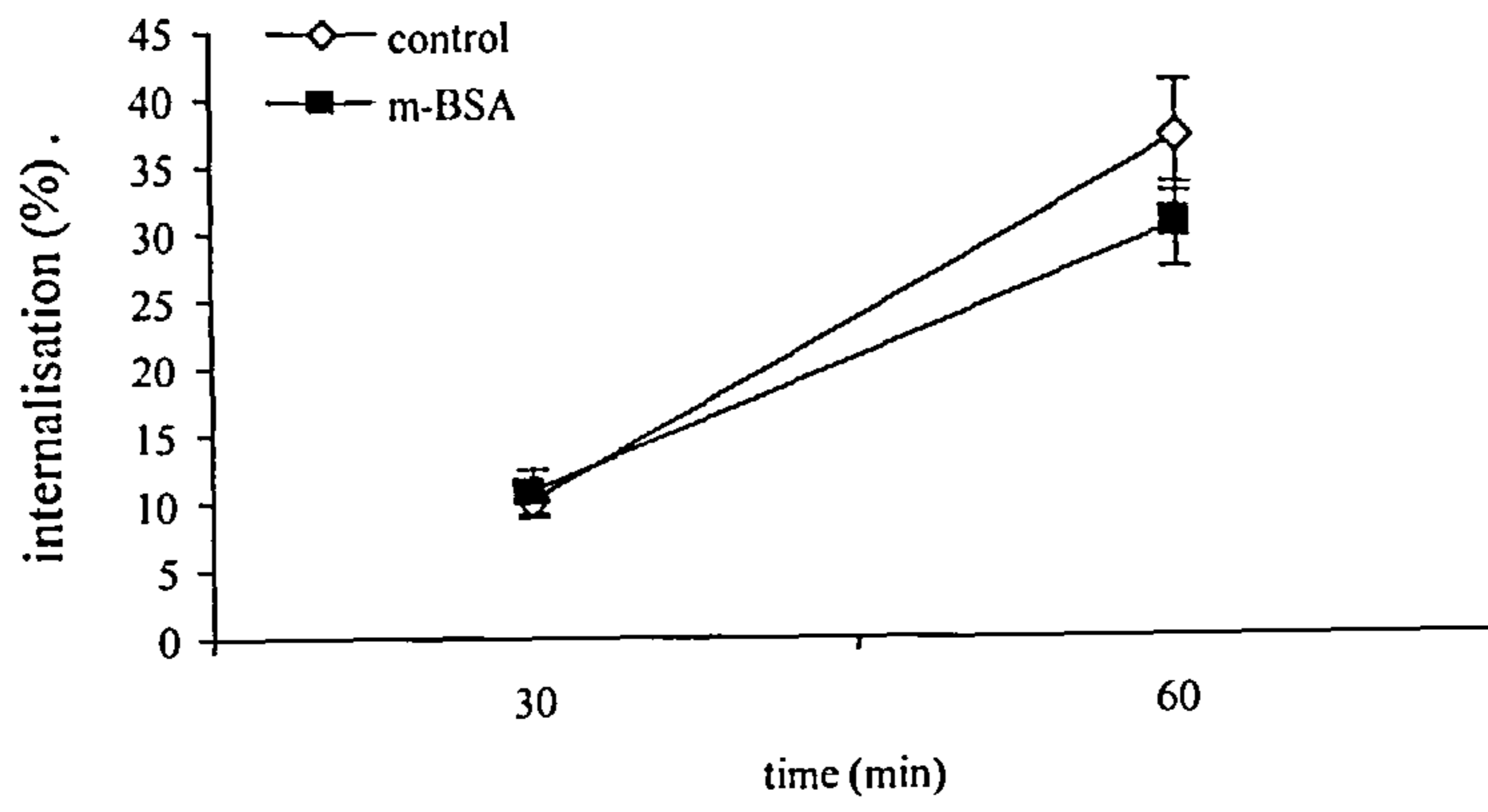
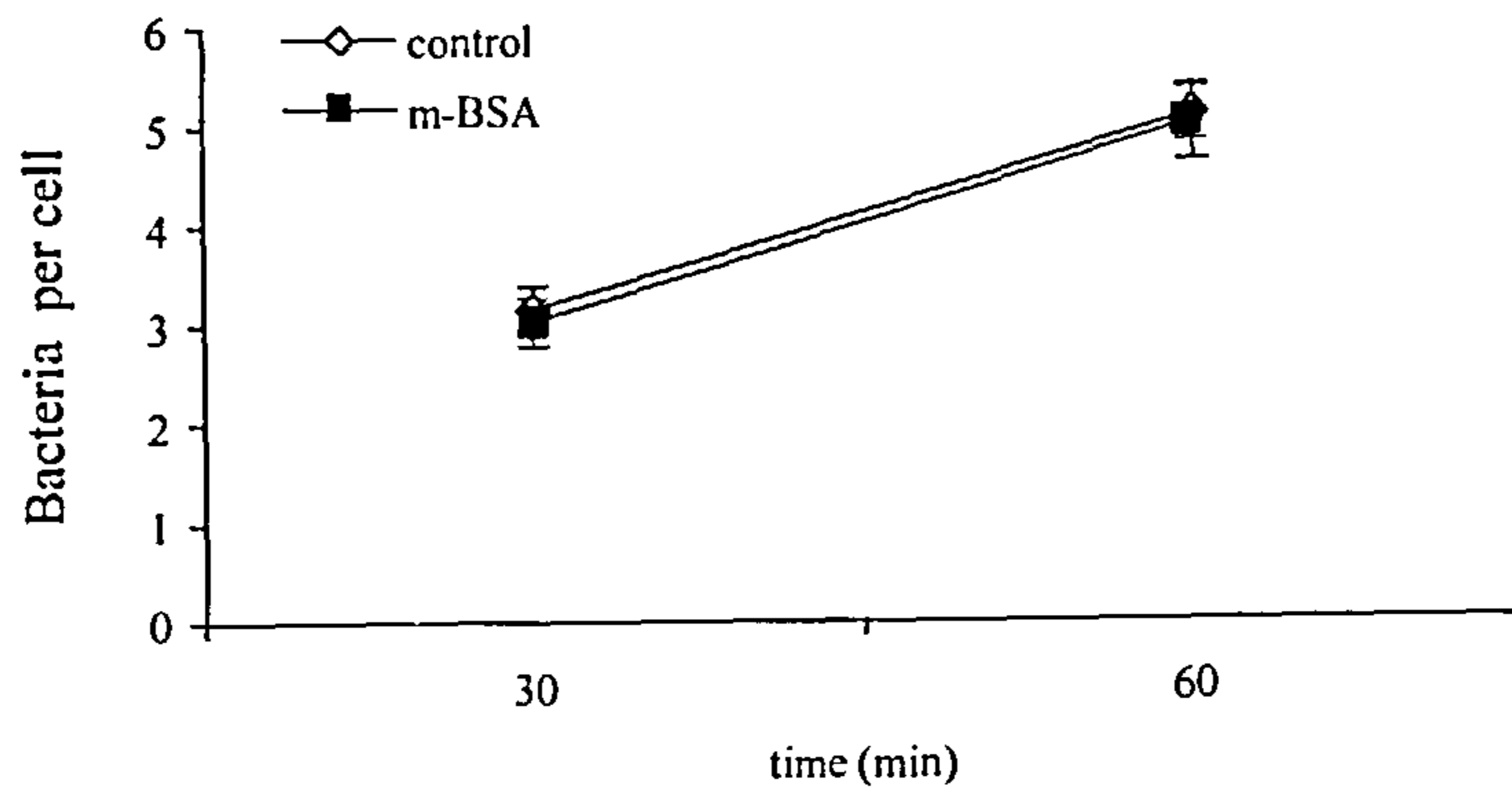
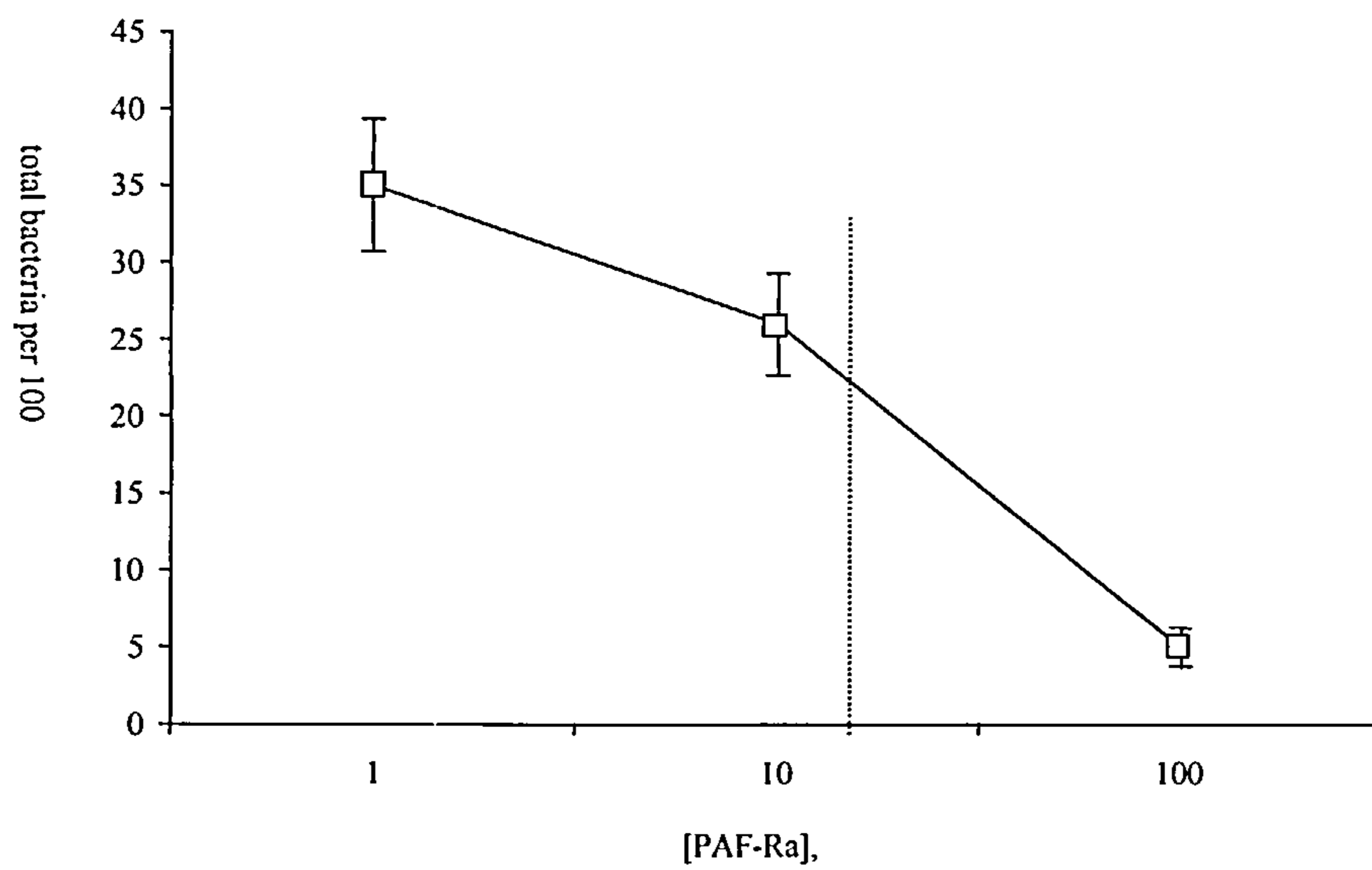


Figure 6.12

Platelet activating factor receptor inhibition

Platelet activating factor receptor (PAF) on MDM blocked using a range of concentrations of receptor antagonist (PAF-Ra). Macrophages were inoculated with *N. meningitidis* H44/76, in the absence of serum, and incubated for 60min at 4°C. Vertical dotted line indicates point after which morphological deterioration of MDM is apparent. Data are mean values, and values in parenthesis are \pm standard mean of error. Results obtained from three donors and repeated three times.



Platelet-activating factor receptor		
[PAF-Ra] nM	adherence ratio	Population m ϕ (%)
control	2.62 (0.1)	12.2 (1.33)
1	2.89 (0.2)	12.06 (1.32)
10	2.49 (0.2)	10.12 (0.88)
100	1.55 (0.24)	3.11 (0.7)

6.3 Summary and discussion

Observations thus far suggest that the two processes of binding and internalisation of *N. meningitidis* H44/76 by MDM, under non-opsonic conditions, are governed by different groups of receptors. Blockade of macrophage SR-A, using Poly I, demonstrated that *N.meningitidis* adhered to macrophages via specific binding receptors that were unaffected by Poly I treatment. It would also appear that blocking SR-A on the surface of the cells significantly impaired phagocytosis of *N. meningitidis*, but the percentage population of cells able to directly interact with meningococci remained the same irrespective of Poly I treatment. Differentiated THP-1(dTHP-1) cells were again found to be a useful tool, as PMA-treatment led to the scenario of bacterial binding and uptake that could be inhibited using Poly I.

SR-A-dependent phagocytosis appeared to be affected by incubation time and concentration of Poly I, but interactions between meningococci and SR-A were quite specific as flow cytometry results identified Poly I as an effective inhibitor of SR-A ligand (DiI-acLDL) recognition. Differentiating between polyanionic sensitive receptors and SR-A of human macrophages using current methods is not easy. Results based on both primary macrophages and dTHP-1 cells revealed that phagocytosis of meningococci is inhibited to varying degrees by Poly I treatment. Bacterial internalisation by dTHP-1 cells was reduced by 24.2% whereas uptake by MDM fell by almost 60%. The differences between the cell types suggests that the contribution of SR-A in meningococcal phagocytosis varies (see Chapter 3), and the fact that Poly I did not abolish bacterial uptake implies that although SRA can be an important receptor for *Neisseria*, it is not an exclusive port of entry. The reduction in SRA-mediated inhibition by dTHP-1 cells may be due to the action of PMA, which is thought to be effective in altering the expression of macrophage receptors, or may alternatively stimulate the over-expression of non-phagocytic receptors that could compete and bind the bacteria. Differences in bacterial internalisation taken in conjunction with

changes in TNF- α production following a range of concentrations of Poly I (Fig 6.6 & 6.7) allows us to suggest that levels of TNF- α play some part in the loss of effectiveness of Poly I at higher concentrations.

Blocking of multiple receptors suggested that in the absence of CD14 less organisms adhere to fewer macrophages, but are internalised effectively if SRA is available. In the absence of SRA bacteria can adhere to the macrophages well, but subsequent internalisation is impaired. Notably, incubation of MDM in the presence of anti-CR3 mAb (2LMP19c) Poly I has no effect on meningococcal internalisation, suggesting that in the absence of both SRA and CR3 binding sites another receptor is able to take over meningococcal internalisation. Perhaps CD14 facilitates the transfer of organisms to another phagocytic receptor or a separate and available CR3 binding site is being utilised. Upregulation of CD14 in response to endotoxin has been previously reported, (Marchant, Duchow et al. 1992; Matsuura, Ishida et al. 1994) but upregulation of human SR-A in response to meningococcal LPS has not, and although the limited results in this chapter did not indicate this, further experiments should be carried out using a range of concentrations of LPS over time. Subsequent survival of internalised bacteria within macrophages, using established methods, was difficult to measure as relatively few meningococci were internalised under non-opsonic conditions. Previous observations showed that the numbers of bacteria bound per cell was self-limiting, as no more than approx 3 bacteria per cell bound to MDM in the absence of serum, even if the MOI was 200:1 (data not shown). It has also been suggested that Poly I may interact with other members of the scavenger receptor family, CD36 and MARCO, but neither are known to play a role in bacterial phagocytosis, and no evidence exists to show CD36 binds bacteria, and MARCO is only expressed in a small subset of macrophages (Elomaa, Kangas et al. 1995). A number of factors may affect this data including, levels of SRA expression, recycling of receptor, clustering or limiting levels of bacteria or intracellular molecules.

It has been established that SRA could bind gram positive organisms (Thomas, Li et al. 2000) but it was not previously known that SRA could also recognise and ingest whole gram-negative bacteria. This *in vitro* study, and *in vivo* work carried out by Peiser *et al* (Peiser, de Winther et al. 2002) in mice have observed that SRA recognise *N. meningitidis*, but this study suggests that human SRA is not a significant binding receptor for meningococci, a role which this study suggest is filled by CD14. Dual blockade of receptors also supported this hypothesis, but SRA-mediated uptake did not appear to take place on nystatin- or β -cyclodextrin-depleted cholesterol-rich lipid rafts.

Neither mannose or PAF receptors appeared to play a role in bacteria-macrophage interactions here; but as a general MR blocking agent mBSA may have bound to mannose receptors alone, but may have also adhered to the lectin-binding site of CR3, which recognises a wide range of saccharides. This study indicates that the initial recognition and subsequent phagocytosis of *N. meningitidis* H44/76 are quite separate processes, governed by different receptors. If specific receptor(s) exist through which *Neisseria* can enter immune cells, such as macrophages or dendritic cells and subsequently remain or proliferate without alerting the necessary cytotoxic machinery, then inhibition of these receptors may leave the bacteria more susceptible to innate immune responses and inhibit bacterial invasion. Such avoidance of the cytotoxic machinery of immune cells is a tactic employed by certain facultative intracellular organisms, a number of which are known to “shelter” inside macrophages, and survival of particular microbial pathogens in macrophages is thought to be reliant on the selective exploitation of specific phagocytic receptors that do not induce signalling cascades or cytotoxic mechanisms, such as pro-inflammatory cytokine release and phagolysosome maturation. Therefore entry via SR-A could be viewed as a relatively “safe” portal of entry, as SR-A has been found to suppress rather than induce a range of pro-inflammatory cytokine and microbicidal signalling mechanisms. It is likely that further studies will reveal other microorganisms that interact with this receptor.

Chapter 7

Summary of results and future work

Macrophages are considered the first line of defence against invading *Neisseria* species in the upper respiratory tract, and it is therefore important to consider the question of how these distinctive organisms are able to overcome a number of innate defence systems present in their environment. Macrophages express an array of surface receptors; multiple receptors can be involved in the processing of single bacteria cells. These events are initiated by the crucial step of ligand recognition via specific receptors. The relationship between meningococci and specific receptors may contribute to host colonisation, therefore this study focused on receptor-mediated recognition of *N. meningitidis* by uncoupling the sequential processes of adherence, internalisation and intracellular trafficking by macrophages.

Once meningococci are in the bloodstream complement-mediated responses are the first line of defence, and are usually dependent on bacterial lysis by C5b to C9 complement components. If organisms can evade such an attack then they are able to proliferate relatively unhindered, and reach concentrations that activate a more pronounced complement-mediated response that can lead to tissue damage by neutrophil activation. Hence the contribution of complement and immunoglobulin G to macrophage recognition of meningococci was examined. Opsonisation of bacteria, using serum obtained from an late-complement deficient disease (LCCD) patient, resulted in a 2-fold increase in bacterial adherence to the surface of the macrophages, compared to control organisms. Adherence of organisms opsonised using serum deplete of IgG was significantly reduced to levels equivalent with non-opsonised control bacteria; suggesting that meningococcal adherence to primary human macrophages is a Fc γ receptor-

mediated process. Subsequent internalisation was also measured, and though opsonisation enhanced bacterial uptake, the presence or absence of immunoglobulin did not alter the levels of phagocytosis, implying that non-Fc γ opsonic receptors, such as the complement receptors, are likely to play a more prominent role in opsonin-dependent meningococcal phagocytosis. A similar pattern of adherence and uptake had previously been observed in work carried out with *S. pneumoniae* and alveolar macrophages (Gordon, Irving et al. 2000).

Subsequent experiments were carried out to further explore the involvement of complement receptor, specifically CR3, in bacterial phagocytosis in the presence of serum opsonins using an anti-CD11b monoclonal antibody to block an epitope of the I domain. Previous studies with *Mycobacteria* (Peyron, Bordier et al. 2000) had found this antibody successful in inhibiting bacterial uptake, but no difference in meningococcal adherence or internalisation was reported; the only reduction was in percentage population of macrophages directly interacting with bacteria. This may be due to the fact that I-domain of CR3 has many epitopes to different micro-organisms bind, and so future work might involve the use of a panel of anti-CD11b monoclonal antibodies that recognise different epitopes on the I-domain; though some anti-I domain antibodies are also unsuitable as LPS was shown to displace the binding of M1/70, an anti-I domain mAb, to macrophages (Matsuno, Aramaki et al. 1998) and I domain mAbs do not inhibit binding of β -glucans (Thornton, Vetvicka et al. 1996). Measurement of the concentration of other serum opsonins, such as MBL could also be performed as this may effect immune serum-mediated bacterial uptake; coupled with this the degree of opsonin deposition on meningococci would be useful information. Lipid raft disruption using Nystatin also appeared to reduce recognition of opsonised bacteria, which suggests that uptake takes place on these cholesterol-rich microdomains that are utilised by a number of invasive pathogenic microorganisms as a means of avoiding immune detection. This may be a strategy employed by *N. meningitidis* but at the moment the tools for studying lipid raft involvement are fairly crude (see Chapter 5).

Once in the nasopharynx, meningococci are likely to be exposed to resident macrophages and dendritic cells which are widely distributed extravascularly where opsonins may be low or absent, and in the liver, adrenal glands and choroid plexus they would be in direct contact with blood and CSF (Gordon, Lawson et al. 1992): therefore non-opsonic interactions of serogroup B *N. meningitidis* were examined. The lectin-binding site of CR3, to which LPS can directly bind, was blocked using NADG, as well as monoclonal antibody. *N. meningitidis* did not appear to be able to adhere to macrophages via this binding site of CR3. Use of a wider range of monosaccharides and polysaccharides to block the lectin site may be helpful; and a number of anti-I domain antibodies have also been found to block the lectin site and could be utilised. It might also be of interest to prime macrophages with zymosan (contains β -glucan) and observe if this affects on uptake of opsonised meningococci. Peripheral blood monocytes obtained from LAD patients may indicate if reduction or absence of complement receptors enhances susceptibility to meningococcal disease.

By comparing meningococci with two other *Neisseria* species, *N. lactamica* and *N. animalis*, which are not known to be pathogenic in humans I observed that these two strains were bound and internalised by macrophages more efficiently than the pathogenic strains of *N. meningitidis* using this assay. The *N. meningitidis* strains used are capsulate and express both Opa and PilC, whereas *N. lactamica* lacks Opa expression and *N. animalis* expresses neither Opa nor PilC. Of all three *Neisseria* species examined, *N. animalis* was internalised most effectively by MDM, which is consistent with the fact that this strain has never been isolated in humans but is more usually found in mammals such as guinea pigs, and is consequently likely to be destroyed by the cell. *N. lactamica* was found to adhere most efficiently to macrophages, but internalisation was similar to that of *N. meningitidis*. As *N. lactamica* is a non-pathogenic organism usually carried during early childhood, and this carriage is associated with an increase in antibody titres to pathogenic meningococci strains (Zorgani, James et al. 1996),

perhaps internalisation and degradation of the non-pathogen *N. lactamica* is beneficial to the host, by permitting the induction of an acquired immune response that is cross-reactive with *N. meningitidis*.

Although a relatively rare occurrence, the fact meningococci are able to avoid the gauntlet of innate immune responses, suggests that either host cells fail to recognise and destroy the invasive bacteria, or that virulence determinants on the outer surface of the microbes are successful in preventing direct recognition. I therefore examined the role of meningococcal LPS in host cell recognition. The use of *lpxA* mutant of *N. meningitidis* suggested that non-opsonic phagocytosis, but not adherence, was dependent to a large degree on the presence of LPS, as uptake of the mutant was reduced by 72.5%, and cytokine secretion was also reduced. The fact that uptake was not completely abolished and the mutant organisms stimulated lower levels of cytokine production suggests that other bacterial outer membrane components may play a role in meningococcal-macrophage interactions. Dissection of signal transduction pathways in macrophages could be used to investigate role of meningococcal LPS in greater detail, using LPS-deficient and truncated mutants.

As a result of the findings using *lpxA* mutant in Chapter 4, a number of receptors known to bind LPS were examined in more detail. CD14 and SR-A appeared to contribute most to the non-opsonic adherence and phagocytosis of *N. meningitidis*. All GPI-linked proteins were initially removed from the surface of the cells using PIPLC enzyme, and this resulted in a dramatic reduction in bacterial adherence. Attention was therefore directed at CD14, a GPI-linked pattern recognition receptor (PRR) that was known to bind LPS, but not whole gram-negative bacteria. Inhibition of CD14 resulted in a marked reduction in bacterial association to macrophages that affected subsequent internalisation of bacteria. Human CD14 was also examined in isolation of a number of other LPS receptors, to determine whether this receptor could enhance meningococcal binding to endothelial HeLa cells when transfected. Compared to control cells

over 60% increase in meningococcal binding to CD14-transfected HeLa cells was observed. As the functions of Toll-like receptors and CD14 are intimately linked, and previous work has implicated TLR4/MD2 with CD14 in induction of a pro-inflammatory cytokine response using *N. meningitidis* (Pridmore, Wylie et al. 2001) blockade of macrophage TLRs may shed more light on this relationship; but as yet the relevant blocking monoclonals are not available. Attempts were made to obtain peripheral blood monocytes from PNH patients, as the cells would innately lack GPI-linked proteins to varying degrees depending on the severity of disease, but this proved difficult as it is a relatively rare condition.

Study of bacterial interactions using primary human cells can be problematic, due to the high levels of variation between donors, therefore a suitable cell-line models for the use of *N. meningitidis* studies was sought. As permanent human mature cell lines do not exist, THP-1 cells were differentiated using phorbol myristate acetate (PMA), and numerous studies have shown that the *in vitro* differentiation of THP-1 are an appropriate model to study macrophage-pathogen interactions, as differentiation induces the cells to take on a more macrophage-like phenotype, resulting in the improved phagocytic capability of the cells compared to undifferentiated cells (Tsuchiya, Kobayashi et al. 1982). Following differentiation, THP-1 cells displayed an increased capacity to internalise meningococci was detected and was accompanied by a marked increase in numbers of bacteria adhering to the surface of the cells. Differentiation appears to result in the up-regulation of receptors essential for the capture *N. meningitidis* by macrophages. Observations using MDM cells blocked with Poly I, SRA inhibitor, suggest that phagocytosis of *N. meningitidis* is mediated, to a large degree, by class A scavenger receptors; and the use of PMA-differentiated THP-1 (dTHP-1) cells also supported the hypothesis that the phagocytosis of *N. meningitidis* is governed to a large part by the presence of specific macrophage receptors. The lack of available anti-human SR-A antibodies, meant Poly I was used to block bacterial interactions with class A scavenger receptors, and this

reagent was effective as phagocytosis was inhibited in both MDM and dTHP-1 cells. At concentrations used in study, Poly I blockade had no significant effect on levels of cytokine secreted into the surrounding media suggesting that macrophage cytokine production is not dependent on numbers of bacteria being internalised and is SR-A-independent. It has also been suggested that Poly I may interact with other members of the scavenger receptor family, CD36 and MARCO, but neither are known to play a role in bacterial phagocytosis, and no evidence exists to show CD36 binds bacteria, and MARCO is only expressed in a small subset of macrophages (Elomaa, Kangas et al. 1995). A number of factors may affect this data including, levels of SRA expression, recycling of receptor, clustering or limiting levels of bacteria or intracellular molecules. Animal studies suggest that scavenger receptors are important in bacterial infections, including *Neisseria* (Suzuki, Kurihara et al. 1997; Thomas, Li et al. 2000; Peiser and Gordon 2001), as complement components are widely distributed *in vivo*, it is less problematic to assess the contribution of non-opsonic receptors *in vitro*.

Resident macrophages have reduced microbicidal mechanisms available than activated macrophages, and uptake of *N. meningitidis* by these cells via SR-A might provide some protection against complement, IgA or clearance. CD14 expression is reduced on these tissue macrophages (Landmann, Muller et al. 2000) but SR-A expression is high (Gough, Greaves et al. 1999), which suggests these cells might be less sensitive to endotoxin than monocytes or activated macrophages and might help prevent immune activation by scavenging less LPS in a CD14-independent manner via SR-A.

Targeting molecules for SR-mediated internalisation is followed by the display of modified specific antigens to T-cells by MHC class I (Abraham, Singh et al. 1995; Bansal, Mukherjee et al. 1999). Cultured dendritic cells can express SR-A and phagocytic cells, capable of antigen presentation and stimulation of naïve T cells (Platt, da_Silva et al. 1999). Further study with these cells should help determine if SR-A are involved in these processes. Further examination of

receptor signalling and cell biology is required to shed more light on role of SR-A in host defence.

Mucosal damage may alter invasion and colonisation of meningococci and activities such as smoking are associated with increased risk of invasive disease. Activated macrophages are also more likely to be recruited to sites of mucosal damage, and could help explain the increased risk of infection to smokers (Brandtzaeg 1995) and if they internalise *N. meningitidis* could traffic the organisms to the parts of the body. Dendritic cells, which may also express SR-A (Platt, da_Silva et al. 1999), may also be used as a vehicle for bacteria to move to other parts of the body, so interactions of meningococci with these cells should also be examined. Further work to identify whether other *Neisseria* species and strains, such as *N. lactamica*, are also internalised via SR-A would help determine if indeed the ligand recognised is conserved across species and thus a general receptor for colonisation. If this were the case, diseases caused by pathogenic *Neisseria* may be a result of other virulence mechanisms. Following immune activation of macrophages their role in meningococcal pathogenesis may become more protective, as scavenger receptors may function in macrophage control of inflammation, (reviewed in Chapter 1) as high levels of LPS are correlated with disease severity and are characteristic of MCD (Brandtzaeg 1995). Excess meningococcal LPS would also be in contact with Kupffer cells and marginal spleen zone macrophages in the spleen, which express high levels of SR-A (Hughes, Fraser et al. 1995) but not CD14. These macrophages would be well placed to clear excess LPS from the circulation without release of pro-inflammatory molecules and help prevent septicaemia.

Further experiments that could be undertaken, includes the use of FRET analysis to study the molecular proximity of CD14 and SR-A on the surface of macrophages, and explore the possibility that SR-A may exist as part of a more complex signalling complex. Also studies carried out by Yamamoto and colleagues (Yamamoto, Ebe et al. 1999) suggested that CD14 and SR-A receptor

expression of alveolar macrophages were upregulated in response to endotoxin exposure. Specifically, a significant number of CD14 single positive and SR-A single positive cells were observed over time; and a minor population of CD14/SR-A double positive cells were also noted. As this thesis indicates the possible involvement of both CD14 and SR-A in macrophage interactions with meningococci, I performed preliminary experiments by exposing MDM cells to purified LPS of *N. meningitidis* H44/76 before incubating with SR-A ligand DiI-acLDL or labelled CD14 mAb, and analysing by flow cytometry for increases in receptor expression: unfortunately this set of experiments was unsuccessful. It would also be of interest to attempt to characterise events governing signal transduction for SR-A receptors in response to meningococcal LPS. Measurement of nitric oxide production of Poly I treated cells to further examine the role of SR-A in host cytotoxic responses. Initial genetic studies linking SR-A with disease, by studying polymorphisms in human receptor gene to determine a link with meningococcal infection has yet to be completed, and this work could be expanded to include CD14.

Many aspects of macrophage-meningococcal interactions have yet to be clarified, including the roles of SR-A, CD14 and CR3 on activated macrophages and dendritic cells, and the role of lipid-rich rafts in these interactions should also be studied in more detail.

Chapter 8

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Poster

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Poster and short presentation

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