

Dectin-1: receptor internalisation, trafficking and biological effects in macrophages

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For my parents

Abstract

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In host defence, pattern recognition plays an essential role by enabling the immune system to discriminate self from pathogenic non-self. Pattern recognition is mediated by leukocyte expressed pattern recognition molecules (PRMs), which recognise pathogen associated molecular patterns (PAMPs) on pathogens. Phagocytosis is a critical event for anti-microbial defence and its contribution is not limited to the clearance and killing of pathogens, but extends to the activation of adaptive immunity through production of pro-inflammatory mediators and antigen presentation. Anti-fungal immunity is extremely efficient and operates via recognition, phagocytosis and killing of fungal pathogens by leukocytes. We have examined Dectin-1, a non-opsonic pattern recognition receptor that recognises live fungi and fungal derived particles and that is highly expressed on various leukocyte populations. We wanted to establish whether Dectin-1 contributes to anti-fungal defence by analysing various aspects of the receptor biology.

Using both confocal microscopy and flow-cytometry, we demonstrate that Dectin-1 is a phagocytic receptor. Furthermore, using cell lines expressing receptor mutants, we show that this capacity is mediated by the membrane proximal tyrosine residue located in the ITAM-like motif. This makes Dectin-1 the first described phagocytic leukocyte expressed receptor for unopsonised fungi and fungal derived particles, and the first pattern recognition receptor that mediates phagocytic uptake through a tyrosine based motif. We demonstrate that the mechanisms by which Dectin-1 mediates cytoskeletal activation and actin polymerisation are novel, and not shared with the canonical ITAM containing Fc γ R_s. In particular the observation that Syk kinase plays no role in Dectin-1 mediated phagocytosis in macrophages. We show that Dectin-1 mediates cellular activation in response to zymosan particles and that these β -glucan dependent biological effects require collaboration with toll-like receptors (TLRs) at the cell surface. We also show that ligand size determines intracellular receptor trafficking following internalisation. Furthermore, we show that when biologically active soluble glucans are internalised by Dectin-1, the receptor is retained intracellularly yet, when biologically silent glucans are used, Dectin-1 is recycled. Dectin-1 is thus established as both an important phagocytic fungal pattern recognition receptor with pro-inflammatory abilities and an additional tool with which to study the diversity of signalling processes associated with leukocyte expressed receptors.

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Abbreviations used in this thesis

2A11	Rat anti-murine Dectin-1 monoclonal antibody, IgG2b subclass
3T3-D1	NIH-3T3 cells stably expressing full-length Dectin-1
Ab	Antibody
ARF	ADP ribosylation factor
BCA	Bicinchoninic acid
BMDM	Bone marrow derived macrophages
BSA	Bovine serum albumin
CHO	Chinese hamster ovary cell line
CLEC	C-type lectin-like receptor
CT-HA	C-terminal HA tag
DC	Dendritic cell
ddH₂O	Distilled deionised water
DMSO	dimethylsulfoxide
dNTPs	Deoxynucleotide triphosphates
ECL	Enhanced chemoluminescence
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FcγR	Immunoglobulin Fc-gamma receptor
Fig.	Figure
FITC	Flourescein isothiocyanate
FMLP	formyl-methionyl-leucyl-phenylalanine
g	Gravity
G418	Geneticin
GEF	GTP exchange factor
GROα	growth-related gene product α
HRP	Horseradish peroxidase
IL	interleukin
IP-10	interferon- γ -inducible protein of 10 kDa
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunireceptor tyrosine-based inhibition motif
kDa	Kilodalton
LAM	Lipoarabinomannan
LCM	L929 cell conditioned medium
LOX	oxidised low-density lipoprotein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LTB₄	leukotriene B ₄
mAb	monoclonal antibody
MARCKS	myristoylated, alanine-rich C kinase substrate
MCP-1	monocyte chemotactic protein-1
M-CSF	Macrophage colony stimulating factor
MIP-1α	macrophage inflammatory protein-1 α
Mphi	Thioglycollate-elicited macrophages
MyD88	myeloid differentiation factor 88
NK	natural killer
NKRP	natural killer cell receptor protein

NT-HA	N-terminal HA tag
PAGE	Polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular pattern
PBS	Phosphate buffered saline
PE	Phycoerythrine
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C
PMN	Polymorphonuclear leukocyte
PRM	pattern recognition molecule
PRR	pattern recognition receptor
RAW-D1	RAW 264.7 cells overexpressing full-length epitope tagged Dectin-1
SDS	Sodium dodecyl sulphate
Syk	p72Syk kinase
Th1	type 1 T-helper cells (IFN- γ secretion)
Th2	type 2 T-helper cells (IL-4, IL-5 production)
TIRAP	Toll-interleukin 1 receptor (TIR) domain-containing adapter protein
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
Trif	Toll/IL-1 receptor (TIR) domain-containing adaptor
Tris	Tri(hydroxymethyl)aminomethane
TRITC	Rhodamine
WASP	Wiscott Aldrich Syndrome Protein
WT	Wild type

Table of contents

Chapter 1: Introduction

1.1	Innate immunity and its role in host defence.....	1
1.2	Cells of the innate immune system.....	2
1.2.1	Macrophages and their roles in immunity and homeostasis.....	2
1.2.2	Neutrophils, the front line of host defence.....	7
1.2.3	Natural killer cells support inflammation.....	9
1.2.4	Dendritic cells, a bridge between innate and adaptive immunity.....	11
1.3	Innate immune and phagocytic receptors found on leukocytes.....	14
1.3.1	Phagocytosis promoting receptors.....	15
1.3.2	Self vs non-self discrimination: the TLRs.....	15
1.4	PAMPS and their effects on immune responses.....	19
1.4.1	Lipopolysaccharide.....	20
1.4.2	Fungal derived carbohydrates.....	21
1.4.2.1	Beta Glucans.....	21
1.4.2.2	Mannans.....	22
1.4.2.3	Other microbial PAMPS.....	22
1.5	Mechanisms of phagocytosis in macrophages.....	23
1.5.1	Phagocytic signalling.....	23
1.5.2	Fc γ Receptor mediated phagocytosis.....	24
1.5.2.1	Early signalling events.....	25
1.5.2.2	Signals leading to cytoskeletal remodelling.....	26
1.5.2.3	Signals leading to actin nucleation.....	27
1.5.2.4	Membrane remodelling, pseudopod extension and phagosome closure.....	28
1.5.2.5	Macrophage Fc γ Receptor mediated inflammatory signalling.....	29
1.5.2.6	Negative regulation of phagocytic signalling.....	30
1.5.3	Complement receptor-3 mediated phagocytosis.....	30
1.5.3.1	Cells expressing CR3 require activatory signals to induce phagocytosis....	31
1.5.3.2	CR3 phagocytic mechanisms differ from those of Fc γ Receptors.....	32
1.5.3.3	CR3 mediated cytoskeletal signalling.....	32
1.5.3.4	CR3 mediated inflammatory signalling.....	34
1.5.4	Other integrins.....	35
1.5.5	Lectin-mediated phagocytosis.....	36
1.5.5.1	Mannose receptor.....	36
1.5.5.2	DC-SIGN.....	37
1.5.6	Scavenger receptor class A mediated phagocytosis.....	38
1.5.7	IgG superfamily adhesion receptors.....	38
1.6	Phagosomal maturation.....	39
1.7	Dectin-1.....	41
1.7.1	Innate immune responses to fungal pathogens.....	41
1.7.2	The identification of murine Dectin-1 as a fungal receptor.....	44
1.7.3	Dectin-1 is expressed on the monocyte/macrophage and neutrophils.....	45
1.7.4	Dectin-1 is a classic PRR for fungal β -glucans.....	46
1.7.5	Dectin-1, and not CR3, is the principal non-opsonic β -glucan receptor on macrophages.....	48
1.7.6	Human Dectin-1.....	49
1.7.7	Dectin-1 recognises an endogenous ligand on T-cells.....	50
1.8	Unanswered questions.....	52

1.9	Aims of the thesis.....	53
-----	-------------------------	----

Chapter 2: Materials and methods

2.1	Materials.....	56
2.1.1	General materials.....	56
2.1.2	Materials for cell culture.....	56
2.1.3	Materials for cell treatment.....	56
2.1.4	Materials for inflammatory macrophage production.....	57
2.1.5	Materials for cell transfection.....	57
2.1.6	Materials for phagocytic particle labelling and opsonisation.....	57
2.1.7	Materials for molecular biology.....	57
2.1.8	Materials for confocal microscopy.....	58
2.1.9	Materials for flow cytometry.....	58
2.1.10	Materials for protein determination.....	59
2.1.11	Materials for metabolic labelling.....	59
2.2	Solutions.....	59
2.3	Cells.....	61
2.3.1	Primary cells and cell lines.....	61
2.3.2	General cell culture.....	62
2.3.3	Primary murine macrophages.....	62
2.4	Methods.....	64
2.4.1	General methods.....	64
2.4.1.1	Polymerase chain reaction (PCR).....	64
2.4.1.2	Agarose gel electrophoresis.....	65
2.4.1.3	Purification of DNA from agarose gels.....	66
2.4.1.4	Phenol chloroform extraction.....	66
2.4.1.5	Plasmid digestion.....	66
2.4.1.6	Plasmid dephosphorylation.....	67
2.4.1.7	Ligation of plasmid vectors and insert DNA.....	68
2.4.1.8	Transformation and colony selection.....	68
2.4.1.9	Colony PCR.....	69
2.4.1.10	Plasmid mini prep.....	69
2.4.1.11	Plasmid sequencing.....	70
2.4.1.12	Maxipreps of bacterial transformants.....	70
2.4.1.13	Isolation of genomic DNA.....	72
2.4.1.14	Ployacrylamide gel electrophoresis (PAGE).....	73
2.4.1.15	Western blotting.....	74
2.4.1.16	Enzyme-linked Immunosorbent Assay (ELISA).....	74
2.4.1.17	Antibody testing.....	75
2.5	General cell biology techniques.....	76
2.5.1	Transient transfection of constructs using cationic lipids.....	76
2.5.2	Electroporation.....	77
2.5.3	Virus production.....	77
2.5.4	Retroviral transduction of fibroblast and macrophage cell lines.....	78
2.5.5	Flow cytometry.....	78
2.5.6	Confocal microscopy.....	80
2.5.7	Synchronising phagocytic particle uptake.....	81
2.5.8	Inhibition of protein synthesis.....	81
2.5.9	Inhibitor treatments of cells.....	82

2.6	Techniques specific to this thesis.....	83
2.6.1	Generation of cell lines stably expressing Dectin-1 molecules.....	83
2.6.1.1	HA tagged plasmid production.....	83
2.6.1.2	Dectin-1 mutant receptor generation by PCR.....	84
2.6.1.3	Transduction of cell lines.....	86
2.6.1.4	Cell line characterisation by flow cytometry.....	86
2.6.2	Comparative internalisation assay.....	87
2.6.2.1	Sheep red blood cell (SRBC) membrane labelling and opsonisation.....	87
2.6.2.2	Cell plating.....	87
2.6.2.3	Inhibitor treatments.....	87
2.6.2.4	Synchronised particle phagocytosis.....	88
2.6.2.5	Flow cytometry.....	88
2.6.3	Receptor recycling assays.....	88
2.6.3.1	Cells and cell culture.....	88
2.6.3.2	Inhibition of protein synthesis.....	89
2.6.3.3	Low pH elution of bound ligands.....	90
2.6.3.4	Ligand induced receptor internalisation.....	90
2.6.4	Dominant negative RhoGTPase assay.....	91
2.6.4.1	Plasmid preparation.....	91
2.6.4.2	Transfection of plasmids.....	91
2.6.4.3	Zymosan particle labelling.....	92
2.6.4.4	Three-colour flow cytometry.....	92
2.6.5	Analysis of the biological effects of zymosan.....	93
2.6.5.1	TNF- α determination.....	93
2.6.5.2	Transfection of epitope tagged TLR constructs and confocal microscopy..	93
2.6.5.3	Inhibitor studies.....	94
2.7	Statistical analysis.....	94

Chapter 3: Studies in NIH-3T3 cell models

3.1	Introduction.....	96
3.2	Aims of the study.....	97
3.3	Dectin-1 is a phagocytic receptor.....	98
3.4	Internalisation of zymosan particles.....	99
3.4.1	Development of a quantitative assay to measure phagocytic efficiency....	99
3.4.2	Dectin-1 internalises bound particles efficiently.....	99
3.5	Establishing 3T3-D1 mutant cell line.....	100
3.5.1	Sequence alignments.....	100
3.5.2	Synchronised particle binding.....	101
3.6	Effects of cytoplasmic tail mutation on Dectin-1 mediated phagocytosis..	102
3.7	Dectin-1 cytoskeletal signalling in a non-phagocyte model.....	103
3.8	The Rho GTPases Cdc42 and Rac-1 mediate cytoskeletal activation.....	105
3.8.1	Three colour flow cytometry.....	106
3.8.2	Rac-1 and Cdc42, but not RhoA play a role in zymosan phagocytosis....	107
3.9	Discussion.....	107

Chapter 4: Dectin-1 mediated phagocytosis in macrophages

4.1	Introduction.....	112
4.2	Aims of the study.....	113

4.3	Results.....	113
4.3.1	Modification of the Dectin-1 cytoplasmic tail results in diminished phagocytic uptake by macrophages.....	113
4.3.2	RAW 264.7 cell lines over-expressing HA-tagged Dectin-1 show enhanced recognition and phagocytosis of β -glucan rich particles.....	115
4.3.3	Sheep red blood cell phagocytosis by Fc γ Rs in RAW-D1 cells.....	117
4.3.4	Dectin-1 mediated phagocytosis is Syk independent in macrophages.....	118
4.4	Discussion.....	119

Chapter 5: Dectin-1 mediates the biological effects of β -glucans

5.1	Introduction.....	124
5.2	Aims of the study.....	125
5.3	Results.....	125
5.3.1	Zymosan-induced TNF- α production by murine macrophages is β -glucan dependent.....	125
5.3.2	Zymosan-induced TNF- α production increases with Dectin-1 expression.....	126
5.3.3	Dectin-1 also mediates TNF- α production in response to live yeast.....	126
5.3.4	Zymosan induced TNF- α production depends on the TLR pathway.....	127
5.3.5	Dectin-1 and TLR 2/6 co-localise in nascent phagosomes.....	127
5.3.6	Increased availability of TLR2 ligand does not increase TNF- α production.....	128
5.3.7	The cytoplasmic tail of Dectin-1 is essential for zymosan induced TLR activation.....	128
5.3.8	Dectin-1 and TLR mediated TNF- α production does not depend on phagocytosis.....	129
5.3.9	Kinases and GTPases responsible for Dectin-1 mediated cytoskeletal rearrangements play no role in zymosan mediated TNF- α production.....	130
5.4	Discussion.....	131

Chapter 6: Dectin-1 receptor trafficking in response to β -glucan ligands

6.1	Introduction.....	137
6.2	Aims of the study.....	140
6.3	Results.....	140
6.3.1	Particulate ligands of Dectin-1 are not released from the receptor at low pH.....	140
6.3.2	Primary macrophages retain Dectin-1 intracellularly following internalisation of particulate ligands.....	141
6.3.3	Dectin-1 trafficks to to lysosomes along with its particulate cargo.....	142
6.3.4	Size determines intracellular trafficking of Dectin-1 following soluble ligand mediated internalisation.....	143
6.3.5	Internalised soluble ligands do not traffic to lysosomes.....	144
6.4	Discussion.....	145

Chapter 7: Final summary, discussion of results and future work

7.1	Original aims.....	148
-----	--------------------	-----

7.2	Summary of key findings.....	148
7.2.1	Dectin-1 is a phagocytic receptor.....	149
7.2.2	Dectin-1 mediated phagocytosis depends on the membrane proximal tyrosine in the ITAM-like motif.....	150
7.2.3	Dectin-1 mediated phagocytic signalling differs from that of Fcγ and Complement receptors.....	150
7.2.4	Dectin-1 mediates biological responses to β-glucans in macrophages.....	151
7.2.5	Dectin-1 does not recycle from phagosomes.....	152
7.2.6	Dectin-1 mediated endocytosis results in differential, ligand size dependent, intracellular receptor trafficking.....	153
7.3	Working models.....	154
7.3.1	Dectin-1 mediated signalling.....	154
7.3.2	Dectin-1 trafficking following internalisation.....	156
7.4	Future work.....	157
7.5	Closing remarks.....	158

Chapter 8: References

8.1	References.....	160
-----	-----------------	-----

Appendix

A1	Published work.....	180
A2	Work in submission	

Chapter 1

Introduction

1.1 Innate immunity and its role in host defence

The primary role of the immunity is host defence and, in vertebrates, immune responses to microbial pathogens are reliant on both the innate and adaptive components of the immune system (Hoffmann, Kafatos et al. 1999). The innate immune system is ancient and phylogenetically conserved in all multi-cellular organisms while only vertebrates have an adaptive system. The first line of host defence is the innate response, which reacts very rapidly and is mediated primarily by leukocytes such as neutrophils and macrophages that can phagocytose and kill pathogens. These cells also coordinate subsequent host responses by synthesising and releasing a wide range of inflammatory mediators and cytokines (Aderem and Underhill 1999).

The main challenge to the immune system is how, with the use of a limited number of germline-encoded receptors, to discriminate diverse pathogens from self. This difficulty is compounded by the capacity of pathogens to mutate in response to selective pressure. In order to meet the challenge, multi-cellular organisms evolved a variety of receptors that recognise conserved motifs only found on pathogens. These motifs are called pathogen associated molecular patterns (PAMPS) and are recognised by what are known as pattern recognition molecules (PRMs) (Janeway 1992), which include cell surface expressed pattern recognition receptors (PRRs) (Janeway and Medzhitov 2002). Recognition of pathogens by the innate system leads to a rapid inflammatory response and containment of the challenge. This process usually occurs without recourse to adaptive immunity, that only becomes involved when the innate immune response is unable to contain the infection (Janeway and Medzhitov 2002).

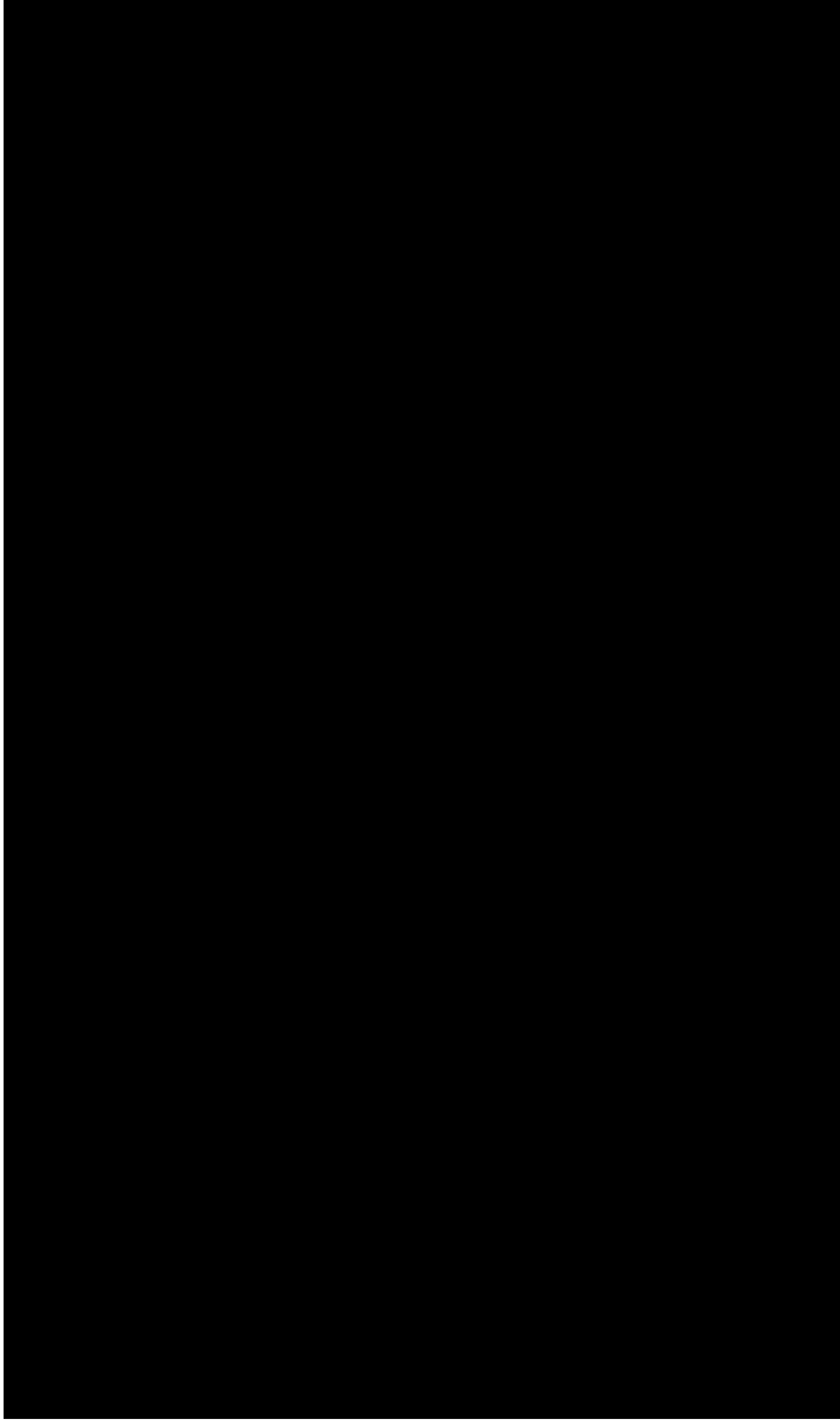
The PAMPS recognised by PRMs are not only essential structural components of pathogens, but are often specific to particular groups of pathogens, for instance mannans and beta-glucans in yeast cell walls, lipopolysaccharide (LPS) in Gram-negative bacteria, lipoteichoic acid (LTA) in Gram-positive bacteria and lipoarabinomannan (LAM) in mycobacteria (Aderem and Ulevitch 2000). The innate immune system thus not only distinguishes self from pathogenic non-self, but can also discriminate between different classes of pathogens. PRMs exist in a variety of forms including soluble or secreted molecules, molecules expressed on the cell surface, and intracellular molecules (see **table 1.1**). Over the past few years the numerous membrane bound PRRs have aroused much interest, with particular attention directed at the family of Toll like receptors (TLRs).

1.2 Cells of the innate immune system

Central to the mammalian innate immune defences are the professional phagocyte populations consisting of dendritic cells (DCs), monocyte/macrophages and polymorphonuclear leukocytes (PMNs, also known as neutrophils) and cytokine producing NK cells. These cells defend the host against pathogens by internalising and killing microbes, initiating and regulating inflammation as well as maintaining homeostasis by clearing cellular debris and shutting down unnecessary inflammatory processes by scavenging mediators. Innate immune cells have developed some degree of specialisation in terms of their functions in host defence and homeostasis and these will be briefly reviewed.

1.2.1 Macrophages and their roles in immunity and homeostasis

Table 1.1 Microbial Pattern Recognition Molecules (Modified from Brown et al 2003)



Macrophages play a central role in the innate recognition of a range of pathogens and in the modulation of the subsequent effector mechanisms. Blood monocytes are derived from bone marrow precursors called monoblasts and promonocytes and mature into macrophages in the host tissues (Ross and Auger 2002). In order to reach the tissues monocytes must leave the blood stream; this requires adhesion to blood vessel walls, a function fulfilled by a number of surface receptors that include β -integrins. After adhesion, monocytes pass between endothelial cells by diapedesis and migrate into the tissues where differentiation into mature macrophages occurs (Muller and Randolph 1999). Factors in the local microenvironment appear to contribute to the wide morphological and functional heterogeneity displayed by these mature macrophages (Gordon 1999). Resident populations of macrophages are found near or at all portals of entry for pathogens such as the gut, lungs or skin, as well as in the stroma of organs central to host defence such as the liver, spleen, bone marrow and lymph nodes. As part of the brain parenchyma microglial cells provide both functional support for neurones as well as acting as immune sentinels (Crocker, Morris et al. 1991; Perry, Andersson et al. 1993). Monocytes can also differentiate into dendritic cells (DCs) (section 1.2.4) that have the unique ability to mature in response to various stimuli, migrate out of the tissues to secondary lymphoid organs and prime naïve T cells (Mellman and Steinman 2001).

Macrophages either directly recognise pathogens through their PRRs or indirectly by recognising opsonins like antibodies or C3 coating the microbial surface. The expression of PRRs varies in different macrophage populations and is also subject to regulation by cytokines and other immune-modulating agents such as LPS (McKnight and Gordon 1998). Macrophage PRRs, in addition to mediating pathogen recognition,

can also determine the mechanism and route of cellular uptake as well as the subsequent macrophage responses. Multiple PRRs and opsonic receptors can recognise a single pathogen, so internalisation and inflammatory activation are likely to be the aggregate of these interactions (Ernst 1998; Underhill and Ozinsky 2002).

Recognition of a microbe by a macrophage generally leads to internalisation and killing of the pathogen. Macrophages express a multitude of innate immune receptors and have a prodigious capacity to ingest microbes. When microbes are larger than 0.5 μ m, internalisation occurs through the actin-dependent process of phagocytosis (**Fig. 1.2.1**) (Aderem and Underhill 1999). The best understood mechanisms of phagocytosis are those mediated by the antibody Fc γ (Fc γ Rs) and complement receptors (CRs) discussed in detail in 1.5.2 and 1.5.3. Phagocytosis in macrophages occurs through distinct mechanisms, driven specifically by the particular receptors ligated. Microbes can also be ingested by other mechanisms like macropinocytosis (**Fig. 1.2.1**), an actin dependent process whereby membrane ruffles form large random vacuoles that can surround particles bound to the cell surface (Rittig, Wilske et al. 1999).

Particles smaller than 0.5 μ m are internalised by a process called endocytosis that is largely independent of actin polymerisation. 'Endocytosis' encompasses several diverse mechanisms (**Fig. 1.2.1**) by which cells internalize macromolecules into transport vesicles derived from the plasma membrane. Endocytosis regulates entry into the cell and plays a crucial role in development, the immune response, neurotransmission, intercellular communication, signal transduction, and cellular and organismal homeostasis. Endocytosis contributes to cellular homeostasis by helping to transport

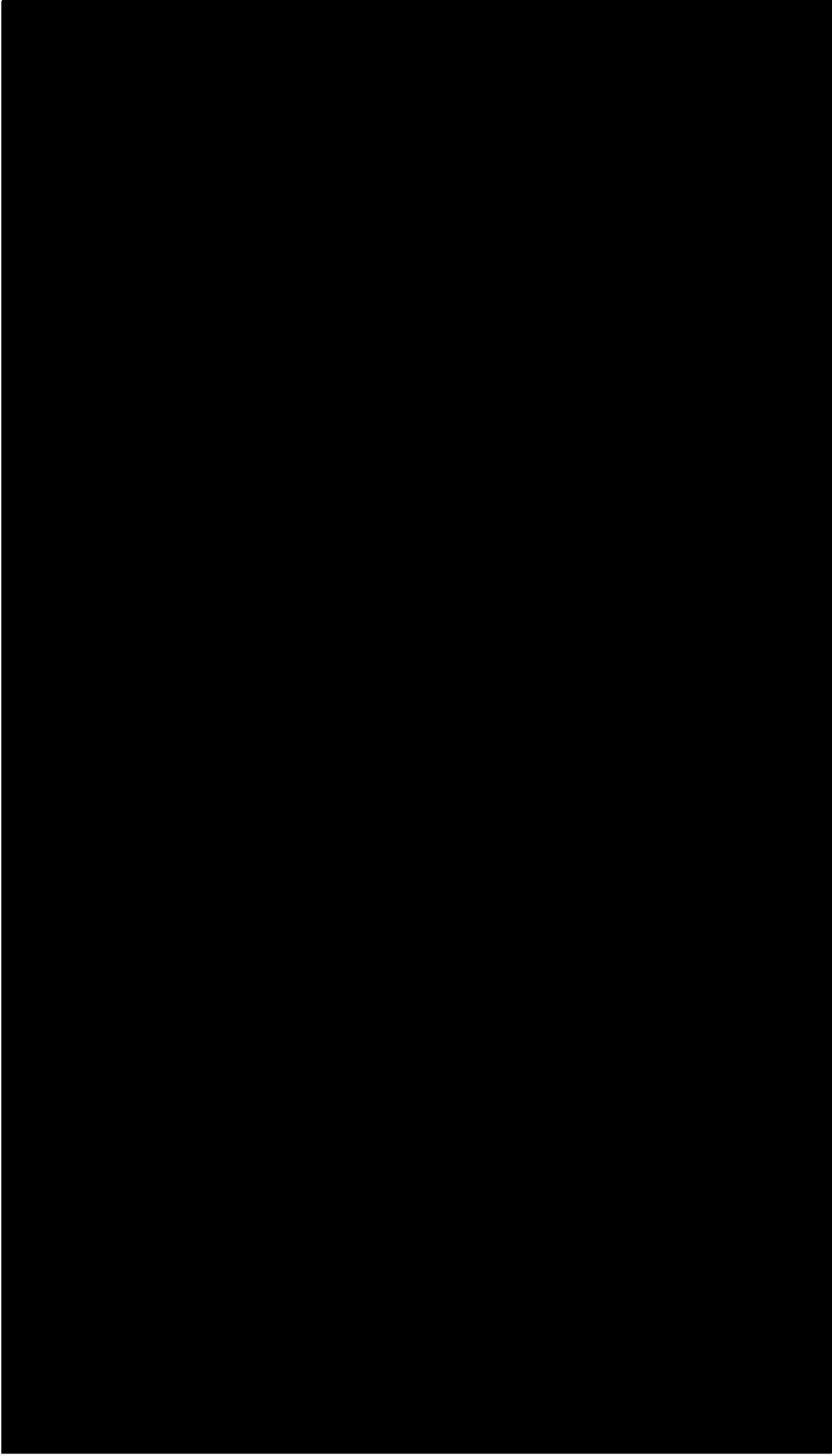


Figure 1.2.1 (Modified from Brown et al 2003)

Macrophages have a number of size-dependent mechanisms to internalise extra-cellular material at their disposal. Larger particles are internalised in an actin-dependent fashion by phagocytosis and phagosomes traffic to the peri-nuclear region of macrophages, interacting with the endocytic machinery along the way to acquire the characteristics of mature phagosomes. Macropinocytosis is also actin-dependent, but is not driven by particular receptor binding events, and serves to sample the extra-cellular environment. Macropinosomes join the endocytic pathway. Endocytosis transports smaller ligands and is usually driven by the ligation of receptors congregated in clathrin-coated pits. Endocytosis is actin-independent and internalised endosomes interact with other endosomes, macropinosomes, the golgi apparatus and phagosomes. Endosomes also mature through the acquisition of components of lysosomes by fusion events. Caveolae are unusual types of endosomes that are formed in lipid rich membrane domains by caveolin proteins, and caveolae mostly function in nutrient transport.

nutrients, clear debris released by infection or to modulate inflammation by scavenging inflammatory mediators such as LPS (Conner and Schmid 2003).

Microbes internalised by phagocytosis are exposed to an increasingly hostile environment. Phagosome membranes change through a number of sequential steps that result from interactions with the endocytic pathway, and perhaps also the endoplasmic reticulum (**Fig.1.2.1**), in a process referred to as maturation that is discussed in more detail later (Desjardins, Huber et al. 1994; Beron, Alvarez-Dominguez et al. 1995; Desjardins, Nzala et al. 1997; Lovdal, Andersen et al. 2000; Garin, Diez et al. 2001). As phagocytosed microbes are transported from the periphery to the perinuclear regions of the macrophage, their phagosomes acquire a hydrogen pump that lowers the pH, as well as a number of hydrolytic enzymes such as cathepsin-D. In response to signals emanating from the receptors recognising the pathogen, microbes are exposed to reactive oxygen intermediates (ROIs) generated by a phagosomal membrane-associated protein complex called the phagocyte NADPH oxidase (phox) usually resulting in microbial killing (Hampton, Kettle et al. 1998; Karlsson and Dahlgren 2002). Another anti-microbial enzyme, inducible nitric oxide synthetase (iNOS) is recruited to phagosomes and its activity induced by cytokines and microbial products such as LPS (MacMicking, Xie et al. 1997). In the peri-nuclear region the final stages of maturation occur and phagosomes fuse with lysosomes generating a highly degradative environment in which microbes are ultimately killed and digested (Brown and Gordon In Press).

Macrophages need to be activated to optimise their capacity to control infection, and both microbial products and cytokines drive this activation. Three activated

macrophage phenotypes have been described and these are termed innate, classical and alternative activation, respectively. Innate activation occurs in response to intact organisms as well as microbial products and results in changes in pro- and anti-inflammatory metabolite secretion and in increased expression of co-stimulatory molecules and PRRs such as scavenger receptors (Gordon 2001). Classical activation occurs in response to IFN- γ produced by NK and T-cells that are responding to antigen presenting cell (APC) cytokines, and leads to increased expression of MHC class II and iNOS and primes macrophages to secrete high levels of inflammatory mediators in response to microbial stimuli (Gordon 1999; Brown and Gordon In Press). Alternative activation refers to the phenotype induced by IL-4 and IL-13 with elevated expression of MHC class II and MMR as well as cell fusion leading to giant cell formation (Gordon 2003). Activated macrophages produce a large variety of proteins involved in host defence; these include products toxic to microbes, opsonic complement proteins, cytokines, chemokines and MHC proteins involved in antigen presentation. Eventually activated macrophages need to be deactivated to minimise damage to host tissues, and this is achieved by cytokines such as IL-10 and type 1 interferons as well as by inhibitory receptors including SIRP α (Smith, Patel et al. 2003; Brown and Gordon In Press).

Macrophages are also a central element in tissue homeostasis, adapted to their local microenvironment and contributing to its maintenance. Resident tissue macrophages for instance contribute to haematopoiesis, surfactant metabolism, neurotransmitter metabolism and bile pigment formation. Furthermore macrophages express receptors that allow them to remove senescent and apoptotic cells from the circulation and scavenge a wide variety of ligands from tissue fluids that include products resulting

from normal metabolism as well as products arising from inflammatory responses such as those released by activated neutrophils and microbes. Disturbances in these homeostatic functions can result in persistent macrophage activation leading to chronic inflammation and subsequent pathology (Brown and Gordon In Press).

1.2.2 Neutrophils the front line of host defence

Neutrophils like macrophages are professional phagocytes and are highly efficient at engulfing pathogens and cell debris. Neutrophils derive from bone marrow myeloblasts and are released into the circulation as mature polymorphonuclear neutrophils (Faurischou and Borregaard 2003). The neutrophil is the body's first line of defence against microorganisms and a critical effector cell in both humeral and innate immunity playing vital roles in phagocytosis and bacterial killing. Neutrophils are recruited to sites of inflammation from the blood stream by chemoattractants including TNF- α , IL-6, IL-8, G-CSF, FMLP, C5a and LTB₄ and become activated during the selectin and integrin mediated processes of adhesion and diapedesis (Burg and Pillinger 2001). Neutrophil stimulation results in extracellular granule secretion, which constitutes a central innate immune weapon. The other mechanism of killing microbes is by phagocytosis, a process similar to that of macrophages and that also involves neutrophil granules (Burg and Pillinger 2001). Neutrophils produce lower amounts of cytokine per cell than macrophages, but their high numbers make them a physiologically important source (Denkers, Del Rio et al. 2003). Cytokines and chemokines synthesised and secreted by activated neutrophils include IL-8, IL-12, GRO α , MIP-1 α , IP-10 and MIG (Scapini, Lapinet-Vera et al. 2000)

Neutrophils are refractory to genetic manipulation and consequently much of what is known about their phagocytic functions has been inferred from studies in macrophages (Lee, Harrison et al. 2003). Neutrophils express a collection of PRRs and phagocytic receptors very similar to those found on macrophages, and these receptors appear to utilise, as far as is known, the same mechanisms of particle internalisation. The process of phagosome maturation however, differs substantially (Lee, Harrison et al. 2003). Whereas macrophage phagosomes mature by fusing with components of the endocytic machinery (**Fig. 1.2.1**) in a process accompanied by a progressive lowering of luminal pH, neutrophil phagosomes behave differently. Mature neutrophils are not endocytic cells and generally lack endosomes and lysosomes (Lee, Harrison et al. 2003), but have many secretory vesicles and granules, that are rapidly mobilised in a calcium and microtubule dependent fashion to fuse with phagosomes (Jaconi, Lew et al. 1990; Lundqvist-Gustafsson, Gustafsson et al. 2000; Tapper, Furuya et al. 2002). This secretion is focal, being restricted to the phagosomal membrane, rather than the generalised plasma membrane secretion seen in response to soluble ligands (Tapper, Furuya et al. 2002). In this way neutrophil phagosomal membranes remodel and acquire the lethal NADPH oxidase and V-ATPase complexes (Lee, Harrison et al. 2003). Surprisingly V-ATPase activity does not lead to progressive acidification of the phagosomal lumen, an effect thought to be due to the very high levels of NADPH oxidase activity that raise pH by consuming hydrogen ions (Jankowski and Grinstein 2002). The low intra-luminal pH that characterises mature macrophage phagosomes is thus not present in neutrophils suggesting that perhaps a different form of maturation may be occurring, or that killing rather than classical phagosomal maturation and microbe degradation is more central to their function.

Neutrophil activity is usually limited by phagocytosis-induced apoptosis and complement receptor 3 (CR3) (discussed in 1.5.3) has been implicated in both this apoptotic process and in subsequent clearance of effete neutrophils by macrophages (Coxon, Rieu et al. 1996).

1.2.3 Natural killer cells support inflammation

Natural killer cells are a sub-population of bone marrow derived lymphocytes that were initially characterised by their ability to kill tumour cells in an MHC-independent fashion (Trinchieri 1989). Subsequently it was established that these cells also enhance innate resistance to viruses, fungi, parasites and bacteria by secreting cytokines, lysing host cells harbouring intracellular pathogens and inhibiting the growth of microbes (Bancroft 1993). This innate immune role is mediated by two response and effector mechanisms, the first being cytokine induction of NK cell cytokine production, the second, activation of NK cells by targets leading to cytolytic activity and cytokine production (Yokoyama and Scalzo 2002).

In response to chemokines such as MDC and MCP-1 NK cells migrate from the blood vessels to target tissues (Allavena, Bianchi et al. 1994; Godiska, Chantry et al. 1997). NK cells are major producers of IFN- γ and are the first cellular source of IFN- γ in lymph nodes following bacterial infection. The NK cell derived IFN- γ plays an important role in both activating macrophages before the development of a antigen specific T-cell response and promoting a Th1 biased immune activation (Dunn and North 1991; Scharon and Scott 1993). This cellular IFN- γ production depends on macrophage derived cytokines, IL-12 in particular but also TNF- α (Bancroft, Sheehan et al. 1989; Tripp, Wolf et al. 1993). Macrophage derived IL-12 also enhances NK cell

cytolytic activity (Perussia, Chan et al. 1992). Dendritic cells, though perhaps less centrally than macrophages, can also activate NK cells following microbial stimulation by secreting both IL-12 and IL-2 (Mellman and Steinman 2001; Granucci, Zanoni et al. 2003). During viral infection a sub-population of dendritic cells, the plasmacytoid DCs act as the major source of type I IFNs and IL-12 that activate NK cells (Colonna, Krug et al. 2002). Activated NK cells can also produce many other cytokines involved in the regulation of inflammation including TNF- α , IL-1 β , GM-CSF and TGF- β 1(Perussia 1991).

NK cells can also be activated by targets they recognise through surface expressed receptors, and such targets include cells infected by viruses, bacteria and protozoa. Recognition by NK cells leads to both the lysis of infected cells following release of cytoplasmic granules and to inflammatory cytokine production (Yokoyama and Scalzo 2002). How NK cells become activated in response to what are predominantly self-protein ligands depends on the balance of activation and inhibition mediated by co-ligated receptors (Biassoni, Cantoni et al. 1996; Idris, Smith et al. 1999). Cytoplasmic motifs central to the activation and inhibition of many important immune cells including leukocytes, B and T cells and NK cells are known as ITAMs (immunoreceptor tyrosine based activatory motifs), discussed in detail in 1.5.2, and ITIMs (immunoreceptor tyrosine based inhibitory motifs), the definition being based on consensus sequences. NK cell receptors including NKR-P1, NKG2C, Ly49D and Ly49H, associate with an ITAM containing molecule DAP12; CD16 and NKR-P1C associate with the ITAM containing CD3 ζ or Fc ϵ R1 γ chains (Anderson, Caligiuri et al. 1989; Chambers, Vujanovic et al. 1989; Lanier, Yu et al. 1989; Arase, Arase et al. 1997; Smith, Wu et al. 1998; Tomasello, Olcese et al. 1998; Wu, Song et al. 1999;

Bakker, Hoek et al. 2000; Wu, Cherwinski et al. 2000). These ITAMs become phosphorylated and recruit the kinases ZAP70 and/or Syk, leading to activatory signals (Lanier, Corliss et al. 1998; Lanier, Corliss et al. 1998). The C-type lectin-like receptor NKG2D is also an activatory receptor, but is unusual in that it is able to transmit activatory signals by associating with DAP10 that does not contain an ITAM but has a PI3K docking site that mediates this function (Wu, Song et al. 1999; Wu, Cherwinski et al. 2000). Opposing these activatory signals are the dominant inhibitory receptors that include the Type I killer Ig-like inhibitory receptors found in humans and the Type II C-type lectin-like molecules CD94, NKG2A and Ly49A all of which contain ITIMs in their cytoplasmic tails. These ITIMS recruit phosphatases that de-phosphorylate the molecules involved in activatory signalling (Yokoyama 1997; Long 1999).

NK cells thus are an essential part of the innate immune system and both by amplifying inflammatory signals from macrophages and by influencing the nature of T-helper responses, furthermore NK cells can identify and help to kill cells infected with a very wide range of pathogens.

1.2.4 Dendritic cells, a bridge between innate and adaptive immunity

Signals resulting from the detection of invading pathogens in the periphery by the innate immune system must be conveyed to the cells of the adaptive immune system in the lymphoid organs. This task is performed exclusively by the dendritic cell (DC) populations of the innate immune system (Schuler and Steinman 1985). Dendritic cells comprise two populations, the first being the myeloid DC precursors in the bone marrow, the other are tissue resident immature DCs that surround portals of pathogen entry (Moll 2003). Myeloid DC precursors can be rapidly recruited to sites of infection

by chemokines produced by tissue macrophages, where they then differentiate into immature DCs that can take up antigen (McWilliam, Nelson et al. 1994). Tissue dendritic cells are the prototypical immature DCs and are close relatives of macrophages, deriving, as macrophages do, from blood monocytes that have migrated into the tissues (Moll 2003).

When immature DCs recognise and internalise microbial structures or receive pro-inflammatory signals, this induces a process of maturation resulting in profound changes in membrane traffic, surface molecule expression, cytokine production and in migration from the tissues to the T-cell areas of secondary lymphoid structures where naïve T-cells can be primed and quality of the T-cell response shaped into distinct effector classes through DC cytokine production (Mellman and Steinman 2001).

DCs accomplish antigen uptake by a number of mechanisms namely macropinocytosis, clathrin-mediated endocytosis and phagocytosis (Mellman and Steinman 2001). DCs can phagocytose microbes or apoptotic cells in an identical fashion to macrophages, but immature DCs are much less efficient at internalising and killing microbes than macrophages are, suggesting that their role in the clearance of pathogens is limited (Blank, Bogdan et al. 1996; Bodnar, Serbina et al. 2001). That said, the derivation of antigenic peptides from phagocytosed microbes or particles is particularly efficient, making it an important mechanism of antigen acquisition (Inaba, Turley et al. 1998). Endocytosis is an important mechanism of microbial antigen acquisition and is usually mediated by PRRs that include the lectins MMR, DEC205 and DC-SIGN (Mahnke, Guo et al. 2000; Engering, Geijtenbeek et al. 2002). In immature DCs, internalised antigens undergo a complex series of inefficient interactions with endosomal and

lysosomal compartments that contain MHC class II molecules that result in prolonged intracellular retention of these antigens (Mellman and Steinman 2001).

DC maturation can be triggered in a number of ways: (a) **Direct activation**. DCs express toll-like receptors (TLRs), discussed in 1.3.2 below, and maturation can thus be triggered in a NF- κ B dependent manner by microbial products such as LPS CpG DNA or ds RNA (Kaisho and Akira 2001) as well as contact with whole bacteria such as *Salmonella typhimurium* (Yrlid and Wick 2002) and heat shock proteins from necrotic cells. (b) **Indirect activation**. Such stimuli include pro-inflammatory cytokines such as TNF- α , IL-18 and IL-1 β (Mellman and Steinman 2001) and chemokines. (c) **Protein Tyrosine Kinase (PTK) and extracellular signal-regulated kinase (ERK) mediated activation**. FcR and TREM/DAP12 ligation (Regnault, Lankar et al. 1999; Bouchon, Hernandez-Munain et al. 2001). (d) **IL-12 activated NK cells**. Cell-cell contact event mediated by TNF- α and IFN- γ (Zitvogel 2002).

The maturation process involves a dramatic reduction in endocytic capacity, a decrease in the expression of the chemokine receptors CCR1, CCR2, CCR5 and CCR6 (Dieu, Vanbervliet et al. 1998; Saeki, Moore et al. 1999), an increase in the expression of homing chemokine receptor CCR7 (Saeki, Moore et al. 1999), increased efficiency in processing of retained or internalised antigens and expression at the cell surface of antigen loaded MHC class II and MHC class I molecules along with the co-stimulatory molecules CD80, CD86 and CD40 (Banchereau, Briere et al. 2000). DCs migrate to lymph nodes where they interact with T-cells, priming them (Mellman and Steinman 2001). During maturation DC cytokine production is also induced and the production

IL-12, IL-18 or IL-10 enables DCs to drive the T-cell mediated immune response towards a Th1 or a Th2 type (Moll 2003).

DCs have another discriminatory capacity, the ability to distinguish between different forms of the same pathogen and to elicit appropriate T helper responses. The yeast forms of *Candida albicans* and *Aspergillus fumigatus* for example elicit IL-12 mediated Th1 responses whereas the hyphal forms of these two inhibit DC production of IL-12 triggering a non-protective IL-4 mediated Th2 response (d'Ostiani, Del Sero et al. 2000; Bozza, Gaziano et al. 2002). The implications of these observations remain to be established.

DCs are thus professional phagocytes whose primary role is to use internalised antigens to elicit appropriate T cell activation, so linking innate immune defences to adaptive immunity.

1.3 Innate immune and phagocytic receptors found on leukocytes

As already mentioned, the innate immune system includes a multitude of PRMs, many of which are pattern recognition receptors (PRRs) found on leukocytes (**Table 1.1**). Cells of the innate immune system are bristling with surface expressed receptors, both opsonic and non-opsonic, waiting to engage microbial components in the serum or tissues. Many of these receptors promote the uptake of microbes or microbial components by phagocytosis or endocytosis. Another abundant family of PRRs are the Toll-like receptors (TLRs) that are essential for self-vs non-self discrimination and although TLR signalling have been shown to promote bacterial phagocytosis (Doyle,

O'Connell et al. 2004), TLRs are generally thought of as non-phagocytic receptors (Romani 2004).

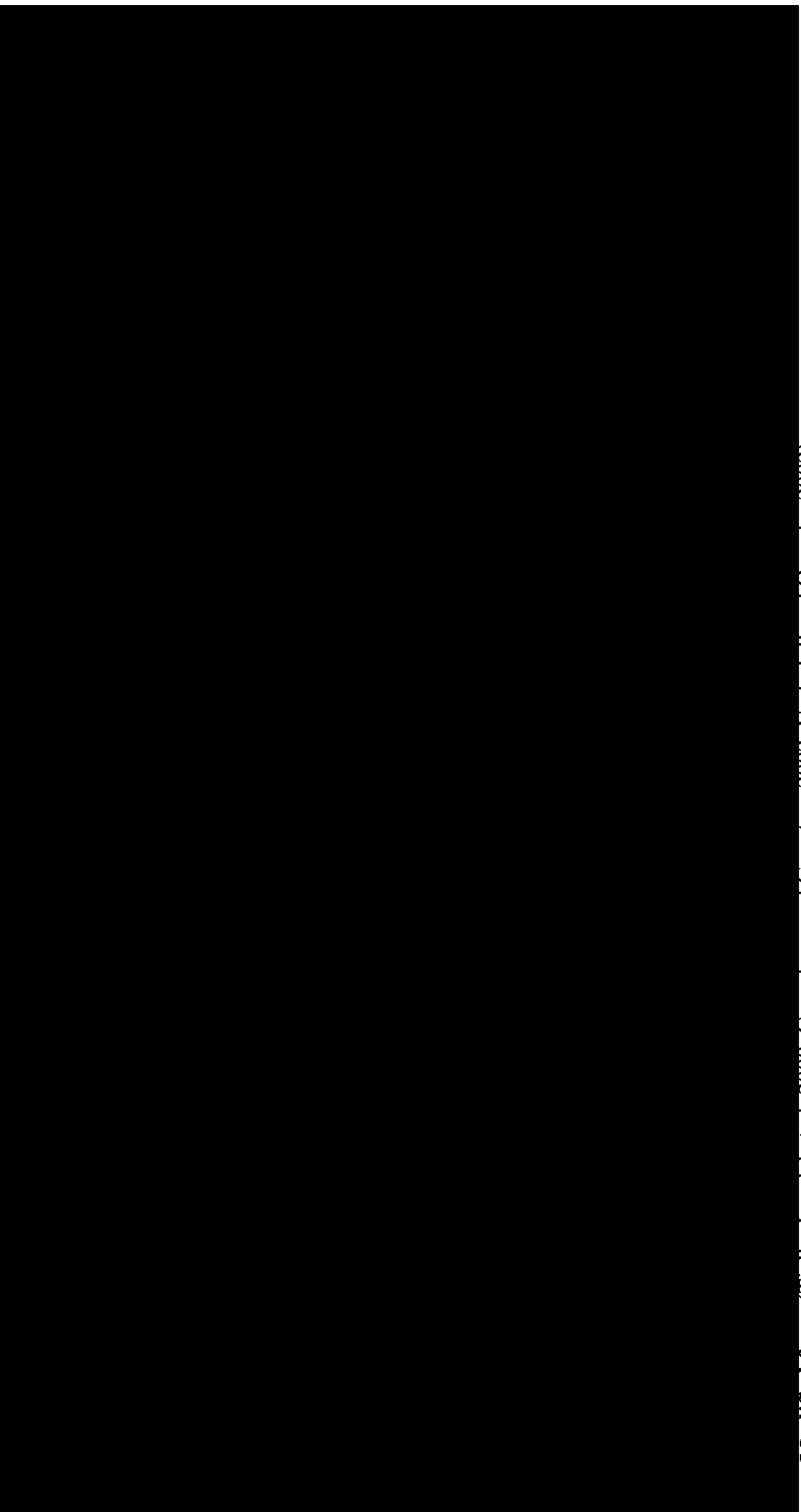
1.3.1 Phagocytosis promoting receptors

Leukocytes express a variety of surface receptors (**Table 1.3.1**) that either directly, or in co-operation with other receptors or stimuli mediate the phagocytosis of microbes and apoptotic cells. The opsonin-dependent receptors only indirectly recognize microbes, recognizing instead antibodies, complement or other opsonins coating the surface of microbes. Although there are phagocytic opsonin-independent PRRs such as CR3, many opsonin-independent microbial receptors such as scavenger receptor (see 1.5.1.5 below) and CR4 (Zaffran, Zhang et al. 1998) seem to mediate microbial adhesion rather than phagocytosis. Several of the listed phagocytic receptors will be discussed in detail in section 1.5 below.

1.3.2 Self vs non-self discrimination: the Toll-like receptors

How PRRs convert the information gleaned from recognition of a pathogen to an appropriate cellular response has been a subject of intensive investigation. Two principal classes of PRRs have been proposed: those that mediate phagocytic uptake, and those that lead to activation of pro-inflammatory pathways (Aderem and Ulevitch 2000). Most PRRs do not possess the cytoplasmic motifs shown to activate pro-inflammatory responses, and only with the description of a family of proteins originally identified in *Drosophila*, the Toll-like receptors (TLRs), did it become clearer how innate immune activation occurred in response to PAMPs (Aderem and Ulevitch 2000; Medzhitov 2001; Beutler, Hoebe et al. 2003; Underhill 2003). The Toll receptors are conserved from *Drosophila* to humans and there are 9 TLRs in mice and 10 in humans.

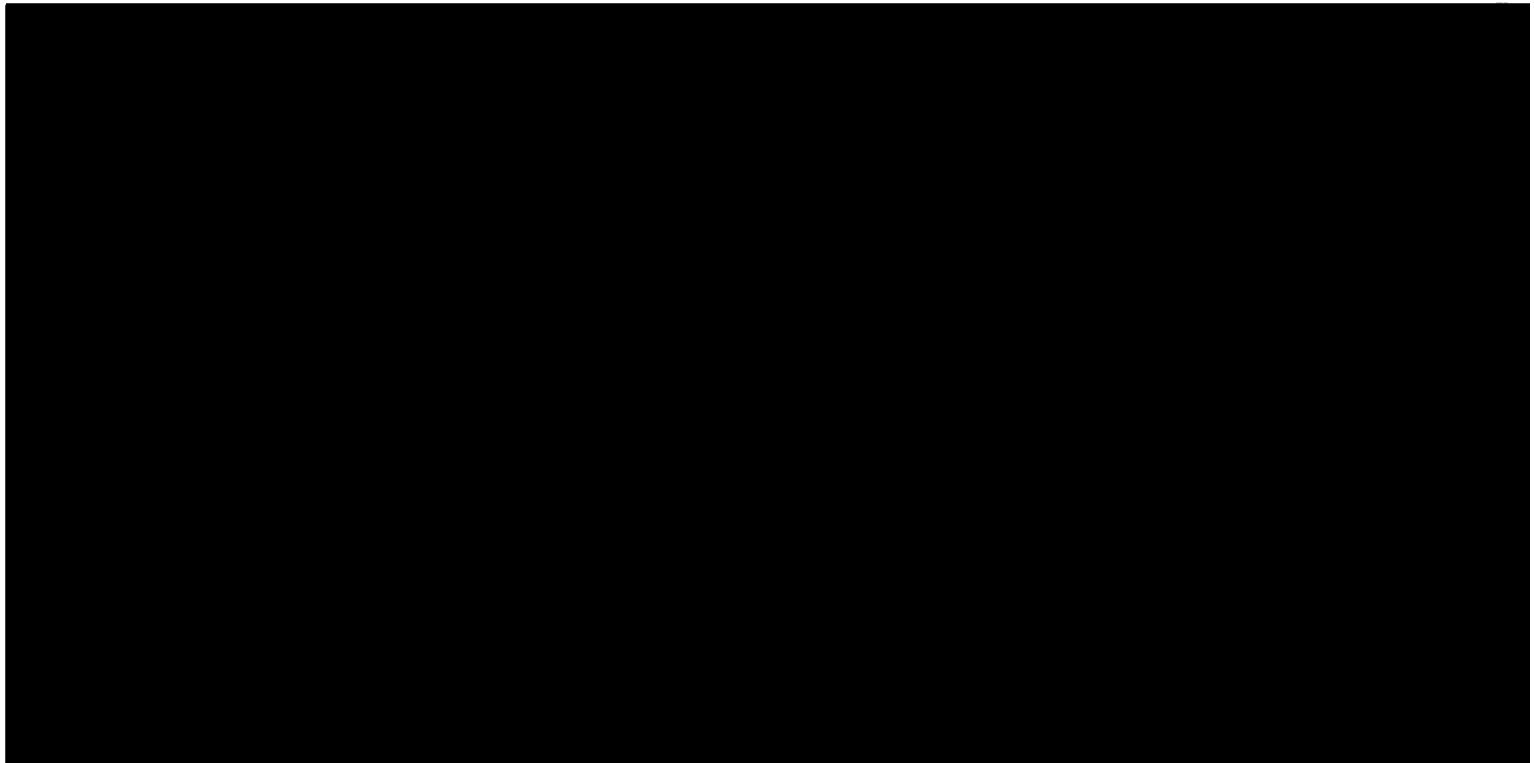
Table 1.3.1 Mammalian macrophage expressed innate immune receptors that promote phagocytosis



Modified from (Tjelle, Lovdal et al. 2000; Greenberg and Grinstein 2002; Underhill and Ozinsky 2002)

It has been discovered that each TLR recognizes a restricted subset or even a single molecule produced by microbes (**Table 1.3.2**) and it is now accepted that the TLRs are the principal signalling molecules through which mammals sense infection (Beutler, Hoebe et al. 2003).

Table 1.3.2



Modified from (Takeda, Kaisho et al. 2003; O'Neill 2004)

What is not clearly established is whether TLRs can directly recognise their ligands as some studies suggest (Lien, Means et al. 2000; Poltorak, Ricciardi-Castagnoli et al. 2000; Sato, Sano et al. 2003), or whether an accessory molecule such as MD-2 or an intermediary similar to *Drosophila* Spaetzle performs this function (Akashi, Nagai et al. 2001; Medzhitov 2001; Viriyakosol, Tobias et al. 2001; Hoffmann and Reichhart 2002). The signalling pathways of the TLRs have now been established in some detail, for recent reviews see (Akira 2003; Beutler, Hoebe et al. 2003). All TLRs, with the exception of TLR-3, share a common signalling pathway that depends on the adaptor myeloid differentiation factor 88 (MyD88) as illustrated in **Fig.1.3.2**. This signalling pathway is also shared by the IL-1 receptor. All TLR mediated pro-inflammatory cytokine production in response to microbial recognition is critically dependent on

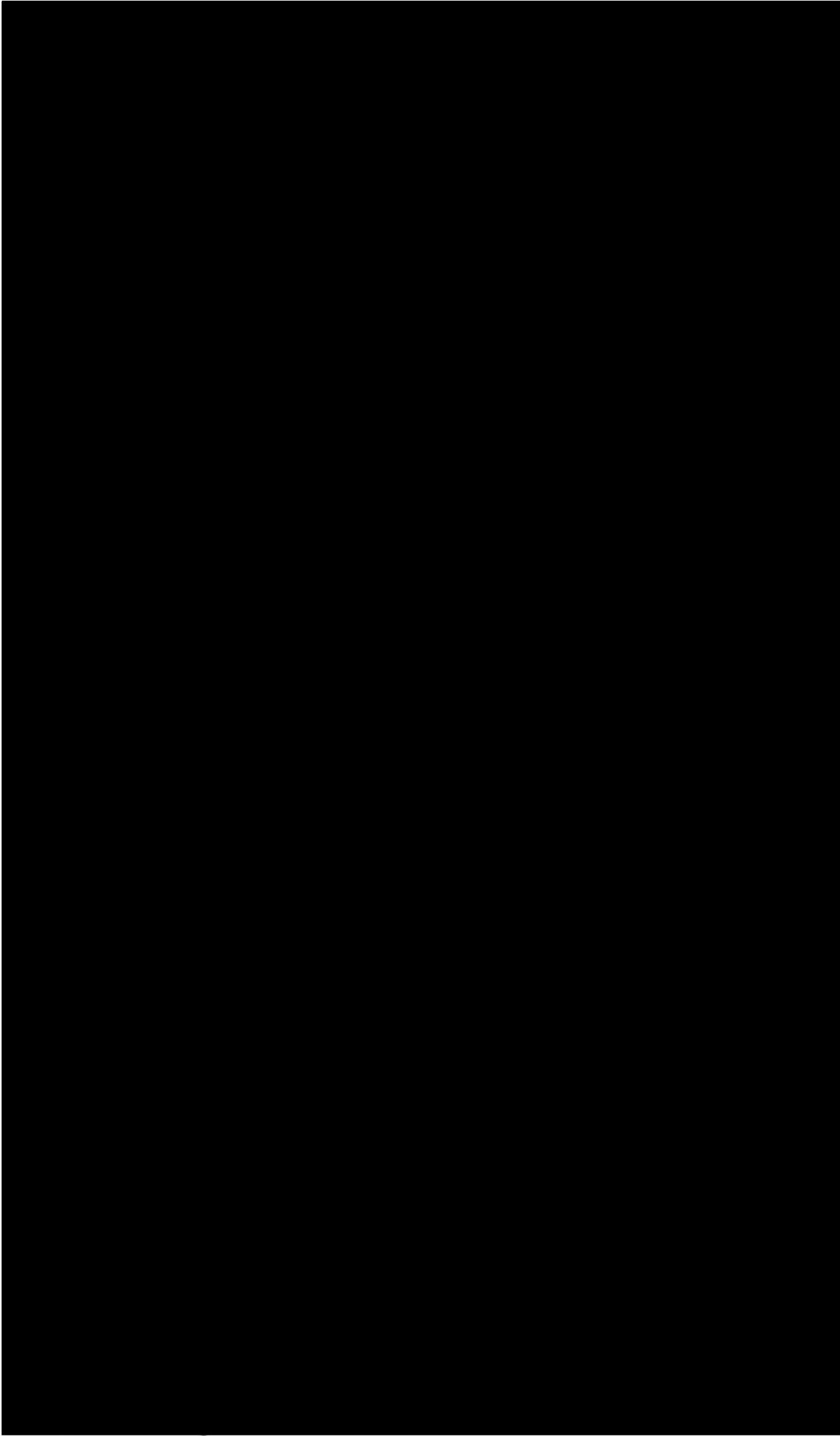


Figure 1.3.2 (Modified from Akira, Takeda et al. 2001; Takeda, Kaisho et al. 2003)

The Toll-like receptor signalling pathways. Most of the TLRs utilise a common downstream signalling pathway that proceeds from the receptor to MyD88, IRAK4, TRAF-6 and to NFκB and AP-1 activation. TLR 3 has a MyD88 independent pathway that activates IRF-3 and TLR4 has both a MyD88 dependent as well as a MyD88 independent pathway that both culminate in the activation of NFκB. From this model it is unclear how ligand specific signalling can be achieved.

MyD88 and its downstream mediators IRAK-4 and TRAF-6 that activate JNK and NFκB (Akira 2003). The importance of this pathway to host defence against a wide range of organisms was demonstrated when it was shown that MyD88-deficient macrophages are completely unresponsive to immunostimulatory components including LPS, peptidoglycan, lipoproteins, CpG DNA, flagellin, and imidazoquinolines, suggesting an essential role of MyD88 in mediating all inflammatory TLR responses (Akira 2003). More recently TLR-4 and TLR-3 mediated MyD88 independent pathways have been described (Akira 2003; Akira, Yamamoto et al. 2003) but these remain poorly characterised and mostly do not induce pro-inflammatory gene expression.

An important unresolved question is how, given the common MyD88 dependent signalling pathway shared by almost all the TLRs (**Fig.1.3.2**), discriminatory signals are transmitted from the TLR that has recognised its ligand to the cell nucleus. In the first instance some TLRs show a high degree of promiscuity making discrimination between pathogens less precise and in the second instance all the TLRs, with the exceptions of TLR-4 and TLR-2 that additionally require the adaptor TIRAP, and TLR-3 that senses viral RNA, signal exclusively through the Myd88, IRAK-4, TRAF-6 pathway to activate NFκB and JNK. The TLR-3 and TLR-4 signalling pathways are more complex, also having a MyD88 independent pathway that signals through the MyD88 like molecule, Trif and a protein encoded by the gene *Lps2* to the transcription factor IRF-3 (**Fig.1.3.2**) (Doyle, Vaidya et al. 2002; Yamamoto, Sato et al. 2002; Beutler, Hoebe et al. 2003). Furthermore studies have shown or suggested that TLR independent sensing mechanisms for the prototypical TLR-4 ligand, LPS, exist and that LPS can also be recognised by multiple other surface and an intracellular protein (Nod1) that are able to

activate the transcription factor NF κ B in a TLR independent fashion (Girardin, Tournebize et al. 2001; Inohara, Ogura et al. 2001; Triantafilou, Triantafilou et al. 2001; Chamaillard, Hashimoto et al. 2003; Inohara and Nunez 2003). Transcription factors not activated in the TLR-mediated signalling pathway such as members of the STAT family can be activated by both bacterial infection and LPS stimulation of macrophages, influencing expression of IFN-regulated genes (Tsukada, Waterman et al. 1996; Ohmori and Hamilton 2001).

Despite these reservations many authors believe that TLR mediated signalling is the primary mechanism of pathogen detection. A number of mechanisms have been proposed by which TLRs might discriminate between pathogens and include TLRs functioning in a combinatorial repertoire (Ozinsky, Underhill et al. 2000) whereby activation of several TLR signalling pathways could fulfil this function. This combinatorial mechanism still does not avoid the problem of the extensive overlap between TLR signalling pathways. More recently others have now shown that the recruitment of additional adaptors such as TIRAP (downstream of MyD88) and Trif by particular TLRs can activate additional signalling pathways (Yamamoto, Sato et al. 2002; Beutler, Hoebe et al. 2003; Yamamoto, Sato et al. 2003). Trif recruitment helps to explain the MyD88 independent pathway attributed to TLR-3 and TLR-4, yet other TLRs have known ligands that are not recognised by either TLR 3 or TLR 4 yet presumably have a role in pathogen discrimination that depends on the shared MyD88 signalling pathway. Furthermore, TLR-4 has both many ligands, and at least 2 signalling pathways, so how this molecule can relay specific signals in response to a particular ligand remains unclear. Other speculative mechanisms include each TLR having individual pathways in addition to the common MyD88 pathway, recruitment of

different ratios of TLRs to pathogens or different temporal relations in recruitment (Beutler, Hoebe et al. 2003; Takeda, Kaisho et al. 2003). All of these proposals seek to attribute pathogen specific inflammatory cell activation solely or primarily to TLR mediated recognition of PAMPS. An unexplored area is whether given the multiplicity of receptors that can recognise microbes, any of the non-TLR PRMs transmit signals that assist the TLR system in discriminating between pathogens and so help to regulate subsequent cellular activation.

Although it has been suggested that PRRs mediate either inflammation or uptake by endocytic or phagocytic mechanisms in response to ligation (Aderem and Ulevitch 2000), the biological roles of many PRRs are not well understood. Scavenger receptors and lectins such as the MMR (Figdor, van Kooyk et al. 2002) may be efficient at endocytosing soluble ligands, but the evidence demonstrating that they are truly phagocytic remains unconvincing. Whether such receptors function primarily to tether particulate ligands to cells or whether they also play a part in any subsequent inflammatory response has also not been clearly established. Furthermore, the TLRs are not the only PRRs capable of mediating inflammatory signals. The Nod receptors (Inohara and Nunez 2003) as well as the TREMs (Bleharski, Kiessler et al. 2003; Daws, Sullam et al. 2003) can induce inflammatory responses in macrophages.

1.4 PAMPS and their effects on immune responses

It is the current consensus that pathogen associated molecular patterns (PAMPs) elicit immunologically appropriate responses when they are recognised by a PRR. In most cases both the PRR and the cells involved in the biological responses to a given PAMP were known before any connection between receptor and ligand was established. Of the

many known PAMPS, lipopolysaccharide (LPS) has been the tool most widely used to elucidate the nature of innate responses to pathogens (Beutler and Rietschel 2003).

1.4.1 Lipopolysaccharide

LPS is the prototypical bacterial toxin; however the sources, effects and molecular nature of LPS derived from gram-negative bacterial cell walls were known long before the cells and receptors involved in mediating its effects were discovered. Richard Pfeiffer described heat-stable 'endotoxin', derived from *Vibrio cholerae* for the first time in 1894, and its purified form named LPS by Lüderitz and Westphal in 1947 (Beutler and Rietschel 2003). By this time it was known that injections of bacterially derived LPS lead to fever and that large doses were fatal in animal models but also that lower doses had therapeutic anti-tumour effects and stimulated the immune system in general, leading its use in 'fever therapy' (Beutler and Rietschel 2003). Only in 1985 was it shown that Lipid A was the active component of LPS (Galanos, Luderitz et al. 1985; Shiba and Kusumoto 1986) and that LPS effects were mediated by the macrophage derived cytokine, TNF (Beutler, Milsark et al. 1985). The recognition of LPS by the soluble PRR Lipopolysaccharide-binding protein (LBP) and the membrane bound PRR CD14 were described in 1986, 1990 respectively (Tobias, Soldau et al. 1986; Wright, Ramos et al. 1990). The role of the TLRs in LPS sensing was first proposed by Medzhitov in 1997 (Medzhitov, Preston-Hurlburt et al. 1997). Understanding the receptors and signalling pathways responsible for the effects of LPS developed piecemeal over nearly a century but eventually provided the crucial insights required to understand how innate immune sensing might operate and a template for the analysis of other PAMPS.

1.4.2 Fungal derived carbohydrates

Like LPS, fungal derived carbohydrates have well documented effects on the immune system and zymosan, that is rich in both α -mannan and β -glucan, was shown in 1959 to have activatory effects on cells of the innate immune system (Benacerraf, Thorbecke et al. 1959).

1.4.2.1 Beta Glucans

The fungal derived β -glucan rich particle zymosan was found to have potent stimulatory effects on the immune system and these were described before the active components or the cells and receptors involved in their recognition were identified or the mechanisms of their biological effects elucidated. Beta-glucans are major structural components of fungal cell walls and are also released into the systemic circulation during mycotic infections, making them widely available to the immune system (Miyazaki, Kohno et al. 1995; Kami, Tanaka et al. 2000). The important structural features of immunomodulatory fungal β -glucans will be discussed in section 1.6 below. In 1961 Riggi and DiLuzio reported that the glucan component was responsible for the reticuloendothelial stimulating activity of zymosan particles (Riggi and Di Luzio 1961). How this occurred was clarified when it was shown that administration of β -glucans stimulates innate immunity by inducing phagocyte activation and the production of several pro-inflammatory mediators, TNF- α in particular (Czop and Austen 1985; Browder, Williams et al. 1990). Crucially, the immunomodulatory effect of β -glucans was found to depend on glucan size, with glucans smaller than 7.7 kDa unable to induce protective effects whereas glucans of 500kDa were the most potent (Kulicke, Lettau et al. 1997; Lowe, Rice et al. 2001).

How the innate immune system recognizes and responds to glucans has only become clearer quite recently. The mechanisms of β -glucan action involves interaction with membrane bound receptors expressed on macrophages (Czop and Austen 1985; Muller, Rice et al. 1996). The major β -glucan receptor on macrophages was later identified to be Dectin-1 (discussed in detail below) (Brown and Gordon 2001; Brown, Taylor et al. 2002). Dectin-1 is, however, not the only macrophage expressed β -glucan receptor known, and CR3 as well as scavenger receptor are capable of binding β -glucans (Ross, Cain et al. 1987; Rice, Kelley et al. 2002). β -glucan receptors are also not restricted to immune cells and dermal fibroblasts, vascular epithelial cells, epithelial cells and anterior pituitary all have β -glucan recognition capacity (Williams, Rice et al. In Press). β -Glucans are thus important PAMPS with major effects on the immune system but the mechanisms of action of these molecules remain to be described.

1.4.2.2 Mannans

Fungal mannans and mannoproteins have immunomodulatory properties and can result in both suppressive and stimulatory effects on the immune system (Garner, Childress et al. 1990; Delfino, Cianci et al. 1996; Gomez, Torosantucci et al. 1996; Delfino, Cianci et al. 1997; Tzianabos 2000; Mansour, Schlesinger et al. 2002). Mannans stimulating the immune system interact with phagocyte receptors such as the mannose receptor and induce production of pro-inflammatory cytokines such as TNF- α and IL-6 (Garner and Hudson 1996; Tzianabos 2000). Suppressive effects on immune responses are less well understood but are thought to involve interactions with T-cells (Garner, Childress et al. 1990; Tzianabos 2000).

1.4.2.3 Other microbial PAMPS

Many other microbial PAMPS such as lipoteichoic acid (LTA), peptidoglycan and CpG DNA are recognised by cells of the innate immune system. Many of these products are TLR ligands (see Table 1.3.2) whilst some are recognised by other PRRs. As these molecules are not directly relevant to the subject of this thesis, they will not be discussed further.

1.5 Mechanisms of phagocytosis in macrophages

Cells use a variety of strategies to internalise particles and solutes, these include pinocytosis, caveolae, receptor-mediated endocytosis and phagocytosis (**Fig. 1.2.1**). Innate immunity is critically dependent on phagocytosis, a phylogenetically conserved process by which leukocytes ingest particles or microbes larger than 500nm - 1µm (Greenberg and Grinstein 2002). Phagocytosis by leukocytes fulfils two essential immune functions; firstly it leads to the killing and digestion of microbial pathogens, and secondly allows microbial antigens to be directed to the antigen presentation pathway (Greenberg and Grinstein 2002). The events that characterise phagocytic uptake are: particle binding leading to clustering and signalling, activation of the cytoskeleton leading to localised actin assembly, dynamic membrane changes facilitate the formation of extensions or invaginations leading to phagosome closure, detachment of the phagosome from the cell surface followed by de-polymerisation of cytoskeletal components to allow subsequent membrane fusion events with components of the endosomal compartment to occur, permitting phagosome maturation.

1.5.1 Phagocytic signalling

Following recognition, ligand binding to the surface of macrophages leads to receptor clustering, initiating the process of particle or microbe engulfment. The internalisation

process is dependent on the specific receptor or receptors ligated and the signalling intermediates subsequently recruited that direct receptor-specific uptake mechanisms. The best-described phagocytic mechanisms are those attributed to the opsonic FcγRs and CRs. Non-opsonic PRRs to which phagocytosis of pathogens have been attributed are the macrophage mannose receptor (MMR), DC-SIGN, CAECAM3 and the scavenger receptor class A (SR-A) (Ezekowitz, Sastry et al. 1990; Ezekowitz 1992; Stahl and Ezekowitz 1998; Platt, da Silva et al. 1999; Popp, Dehio et al. 1999; Peiser, Gough et al. 2000; Underhill and Ozinsky 2002; Cambi, Gijzen et al. 2003). Many other macrophage receptors have been implicated in promoting phagocytic uptake; these are listed in **Table 1.3.1**. As can be seen, many of these receptors recognise self-ligands rather than PAMPS.

1.5.2 Fcγ Receptor mediated phagocytosis

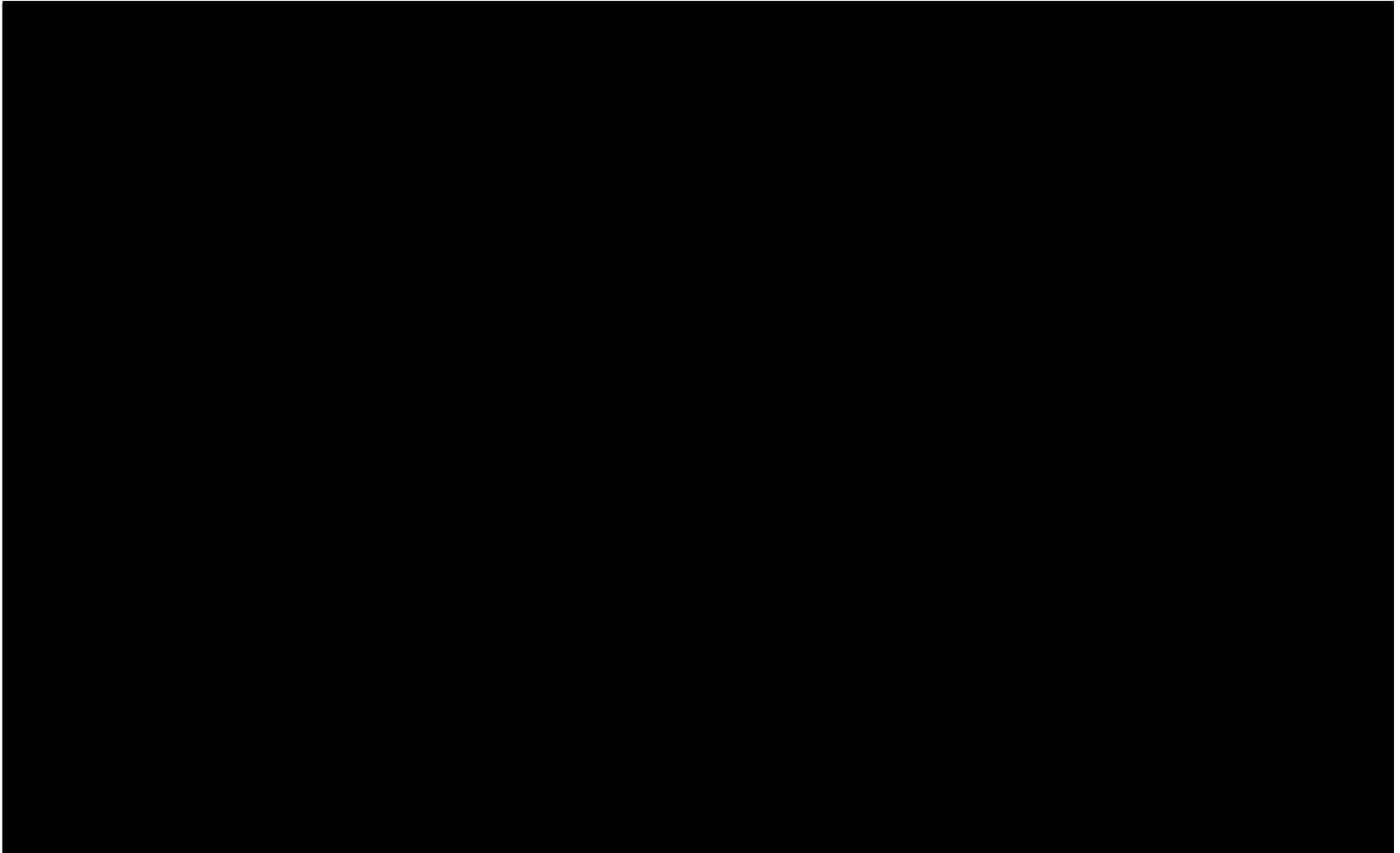
Our understanding of the signalling pathways leading to phagocytosis relies heavily on studies of Fcγ receptors. FcγRs recognize IgG, CRP and SAP opsonised particles (Bharadwaj, Stein et al. 1999; Bharadwaj, Mold et al. 2001; Mold, Gresham et al. 2001; Ravetch and Bolland 2001). In addition to phagocytosis, FcγRs mediate numerous other biological responses including immune complex clearance, antibody-dependent, cell-mediated cytotoxicity, release of inflammatory mediators, regulation of lymphocyte proliferation and differentiation and regulation of antibody production (Raghavan and Bjorkman 1996).

FcγRs can be divided into two groups, those that activate effector functions, and those that inhibit them (Aderem and Underhill 1999). Activatory murine FcγRs are

heterodimers consisting of a ligand binding α chain that forms a signalling complex with a pair of Fc γ chains (**Fig 1.5.2a**) (Daeron 1997). Humans also express these heterodimers, but the predominant activatory human Fc γ R is Fc γ RIIA (**Fig 1.5.2a**), a single chain molecule (Daeron 1997). Fc γ chains contain motifs referred to as immunoreceptor tyrosine based activation motifs (ITAMs), which are also found on the ζ chains of T-cell receptors, and M-antigen B cell receptors (Park, Murray et al. 1993). ITAMs consist of paired tyrosine motifs separated by a restricted number of random amino acid residues, conforming to the consensus YxxLx₅₋₁₂Yx₂₋₃L/I with the prototypical Fc γ -chain sequence being YxxLx₇YxxL (Reth 1989; Cox and Greenberg 2001). These ITAM motifs are the critical mediators of Fc γ R functions (Daeron 1997). **Fig. 1.5.2b** provides a diagrammatic representation of the discussion contained in 1.5.2.1-1.5.2.4 below.

1.5.2.1 Early signalling events

Fc γ R mediated phagocytosis is usually initiated by receptor clustering following antibody-opsonised particle binding and all subsequent events are critically dependent on tyrosine phosphorylation (Greenberg, Chang et al. 1993; Indik, Park et al. 1995). The ITAMs of clustered or cross-linked receptors are tyrosine phosphorylated by members of the Src family tyrosine kinases (most probably *Lyn*) (Chimini and Chavrier 2000; Fitzer-Attas, Lowry et al. 2000; Majeed, Cavegion et al. 2001) in a process that may involve localisation to membrane domains known as lipid rafts (Greenberg and Grinstein 2002; Van Laethem and Leo 2002). There is substantial redundancy amongst these mediators of ITAM phosphorylation and it appears that the six Src members expressed in haematopoietic cells can substitute for one another (Majeed, Cavegion et al. 2001). Phosphorylated ITAMs then serve as high affinity binding sites for the



Modified from (Daeron 1997)

Figure 1.5.2a

Activatory Fc γ receptors come in a variety of forms: I) The common feature of the cytoplasmic tails of these receptors is the presence of ITAMs, usually in the form of paired Fc γ chains that associate with the ligand binding Fc α chain. II) The prototypical ITAM is illustrated YxxLx₇YxxL. III) Human Fc γ RIIA is unusual as it is the only single-chain ITAM containing Fc γ R, and the ITAM has an unusually large spacing (x₁₂) between the tyrosine residues.

Figure 1.3.ZD (Compiled from: Castellano 2001, Chimini 2000, Greenberg 2002, Cox 2001, Garcia-Garcia 2002, Takenawa 2001, Allen 1995, Carballo 1999, Aderem 1992, Nagaishi 1999)

A diagrammatic representation of the molecules that have been shown to participate in FcγR mediated phagocytosis and their interactions with one another. Detailed discussion is found in the text.

tandem SH2 domains of the tyrosine kinase Syk (Ravetch 1994). There is compelling evidence that the recruitment of Syk is absolutely required for IgG-opsonised particle internalisation (Crowley, Costello et al. 1997; Kiefer, Brumell et al. 1998). Syk becomes tyrosine phosphorylated, probably by a Src kinase, thereafter it is thought to recruit the p85 subunit of PI3 kinase (PI3K), leading to increased local production of PIP₃ that plays a role in pseudopod extension (De Camilli, Emr et al. 1996; Rittig, Wilske et al. 1999; Greenberg and Grinstein 2002). PI3K may also be recruited directly to the Src kinase *Lyn* through an adapter protein called Cbl that interacts with the SH3 domain of *Lyn* (not shown) (Daeron 1997; Suzuki, Kono et al. 2000). The extent to which PI3K becomes activated seems to depend on particle size, with particles smaller than 3µm across being less dependent on its activity (Araki, Johnson et al. 1996; Cox, Tseng et al. 1999).

1.5.2.2 Signals leading to cytoskeletal remodelling

Signalling events downstream of Syk and PI3K are less clearly defined and no final consensus view has yet been achieved. Despite this reservation the central players as well as some of the interactions between them have been defined. The actin polymerisation required for phagocytic uptake to take place is driven by small GTPases including the Rho and ARF families (Caron and Hall 1998; Greenberg and Grinstein 2002). The PI3K product, PIP₃, is thought to facilitate attachment of the guanine-nucleotide exchange factor (GEF) Vav to the cell membrane where it activates the small Rho GTPase Rac-1 (Chimini and Chavrier 2000; Patel, Hall et al. 2002). Activated Rac-1 activates the serine/threonine kinase PAK-1, which facilitates stress fibre and focal adhesion disassembly, a process essential for increasing membrane mobility and ruffling prior to phagocytosis, and may also activate the actinomyosin

contractility required for phagosomal closure (Chimini and Chavrier 2000). Another small Rho GTPase, Cdc42 becomes activated by an unknown GEF that may be directly activated by the Src kinases (Chimini and Chavrier 2000).

1.5.2.3 Signals leading to actin nucleation

To initiate actin nucleation activated Cdc42 co-operates with the membrane lipid PI(4,5)P₂ to recruit Wiscott-Aldrich syndrome protein (WASP) permitting its activation by unfolding (Takenawa and Miki 2001). Unfolded WASP provides binding sites for a protein complex called Arp2/3 that triggers actin polymerisation and membrane extension in the form of filopodia (Chimini and Chavrier 2000; Takenawa and Miki 2001). Rac also participates in the recruitment of the Arp 2/3 complex by binding to another WASP family protein called WAVE2 through an intermediary, insulin receptor substrate protein of 53 kDa (IRSp53) (Lorenzi, Brickell et al. 2000; Takenawa and Miki 2001). There may be Arp2/3 independent mechanisms of actin assembly thought to be mediated through Rac driven PI(4,5)P₂ production, or through increasing actin turnover by activation of the actin severing proteins cofilin and gelsolin (Arcaro 1998; Cox and Greenberg 2001).

Another family of GTPases, the ARFs play a role in most membrane trafficking events (Roth and Sternweis 1997) and ARF6 is known to play a role in FcγR mediated phagocytosis (Zhang, Cox et al. 1998). There appear to be several mechanisms by which ARF6 influences cytoskeletal events. Firstly by, inducing Rac activation and distribution to the plasma membrane by releasing it from its cytosolic complex with Por1/Arfaptin2 (Tarricone, Xiao et al. 2001), and secondly by acting downstream of activated Rac to increase PIP₂ generation at the plasma membrane, either by direct or

PLD dependent activation of P(4)I5K, a step that stimulates actin filament growth (Honda, Nogami et al. 1999; Radhakrishna, Al-Awar et al. 1999; Zhang, Calafat et al. 1999; Rohatgi, Ho et al. 2000; Santy and Casanova 2001). Downstream effectors of the three Rho GTPases Rac-1 and Cdc42 and RhoA activate Cofilin, an actin depolymerizing protein that contributes to actin remodelling by increasing actin filament turnover. Rac effectors activate LIM kinase 1, Cdc42 effectors activate LIM kinase 1 and 2 and the Rho effector ROCK activates LIM kinase 2 (Greenberg and Grinstein 2002).

1.5.2.4 Membrane remodelling, pseudopod extension and phagosome closure

Professional phagocytes have a very high capacity to internalise particles with the consequence that macrophages can internalise their entire surface area within 30 minutes (Cox, Lee et al. 2000). The observation that this occurs without a reduction in surface area implies that an internal source of membrane is needed to replenish the membranes that form phagosomes. Active recruitment of endomembranes sourced from the endoplasmic reticulum (Garin, Diez et al. 2001) and lysosomal compartments (Sibley and Andrews 2000) has been shown.

Membrane remodelling depends on another proposed downstream effector of Syk, phospholipase C (PLC) that may contribute to actin uncapping by activating both Gelsolin through its product IP₃ and protein kinase C (PKC) through its product DAG. PKC localises to nascent phagosomes and is necessary for the completion of particle internalisation. The mechanism of PKC activity remains unknown but may, for certain ligands, be through the PKC substrate MARCKS (Aderem and Underhill 1999; Greenberg and Grinstein 2002). PI3K plays an important role in the membrane

remodelling associated with pseudopod extension by collaborating with PLA2, the SNARE apparatus and perhaps dynamin2/amphiphysin to activate an exocytic event that may involve the Rab11 recycling endocytic pathway (Cox and Greenberg 2001). PI3K is also thought to facilitate phagosome closure, perhaps by recruiting unconventional myosins to the neck of the phagosome (Araki, Johnson et al. 1996; Swanson, Johnson et al. 1999).

Actin polymerization is the central process responsible for phagocytic uptake, but it has long been speculated that actin polymerization was insufficient to drive pseudopod extension and particle internalization. The myosins are molecular motors that accumulate in phagocytic cups and have been speculated to act as mechanical motors during particle internalization (Stendahl, Hartwig et al. 1980; Valerius, Stendahl et al. 1981). It has recently been shown that myosins and microtubules contribute to the membrane recycling that facilitates internalization (Damiani and Colombo 2003).

1.5.2.5 Macrophage Fc γ R mediated inflammatory signalling

As mentioned earlier, Fc γ R cross-linking not only mediates particle internalization, but also induces the production of inflammatory mediators such as reactive oxygen intermediates and arachidonic acid metabolites (Wright and Silverstein 1983; Aderem, Wright et al. 1985). Pro-inflammatory mediators that are released in response to Fc γ R ligation include TNF- α , IL-1 β , IL-6 as well as chemokines and growth factors (van de Winkel and Anderson 1991). TLR recruitment is a general feature of phagocytosis and it is possible that antibody opsonised microbes binding simultaneously to both Fc γ R and TLRs may synergistically activate inflammatory responses (Ozinsky, Underhill et al. 2000). Thus although they can be separated for the purposes of study, Fc γ R

mediated phagocytic and inflammatory signalling pathways are intimately connected and share many early signalling molecules.

1.5.2.6 Negative regulation of phagocytic signalling

Failure to regulate pro-inflammatory signals generated by phagocytosis could result in excessive inflammation therefore inhibitory signals need to be generated. The efficiency of Fc γ R mediated phagocytosis is regulated by co-ligation of inhibitory Fc γ Rs (Fc γ RIIB) that contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that recruit the phosphatase SHIP that blocks phosphoinositide signalling (Ravetch and Bolland 2001). The relative expression of activatory and inhibitory Fc γ Rs determines the threshold for both phagocytosis and inflammatory responses to IgG-opsonised particles (Underhill and Ozinsky 2002).

1.5.3 Complement receptor-3 mediated phagocytosis

Complement receptor 3 (CR3) is an integrin expressed on phagocytes and NK cells and can function as both an opsonic and a non-opsonic pattern recognition receptor. It is the most promiscuous integrin recognising a multitude of endogenous ligands, including ECM proteins, coagulation proteins, ICAM-1 and -2 and complement C3bi, as well as diverse protein and non-protein microbial ligands (Plow and Zhang 1997). These ligands include the hookworm protein NIF, LPS, lipophosphoglycan, acylpolygalactoside, mycobacterial polysaccharides, zymosan particles and β -glucans (Ehlers 2000). CR3 also mediates a wide variety of leukocyte functions including cell adhesion, cell migration, microbe recognition and phagocytosis, and cellular activation (Ehlers 2000).

1.5.3.1 Cells expressing CR3 require activatory signals to induce phagocytosis

Most studies of CR3 mediated phagocytosis use complement-opsonised particles as tools. Complement proteins non-specifically opsonise bacteria, designating them for phagocytic uptake by the C3b and C3bi receptors on macrophages. Several macrophage expressed complement receptors are involved in this process including CR1, CR3 and CR4 (Sengelov 1995; Carroll 1998). CR1 primarily mediates particle binding, whereas CR3 and CR4 are heterodimeric integrins that share a β -chain, specifically bind to C3bi and mediate particle internalisation (Aderem and Underhill 1999). When ligated CR3 receives an activatory signal, this leads to an increase in the number of receptors at the cell surface (Berger, O'Shea et al. 1984; Sengelov, Kjeldsen et al. 1993; Blystone, Slater et al. 1999), as well as increasing the affinity of these receptors (Jones, Knaus et al. 1998) allowing them to trigger phagocytosis (Pommier, Inada et al. 1983; Wright and Griffin 1985).

Unlike Fc γ Rs, CRs are not constitutively active for phagocytosis and do not internalise particles in the absence of additional stimuli (Pommier, Inada et al. 1983; Wright, Craigmyle et al. 1983). Examples of such stimuli are PKC activators such as PMA, cytokines such as TNF- α and GM-CSF, attachment to fibronectin or laminin coated substrata, microbial products such as LPS and ligation of Fc γ Rs (**Fig. 1.5.3**) (Aderem and Underhill 1999; Underhill and Ozinsky 2002). The mechanisms by which CR3 becomes activated involves either receptor clustering and ligation of co-receptors such as the Fc γ R leading to PI3K activation and actin cytoskeletal reorganisation which releases CR3 from its cytoskeletal constraints, or (in PMNs at least) is PI3K independent and mediated by G protein-coupled receptors or TLRs leading to the activation of p38 MAPK that in turn activates the small Rho GTPase Rap-1 (Jones,



Figure 1.5.3 (Compiled from: Schmidt, Caron, Hall 2001, Berton, Lowell 1999, Jongstra-Bilen, Harrison, Grinstein 2003, Turner 2000).

A diagrammatic representation of the molecules that have been shown to participate in Complement Receptor mediated phagocytosis and their interactions with one another. Detailed discussion is found in the text.

Knaus et al. 1998; Castellano, Chavrier et al. 2001; Schmidt, Caron et al. 2001; Jongstra-Bilen, Harrison et al. 2003).

1.5.3.2 CR3 Phagocytotic mechanisms differ from those of Fc γ R

Although like FcR mediated phagocytosis CR-mediated phagocytosis in macrophages is an actin dependent process, complement-opsonised particles are internalised through a different mechanism of actin regulation (Kaplan 1977; Allen and Aderem 1996). Whilst Fc γ R-mediated phagocytosis results in the formation a continuous F-actin cup from which pseudopods extend above the cell surface to tightly engulf the particle, CR-mediated phagocytosis is a more passive process where particles appear to sink into the cell without pseudopod formation surrounded by a less tightly opposed membrane that makes point like contact with the particle suggesting focal complexes of actin polymerisation that are very reminiscent of integrin mediated cellular adhesion complexes (Kaplan 1977; Allen and Aderem 1996). The certainty of this classical distinction has been questioned in a recent study that showed that opsonised zymosan phagocytosis by CR3 expressing CHO cells conformed to this paradigm, but unopsonised zymosan internalisation was accompanied by pseudopod extension (Le Cabec, Carreno et al. 2002). As may be the case for Fc γ Rs, CRs must cluster in lipid rafts for signalling to be effective (Del Pozo, Alderson et al. 2004).

1.5.3.3 CR3 mediated cytoskeletal signalling

Less is known about the signalling processes involved in CR mediated phagocytosis than is known about Fc γ R-mediated signalling. Major differences when compared to Fc γ Rs have been demonstrated, the most significant being that CR mediated phagocytosis appears to be independent of tyrosine phosphorylation yet, like Fc γ Rs, is

dependent on PKC, furthermore, unlike Fc γ Rs, CRs require intact microtubules to accomplish particle internalisation suggesting that vesicle trafficking may be essential (Allen and Aderem 1996). An additional immunologically significant difference is that CR mediated phagocytosis does not trigger an inflammatory mediator response (Wright and Silverstein 1983; Aderem, Wright et al. 1985), making its mediators of actin polymerisation the targets of pathogens like *Shigella* and *Salmonella* (Hardt, Chen et al. 1998; Mounier, Laurent et al. 1999).

These differences are due to the involvement of different signalling mediators and CR mediated signalling is thought to proceed as illustrated (**Fig.1.5.3**). Being an integrin, the cytoplasmic tail of CR3 is attached to the actin cytoskeleton through the protein talin, and this connection must be disrupted before phagocytosis can take place (Berton and Lowell 1999). When complement opsonised particles bind to CR3, it has been shown that a variety of cytoskeletal proteins including F-actin, vinculin, paxillin, α -actinin and phosphotyrosine containing proteins are enriched in the point-like contact zones between particle and cell membrane (Allen and Aderem 1996). Although the mechanisms of this recruitment are unclear this process is PKC dependent (Allen and Aderem 1996). Interestingly, most of the proteins recruited to these focal contacts are also essential for CR3 mediated adhesion and the similarity between these two processes is striking (Castellano, Chavrier et al. 2001).

On CR3 cross-linking, the peri-nuclear scaffolding protein paxillin is thought to be recruited and to become serine phosphorylated in a process mediated by an unknown kinase (Ojaniemi, Martin et al. 1997). Paxillin is linked to the actin cytoskeleton by vinculin and paxillin also attaches to microtubules to facilitate membrane transport

necessary to form phagosomes (Turner 2000). Paxillin activates membrane bound PLC- γ , which activates PKC leading to activation of PLD, a product of which DAG in turn further activates PKC setting up a positive feedback loop. In a process mediated by a small GTPase that controls cell adhesion, Rap-1, the third member of the Rho GTPase family RhoA is recruited to the receptor complex (Caron and Hall 1998; Caron, Self et al. 2000). Whether there is also a RhoA GEF downstream of the tyrosine kinases or of PI3K as is the case for Rac-1 is unknown, but tyrosine phosphorylation is thought not to be essential (Allen and Aderem 1996). CR3 mediated actin polymerisation depends on RhoA yet the process linking activated RhoA to the activation of the Arp2/3 complex is not well understood (Castellano, Chavrier et al. 2001). It has been suggested that activated RhoA may recruit and activate PIP5K through its downstream effector known as Rho kinase (ROCK) leading to increased PIP₂ production in the cell membrane. PIP₂ is known to be involved in facilitating the recruitment of the Arp2/3 complex, actin filament uncapping and actin polymerisation (Caron, Self et al. 2000; Castellano, Chavrier et al. 2001). Furthermore RhoA mediated control of actin depolymerisation may be mediated by ROCK mediated phosphorylation of LIMK2 that leads to phosphorylation of cofilin the actin depolymerising protein (Castellano, Chavrier et al. 2001). Interestingly, the other small Rho GTPases, Rac-1 and Cdc42, play no role in CR mediated phagocytosis (Caron and Hall 1998).

1.5.3.4 CR3 mediated inflammatory signalling

Like the Fc γ R, CR3 can induce NADPH recruitment and inflammatory signal production, but these effects are context dependent. In macrophages CR3 ligation does not lead to activation of the NADPH oxidase, whereas in neutrophils it does (Wright and Silverstein 1983; Yamamoto and Johnston 1984; Lofgren, Serrander et al. 1999;

Serrander, Larsson et al. 1999). CR3 mediated inflammatory signalling is also complex and remains poorly described. CR3 mediated phagocytosis in macrophages does not lead to arachidonic acid metabolite production (Aderem, Wright et al. 1985), yet CR3 mediated adhesion to either CR3 ligands or antibodies leads to induction of multiple genes including TNF, IL-1, chemokines, tissue factor and transcription-associated proteins (Berton and Lowell 1999).

1.5.4 Other integrins

Fibronectin and vitronectin can promote phagocytosis in a manner analogous to complement, by non-specifically opsonising pathogens. The opsonised pathogens can then be recognized by integrins (**table 1.3.1**), and uptake triggered by a second signal such as PKC activation (Blystone, Graham et al. 1994; Blystone, Slater et al. 1999). The signalling mechanisms involved in this process remain to be described.

The role of integrins in regulating both phagocytic uptake and inflammatory signalling is more complex however, and the combinations of integrins that recognise a particle influence uptake and cellular activation. So whilst $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins promote uptake of fibronectin and vitronectin opsonised pathogens, when serine phosphorylated $\alpha_v\beta_3$ associates with CD47 and its ligand SIRP α both Fc γ R and CR mediated phagocytosis and inflammatory signalling are inhibited (Blystone, Graham et al. 1994; Blystone, Lindberg et al. 1995; Blystone, Slater et al. 1999; Demeure, Tanaka et al. 2000; Oldenborg, Gresham et al. 2001). In contrast $\alpha_v\beta_3$, when associated with $\alpha_v\beta_5$ and CD36 (a class B scavenger receptor), phagocytosis of apoptotic cells is mediated, but anti-inflammatory signals accompany this process (Albert, Pearce et al. 1998; Fadok, Warner et al. 1998).

1.5.5 Lectin mediated phagocytosis

Phagocytic uptake following the recognition of cell surface sugars by lectins was originally called lectinophagocytosis by Sharon and co-workers (Ofek and Sharon 1988). Macrophage expressed lectins that have been implicated in the phagocytic uptake of microbes include CR3 (via its lectin binding site), the MMR, DC-SIGN and potentially the β -glucan receptor Dectin-1 (discussed in 1.6 below) (Aderem and Underhill 1999; Cambi, Gijzen et al. 2003).

1.5.5.1 Mannose receptor

The macrophage mannose receptor (MMR) that recognises mannose and fucose on the surfaces of pathogens (Stahl and Ezekowitz 1998) and along with DC-SIGN are the only lectins thus far shown to mediate phagocytosis see (**Table 1.3.1**) (Ezekowitz, Williams et al. 1991; Cambi, Gijzen et al. 2003). Phagocytosis by MMR was demonstrated in a COS cell system and is inefficient, furthermore it has not been possible to reproduce this phagocytic phenotype in NIH-3T3 fibroblasts (Dr. Phil Taylor unpublished data), making it uncertain whether on macrophages it actually functions as a phagocytic receptor as well as a PRR. MMRs are also endocytic receptors, constitutively internalised without the need for ligand binding to initiate this process. The observations that the MMR is rapidly internalised in a ligand independent fashion and is also readily shed from the cell surface leading to relatively low levels of steady-state expression at the cell surface makes it difficult to understand how it would function in a phagocytic capacity. The cytoplasmic tail is thought to be crucial to mediating both endocytic and phagocytic functions, but little is known about the

molecular mechanisms that lead to receptor internalisation (Ezekowitz, Sastry et al. 1990; Stahl and Ezekowitz 1998).

It was thought that the phagocytic mediators recruited to unopsonised zymosan phagosomes in macrophages were recruited by the mannose receptor (Speert and Silverstein 1985; Allen and Aderem 1996). These include F-actin, talin, PKC α , MARCKS and Myosin I but not vinculin and paxillin (Allen and Aderem 1996). Furthermore pro-inflammatory signals have been shown to be generated upon MMR ligation including IL-1 β , IL-6, GM-CSF, TNF- α and IL-12 (Aderem and Underhill 1999). It is thus believed that, like Fc γ R mediated phagocytosis, MMR mediated phagocytosis is a pro-inflammatory process. The observation that CR3 can also recognize the β -glucan component of unopsonised zymosan particles (Ross 2000) and may therefore participate in zymosan particle recognition as well as evidence that will be presented regarding Dectin-1 functions make it doubtful whether the MMR plays the role of either a phagocytic or a pro-inflammatory receptor in macrophages.

1.5.5.2 DC-SIGN

DC-SIGN and DC-SIGNR are type II C-type lectins that mediate cell-cell interactions within the immune system by binding to intercellular adhesion molecule-3. These molecules also have other ligands and DC SIGN was initially implicated in transporting HIV from the periphery to T-cell areas in lymph nodes (Steinman). More recently DC-SIGN and DC-SIGNR have been shown to recognise mannan containing ligands (Mitchell, Fadden et al. 2001). Furthermore it has now been demonstrated that DC can function as a phagocytic receptor for fungi on DCs (Cambi, Gijzen et al. 2003). The mechanisms of phagocytic uptake remain to be described.

1.5.6 Scavenger receptor class A (SR-A) mediated phagocytosis

Scavenger receptors were originally defined as receptors that could bind and internalise modified lipoproteins such as acetylated low-density lipoprotein (LDL), but were later found to bind to a wide range of ligands that include LPS, polyribonucleotides and silica particles (Platt, da Silva et al. 1999). Two SRs that participate in phagocytosis have been described: SR-A, a transmembrane homo-trimer that can bind to whole bacteria as well as the microbial cell wall components LTA and LPS (Dunne, Resnick et al. 1994; Hughes, Fraser et al. 1995; Peiser, Gough et al. 2000) and MARCO (macrophage receptor with collagenous structure) another class A scavenger receptor that binds particles including gram positive and negative bacteria as well as latex beads although the precise ligands remain unknown (Palecanda, Paulauskis et al. 1999). Whilst it is clear that SRs participate in phagocytosis of microbes, it has been suggested that their role may be restricted to binding particles to phagocytes whilst co-receptors generate the internalisation signals (Underhill and Ozinsky 2002). In contrast others have shown that CHO cells expressing SR-A are able to internalise particles by phagocytosis at a low level, but the mechanism remains unknown (Peiser, Gough et al. 2000).

1.5.7 IgG superfamily adhesion receptors

A recently described family of type I single chain receptors expressed in granulocytes and epithelial cells, the carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs) (**Table 1.2.1**) have also been shown to include a phagocytic, opsonin independent PRR. Granulocyte expressed CEACAM3 is a receptor that contains an ITAM-like motif in the cytoplasmic tail and binds to the Opa adhesins of pathogenic

Neisseria species (Popp, Dehio et al. 1999). Ligation of CEACAM3 in transfected epithelial cells induces Src family kinase dependent phosphorylation of the ITAM-like motif leading to Rac-1 and Cdc42 activation that mediate localised actin reorganisation culminating in microbe internalisation (Billker, Popp et al. 2002; Schmitter, Agerer et al. 2004). Interestingly, unlike in epithelia, in granulocytes Cdc42 does not seem to play a role in CEACAM3 mediated phagocytosis of pathogens and only Rac becomes activated, furthermore it is speculated that Syk kinase recruited to the phosphorylated ITAM motif is most likely the mediator of Rac and PLC activation (Billker, Popp et al. 2002; Schmitter, Agerer et al. 2004).

1.6 Phagosome maturation

As discussed earlier, some aspects of the phagosome maturation process differ slightly amongst the innate immune cells. For the purposes of this discussion phagosome maturation, as it is understood in macrophages, will be described. Studies involving phagosomal maturation have utilized both Fc γ R and CR3 generated phagosomes and no significant differences in the maturation process have been described suggesting a highly conserved process. That there may be some subtle differences to other phagocytic receptors can of course not be excluded.

Once a phagosome had undergone scission from the plasma membrane and the surrounding actin structures have disassembled it undergoes a series of rapid membrane remodeling events that alter its composition, and these are collectively referred to as maturation (**Fig. 1.2.1**). Nascent phagosomes are unable to kill pathogens yet, once formed, phagosomes travel within the cell, assembling the elements of the superoxide ion producing NADPH oxidase, acquiring the pH lowering V-ATPase, fusing with

components of the endocytic apparatus and maturing as the molecules associated with the phagosomal membrane change to reflect a progressively modified and lethal internal milieu (DeLeo, Allen et al. 1999; Tjelle, Lovdal et al. 2000; Vieira, Botelho et al. 2002). In quiescent cells components of the NADPH oxidase system are distributed around phagosomal membranes and in the cytosol. Fc γ R mediated phagocytosis leads to the activation of many kinases and some of these, PI3K, PKC, PKA and PAK have been implicated in phosphorylating p47^{phox}, that is responsible for recruiting the cytoplasmic components of the oxidase to phagosomal membranes (DeLeo, Allen et al. 1999; Segal, Wientjes et al. 1999; Underhill and Ozinsky 2002). Once assembled, NADPH oxidase generates reactive oxygen ions within the phagosome, usually killing the microbe. Phagosome formation however is not a prerequisite for NADPH mediated superoxide production as it can also be activated by various soluble stimuli (Segal, Wientjes et al. 1999). Phagosomal pH progressively falls from the near neutral extracellular environment to less than pH5 in the phagolysosome. This process is achieved by the rapid insertion of a multi-subunit transmembrane proton pump called the vacuolar (V) ATPase through fusion events with endomembrane organelles (Nanda, Brumell et al. 1996). Nascent phagosomes have membranes rich in both the class I PI3K product PI(3,4,5)P₃ and the PI(4)5K product PI(4,5)P₂, yet as soon as scission has taken place these disappear and are replaced by the class III PI3K product PI(3)P suggesting differential recruitment of PI3Ks (Vieira, Botelho et al. 2001). The role of PI(3)P is not clear, but it be to recruiting proteins such as EE1 that interacts with Rab5 (**Fig. 1.2.1**) to mediate endosome-endosome fusion, the same process that drives phagosome maturation (Christoforidis, McBride et al. 1999; Christoforidis, Miaczynska et al. 1999; Lawe, Patki et al. 2000). More mature phagosomes accumulate late endosomal markers such as Rab7 that may be a prerequisite for fusion with lysosomes

to form mature phagolysosomes in which ingested particles are ultimately destroyed (Roberts, Barbieri et al. 2000; Garcia-Garcia and Rosales 2002)

1.7 Dectin-1

1.7.1 Innate immune responses to fungal pathogens

Throughout our lives we are continuously exposed to fungi. Fungal infections now represent over 10% of all nosocomial infections (van Burik and Magee 2001), and mortality from opportunistic fungal infections still exceeds 50%. Immune systems as diverse as those of fruitflies and mammals have developed potent defences against fungal invasion, some of which are highly conserved. Fungi are eukaryotes, sharing many biological processes with multicellular organisms and have in turn developed sophisticated strategies for subverting or evading host immune responses (Romani 2004). Furthermore, the nature of host immune response influences the clinical form of fungal disease, for example whether the transition from commensalism to infection occurs with *Candida albicans* (Fidel and Sobel 1994).

The purpose of the innate immune response to fungal challenge is twofold, firstly the killing and removal of the pathogen, and secondly, instruction of the adaptive immune response (Romani 2004). Central to mammalian innate and acquired defences against these pathogens are the professional phagocyte populations comprising macrophages, neutrophils and dendritic cells (Mansour and Levitz 2002). NK cells, $\gamma\delta$ T cells and epithelial cells also contribute to defences (Romani 2004).

Professional phagocytes recognise fungi directly through PRRs that include the TLRs. Phagocytes have intrinsic anti-fungal activity, and this activity is enhanced by humeral

elements such as opsonins including complement, the collectins and antibodies as well as cytokines including IFN- γ , TNF- α , IL-12 and GM-CSF (Mansour and Levitz 2002; Romani 2004). Whilst macrophages can present antigens to T-cells their central roles seem to be the PRR mediated recognition, phagocytosis and killing of fungal pathogens accompanied by pro-inflammatory cytokine production, granuloma formation and the maintenance of latency (Mansour and Levitz 2002). Neutrophils kill fungi following phagocytic uptake, but can also do so by secreting granules containing defensins and perhaps by influencing T-helper cell responses (Mencacci, Montagnoli et al. 2002). Dendritic cells play a complex role in fungal defence and are able to distinguish not only between fungal pathogens, but also between fungal life cycle forms (yeast vs hyphal). DCs appear to distinguish the fungal form with the use of TLRs, and tailor subsequent CD4+ T helper responses to a Th-1 phenotype (protective) in response to yeast and to a Th-2 phenotype (non-protective) in response to hyphal forms (Claudia, Bacci et al. 2002; Romani 2004). In **Fig. 1.7.1a** some of the innate immune elements involved in driving fungal defences are shown (Romani 2004). As indicated, the TLRs that play a role in the recognition of fungi seem to induce inflammatory responses and Th-1 differentiation in a predominantly Myd88 dependent fashion. A study however has also suggested that the mould *Aspergillus fumigatus* can induce innate defences in a Myd88 independent fashion supporting a role for TLR4 in this process (Marr, Balajee et al. 2003). TLR2 in particular and also TLR4 are thus central to controlling fungal infections (Netea, Van Der Graaf et al. 2002). The role of Dectin-1 in the process will be discussed below and in chapter two.

The control of fungal growth is the result of oxidative and complementary non-oxidative mechanisms. Oxidative mechanisms include the enzymes NADPH oxidase

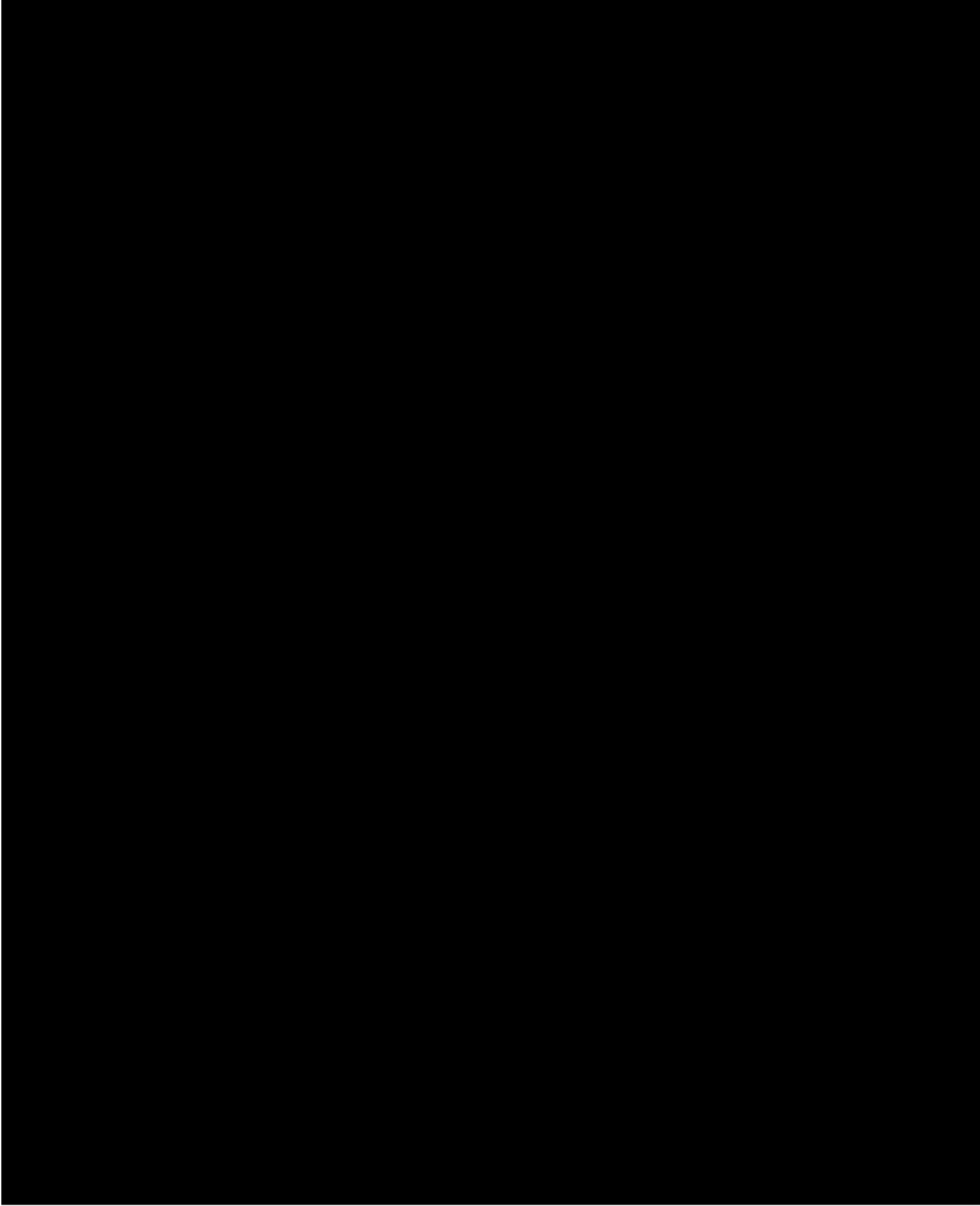


Figure 1.7.1a (Modified from (Romani 2004))

Innate immune PRRs involved in the activation of antifungal effector functions in phagocytes. All anti-fungal effector functions involve the canonical signalling pathway for mammalian TLRs requiring the adaptor MyD88 to drive pro-inflammatory gene activation. The role of Dectin-1 in TLR mediated pro-inflammatory gene activation will be discussed in detail in chapter two.

and iNOS, which produce reactive oxygen intermediates (ROIs) that damage fungi by protein modification, nucleic-acid damage and lipid peroxidation (Mansour and Levitz 2002). Non-oxidative mechanisms include degranulation and the release of effector molecules, defensins, neutrophil cationic peptides and iron sequestration (Romani 2004). Opsonins including antibodies, collectins and complement not only promote recognition of fungi, but also influence inflammatory and adaptive immune responses by regulating cytokine secretion and co-stimulatory molecule expression by phagocytes (Romani 2004). Phagocyte produced IL-12 together with IL-18 induce NK cells and T-cells to produce IFN- γ , a key cytokine in the innate control of fungal infections (Romani, Puccetti et al. 1997). IFN- γ stimulates phagocyte migration, adherence, phagocytosis and oxidative killing as well as sustaining Th-1 cell reactivity by maintaining IL-12 responsiveness in CD4+ T cells (Romani 2004).

The discovery of TLRs shed light on how inflammatory signals are generated in response to microbes. Despite these discoveries the innate recognition of fungal pathogens or their components remains incompletely understood. During both localised or systemic fungal infections fungal cell wall components are released into the systemic circulation implying that, if detected by the immune system, a systemic response might result (Miyazaki, Kohno et al. 1995; Kami, Tanaka et al. 2000). The direct recognition of fungi and yeast-derived particles by macrophages has been attributed to a variety of PRRs including mannose receptor, complement receptor 3 (CR3), lactosylceramide, scavenger receptors and a putative β -glucan specific receptor (**Fig. 1.7.1b**) (Brown and Gordon 2003; Herre, Gordon et al. 2003). Although it remains to be established which of these receptors are central to the recognition process, yeast cell wall components (**Fig. 1.7.1c**) are known to activate the immune system. The yeast derived particle

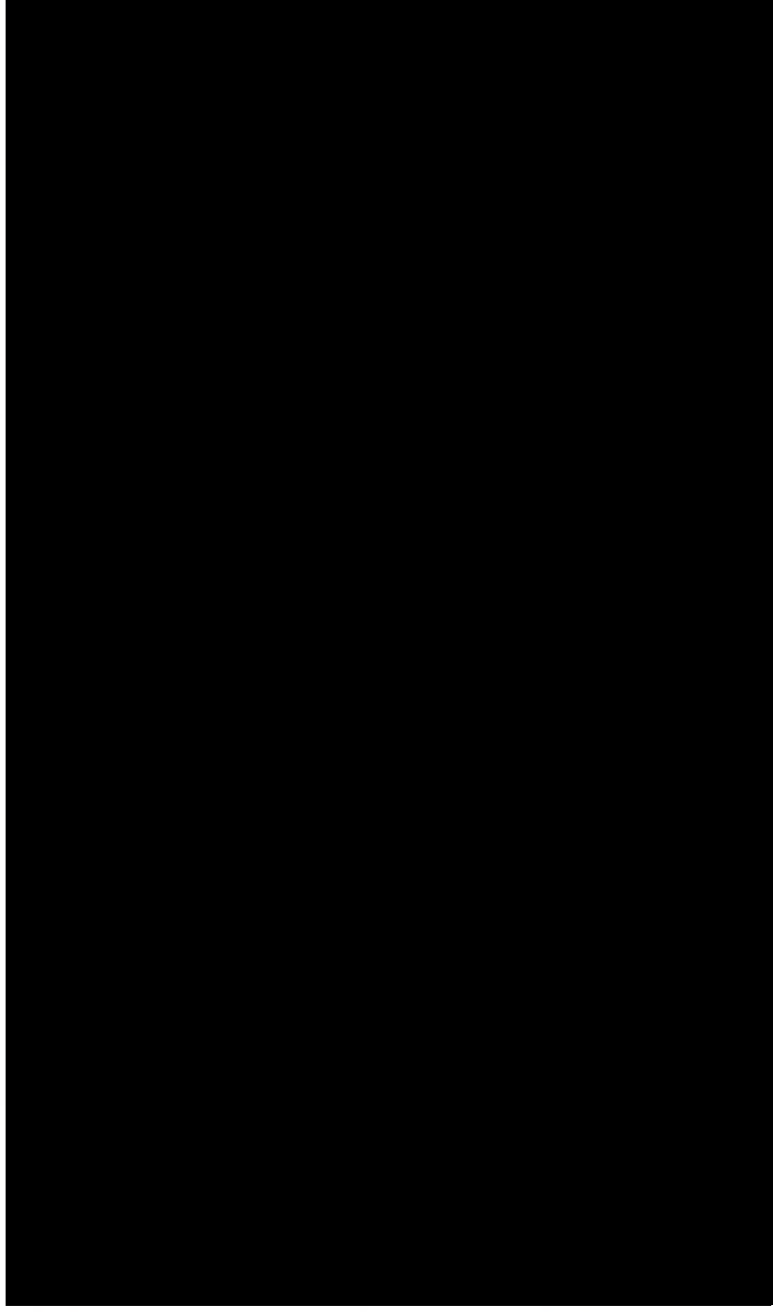


Figure 1.7.1b (Modified from Herre et al 2003)

Macrophages express a number of receptors capable of recognising fungal carbohydrates. Of the receptors illustrated, Dectin-1, scavenger receptor and CR3 have all be shown to recognise fungal derived β -glucans.

Figure 1.7.1c (Modified from Herre et al 2003)

A diagrammatic representation of the composition of fungal cell walls. Fungal cell walls comprise predominantly β -glucans and mannoproteins, with the mannoproteins potentially shielding the β -glucan component from immune recognition. Some of these fungal carbohydrates are also released from the yeast cell wall into the systemic circulation during mycotic infections.



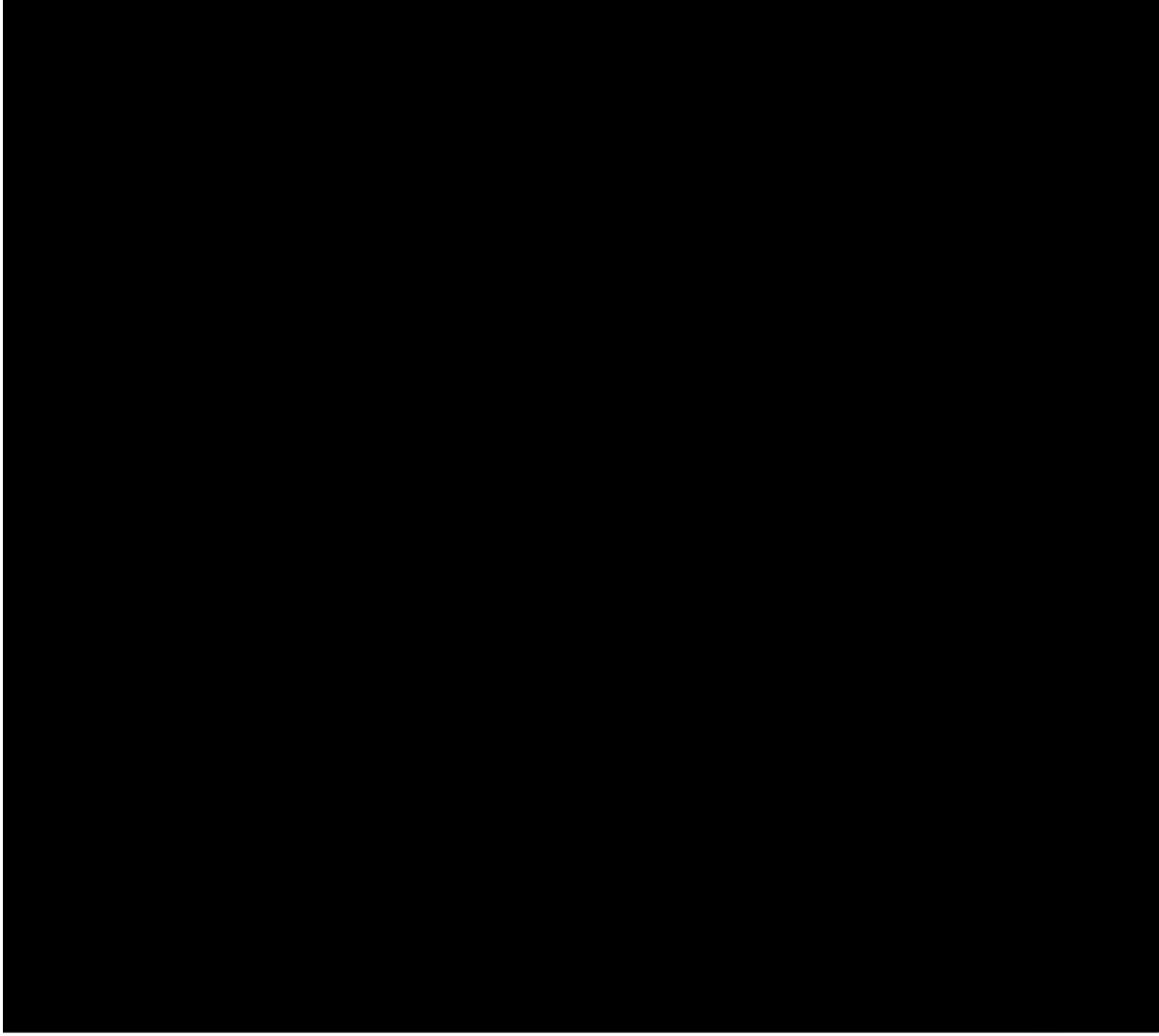
zymosan, composed principally of β -glucan polymers, α -mannan, protein and lipid (Di Carlo and Fiore 1958), was first shown in the 1950s to activate cells of the reticuloendothelial system (Benacerraf, Thorbecke et al. 1959). Cell wall β -glucans (**Fig. 1.7.1c**) were subsequently identified as the active component (Di Luzio and Riggi 1970), and since this discovery, have been shown to act as potent biological response modifiers with various effects on the immune system such as anti-tumour activity as well as anti-infective activities that include protection against fungal, bacterial, viral and protozoal infections (Kokoshis, Williams et al. 1978; Cook, Holbrook et al. 1982; Itoh 1997; Williams 1997; Ross, Vetvicka et al. 1999). As evidence from a number of studies suggested the existence of a specific non-opsonic leukocyte β -glucan receptor (Goldman 1988; Czap and Kay 1991), an attempt was made to identify this molecule.

1.7.2 The identification of murine Dectin-1 (Dendritic cell-associated C-type lectin) as a fungal receptor

Screening of a RAW 264.7 derived cDNA library expressed in fibroblasts for zymosan binding identified a single sequence that encoded this capacity. This sequence was homologous to the murine NK C-type-lectin-like receptor called Dectin-1 (Ariizumi, Shen et al. 2000; Brown and Gordon 2001). Dectin-1 was originally isolated by subtractive cloning from a murine epidermal dendritic cell (DC) line, and was thought to be a DC restricted molecule that recognized an endogenous ligand on T cells (Ariizumi, Shen et al. 2000). Structurally Dectin-1 has a predicted mass of 28kDa and is a type II membrane receptor with a cytoplasmic domain containing an ITAM-like motif, a single trans-membrane domain, a stalk and a single C-Type lectin like domain with two probable N-glycosylation sites (**Fig. 1.7.2**) (Ariizumi, Shen et al. 2000; Brown and Gordon 2001). Dectin-1 has homology with the NK C-type lectins LOX-1, NKG2D and Ly49 and the gene is found in the NK receptor locus on murine

Figure 1.7.2 (Modified from Herre et al 2003)

A diagrammatic representation of murine Dectin-1. Dectin-1 is a type II membrane molecule that has a single NK C-type lectin-like extracellular domain with two potential glycosylation sites, a stalk region, a transmembrane region and a cytoplasmic tail that encodes an ITAM-like motif with the sequence: **YxxIx₂YxxL**.



chromosome 6f3 (130.3Mb). What was unusual is that NK C-type lectins generally recognize endogenous proteins, MHC class I in particular (Yokoyama and Plougastel 2003), and with some exceptions (Drickamer 1993), lack any carbohydrate binding activity. Furthermore, the lectin domains of both NK C-type lectins and Dectin-1 lack the residues required for calcium co-ordination, normally required for carbohydrate binding in classical C-type lectins (Weis, Taylor et al. 1998; Ariizumi, Shen et al. 2000), an observation later confirmed (Brown and Gordon 2001). Thus despite the high structural similarity between Dectin-1 and NK C-type lectins, marked functional differences seem to exist.

1.7.3 Dectin-1 is expressed on the monocyte/macrophage and neutrophil lineages

β -Glucan receptor activity has been reported on a variety of leukocytes, including monocytes, macrophages, neutrophils and Langerhans cells, which have a wide tissue distribution (Czop and Austen 1985; Goldman 1988; Reis e Sousa, Stahl et al. 1993; Williams 1997). Northern blotting found Dectin-1 expression in liver, lung, thymus, stomach, spleen, small intestine, kidney and heart, compatible with a leukocyte restricted distribution (Ariizumi, Shen et al. 2000; Brown and Gordon 2001). Flow cytometric analysis using the monoclonal antibody 2A11 revealed that splenic DCs had heterogeneous surface expression of Dectin-1, but in contrast to earlier reports suggesting that Dectin-1 was a DC restricted molecule (Ariizumi, Shen et al. 2000), it was found that splenic neutrophils, macrophages and monocytes also exhibited high levels of Dectin-1 surface expression (Taylor, Brown et al. 2002). Freshly isolated splenic NK cells that have been shown to recognize β -glucans (Ross and Vetvicka 1993; Williams 1997), however showed no significant Dectin-1 expression. It may be that expression of Dectin-1 on the surface of NK cells is regulated by activation, but

this remains to be established. Dectin-1 is also expressed on the surface of a Gr-1⁺ subset of splenic T cells and although unexpected, expression of other NK-like C-type lectins on this population is not without precedent (McMahon and Raulet 2001).

Dectin-1 expression is not only found in splenic cell populations, and murine peripheral blood neutrophils and monocytes also express high levels of Dectin-1. Bone marrow derived neutrophils could be divided into two populations, those expressing high levels of Dectin-1, speculated to be more mature, and the remainder expressing intermediate or low levels. In the bone marrow, the highest levels of Dectin-1 surface expression is found on cells of the monocyte/macrophage lineage (Taylor, Brown et al. 2002). Dectin-1 expression on tissue macrophages and cell populations present in inflammatory infiltrates showed high levels of surface expressed Dectin-1 on freshly isolated thioglycollate-elicited, biogel-elicited and alveolar macrophages. In comparison, expression on resident peritoneal macrophages was low at the time of isolation but increased over time in culture. Neutrophils, also present in the peritoneal exudates, showed similarly high levels of surface Dectin-1, whereas recruited eosinophils showed no detectable expression (Taylor, Brown et al. 2002). These studies show that murine Dectin-1 is not restricted to DCs, but is widely expressed on leukocytes with the highest surface expression being on monocytes, macrophages and neutrophils, findings consistent with the previously reported cellular distribution of the phagocyte β -glucan receptor (Czop and Austen 1985; Goldman 1988; Czop and Kay 1991).

1.7.4 Dectin-1 is a classic PRR for fungal β -Glucans

Dectin-1 was identified on the basis of its ability to recognize β -glucan rich zymosan particles (**Fig.1.7.4a**) (Brown and Gordon 2001; Herre, Gordon et al. 2003). As zymosan is a complex particle containing a number of different polysaccharides, which may have been involved in these interactions, the carbohydrate specificity of Dectin-1 was characterised. It was found that zymosan recognition by Dectin-1 could be inhibited by β -1,3 and 1,6 linked glucan polymers (**Fig.1.7.4b**) derived from fungi, plants and bacteria but not by glucan monomers or polymers of seven or fewer β -1,3 linked glucose units. Polymers with different linkages, such as β -1,4 linked cellulose or α linked mannan, had no inhibitory effect (Brown and Gordon 2001). Yeast-derived laminarin and glucan phosphate, previously shown to bind to the β -glucan receptor on monocytes and macrophages (Muller, Rice et al. 1996), proved to be the most effective inhibitors. Dectin-1 thus has the features of a classic PRR, recognizing highly conserved β -1,3 and β -1,6 linked glucans from a variety of sources.

It has been suggested that the recognition and phagocytosis of intact yeast, including *Saccharomyces cerevisiae*, *Pneumocystis carinii* and *Candida albicans*, depends on their cell wall carbohydrates, including mannans and β -glucans (Giaimis, Lombard et al. 1993). It was demonstrated that Dectin-1 is able to recognize these yeasts in a β -glucan dependent fashion (Brown and Gordon 2001; Steele, Marrero et al. 2003) and this process is enhanced by opsonisation of zymosan with Pentraxin 3 (Diniz, Nomizo et al. 2004). Interestingly, the non-pathogenic *S. cerevisiae* was recognized more efficiently by Dectin-1 than the opportunistic fungal pathogen *C.albicans*. In addition, pathogens including *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Aspergillus fumigatus* were not recognized by this receptor, despite possessing β -glucans in their cell wall (Bartnicki-Garcia 1968). It has therefore been proposed that

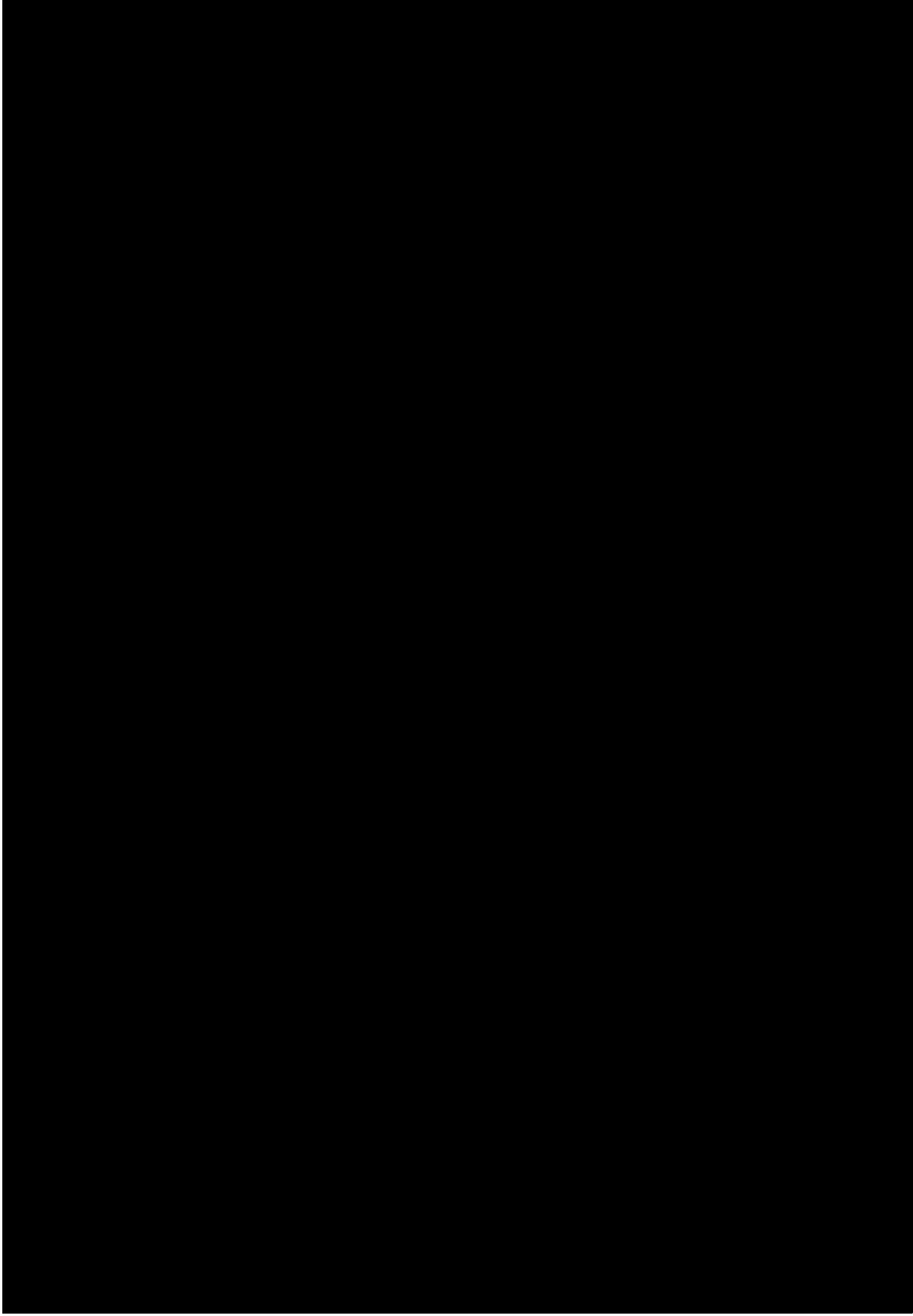


Figure 1.7.4a (Reproduced from Herre et al. 2003)

The effects of Dectin-1 expression on fluorescent zymosan particle binding on ice. Actin is shown by TRITC-Phalloidin staining in red, and FITC labelled zymosan particles in green. I) NIH 3T3 fibroblasts transduced with empty vector. II) NIH 3T3 cells stably expressing full length Dectin-1.

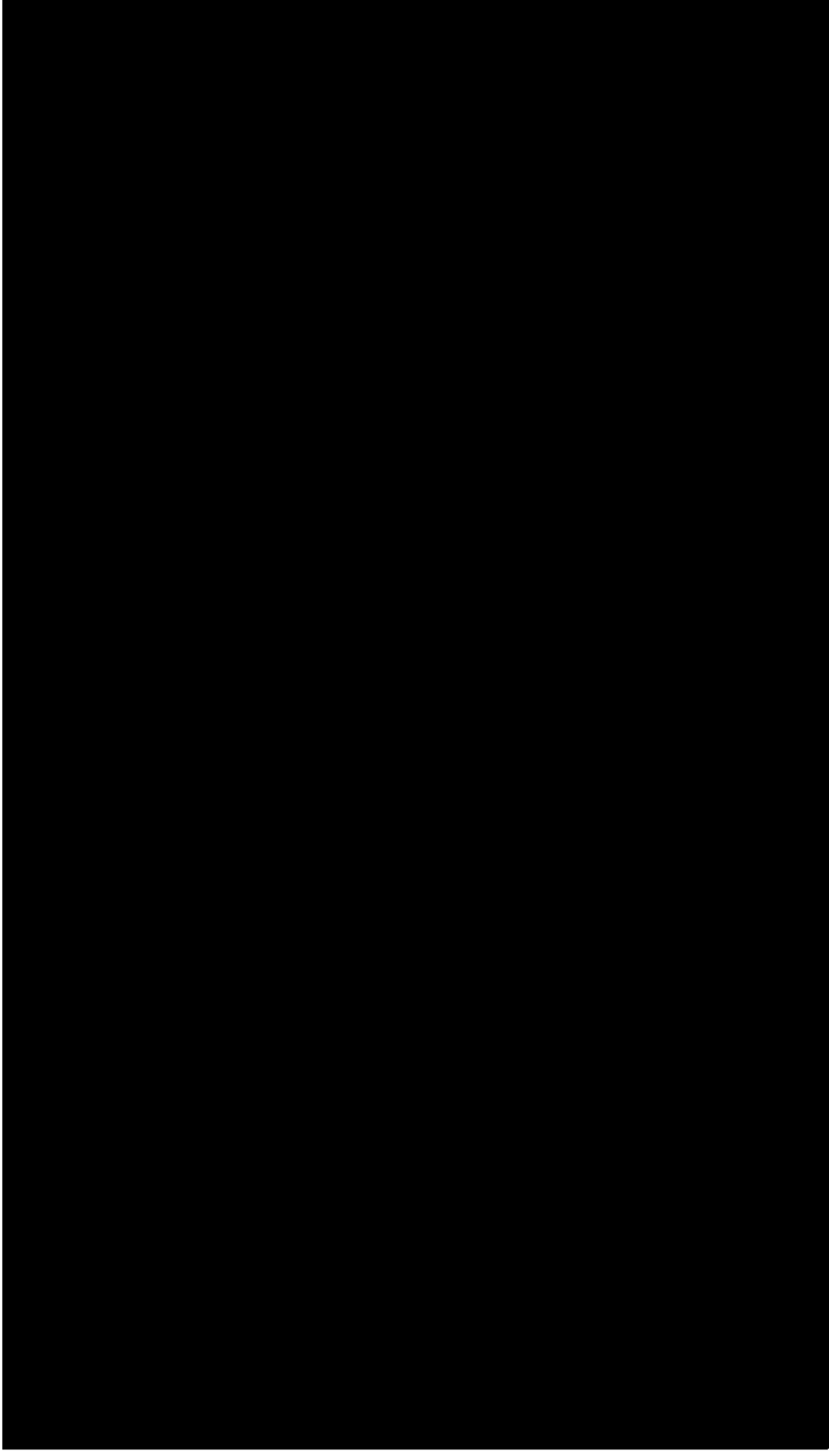


Figure 1.7.4b (Reproduced from Herre et al. 2003)

The molecular structure of immunomodulatory β -glucans recognised by Dectin-1. These glucans can be both linear and branched.

pathogens may mask their β -glucans in order to avoid immune recognition through Dectin-1 (Brown, Taylor et al. 2002).

1.7.5 Dectin-1, and not CR3, is the principal non-opsonic β -glucan receptor on macrophages

In addition to Dectin-1, a number of other macrophage receptors have been shown to recognize zymosan, including CR3 and the macrophage mannose receptor (Ross, Cain et al. 1987; Ezekowitz, Sastry et al. 1990). CR3, in particular, has a lectin domain that recognizes β -glucans, and has been postulated to be the major β -glucan receptor on phagocytes (Ross, Cain et al. 1987). Zymosan recognition by primary macrophages in the absence of complement has been shown to be β -glucan dependent, eliminating the mannose receptor as a major player in this process (Czop and Austen 1985; Goldman 1988; Brown, Taylor et al. 2002). Furthermore, neither the inhibition of the CR3 lectin domain in WT macrophages by methyl glucoside or monoclonal antibody 5C6 (Rosen and Gordon 1987; Xia, Vetvicka et al. 1999), nor using primary cells from CD11b^{-/-} mice lead to any reductions in β -glucan dependent zymosan recognition (Brown, Taylor et al. 2002). Instead, the recognition of unopsonised zymosan could be strongly inhibited by the anti-Dectin-1 mAb (2A11) to a level comparable to that achieved by soluble β -glucans, suggesting that Dectin-1 was the major β -glucan receptor on primary macrophages, mediating the non-opsonic recognition of zymosan.

As mentioned previously, resident peritoneal macrophages express lower levels of Dectin-1 than the elicited macrophages. Zymosan binding in these cells depends in part on a mannan inhibitable component not attributable to the mannose receptor (Taylor, Brown et al. 2002). This suggests that another, undefined non-opsonic mannose recognition mechanism is present in these cells. Potential candidates that have shown

mannose binding capabilities include DC-SIGN/DC-SIGNR (Mitchell, Fadden et al. 2001), Endo 180 (East, Rushton et al. 2002) and SIGNR1 (Taylor, Brown et al. 2004). These observations might help to explain the reported contribution of a mannan-dependent receptor in zymosan and yeast recognition (Sung, Nelson et al. 1983) and point to the existence of significant heterogeneity amongst myeloid cells, most likely representing specialization for particular functions.

In vivo most pathogens become opsonised, allowing recognition through complement receptors. It had been observed that soluble β -glucans were able to partially inhibit the binding of complement opsonised zymosan particles to primary macrophages, and involvement of Dectin-1 in this process was later confirmed (Brown, Taylor et al. 2002). Thus Dectin-1 also contributes to the recognition of opsonised β -glucan containing particles. These observations clarify previous reports that show a lack of specificity of C3 opsonised zymosan for CR3 in macrophages (Sung, Nelson et al. 1983).

1.7.6 Human Dectin-1

The human homologue of Dectin-1 (hDectin-1) has been identified (Hermanz-Falcon, Arce et al. 2001; Willment, Gordon et al. 2001; Yokota, Takashima et al. 2001) and is schematically represented in (**Fig. 1.7.6**) (Herre, Gordon et al. 2003). The gene is expressed as two predominant transcripts, a full-length transcript (hDectin-1 A) containing a stalk region and resembling murine Dectin-1, and a smaller transcript (hDectin-1-B) lacking the stalk region (Willment, Gordon et al. 2001; Yokota, Takashima et al. 2001). Several other additional transcripts, which are the result of alternative splicing, have been identified, but their significance remains to be

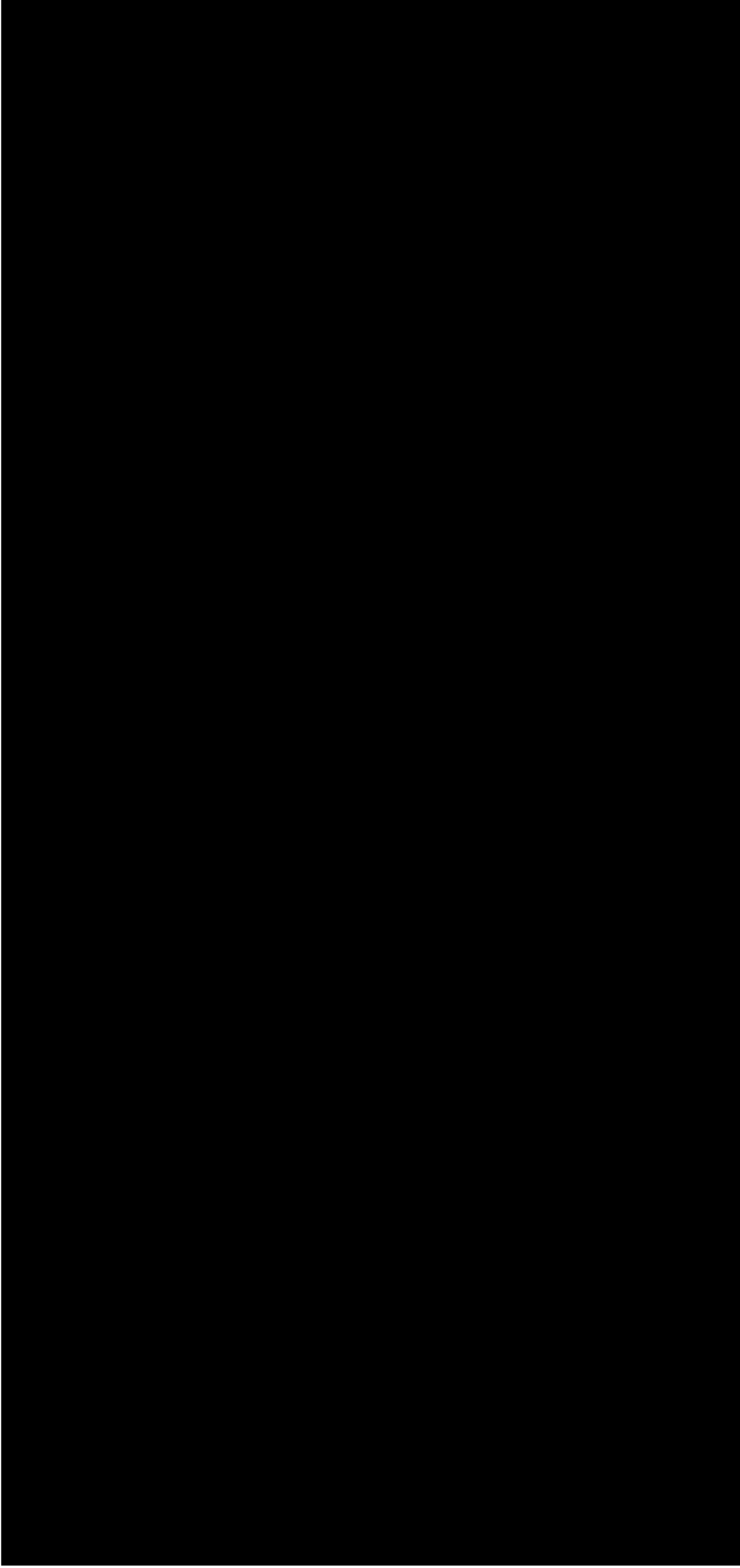


Figure 1.7.6 (Modified from Herre et al. 2003)

Diagrammatic representations of the isoforms of human Dectin-1. Isoforms A and B are the predominant transcripts and differ regarding the presence of a stalk region. The expression patterns of the isoforms of Dectin-1 will only be known when antibodies become available, and the roles of the splice variants C-H remain to be established.

established (Willment, Gordon et al. 2001). In contrast to the murine receptor, hDectin-1 has only one predicted N-linked glycosylation site. The significance of these glycosylation differences, if any, has yet to be determined. The cellular and tissue expression patterns for hDectin-1 by RT-PCR and Northern blotting show a leukocyte restricted distribution with some groups finding highest expression in polymorphonuclear leukocytes and mononuclear cells, that increased on differentiation to macrophages and neutrophils respectively (Willment, Gordon et al. 2001) and others report finding high expression of Dectin-1 on DCs and little on monocytes (Yokota, Takashima et al. 2001; Grunebach, Weck et al. 2002). These differences will only be resolved when monoclonal antibodies to hDectin-1 become available. An interesting finding that only the B isoform was detectable in mononuclear cells, monocytes, and macrophages, whereas, both A and B isoforms were expressed in polymorphonuclear cells (Willment, Gordon et al. 2001) suggests that splicing events might be regulated in a cell-type specific way (Willment, Gordon et al. 2001). hDectin-1 is very similar to the murine receptor, in terms of its carbohydrate specificity and pattern recognition functions (Willment, Gordon et al. 2001). Both receptors act as PRRs and recognize β -1,3 and 1,6 linked glucans from a variety of sources and intact yeast. The presence or absence of the stalk, in the two major isoforms, did not significantly affect these functions. Taken together, these findings suggest that the human homologue of Dectin-1 is likely to be the functional equivalent of the murine receptor.

1.7.7 Dectin-1 recognises an endogenous ligand on T cells

The recognition of endogenous ligands is a common feature of PRRs (Gordon 2002), and murine Dectin-1 was initially described as a receptor recognizing an endogenous ligand on activated T cells (Ariizumi, Shen et al. 2000). Fibroblasts expressing murine

Dectin-1 bind T-cells, and it was found that this recognition is carbohydrate independent (Brown, Taylor et al. 2002; Herre, Gordon et al. 2003). What remains uncertain is whether T-cell activation state contributes to Dectin-1 mediated recognition as some report that it does (Ariizumi, Shen et al. 2000), and others found no such requirement (Brown, Taylor et al. 2002). hDectin-1 can also recognize T cells in a β -glucan independent fashion (Willment, Gordon et al. 2001) but only one of four T cell lines tested was actually recognised, suggesting that the ligand might be restricted to a specific subset of T cells. Thus Dectin-1 has at least two ligand-binding sites, one that recognizes an endogenous ligand, and another that recognizes β -glucans.

It has been proposed that Dectin-1 may act as a T-cell co-stimulatory molecule (Ariizumi, Shen et al. 2000; Grunebach, Weck et al. 2002). Bacterially produced soluble Dectin-1 stimulates the proliferation of T lymphocytes in the presence of sub-optimal concentrations of anti-CD3 antibody (Ariizumi, Shen et al. 2000). In addition, HeLa cells expressing the hDectin-1 B isoform, acquire stimulatory properties proportional to their level of hDectin-1 surface expression despite not expressing co-stimulatory molecules, such as CD80 and CD86 (Grunebach, Weck et al. 2002). Both CD4+ and CD8+ T lymphocytes could be induced and this was accompanied by increases in the activation markers CD25, CD69 and CD154 and by cytokine production (Grunebach, Weck et al. 2002). These data suggest that Dectin-1 expressing cells may be able to influence the activation state of T cells *in vivo*.

Although the T cell ligand remains unknown it may, given the similarity of Dectin-1 to the NK C-type lectin receptors, be an MHC-I-like molecule. Furthermore ligation of NK C-type lectins by the target MHC class I proteins can lead to either activatory or

inhibitory signals, depending on whether the cytoplasmic tail of the NK-receptor contains an ITAM or an immunoreceptor tyrosine-based inhibitory motif (ITIM) (Yokoyama and Plougastel 2003). As Dectin-1 contains an ITAM-like motif, with the potential to transmit stimulatory signals, the interaction of Dectin-1 with T cells may also lead to changes in the activation state of the Dectin-1 expressing cell. A further possibility is that Dectin-1, which is also expressed at high levels on the surface of dendritic cells, plays a role analogous to that of the C-type lectin DC-SIGN, a molecule that stabilises interactions with T-cells by binding to ICAM-3 (Figdor, van Kooyk et al. 2002).

1.8 Unanswered questions regarding Dectin-1 function

Pattern recognition by innate immune receptors is central to the activation of appropriate immune responses. It has been established that Dectin-1 mediates recognition of fungal β -glucans by macrophages and that it is a major β -glucan receptor on these cells (Brown and Gordon 2001; Brown, Taylor et al. 2002). To enhance our understanding of the biological roles of Dectin-1 a multitude of unanswered questions remain.

It is important to establish whether Dectin-1 is both a phagocytic and/or an endocytic receptor, because these processes are relevant to immune activation and pathogen killing. Any phagocytic and endocytic ligand trafficking should be analysed to establish whether novel compartments are generated. Furthermore, should Dectin-1 be found to be phagocytic, the mechanisms of interaction with the cytoskeleton should be established and as it contains an ITAM-like motif, these should be compared to the canonical Fc γ Rs. As fungal particles induce pro-inflammatory mediator production, it

should be established whether Dectin-1 plays a role in this process, or whether these effects are solely due to the TLRs. Dectin-1 *in vivo* will almost certainly be part of a multi-receptor complex, therefore potential interactions with other pro-and anti-inflammatory receptors are important. Identifying and determining the significance of the T-cell ligand will provide further insight into the immunological roles of Dectin-1. Finally, and perhaps most centrally, the *in vivo* role of Dectin-1 in host defence against fungal and other pathogens needs to be defined with the use of receptor deficient mice when these become available.

1.9 Aims of this thesis

Specific aims are featured in the introduction to each results chapter, and more global aims are discussed here. This thesis aims to provide an analysis of a number of aspects of Dectin-1 function. The six major aims of this thesis can be defined as follows:

1. Phagocytic processes are central to the function of innate immune cells; yet leukocyte expressed receptors are difficult to study in isolation when expressed on their native populations. Epithelial cell or fibroblast derived non-phagocyte cell lines are useful tools for the examination of specific functions in particular receptors. To study Dectin-1 outside of its normal cellular context, non-phagocyte cell lines that express the receptor, both in its native form and as cytoplasmic tail mutants, will be generated and characterised. Furthermore the role of the cytoplasmic tail in mediating phagocytosis will be analysed.

2. Innate immune responses to pathogens include elimination by internalisation as well as the release of appropriate signals to the other elements of the innate and adaptive

immune system. It is therefore important that findings made in non-phagocytes be reproduced in phagocytes, the native cell populations expressing Dectin-1. To address this issue, macrophage populations stably over-expressing both native and cytoplasmic tail mutant Dectin-1, will be analysed for effects on particulate ligand internalisation. Furthermore the roles of mediators linking signals emanating from the cytoplasmic tail to the reorganisation of the cytoskeleton will be studied.

3. Phagocytosis is often accompanied by inflammatory mediator production, and this plays an essential role in shaping the emerging immune responses. Yeasts are potent inducers of pro-inflammatory mediators, and the role of Dectin-1 in promoting the release of such molecules will be analysed. The TLRs are thought to be the central players in mediating pathogen specific inflammatory responses, and an attempt will be made to integrate TLR and Dectin-1 functions.

4. Phagocytic receptors, internalised along with their microbial ligands often end up in lysosomes and are degraded, providing a mechanism of switching off receptor signalling. Lectins, however, often recycle, therefore the intracellular path Dectin-1 following particle mediated internalisation is analysed in primary cells and cell lines.

5. Many phagocytic receptors can also mediate endocytosis, and the cytoplasmic tail of endocytic receptors influences both intracellular ligand destination and whether the receptor recycles. Furthermore, molecular size has been shown to determine whether soluble fungal carbohydrates prime subsequent immune responses. To establish whether there is a relationship between molecular size and intracellular receptor

trafficking, Dectin-1 intracellular trafficking following internalisation soluble ligands of differing sizes will be analysed.

6. To integrate the data derived from the analysis of multiple cellular sources requires models. Working models will be proposed for Dectin-1 mediated phagocytic pathways and inflammatory signalling and for intracellular receptor trafficking following internalisation by ligands of differing sizes.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 General materials

Analytical grade chemicals were purchased from Sigma-Aldrich (Dorset,UK), Gibco/Invitrogen Corporation (Paisley, UK) and Calbiochem (Nottingham, UK). Buffers and solutions prepared from these chemicals and used in this thesis are listed in Materials Appendix. Autoradiography film was from Kodak.

2.1.2 Materials for cell culture

Cell lines were obtained from the cell bank at the Sir William Dunn School of Pathology and the ATCC (LGC Promochem, Teddington, UK). Media, antibiotics, L-Glutamine and trypsin for cell culture were from Gibco/Invitrogen Corporation (Paisley, UK). Lidocaine, G418 (geneticin) and trypan blue were from Sigma-Aldrich (Dorset, UK). Foetal Calf Serum (FCS) was from PAA Laboratories GmbH (Austria) and was heat inactivated at 56°C for 30 minutes and stored at 4°C before use. Tissue culture plastic was from BD Biosciences (Oxford, UK), BDH (Dorset, UK), Corning Corporation (New York, USA) and Fisher Scientific (Leicestershire, UK).

2.1.3 Materials for cell treatment

Pharmacological inhibitors of cellular signalling processes were from Calbiochem (Nottingham, UK) and Sigma-Aldrich (Dorset, UK). Zymosan particles were from Molecular Probes (Leiden, Netherlands), Laminarin was from Sigma-Aldrich (Dorset, UK), glucan phosphate was provided by a collaborator, Dr D.L. Williams (East Tennessee State University, Johnson City, Tennessee, USA), Mannan was from Sigma-

Aldrich (Steinberg, Germany) and Dextran 40kDa was from Fluka (Shaftesbury, UK). LPS was from Sigma-Aldrich (Dorset, UK). DMSO was from Sigma-Aldrich (Steinheim, Germany).

2.1.4 Materials for inflammatory macrophage production

Brewer Thioglycollate Medium was from DIFCO Laboratories (Detroit, USA). This broth was aged at room temperature for at least on month prior to use.

2.1.5 Materials for cell transfection

Transfection reagents: Lipofectamine was from Gibco/Invitrogen Corporation (Paisley, UK), Fugene 6 was from Roche (Indianapolis, USA), GenePORTER 2 was from Gene Therapy Systems (San Diego, USA), GeneJuce was from Novagen (Madison, USA). Cuvettes with 0.4cm electrode gap for electroporation were from BioRad Laboratories (Hertfordshire, UK). OPTI-MEM reduced serum medium was from Gibco/Invitrogen Corporation (Paisley, UK).

2.1.6 Materials for phagocytic particle labelling and opsonisation

Sheep red blood cells (SRBCs) were from TCS Biosciences (Buckinghamshire, UK). PKH2 membrane labelling kit was from Sigma-Aldrich (Steinheim, Germany). Rabbit anti-Sheep red blood cell stroma was from Sigma-Aldrich (Steinheim, Germany). Zymosan particles (Molecular Probes) were labelled with FITC (Sigma) using a carbonate buffer protocol.

2.1.7 Materials for molecular biology

Enzymes and reagents for nucleic acid processing were from Promega (Southampton, UK), Roche (East Sussex, UK), Stratagene (La Jolla, USA), Gibco/Invitrogen Corporation (Paisley, UK), Boehringer Mannheim (Germany), New England Biolabs (Hertfordshire, UK). Oligonucleotides were from Sigma-Genosys (Cambridgeshire, UK). Standard agarose was from Amresco (Ohio, USA). DNA ladders were from Gibco/Invitrogen Corporation (Paisley, UK). Top 10 competent cells were from Gibco/Invitrogen Corporation (Paisley, UK). The pBluescript SK and pFBNeo vectors were from Stratagene (La Jolla, USA). Kits for DNA minipreps and gel purification were from Qiagen (West Sussex, UK).

2.1.8 Materials for confocal microscopy

Microscope slides and 13mm thickness #1 coverslips were from BDH (Dorset, UK). EM grade paraformaldehyde (16%) was from Electron Microscopy Sciences (Ft. Washington, USA). Hepes buffer was from Gibco/Invitrogen Corporation (Paisley, UK). Aqua PolyMount was from Polysciences Incorporated (Warrington, USA). Antibodies were from Covance (Richmond, USA), Developmental Studies Hybridoma Bank (Iowa City, USA), Sigma-Aldrich (Dorset, UK), Jackson ImmunoResearch (West Grove, USA) and Invitrogen (Paisley, UK). Goat serum was from Sigma-Aldrich (Dorset, UK).

2.1.9 Materials for flow cytometry

V bottom 96 well plates were from Greiner (Germany). Goat and rabbit sera were from Sigma-Aldrich (Dorset, UK). Cell lifters were from Corning (New York, USA). Formaldehyde solution was from Sigma-Aldrich (Dorset, UK). Antibodies were from Covance (Richmond, USA), Developmental Studies Hybridoma Bank (Iowa City,

USA), Molecular Probes (Leiden, Netherlands), Serotech (Oxford, UK), Abcam (Cambridge, UK), Jackson ImmunoResearch (West Grove, USA) and Invitrogen (Paisley, UK) and Leinco Technologies (St.Louis, USA), 2A11 was provided by Dr. Delyth Reid (Jenner Institute, London).

2.1.10 Materials for protein determination

The BCA protein assay reagent kit was from Pierce (Rockford, USA). Antibodies were from Abcam (Cambridge, UK) and Sigma-Aldrich (Dorset, UK). Mouse TNF- α ELISA kit was from Pharmingen (Oxford, UK). HybondC+ membranes, ECL reagents and rainbow molecular weight markers were from Amersham Biosciences (Buckinghamshire, UK). Acrylogel 2.6 (30%) for SDS polyacrylamide gel electrophoresis (PAGE) was from BDH (Dorset, UK). GammaBind Plus Sepharose (20%) was from Amersham Biosciences (Buckinghamshire, UK).

2.1.11 Materials for metabolic labelling

L- [35 S]Methionine (in vitro translation grade) was from Amersham Biosciences (Chalfont St.Giles, UK). Methionine free medium was from Gibco/Invitrogen Corporation (Paisley, UK). EN 3 HANCE scintillation fluid was from NEN Life Sciences (Geneva, Switzerland). Cycloheximide was from Sigma-Aldrich (Steinheim, Germany).

2.2 Solutions

Annealing buffer: 5ml 1M Tris, 10ml 5M NaCl, 1ml 0.5M EDTA and 34ml DEPC-treated water

Carbonate buffer#1: 5.3% (w/v) Na $_2$ CO $_3$

Carbonate buffer#2: 4.2% (w/v) Na_2HCO_3

Confocal Block: PBS, 5% goat serum, 1% BSA

Confocal Fix: dilute 16% EM grade paraformaldehyde solution to 2% with 200mM HEPES/PBS

Confocal Perm: PBS, 0.2% Triton X100, 1% BSA

Confocal Quench: 50mM NH_3Cl

CsCl solution#1: 9g D-Glucose, 25ml 1M Tris HCl (pH 8.0), 20ml 0.5M EDTA (pH8.0), 20 μl RNase A, ddH₂O to 1L final volume

CsCl solution#2: 2ml 10N NaOH, 10ml 10% SDS, ddH₂O to a final volume of 100ml

CsCl solution#3: 147.21g potassium acetate, 57.19g 100% glacial acetic acid, ddH₂O to a final volume of 500ml

FACS Block: PBS, 5% heat inactivated rabbit or goat serum, 0.5% BSA, 5mM EDTA, 2mM NaN_3 (+5 $\mu\text{g}/\text{ml}$ 24G2 antibody when used for macrophages)

FACS Fix: 2% (v/v) formaldehyde in PBS

FACS Perm: FACS Block, 0.5% Saponin

KOAc solution: 29.4g potassium acetate, 11.5ml glacial acetic acid per 100ml (pH4.8)

Lidocaine/EDTA: 4mg/ml Lidocaine, 10mM EDTA (pH8.0) in PBS, filter sterilised

Low pH elution solutions: PBS, HCl titrated to pH's of 6,5,4,3 and 1.7

Lysis Buffer: PBS, 3% Triton X100

PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.3

T₁₀E₁₀: 10mM Tris (pH8.0), 10mM EDTA (pH8.0)

TBE (1x): 0.09M Tris, 0.09M boric acid, 2mM EDTA

Thioglycollate solution: Boil 40.5g Brewers Thioglycollate in 1 litre of distilled water until dissolved, autoclave for 15 minutes and then age for 1 month.

Trypsin/EDTA: 0.05% trypsin solution, 5mM EDTA (pH8.0) in PBS, filter sterilised

Turks solution: 1% glacial acetic acid, 0.01% (w/v) gentian violet in ddH₂O

Western-Blot blocking buffer: PBS, 5% (w/v) milk powder, 0.1% Tween 20

Western-Blot Lysis buffer: 50mM Tris (pH7.4), 150mM NaCl, 0.1% SDS (w/v), 1% Triton X-100 (v/v)

Western-Blot transfer buffer: 25mM Tris, 90mM glycine, 20% methanol

Western-Blot wash buffer: PBS, 0.1% Tween 20

2.3 Cells

2.3.1 Primary cells and cell lines

1. NIH 3T3 (ATCC#CRL-1658):

Murine embryonic fibroblasts. Adherent cells grown in DMEM and lifted with trypsin/EDTA.

2. RAW 264.7 (ATCC#TIB71)

Murine macrophage cell line derived from ascites of a murine intraperitoneal tumour induced by injection of Abelson murine leukaemia virus. Adherent cells grown in RPMI-1640 and lifted with lidocaine/EDTA.

3. CHO-K1 (ATCC#CCL61)

Chinese hamster ovary epithelial cell line. Adherent cells grown in Hams F12 medium and lifted using trypsin/EDTA.

4. Thioglycollate elicited macrophages

Macrophages from C57/BL/6 mice. Grown in RPMI-1640 and lifted using a cell lifter.

5. Bone marrow elicited macrophages

Macrophages from C57BL6/SJL mice. Grown in Petri dishes in RPMI supplemented with 20% L-cell conditioned medium (a source of M-CSF) and lifted by pipetting with lidocaine/EDTA.

2.3.2 General cell culture

Cells were grown in the appropriate media detailed above. Unless otherwise stated, media were supplemented with 10% heat-inactivated FCS, 2mM L-glutamine, 250U/ml penicillin G sodium and 250µg/ml streptomycin sulphate. Cells were cultured on tissue culture plastic in a 5% CO₂, 95% humidity incubator at 37°C and routinely passages every 3-4 days. Reagents used for lifting specific types of adherent cells from tissue culture plastic are described above. All PBS used was tissue culture tested from Gibco/Invitrogen. Prior to plating, cells were always counted in the presence of 1:1000 trypan blue to ensure that equal numbers of viable cells were used. Cells were generally plated for experiments at concentrations that resulted in low confluency (15-20%) for confocal and high confluency (80-100%) for flow cytometry and cytokine experiments at the time of assay. After plating, cells were incubated overnight before being used in experiments to allow Dectin-1 levels to be re-established on the cell surface.

2.3.3 Primary murine macrophages

All experiments were carried out according to Home Office guidelines under the appropriate project license and with local ethical approval. Primary macrophages were isolated from C57BL/6 mice. These animals were housed at the Sir William Dunn School of Pathology. Cells derived from both male and female mice were pooled for all experiments to minimise any sex bias. The ages of the mice varied from 8-15 weeks.

Primary macrophages were harvested from the peritoneum after intra-peritoneal injection of Brewer's Thioglycollate broth, which acts as an inflammatory stimulus resulting in the recruitment of macrophages. Mice were given peritoneal injections of 1ml thioglycollate and four days later euthanasia was performed using CO₂ asphyxiation and cervical dislocation. Peritoneal cavities were lavaged using cold PBS/5mM EDTA to increase yield and reduce cell clumping. Lavage fluid was then centrifuged at 400g for 5 minutes and supernatants discarded before re-suspending cells in cold medium. Cells were counted using 50% (v/v) Turk's solution, which causes membrane blebbing in macrophages allowing these cells to be discriminated from other cells present in the exudates. These inflammatory macrophages do not proliferate and were routinely plated at $.25 \times 10^6$ per well in 24 well plates, 0.5×10^6 per well in 12 well plates and 2×10^6 per well in 6 well plates to provide a confluent monolayer. Two hours after initial plating, cells were washed with warm medium to remove non-adherent cells and returned to the incubator for overnight incubation. This method provides a cell purity of >95% (Dr. P.R. Taylor, personal communication).

Bone marrow derived macrophages (BMDMs) were derived from C57BL6/SJL Syk deficient and control mice. Syk deficient bone marrow-derived macrophages (BMDM) were generated from chimeric mice, as described (Colucci, Turner et al. 1999). In brief, foetal livers from CD45.2 Syk^{+/-} x Syk^{+/-} matings, were removed from d16.5 embryos and the foetal liver cell suspensions from Syk^{-/-} and Syk-sufficient womb-mates were then used to reconstitute irradiated CD45.1 C57BL6/SJL recipients. Bone marrows were harvested 4-6 weeks after reconstitution, and checked by FACS for CD45.2 expression. Femurs were harvested from mice and marrow washed out of the femoral cavities using a 26G needle and syringe filled with sterile cold PBS/5 μ M EDTA. Cells

were collected in a cell strainer and forced through the strainer using a syringe plunger. Bone marrow cells were then pelleted at 250g x 5 minutes before resuspending in fresh cold medium containing 20 % L-cell conditioned medium (LCM). Cells were then transferred to Petri dishes and allowed to adhere for two days in the incubator. Thereafter non-adherent cells were removed and fresh medium added. Fresh medium was then added every day to ensure optimal concentrations of M-CSF were present to stimulate cell proliferation. When cells approached confluence, they were lifted and split into two dishes to minimize contact inhibition. Cells were plated for experiments at day 7 and 8 after isolation. BMDMs were used at the same number of cells per well as Thioglycollate elicited macrophages.

All cells were washed 3x with fresh medium prior to all subsequent experiments to remove both dead cells and local opsonins secreted by macrophages.

2.4 Methods

2.4.1 General methods

2.4.1.1 Polymerase chain reaction (PCR)

25 μ l PCR reactions containing dATP, aTTP, dCTP and dGTP each at 0.2mM, primers (0.5 μ M), 1.5mM MgCl, 1x manufacturers' PCR buffer (20mM Tris-HCl (pH8.4), 50mM Kcl), 0.5 units of Taq polymerase (Gibco Life Technologies) and 100ng of template DNA were performed using a hot-lid thermo cycler. Cycling parameters for DNA amplification were dependent on primers and cDNA, however, standard cycle parameters involved a denaturation step (94°C for 90 seconds) followed by 30-40 cycles of denaturation (94°C for 30 seconds), annealing (50°C for 30 seconds), extension (72°C for 1 minute per kb of template). After cycling the reaction was

incubated at 72°C for a further 6 minutes and then maintained at 4°C. 5µl from each reaction was subsequently analysed by agarose gel electrophoresis or stored at -20°C until use.

High fidelity PCR was used for to amplify constructs to be used for the generation of the new cell lines. Proof-reading Advantage-HF polymerase (Clontech) and manufacturers' buffers were used. Cycling parameters used involved: denaturation step (94°C for 90 seconds) followed by 30-40 cycles of denaturation (94°C for 30 seconds), 5 cycles of 94°C for 30 seconds and extension at 72°C, 5 cycles of 94°C and extension at 70°C followed by 30 cycles of 94°C for 30 seconds and annealing at 68°C.

2.4.1.2 Agarose gel electrophoresis

The products of PCR reactions and RE digests were resolved by agarose gel electrophoresis for the purposes of analysis and purification. Gels were prepared by dissolving electrophoresis grade agarose in 0.5X TBE buffer to a final concentration of 1.0%. Ethidium bromide was added to the dissolved agarose once it had cooled to a final concentration of 0.5µg/ml. Ethidium bromide intercalates between base pairs of nucleic acids leading to enhanced fluorescence under UV light allowing bands to be clearly identified.

Blue Juice gel loading buffer (Invitrogen) was added at 1:10 dilution to samples before they were loaded onto the gel. A DNA size marker, Quanti-ladder from Origene (Rockville, USA) was also added for identification of fragments of specific size. Gels were run at constant voltage of 110V at 25mA, which gave good separation of products. Gels were visualised using a short wavelength (312nm) UV transilluminator,

or a hand-held long wavelength (366nm) UV transilluminator when it was necessary to limit damage to DNA products intended for use in cloning.

2.4.1.3 Purification of DNA from agarose gels

DNA to be used for cloning was purified from other reaction components by agarose gel electrophoresis as described above. DNA was excised from the agarose gel under long wave (366nm) UV transillumination, weighed and extracted from the agarose using the Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturers' protocol. Eluted DNA was resuspended in buffer containing 10mM Tris (pH8.5).

2.4.1.4 Phenol chloroform extraction

Phenol chloroform extraction was performed to remove all traces of alkaline phosphatase from dephosphorylated plasmid digests prior to ligation. A pre-prepared phenol/chloroform/isoamyl alcohol (25:24:1) pH8 mix was purchased from Sigma. An equal volume phenol chloroform was added to the nucleic acid sample and mixed by vortexing. The aqueous phase containing the nucleic acid was transferred to a clean eppendorf tube and the nucleic acid was precipitated using 0.1 volume of 3M sodium acetate (pH5.2) and 2 volumes 100% ethanol. When nucleic acid concentration was predicted to be low, 1µl biochemical grade glycogen (Roche) was added to make it possible to see the pellet. The nucleic acid was pelleted by centrifugation at 13000rpm for 15 minutes in an Eppendorf 5417R centrifuge at 4°C and then washed in 70% ethanol to remove residual salts. After air-drying, the nucleic acid pellets were either suspended in DEPC treated ddH₂O or T₁₀E_{0.1}.

2.4.1.5 Plasmid digestion

RE digests of DNA inserts and plasmid vectors were performed at 37°C in the appropriate buffers supplied by the manufacturers (New England Biolabs or Boehringer Mannheim). Manufacturers' protocols were consulted for guidelines on the usage of each enzyme. In general 25-50ng plasmid DNA was digested with 10U enzyme in the presence of BSA to inhibit denaturation of the enzyme. The proportion of RE in the total reaction mix was never allowed to exceed 10% to prevent glycerol interference with the reaction. DNA was digested for 2 hours at 37°C to ensure maximal digestion. The digestion reaction was terminated by heat inactivation of the enzyme or phenol-chloroform as appropriate. When plasmids needed to be digested with 2 different RE, manufacturers' protocols were consulted to determine whether both digests could be performed in the same buffer. When this was not possible, sequential digestions were performed with the DNS being phenol-chloroform extracted and examined by agarose gel electrophoresis before the second digest was performed. The success of every RE digest was checked by gel electrophoresis. This also served to purify the desired insert or cut vector, which could then be excised, and gel purified.

2.4.1.6 Plasmid dephosphorylation

The plasmids used in this thesis were always digested with 2 different REs to create non-compatible 5' and 3' termini, which prevent the plasmid from re-circularising by self-ligation in the presence of the ligase enzyme. To prevent self-ligation by incompletely digested plasmids it is necessary to remove 5' phosphate groups from their termini. Removal of these groups means that plasmids can only ligate to insert DNA that contains 5' phosphate groups. The dephosphorylation was performed using 2U of calf alkaline phosphatase (Roche) in the presence of manufacturer-supplied

buffer. The reaction was incubated at 37°C for 60 minutes before phenol chloroform extraction.

2.4.1.7 Ligation of plasmid vectors and insert DNA

Ligations in this thesis were either carried out between annealed oligonucleotides and digested plasmid vector, Between PCR product and digested vector or between gel-purified inserts, excised from one vector, and ligated into another. Optimal insertion occurs when the ratio of insert to plasmid vector is 2:1. The lower the ratio, the lower the risk of oligomeric inserts. Colonies resulting from the lowest insert to plasmid ratio were deemed to least likely to contain oligomeric inserts and were carried forward in cloning experiments.

Generally 25ng of plasmid were ligated to 0, 25, 75 and 250ng insert DNA in 20µl reactions containing 1U T4 DNA ligase (Roche) and the manufacturer supplied buffer. A no plasmid DNA control was also included to observe background self-ligation and *E.Coli* growth or the presence of contaminating plasmids. Ligations were performed overnight at 12°C in a MJ Research thermal cycler. The reaction was terminated by storing ligation products at -20°C.

2.4.1.8 Transformation and colony selection

Top10 competent RecA⁻ *E.Coli* were purchased from Invitrogen and stored in aliquots at -80°C until used. Competent cells were thawed on ice and kept on ice throughout the transformation to maximise competency. Typically 1µl ligation mix was added to 15µl competent cells and incubated for 30 minutes on ice. Transformation was achieved by heat shocking competent cells at 42°C for 30 seconds, followed by transfer to ice for

2 minutes. 285µl SOC medium was added to the cells and they were incubated at 37°C on a shaking platform for 1 hour. This allows cells to begin transcription of the ampicillin resistance gene prior to being added to dry ampicillin-containing (100µg/ml) agar plates and incubated at 37°C overnight.

All plasmids used contained ampicillin resistance cassettes thus only bacteria containing successfully ligated plasmids could grow to generate colonies on the ampicillin containing agar plates. It is possible for digested plasmids to circularise during ligation without incorporating insert and thus to grow into colonies, furthermore non-transformed bacteria can grow if local ampicillin concentrations are depleted by the diffusion of β -lactamase secreted by successful transformants resulting in satellite colonies. The latter problem can be avoided by keeping incubation times short and by careful colony selection, but the former only by colony PCR and plasmid sequencing.

2.4.1.9 Colony PCR

Colonies were screened by PCR prior to selection to confirm that they carried an insert. PCR is carried out using primers designed to amplify the insert of interest. Carefully touching a colony with a pipette tip, and then transferring the tip directly to a tube containing PCR master mix, provides the bacterial template. Colonies tested are numbered allowing for later identification of those yielding positive results. These PCR reactions confirm that a plasmid carries an insert, and that the insert is the correct size, but cannot establish whether the insert has the correct orientation or sequence. Insert positive colonies were selected and grown up for mini prep and sequencing.

2.4.1.10 Plasmid mini prep

In order to generate enough plasmid DNA for analysis by RE digest and sequencing, the selected colony PCR positive colonies were 'miniprepped'. Colonies were picked with a culture loop and added to 500µl LB broth. The colony was then distributed throughout this volume by pipetting before transferring this volume to 50ml of LB medium containing 50µg/ml ampicillin and then incubating on a shaking platform at 37°C overnight. The culture was pelleted at 5000rpm for 10 minutes and then processed using a QIAprep Spin Miniprep Kit (Qiagen) and the purified plasmid DNA was resuspended in 50µl buffer containing 10mM Tris (pH8.5). This DNA was pure enough for direct sequencing to confirm the fidelity of the inserted sequence.

2.4.1.11 Plasmid sequencing

Automated sequencing based on the Sanger method was carried out at the Sir William Dunn School of Pathology or the Department of Biochemistry, Oxford University. Forward and reverse primers specific for the multiple cloning region of the plasmid, or the insert were either purchased or designed so that insert sequence and orientation with respect to plasmid could be determined. Sequence profiles were analysed using the BLAST programme for comparison with the RAW derived Dectin-1 sequence. Where discrepancies were observed, sequence traces were consulted. Only DNA with no sequence errors was carried forward for maxiprepping.

2.4.1.12 Maxipreps of bacterial transformants

Sequence verified miniprep DNA was used in large-scale DNA preps (maxipreps) to obtain sufficient yields for multiple transfection experiments. Top10 competent *E.coli* were transformed with 1µl miniprep DNA and added to autoclaved large volume flasks containing 1000ml of LB broth with 100µg/ml ampicillin and incubated at 37°C on a

shaking platform overnight. The bacteria were pelleted by centrifugation at 6000rpm for 15 minutes at 4°C in a Beckman J2-21 centrifuge.

To save costs and to recover highly pure plasmid DNA, it was elected to use caesium chloride gradients (CsCl) (Birnboim 1983) rather than commercial maxiprep kits. The bacterial pellet was initially resuspended in 5 ml CsCl Solution I, brought to 15ml. The EDTA content of this solution helps to damage the integrity of bacterial walls and membranes. After 30 minutes at 4°C, the bacteria were lysed by the addition of 30ml CsCl Solution 2. This solution contains both SDS detergent and NaOH and raises the pH of the suspension to around pH12. At this pH following a short incubation, non-supercoiled (bacterial genomic) DNA is denatured, but supercoiled plasmid DNA is not. After a maximum of 5 minutes, the lysis conditions are neutralised by the addition of 22.5ml CsCl Solution 3 and thorough mixing. The neutralised suspension was kept at 4°C for 30 minutes. During this incubation denatured genomic DNA precipitates with the detergent. The suspension is then centrifuged at 12000g for 10 minutes in a pre-cooled Beckman J2-21 centrifuge and the supernatants filtered through cell filters (Falcon). The DNA was precipitated by the addition of 0.7 volumes of Isopropanol and kept on ice for 60 minutes before centrifugation at 12000g for 30 minutes in a 4°C centrifuge. Pellets were carefully rinsed in cold 70% ethanol, air dried and then resuspended in 4ml T₁₀E₁.

For caesium chloride purification of the plasmid DNA, 5.5g of biochemical grade CsCl was added to the plasmid solution and mixed in a 37°C shaker for a few minutes until dissolved. 200µl of a 10mg/ml ethidium bromide solution was added to this solution. Using a refractometer, and through the addition of T₁₀E₁, the final refractive index was

adjusted to 1.389 which corresponds to approximately 1g/ml final concentration of CsCl. Solutions were transferred to Beckman heat-sealable ultracentrifuge tubes and sealed. These were centrifuged at 85000rpm in a Beckman TL-100 ultracentrifuge at 25°C for 16 hours. Tubes were decompressed by insertion of a 21G needle at the top and then the lower DNA band, that contains closed circular plasmid DNA was extracted with a syringe and needle. The lower band is formed by supercoiled DNA, which intercalates less ethidium bromide than linear fragments (upper band), making it less dense in CsCl and accounting for its lower position. Ethidium bromide was subsequently removed from the plasmid DNA by repeated extractions with an equal volume of water-saturated butanol, until all the pink colour was removed. The plasmid DNA was then diluted in an equal volume of ddH₂O and precipitated in 2 volumes of ethanol. The resulting pellet was washed with 70% ethanol and resuspended in 2ml T₁₀E₁₀. Any contaminating RNA was removed by incubation with 20µg/ml RNase A at 37°C for 15 minutes. Contaminating protein was removed by the addition of SDS at a final concentration of 0.1% (w/v), followed by two rounds of phenol-chloroform extraction. The purified plasmid DNA was finally precipitated with 0.1 volume 3M sodium acetate (pH5.2) and two volumes ethanol (as previously described), washed in 70% ethanol and resuspended in ddH₂O.

2.4.1.13 Isolation of genomic DNA

In order to confirm that the newly generated cell lines expressed the entire sequence introduced by retroviral transduction genomic DNA was isolated. Cells were lifted, pelleted at 250g for 5 minutes and lysed in cell lysis buffer (CLB) which had been supplemented with 50µg/ml protease K (BDH). Lysates were then incubated on a shaking platform at 55°C overnight.

Genomic DNA was extracted by adding an equivalent volume of phenol to lysates, shaking and transferring to phase lock tubes. Tubes were centrifuged for two minutes at 13000rpm in a MSE Harrier 18/80 centrifuge and supernatants removed and placed in a fresh phase lock tube. An equal volume of chloroform:isoamyl alcohol was added and the tubes shaken and centrifugation repeated. The aqueous phase was transferred to a new eppendorf tube, 1/10 volume 3M (pH5.2) sodium acetate was added followed by an equal volume isopropanol. Gentle shaking precipitated the DNA which was pelleted, washed with 70% ethanol, air dried, and resuspended in T₁₀E₁₀ prior to use. Sequence integrity was established by PCR using proprietary primers that flank the retroviral insert. Empty vector transduced cells served as a negative PCR control.

2.4.1.14 Polyacrylamide gel electrophoresis (PAGE)

Whole cell extracts were prepared by lysing cells using lysis buffer supplemented with protease inhibitors (2µg each of aprotonin, leupeptin and trypsinase and 1mM PMSF) immediately prior to use. Cells were washed with cold PBS and lysed at 4°C for 30 minutes. Extracts were harvested by scraping with a syringe plunger and then centrifuged for 15 minutes at 4°C to remove cellular debris. Supernatants were collected and frozen at -80°C prior to use.

The protein concentration of cell extracts was determined by BCA assay (Pierce) according to the manufacturers' instructions. Cell extract samples containing 25µg protein were loaded with sample buffer onto a 10% (w/v) acrylamide gel alongside molecular weight markers (Amersham Biosciences). Gels were run in running buffer at 20mA until the dye front had run off the end of the resolving gel.

2.4.1.15 Western blotting

Following PAGE electrophoresis, resolved polypeptides were transferred to HybondC+ nitrocellulose membranes (Amersham Biosciences) by applying a constant current of 300mA for 1 hour. Membranes carrying trapped polypeptides were blocked for 1 hour using Western-Blot Blocking buffer. The milk proteins reduce non-specific interactions between antibody and membrane by saturate potential binding sites without compromising specific antibody-ligand interactions.

Primary antibodies were diluted between 1/100 and 1/5000 (depending on the manufacturers' recommendations) in blocking buffer. Membranes were incubated with primary antibodies for 1 hour at room temperature with gentle agitation, then washed three times with blocking buffer. Secondary HRP conjugated antibodies (Jackson ImmunoResearch) were typically used at 1/5000 in blocking buffer and added to membranes for 1 hour with agitation followed by washing with Western-Blot wash buffer. A final PBS only wash was carried out to remove any traces of detergent. The ECL (Amersham Life Sciences) was used to detect specific antibody binding to the membranes and emitted light detected on X-ray film (Kodak) following incubation times that varied between 10 seconds and 5 minutes.

2.4.1.16 Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked Immunosorbent Assays (ELISAs) were performed to measure the level of TNF- α secreted into the culture supernatant over time by primary murine macrophages and murine cell lines treated with particulate and soluble glucans as well as LPS (Sigma) as a control. Thioglycollate-elicited, bone marrow derived, or Dectin-1

expressing RAW 264.7 macrophages were plated at 2.5×10^5 per well in triplicate in 24 well plates in the appropriate medium and treated as required. After 3 hours in the case of Thioglycollate and RAW macrophages and after overnight culture in BMDMs aliquots of 150 μ l cell supernatant were taken and stored at -80°C .

ELISA was carried out with 100 μ l of supernatant using a OPTeia Mouse TNF- α Set (Pharmingen). Every plate included a serial dilution of standards, in duplicate, that were analysed alongside cell supernatants. Medium only controls for background signal were also included. After termination of the reaction with stop solution (dilute hydrochloric acid), sample absorbance was read at 450nm (test) and 570nm (reference) wavelengths on an Anthos ht11 plate reader. Corrected absorbance was obtained by subtracting the reading at 570nm from the reading at 450nm. Levels of TNF- α were quantified by interpolation from the standard curve results plotted using Microsoft Excel.

2.4.1.17 Antibody testing

To study Dectin-1 expression at the protein rather than mRNA level a monoclonal antibody was developed (in collaboration with Dr. Delyth Reid, Jenner Institute, Compton). The antibody was raised to NIH-3T3 cells stably transfected with Dectin-1. Following screening of rat hybridomas an antibody, 2A11, was found to recognise a fixation sensitive epitope on Dectin-1. This antibody is an IgG2b subclass and its epitope resides in a region of the carbohydrate recognition domain such that the antibody very effectively blocks ligand recognition (Brown, Taylor et al. 2002).

To define the late-endosomal and lysosomal compartments of macrophages, the marker LAMP-1 was selected (Pitt, Mayorga et al. 1992). The hybridoma producing this

antibody was purchased from the Developmental Studies Hybridoma Bank at the University of Iowa. To produce antibody stocks, the hybridoma was grown up in 2000ml DMEM¹⁰ and allowed to grow until the medium was depleted and all cells had died. Supernatants were cleared of cellular debris by centrifugation at 1000g for 10 minutes and were then 0.22µm filtered. Filtered supernatants were run over a Protein G Sephrose column and fractions of purified antibody were eluted from the column using 50mM glycine pH2.5 into an equal volume of 1M Tris pH8 to neutralise the elution buffer. Eluted fractions were analysed by spectrophotometry at 230 and 260nm (on a Beckman Coulter DU 800 spectrophotometer) and antibody concentration was quantified according to the formula (Absorbance at 230nm * 183) – (Absorbance at 260nm * 78.3). The 6 fractions containing the highest antibody concentrations were pooled, injected into a 10kDa cut off dialysis cassette (Perbio, Cheshire, UK) and dialysed against 2 litres of PBS overnight. Quantitation of antibody concentration was repeated by both spectrophotometry and BCA protein assay. Purified antibodies were used at 10µg/ml and were tested on fixed, permeabilised macrophages and analysed by flow cytometry as previously described.

2.5 General cell biology techniques

2.5.1 Transient transfection of constructs using cationic lipids

The plasmid constructs described above were transfected into NIH-3T3 cells using Lipofectamine according to the manufacturers' protocol that had been optimised for transfection efficiency by varying the relationship between DNA and cationic lipid (data not shown). 5×10^4 NIH-3T3 cells were seeded onto an acid treated 13mm coverslip and incubated overnight. The following morning a transfection mix was prepared by adding 1µg of plasmid DNA and 5µl of Lipofectamine to 0.75ml

OPTIMEM and allowing to stand for 15 minutes. Serum was removed from cells by washing three times with PBS and then the transfection mix was added and cells incubated at 37°C overnight. The following morning cells were processed for confocal microscopy as described below.

2.5.2 Electroporation

RAW 264.7 macrophages were grown to 80% confluence, lifted and pelleted at 250g for 5 minutes. Cells were resuspended, counted and counts adjusted to 25×10^6 /ml. 200µl aliquots of this suspension were transferred to eppendorf tubes and 10µg of plasmid DNA dissolved in 50µl PBS was added and allowed to stand for 10 minutes at room temperature. The entire volume was then transferred to a 0.4cm gap cuvette (BioRad) and electroporated at settings of 975µF and 280V for 40 milliseconds using a Gene Pulser apparatus. Cells were then immediately transferred to 5 ml fresh culture medium containing 20% FCS and pelleted at 600g for 5 minutes. Cells were resuspended in fresh medium containing 20% FCS, counted and plated onto 13mm coverslips as describe above. Cells were incubated for two hours before medium was replaced again. All assays were performed 24 hours after transfection.

2.5.3 Virus production

Inserts encoding recombinant Dectin-1 molecules were excised from the PSK vector by RE digestion and ligated into a retroviral vector, pFB Neo (Stratagene). Following ligation, colony selection and sequencing, vectors containing various Dectin-1 mutant sequences were transfected into a packaging cell line Φ NXeco (a gift from Dr Gary Nolan, Stanford, CA). On Day 1, recently passaged Φ NXeco cells were plated at 2×10^6 per well in 6 well plates overnight in 3ml DMEM medium. The following day (Day 2)

the medium was gently replaced to avoid detaching cells to a final volume of 2ml per well. Φ NXeco cells were then transfected according to the manufacturers' protocol with Fugene 6 using 1 μ g DNA and 6 μ l Fugene per well and incubated overnight at 37°C. On Day 3 plates were transferred to a 32°C incubator in a CO₂ purged airtight box. On Day 4 supernatants were harvested and polybrene, which reduces electrostatic repulsive forces between membranes was added at 5 μ g/ml. Supernatants were then filtered through a 0.45 μ m filter and aliquots of 2 ml were stored in cryovials at -80°C prior to use.

2.5.4 Retroviral transduction and selection of fibroblast and macrophage cell lines

On Day 1 NIH 3T3 cells and RAW 264.7 cells were plated at 2×10^5 cells per well in 6 well plates overnight. The following morning cells were washed using fresh medium and the medium was replaced with 1.5ml of the filter sterilised viral supernatants. Plates were then spun at 2500rpm at 25°C for 2 hours to bring cells and viral particles into contact with one another. Plates were then returned to the 37°C incubator overnight. On day 3 the medium was replaced with fresh DMEM and on Day 4 the medium was replaced with selection medium: DMEM containing 600 μ g/ml G418. Cells were kept under selection until transduced colonies became clearly visible before lifting and transferring cells initially to 25cm² tissue culture flasks and later to T175 flasks. Initial attempts to generate RAW cell lines resulted in low transduction efficiency and relatively few colonies. We found that transduction efficiency could be markedly enhanced if the cells were first pre-treated with tunicamycin (Sigma), which reduces the extent to which surface proteins are N-glycosylated, prior to transduction.

2.5.5 Flow cytometry

In this thesis extensive use is made of flow-cytometry based assays. General principles will be discussed here and more assay-specific details in the results chapters. Flow cytometry makes it possible to examine fluorescently tagged features of a cell population on a cell-by-cell basis, allowing quantitation of cellular characteristics as well as revealing variability within populations.

In the majority of assays cells were stained prior to fixation to preserve sensitive epitopes, and this staining was performed on adherent cells. Staining was performed at 4°C to limit membrane mobility and cells were blocked with FACS block buffer containing either 5% rabbit or 5% goat serum, depending on the secondary antibody used, to reduce non-specific staining. In experiments using macrophages, the antibody 24G2 that blocks FcγRII/III was added to FACS block to reduce non-specific background staining. Cells were always lifted prior to fixation to prevent clumping. In cases where cells were fixed prior to staining, permeabilisation was achieved by adding Saponin to FACS block for 30 minutes prior to the addition of antibodies. All primary antibodies were used at 10µg/ml and cells were washed three times with FACS block between staining steps. Secondary antibodies were used at between 1:100 and 1:200. All experiments using monoclonal antibody staining contained cell populations stained with isotype matched control antibodies. Where polyclonal antibodies were used, secondary only controls were performed. Incubation times were typically 60 minutes for primary antibody and 45 minutes for secondary antibody staining. In three colour experiments, where cells were stained for surface as well as intracellular epitopes, surface staining was performed at 4°C followed by lifting, fixation, permeabilisation, a repeat blocking step, and a second round of staining and a fixation.

2.5.6 Confocal microscopy

Optical sectioning of cells by confocal microscopy allows for more accurate analysis of the spatial relationships between sub-cellular compartments than traditional fluorescence microscopy. Nonetheless, the practical resolution limit of conventional confocal microscopy is about 200nm, a limit imposed by the wavelengths of visible light. Co-localisation of fluorescence signals from separately labelled sub-cellular compartments thus indicates a high probability of an interaction rather than definitive proof.

To provide a better surface for adhesion, cells were grown on 13mm coverslips that had been treated overnight in 70% nitric acid (BDH). Cells were generally plated at a low density, typically 5×10^4 per coverslip to minimise cell membrane overlap and incubated overnight before use in experiments. Cells were fixed using a 2% solution of paraformaldehyde diluted in 200mM HEPES buffer for 20 minutes. To reduce background staining, free aldehyde groups were quenched using confocal quench solution for 10 minutes. Permeabilisation was achieved by incubating cells for 5 minutes with a 0.2% Triton X100 solution containing 1% BSA in PBS. Cells were then washed with PBS to remove the detergent and blocked with confocal block for 30 minutes prior to antibody staining. Primary antibodies were added to confocal block solution at 10 μ g/ml and incubated for 60 minutes, and secondary antibodies were used at 1:200 for 40 minutes. Where possible, directly conjugated antibodies were used to further reduce background. Washes between staining steps were performed with confocal block solution. Following staining coverslips were washed three times with PBS and once with ddH₂O to prevent salt crystal formation. Coverslips were then inverted onto drops of polymerising mountant (Aqua Polymount) and allowed to

polymerise for at least 4 hour prior to confocal imaging. Images were captured on a BioRad 1024 confocal microscope and processed using Adobe Photoshop 6.0.

2.5.7 Synchronising phagocytic particle uptake

Synchronised uptake of phagocytic particles was essential for the majority of experiments in this thesis. Synchronisation was achieved by two different techniques. For flow cytometry based assays cells were cooled to 4°C for 30 minutes prior to the addition of phagocytic particles that were allowed, under gravity, to settle on cells for 60 minutes before thorough washing with cold medium and placing cells in a 37°C incubator or warm bath. Confocal experiments required a different technique as cooling cells attached to glass coverslips results in the detachment and loss of a large proportion of cells. Coverslips were washed with warm medium and a suspension of particles in pre-warmed medium was added and plates were immediately spun at 1000rpm for 60 seconds to bind particles to cells. On removal from the centrifuge, coverslips were immediately washed with warm medium to remove unbound particles and returned to the incubator for the designated time periods before fixation and processing as described.

2.5.8 Inhibition of protein synthesis

Metabolic labelling experiments were carried out to establish doses of the peptidyl transferase inhibitor cycloheximide that would effectively prevent *de novo* protein synthesis within murine primary macrophages. The time course for inhibition of protein synthesis was 2 hours, and cycloheximide concentrations within the range selected from the literature (Paradisi, D'Onofrio et al. 1979) was used. An untreated sample was used as a control.

C57/BL/6 thioglycollate-elicited macrophages were plated at 2×10^6 cells per well in a 6 well plate and cultured overnight in methionine free DMEM supplemented with 2mM L-glutamine, 250U/ml penicillin G sodium and 250 μ g/ml streptomycin sulphate. The following morning cells were washed in methionine free medium and incubated for 2 hours with fresh methionine-free medium containing 10 μ g/ml cycloheximide. Thereafter 100 μ Ci 35 S-methionine (Amersham) per well was added and cells were incubated for two hours at 37°C. Methionine is an essential amino acid therefore any newly synthesised protein would incorporate the radiolabelled amino acid.

After 2 hours of incubation cells were cooled to 4°C to prevent any further protein synthesis. Cells were washed three times with fresh, cold, medium and then with cold PBS prior to the addition of 500 μ l lysis buffer. Cell lysates were added to scintillation enhancing fluid in scintillation counter tubes and the incorporation of radio labelled amino acid was quantified using a Beckman LS 5000CE scintillation counter.

2.5.9 Inhibitor treatment of cells

Soluble inhibitors of the ligand-binding site of Dectin-1 were always used at 100 μ g/ml and incubation was performed at 4°C for 60 minutes before either warming cells to 37°C in the presence of inhibitor, or washing away soluble inhibitor prior to addition of particulate ligands at 4°C. Soluble inhibitors used were Laminarin, glucan phosphate, mannan and a control glucan, Dextran 40. Pharmacological inhibitors of receptor signalling were all solubilised in DMSO with the exception of Toxin B that was solubilised in sterile tissue culture grade water from Sigma-Aldrich (Dorset, UK).

Inhibitors were used at the concentrations indicated in Table 1. Incubation periods are also indicated.

Table 2.5.9

Inhibitor	Doses tested	Dose used	Incubation time	References
Cytochalasin D	1-5 μ M	2 μ M	40 minutes	- (Greenberg, Chang et al. 1994)
Genistein	150 μ M	150 μ M	10 minutes	- (Itoh, Suzuki et al. 2002)
PP1	12.5-50 μ M	15 μ M	45 minutes	- (Majeed, Caveggion et al. 2001)
PP2	12.5-50 μ M	15 μ M	45 minutes	- (Hu, Punturieri et al. 2002)
PP3	12.5-50 μ M	15 μ M	45 minutes	- (Hu, Punturieri et al. 2002)
Piceatannol	10-50 μ M	50 μ M	45 minutes	- (Majeed, Caveggion et al. 2001)
Wortmannin	1ng/1 μ g/ml	100ng/ml	45 minutes	- (Cox, Tseng et al. 1999)
Chelerythrine	10 μ M	10 μ M	30 minutes	- (Itoh, Suzuki et al. 2002)
Toxin B	1-100ng/ml	100ng/ml	120 minutes	- (Massol, Montcourrier et al. 1998)

PP1=[4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo[3,4d] pyrimidine]

PP2=[4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo(3,4-d)pyrimidine]

PP3=[4-amino-7-phenylpyrazolo(3,4-d)pyrimidine],

Concentrations of reagents added to cells were selected on the basis of manufacturers' advice and published literature. Preliminary experiments were performed to ensure no loss of viability amongst treated cells as judged by microscopic observation and trypan blue exclusion as well as overnight culture following removal of inhibitor.

2.6 Techniques specific to this thesis

2.6.1 Generation of cell lined stably expressing Dectin-1 molecules

2.6.1.1 HA tagged plasmid production

The pBluescript SK phagemid (Stratagene) was digested using the restriction enzymes (REs) Sall and XmaI or Not-I and XmaI and gel purified as described earlier. Oligonucleotide adapters were ordered from Sigma Genosys Ltd. (**Table 2**) and diluted to 100nmol/ml working solution.

Table 2.6.1.1

Cytoplasmic HA tag (N-Terminal)	
<i>Restriction enzyme site:</i> <i>SalI</i>	<i>Kozak</i>
<i>HA</i>	<i>XmaI</i>
Oligo Set 1: 5' P ^o - <u>TCGAC</u> CCA CCATG GGA TAC CCA TAC GAT GTT CCG GAT TAC GCT <u>C</u>	
3'	<u>G</u> GGT GG TAC CCT ATG GGT ATG CTA CAA GGC CTA ATG CGA <u>GGGCC</u> -P ^o

Extracellular HA tag (C-Terminal)	
<i>Restriction enzyme site:</i> <i>XmaI</i>	<i>HA</i>
<i>Stop</i>	<i>NotI</i>
Oligo Set 2: 5' P ^o - <u>CCGGG</u> TAC CCA TAC GAT GTT CCG GAT TAC GCT <u>TAA</u> <u>GC</u>	
3'	<u>C</u> ATG GGT ATG CTA CAA GGC CTA ATG CGA <u>ATT</u> <u>CGCCGG</u> -P ^o

These adapters were annealed by mixing 20µl of each adapter solution with 5µl 10X annealing buffer and 5µl DEPC water, heating the solution to 65°C for 10 minutes in a thermal cycler, and then cooling slowly to room temperature over 2 hours. Ligated adapters were then stored at -20°C. Adapters were then ligated into the cut vector as described below, grown in bacteria and isolated by miniprep. The new HA containing vectors are referred to as PSK-3'HA and PSK 5'HA

2.6.1.2 Dectin-1 mutant receptor generation by PCR

Dectin-1 was amplified by PCR from a pCDNA3.1 vector (from Gordon Brown) using the sets of primers illustrated in table 3 to introduce a new restriction site, XmaI into the Dectin-1 sequence.

Table 2.6.1.2a

5' AAA <u>CCC GGG</u> AAA TAT CAC TCT CAT ATA GAG	T _m complementary=54, T _m total=84
<i>XmaI</i>	
5' TTT <u>GGA TCC</u> TTT ACA GTT CCT TCT CAC AG	T _m complementary=56, T _m total=82
<i>BamHI</i>	
5' AAA <u>CCC GGG</u> CAG TTC CTT CTC ACA GAT ACT	T _m complementary=50, T _m total=90
<i>XmaI</i>	
5' AAA <u>GAA TTC</u> GCC CTG TGA AGC AAT G	T _m complementary=50, T _m total=72
<i>EcoRI</i>	

PCR conditions were optimised using melting temperatures (T_m) as a guide. High fidelity PCR kit Advantage-HF-2 (Clontech) was used according to manufacturers' protocol. PCR products were gel purified and ligated into a TA cloning vector (Invitrogen). Reactions were allowed to proceed for 30 minutes at room temperature

and terminated by storage at -20°C. Top10 bacteria were transformed using TA vectors containing PCR products and grown on agar plates containing ampicillin. Colonies were selected, grown in broth and plasmid DNA extracted by miniprep as described. Plasmids were then sequenced using proprietary primers to confirm sequence fidelity.

TA cloning vectors containing correct sequences and the two PSK-HA vectors were digested with the appropriate enzymes and gel purified. The PSK HA vectors were dephosphorylated and ligated with the appropriate inserts to yield Dectin-1 constructs that contained 5' (cytoplasmic) and 3' (extracellular) HA tags. These plasmids were grown in bacteria and colony PCR was performed to confirm the presence of inserts. These colonies were subsequently grown in bacteria, and plasmids isolated by miniprep and sequencing performed using proprietary T3 and T7 primers to confirm fidelity. Plasmids containing 3' and 5' HA tagged Dectin-1 tested by transient transfection into NIH-3T3 cells and confocal microscopy, prior to being subcloned into the pFBNeo retroviral vector.

The 3'HA tagged Dectin-1 construct was selected as the template for the generation of cytoplasmic tail mutated receptors. PCR was used to replace the bases TAT encoding the cytoplasmic tyrosine residues with TTC encoding phenylalanine. PCR was also used to create a cytoplasmic tail truncated receptor. Table 4 lists the primers used for the PCR reactions.

Table 2.6.1.2b

Y1	AAA <u>CCC GGG</u> CAG TTC CTT CTC ACA GAT ACT <i>XmaI</i>
	AAA <u>GAA TTC</u> GCC CTG TGA AGC AAT GAA ATA TCA CTC TCA TAT AGA GAA TCT GGA TGA <i>EcoRI</i>
	AGA TGG <u>ATT TAC</u> TCA A <i>Y→F</i>

Y2 & Y1Y2	AAA <u>CCC GGG</u> CAG TTC CTT CTC ACA GAT ACT <i>XmaI</i> AAA <u>GAA TTC</u> GCC CTG TGA AGC AAT GAA <u>ATT TCA</u> CTC T <i>EcoRI</i> <i>Y→F</i>
Truncation	AAA <u>CCC GGG</u> CAG TTC CTT CTC ACA GAT ACT <i>XmaI</i> AAA <u>GAA TTC</u> TCT TCA CCT TGG AGG CCC ATT EcoR1

These constructs were sequenced to confirm fidelity and subcloned into the pFBNeo vector, grown in bacteria, minipreped and stored at -80°C prior to use.

2.6.1.3 Transduction of cell lines

Raw 264.7 and NIH-3T3 cell lines were transduced as described above and placed under selection in G418 containing medium.

2.6.1.4 Cell line characterisation by flow cytometry

As Dectin-1 is trypsin sensitive, cells that had been under selection for several weeks were lifted using lidocaine/EDTA, pelleted at 260g for 5 minutes, resuspended in cold FACS block and counted. Samples of 1×10^7 cells in 100 μl were placed into V bottom wells of a 96 well plate and blocked for 30 minutes at 4°C prior to the addition of monoclonal antibodies against Dectin-1 (2A11) and the HA tag (HA11)(Covance) at 10 $\mu\text{g/ml}$. Cells were stained at 4°C for 60 minutes prior to pelleting at 300g for 10 minutes. Cells were washed three times with cold FACS block and then resuspended in 100 μl prior to the addition of phycoerythrine (PE) labelled secondary anti-rat and anti-mouse (Leinco) respectively. Secondary antibodies were incubated at 4°C for 45 minutes before washing three times. Cells were resuspended in cold PBS prior to fixation through the addition of an equal volume of 2% formaldehyde solution (Sigma). Cells expressing an intracellular HA tag were essentially processed in a similar manner, these cells were fixed for 30 minutes directly following the first blocking step. These

fixed cells were then permeabilised in FACS block containing 0.5% saponin and then stained as above. 2A11 was not used on these fixed cells as the epitope is fixation sensitive, HA11 staining revealed tag expression. Fixed cells were analysed using a BD FACSCalibur 4 colour flow cytometer and data processed using CellQuest software.

2.6.2 Comparative internalisation assay

2.6.2.1 Sheep red blood cell (SRBC) membrane labelling and opsonisation

200µl sheep red blood cells (TCS Biosciences) were suspended in 5ml PBS and counted. 1×10^8 cells were transferred to a round bottom polypropylene tube and pelleted at 400g for 5 minutes. These cells were then labelled with a PKH2 fluorescent membrane dye (Sigma) according to the manufacturers' protocol and pelleted at 800g for 5 minutes and washed three times with PBS. Labelled SRBC pellets were then suspended on 2ml PBS to which was added 1:500 anti-SRBC stroma (Sigma) for 60 minutes at 37°C, resulting in sub-agglutinating opsonisation. SRBCs were then pelleted at 800g for 5 minutes and washed three times with PBS and once with Medium before being resuspended in medium at 1×10^8 /ml. Prior to use phase contrast and fluorescent microscopy were used to ensure that labelling was successful and that no agglutination had occurred.

2.6.2.2 Cell plating

NIH-3T3 and RAW 264.7 cell lines expressing Dectin-1 full-length and mutant receptors were plated at a density of 5×10^5 per well in 12 well plates overnight.

2.6.2.3 Inhibitor treatments

In experiments where inhibitors were used, these were used at the concentrations and for the times indicated in table 1.

2.6.2.4 Synchronised particle phagocytosis

Synchronous uptake of 10 Zymosan FITC particles (NIH-3T3 cells) or either Zymosan particles or SRBC (RAW cells) per cell was initiated as described earlier. Cell lines were permitted to internalise particles for either 60 minutes (NIH-3T3 cells) or 30 minutes (RAW cells). The internalisation process was terminated both by cooling cells to 4°C, and blocking cells with cold Blocking buffer containing sodium azide.

2.6.2.5 Flow cytometry

Cells were blocked at 4°C with FACS block for 30 minutes prior to the addition of Zymosan opsonising antibody (Molecular Probes) at 1:1000 (in the case of zymosan particles) or no primary antibody (in the case of opsonised sheep red blood cells). Antibodies were incubated with cells for 60 minutes at 4°C prior to washing three times with cold FACS block. Secondary APC conjugated anti-rabbit antibody (Molecular Probes) was added at 1:200 for 45 minutes at 4°C before washing three times with FACS block and once with PBS. Cells were then lifted following 10 minutes of incubation with cold Lidocaine/EDTA using a cell lifter and disaggregated by pipetting. An equal volume cold FACS fix was added to the cell suspensions and cells were fixed for 60 minutes prior to analysis by two colour flow cytometry as described in the results.

2.6.3 Receptor recycling assays

2.6.3.1 Cells and cell culture

Thioglycollate elicited macrophages were isolated as described above. These macrophages were plated in 24 well plates at

2.6.3.2 Inhibition of protein synthesis

Metabolic labelling experiments were carried out to establish doses of the peptidyl transferase inhibitor cycloheximide that would effectively prevent *de novo* protein synthesis within murine primary macrophages. The time course for inhibition of protein synthesis was 2 hours, and cycloheximide concentrations within the range selected from the literature (Paradisi, D'Onofrio et al. 1979) was used. An untreated sample was used as a control.

C57/BL/6 thioglycollate-elicited macrophages were plated at 2×10^6 cells per well in a 6 well plate and cultured overnight in methionine free DMEM supplemented with 2mM L-glutamine, 250U/ml penicillin G sodium and 250 μ g/ml streptomycin sulphate. The following morning cells were washed in methionine free medium and incubated for 2 hours with fresh methionine-free medium containing 10 μ g/ml cycloheximide. Thereafter 100 μ Ci 35 S-methionine (Amersham) per well was added and cells were incubated for two hours at 37°C. Methionine is an essential amino acid therefore any newly synthesised protein would incorporate the radiolabelled amino acid.

After 2 hours of incubation cells were cooled to 4°C to prevent any further protein synthesis. Cells were washed three times with fresh, cold, medium and then with cold PBS prior to the addition of 500 μ l lysis buffer. Cell lysates were added to scintillation enhancing fluid in scintillation counter tubes and the incorporation of radio labelled amino acid was quantified using a Beckman LS 5000CE scintillation counter.

2.6.3.3 Low pH elution of bound ligands

Zymosan particles were bound, at a ratio of 25 particles per cell, to triplicate wells of Dectin-1 expressing CHO cells plated at a density of $.25 \times 10^6$ per well, at 4°C for 60 minutes. Cells were then washed three times for 30 seconds with either cold medium only, or with cold PBS the pH of which had been adjusted to 6,5,4,3,2,1.7 and 1. Following these washes, the cells were lysed using lysis buffer and the fluorescence was read on a Titertec Fluoroscan II fluorimeter. Monoclonal antibody to Dectin-1 was bound to the same cells at 4°C and cells were washed with PBS prior to washing three times with PBS the pH of which had been adjusted to 1.7. These cells were then stained with a PE-conjugated anti-Fc secondary antibody (Leinco) and cells were processed for flow cytometry as described.

2.6.3.4 Ligand induced receptor internalisation

Thioglycollate-elicited macrophages plated, in triplicate, at a density of $.25 \times 10^6$ per well in 24 well plates and incubated overnight. The following morning cells were washed and medium replaced, with designated cells receiving medium containing 50µg/ml cycloheximide, and incubated at 37°C for 2 hours. Plates were removed from the incubator and cooled to 4°C and washed three times with cold medium. Both soluble and particulate ligands were bound to cells at 4°C for 60 minutes. These plates were then placed in a pre-warmed 37°C warm bath for 10 minutes to allow synchronous internalisation of ligated receptors to occur. Following this incubation step, plates were again rapidly cooled to 4°C by washing three times with ice-cold medium that also removed unbound and non-internalised glucans. Medium was replaced with fresh cold medium containing cycloheximide where necessary. Control plates were kept cold and

remaining plates were placed in a 37°C incubator for between 10-160 minutes. Following incubation, plates were again cooled to 4°C, washed three times with cold medium to remove secreted opsonins, and kept cold for the remainder of the assay. Selected control wells were blocked with anti-Dectin-1 monoclonal antibody 2A11 at 10µg/ml for 60 minutes on ice prior to washing away unbound antibody. All wells then received 50 particles per cell FITC zymosan particles (Molecular Probes) suspended in 500µl ice-cold medium for 60 minutes. Cells were then washed 5 times to remove unbound particles and lysed with 200µl lysis buffer and a 1ml syringe plunger. Lysates were transferred to a black fluorimeter plate and fluorescence read at 488nm.

2.6.4 Dominant negative RhoGTPase assay

2.6.4.1 Plasmid preparation

The plasmids used were grown from mini-prep samples and were isolated by CsCl gradient purification, as previously described, to yield contaminant free DNA.

2.6.4.2 Transfection of plasmids

NIH-3T3 Dectin-1 expressing cells were plated at 1×10^6 per well in 6 well plates and incubated overnight. Using manufacturers' protocols these cells were transfected with the Cdc42 dominant negative construct. Various transfection reagents and DNA concentrations (1-4µg/well) in either serum containing or serum free OPTIMEM medium were tested. Only GenePorter 2 was found to result in acceptable levels of transfection as illustrated in the results. A plasmid designated Tm-7 (a gift from Dr. John Davies, Oxford, UK) was used as an irrelevant myc-tagged protein control to measure the effect of transfection on internalisation of bound zymosan particles.

2.6.4.3 Zymosan particle labelling

50mg of unlabelled zymosan particles (Molecular Probes) were suspended in 5ml sterile ddH₂O and sonicated for 6 minutes to disaggregate particles. Zymosan particles were then pelleted at 2500rpm for 2 minutes and resuspended in 9ml 0.15M NaCl to which was added 1ml labelling buffer. Labelling buffer comprised 0.58ml Carbonate buffer#1 and 1ml Carbonate buffer#2. FITC (Sigma) particles were dissolved in DMSO to a final concentration of 1mg/ml and this solution was titrated into 1ml aliquots of the 5mg/ml suspension of zymosan. Final concentrations of FITC ranging between 1 and 50µg/ml were used and particles were incubated in the dark for 2 hours followed by thorough washing of particles in sterile ddH₂O. Labelled particles were suspended in ddH₂O and counted prior to testing by flow cytometry as described in the results. Particles labelled with 10µg/ml FITC (FITC-10 zymosan) were selected for use.

2.6.4.4 Three-colour Flow cytometry

24 hour following transfection, NIH-3T3 Dectin-1 expressing cells were incubated with cytochalasin D (controls) or fresh medium at 37°C for 60 minutes, cooled to 4°C and washed three times with cold medium. Media were replaced with 1.0ml cold medium containing 10 particles per cell FITC-10 zymosan and incubated for 60 minutes at 4°C, then washed three times with cold medium. Cells were then processed as for the comparative internalisation assay described above.

Following 60 minutes of fixation, cells were washed three times with FACS Block and permeabilised with FACS Perm buffer and stained with anti-myc (Abcam) at 10µg/ml for 60 minutes at room temperature. Cells were then washed three times with FACS Perm and incubated with 1:100 PE conjugated anti-mouse Fc (Jackson

Immunoresearch) for 45 minutes. Cells were then washed three times with FACS Perm, once with PBS and fixed again with FACS Fix.

Controls used to compensate the flow cytometer against bleed through were:

- Cells with bound particles (Green)
- Cells expressing myc tagged dominant negative plasmid only (Red)
- Cells expressing myc tagged dominant negative plasmid and having bound particles (Red and Green).

These controls were performed during every experiment to ensure the minimum interference between channels. Cells transfected with DN-RhoGTPases were then analysed on the compensated flow cytometer as described in the results.

2.6.5 Analysis of the biological effects of zymosan

2.6.5.1 TNF- α determination

TNF- α secreted by macrophages stimulated with glucans was measured by ELISA as described above.

2.6.5.2 Transfection of epitope tagged TLR constructs and confocal microscopy

Dominant negative TLR constructs were obtained from Dr. Emmanuelle Caron (Imperial College, London) and RAW-D1 macrophages were transfected by electroporation as described above, plated on glass coverslips and incubated for 24 hours. Zymosan particles phagocytosis was synchronised by centrifugation as previously described and cells were allowed to internalise particles for 5 minutes prior to fixation and permeabilisation. Permeabilised cells were stained with an anti-V5

primary antibody (Abcam) and a Cy3 conjugated secondary antibody (Jackson ImmunoResearch) this was followed by staining with a 488 Alexa conjugated anti-HA antibody (Covance). Coverslips were then mounted and analysed as previously described.

2.6.5.3 Inhibitor studies

Macrophages were plated in triplicate wells as described, earlier then incubated with inhibitors (as described previously), prior to incubation with zymosan particles and determination of secreted TNF α by ELISA. Thioglycollate elicited macrophages were incubated for 3 hours with zymosan, and bone marrow derived macrophages overnight.

2.7 Statistical analysis

As a measure of the variability amongst samples and sample populations, to statistical analyses were performed using either Microsoft Excel or GraphPad Prism. The standard deviation (SD) was calculated where it was necessary to determine the spread of two data sets around the mean. The formula used for this calculation is:

$$SD = \sqrt{\frac{\sum d^2}{n-1}}$$

Where d = deviation for each data point

n = number of data points

Σ = sum of

$\sqrt{\quad}$ = square root

Where there are more than 2 data points within a series, the standard error of the mean (SEM) was calculated to give a more accurate estimate of the variation around the mean. The formula used for this calculation is:

$$\text{SEM} = \frac{\text{SD}}{\sqrt{\text{sample size}}}$$

The SD or SEM of data is represented as error bars in figures and the details are contained within figure legends.

In some cases it was necessary to employ statistical methods to determine whether differences between data sets were significant. Where there were only two sets of data, unpaired t tests were performed. Where there were multiple data sets, one-way analyses of variance (ANOVA) were performed to ensure the most stringent comparison of the data. Both tests were carried out using GraphPad Prism software. Two tailed P values (probability of the difference seen being due to chance) are shown where appropriate according to the key:

- * P = 0.01-0.05
- ** P = 0.001-0.01
- *** P < 0.001

P values above 0.05 were considered to indicate that the difference was not significant (N/S).

Chapter 3

Studies in NIH-3T3 cell models

3.1 Introduction

Leukocytes including macrophages and neutrophils play a central role in innate host defence against microbial infection. The ability of these cells to recognise and rapidly internalise pathogens resulting in microbial killing is a major weapon in their armoury. There are few well-described phagocytic receptors, and these include the opsonic Fc γ and complement receptors and perhaps several innate immune receptors including the MMR, SR-A and CEACAM-3, which are much less well studied. Direct recognition of fungal pathogens has been attributed to various receptors including Dectin-1, and phagocytosis of fungal pathogens by CR3 and the MMR has been demonstrated (Ezekowitz, Sastry et al. 1990; Brown and Gordon 2001; Taborda and Casadevall 2002). Dectin-1 directly recognises fungi (**figs. 3.1 and 3.3b**) and fungal derived particles (**figs. 3.3d and 3.3e**) in a β -glucan dependent fashion and is highly expressed on leukocyte populations (Brown, Taylor et al. 2002). The cytoplasmic tail of Dectin-1 contains an ITAM-like motif (Ariizumi, Shen et al. 2000), similar to classical ITAMs that are usually associated with phagocytic and activatory receptors. These features make it possible that Dectin-1 plays a role in innate anti-fungal defences and the presence of an ITAM-like motif suggests Dectin-1 may be capable of mediating the phagocytosis of such pathogens. We were thus interested in understanding whether Dectin-1 functioned as a phagocytic receptor and whether its ITAM-like motif mediated this process.

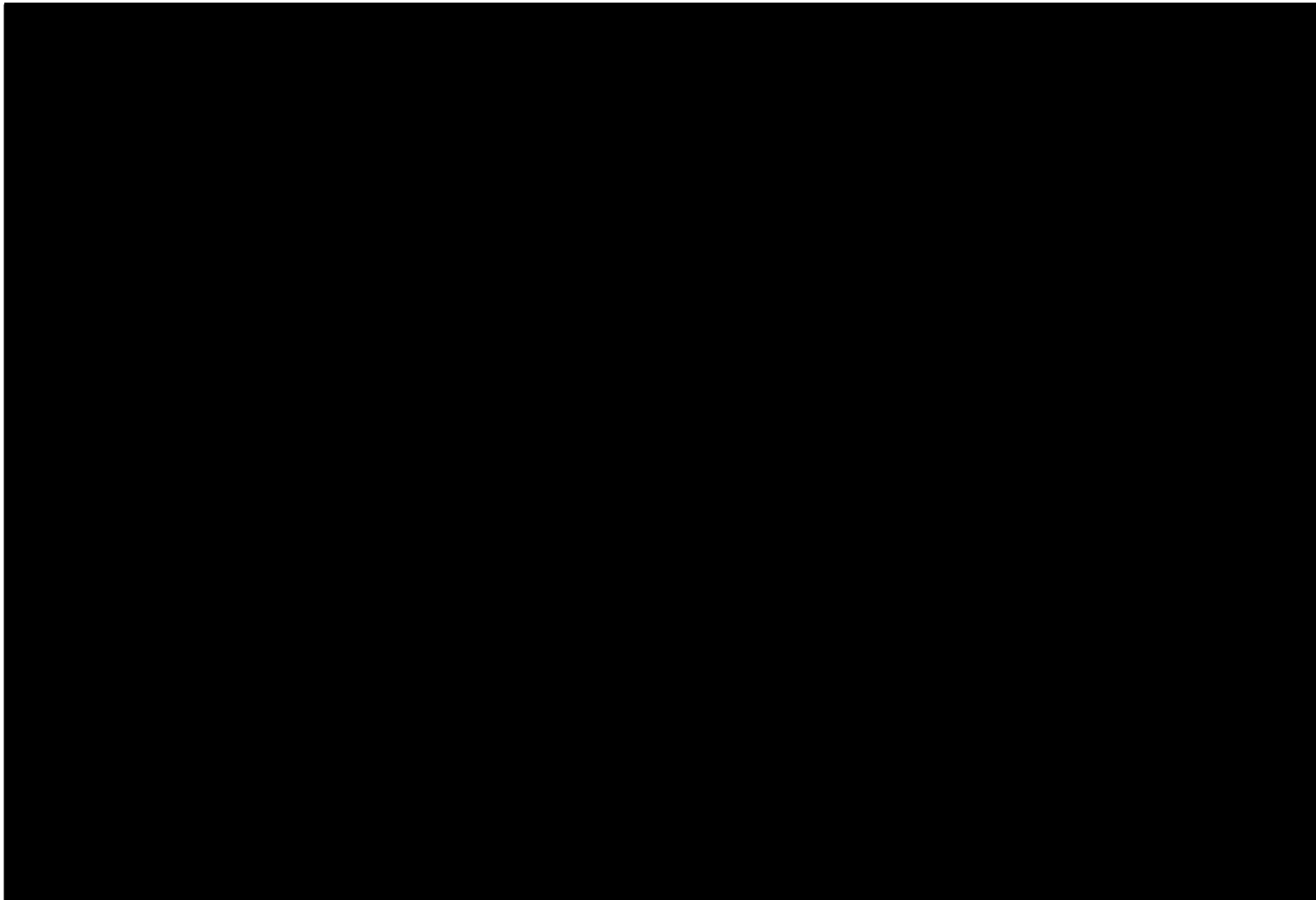


Figure 3.1

a) (Reproduced from Herre et al. 2003) Binding at 4°C of fluorescent *S.cerevisiae* to RAW macrophage and NIH 3T3 cell lines. Wild type NIH 3T3 cells bind poorly to yeast, and NIH 3T3 cells expressing Dectin-1 show high levels of binding. This binding can be competed effectively by 100µg/ml of the soluble β-glucans Laminarin (lam) and glucan phosphate (gluP), but not by high concentrations (1mg/ml) of mannan (man). RAW macrophages, that express low levels of endogenous Dectin-1 are shown for comparison. b) (Reproduced from Brown and Gordon 2001) Binding of FITC labelled zymosan particles to RAW and NIH 3T3 cell lines. Again, wild type NIH 3T3 cells bind low levels of particles and the Dectin-1 expressing cells much higher levels. This recognition of Dectin-1 is also β-glucan dependent, and mannan independent.

It has been the convention to study leukocyte-expressed receptors in non-leukocyte cell lines in order to reduce the chances of interference by other surface expressed molecules. This has, in particular, been the case when studying phagocytic receptors including those described in Chapter 1. Fibroblast cell lines such as NIH 3T3 and COS have been used to study phagocytic uptake by FcγRs, CR3, scavenger receptors and mannose receptor (Ezekowitz 1992; Kruskal, Sastry et al. 1992; Krauss, PooH et al. 1994; Socolovsky, Hockaday et al. 1994; Worth, Mayo-Bond et al. 1996; Carroll 1998; Fong and Le 1999; Cox and Greenberg 2001; Huang, Liu et al. 2001). The functions of phagocytic receptors are most commonly analysed by generating a panel of cell lines expressing receptors with targeted deletions of consensus sequences known to be essential for mediating downstream signalling and comparing the effects on various measures of function. As discussed in Chapter 2.6.1, we generated NIH-3T3 cell lines stably expressing either full-length Dectin-1 or one of a series of different cytoplasmic tail mutations.

3.2 Aims of study

The aims of this study were to analyse the phagocytic capacity of Dectin-1 out of its cellular context by expressing the receptor in a poorly phagocytic cell line and to define the cytoplasmic domains and any proximal signalling intermediaries involved in this process. If Dectin-1 were found to be truly phagocytic, it would be a very useful tool to study phagocytic mechanisms particularly as the prototypical Fcγ and Complement receptors exhibit different phagocytic phenotypes. Such a study may help to establish whether every phagocytic receptor has its own unique phagocytic pathway, or whether receptors could be classified based on shared uptake mechanisms.

3.3 Dectin-1 is a phagocytic receptor

A NIH 3T3 cell line was retrovirally transduced with viral particles encoding full-length Dectin-1 and placed under selection until a population stably expressing high levels the receptor developed (3T3-D1 cells). This Dectin-1 expression is illustrated in **fig. 3.3a**. The expression shown is on live cells implying good levels of surface expressed Dectin-1. WT cells only show background levels of antibody binding.

It is well established that the phagocytic process involved in yeast and yeast derived particle internalisation is dependent on actin polymerisation (Allen and Aderem 1996; Castellano, Chavrier et al. 2001). In order to establish whether 3T3-D1 cells were indeed phagocytic for zymosan particles, confocal microscopy studies were carried out. Fluorescently labelled unopsonised zymosan particles were added to cells in warm medium and unsynchronised binding and uptake allowed to proceed. TRITC-phalloidin staining revealed polymerised actin.

As is evident from the image of this 3T3-D1 cell (**fig. 3.3b**), yeast conidia induce active polymerisation of actin at sites of yeast binding (white arrows), leading to membrane extensions that eventually completely surround the conidium (top arrow). 3T3-D1 cells also bind zymosan particles in an exclusively Dectin-1 dependent fashion as demonstrated by competing binding with soluble glucans (**fig. 3.3c**). Dectin-1 mediated actin polymerisation in response to zymosan particles is clearly evident (**figures 3.3d and 3.3e**). The polymerised actin surrounding the particle shown in both the magnified box (**fig. 3.3d**) and (**fig 3.3b**) is strong evidence that an active, actin-based internalisation process is taking place.

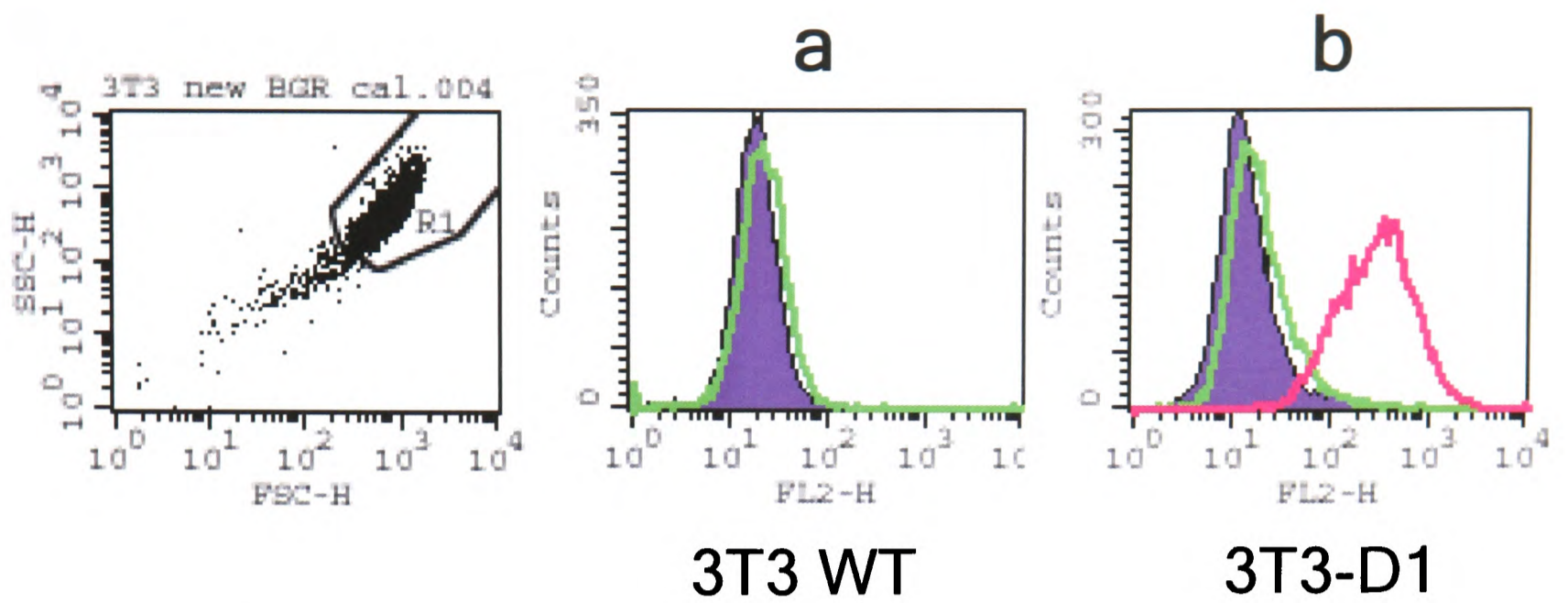


Figure 3.3a

Expression of Dectin-1 by 3T3-D1 cells. a) NIH 3T3 cells are not recognised by the 2A11 monoclonal antibody. The green line shows 2A11 staining. b) 3T3-D1 cells are not recognised by an IgG2b isotope control antibody shown in green, but express high levels of Dectin-1 (shown in pink) when stained with 2A11.

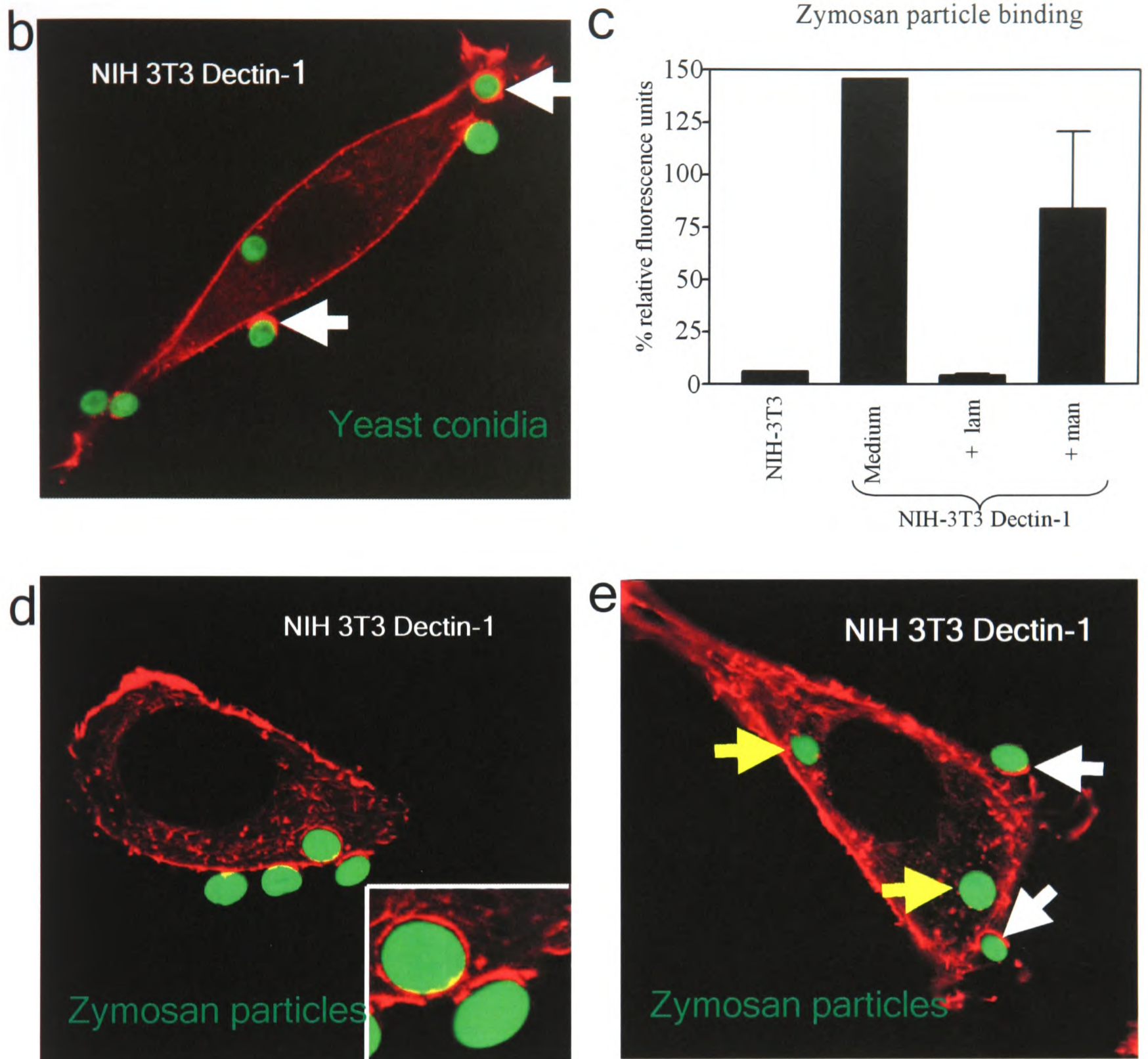


Figure 3.3b-e

Phagocytosis of yeast and yeast derived particles by 3T3-D1 cells (in collaboration with Dr. G.D.Brown). b) Following unsynchronised exposure, yeast conidia are recognised by 3T3-D1 cells and induce actin polymerisation at sites of contact (white arrows). c) Zymosan particle recognition is β -glucan dependent. d and e) 3T3 D-1 cells also show actin polymerisation at points of contact with zymosan particles (expanded view and white arrows). e) Particles indicated with yellow arrows may be either internalised or lying on top of cells.

3.4 Internalisation of zymosan particles

3.4.1 Development of a quantitative assay to measure phagocytic efficiency

Microscopy has been used to quantify the effects of interventions on phagocytic efficiency (Caron and Hall 1998) but **fig. 3.3e** reveals a limitation of confocal microscopy when trying to quantify phagocytic efficiency. Whilst active phagocytosis can readily be visualised as polymerised actin, this network is rapidly released from the phagosomal membrane on completion of phagocytosis. Thus the two particles that appear to be within the fibroblast (yellow arrows) may actually be lying on top, and the resolution of confocal microscopy does not allow a clear distinction to be made in adherent spread cells. In order to avoid these problems, a quantitative flow-cytometry based assay, which can measure the extent to which Dectin-1 bound particles are internalised, was developed.

The design of this assay is illustrated in **figure 3.4a** and described in chapter 2.5.7. and 2.6.2. Synchronous uptake of cell bound zymosan particles is initiated by warming to 37°C. Particles that have been completely internalised are not accessible to polyclonal anti-zymosan antibodies and are consequently not labelled with the APC conjugated secondary antibody, whereas the cytochalasin D treated cells that cannot internalise particles are strongly APC labelled. The proportion of cells able to fully internalise Dectin-1 bound zymosan particles over a defined time period can now be quantified by two-colour flow cytometry.

3.4.2 Dectin-1 internalises bound particles efficiently

In addition to Dectin-1, human DC-SIGN and its homologue DC-SIGNR have both been shown to recognise yeast (Mitchell, Fadden et al. 2001), and DC-SIGN has been

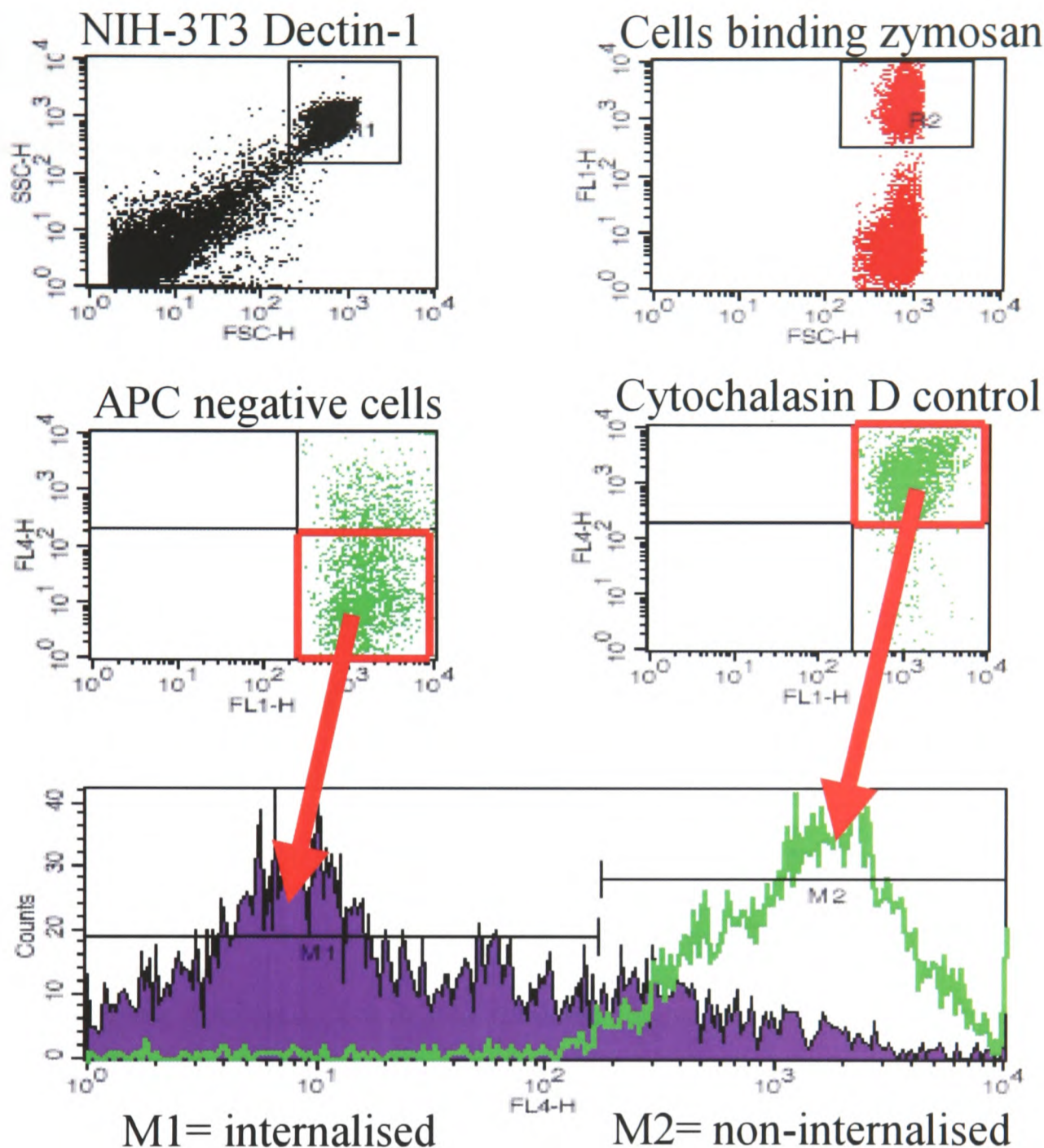


Figure 3.4a

Comparative uptake assay. Control cells are incubated with $2\mu\text{M}$ cytochalasin D for 40 minutes before placing at 4°C . Zymosan particles are allowed to bind to 3T3-D1 cells on ice and unbound particles are washed away prior to warming of cells to 37°C for 60 minutes. After 60 minutes cells are placed at 4°C and stained with an anti-zymosan polyclonal antibody and an APC conjugated secondary antibody. Cells are then detached and fixed. The cell population that bound particles (R2) was gated and the degree to which these cells were also APC positive was analysed. This was compared to the extent to which cytochalasin D treated, particle positive cells were APC positive. The histogram shows an overlay of the two APC histograms. M1 shows the cell population that is APC negative and has internalised particles. M2 shows cells that have not internalised particles.

shown to mediate limited phagocytosis of *C.albicans* (Cambi, Gijzen et al. 2003). SIGN-R1 is another lectin expressed by murine macrophages and is the homologue of DC-SIGNR (Taylor, Brown et al. 2004). SIGN-R1 also recognises fungal derived zymosan particles in a mannose dependent fashion (Taylor, Brown et al. 2004). When SIGN-R1 was stably expressed in NIH-3T3 cells (3T3-SIGNR1) binding of zymosan particles on ice was found to be at similar levels to that of 3T3-D1 cells (**fig. 3.4b**). When the extent of phagocytic uptake between these two receptors was compared, however, it is evident that Dectin-1 is considerably more efficient at mediating internalisation than SIGN-R1 (**fig. 3.4c**) (Taylor, Brown et al. 2004). SIGN-R1 recognises fungal particles and as expected demonstrates some phagocytic capacity. These data demonstrate that simple recognition of zymosan particles by a receptor with phagocytic potential, even when highly expressed by NIH-3T3 fibroblasts is insufficient to mediate efficient particle internalisation (as indicated by a failure to exclude APC staining) under the assay conditions used. Thus Dectin-1 is not only a phagocytically competent receptor, but is also very efficient at mediating this process.

3.5 Establishing 3T3-D1 mutant cell lines

3.5.1 Sequence alignments

The prototypical phagocytic FcγR requires the ITAM-containing γ-chains to enable signalling to occur. There is only one single chain FcγR, the human FcγRIIA that also encodes an ITAM motif, but with increased spacing between the two YxxL sequences as shown in **fig. 3.5.1a**. In the case of the paired Fcγ chains, FcγRIIA and Fcγ chimaeric molecules, both membrane proximal and distal tyrosine residues are essential for ITAM mediated phagocytic and activatory signalling (Daeron 1997). Only one other phagocytic Type 1 leukocyte receptor containing an ITAM-like sequence has been

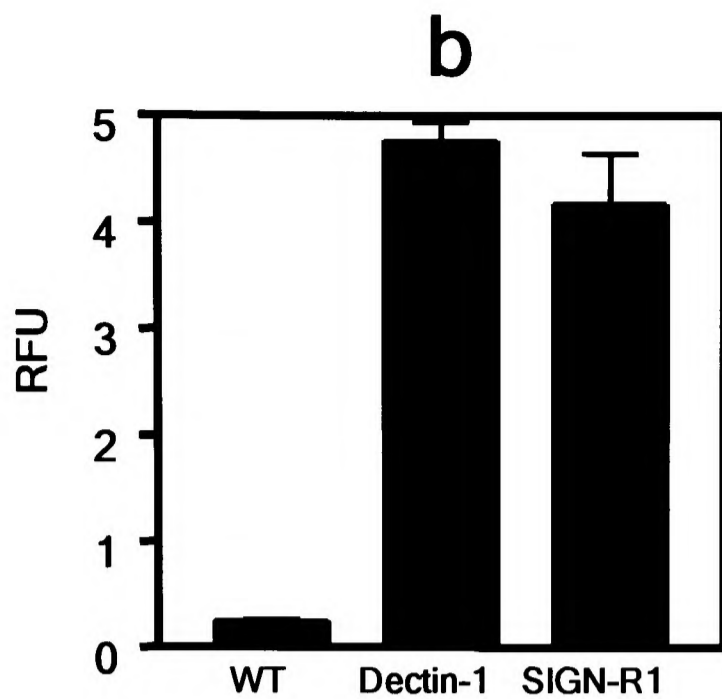


Figure 3.4b

3T3-D1 cells and NIH 3T3 cells stably expressing SIGN-R1 are able to bind FITC-zymosan particles equally well on ice.

c

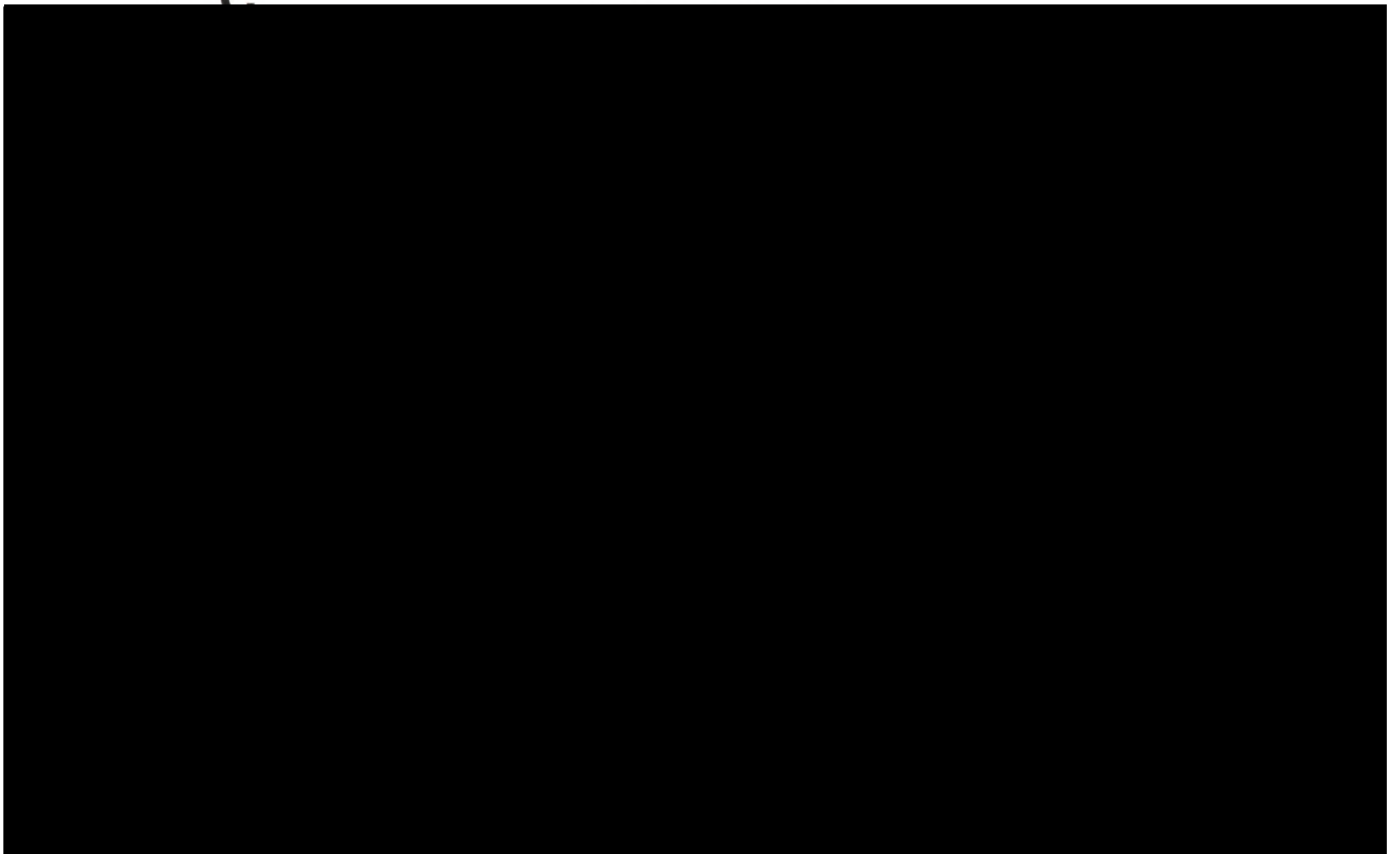


Figure 3.4c (From Taylor et al. 2004)

3T3-D1 and 3T3-SIGN-R1 cells show differing capacities to internalise bound particles. The upper 3 panels show that 3T3-D1 cells are predominantly APC negative (red boxes) compared to the cytochalasin D control when compared to the lower panels (3T3-SIGN-R1 cells). The histograms provide a quantitative comparison.

Cytoplasmic tails of ITAM and ITAM-like motif containing receptors

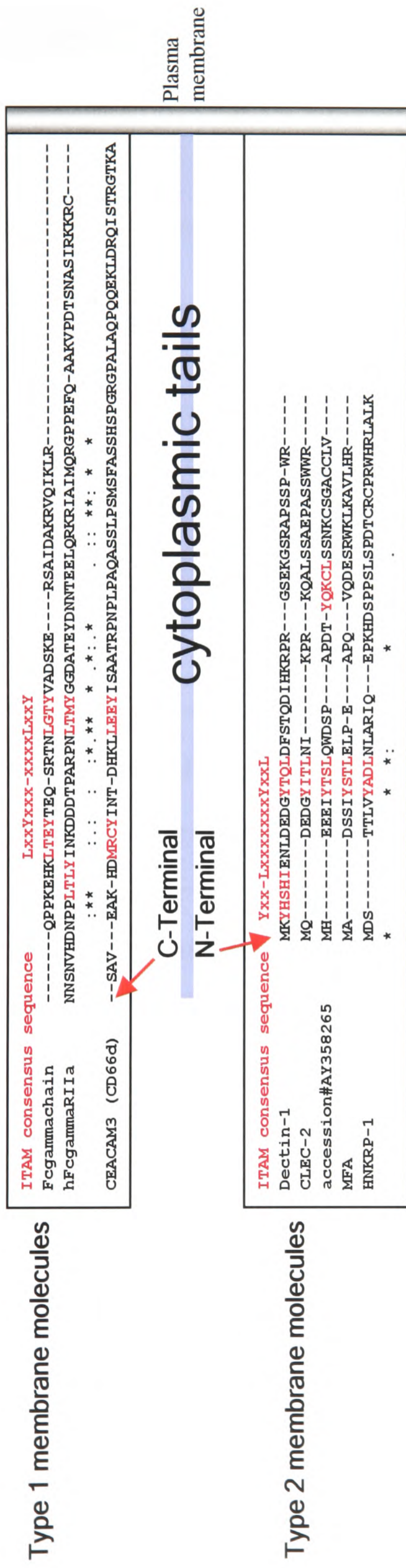


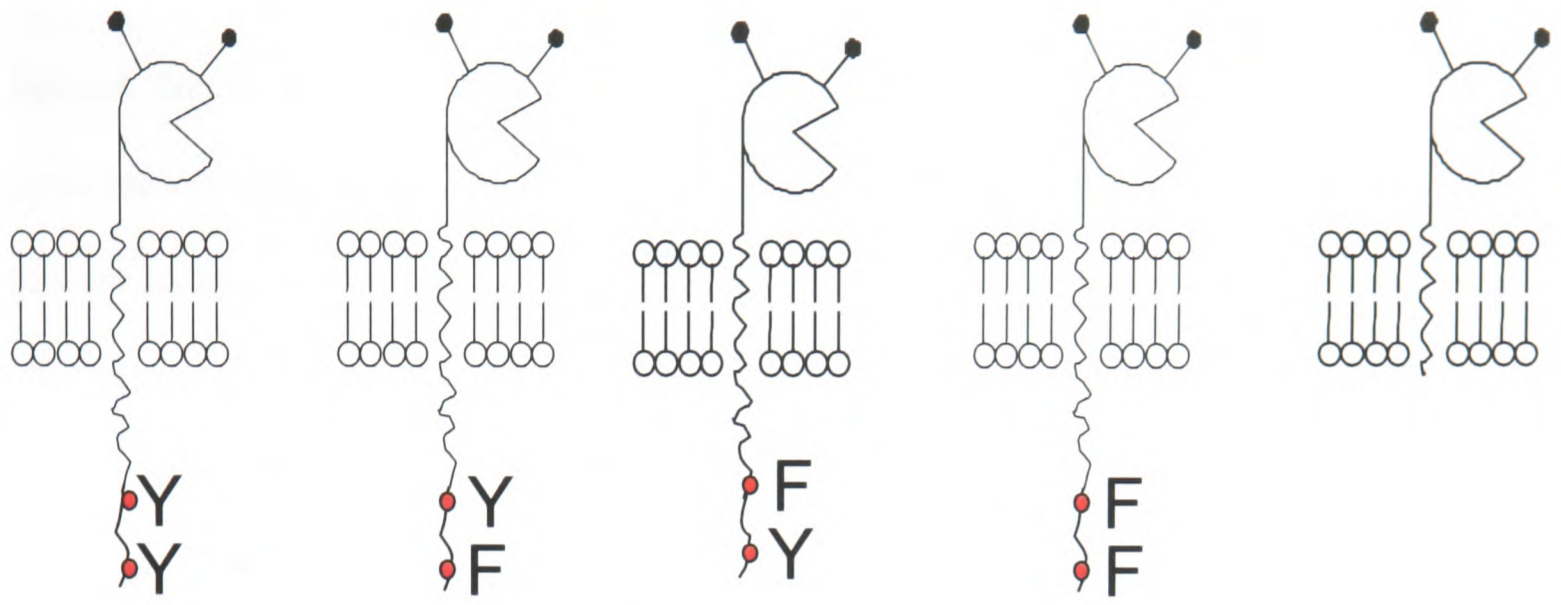
Figure 3.5.1a

Receptors containing cytoplasmic ITAMs and ITAM-like motifs. The prototypical ITAM containing receptor chain is the Type 1 Fcγ chain. The single chain ITAM containing FcγRIIA receptor has an increased spacing between the YxxL sequences. CEACAM3 contains a methionine residue instead of a lysine in its membrane distal YxxM, making it an ITAM-like motif. There are several Type II membrane molecules that contain YxxL sequences in their cytoplasmic tails, but with the exceptions of Dectin-1 and accession number #AY358265, these are single YxxLs. Amongst these receptors only Dectin-1 has a known ligand. The Dectin-1 ITAM-like sequence shows conventional spacing between the tyrosine residues, but the membrane distal sequence YxxxI differs from the consensus. Whether ITAM-like motifs can interact with the same mediators as classical ITAMs remains unknown.

described to date, CEACAM3 (**fig. 3.5.1a**) (Schmitter, Agerer et al. 2004), but the role of the tyrosines in mediating phagocytosis by this receptor is currently undefined. What these alignments aim to illustrate is that, although the presence of tandem YxxL/I sequences is critical for phagocytosis and cellular activation, the spacing between them seems somewhat flexible.

In contrast, Dectin-1 is a type II membrane molecule, so the orientation of the ITAM-like motif is the reverse of that found in type I molecules (**fig. 3.5.1a**). CLEC-2, accession number AY358265, MFA and HNKRP-1 are all NK C-type lectin-like molecules, and CLEC-2 is closely related to Dectin-1. These alignments show that YxxL sequences are present in the cytoplasmic tails of all of these molecules, yet their significance remains to be determined. As the only putative signalling sequence identified in the cytoplasmic tail of Dectin-1 was the ITAM-like motif, we decided to target the two tyrosine residues for mutation. **Figure 3.5.1b** illustrates the recombinant Dectin-1 molecules generated from full-length Dectin-1 by PCR and stably expressed in NIH-3T3 cells by retroviral transduction. Full-length denotes wild-type Dectin-1 as isolated from a RAW 264.7 cell line generated cDNA library (Brown and Gordon 2001), Y1 refers to substitution of the membrane distal tyrosine with phenylalanine, Y2 to substitution of the membrane proximal tyrosine, Y1Y2 to substitution of both tyrosines and TRUNC to a complete truncation of the cytoplasmic tail. These cell lines should allow analysis of the roles of the tyrosines present in the cytoplasmic tail in particle uptake.

3.5.2 Synchronised particle binding



$Y_{xxx}I_{x_7}Y_{xx}L$ $F_{xxx}I_{x_7}Y_{xx}L$ $Y_{xxx}I_{x_7}F_{xx}L$ $F_{xxx}I_{x_7}F_{xx}L$ Truncation
 (Full-length) (Y1 mutant) (Y2 mutant) (Y1Y2 mutant)

Figure 3.5.1b

The recombinant Dectin-1 molecules expressed in NIH-3T3 cells. Full length refers to wild-type Dectin-1 as cloned from mRAW 264.7 cells. Y1 mutant has the membrane distal tyrosine mutated to a phenylalanine. Y2 has the membrane proximal tyrosine mutated. Y1Y2 mutants both tyrosine residues mutated. Truncation has the entire cytoplasmic tail deleted. These molecules were all stably expressed in NIH-3T3 cells by retroviral transduction.

A potential problem when comparing different cell lines is the extent to which they bind particles (a reflection of receptor expression at the cell surface). We therefore compared the zymosan binding capacity of the cell lines generated by measuring fluorescent zymosan particle binding at 4°C at a ratio of 10 particles per cell. **Figure 3.5.2a** shows that for the four cell lines used in subsequent experiments particle binding on ice, when quantified by flow cytometry, was found to be very similar, and pre-treatment with cytochalasin D did not affect binding either. In an independent experiment (**fig. 3.5.2b**) the zymosan binding capacity of the two other cell lines (Y1 and Y1Y2) was also measured and found to be adequate. These data suggest that the six cell lines generated are able to bind zymosan particles similarly well under assay conditions, thus implying that robust comparisons can be made between the cell lines as to the extent to which bound zymosan particles are internalised.

3.6 The effects of cytoplasmic tail mutation on Dectin-1 mediated phagocytosis

Using the comparative internalisation technique described in (2.6.2 materials and methods) and 3.4 above, the effects of the cytoplasmic tail mutations shown in **fig. 3.5.1b** were examined. Synchronised uptake of bound particles was allowed to proceed over 60 minutes prior to staining of exposed particles and analysis by flow cytometry. The flow cytometric profiles of three cell lines and controls from a single experiment are shown in **fig. 3.6a**. The control cytochalasin D profile shows un-internalised particle staining, whilst the Dectin-1 profile shows full-length Dectin-1 mediated internalisation. Substitution of the membrane proximal tyrosine residue resulted in the almost complete abrogation of particle internalisation, and complete cytoplasmic tail truncation resulted in only a small additional reduction in internalisation. This experiment was repeated on three separate occasions and combined data are presented

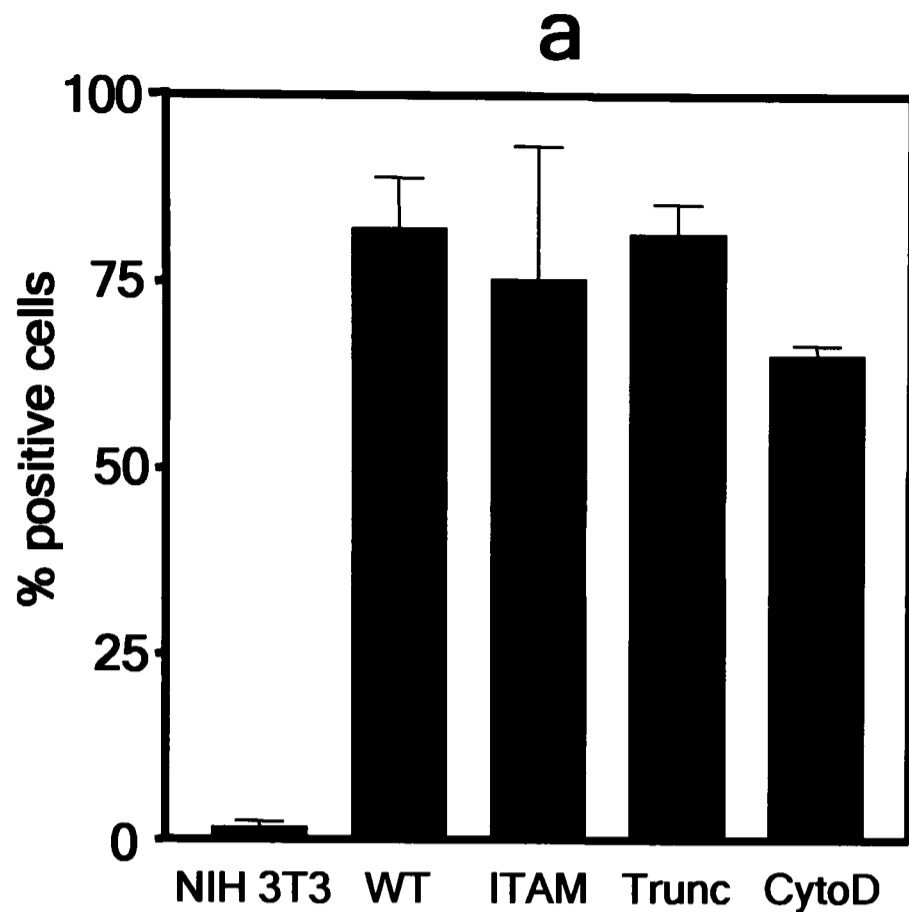


Figure 3.5.2a

Cold binding of FITC zymosan to 3T3 cell lines. 10 particles per cell zymosan bound to cells at 4°C prior to lifting, fixation and flow cytometric analysis. The levels of zymosan binding between the various cell lines are very similar, and cytochalasin D treatment does not reduce binding significantly.

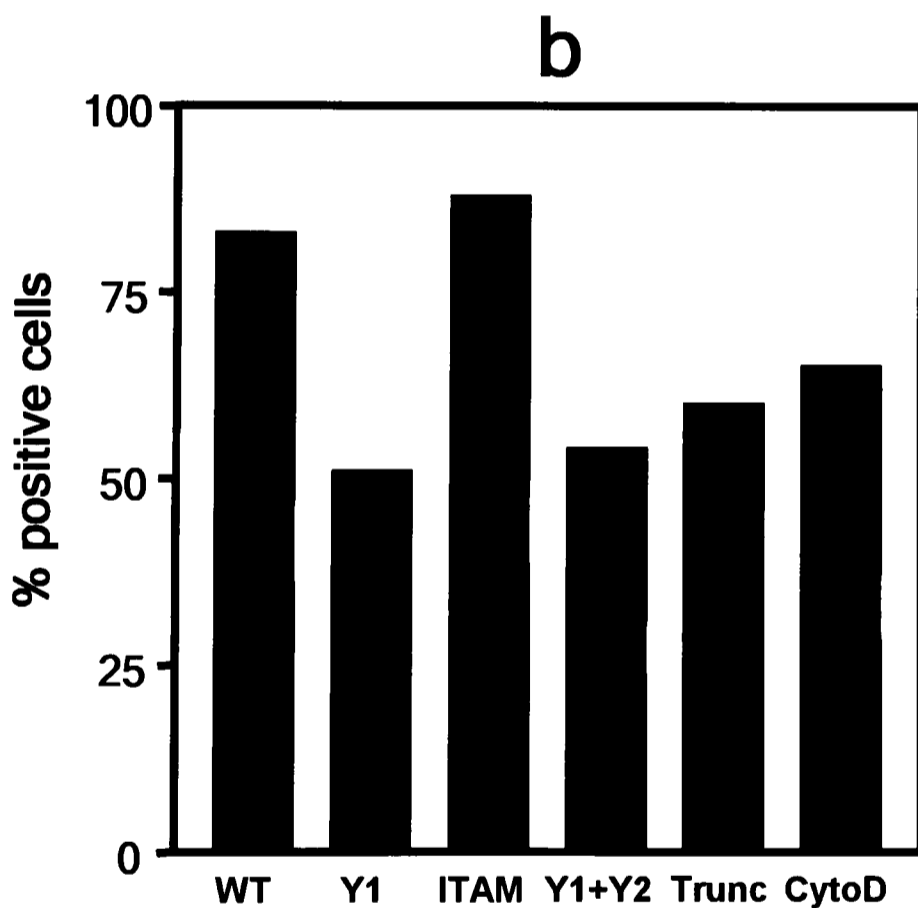


Figure 3.5.2b

Independent experiment of the binding of FITC-zymosan to all 3T3 cell lines generated at 4°C. 10 particles per cell zymosan bound to cells on ice prior to lifting, fixation and flow cytometric analysis. The levels of zymosan binding in the Y1 and Y1Y2 cell lines are slightly lower than for the other cell lines, but at least 50% of cells bind particles.

in **fig. 3.6b**. The chart shows that a single amino acid residue in the ITAM-like motif is the major mediator of phagocytic uptake by Dectin-1, and that other components of the cytoplasmic tail make a small additional contribution. The two additional cell lines generated (3T3-Y1 and 3T3-Y1Y2) were also tested in an independent experiment **fig. 3.6c** and it was found that the Y1 cell line had little effect on internalisation and that substitution of both tyrosines in the Y1Y2 cell line had no additive effect when compared to substitution of the membrane proximal tyrosine (Y2) only. These findings suggest that the ITAM-like motif of Dectin-1 is responsible for mediating phagocytic uptake of fungal particles in a tyrosine dependent fashion and thus may share other similarities with the canonical Fc γ R ITAMs.

3.7 Dectin-1 cytoskeletal signalling in a non-phagocyte model

The ITAM-like motif of Dectin-1 is similar to the canonical ITAMs and the essential role of the tyrosine embedded in the membrane proximal YxxL sequence suggests that tyrosine phosphorylation may play a role in transmitting activatory signals to the actin cytoskeleton. Furthermore it has been shown by immunoprecipitation that Dectin-1 becomes tyrosine phosphorylated on receptor ligation (Gantner, Simmons et al. 2003). We thus decided to test the effects of a variety of established pharmacological inhibitors of phagocytic uptake on the internalisation efficiency of 3T3-D1 cells. The selection of inhibitors was guided by the possibility that Fc γ R ITAMs and ITAM-like motifs might utilise the same early signalling intermediates. **Table 2.5.9** lists the inhibitors used and the concentrations tested. The mediators of phagocytic signalling targeted were cellular tyrosine kinases, the Src family kinases, Syk, PI3K, PKC and the Rho family of small GTPases (for detailed descriptions see chapter 1.5.2). Briefly, genistein is a broad-spectrum tyrosine kinase inhibitor, PP1 and PP2 are specific

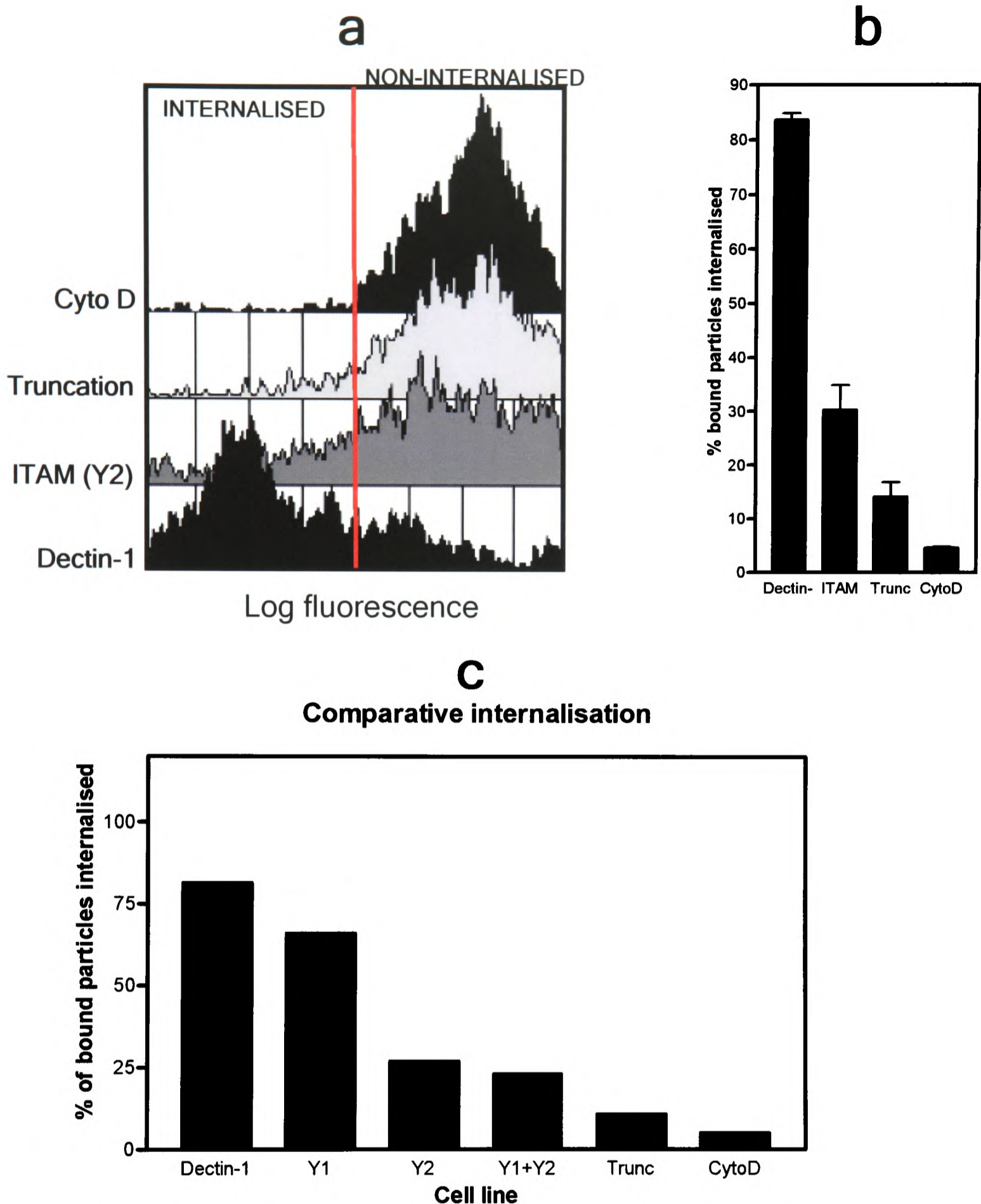


Figure 3.6a-c

Tyrosine substitutions in the cytoplasmic tail of Dectin-1 render the receptor non-phagocytic. a) Flow cytometry profiles from a representative experiment showing APC positive and negative zymosan particles bound and internalised by 3T3-D1 cells. Cytochalasin D profile is the control for non-internalised particles. b) Combined data from 3 independent experiments showing quantitative effects of cytoplasmic tail mutation on phagocytic internalisation. c) An independent experiment quantifying the effects in all the NIH-3T3 Dectin-1 mutant cell lines generated on zymosan particle internalisation.

inhibitors of the Src family tyrosine kinases that are responsible for phosphorylating FcγR ITAMs, and PP3 is an inactive analogue. Piceatannol is a selective inhibitor of Syk kinase, the essential mediator of FcγR signalling, Wortmannin inhibits PI3K responsible for membrane remodeling and phagosomal closure, Chelerythrine inhibits PKC also involved in phagosome closure and Toxin B inhibits the Rho family of small GTPases that include RhoA, Rac-1 and Cdc42 that mediate cytoskeletal remodelling.

To establish inhibitor doses that could inhibit zymosan particle phagocytosis, we were guided by the literature, and performed limited titrations. **Figures 3.7a** and **3.7b** illustrate titrations for several of the listed inhibitors. From these titrations it can be seen that, at equivalent doses, PP1 is less efficient than PP2 at inhibiting particle internalization, and the control, PP3, has no effect. The effect of piceatannol is only evident at higher doses, but inhibition is nearly complete at the maximum dose tested. Toxin B proved to be a very effective inhibitor in 3T3-D1 cells. To minimize toxic effects on cells, doses were kept to the minimum effective dose. **Figure 3.7c** shows the effects of the pharmacological inhibitors described above on the internalization of fluorescently labeled zymosan particles. The data were compiled from 3 independent experiments and represented as percentage internalization compared to incubation with medium only. As all the inhibitors with the exception of Toxin B are dissolved in DMSO, the DMSO bar represents a control for carrier alone. The actin-depolymerising agent cytochalasin D represents the internalization-inhibited control. The broad-spectrum tyrosine kinase inhibitor genistein was used to demonstrate that Dectin-1 mediated phagocytosis depended on tyrosine phosphorylation. Although the inhibition noted is not as complete as that of cytochalasin D, a 70% reduction in internalization over controls was noted suggesting that tyrosine phosphorylation plays a central role in

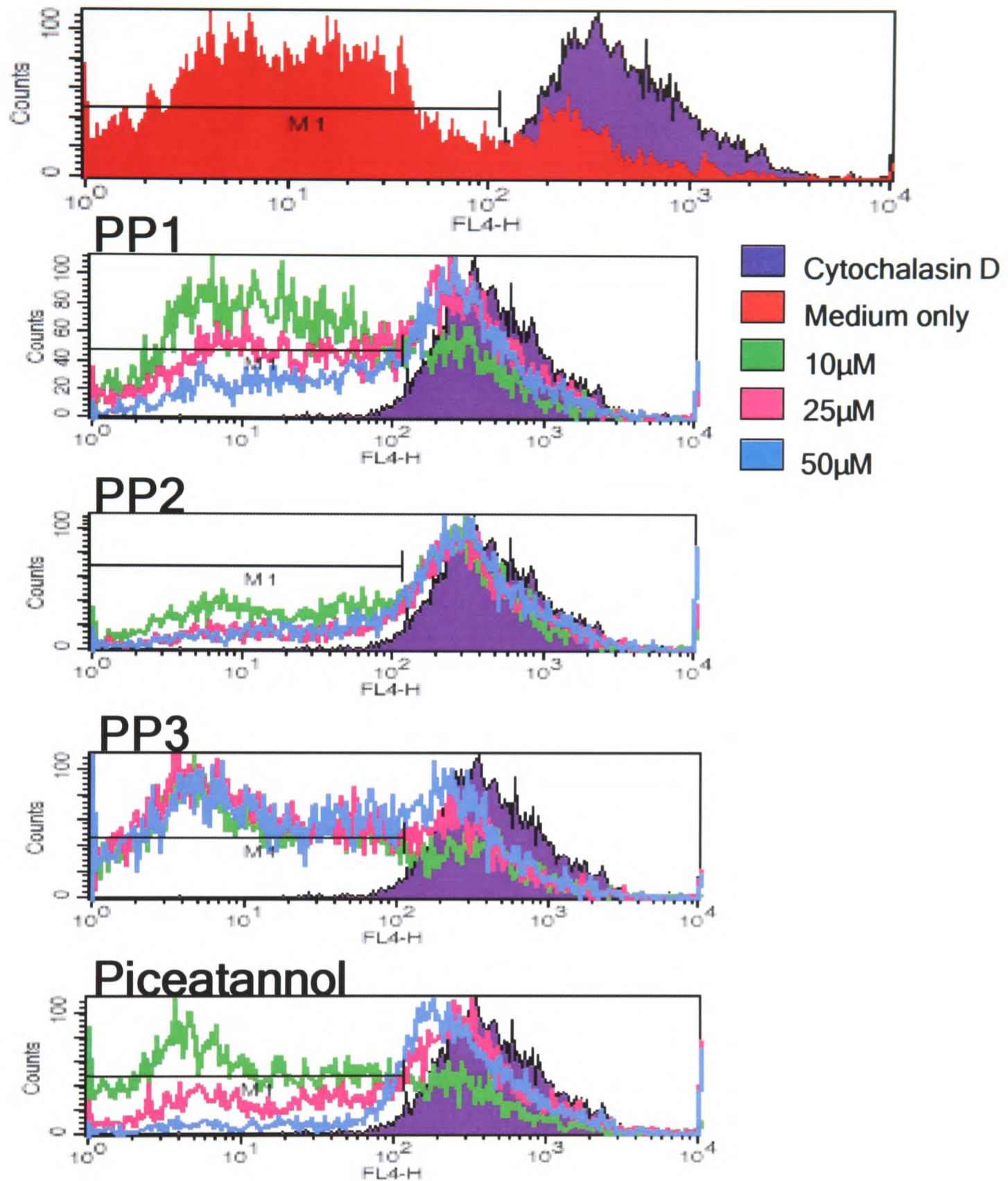


Figure 3.7a

Effects of inhibitors of tyrosine kinases involved in the phosphorylation of ITAMs on Dectin-1 mediated phagocytosis. The top histogram shows the effect of cytochalasin D on Dectin-1 mediated phagocytosis. The remaining histograms show the effects of various concentrations of inhibitor in phagocytosis by 3T3-D1 cells. PP1 and PP2 inhibit Src family kinases, PP3 is an inactive analogue and piceatannol inhibits Syk kinase. PP2 potently inhibits phagocytic uptake even at lower doses whilst Piceatannol is effective at the upper levels of the dose range.

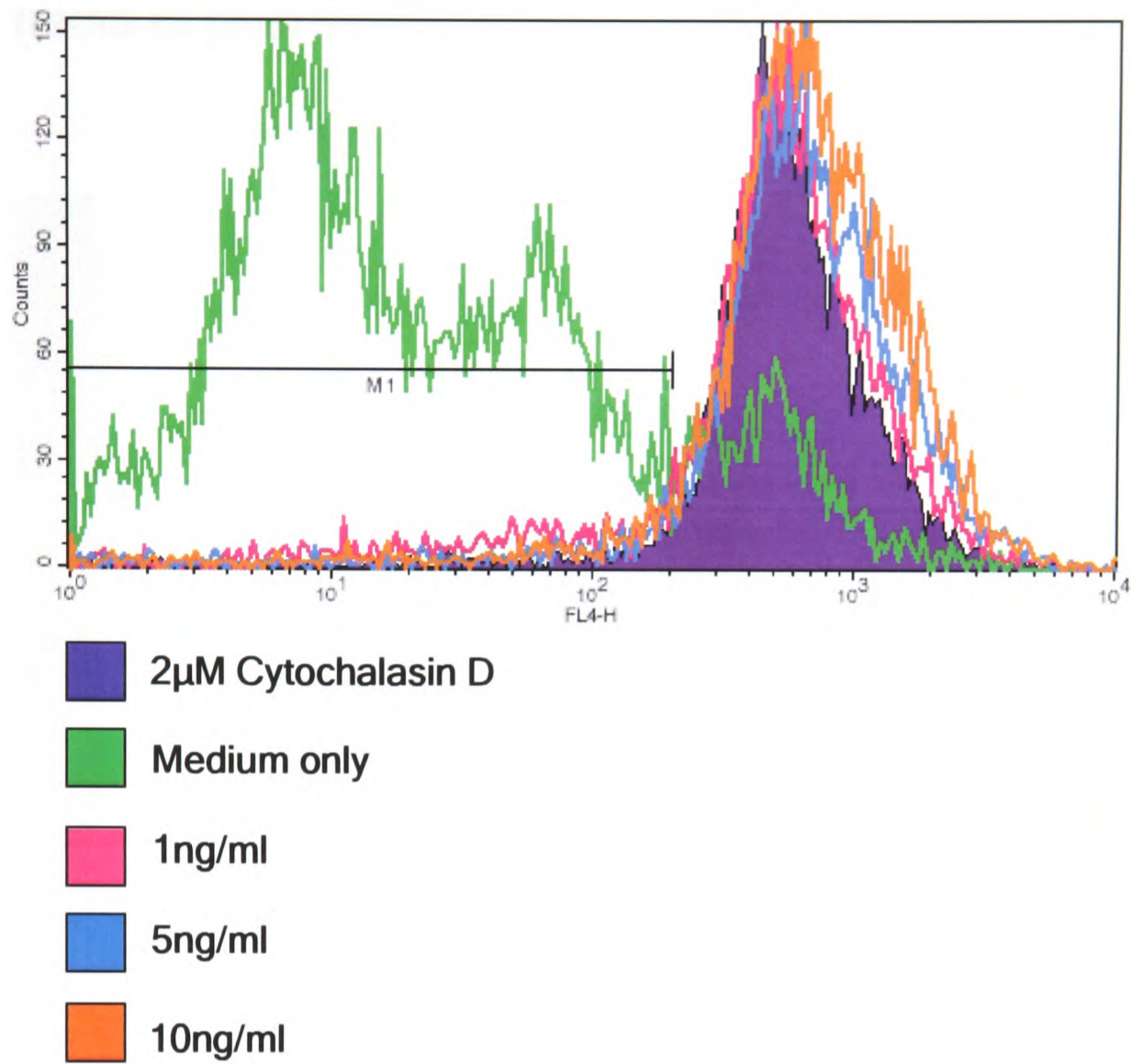


Figure 3.7b

Effects of Toxin B on FITC zymosan particle internalisation. Toxin B is a very effective inhibitor of particle internalisation, even at low doses.

Effects of pharmacological inhibitors on phagocytic uptake

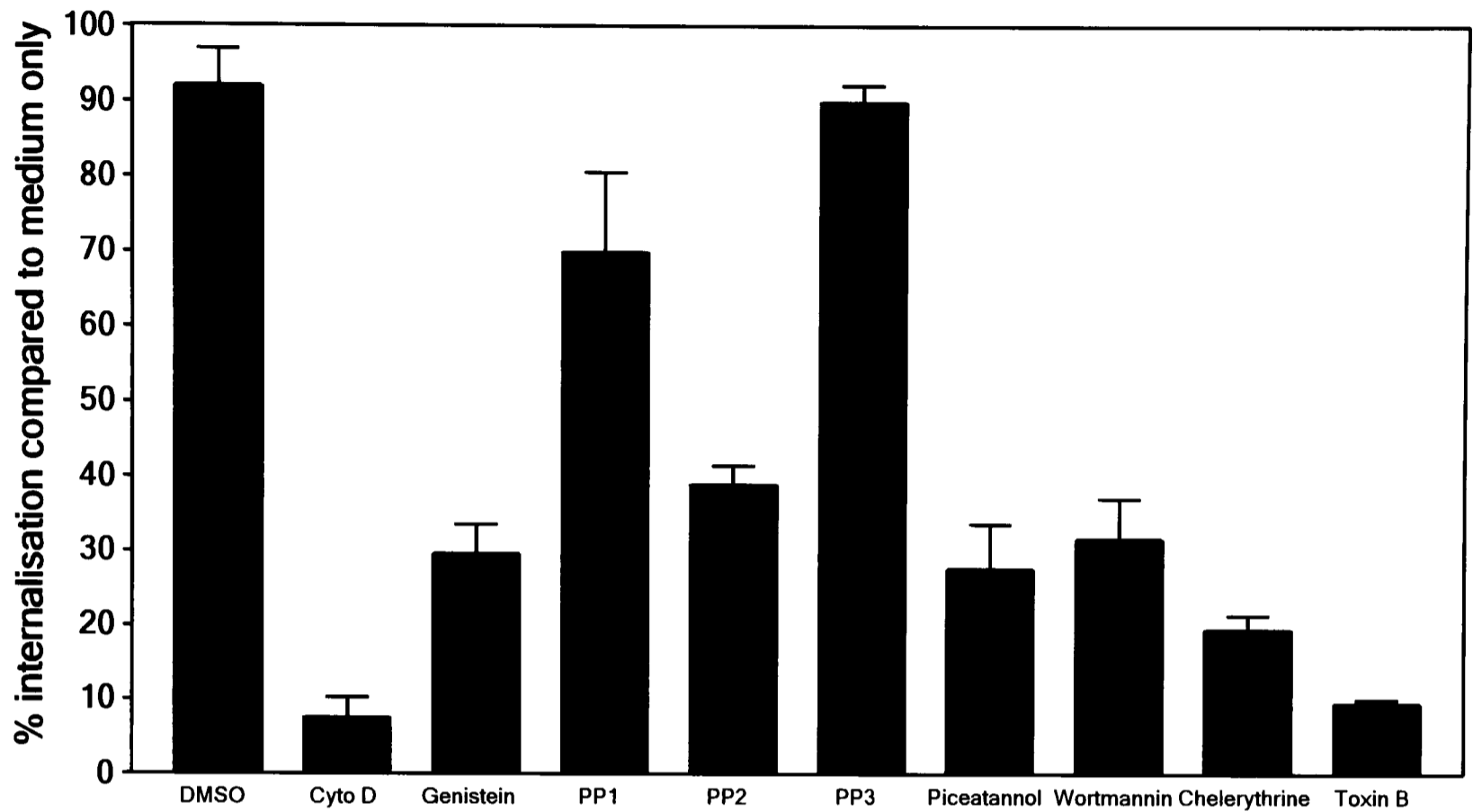


Figure 3.7c

Effects of inhibitors of molecules known to play a role in Fcγ and Complement receptor mediated phagocytosis. Data compiled from 3 independent experiments using 3T3-D1 cells and FITC zymosan particles.

Dectin-1 mediated cytoskeletal activation. NIH-3T3 cells express PP2 inhibitable Src-family kinases (Gilmore, Stutts et al. 2001) and although PP1 at equivalent doses was a less potent inhibitor of Src-family kinases than PP2 (see also **fig. 3.7a**), PP2 was able to inhibit zymosan internalization with virtually identical efficiency to genistein. This suggests that Src family kinases, or closely related kinases inhibited by PP2 play an important role in phosphorylating the membrane proximal tyrosine of Dectin-1. PP3 the inactive analogue had no effect. Several fibroblast cell lines express Syk kinase (Yanagi, Inatome et al. 2001; Hinkovska-Galcheva, Boxer et al. 2003; Miah, Sada et al. 2004), and higher doses of piceatannol inhibited phagocytosis by 3T3-D1 cells resulting in a marked reduction in zymosan internalization. In fibroblasts PI3K has been shown to be activated downstream of Syk (Hinkovska-Galcheva, Boxer et al. 2003) and to be important for membrane trafficking and phagosomal closure. The PI3K inhibitor, wortmannin, strongly but not completely inhibited zymosan particle internalization. PKC is essential for both Fc γ R and CR3 mediated phagocytosis (Allen and Aderem 1996), and after chelerythrine treatment, Dectin-1 mediated phagocytosis was also markedly inhibited. These three kinases thus appear to play a role in Dectin-1 mediated phagocytosis. Toxin B inhibited particle internalization with efficiency equal to that of cytochalasin D, even at doses as low as 1ng/ml, demonstrating that the small Rho GTPases are absolutely required to mediate the actin polymerization that enables particle uptake.

3.8 The Rho GTPases Cdc42 and Rac-1 mediate cytoskeletal activation

Receptor mediated actin polymerization and phagocytosis requires the small Rho GTPases Rac-1 and Cdc42, and RhoA (Caron and Hall 1998). It was found that receptors selectively recruit these GTPases to activate the cytoskeleton, and Fc γ R

recruits Rac-1 and Cdc42 and CR3 recruits RhoA (Caron and Hall 1998). Dectin-1 mediated phagocytosis also depends on these small GTPases (**figs. 3.7c**), and we wanted to establish whether Dectin-1 relied on any particular combination of these to activate the cytoskeleton. In an isolated experiment **fig. 3.8a**, the effects of the DN-Rho GTPases were tested on 3T3-D1 cells by confocal microscopy and manual counting of particle internalization by transfected cells. As we were concerned about the limitations of confocal microscopy in distinguishing internalized from non-internalised particles, we wanted to establish a less observer-dependent assay.

3.8.1 Three colour flow cytometry

A quantitative 3-colour flow cytometry based assay was developed for this purpose **fig. 3.8b**. 3T3-D1 cells were transfected with myc-tagged dominant negative Rho GTPases and specially labeled, low-fluorescence, zymosan particle internalization measured. The cell population was selected based on size and granularity and then those cells binding fluorescent zymosan particles were selected. This population was divided according to their expression of dominant negative Rho GTPase as measured by the signal above the background in empty vector transfected cells. The extent to which phagocytosis is reduced in both the untransfected and transfected populations was then quantified. Several transfection regimes were attempted in order to achieve acceptable levels of expression of DN-Rho GTPases in 3T3-D1 cells, and these are illustrated in **fig. 3.8c**. As can be seen, only the GenePorter transfection system under serum free conditions resulted in sufficient numbers of transfected cells to conduct the experiment. **Figure 3.8d** illustrates why low intensity labeled FITC zymosan particles had to be used rather than the commercially labeled particles. The amount of signal bleed-through from the commercially labeled particles into the FL-2 channel (used to detect the DN-Rho

Microscopy-based assay

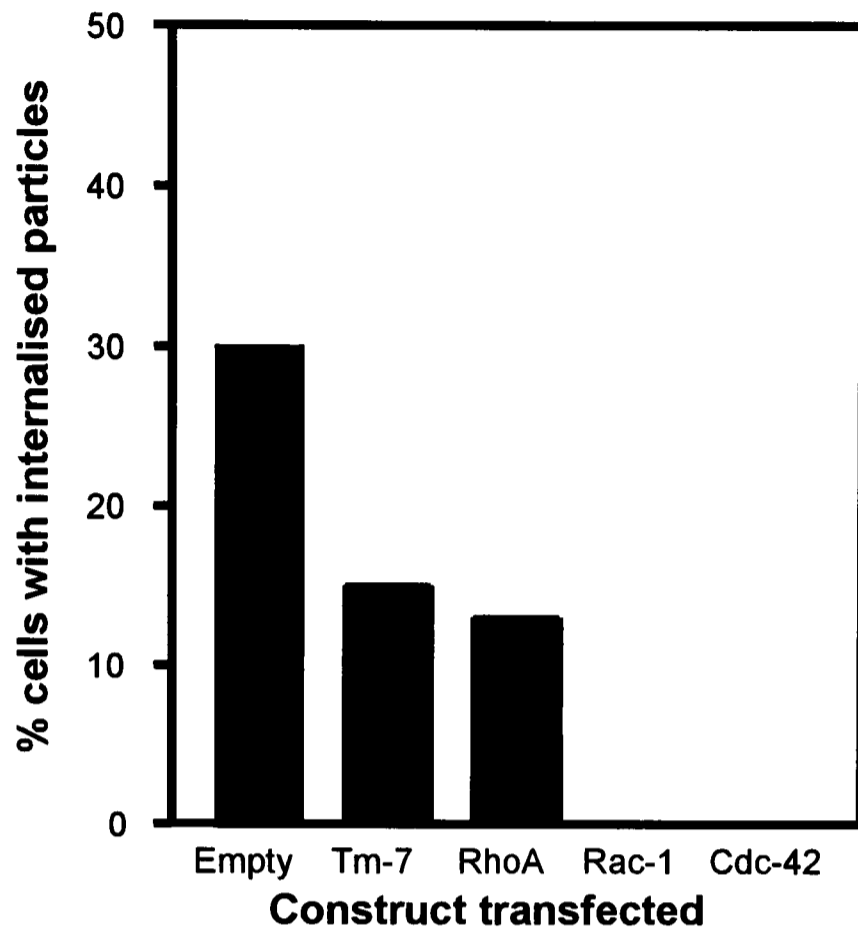


Figure 3.8a

DN Rho-GTPase effects on FITC zymosan particle phagocytosis. a) Confocal microscopy based assay showing that DN-Rac-1 and Cdc-42 prevented Dectin-1 mediated zymosan particle phagocytosis whereas Rho A and the irrelevant tagged control protein both showed reduced but not inhibited internalisation.

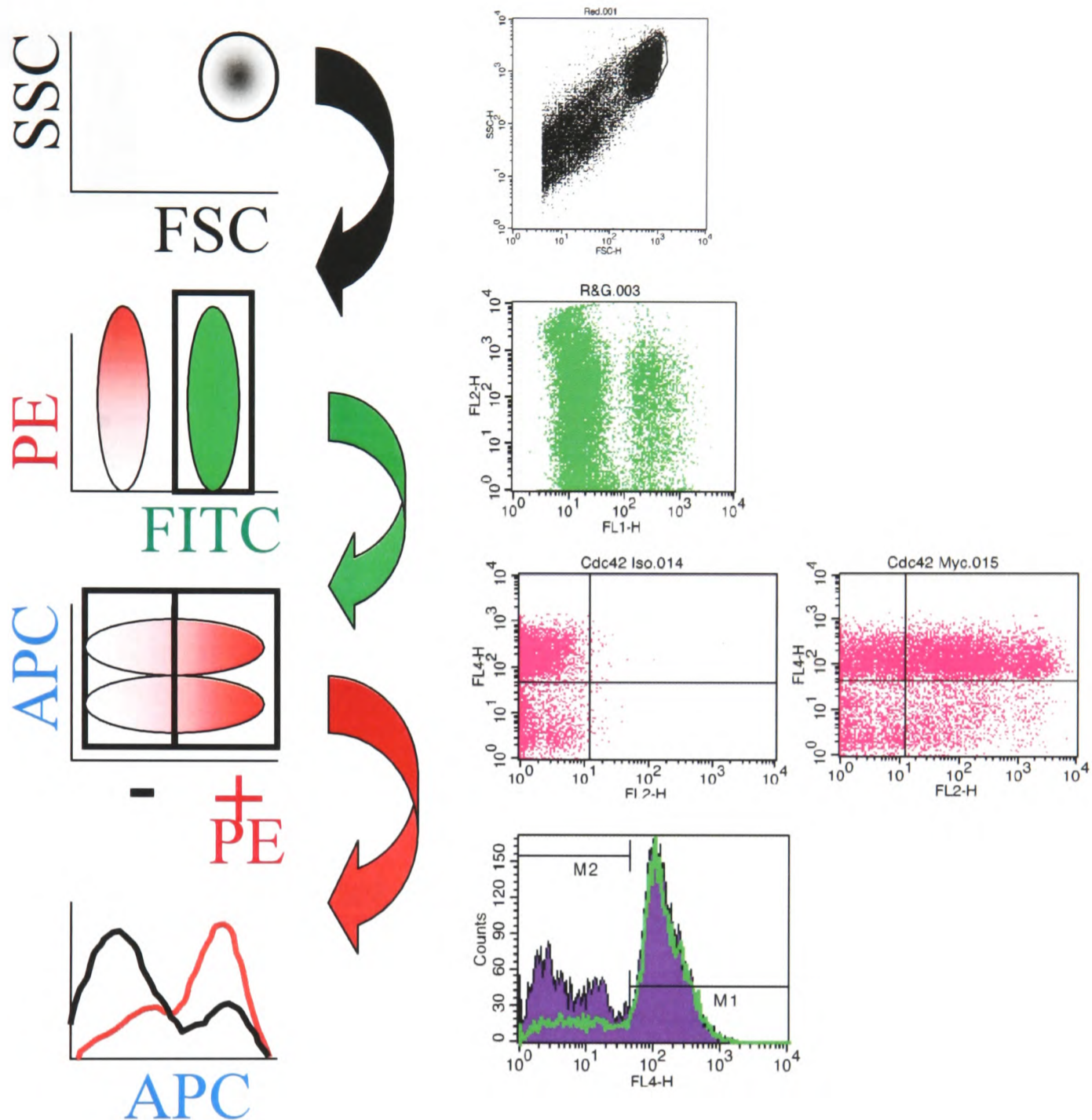


Figure 3.8b

Method for flow cytometric quantitation of the effects of DN-Rho GTPases. Specially labelled FITC zymosan particles (described in fig 3.8c and 3.8d) are bound to 24 hour DN-Rho GTPase transfected 3T3-D1 cells on ice. Cells are then allowed to internalise particles for 60 minutes at 37°C prior to staining exposed zymosan particles with APC followed by lifting and fixing cells. Fixed cells were then permeabilised and stained for DN-Rho GTPase V5 tag expression before fixing again and analysing by flow cytometry. The population of cells binding zymosan particles was selected and the cells expressing the DN constructs were compared to the untransfected cells in the same sample providing an internal control. This permitted the change in phagocytic capacity to be quantified and compared.

C

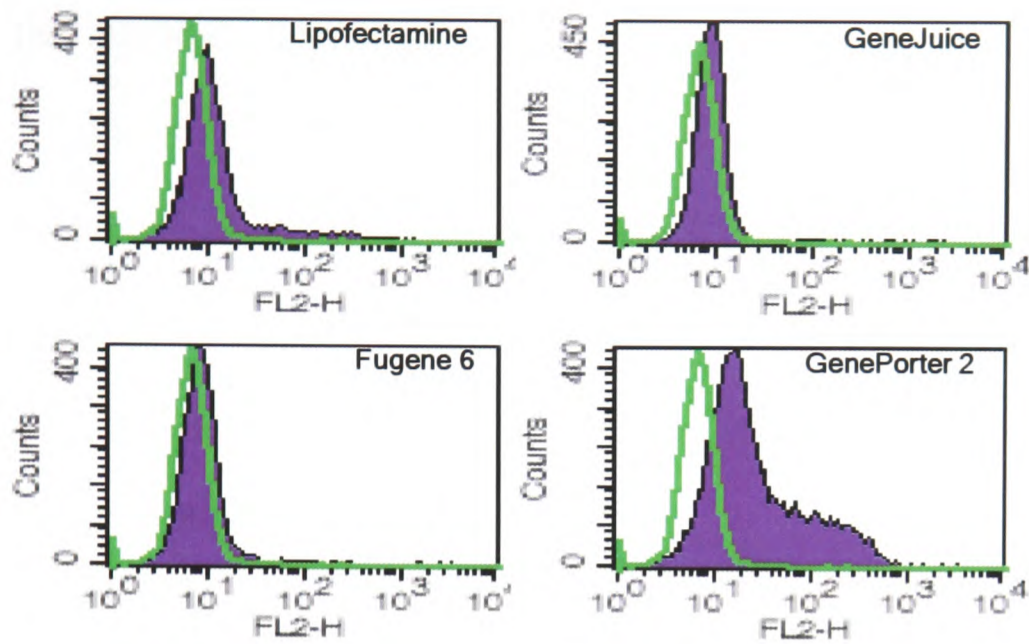


Figure 3.8c

Transfection reagent efficiency at achieving DN-RhoGTPase express 3T3-D1 cells. GenePorter2 was the only transfection reagent resulting sufficiently high transfection to attempt experiments.

d

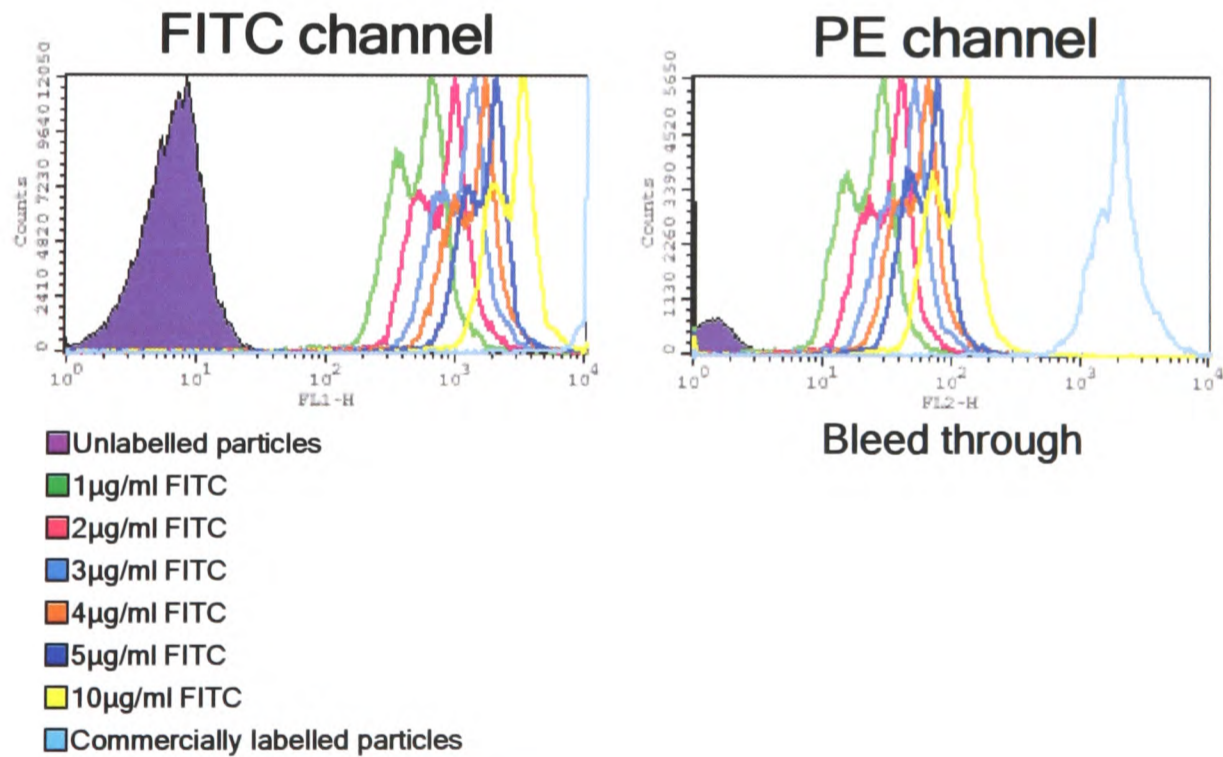


Figure 3.8d

FITC-labelling of zymosan particles. The best FL-1 signal to FL-2 bleed-through ratio was achieved with 10 $\mu\text{g/ml}$ FITC incubation.

GTPase tag) is shown on the right, and the FITC signal on the left. As can be seen even very low level labeling of zymosan particles resulted in a good signal compared to unlabelled particles. The effect of various labeling intensities on particles bound to 3T3-D1 cells was also examined (data not shown). What we found was that autofluorescence associated with cells meant that zymosan particles need to be labeled to an intensity achieved by at least 10 μ g/ml FITC, to enable us to distinguish cells binding a single particle from the background cellular signal in the FITC channel, and select them for analysis.

3.8.2 Rac-1 and Cdc42, but not RhoA play a role in zymosan phagocytosis

Using the assay described above, we analysed the effects of dominant negative Rho-GTPases on zymosan phagocytosis in 3T3-D1 cells. The cumulative data of at least 3 separate experiments is presented in **fig 3.8e**. The control bar represents empty vector transfected cells taking up bound particles. Tm-7 represents an irrelevant myc tagged protein control, and expression of this molecule lead to no significant change in the extent to which bound particles were internalized. Similarly DN-RhoA did not affect particle internalization. Both DN-Rac-1 and DN-Cdc42 lead to significant reductions in particle internalization, suggesting that like Fc γ Rs, Dectin-1 mediates cytoskeletal activation through these two GTPases. These data show the same pattern as those shown in **figure 3.8a**, confirming that the approach used was valid.

3.9 Discussion

The primary aims of this chapter were to analyse the phagocytic capacity of Dectin-1, to identify elements of the cytoplasmic tail that regulate this activity and to attempt to identify some of the molecules that might play a role in transducing the signals

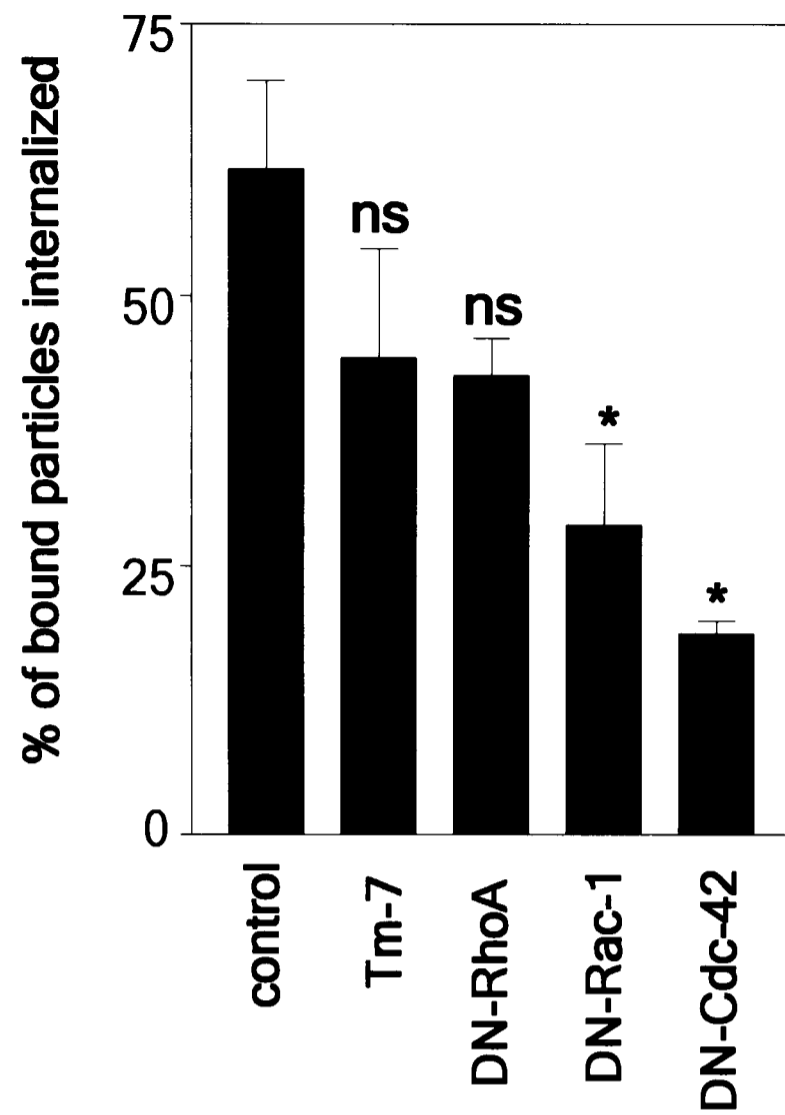


Figure 3.8 e

DN Rho-GTPase effects on FITC zymosan particle phagocytosis. Cumulative data from 3 separate experiments showing that Rac-1 and Cdc-42 are the GTPases that mediate actin cytoskeletal rearrangement following particle uptake by Dectin-1.

emanating from it. It was important in the first instance to study Dectin-1 out of its normal cellular context by using a non-phagocyte cell model that does not express endogenous receptors capable of binding the particulate ligand used. A further aim was to establish robust assays that could be used in the study of other cell types such as macrophages.

We found that Dectin-1 confers phagocytic ability for zymosan particles on an otherwise poorly phagocytic cell line. This makes Dectin-1 the first truly phagocytic fungal PRR described, and extends its role beyond mere fungal recognition. The phagocytic ability of Dectin-1 was found to depend on the ITAM-like motif in the cytoplasmic tail, with the membrane proximal tyrosine being the crucial residue. This dependence on tyrosine residues to mediate phagocytosis is a property Dectin-1 shares with the ITAM containing FcγRs. What is clearly different though is that only one of the tyrosine residues is responsible for mediating phagocytic uptake by Dectin-1, whereas both are required for the same function in FcγRs (Daeron 1997). The signals that link FcγR ITAMs to cytoskeletal activation have been defined in much detail, even if gaps still exist. We inhibited established elements of this signalling pathway in an attempt to compare Dectin-1 mediated phagocytosis of zymosan to FcγR phagocytosis. This data is summarised as a tentative model for Dectin-1 cytoskeletal signalling, that can be used both for comparative purposes and as a basis for the analysis of more complex cell models, as follows: when Dectin-1 is cross-linked by a particulate ligand, the membrane proximal tyrosine of its ITAM-like motif becomes phosphorylated through the action of a Src family kinase. Thereafter Syk kinase becomes activated and recruits PI3K, the products of which facilitate the recruitment of both PKC and the GEFs responsible for activating the Rho GTPases Rac-1 and Cdc42. The activated Rho

GTPases then mediate actin polymerisation leading to rapid particle internalisation. These data strongly suggest that, in NIH-3T3 fibroblasts, the pathway linking the Dectin-1 ITAM-like motif to the actin cytoskeleton is very similar to that linking FcγR ITAMs to the same structure. The significance of this finding is that, although there appear to be differences between Dectin-1 and FcγRs with regard to the number of tyrosine residues required to mediate the signals that lead to actin polymerisation, the intermediaries of this process are shared. This may imply that tyrosine based phagocytic signalling to the cytoskeleton is a conserved process not dependent on the receptors involved.

The establishment of a quantitative assay for measuring the extent to which bound particles are internalised is an important tool. This assay is sensitive enough to discriminate the extent to which a single zymosan particle bound to a cell is internalised (data not shown); yet can be used to examine large numbers of cells and has the additional potential to compare different model particles.

A limitation of taking a receptor out of its cellular context is that fibroblasts are not professional phagocytes and play little role in the elimination of pathogens, and consequently may not be endowed with the same phagocytic machinery as leukocytes. The observation that the extent of inhibition observed with the other inhibitors used was, with the exception of Toxin B, incomplete might be as a result of a number of factors. Firstly the dose may not have been sufficiently high to ensure complete inhibition, and secondly the function of the targeted kinase may have been redundant in fibroblasts. For instance, 3T3-D1 cells do not express haematopoietic Src kinases, instead expressing several other Src family members, that may not be as effectively

inhibited by PP2 (Courtneidge, Fumagalli et al. 1993; Twamley-Stein, Pepperkok et al. 1993). We were also unable to detect expression of either Syk kinase or Zap70 in 3T3-D1 lysates, when we performed western blotting (see **fig. 4.1.3.4d-e**), suggesting that expression levels are very low or absent. Furthermore piceatannol is a selective rather than a specific inhibitor of Syk and has been shown to affect other intracellular phosphorylation events non-specifically (Wang, Lu et al. 1998; Ashikawa, Majumdar et al. 2002). This suspicion is supported by **fig. 3.7a** that shows lower doses of piceatannol having little effect on particle internalisation. We had expected more complete inhibition following wortmannin and chelerythrine treatment, and there is no clear explanation for this. It has been noted by others, however, that phagocytosis of collagen coated latex particles by fibroblasts could only be inhibited 80% by PKC inhibition (Knowles, McKeown et al. 1991), suggesting fibroblasts may have additional mechanisms to internalise particles.

The Rho-GTPases are essential mediators of actin polymerisation, and consequently inhibition leads to the complete inhibition of zymosan particle internalisation. Dectin-1 mediated cytoskeletal rearrangements thus are regulated by these molecules, as is the case for both Fc γ R and CR3 (Caron and Hall 1998). The less potent inhibition seen when inhibiting the Rho GTPases individually are most likely due to redundancy between Rac-1 and Cdc42 and almost exactly mirror the original results achieved by micro-injection of the same constructs into a COS fibroblast system expressing Fc γ RIIA (Caron and Hall 1998). This strongly suggests that the Rho GTPases Rac-1 and Cdc-42 rather than Rho-A play a role in Dectin-1 mediated cytoskeletal rearrangement.

With the reservations discussed above in mind, the elements of Dectin-1 mediated cytoskeletal signalling in NIH 3T3 fibroblasts that are well supported by the data are that: Dectin-1 requires only the membrane proximal YxxL sequence for phagocytic uptake and probably becomes tyrosine phosphorylated following ligand binding. Further downstream it is likely that PI3K and almost certain that PKC are involved in the Rac-1 and Cdc42 mediated cytoskeletal rearrangements that are required for phagocytic uptake. The kinases involved in the tyrosine phosphorylation of Dectin-1 probably include non-haematopoietic members of the Src family, and the role of Syk in subsequent signal transduction is not determined.

These studies could be extended by looking at intermediate time points and extracting kinetic data. This might reveal additional internalisation mechanisms that can overcome the inhibitor and dominant negative construct effects. Another way to extend this study in the fibroblast model would be express Dectin-1 chimaeric receptors with FcγR cytoplasmic tails to allow inhibitor effects on different receptor signalling pathways to be directly compared, and perhaps the use of COS cells that do express Syk kinase. We decided against this route because Dectin-1 would be best studied in a cell population that natively expresses this receptor such as macrophages. Such a model will be described in chapter 4.

Chapter 4

Dectin-1 mediated phagocytosis in macrophages

4.1 Introduction

Phagocytosis plays a critical role in innate immunity, both by facilitating the removal and killing of pathogens and by priming the adaptive immune response. The phagocytic process is initiated by the cross-linking of an array of dedicated surface receptors, some capable of direct recognition, the so-called pattern recognition receptors (PRRs), and others that recognise opsonins coating the pathogens. Of these receptors, the opsonic Fc γ (Fc γ R) and complement receptors (CRs) are the best described and exhibit different phagocytic mechanisms and subsequent cellular responses which reflect important differences in their signalling pathways (Caron and Hall 1998; Aderem and Underhill 1999). Non-opsonic PRRs, such as the macrophage mannose receptor, scavenger receptors and recently CEACAM3 (Stahl and Ezekowitz 1998; Peiser, Gough et al. 2000; Schmitter, Agerer et al. 2004), have also been suggested to possess phagocytic capacity, but the mechanisms underlying these activities are less clear.

Phagocytosis by Fc γ R, after ligand binding, is thought to be initiated by Src kinase-mediated tyrosine phosphorylation of the receptor ITAM domains leading to the recruitment of p72Syk, a protein tyrosine kinase which is required for subsequent cellular activation and ligand internalisation (Crowley, Costello et al. 1997; Aderem and Underhill 1999) see also chapter 1.5.2. Although the exact downstream pathways leading from Fc γ and other receptors to actin polymerisation and phagocytosis is currently unclear, other molecules, including phosphatidylinositol (PI)-3 kinase, protein kinase C (PKC) and the Rho GTPases, are known to be involved (Aderem and Underhill 1999). In chapter 3 we showed that, in NIH-3T3 cells, Dectin-1-mediated

phagocytic uptake through the conserved cytoplasmic ITAM-like motif and resembled Fc γ Rs in the way signals were relayed from receptor to actin-cytoskeleton.

It has previously been shown that in macrophages the phagocytosis of unopsonised yeast occurs with kinetics equal to or greater than those of Fc γ R mediated phagocytosis of opsonised particles in the same cells (Camner, Lundborg et al. 2002). We determined whether, in macrophages, Dectin-1 could also internalise β -glucan ligands, and show here that over-expression of this receptor greatly enhances the efficiency of macrophage phagocytosis of yeast-derived particles. Furthermore, we show that although this activity was dependent on the ITAM-like motif in the cytoplasmic tail, the mechanism of Dectin-1 mediated phagocytosis in macrophages is distinct from that mediated by any other receptor characterised, including the Fc γ R and CR.

4.2 Aims of the study

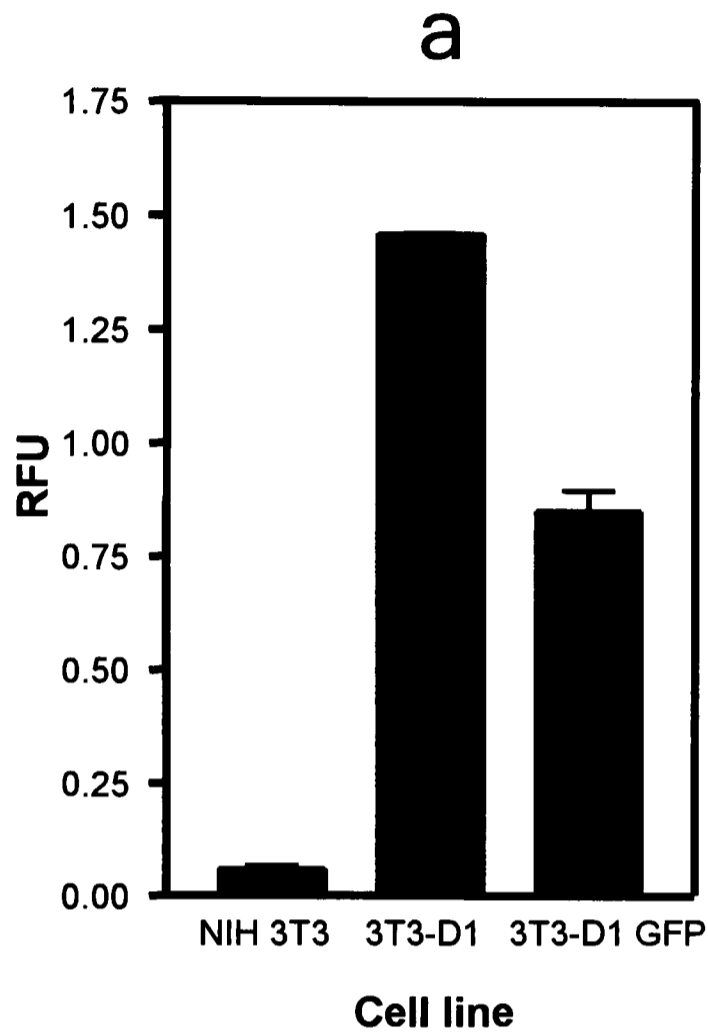
The aims of this study were threefold: firstly, to generate and characterize macrophage cell lines over-expressing epitope-tagged Dectin-1. Particular attention was paid to tag location to minimize interference with receptor function; secondly to analyze Dectin-1 cytoplasmic tail functions by mutational analysis in the correct cellular context; finally to confirm our findings in NIH-3T3 cells with regard to identifying some of the signalling mediators involved in relaying signals from the cytoplasmic tail of Dectin-1 to the actin cytoskeleton by comparing Dectin-1 to the well-defined Fc γ R.

4.3 Results

4.3.1 Modification of the Dectin-1 cytoplasmic tail results in diminished phagocytic uptake of zymosan by macrophages

We had generated NIH-3T3 cell lines stably expressing Dectin-1GFP (provided by Dr. G.D.Brown) in order to be able to study receptor trafficking by microscopy. When we compared the phagocytic efficiency of this cell line to that of 3T3-D1 cells we found that despite recognizing zymosan particles efficiently (**fig. 4.3.1a**), ligation of the GFP tagged receptor was accompanied by poor internalization of bound particles (**fig. 4.3.1b**). This suggested that modification of the cytoplasmic tail of Dectin-1 might interfere with receptor signalling. As we wanted to be able to follow Dectin-1 by confocal microscopy, and our monoclonal antibody to Dectin-1 extracellular domain does not recognise fixed receptor, we elected to generate HA epitope-tagged receptor constructs. RAW 264.7 macrophages express low levels of endogenous Dectin-1, therefore we selected this cell line as a model in which to overexpress this receptor. We designed two different recombinant Dectin-1 molecules, one with a cytoplasmic HA tag (NT-HA) and another with an extracellular HA tag (CT-HA). These receptors were stably over-expressed in RAW 264.7 cells by retroviral transduction and early in selection receptor expression was already good for both constructs (**fig. 4.3.1c**). When we stained both live and fixed, permeabilised cells from both cell lines for HA tag expression (**fig. 4.3.1d**) we found that in unpermeabilised cells, the C-terminal extracellular tag was expressed at high levels on the cell surface and that following permeabilisation, an increase in signal indicative of a cytoplasmic pool was noted. On the contrary, unpermeabilised N-terminal intracellular tagged cells, as expected, showed very little signal and following permeabilisation, good levels of tag expression were noted, although lower than for the extracellular tag. As we were concerned about potential cytoplasmic tag interference with receptor signalling processes, we examined NT-HA tagged cells by microscopy. **Figure 4.3.1e** shows confocal micrographs of cell fields stained for HA tag expression. As is evident, the receptor distribution seems to

Comparative cold binding of FITC zymosan



Effects of Dectin-1 cytoplasmic tail modification

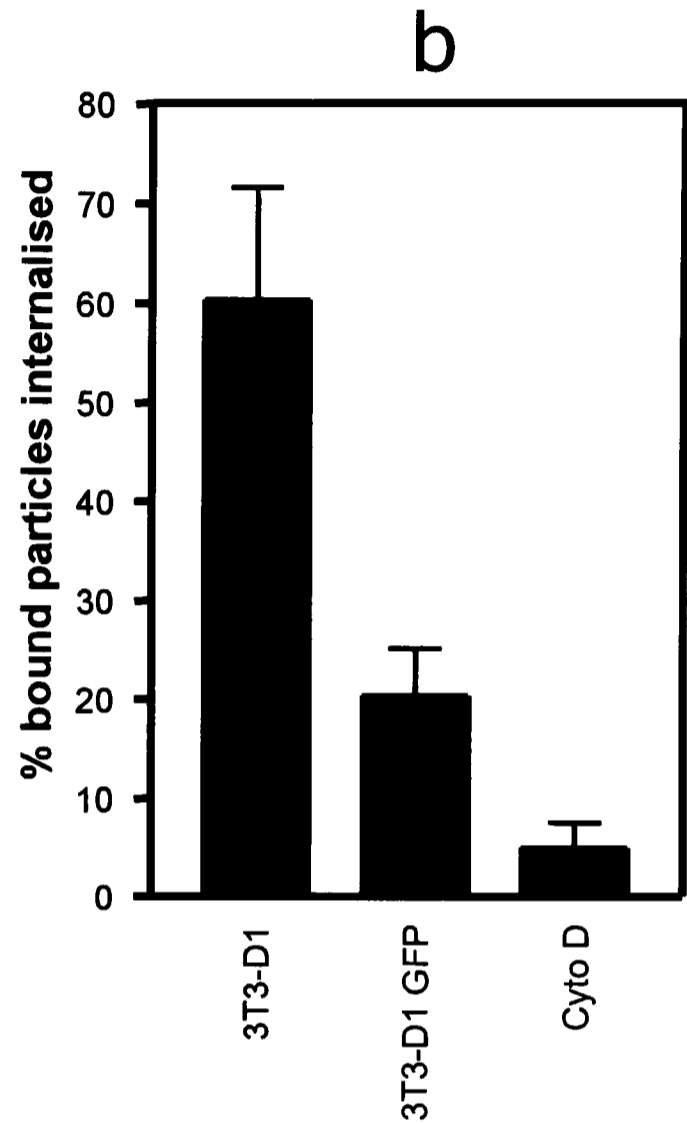


Figure 4.3.1a-b

Alterations to the cytoplasmic tail of Dectin-1 result in decreased phagocytosis. a) 3T3-D1 and 3T3-D1 GFP expressing cells recognise zymosan particles with high efficiency. b) 3T3-D1 GFP cells are much less efficient at phagocytic uptake of bound particles when compared to 3T3-D1 cells.

RAW CT-HA Dectin-1

RAW NT-HA Dectin-1

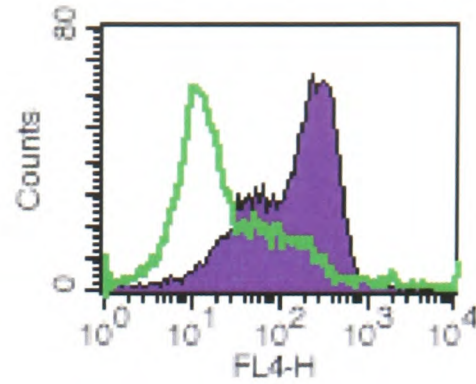
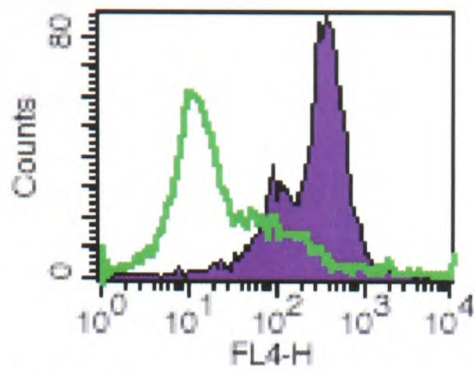
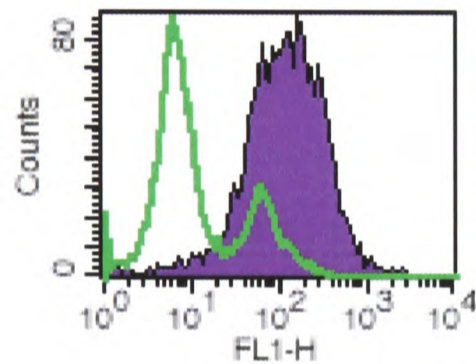
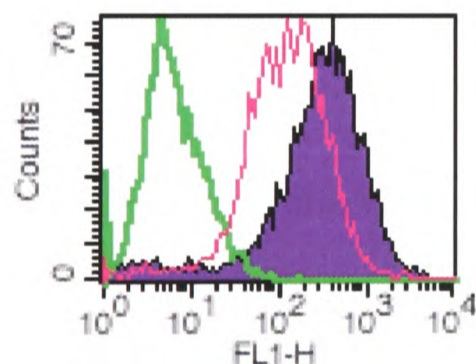


Figure 4.3.1c

Expression of Dectin-1 on the cell surface of retrovirally transduced RAW 264.7 cells as measured by flow-cytometry using the 2A11 monoclonal antibody. Left hand panel shows Dectin-1 with an extracellular HA tag (CT-HA) in purple. Right hand panel shows Dectin-1 with a cytoplasmic HA tag (NT-HA). Isotype matched controls are shown in green.

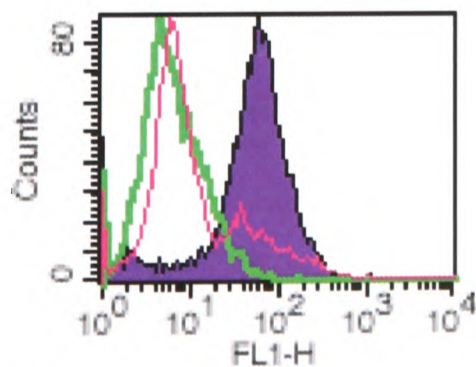


■ Isotype matched control antibody
 ■ Live cell anti-Dectin-1 mAb staining



RAW CT-HA

■ Isotype matched control antibody
 ■ Unpermeabilised anti-HA staining
 ■ Permeabilised anti-HA staining



RAW NT-HA

Figure 4.3.1d

HA tag expression by flow cytometry: surface vs intracellular tag. Top histogram shows unfixed 2A11 staining of the CT-HA cell line (purple). Middle panel shows live, unpermeabilised CT-HA cell after anti-HA staining (pink) and permeabilised HA staining (purple). Lower panel shows unpermeabilised NT-HA cells (pink) and permeabilised cells (purple).

differ between the C-terminal and N-terminal tagged molecules, with the N-terminal tagged receptors having a predominantly vesicular, cytoplasmic, distribution and the C-terminal tagged receptors a more cell membrane bound expression. When we compared zymosan particle recognition by these two receptors we found them to be similar (**fig. 4.3.1f**), suggesting that the extracellular tag did not significantly interfere with ligand recognition. When, however, we compared the phagocytosis of zymosan particles over 30 minutes by confocal microscopy (**figs. 4.3.1g-h**) we found that, whilst the vast majority of particles bound by the CT-HA receptor were successfully internalized (**fig. 4.3.1g**), whilst the NT-HA receptor (**fig. 4.3.1h**) bound many particles yet internalized a lower proportion. To confirm this finding we then compared phagocytic internalization in a flow cytometry based assay (**fig. 4.3.1i**) and found that the NT-HA cell line appeared to be less efficient at internalizing bound particles. Based on these observations we thus decided to use only recombinant molecules with extra-cellular HA tags for our further studies.

4.3.2 RAW 264.7 cell lines over-expressing HA-tagged Dectin-1 show enhanced recognition and phagocytosis of β -glucan rich particles

Recombinant Dectin-1 molecules as illustrated in **fig. 4.3.2a** were stably expressed in RAW 264.7 macrophages by retroviral transduction. After selection, Dectin-1 expression was found to be high when compared to wild type RAW cells (**fig. 4.3.2b**) and remained very stable over time. The HA tag did not interfere with recognition by the monoclonal antibody against the extracellular domain of Dectin-1 as shown in this figure, and also did not interfere with zymosan particle recognition (**fig. 4.3.2c**) which was largely similar amongst the cell lines.

CT-HA Dectin-1

NT-HA Dectin-1

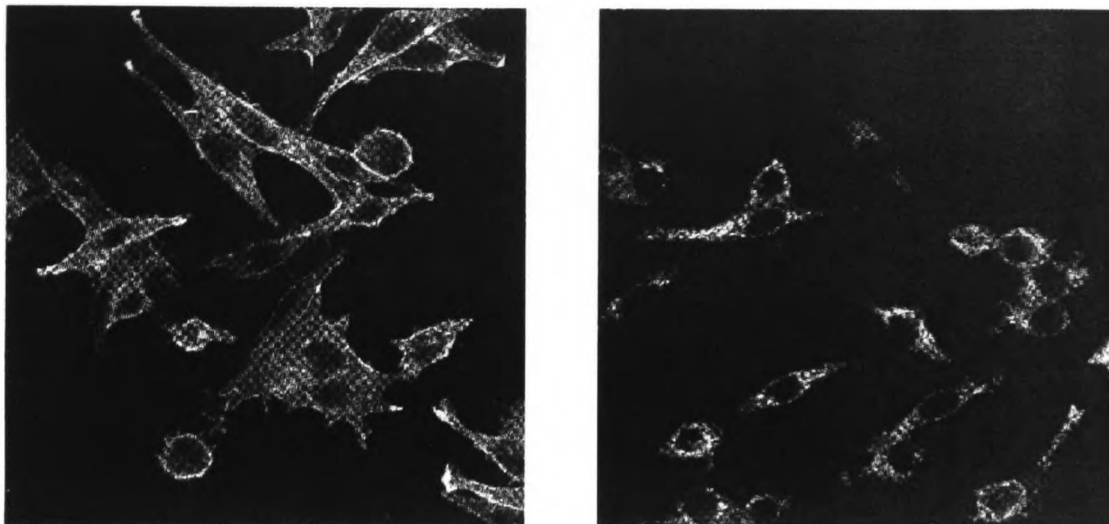


Figure 4.3.1e

CT-HA and NT-HA Dectin-1 expressing RAW 264.7 macrophages show differing receptor distribution. CT-HA cells (left) show more surface expressed Dectin-1 than NT-HA (right) cells.

Cold binding of FITC zymosan to CT and NT tagged Dectin-1 expressing RAW cells

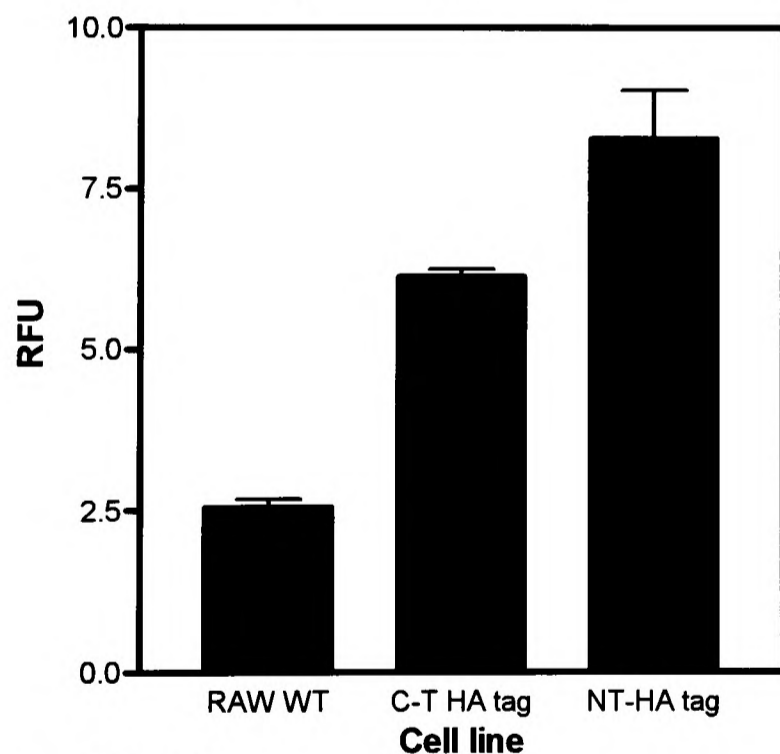


Figure 4.3.1f

Comparative zymosan binding at 4°C and internalisation at 37°C. Zymosan particle recognition at 4°C is efficient for both CT-HA and NT-HA tagged RAW-D1 cell lines.

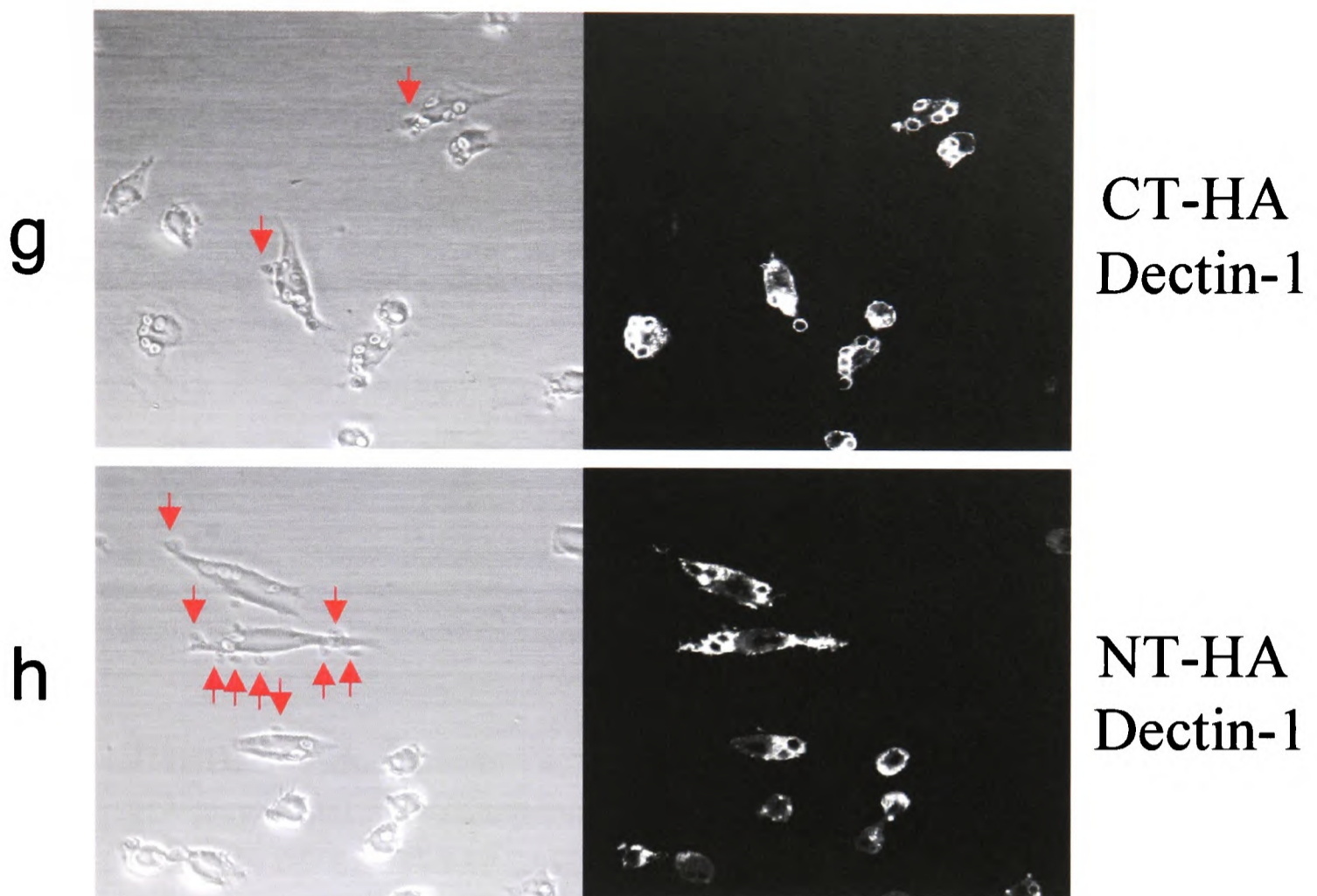


Figure 4.3.1g-h

Receptor tag location influences phagocytic uptake of zymosan particles. g) Upper panels are confocal images of a cell field following zymosan binding and 30 minutes of synchronised particle uptake in CT-HA tag expressing RAW-D1 cells. h) Lower panels represent phagocytic internalisation by NT-HA RAW-D1 cells. Red arrows indicate bound but not internalised particles.

Effect of cytoplasmic HA tag in RAW 264.7 cells

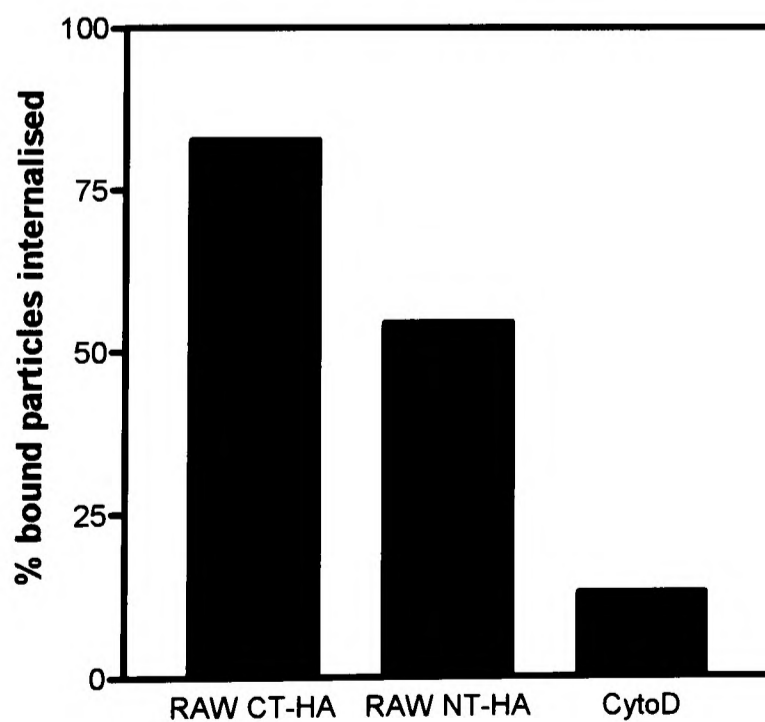


Figure 4.3.1i

Comparative zymosan internalisation at 37°C. Zymosan particle internalisation is better when the tag is extracellular.

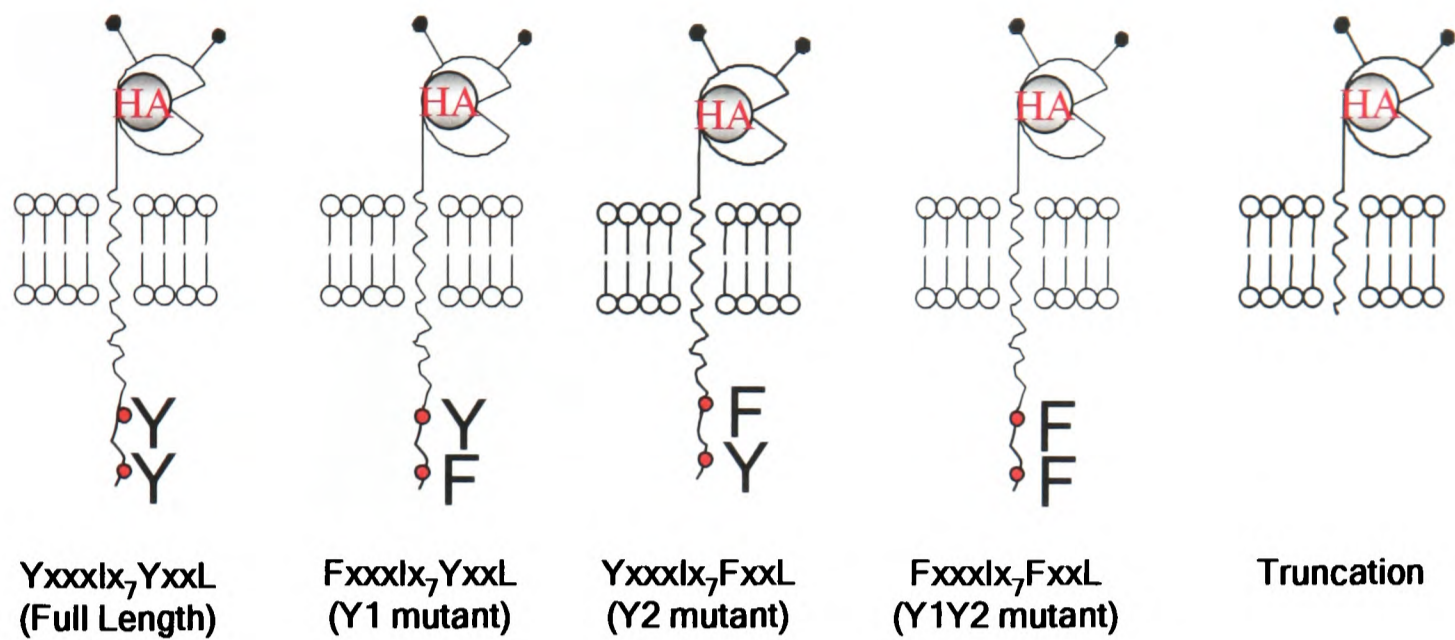


Figure 4.3.2a

HA tagged cell lines generated. 5 CT-HA tagged Dectin-1 expressing RAW 264.7 cell lines were generated by retroviral transduction.

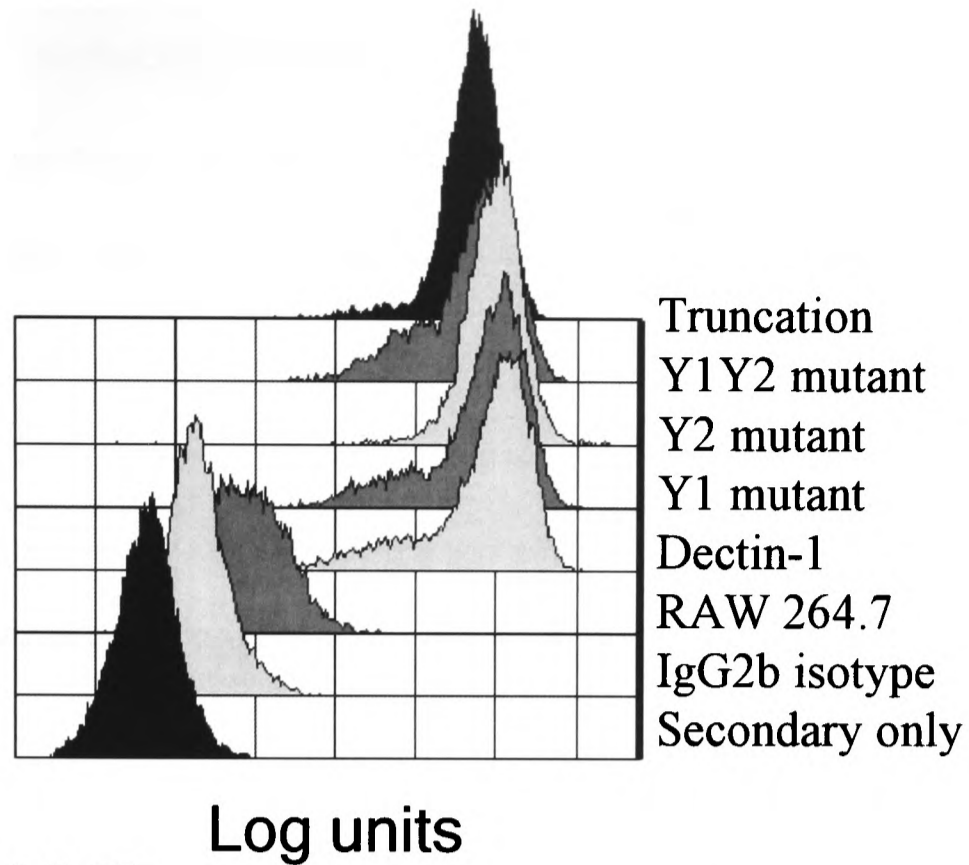


Figure 4.3.2b

Expression of Dectin-1 on the cell surface of RAW 264.7 cells expressing recombinant Dectin-1 molecules.

Comparative cold FITC zymosan binding

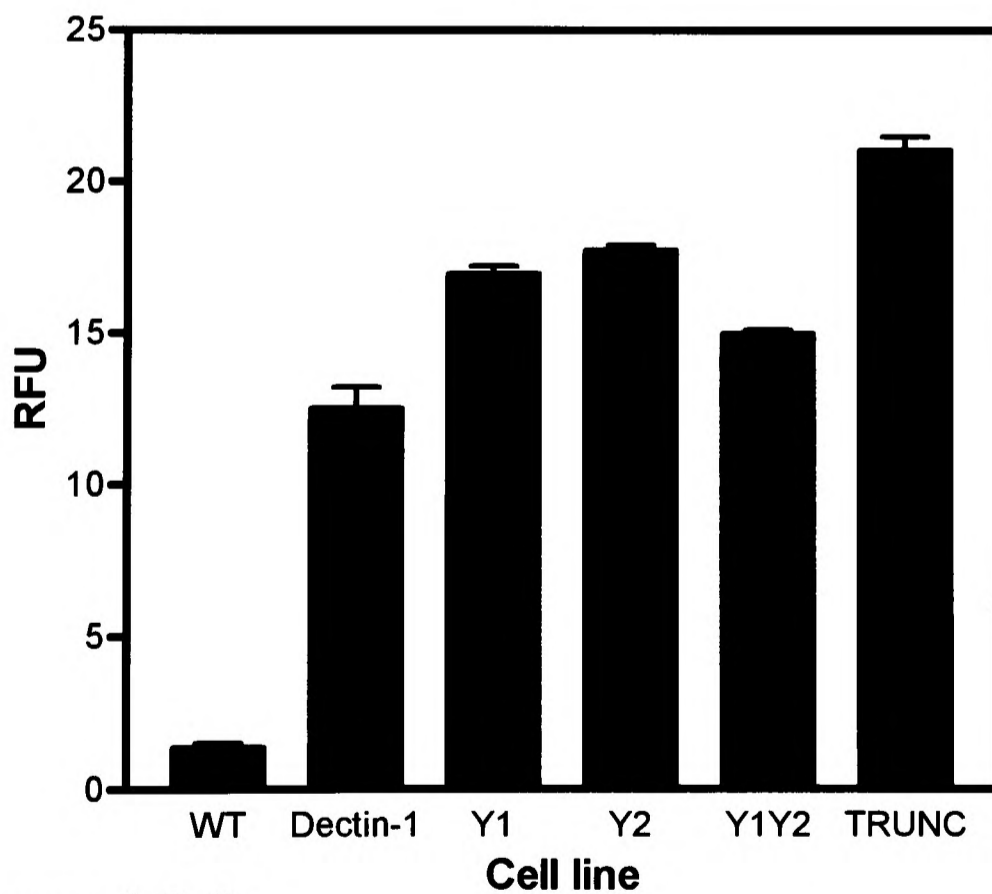


Figure 4.3.2c

FITC zymosan particle recognition by RAW-D1 cell lines. Particle recognition was performed at 4°C over 60 minutes. Binding levels between cell lines is largely similar, and at much higher levels than untransduced cells.

For the purposes of microscopy we needed to find a particle to cell ratio that would allow the widely dispersed cells on a coverslip to encounter and bind a zymosan particle during the brief centrifugal synchronization prior to particle internalization. To establish an appropriate ratio we used FITC zymosan particles, brought into contact with cells by brief centrifugation, followed by washing with warm medium. We tested various conditions and found that 8 particles per cell provided most cells within a cell field the opportunity to bind at least 1 particle (**fig. 4.3.2d**).

In order to examine the effects of the various cytoplasmic tail mutations we had generated on zymosan particle phagocytosis in Dectin-1 expressing RAW 264.7 cells, we overlaid them with a suspension of unlabelled zymosan particles in warm medium, at a ratio of 8 particles per cell, synchronized particle uptake by centrifugation and compared the cell lines in terms of their ability to internalize bound particles (**fig. 4.3.2e**). RAW 264.7 wild type cells are shown as a control (**fig. 4.3.2f**). Full-length Dectin-1 over-expression results a large increase in both zymosan particle binding and internalization over WT RAW 264.7 cells. Furthermore, when the membrane distal tyrosine residue is mutated (ITAM Y1 panel), no reduction in particle internalization is seen. When, however, either the membrane proximal tyrosine residue (ITAM Y2), both tyrosine residues (ITAM Y1Y2), or the cytoplasmic tail are deleted, no phagocytic uptake is seen despite abundant particle binding. Comparison of particle internalization by flow cytometry (**fig. 4.3.2g**) shows that, indeed, across a large sample population, the membrane proximal tyrosine (Y2) does determine phagocytic uptake of zymosan particles by Dectin-1 in macrophages. These data confirm that the observations we made in NIH-3T3 cells in chapter 3 regarding the functions of the cytoplasmic tail of Dectin-1, also apply to macrophages. These data also provide the basis for examining

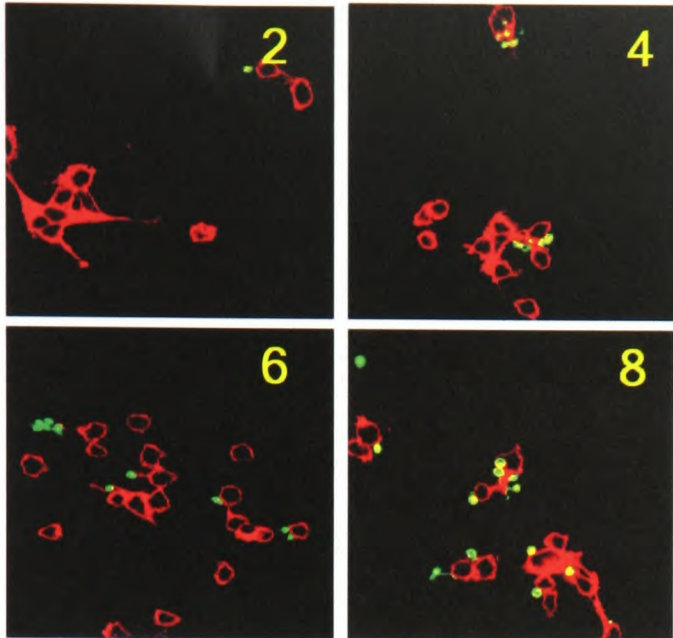


Figure 4.3.2d

Establishing particle to cell ratio for phagocytosis of zymosan particles synchronised by centrifugation. Various ratios of FITC zymosan to cells (2:2 – 8:1) were tested to achieve binding levels sufficient to allow a large proportion of cells to bind at least 1 particle. 8 particles per cell provided a high proportion of cells the opportunity to bind.

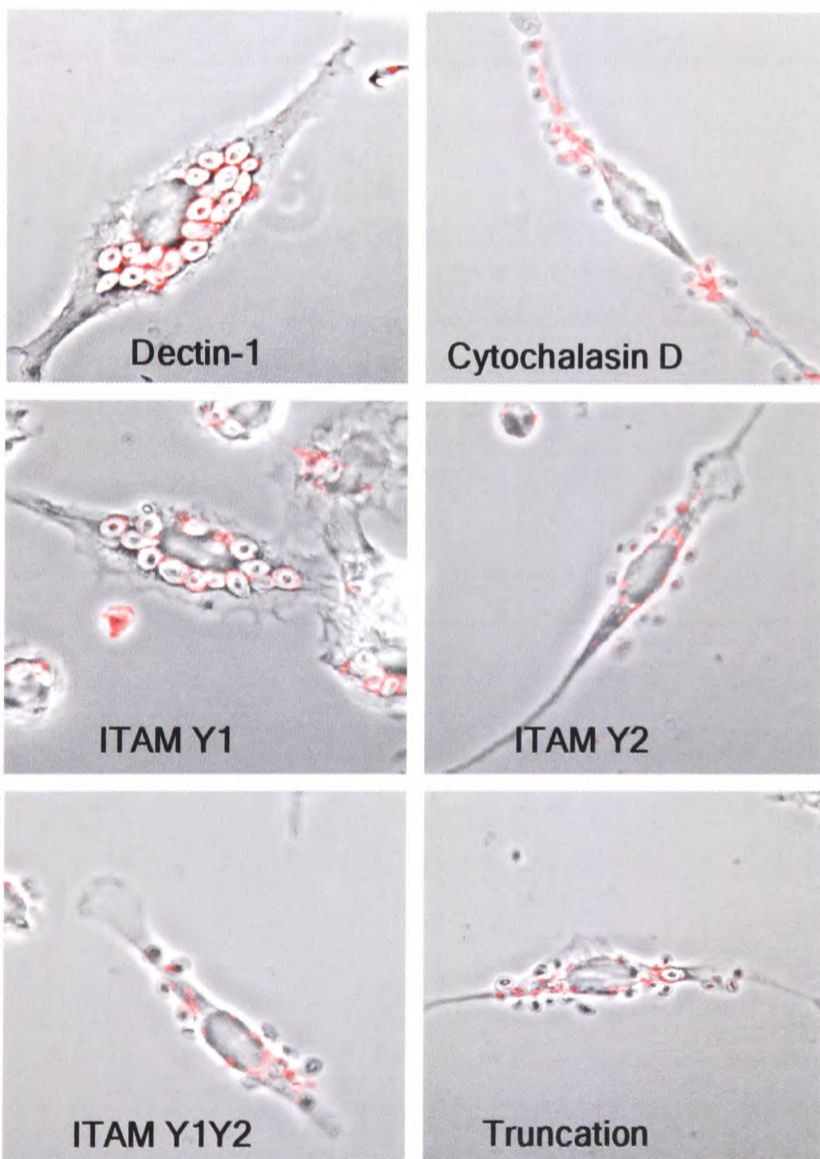


Figure 4.3.2e

Mutation of the membrane proximal tyrosine residue (ITAM Y2) abrogates Dectin-1 mediated particle phagocytosis in macrophages. Cells from the RAW cell lines generated were synchronised with 10 particles per cell unlabelled zymosan and allowed to internalise for 30 minutes at 37°C, after which cells were fixed and stained for Dectin-1 receptor HA tag. Only cells expressing full-length and membrane distal tyrosine mutated Dectin-1 retained the capacity to internalise bound particles.

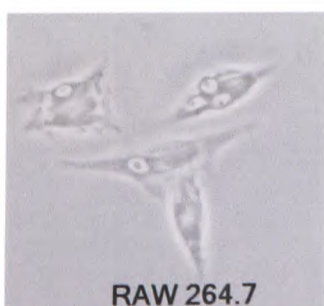


Figure 4.3.2f

Wild type RAW 274.7 internalise zymosan particles but with much reduced efficiency. RAW cells synchronised with 10 particles per cell unlabelled zymosan were allowed to internalise particles for 30 minutes. The majority of RAW cells did not internalise particles, and those that did, internalised few when compared to the RAW-D1 cell line.

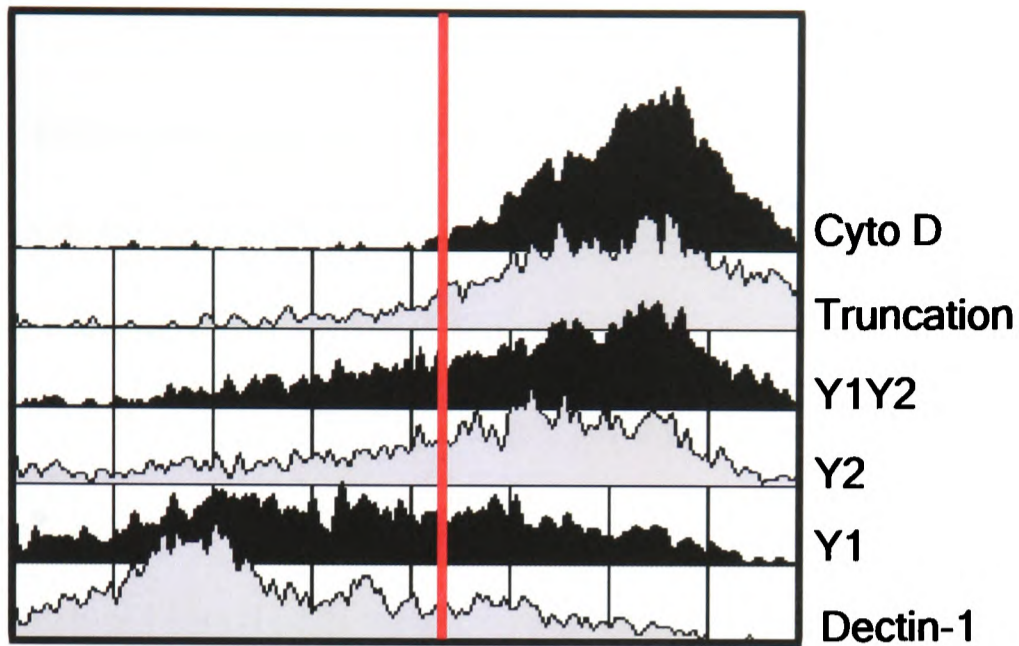


Figure 4.3.2g

Flow cytometric quantitation of RAW-D1 expressing cell lines confirms the phenotype seen during confocal studies. RAW-D1 cell lines were allowed to bind 10 particles per cell FITC-zymosan at 4°C and were then allowed to internalise particles for 30 minutes at 37°C. Comparative uptake was measured by flow cytometry as before. The full-length and Y1 mutant cell lines were competent in phagocytosis, yet the other cell lines showed dramatic reductions in particle internalisation.

whether the mediators that link Dectin-1s' cytoplasmic tail to the actin cytoskeleton are shared with FcγRs.

4.3.3 Sheep red blood cell phagocytosis by FcγRs in RAW-D1 cells

In order to establish whether Dectin-1 and FcγRs also shared similar signalling pathways in macrophages, we set up a phagocytic assay that allowed us to directly compare Dectin-1 mediated and FcγR mediated phagocytosis in the RAW-D1 cell line. As the phagocytic ligand for FcγRs we used antibody opsonised sheep red blood cells (SRBCs). The sheep red blood cells were labeled with a fluorescent membrane dye (**fig. 4.3.3**) and then opsonised with polyclonal antibodies at sub-agglutinating concentrations. As the SRBCs are opsonised with rabbit polyclonal antibodies, antibodies bound to the SRBC surface could be stained using the same APC conjugated anti-rabbit secondary antibody, that we used to label antibody coated zymosan particles, in the flow cytometry assays. This allowed us to use virtually identical flow-cytometry based assays to compare the extent of zymosan particle or SRBC internalization by RAW-D1 macrophages. The figure shows that opsonised SRBCs, labeled to an intensity that results in no transfer of membrane dye to macrophage membranes as assessed by fluorescence microscopy (data not shown), demonstrate a large shift in fluorescence when compared to the unlabelled controls (central histogram). Subsequent APC labeling was even more effective (right histogram). Opsonised, fluorescent SRBCs were then used as a phagocytic ligand for FcγRs in parallel assays with the Dectin-1 phagocytic ligand, FITC labeled zymosan, and as before, particle internalization was measured by the extent to which either phagocytic particle acquired APC secondary antibody staining.

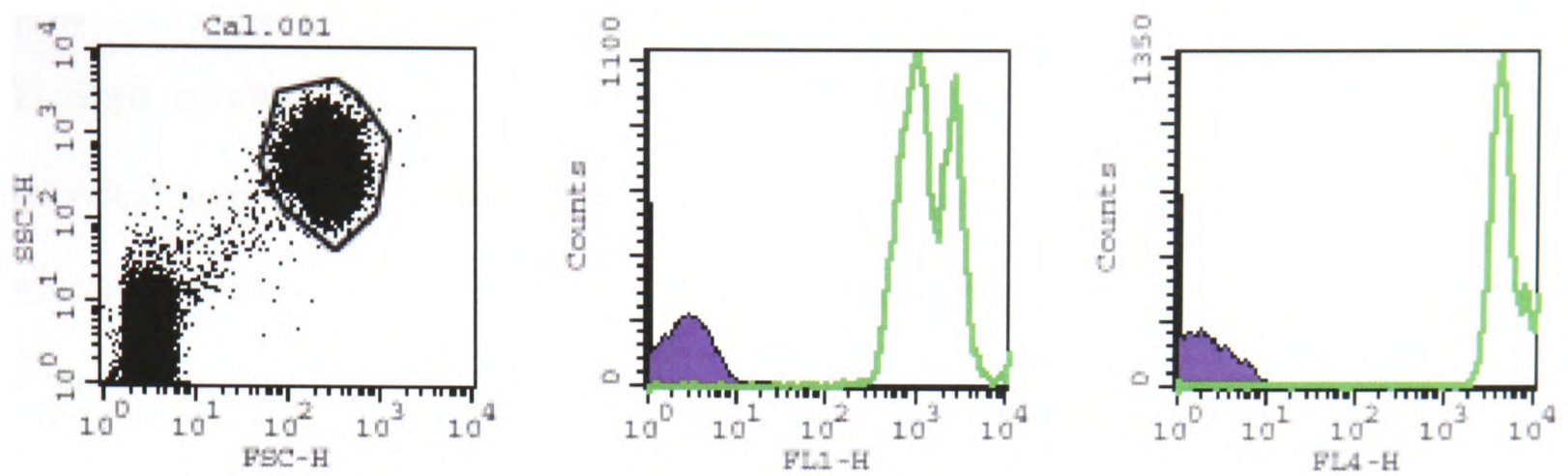


Figure 4.3.3

Sheep red blood cell membrane labelling, opsonisation and APC labelling. Sheep red blood cells were labelled with a green PKH2 dye (central panel). Labelling made it possible to distinguish the labelled and unlabelled populations easily. Right hand side panel shows labelled SRBC following sub-agglutinating opsonisation with rabbit anti-SRBC stroma and subsequent staining with an anti-rabbit APC conjugated monoclonal antibody. Again, unopsonised labelled cells showed no APC signal, whilst opsonised cells showed very strong APC labelling.

4.3.4 Dectin-1 mediated phagocytosis is Syk independent in macrophages

Using RAW-D1 macrophages, we examined the effect of the various inhibitors described in Chapter 3, on phagocytosis mediated by Fc γ R and Dectin-1, using equivalent numbers of antibody opsonised sheep red blood cells (SRBC) and zymosan particles per cell, as specific ligands for the Fc γ R and Dectin-1, respectively. The sizes of these two phagocytic particles are very similar and the same particle to cell ratio, primary and secondary antibody concentration was used throughout.

Although all the inhibitors tested, except piceatannol (see below), had a significant inhibitory effect (**fig. 4.3.4a**), they showed marked differences in their effects on particle internalisation mediated by these two receptors. Inhibition of tyrosine kinases (by genistein), the Src kinases (by PP2), PI-3K (by wortmannin), PKC (by chelerythrine) and the Rho GTPases (by Toxin B) almost completely blocked Fc γ R mediated uptake of SRBCs, in contrast, whilst inhibition of tyrosine kinases by genistein and inhibition of PKC by chelerythrine led to similar levels of inhibition of zymosan and SRBC phagocytosis, PP2, wortmannin and Toxin B had only a partial inhibitory effects on Dectin-1 mediated zymosan phagocytosis. More significantly, and in contrast to what was observed in the NIH3T3 cells, the Syk-kinase inhibitor piceatannol had no effect on Dectin-1 mediated zymosan internalisation in the RAW macrophages. As Syk is thought to be an essential kinase required for all ITAM-mediated signalling (Turner, Schweighoffer et al. 2000), we wanted to confirm this finding by examining Fc γ R and Dectin-1 mediated phagocytosis in Syk-deficient bone marrow derived macrophages.

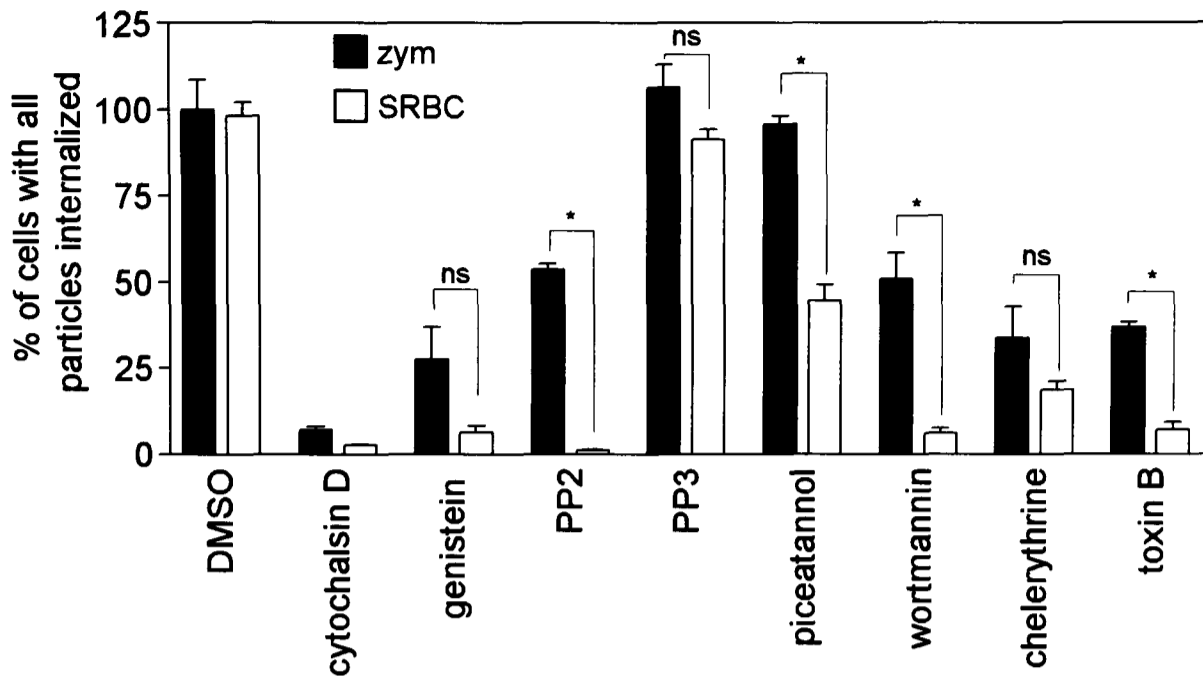


Figure 4.3.4a

Comparison of the effects of inhibitors of kinases and GTPases that play a role in FcγR mediated phagocytosis on Dectin-1 and FcγR mediated phagocytosis. RAW-D1 cells treated with various inhibitors were allowed to internalise labelled, antibody-opsonised SRBC or FITC zymosan particles bound at 4°C for 30 minutes synchronously. Zymosan particles and SRBCs were then stained with antibody and secondary APC conjugated antibody, or APC conjugated secondary antibody only, respectively, prior to quantitation of internalisation by flow cytometry. Results are expressed as a proportion of carrier only controls.

We obtained BMDMs from chimaeric, Syk deficient, mice and determined that these cells were indeed entirely donor derived by staining both control and Syk deficient cells with CD45.1 (**fig. 4.3.4b**) (representing residual cell populations in recipient, CD45.1 positive animal shown in panels on the left) and donor derived CD45.2 cell populations (panels in middle). Using zymosan particles, whose recognition in BMDMs cells is mediated by Dectin-1 (Brown, Taylor et al. 2002), as well as opsonised labeled SRBC, we observed a similar degree of uptake of each type of particle in wild type cells, which could be completely inhibited with cytochalasin D (**fig.4.3.4c**). As has been described previously, the Syk deficient macrophages were unable to mediate phagocytosis through the Fc γ R (**fig. 4.3.4c** & (Crowley, Costello et al. 1997)), but were fully competent in Dectin-1 mediated internalisation. In addition, Syk deficient macrophages were still able to produce the pro-inflammatory cytokine TNF- α in response to zymosan stimulation (**see fig. 5.3.8b-c**). The presence and/or absence of Syk (**fig. 4.3.4d**) and its lymphocyte homologue Zap70 (which should not be expressed in leukocytes, but can substitute for Syk if present) (**fig. 4.3.4e**) in these macrophages and in RAW264.7 cells were confirmed by Western blotting. Thus from these data it appears that Dectin-1, while resembling Fc γ R in NIH3T3 fibroblasts, utilizes different cellular machinery to induce phagocytosis and cellular activation in macrophages.

4.4 Discussion

We have shown that the cytoskeletal rearrangements triggered by Dectin-1 to induce particle internalisation are dependent on a motif in its cytoplasmic tail, which is strikingly similar to ITAM sequences found in a variety of activating immune receptors, including DAP12 and the T-cell-, B-cell- and Fc γ receptors (Turner, Schweighoffer et al. 2000). The motif in the Dectin-1 tail (YxxxI/Lx7YxxL), which is

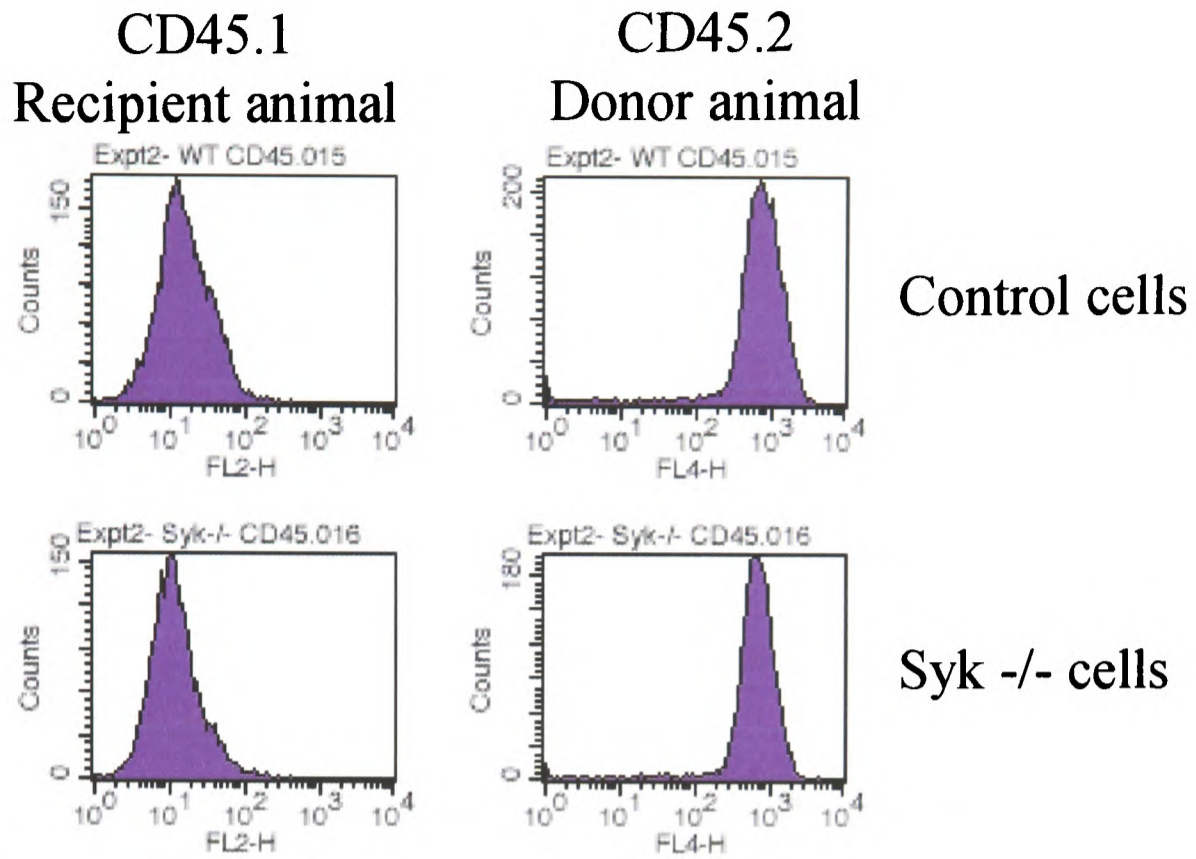


Figure 4.3.4b

Syk deficient bone marrow macrophages from chimaeric mice are entirely donor derived. Recipient irradiated animals expressing myeloid CD45.1 had their bone marrow repopulated by CD45.2 derived foetal liver cells from Syk deficient foetuses. Upper row are control macrophages and lower row Syk deficient macrophages.

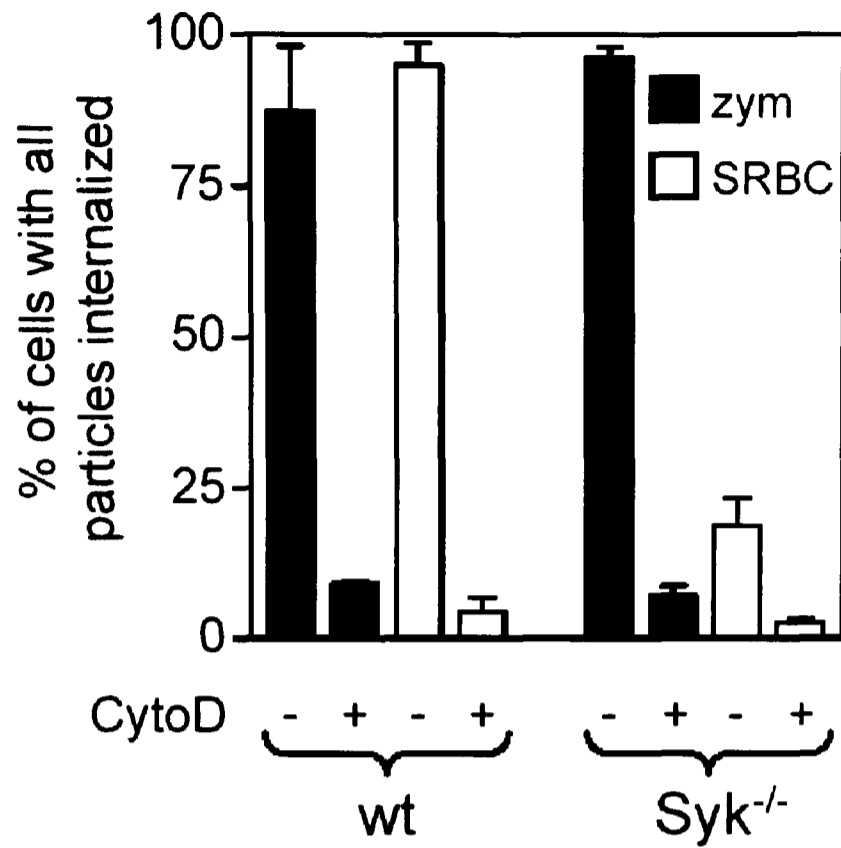


Figure 4.3.4c

Syk is not required for zymosan particle phagocytosis but is essential for Fc γ R mediated phagocytosis. Phagocytosis of zymosan particles in syk deficient and WT BMDMs is equivalent, whereas opsonised SRBC phagocytosis is abrogated in Syk deficient BMDMs.

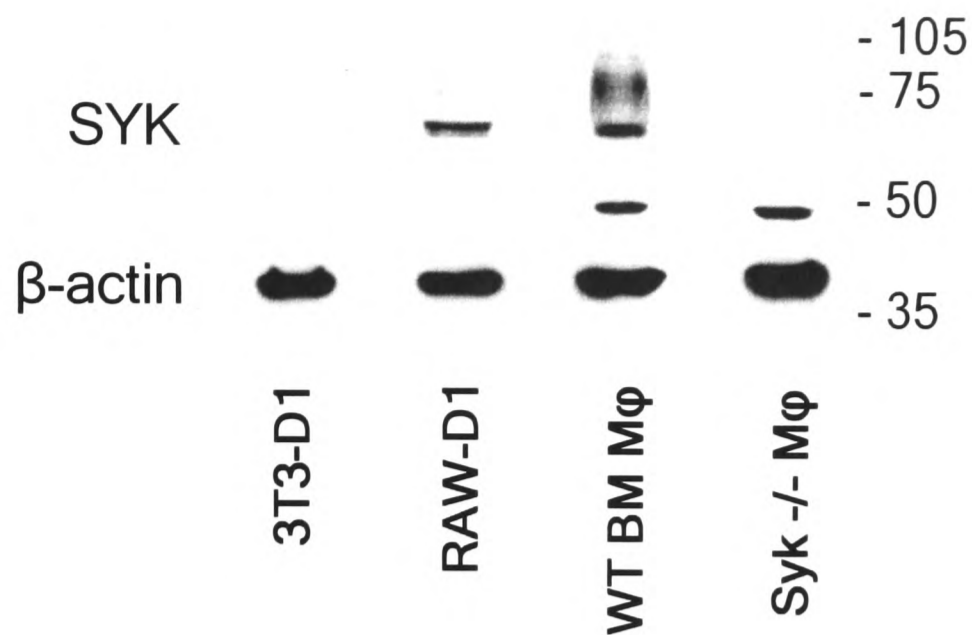


Figure 4.3.4d

Syk deficient BMDMs and NIH-3T3 cells are entirely deficient in Syk whereas WT BMDMs and RAW-D1 cells express Syk. Western blot of whole cell lysates following SDS-PAGE and staining with anti-Syk monoclonal antibody. B-actin is a loading control.

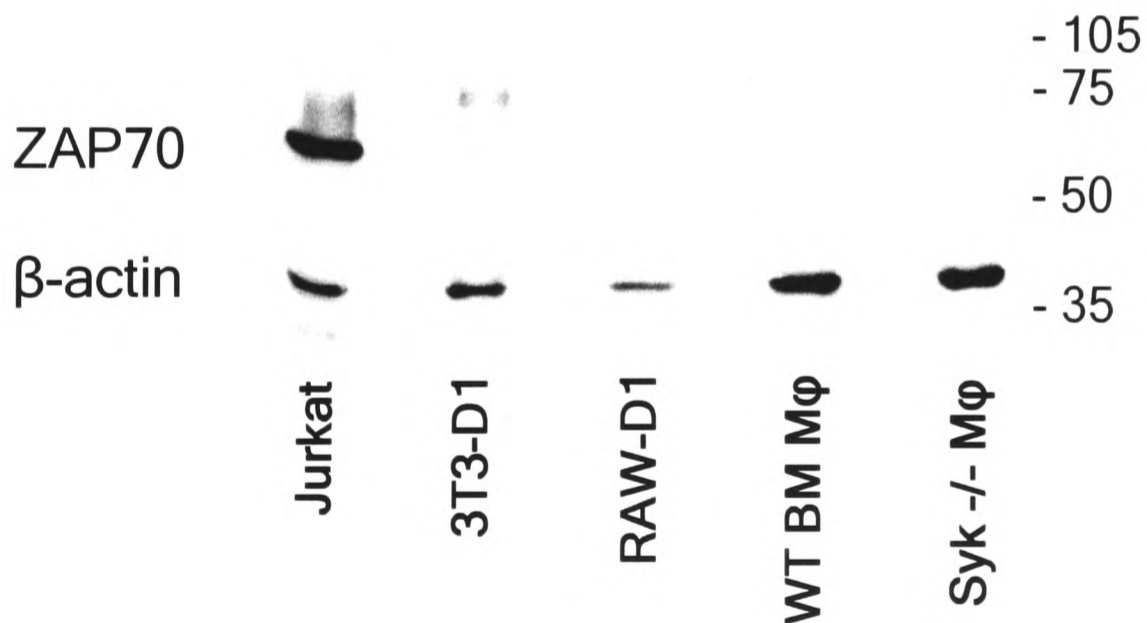


Figure 4.3.4e

All the cell lines used were deficient in ZAP70 a homologue of Syk. Western blot of whole cell lysates following SDS-PAGE and staining with anti-ZAP70 monoclonal antibody. Jurkat T cells were used as a positive control for ZAP70 staining and β -actin as a loading control.

conserved in the mouse, human and monkey receptors, differs only slightly from the canonical ITAM sequence (YxxL/Ix7-12YxxL/I). We speculated that this would imply mechanistic similarity with FcγRs, and particularly to FcγRIIA, a single chain receptor containing an ITAM in its cytoplasmic tail (Daeron 1997). Indeed, our initial analysis in NIH3T3 cells suggested that Dectin-1 was using similar signalling pathways / molecules to mediate phagocytosis. Major differences, however, only became apparent when we directly compared these receptors in macrophages. This highlights an essential requirement to study these processes in the correct cellular context. Dectin-1 becomes tyrosine phosphorylated after ligand binding (Gantner, Simmons et al. 2003), and we have shown that phagocytosis by this receptor was blocked by the non-specific tyrosine kinase inhibitor, genistein. Tyrosine phosphorylation is an initiating event for FcγR, but not CR, mediated phagocytosis and is mediated by the Src family kinases (Aderem and Underhill 1999; Strzelecka-Kiliszek, Kwiatkowska et al. 2002). At least six Src-kinases are known to be expressed in phagocytes, and are essential for FcγR mediated internalisation (**Fig. 4.3.4a** & (Majeed, Cavegion et al. 2001; Garcia-Garcia and Rosales 2002)). Consistent with previous observations of zymosan uptake in macrophages (Majeed, Cavegion et al. 2001), we found that the Src kinases were only partially involved in Dectin-1 mediated uptake, and were not essential, as complete inhibition of uptake was not obtained with the Src-kinase inhibitor PP2. This suggests that other kinase(s) are involved in the phosphorylation of Dectin-1.

In FcγR, Syk is recruited to the phosphorylated ITAMs and is essential for subsequent ITAM mediated signalling events in myeloid cells (Turner, Schweighoffer et al. 2000; Strzelecka-Kiliszek, Kwiatkowska et al. 2002). Strikingly, in macrophages, Dectin-1 mediated phagocytosis did not require Syk, a finding which explains previous

observations on the nature of zymosan uptake by macrophages deficient in this kinase (Crowley, Costello et al. 1997; Kiefer, Brumell et al. 1998). Although only the membrane proximal tyrosine was required for Dectin-1 mediated uptake, whereas both tyrosines are optimally required for Fc γ R mediated phagocytosis, this complete lack of dependence on Syk is surprising, especially as some Fc γ R mediated internalisation can occur in a Syk dependent fashion, with imperfect ITAM sequences (Mitchell, Huang et al. 1994; Indik, Park et al. 1995). However, this does imply that the motif in the cytoplasmic tail of Dectin-1 does not function as a classic ITAM in macrophages. Although uptake in the transduced NIH3T3 fibroblasts was blocked with piceatannol, suggesting a role for Syk in these cells, this inhibitory effect may have been due to non-specific inhibition of other unrelated kinases (Law, Nannizzi-Alaimo et al. 1999). Overall, these findings suggest that in macrophages the initiating events leading to Dectin-1 mediated phagocytosis are likely to involve novel signalling pathway(s) and /or molecules.

We have also examined the contribution of other enzymes known to be involved in receptor-mediated phagocytosis, including PI-3 kinase, PKC and the Rho GTPases. PI-3 kinase is a lipid kinase which is involved in a variety of signalling pathways and is required for Fc γ R and CR mediated phagocytosis in macrophages (Araki, Johnson et al. 1996; Cox, Dale et al. 2001). In Fc γ R mediated uptake, PI-3 kinase has also been shown to be required for pseudopod extension and phagosomal closure (Araki, Johnson et al. 1996; Cox, Tseng et al. 1999). Although inhibition of PI-3 kinase affected Dectin-1 mediated particle internalisation, it did not completely block this process, suggesting that this enzyme was not essential for phagocytosis by this receptor. As wortmannin sensitivity is normally relieved by smaller particles (Cox, Tseng et al. 1999), and as

SRBC are, if anything, slightly smaller than zymosan particles, the effect we observed is unlikely to be due to differences in particle size. This may be a unique feature of Dectin-1 in macrophages, as PI-3 kinase-independent uptake has only been shown previously with Fc γ receptors in monocytes (Garcia-Garcia and Rosales 2002). PI-3 kinase has also recently been implicated in β -glucan mediated immunomodulation *in vivo* (Williams, Li et al. 2004), but the role of Dectin-1 in this response has yet to be established.

PKC comprises a large family of serine/threonine kinases that have been shown to be required for phagocytosis by a number of receptors, including both CR and Fc γ R (Allen and Aderem 1996). PKC is also required by Dectin-1, as phagocytosis by this receptor was blocked by the PKC inhibitor, chelerythrine, consistent with previous reports that this enzyme is required for zymosan uptake by macrophages (Allen and Aderem 1995). Although the exact role of PKC in phagocytosis is unknown, one of its substrates, MARCKS (Myristoylated, Alanine-rich C-Kinase Substrate), possesses actin cross-linking activity that has been shown to be involved in zymosan phagocytosis (Allen and Aderem 1995). Furthermore, as MARCKS-deficient macrophages appear to have partial defects in zymosan phagocytosis only, MARCKS may be specifically induced by Dectin-1 (Carballo, Pitterle et al. 1999). It is also tempting to speculate that PKC is involved in the Dectin-1 mediated induction of the NAPDH oxidase (Gantner, Simmons et al. 2003), as has been demonstrated for complement-opsonised zymosan in neutrophils (Sergeant and McPhail 1997).

The Rho-GTPases are important regulators of the actin cytoskeleton and are involved in many cellular processes, including phagocytosis (Hall 1998). Two distinct

internalisation pathways have been described, that are controlled by different Rho GTPases and which depend on the surface receptor engaged; Type I phagocytosis, which requires Cdc42 and Rac-1 and is utilised by the Fc γ R, and Type II phagocytosis, which requires Rho and is utilised by CR(Caron and Hall 1998). We were able to show that Dectin-1 mediated actin re-organisation is dependent on these GTPases (**fig. 3.8f**), and that the receptor mediates Type I phagocytosis (Caron and Hall 1998) in NIH3T3 cells, requiring Rac-1 and Cdc-42, but not Rho-A. Notably, the Rho GTPase inhibitor, Toxin B, did not completely block Dectin-1 mediated uptake in RAW macrophages when compared to Fc γ R, which may suggest that other mechanisms are also involved in these cells.

To conclude, it seems that it is not ideal to study membrane receptor signalling out of its cellular context as a given receptor may recruit cell-lineage specific mediators to achieve the same outcome in different models. Dectin-1 mediated mechanisms of cytoskeletal activation, whilst dependent on tyrosine phosphorylation of an ITAM-like motif, differ from those attributable to Fc γ Rs, most notably by the lack of requirement of Syk. These observations illustrate the complexity of the phagocytic process when initiated by different macrophage receptors.

Chapter 5

Dectin-1 mediates the biological effects of β -glucans

(In collaboration with G.D.Brown (Brown, Herre et al. 2003))

5.1 Introduction

β -glucans possess anti-inflammatory and anti-tumorigenic properties (Czop 1986; Williams 1997; Ross, Vetvicka et al. 1999) that stem from their ability to activate leukocytes by stimulating their phagocytic activity and their production of reactive oxygen intermediates, inflammatory mediators and cytokines, including TNF- α (Czop 1986; Williams, Mueller et al. 1996). The activatory properties of these fungal-derived carbohydrates, especially particles such as β -glucan rich zymosan (Riggi and Di Luzio 1961), have been widely used to examine the pro-inflammatory responses of phagocytes. Despite almost half a century of investigation (Benacerraf and Sebestyen 1957), however, the receptor or receptors mediating these effects are still undefined. What has been demonstrated is that pro-inflammatory responses to zymosan particles are dependent on the Toll-like receptors (TLRs) (Underhill, Ozinsky et al. 1999; Underhill 2003). Toll like receptors, however have not been shown to directly recognise zymosan particles, making it possible that other receptors co-operate with TLRs to mediate pro-inflammatory responses to fungal derived particles.

As discussed in Chapters 1 and 3, Dectin-1, which was isolated based on its ability to recognise zymosan particles (Brown and Gordon 2001), is a leukocyte expressed receptor for fungal derived β -glucans and a major receptor for zymosan and other β -glucans on macrophages (Brown, Taylor et al. 2002). Dectin-1 encodes an ITAM-like motif in its cytoplasmic tail that is responsible for mediating phagocytic uptake of zymosan particles (Chapters 3 and 4). Thus Dectin-1 recognises zymosan particles and

TLRs mediate pro-inflammatory signalling. This led us to wonder whether Dectin-1 contributed to the cellular responses induced by these carbohydrates.

5.2 Aims of the study

The aims of this study were fourfold: firstly we wanted to establish whether macrophage pro-inflammatory responses to zymosan particles and live yeast pathogens could be mediated by Dectin-1; secondly we wanted to define whether zymosan-induced pro-inflammatory signalling required particle phagocytosis or could be mediated from the site of recognition, the cell surface; thirdly we wanted to establish whether the Dectin-1 cytoplasmic ITAM-like motif played any role in mediating zymosan-induced pro-inflammatory signalling; fourthly we wanted to establish whether Dectin-1 interacted with the Toll-like receptor (TLR) pathway known to be essential for zymosan induced pro-inflammatory signals.

5.3 Results

5.3.1 Zymosan-induced TNF- α production by macrophages is β -glucan dependent

Thioglycollate elicited macrophages (Mphi) have previously been shown to produce TNF- α in response to zymosan particles (Stein and Gordon 1991). To explore the possibility that Dectin-1 was involved in mediating pro-inflammatory responses to zymosan particles we examined the effects of soluble β -glucan inhibitors of Dectin-1 on zymosan induced TNF- α production in primary macrophages. We exposed triplicate wells containing 2.5×10^5 Mphi to soluble β -glucans at 4°C prior to adding an excess of zymosan particles and incubating for 30 minutes at 37°C. Thereafter cells were washed three times, medium was replaced and plates were incubated at 37°C for three hours prior to quantitation of soluble TNF in the supernatants (**fig. 5.3.1**). Glucan phosphate

Zymosan induced cytokine production by Macrophages

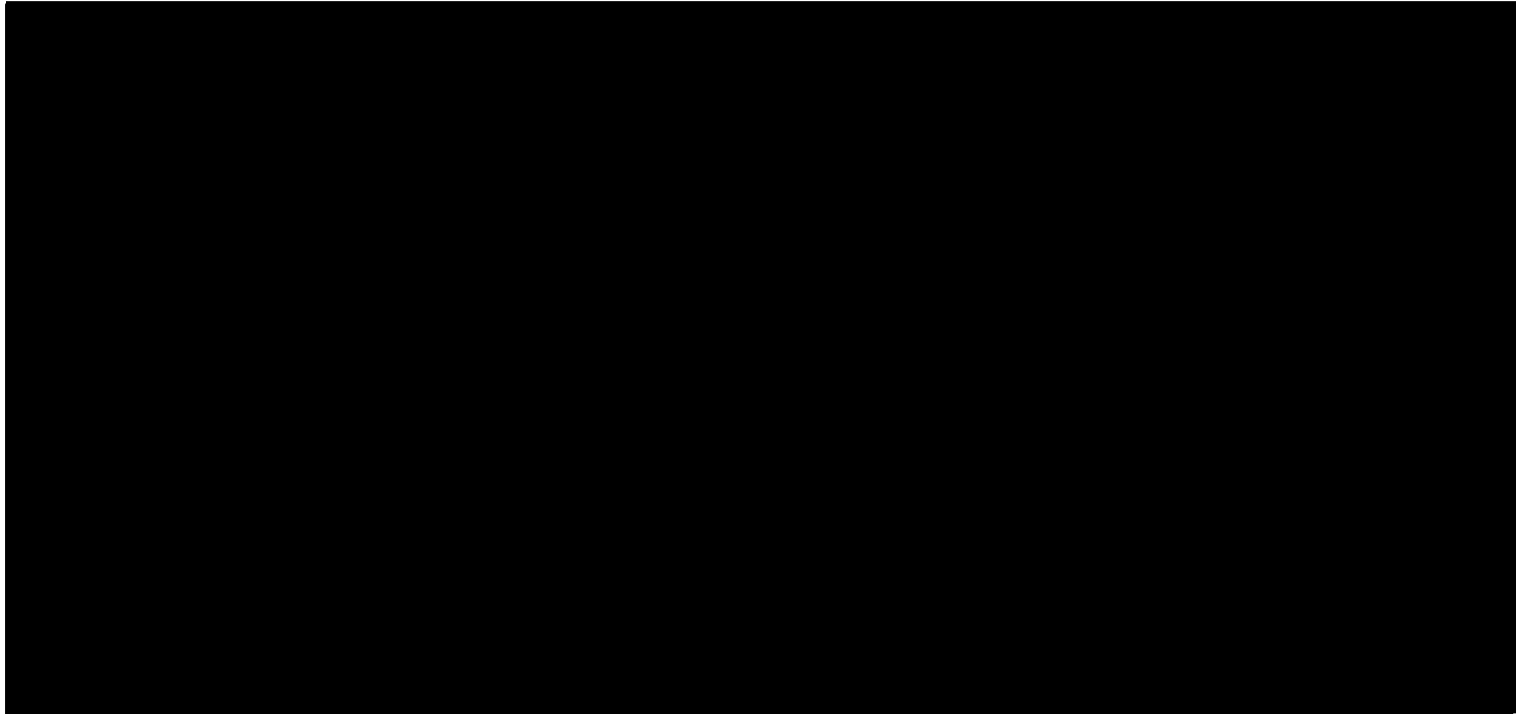


Figure 5.3.1

Effects of the soluble Dectin-1 inhibitor, glucan phosphate, on zymosan induced TNF α production by thioglycollate elicited macrophages. In a 3 hour assay zymosan induces high levels of soluble TNF- α production in these primary inflammatory macrophages, and this production can be abolished by 100 μ g/ml of glucan phosphate. LPS induced TNF- α production is unaffected. (Brown, Herre et al 2003)

abolished zymosan induced TNF- α production yet had no effect on LPS induced TNF- α production. These data suggest that zymosan induced TNF production in these primary macrophages is a β -glucan dependent event.

5.3.2 Zymosan-induced TNF- α production increases with Dectin-1 expression

To establish whether Dectin-1 played a role in the cellular response to zymosan we used the Dectin-1 overexpressing RAW 264.7 macrophage cell line RAW-D1 described earlier. As was shown in **figs. 4.3.2c and 5.3.2a** the levels of zymosan binding are markedly enhanced by the increased Dectin-1 expression. When we compared RAW-D1 cells to empty vector transduced RAW 264.7 cells (RAW-FB) (**fig. 5.3.2b**) we found that TNF- α production increased in both populations as we increased the particle to cell ratio, but that higher levels of Dectin-1 expression markedly enhanced TNF- α production at any given particle to cell ratio tested. Levels of secreted TNF- α in the RAW-D1 population appeared to be approaching saturation at a ratio of 50 particles per cell in this three-hour assay.

5.3.3 Dectin-1 also mediates TNF α production in response to live yeast

In addition to recognising zymosan particles Dectin-1 can recognise intact yeast particles (Brown and Gordon 2001). As successful control of many fungal pathogens depends on TNF- α , we wanted to establish whether Dectin-1 also mediated pro-inflammatory responses to live fungi. We examined TNF- α production in response to intact yeast and yeast pathogens in RAW-D1 and RAW-FB cell lines. When live *C.albicans* and *S.cerivisiae* were added to RAW-D1 and RAW-FB monolayers (**fig. 5.3.3**) we found that both fungi induced TNF- α production. This production could be inhibited using glucan phosphate implying that it was β -glucan dependent.



Figure 5.3.2a-b

Dectin-1 over-expression enhances zymosan induced TNF- α production.
RAW-D1 macrophages (\blacktriangle) both bind (a) and produce (b) substantially higher levels of TNF- α than RAW-FB cells (\blacksquare) at the same particle to cell ratio.
(Brown, Herre et al. 2003)

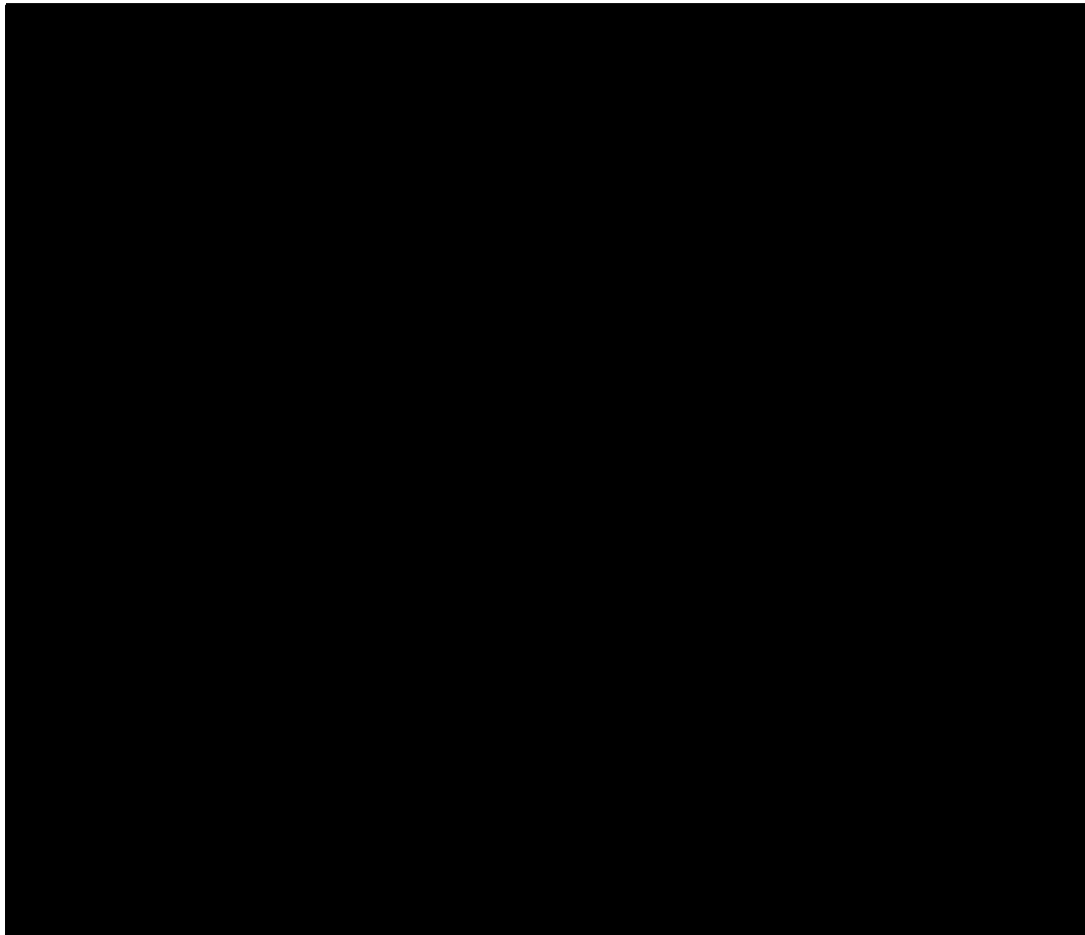


Figure 5.3.3

Dectin-1 induces the production of TNF- α in response to live yeasts. RAW-D1 cells show an increased TNF- α response to (a) live *C.albicans* and (b) *S.cerevisiae* when incubated at a yeast:cell ratio of 5:1. These responses are β -glucan dependent and can be abolished with glucan phosphate. *S.cerevisiae* induces a significantly greater β -glucan dependent TNF- α response than *C.albicans*. (Brown, Herre et al. 2003)

Interestingly, the non-pathogenic *S.cerevisiae* induced higher levels of TNF- α than *C.albicans*.

5.3.4 Zymosan induced TNF- α production depends on the TLR pathway

TLRs are thought to sample the contents of phagosomes, triggering pathogen appropriate responses, and have been shown to be involved in the recognition of fungal pathogens (Netea, Van Der Graaf et al. 2002), zymosan (Underhill, Ozinsky et al. 1999) and other β -glucans (Kataoka, Muta et al. 2002). When we measured zymosan- and LPS-induced TNF- α production in bone marrow derived macrophages from WT control, MyD88 deficient and TLR-2 deficient mice (**fig. 5.3.4a**) we found that zymosan induced TNF- α production depended on both TLR-2 and MyD88 whereas LPS induced TNF- α production depended only on MyD88 (see also **fig. 1.3.2**). LPS, which is recognised by TLR4, induces normal levels of TNF- α in the TLR-2 deficient macrophages. These effects were not due to an impaired ability of macrophages to internalise zymosan particles as neither TLR2 nor Myd88 deficiency had any effect on the phagocytic uptake of zymosan particles (**fig. 5.3.4b**).

5.3.5 Dectin-1 and TLR2/6 co-localise in nascent phagosomes

It has previously been shown that TLR-2 and TLR-6 are recruited to zymosan phagosomes (Underhill, Ozinsky et al. 1999; Ozinsky, Underhill et al. 2000). As we had found that Dectin-1 was present in nascent zymosan phagosomes (**see fig. 6.3.3b**), we were interested in establishing whether, in macrophages, these two receptors co-localised. Epitope tagged TLR2 and TLR6 were transfected into RAW-D1 cells by electroporation, and receptor expression was verified by FACS analysis (data not shown). Transfected cells were seeded onto coverslips overnight before brief,

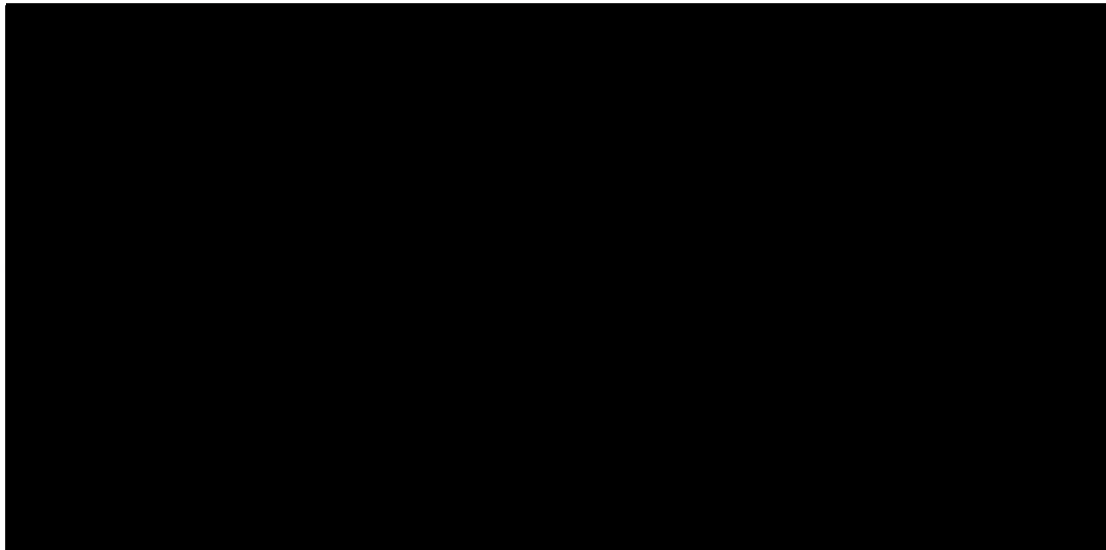


Figure 5.3.4a

TLRs are required for TNF- α production in response to zymosan. BMDM from TLR-2 and MyD88 deficient mice do not produce TNF- α after overnight incubation with unlabelled zymosan. The response to LPS is shown as a control. (Brown, Herre et al. 2003)

TLR-2 or MyD88 deficiency does not impair zymosan phagocytosis

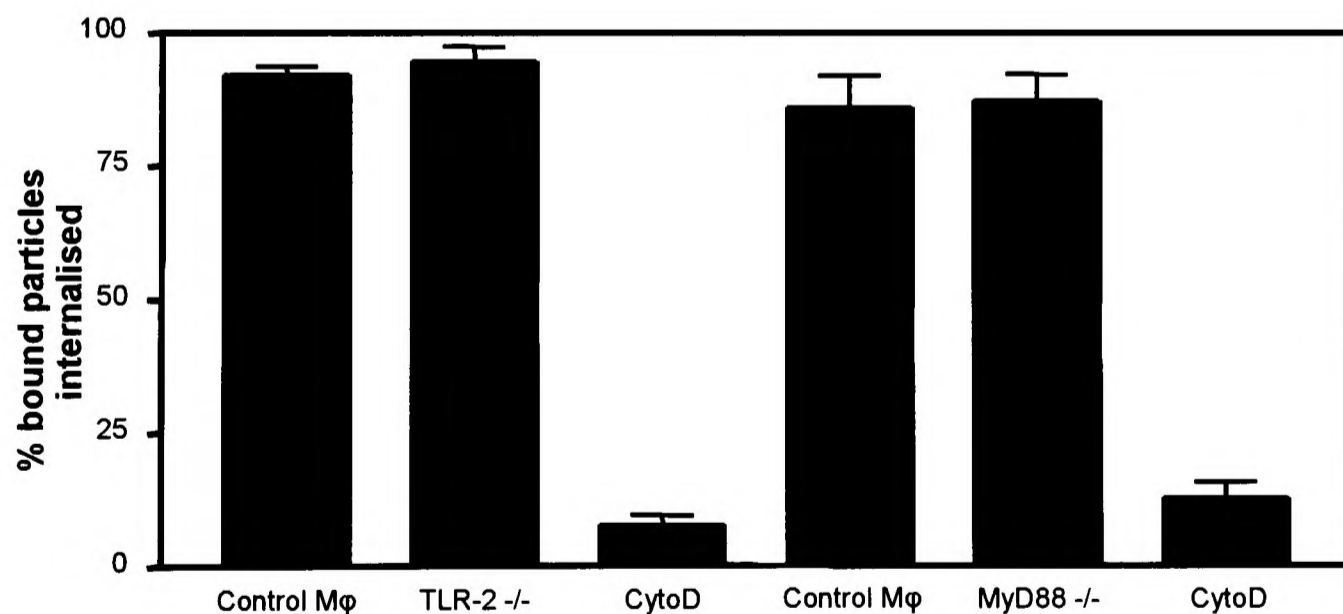


Figure 5.3.4b

TLRs play no role in the phagocytic uptake of zymosan particles. TLR-2 and MyD88 deficient BMDMs show phagocytic capacities equivalent to control macrophages.

synchronised phagocytic uptake of zymosan particles. The confocal images (**fig. 5.3.5a-d**) show that in transfected cells both Dectin-1 and TLR-2 are recruited to nascent zymosan phagosomes. Similarly, TLR-6 also localises to zymosan phagosomes with Dectin-1 (**fig. 5.3.5e-h**). The proximity of the receptors suggests that interactions between them might be possible.

5.3.6 Increased availability of TLR2 ligand does not increase TNF- α production

We showed in **fig. 5.3.2a-b** that increases in Dectin-1 expression lead to markedly increased zymosan particle binding and that this was associated with increased TNF- α production. We were concerned that the increased TNF- α we had observed might have been due to increased availability of zymosan particles to the TLR system rather than because Dectin-1 played a role in pro-inflammatory signalling. To clarify this concern we compared TNF- α production in response to unopsonised and complement opsonised zymosan particles in RAW-FB and RAW-D1 cell lines. We found that, although opsonisation markedly increased zymosan particle binding to RAW-FB cells (**fig. 5.3.6a**), and this binding was β -glucan independent, TNF- α production was only marginally increased (**fig. 5.3.6b**). In the RAW-D1 cells we found that opsonised zymosan particles still produced enhanced levels of TNF- α in a β -glucan dependent fashion. These findings confirm that, rather than the increased availability of zymosan to the TLRs, the high levels of Dectin-1 are responsible for the increased pro-inflammatory cytokine production.

5.3.7 The cytoplasmic tail of Dectin-1 is essential for zymosan induced TLR activation

The data above suggests that Dectin-1 contributes to the TNF- α response to zymosan in macrophages. We showed in chapters 3 and 4 that the ITAM-like motif present in the

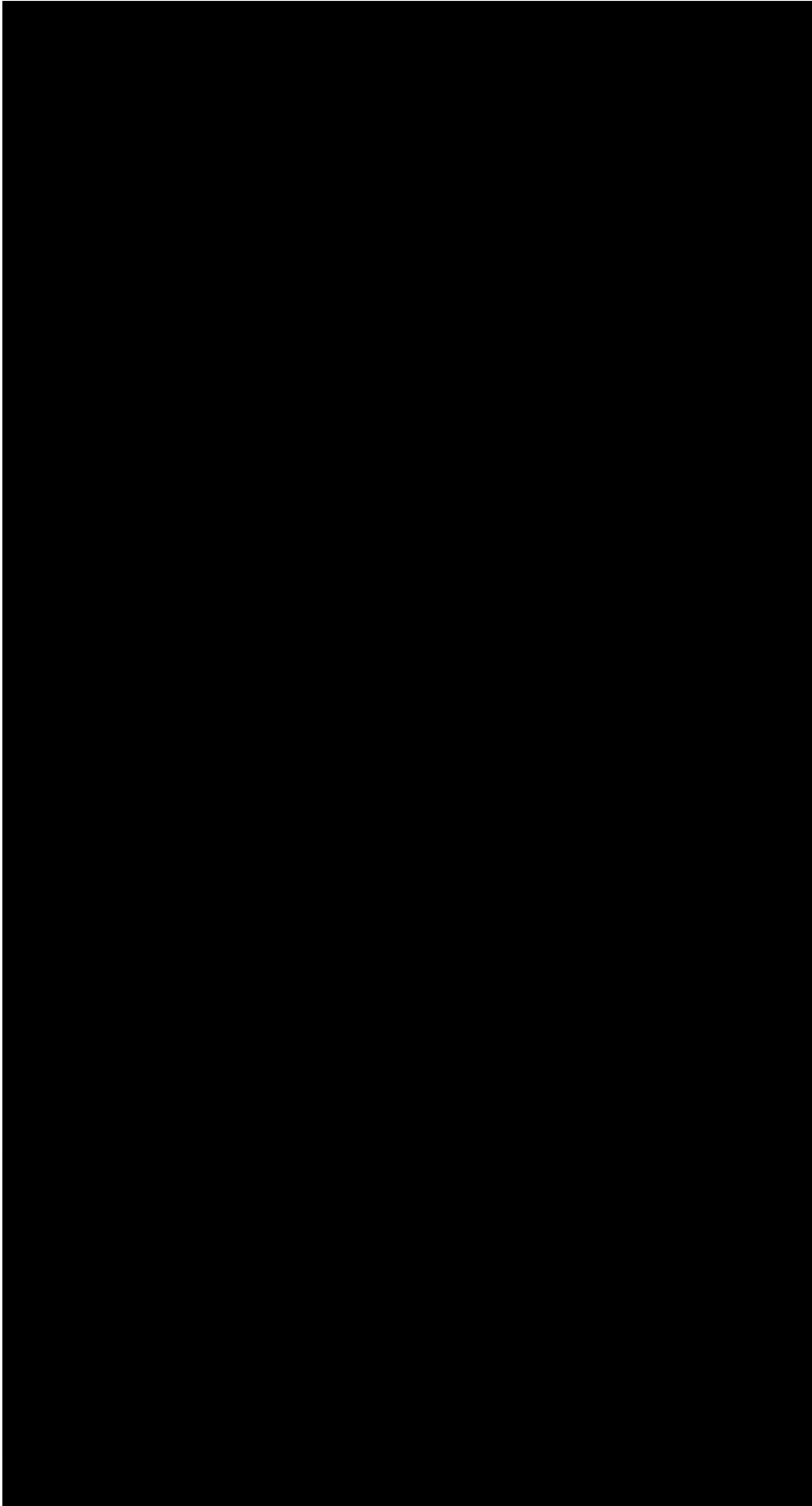


Figure 5.3.5b-i

Dectin-1 and TLR-2 and 6 co-localise in nascent zymosan phagosomes. V5-tagged TLR-2 and 6 (shown in red) and HA-tagged Dectin-1 (green) co-localise within 5 minutes of initiating zymosan uptake. a) Transmission image of two adjacent cells binding zymosan particles. b) Dectin-1 HA staining. c) TLR-2 V5 staining. d) staining showing co-localisation. The cell indicated with the arrow was not transfected with TLR-2 and is presented as a staining control. e) Transmission image of two adjacent RAW-D1 macrophages f) Dectin-1 is present in nascent phagosomes. g) TLR-6 is also present in nascent phagosomes. h) Merge shows that TLR-6 and Dectin-1 co-localise. (Brown, Herre et al. 2003)

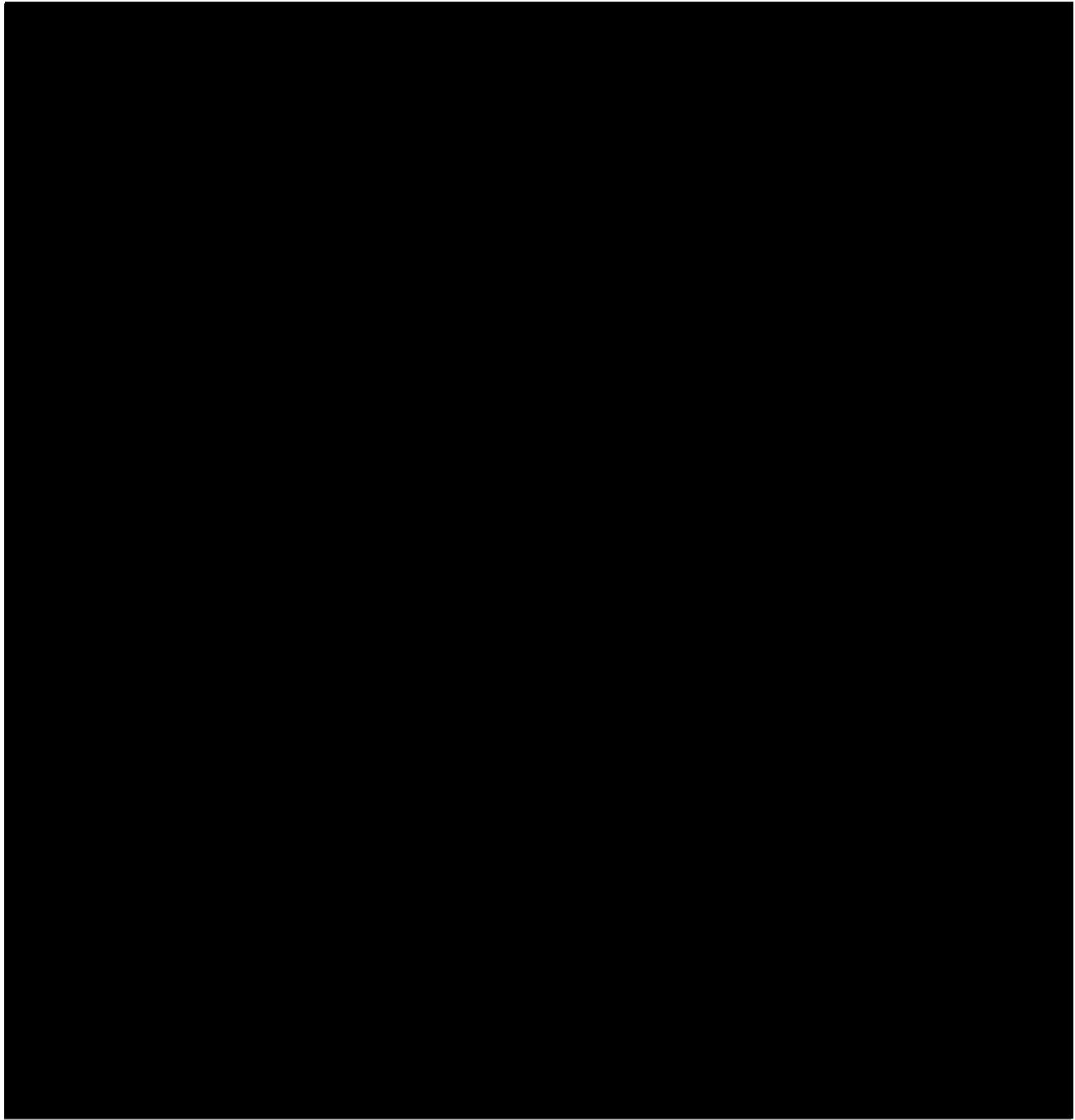


Figure 5.3.6 a-b

The β -glucan dependent TNF α response to zymosan particles is mediated by Dectin-1. a) Complement opsonisation markedly increases binding of zymosan particles to RAW-FB cells, and this binding is β -glucan independent. b) Despite enhanced zymosan availability to RAW-FB cells, there is only a marginal increase in TNF- α production. RAW-D1 cells show enhanced TNF- α responses irrespective of opsonisation status of the zymosan particles. (Brown, Herre et al. 2003)

cytoplasmic tail of Dectin-1 was responsible for phagocytic uptake of zymosan particles. We now wanted to establish whether this motif was also responsible for zymosan induced TNF- α production. When we measured zymosan induced TNF- α production in RAW-FB, RAW-D1, RAW-ITAM Y1Y2 and RAW-Truncation cell lines (**fig. 5.3.7**) we found that the RAW-D1 cell line produced markedly increased levels of TNF- α compared to the RAW-FB vector control, as previously shown (**fig. 5.3.2**) and this increase could be abrogated by glucan phosphate (**fig. 5.3.7**). More importantly, neither the Y1Y2 nor the Truncation mutants produced these elevated levels of cytokine despite binding zymosan particles equally well (**see fig. 4.3.2c**). These data strongly suggest that, in macrophages, TLR-2 mediated TNF- α production in response to zymosan particles depends on both β -glucans and the ITAM-like motif of Dectin-1.

5.3.8 Dectin-1 and TLR mediated TNF- α production does not depend on phagocytosis

The Y1Y2 and Truncation mutant Dectin-1 receptors recognise zymosan particles as well as full-length Dectin-1, but fail to induce phagocytosis (**figs. 4.3.2e & g**). We were concerned that the reason that no TNF production was induced in these cells was due to the lack of phagocytic uptake rather than any defect in signals emanating from the cytoplasmic tails. We thus inhibited phagocytic uptake with the use of cytochalasin D (that has no effect on zymosan particle recognition **fig. 4.3.2c**), and measured zymosan-induced TNF- α production in both RAW-FB and RAW-D1 cell lines (**fig. 5.3.8**). We found that rather than decreasing TNF- α production, cytochalasin D markedly elevated production in both cell lines, providing strong evidence that the TLR mediated pro-inflammatory signals originate at the cell surface.

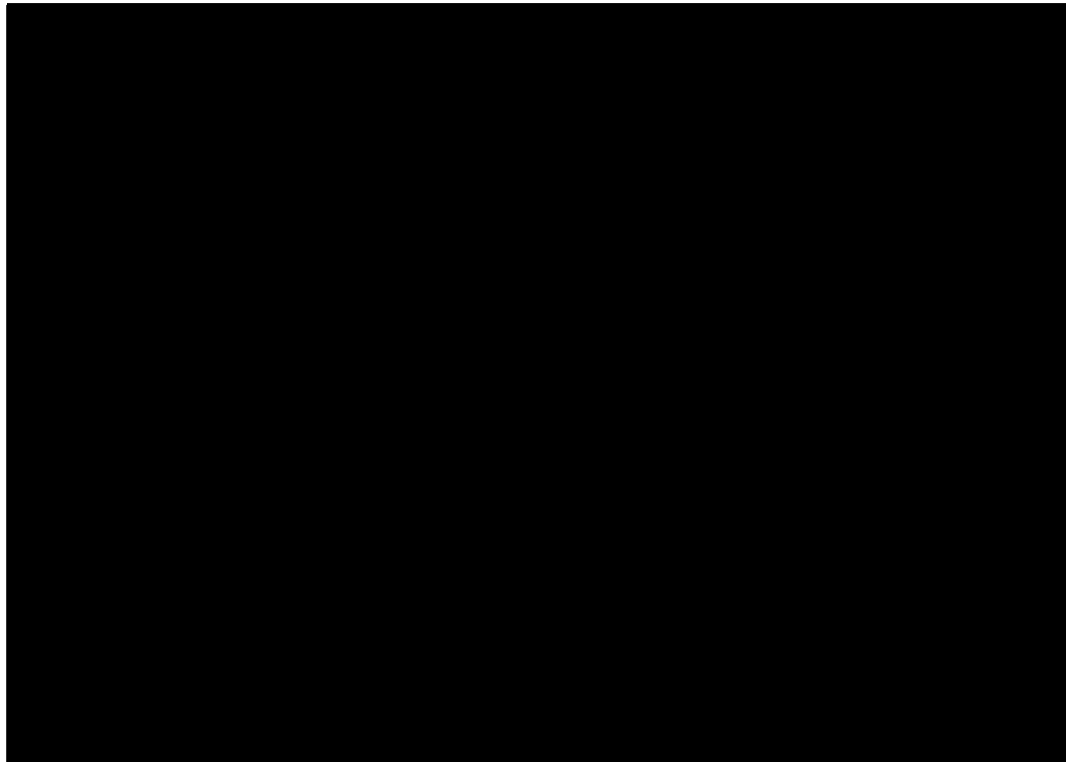


Figure 5.3.7

The cytoplasmic tail of Dectin-1 is involved in mediating the TNF- α response to zymosan. Cell lines expressing Dectin-1 molecules with cytoplasmic tail mutations do not transduce an intracellular signal inducing the TNF- α response to this particle. Cytokine responses can be abrogated by glucan phosphate. (Brown, Herre et al. 2003)

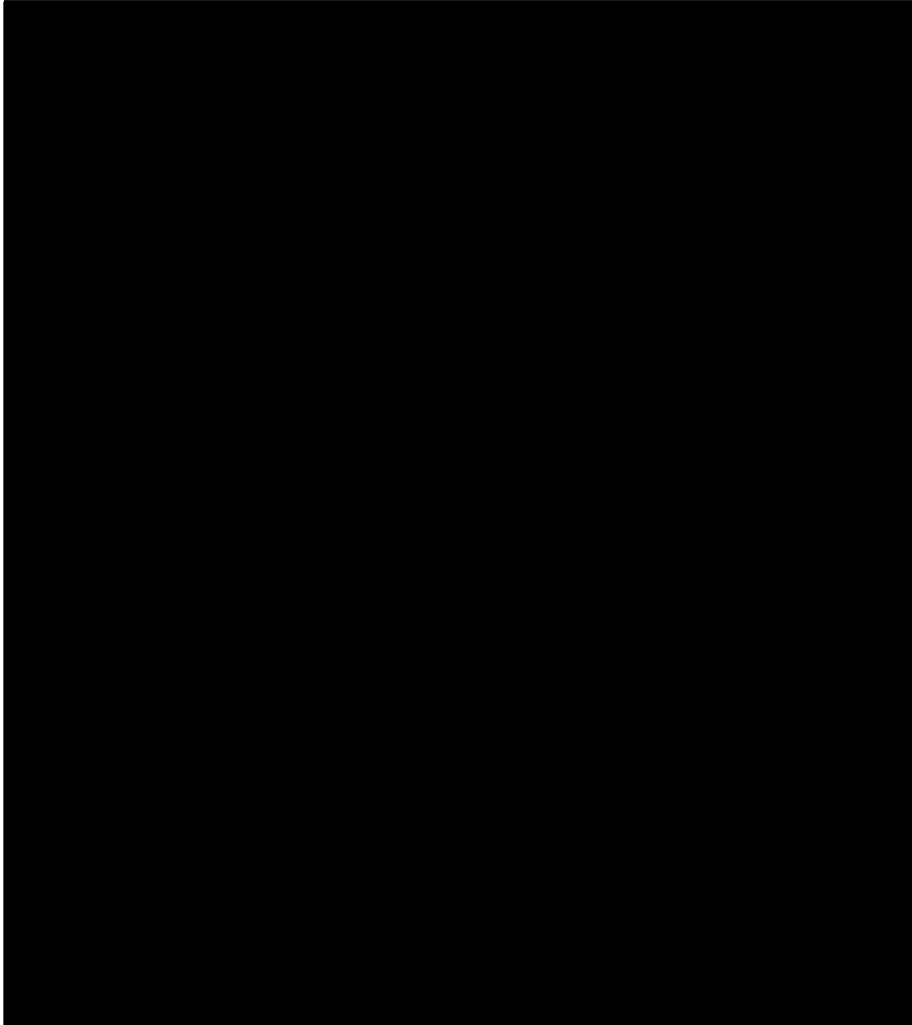


Figure 5.3.8

Phagocytosis is not required for zymosan induced TNF- α production. When the phagocytosis of zymosan particles is inhibited using 5 μ M cytochalasin D, TNF production is markedly increased, and this increase is β -glucan dependent. (Brown, Herre et al. 2003)

5.3.9 Kinases and GTPases responsible for Dectin-1 mediated cytoskeletal rearrangements play no role in zymosan mediated TNF- α production

We have shown that both Dectin-1 mediated zymosan phagocytosis and zymosan induced TNF- α production depend on the cytoplasmic ITAM-like motif. We have also shown that a variety of kinases and GTPases play a role in transmitting signals from the ITAM-like motif to the actin cytoskeleton. We were thus interested to establish whether any of these signalling intermediaries were responsible for linking Dectin-1 ITAM-like motif signalling to the TLRs and TNF- α production. Thioglycollate elicited macrophages were plated overnight and then incubated with various inhibitors, as described earlier (**fig. 4.3.4a**). As described previously, cells were pulsed with zymosan particles for 30 minutes at 37°C, washed and then incubated for three hours with fresh medium before supernatants were assayed for TNF- α (**fig. 5.3.9a**). We found that, as had been the case with RAW-D1 macrophages (**fig. 5.3.8**), cytochalasin D markedly elevated TNF- α production, and glucan phosphate inhibited it. Wortmannin and Toxin B also elevated TNF- α production markedly, suggesting that inhibition of phagocytosis in general has this effect. Unfortunately, although PP1-3 and piceatannol reduced TNF- α production, these results cannot be interpreted as both of these inhibitors also non-specifically inhibit I κ B phosphorylation and thus NF κ B mediated TNF- α production. We thus asked whether, in Syk deficient bone marrow derived macrophages, zymosan was still able to induce TNF- α production. In response to maximal stimulation by LPS, Syk deficient macrophages produce about a third less TNF- α than wild type controls, yet when TNF production was adjusted to LPS induced levels, we found that TNF- α production was β -glucan dependent and that the absence of Syk led to only a small reduction in zymosan induced TNF- α production (**Fig. 5.3.9b**). These data suggest that Syk is not absolutely required for zymosan induced pro-inflammatory cytokine production. Since there are currently no macrophages available that are deficient in all

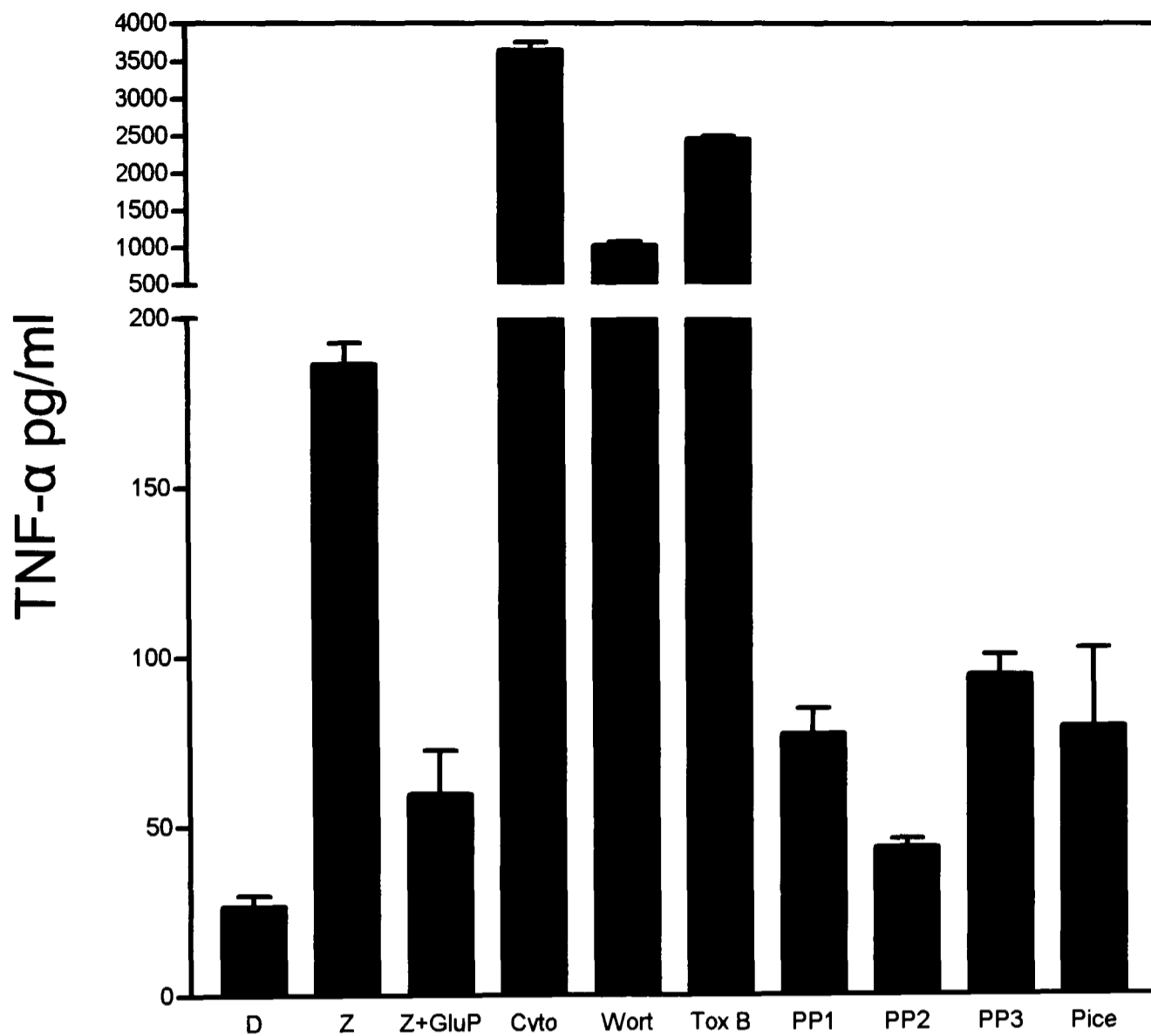


Figure 5.3.9a

Kinases and GTPases responsible for Dectin-1 mediated phagocytosis of zymosan particles are not involved in pro-inflammatory signal induction. In thioglycollate elicited macrophages, inhibitors that interfere with particle internalisation result in large increases in zymosan induced TNF- α secretion. PP1 and PP2 despite interfering with phagocytic uptake as well as piceatannol inhibit NF κ B translocation to the nucleus and thus the reduced TNF- α production is a non-specific effect.

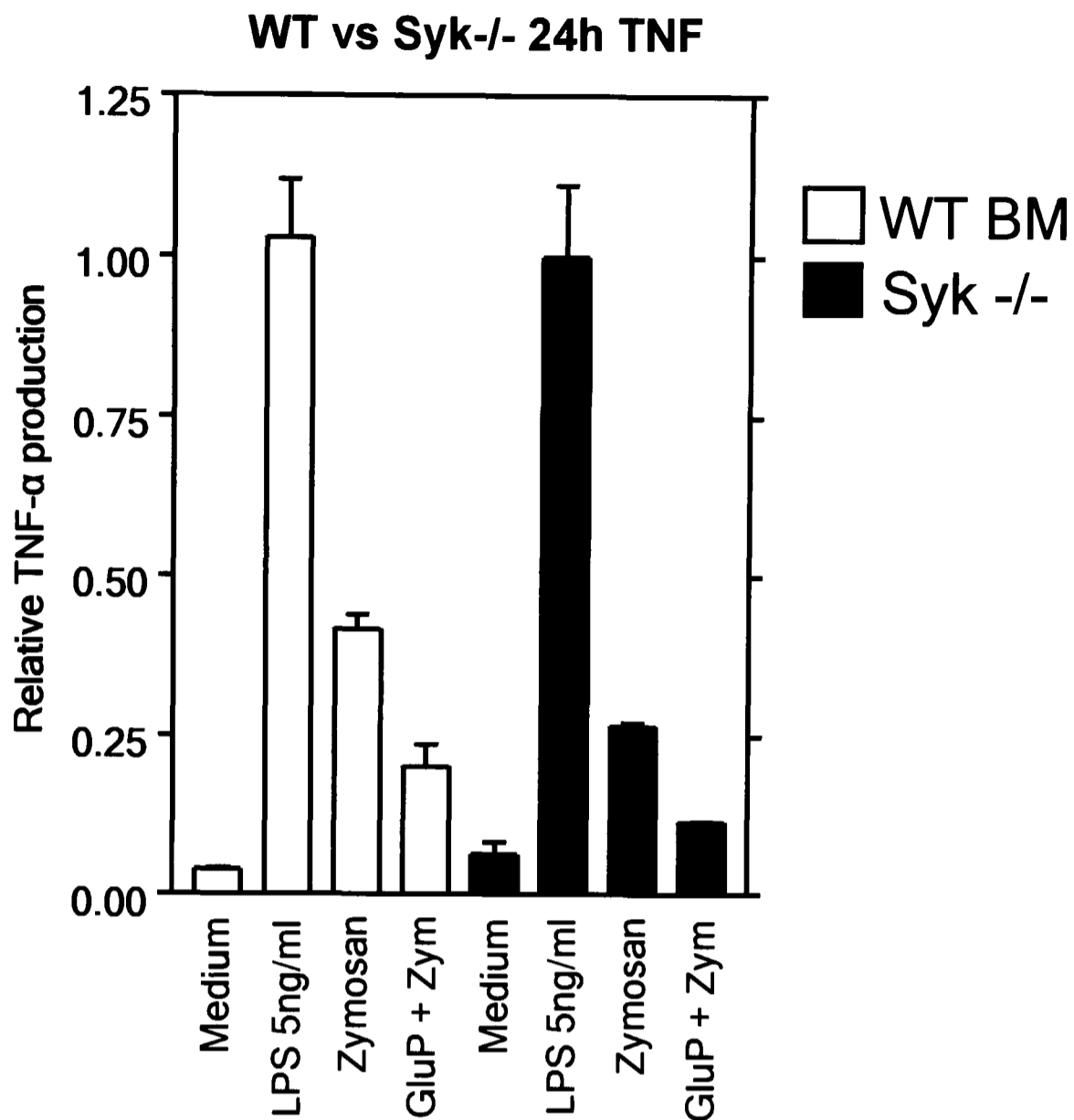


Figure 5.3.9b

Zymosan induced TNF- α production in Syk deficient BMDMs is unaffected. Syk deficient macrophages show a similar pattern of zymosan induced, β -glucan-dependent TNF- α production when compared to wild type controls. TNF- α levels are shown normalised to maximal LPS induced production for comparison between the two macrophage populations.

6 leukocyte expressed Src family kinases (Majeed, Cavegion et al. 2001), this remains an open question.

5.4 Discussion

In this study we define Dectin-1, through its capacity to recognise β -glucans, as an important partner for TLR2 for the production of inflammatory cytokines in macrophages. We demonstrate that both live yeast and yeast-derived particles induce TNF- α production by primary macrophages in a β -glucan and TLR dependent fashion and that cytokine production does not depend on either the phagocytic uptake of particles or on several mediators that play a role in Dectin-1 mediated phagocytosis of zymosan particles. These data provide the first description of a receptor involved in generating a pro-inflammatory response to fungal pathogens, and the collaboration between Dectin-1 and TLR-2 in orchestrating innate immune responses provides a valuable model for elucidating the mechanisms by which multiple innate immune receptors interact during microbial recognition.

It is well established that zymosan particles, that are rich in β -glucans, induce TNF- α production in macrophages (Stein and Gordon 1991; Okazaki, Adachi et al. 1995; Sakashita, Hiyama et al. 2000). It has, however, traditionally been thought that β -glucans found in the cell walls of live fungi are buried and not available for immune recognition (see **fig. 1.7.1c**). We now show that β -glucans are available for recognition on the surface of live yeast. Furthermore, the non-pathogen *S.cerevisiae* is more efficiently recognised and induces higher levels of TNF- α production than the pathogen *C.albicans*, making it possible that fungal pathogens mask their cell wall β -glucan to

avoid immune recognition. These findings suggest that the recognition of β -glucans plays a role in anti-fungal immunity.

We demonstrate that Dectin-1 plays a role in zymosan induced TNF- α production by showing that, in addition to enhancing particle binding to macrophages, increased Dectin-1 expression also enhances TNF- α production in response to these particles. The increases in TNF- α levels are not the product of increased availability of zymosan particles to TLRs. We found that complement opsonised zymosan particles, despite binding in large numbers to RAW-FB and RAW-D1 macrophages, induced only marginal increases in TNF- α production when Dectin-1 recognition was blocked with glucan phosphate. Increased TNF- α production is thus the direct result of higher levels of Dectin-1 expression, implying that its ligation influences anti-fungal responses in macrophages.

Although the recognition and phagocytosis of zymosan particles is independent of the Toll-like receptors (Underhill, Ozinsky et al. 1999; Aderem and Ulevitch 2000; Ozinsky, Underhill et al. 2000; Akira, Takeda et al. 2001; Medzhitov 2001), TLR-2 has been shown to be required for zymosan mediated TNF- α production and anti-fungal defences (Del Sero, Mencacci et al. 1999; Underhill, Ozinsky et al. 1999; Wang, Warris et al. 2001; Bozza, Gaziano et al. 2002). We confirmed that TLR-2 and MyD88 deficient macrophages do not produce TNF in response to zymosan particles. Furthermore we and others (Gantner, Simmons et al. 2003) demonstrate that Dectin-1 co-localises, in the presence or absence of cytochalasin D, with TLR2 in nascent zymosan phagosomes. These observations led us to the hypothesis that Dectin-1 and TLR2 may co-operate to induce TNF- α production. We found this to be the case when

we studied cells that over-express recombinant Dectin-1 with ITAM mutations or cytoplasmic tail truncation, and observed that these cell lines failed to produce TNF- α in response to zymosan despite high levels of particle binding. These findings are supported by the findings that zymosan particle recognition resulted in Dectin-1 tyrosine phosphorylation in a TLR-2 independent fashion and, despite no evidence of any direct interaction between these two receptors, co-expression of Dectin-1 and TLR-2 is required for zymosan induced NF κ B activation (Gantner, Simmons et al. 2003). Furthermore, these authors also show that following ligation by zymosan particles, Dectin-1 co-operates with TLR-2 in the production of another cytokine, IL-12.

It thus appears likely that the cytoplasmic ITAM-like motif of Dectin-1 mediates both phagocytic uptake and pro-inflammatory signalling in a manner analogous to Fc γ Rs (Daeron 1997). As the cytoplasmic tail mutant cell lines are defective in phagocytosis (chapter 4), we needed to ensure that phagocytic uptake of zymosan particles was not required for the production of TNF- α . We showed that when cytochalasin D is used to inhibit the phagocytic uptake of zymosan particles, rather than reduce TNF- α production, instead a very large increase is seen. Importantly, these observations confirm that zymosan mediated induction of TNF- α in macrophages occurs at the cell surface and that retention of ligated receptor at the cell surface enhances pro-inflammatory signalling. These observations also suggests that receptor internalisation may be part of the mechanism of switching off Dectin-1 mediated inflammatory signalling (this will be addressed further in chapter 6). The implications of these findings are that co-operation between Dectin-1 and the TLRs occurs within the complex of receptors assembled following zymosan recognition. Not all anti-fungal defences, however, depend on a collaboration between TLR-2 and Dectin-1, and others

have shown (Gantner, Simmons et al. 2003) that Dectin-1 mediates the production of reactive oxygen intermediates (ROIs) following zymosan phagocytosis in the absence of TLR-2 or MyD88. Interestingly, this zymosan induced ROI production could be enhanced by first priming cells with soluble ligands for either TLR-2 and TLR-4, suggesting wider interactions between Dectin-1 and the TLR system (Gantner, Simmons et al. 2003).

Although Dectin-1 is the first innate immune PRR so far demonstrated to signal through an ITAM-like motif, classical ITAMs are found in many important immune receptors such as Fc γ , T-cell, B-cell and NK activatory receptors. Co-operation between ITAM-containing receptors and the TLRs has been suggested in some previous studies (Sutterwala, Noel et al. 1997; Gerber and Mosser 2001; Leadbetter, Rifkin et al. 2002) implying that functional co-operation between TLRs and immune receptors may be a more general mechanism of cellular activation. Again Dectin -1 appears to share characteristics with Fc γ Rs, and this similarity relies on the ITAM-like motif. We suggested in chapter 4 that phagocytosis mediated by the ITAM-like motif of Dectin-1 showed significant differences to the ITAMs of Fc γ Rs. Are there thus also differences between these two receptors with regard to pro-inflammatory cytokine production? As mentioned earlier, others recently showed that, in addition to TNF- α , Dectin-1 signalling also potentiates IL-12 production by TLR-2 stimuli in both macrophages and dendritic cells (Gantner, Simmons et al. 2003). This finding highlights another significant difference to Fc γ Rs that inhibit IL-12 production in response to TLR stimuli (Gantner, Simmons et al. 2003). Thus despite superficial similarity, Dectin-1 ITAM-like motif based signalling again differs fundamentally from that of Fc γ Rs.

As two different signalling pathways emanate from the cytoplasmic tail of Dectin-1, we wondered whether, as is the case with Syk kinase in FcγRs, there are mediators common to both pathways. We demonstrate that inhibitors of kinases and GTPases shown to play a role in Dectin-1 mediated phagocytosis (**fig. 4.3.4a**) do not appear to play a role in zymosan induced TNF-α production. Indeed both wortmannin and Toxin B induced large increases in cytokine production, suggesting that inhibition of phagocytic uptake of zymosan particles, as following cytochalasin D treatment, may be a mechanism. Another potential mechanism was suggested when it was recently shown that inhibition of PI3K using wortmannin, enhances zymosan induced TNF-α production by blocking the recruitment of the phosphatase SHP-2 by SIRP-α (Smith, Patel et al. 2003). Whilst this may be the case following wortmannin treatment, neither cytochalasin D nor Toxin B have been shown to impair PI3K activity on phagosomes, yet both lead to a similar marked enhancement of zymosan induced TNF-α production. Furthermore, although Rac-1 has been shown to be involved in TLR2-mediated NF-κB activation (Arbibe, Mira et al. 2000), the failure of Toxin B to inhibit zymosan-induced pro-inflammatory cytokine production in macrophages (**fig. 5.3.9a**), suggests no central role for this signalling pathway. The general mechanism for cytochalasin D and Toxin B induced enhancement of TNF-α production may thus still be that failure to internalise particles results in sustained pro-inflammatory signals from the phagosomal cup.

Piceatannol, PP1, PP2 and PP3 have been shown to inhibit IκB phosphorylation (Ashikawa, Majumdar et al. 2002), therefore the reduced levels seen may be due to inhibition of this effector of NFκB. FcγR mediated pro-inflammatory signalling is Syk dependent (Daeron 1997; Ganesan, Fang et al. 2003), yet when we studied Syk

deficient macrophages, these showed only small reductions in zymosan induced TNF- α production compared to wild type controls, suggesting that Syk is unlikely to play a major role in this signalling pathway. The role of Src kinases was not assessed, as macrophages entirely deficient in this family of kinases are not yet available. Thus the ITAM-like motif of Dectin-1 is required and becomes phosphorylated (Gantner, Simmons et al. 2003) during both zymosan phagocytosis and zymosan induced cytokine production. These two signalling pathways however, unlike Fc γ Rs, do not appear to share common early mediators and probably diverge prior to activation of PI3K.

In conclusion, the recognition of microbes by innate immune cells involves multiple receptors, including lectins and TLRs, and the subsequent inflammatory responses depend on both the receptor repertoire of the cell and the functional co-operation between the signals generated downstream of receptor activation. In this way the specific cellular and cytokine responses are tailored to the particular pathogen encountered. In this context we have observed that following recognition of β -glucans, Dectin-1 collaborates with TLRs in promoting anti-fungal defences through cytokine production and, as others have shown (Gantner, Simmons et al. 2003), the subsequent Th-1 focussing of immune responses.

Chapter 6

Dectin-1 receptor trafficking in response to β -glucan ligands

6.1 Introduction

The plasma membrane constitutes the interface between cells and their microenvironment and uptake of nutrients, host defence and all communication amongst cells occurs through it. The concept 'endocytosis' encompasses a variety of mechanisms by which cells internalise particles and macromolecules into plasma membrane derived transport vesicles, these include phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis and clathrin- and caveolin-independent endocytosis (Conner and Schmid 2003).

As discussed earlier, phagocytosis is a receptor-mediated process by which specialised cells engulf large extracellular particles. The phagosomes formed by internalisation undergo maturation, a process involving a series of intracellular fusion and budding events that result in the delivery of particles to compartments rich in lysosomal enzymes where they are digested. Substantial amounts of plasma membrane and many phagosomal proteins, including receptors, rapidly recycle back to the plasma membrane following phagosome maturation (**fig. 6.1**). This membrane transport is particularly active in macrophages that exhibit high phagocytic activity (Damiani and Colombo 2001).

Phagocytosis is a highly regulated process and particular motifs in the cytoplasmic tail of phagocytic receptors, such as the Fc γ Rs, are involved in directing phagosomal contents to particular sub-cellular locations such as lysosomes (Worth, Mayo-Bond et al. 2001; Worth, Kim et al. 2003). Following phagocytic internalisation, the canonical



adapted from (Clemens 1996; de Chastellier *et al* 1995)

Figure 6.1

A simplified diagram illustrating particle phagocytosis and phagosomal membrane remodelling leading to phagolysosome formation. Particles are surrounded by a phagosomal membrane that undergoes extensive remodelling through its interactions with the endocytic compartment. During this process the membrane loses membrane to the recycling compartment sparing proteins from degradation, and acquires lysosomal characteristics that facilitate particle degradation.

FcγRs do not appear to undergo low pH dissociation from their ligands, a pre-requisite for receptor recycling (Mellman, Plutner et al. 1983). Furthermore pharmacological inhibition of membrane recycling did not affect either FcγR expression or particle phagocytosis (Tyteca, Van Der Smissen et al. 2002), suggesting that FcγRs traffic to lysosomal compartments still bound to their ligands. In contrast, MMR mediated particle internalisation is accompanied by rapid receptor recycling to the cell surface (Wilson and Pearson 1986; Pitt, Mayorga et al. 1992; Kang and Schlesinger 1998), sparing it from degradation. Internalisation of a phagocytic receptor therefore has differing consequences for the receptor depending on the sorting motifs present in the cytoplasmic tail.

Ligand-mediated endocytosis is an actin independent process that plays an essential role in cellular homeostasis and is triggered by a diverse group of cell surface receptors, most commonly in a process involving clathrin-coated pits (Ceresa and Schmid 2000; Conner and Schmid 2003). Endocytosis plays an important role in both constitutive nutrient uptake and in the regulation of signal transduction by internalising ligated receptors to either damp down or facilitate receptor signalling (Ceresa and Schmid 2000; McPherson, Kay et al. 2001; Conner and Schmid 2003). Following endocytosis, membrane fusion and budding deliver the membranes and contents of the vesicle through sequential endosomal compartments to their appropriate intracellular destination, which includes recycling back to the plasma membrane or delivery to a lysosomal degradation pathway (**fig. 6.1**).

Receptor internalisation by endocytosis and subsequent trafficking depends on a number of factors including: nature of the ligand, ligand size, receptor targeting to

membrane microdomains and receptor cytoplasmic tail. Endocytosis of ligated Fc γ Rs depends more on the residues that target them to coated pits, than on their cytoplasmic tyrosine residues to mediate endocytic internalisation (Miettinen, Matter et al. 1992) yet receptor trafficking following endocytosis is strictly dependent on the cytoplasmic domains of respective receptors and deletion of the cytoplasmic tail of Fc γ Rs redistributes these receptors from lysosomes (Mellman 1982) to the recycling pathway (van Vugt, Kleijmeer et al. 1999). Following endocytosis of antibody ligands, Fc γ Rs show a retarded trafficking to lysosomes when compared to either scavenger receptors or mannose receptor ligand transport (Lovdal, Andersen et al. 2000), suggesting that non-antigen ligands may be trafficked differently to potentially antigenic ligands. Importantly the size of the ligand endocytosed by Fc γ Rs has an influence on whether this receptor is able to recycle from endosomes, or whether it is degraded along with its cargo, with mono-valent ligands allowing receptor recycling (Ukkonen, Lewis et al. 1986). Functionally, degradation is a biophysical mechanism for attenuating the signalling of activated surface receptors (Ceresa and Schmid 2000).

Although the endocytosis and ligand trafficking of soluble β -glucans have not been described, it is well established that larger soluble β -glucans have priming effects on the immune system, whereas smaller β -glucans do not (Bohn and BeMiller 1995; Kulicke, Lettau et al. 1997; Lowe, Rice et al. 2001). We wondered whether these effects could have something to do with the way β -glucans of different sizes were handled by Dectin-1 expressing cells. To this extent we wanted to analyse aspects of Dectin-1 mediated receptor trafficking by macrophages following both soluble and particulate ligands.

6.2 Aims of the study

We showed in chapters 3 and 4 that Dectin-1 functions as a phagocytic receptor for fungal particles on macrophages. Immune receptors involved in phagocytic uptake, such as the Fc γ R, can usually also function as endocytic receptors, playing additional roles in homeostasis. Consequently we wanted to compare the effects of particulate and soluble ligands on Dectin-1 receptor internalisation and trafficking. The aims of this study were threefold: firstly to establish whether Dectin-1 was a receptor that recycled from phagosomes in a manner similar to other lectins such as MMR, Endo180 or DEC-205 (Stahl 1992; Mahnke, Guo et al. 2000; Howard and Isacke 2002) and other innate immune receptors (Molnar, Hoekstra et al. 1987); secondly to confirm any findings by following Dectin-1 and its recruitment to phagosomes over time by microscopy, as well as cellular localisation and trafficking of the receptor; finally to study the effects of various sizes of soluble ligands of Dectin-1 on the fate of the receptor following ligation.

6.3 Results

6.3.1 Particulate ligands of Dectin-1 are not released from the receptor at low pH

Following internalisation of a particulate or microbial ligand, phagosomes undergo a process of maturation during which the intra-luminal pH falls (Greenberg and Grinstein 2002). The reduction in pH contributes to the disengagement of receptor from ligand, a pre-requisite for receptor recycling (Harding, Levy et al. 1985). Some immune receptors such as Fc γ Rs have been shown not to disengage from their ligands at low pH resulting in their degradation in lysosomes (Mellman 1982; Mellman, Plutner et al. 1983). We therefore wanted to establish whether Dectin-1 could be released from zymosan particles at lowered pH, making recycling possible. We established an assay

using Dectin-1 expressing CHO cells that do not internalise zymosan particles in response to receptor ligation. We bound excess fluorescent zymosan particles to cells at 37°C prior to washing with warm PBS solutions of differing pH (**fig. 6.3.1a**). We found that even at very low pH it was not possible to release all bound zymosan particles, whereas the monoclonal antibody 2A11 was completely released from the cell surface by low pH wash (**fig. 6.3.1b**). These data suggested that it was unlikely that Dectin-1 would be released from zymosan particles during phagosomal acidification.

6.3.2 Primary macrophages retain Dectin-1 intracellularly following internalisation of particulate ligands

In order to establish whether the observation made in 6.3.1 applied to phagocytic cells we established an assay to measure the extent to which Dectin-1 expression on the cell surface recovered following particulate ligand induced receptor internalization. To distinguish recycled from newly synthesized receptors, it was necessary to inhibit protein synthesis by cells, and we established a concentration of cycloheximide that completely inhibited protein synthesis in thioglycollate-elicited macrophages (**fig. 6.3.2a**). To test the principle of our ligand induced receptor-quenching assay, we used our simplest Dectin-1 expressing cellular model, the 3T3-D1 cell line. We tested our assay (**fig. 6.3.2b**) before attempting to use a more representative primary cell population, thioglycollate-elicited macrophages (Mphi) which express high levels of Dectin-1 on the cell surface compared to resident peritoneal macrophages (Taylor, Brown et al. 2002). In the first instance we determined receptor trafficking indirectly, by analysing Dectin-1 recovery at the cell surface, after ligand binding, in the presence or absence of the protein synthesis inhibitor, cycloheximide. The recovery of Dectin-1 at the cell surface was determined by measuring the capacity of the cells to bind fluorescently-labelled zymosan (**fig. 6.3.2c**), over time, and confirmed by flow

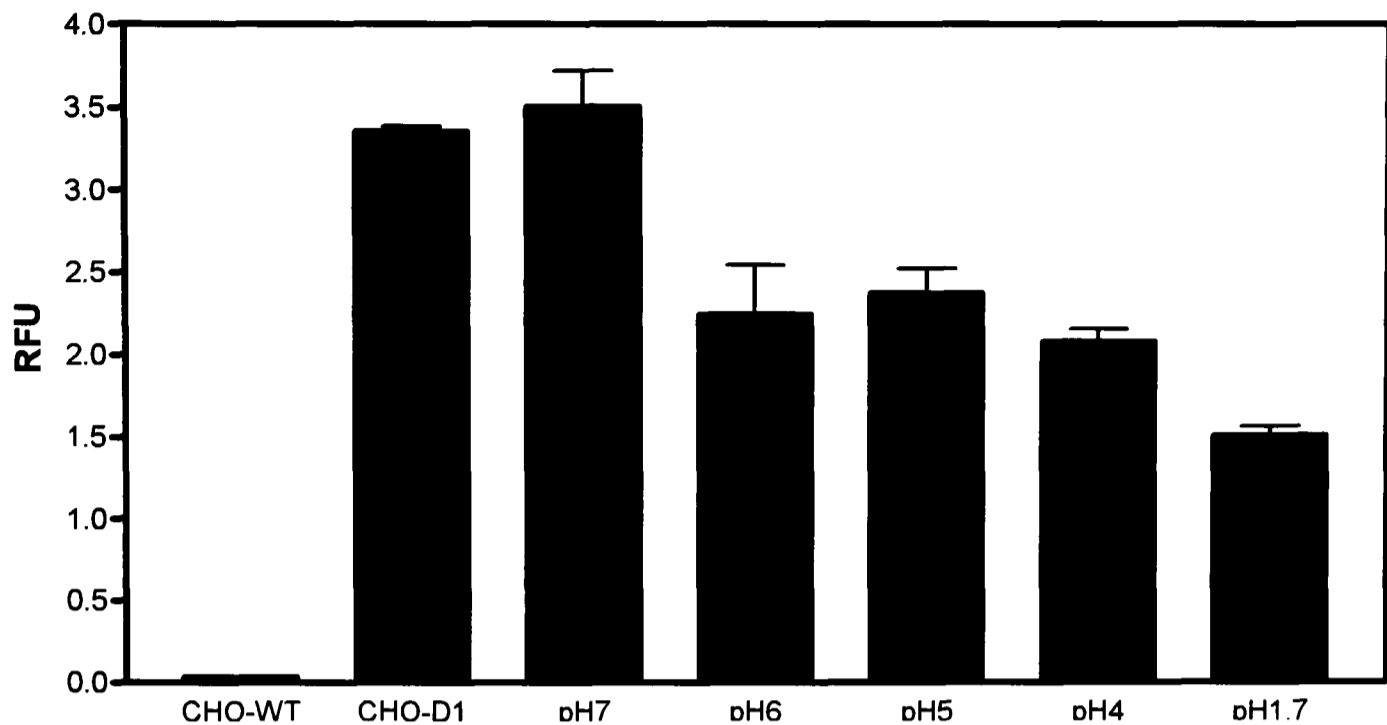


Figure 6.3.1a

Effects of pH on the release of Dectin-1 bound zymosan particles. FITC zymosan particles were bound to CHO-D1 cells at 37°C followed by three 30 second washes with PBS adjusted to the pH shown. Remaining fluorescence was quantified by fluorimetry.

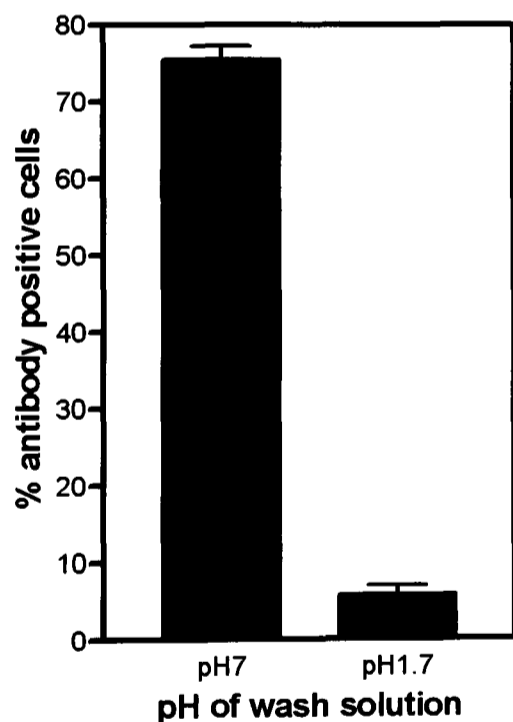


Figure 6.3.1b

Low pH wash releases all Dectin-1 bound monoclonal antibody. Monoclonal antibody 2A11 was allowed to bind to CHO-D1 cells at 4°C to prevent endocytic uptake and cells were then washed three times with cold PBS pH1.7 followed by staining with secondary antibody, lifting, fixing and analysis by flow cytometry.

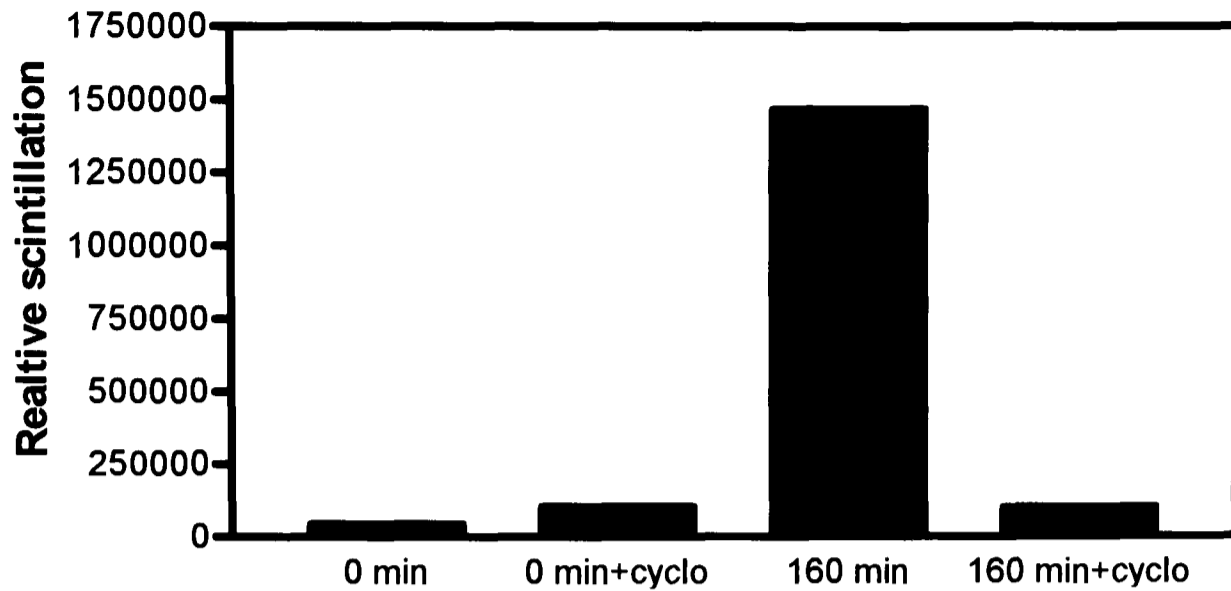


Figure 6.3.2a

Effect of cycloheximide treatment on thioglycollate elicited macrophages by incorporation of S^{35} labelled methionine. Macrophages were plated overnight in methionine free medium, then incubated for 2 hours with cycloheximide followed by cooling to $4^{\circ}C$, washing and replacement of medium with fresh medium containing S^{35} labelled methionine. Cells were then left at $4^{\circ}C$ or warmed to $37^{\circ}C$ for 160 minutes before washing thoroughly and lysing cells. Uptake of S^{35} labelled methionine was quantified by scintillation counter. Cells treated with cycloheximide failed to incorporate any labelled amino acid.

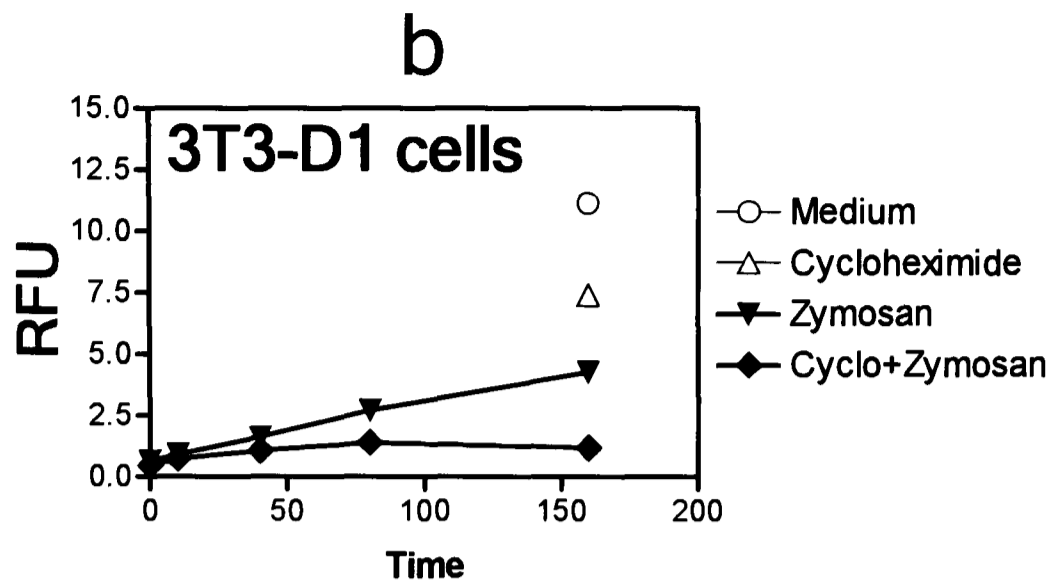


Figure 6.3.2b

Time course of Dectin-1 receptor recovery at the cell surface following particulate ligand mediated internalisation in 3T3-D1 cells. 3T3-D1 cells were allowed to bind excess unlabelled zymosan particles at 4°C followed by brief warming to allow receptor internalisation. Receptor level recovery in the presence or absence of cycloheximide was measured over time using FITC zymosan particles. RFU refers to arbitrary relative fluorescence units as derived from fluorimeter readings.

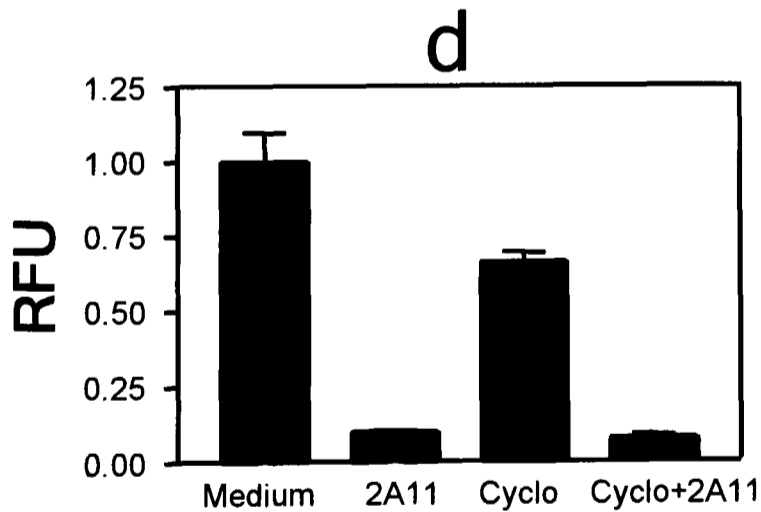
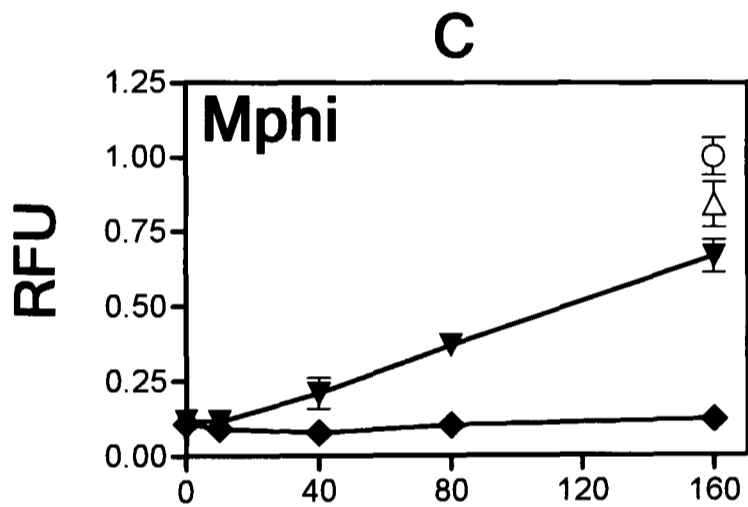


Figure 6.3.2c-d

Receptor recovery in thioglycollate elicited macrophages. Using the same technique as described in 5.3.2a, the recovery in FITC zymosan binding ability was measured over time. c) Zymosan binding recovers following receptor internalisation, but this is blocked by cycloheximide treatment. d) Zymosan binding at the 160 minute time point can be completely abolished by blocking Dectin-1 with the monoclonal antibody 2A11, irrespective of cycloheximide treatment.

cytometry following 2A11 staining prior to cell fixation (data not shown). We found that when both 3T3-D1 and Mphi were pulsed with an excess of unlabelled zymosan, zymosan binding was ablated compatible with rapid receptor internalization. Zymosan binding capacity and receptor expression recovered slowly over time. In the presence of cycloheximide, however, no recovery of zymosan binding was observed. As our measure of Dectin-1 expression was indirect and primary inflammatory macrophages express several receptors which can potentially recognise zymosan particles (Taylor, Brown et al. 2002), we confirmed that the recovery of zymosan binding activity was exclusively due to Dectin-1, by blocking binding with the monoclonal antibody, 2A11 (**fig. 6.3.2d** and (Brown, Taylor et al. 2002; Herre, Gordon et al. 2003)). Thus, these data suggest that after zymosan binding and internalisation, the recovery of Dectin-1 expression at the cell surface is due to new receptor synthesis, consistent with Dectin-1 not recycling from zymosan phagosomes. We next set out to establish to which sub-cellular location internalized Dectin-1 is trafficked.

6.3.3 Dectin-1 traffics to lysosomes along with its particulate cargo

Receptors that do not recycle from phagosomes, such as the Fc γ Rs, are inevitably degraded in the phagolysosomal compartment (Mellman, Plutner et al. 1983). In order to clarify the intracellular path of Dectin-1 following zymosan particle-induced internalization, we next examined zymosan phagosomes in RAW 264.7 macrophages expressing full-length HA-tagged Dectin-1, by confocal microscopy. We used these epitope tagged Dectin-1 expressing cells because the epitope recognized by the anti-Dectin-1 mAb 2A11 is fixation sensitive. The zymosan phagosome is known to mature and fuse with lysosomes (Allen and Aderem 1995), so we followed the synchronous phagocytosis of zymosan over time along with the late endosomal / lysosomal marker

LAMP-1, the effectiveness of which for staining the lysosomal compartment of RAW-D1 cells was assessed by flow cytometry (**fig. 6.3.3a**). We observed that the zymosan particles were rapidly internalised along with Dectin-1, which co-localised with LAMP-1 by 30 minutes (**fig. 6.3.3b**). At time points beyond 30 minutes, the HA-epitope, which is exposed to the phagolysosomal interior, became difficult to detect, suggesting that it was being degraded within this compartment. Thus, Dectin-1 is retained on the phagosomal membrane throughout its maturation to a phagolysosome.

6.3.4 Size determines intra-cellular trafficking of Dectin-1 following soluble ligand mediated internalisation

Dectin-1 is also the major receptor for soluble β -glucans on macrophages (Brown, Taylor et al. 2002), and may be internalised after binding these polymers. As it is thought that the immunomodulatory effects of soluble β -glucans are related to their size (Brown and Gordon 2003), we examined Dectin-1 trafficking after binding of two glucans with differing molecular weight, glucan phosphate and laminarin, and a control glucan Dextran 40. By examining the recovery of surface Dectin-1 in primary macrophages after incubation with excess soluble ligand, as before, we observed that after the addition of the small glucan, laminarin (**fig. 6.3.4a**), the receptor quickly returned to the cell surface, even in the presence of cycloheximide, suggestive of receptor recycling. This finding was further confirmed by staining for Dectin-1 on the cell surfaces of these cells and analysis by flow cytometry (data not shown). In contrast, the levels of surface Dectin-1 did not greatly recover after the addition of the large glucan polymer, glucan phosphate (**fig. 6.3.4b**), even in the absence of cycloheximide. Again this observation was confirmed by flow cytometric analysis (data not shown). Dextran 40 had no influence on Dectin-1 receptor expression in either the cycloheximide treated or untreated cells (**fig. 6.3.4c**); again, all recovery of zymosan

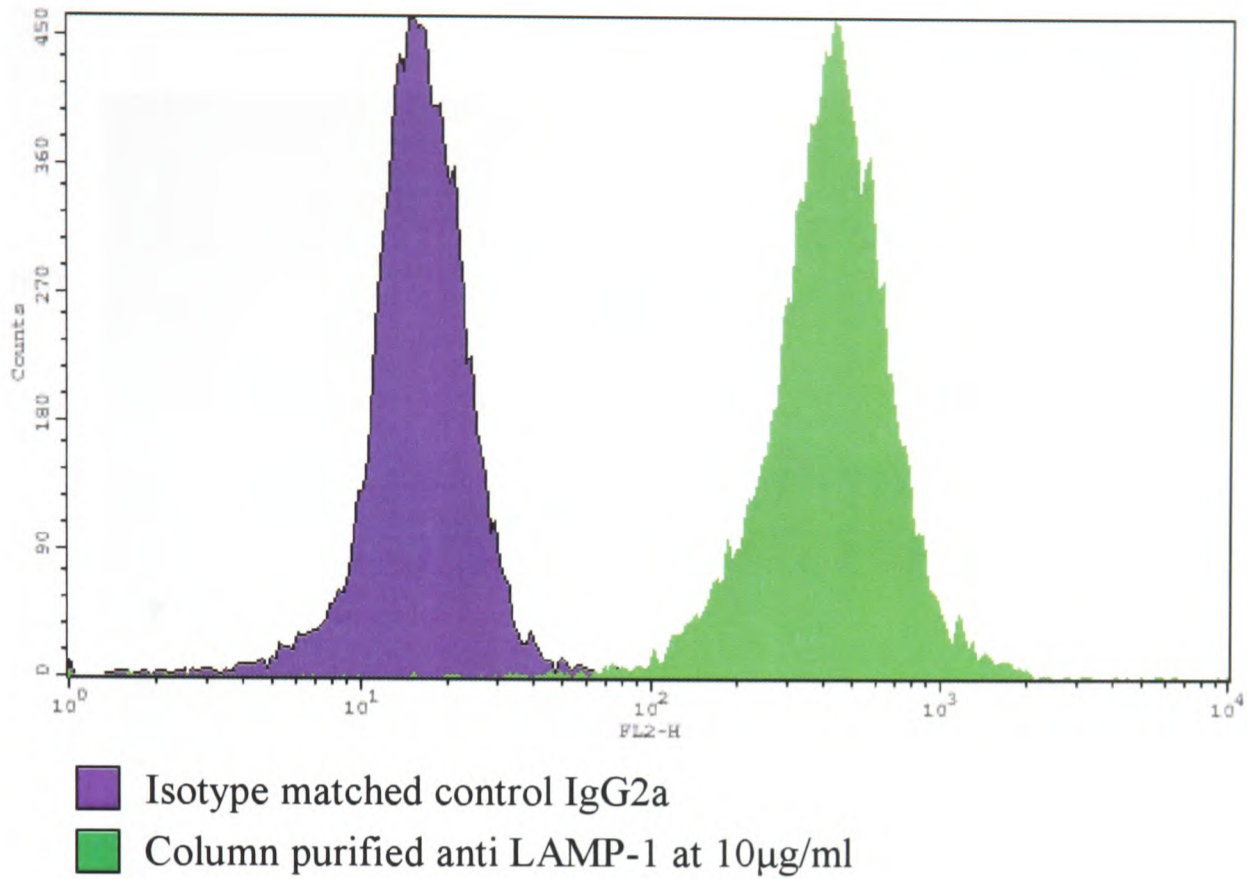


Figure 6.3.3a

LAMP-1 expression by RAW-D1 macrophages. 1D4B hybridoma supernatants were column purified and the antibody tested by flow cytometry to confirm high levels of antigen expression in RAW-D1 cells.

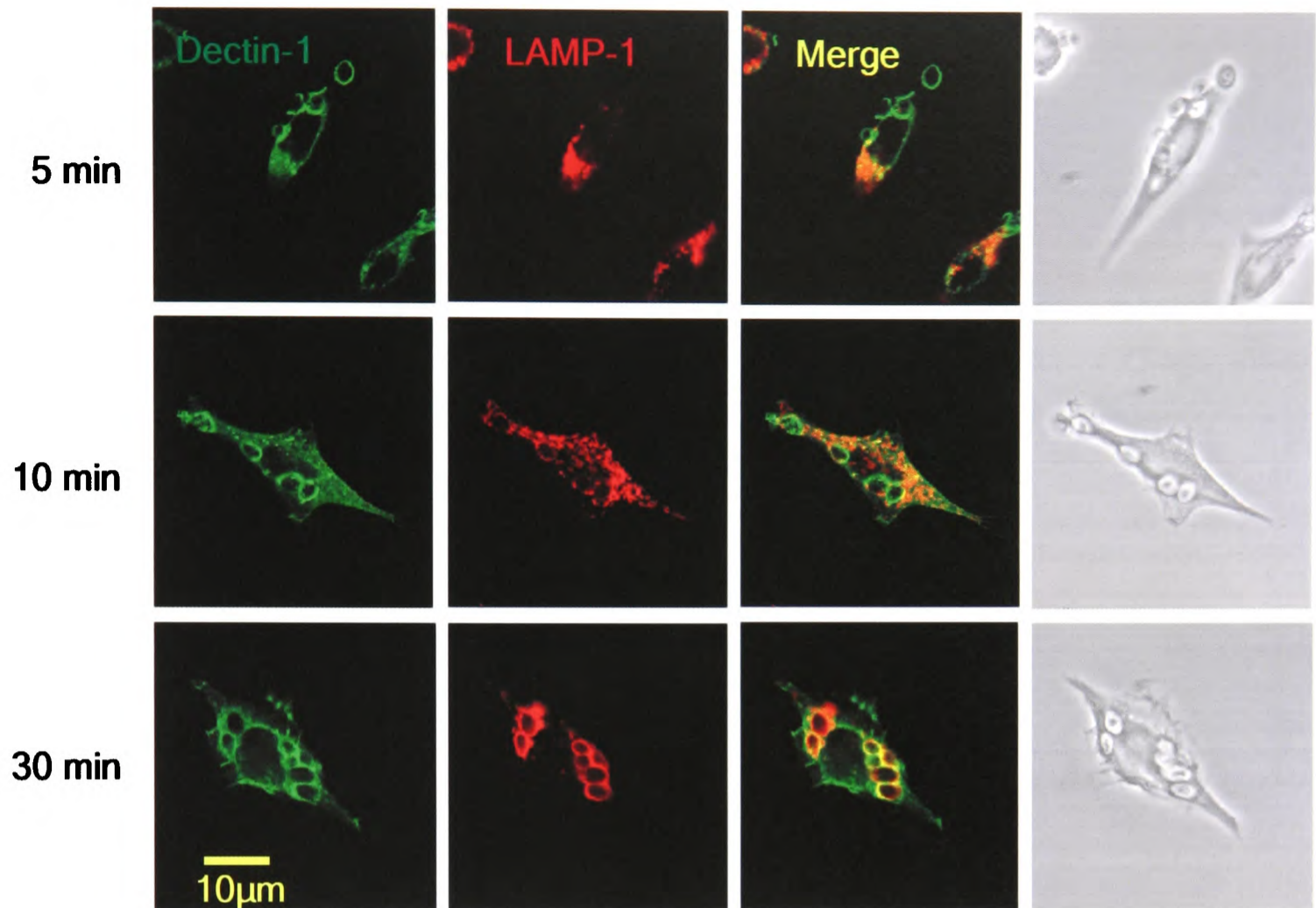


Figure 6.3.3b

Dectin-1 receptor trafficking following zymosan induced receptor internalisation. This time course shows synchronous zymosan phagocytosis and phagosomal trafficking to the lysosomal compartment. Dectin-1 (green) is not released from the phagosomal membrane following internalisation and co-localises with the lysosomal compartment stained with anti-LAMP-1 (red). Over a longer time course (see fig. 5.3.5b) Dectin-1 staining disappears from phagolysosomes, probably due to receptor degradation.

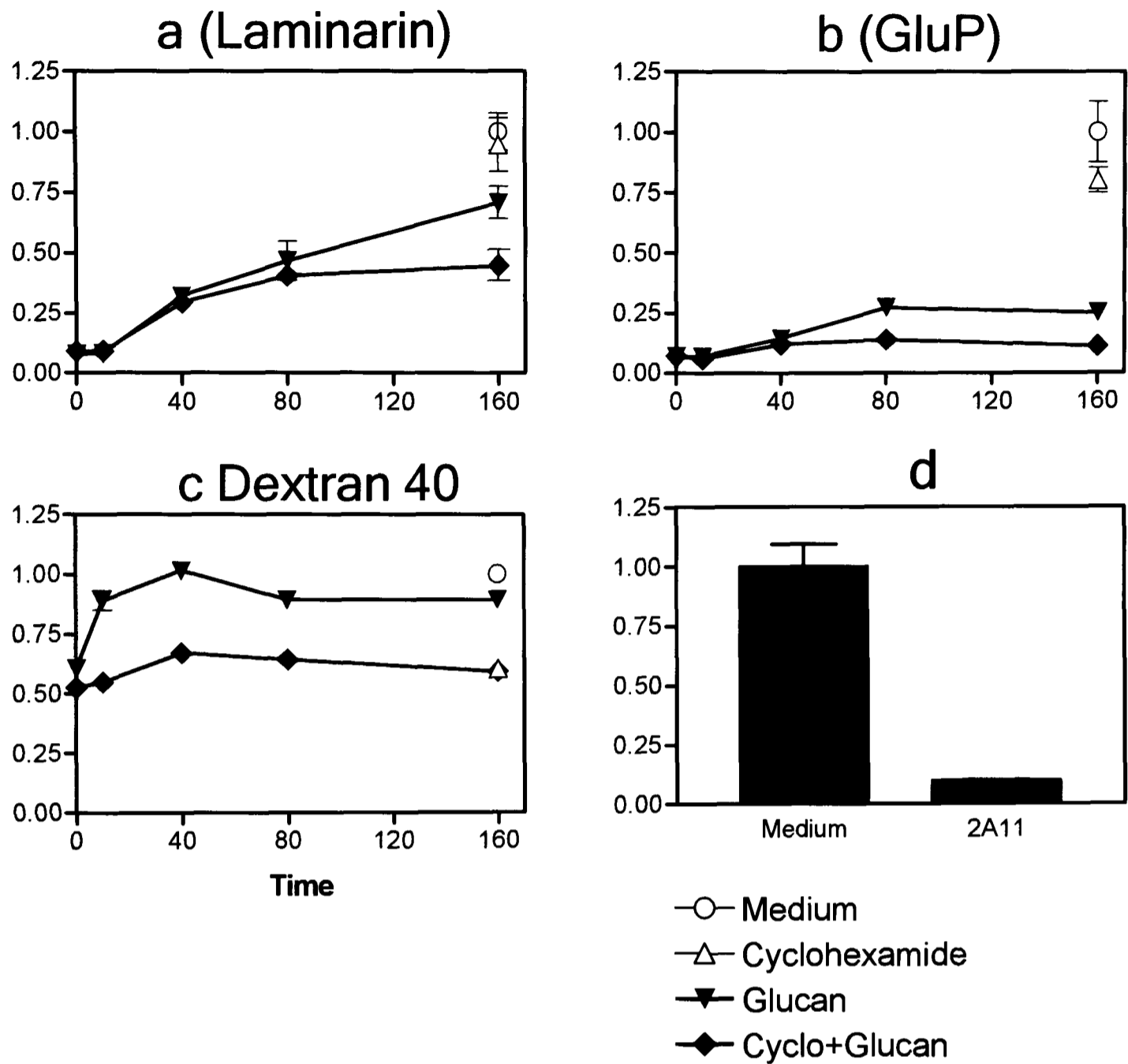


Figure 6.3.4a-d

Dectin-1 receptor recovery at the cell surface following soluble glucan induced receptor internalisation in thioglycollate elicited macrophages. This time course shows recovery in FITC zymosan binding following synchronous endocytosis of soluble glucans. a) Laminarin: Zymosan binding recovers following receptor internalisation, and this is not blocked by cycloheximide treatment. b) Glucan Phosphate (GluP): Zymosan binding recovers very little following receptor internalisation, and this is blocked by cycloheximide. c) Shows the effects of dextran 40, a glucan that is not recognised by Dectin-1. d) A representative bar graph showing that zymosan binding at the 160 minute time point can be completely abolished in all of these experiments by blocking Dectin-1 with the monoclonal antibody 2A11, an effect independent of cycloheximide.

binding capacity following glucan treatment was completely inhibitable by the monoclonal antibody 2A11 (**fig. 6.3.4d**). It thus appears that smaller Dectin-1 ligands are released from the receptor following receptor-mediated endocytosis, but larger ligands result in cytoplasmic retention of internalised receptors.

6.3.5 Internalised soluble ligands show limited trafficking to lysosomes

Soluble glucans larger than 14 kDa have been shown by *in vivo* studies to protect animal models against lethal infections (Williams, Sherwood et al. 1988; Williams, Mueller et al. 1996), suggesting that some aspect of Dectin-1 mediated soluble ligand binding, internalisation or trafficking could mediate these protective responses. We wanted to establish whether, in the case of glucan phosphate, the internalisation of soluble glucans by Dectin-1 expressing macrophages resulted in its trafficking to lysosomes. We pulsed RAW-D1 cells with various glucans and followed Dectin-1 receptor trafficking over time by confocal microscopy. We found that over a 30-minute time course (**fig. 6.3.5a**) a pulse with soluble Dectin-1 ligands resulted in an increase in cytoplasmic vesicles, and disappearance of Dectin-1 from the cell surface. Dextran 40, a glucan that is not recognised by Dectin-1 had no effect, demonstrating that the receptor internalisation was not due to random pinocytic activity. At 30 minutes limited co-localisation with LAMP-1 was seen, suggesting that neither Laminarin nor glucan phosphate-mediated receptor internalisation resulted in the sorting of Dectin-1 endosomes to the degradative pathway. The apparently co-localising vesicles need to be examined at higher resolution to establish whether these are overlapping vesicular compartments, or true co-localisation. Over a longer time course of 160 minutes (**fig. 6.3.5b**) we found that following zymosan particle internalisation (top row), Dectin-1 expression would return to the cell surface by the 160-minute time point, whereas in the

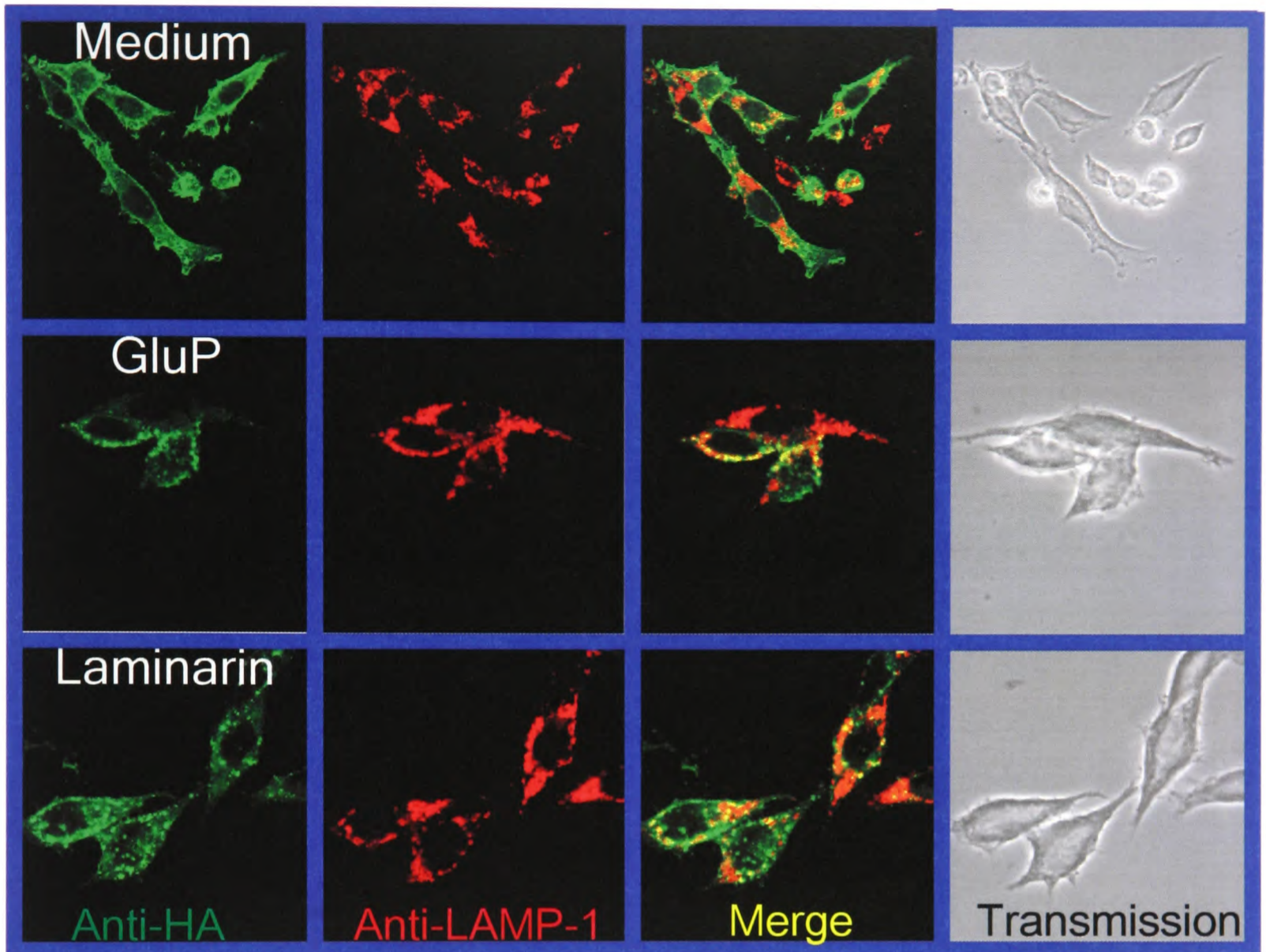


Figure 6.3.5a

Effects of soluble glucans on Dectin-1 trafficking over 30 minutes. Following a brief pulse with soluble glucans, cells were incubated for 30 minutes at 37°C, fixed and stained for Dectin-1 (green) and LAMP-1 (red). (Upper row) Medium alone had no effect on receptor expression at the cell surface. Following uptake of the soluble glucans, glucan phosphate (GluP) (middle row) and Laminarin (lower row), Dectin-1 was redistributed to a vesicular compartment that shows no consistent co-localisation with the lysosomal compartment, although glucan phosphate endosomes seemed to more frequently co-localise with lysosomes.

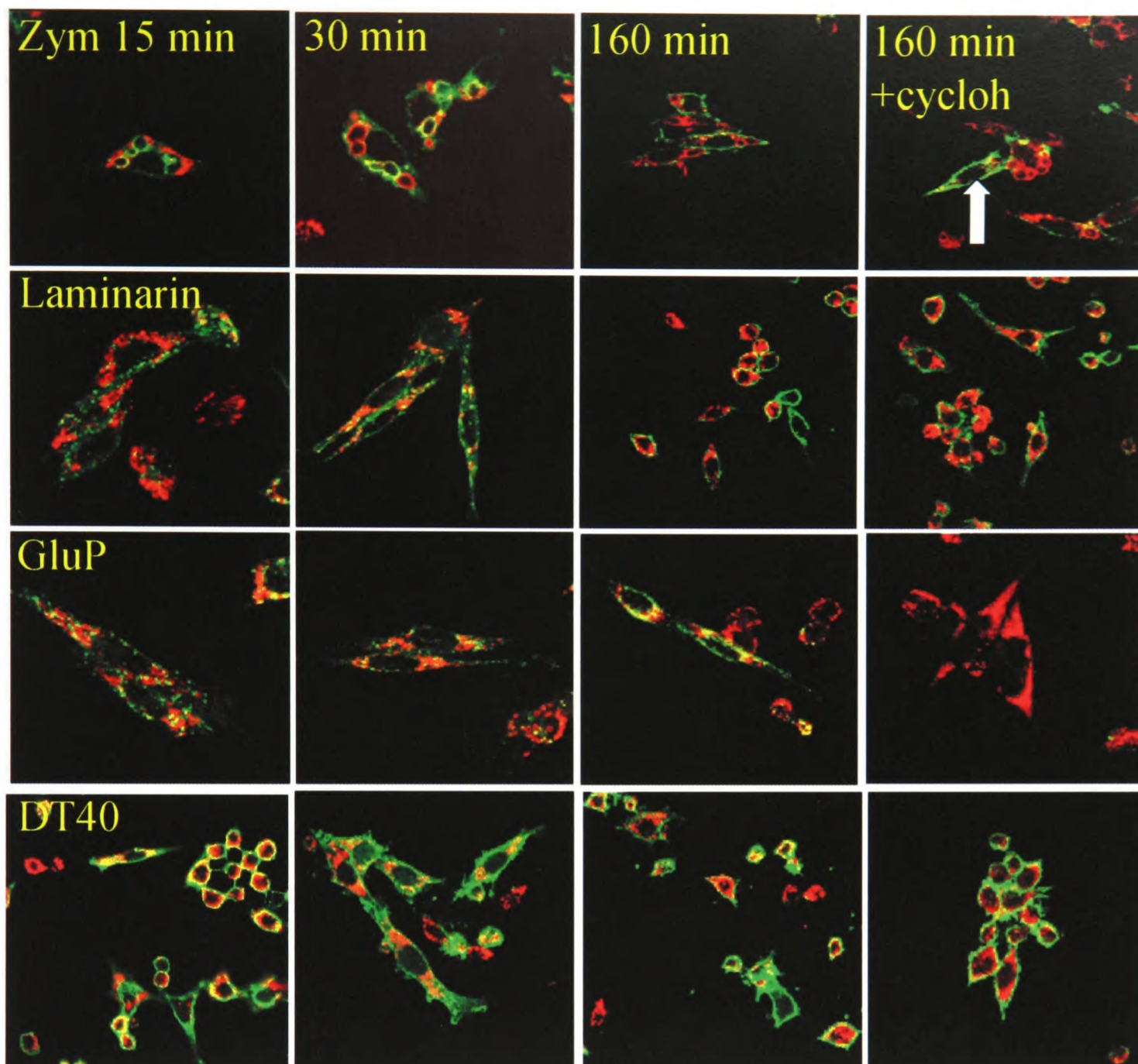


Figure 6.3.5b

Effects of particulate and soluble glucans on Dectin-1 trafficking over a time course of 160 minutes. Cells were either treated or not with cycloheximide. Following synchronous uptake of glucans and varying times at 37°C, cells were fixed and stained for Dectin-1 (green) and LAMP-1 (red). Zymosan phagosomes (top row) lose all Dectin-1 staining by 160 minutes, and receptor levels on the cell surface have recovered in the absence of cycloheximide, and remained high on cells that had not internalised any particles even in the presence of cycloheximide (white arrow). Laminarin endosomes (second row) recover surface expression of Dectin-1 irrespective of cycloheximide treatment. Glucan phosphate endosomes (third row) generally do not recover surface expression of Dectin-1 in the absence of cycloheximide, and there is no recovery following inhibition of protein synthesis. Dextran treatment results in no change in surface expressed Dectin-1 irrespective of cycloheximide treatment. These results are broadly in agreement with the findings in primary cells.

cycloheximide-treated cells poorer surface expression was seen, especially when compared to the bright green control cell (white arrow) that had not internalised any particles. Laminarin endocytosis (second row) was followed by significant receptor recovery to the cell surface by 160 minutes and this recovery was not diminished by cycloheximide, suggesting receptor recycling. Glucan phosphate (third row) resulted in total disappearance of Dectin-1 by 160 minutes in the majority of treated cells, and those cells expressing Dectin-1 showed a vesicular distribution. Cycloheximide treatment resulted in a complete disappearance of Dectin-1. Dextran 40 had no effect on Dectin-1 surface expression over the time course irrespective of the presence of cycloheximide. These data support the observations made in primary cells in 6.3.4. above, suggesting that the route of intracellular trafficking of Dectin-1 depends on the nature of the β -glucan ligand. Soluble glucans appear to determine Dectin-1 receptor trafficking depending on ligand size.

6.4 Discussion

In addition to triggering uptake, the cytoplasmic domains of phagocytic receptors also determine their intracellular fate, although these targeting domains are better defined in endocytic receptors (Bonifacino and Dell'Angelica 1999). Some receptors recycle, such as the macrophage mannose receptor, Dec-205 and Endo180, whereas others, such as the Fc γ R, traffic to lysosomes where they are degraded along with their cargo (Stahl and Ezekowitz 1998; Mahnke, Guo et al. 2000; Worth, Mayo-Bond et al. 2001). We found that the intracellular fate of Dectin-1 depended on the nature of the β -glucan ligand, which is the first demonstration of a receptor with this characteristic. With the particulate ligand, zymosan, Dectin-1 trafficked to lysosomes inducing de novo synthesis of the receptor. On the other hand, little de novo synthesis was observed with

the addition of the soluble glucans. Furthermore, the receptor was observed to recycle with laminarin, but remained in an unidentified intracellular compartment with glucan phosphate. Confocal studies revealed that there may be some delivery of glucan phosphate to the lysosomal compartment within 30 minutes, and after 160 minutes the extensive degradation of Dectin-1 suggests that the receptor eventually ends up in lysosomes. A more direct demonstration of lysosomal delivery would have been provided by the use of specific inhibitors of endocytic transport. The significance of this apparent delay in lysosomal delivery is that it may reflect a role in soluble β -glucan mediated immunomodulation, similar to the situation following immune-complex endocytosis by Fc γ Rs, where the delayed delivery to lysosomes seems to play a role in antigen processing and presentation (Lovdal, Andersen et al. 2000).

Although the basic structures of these two carbohydrates are similar, glucan phosphate is considerably bigger than laminarin (156000 versus 7700 g/mol, respectively) (Mueller, Raptis et al. 2000). It is unclear whether or how these differences in polymer size induce the various intracellular trafficking pathways, but it may be indicative of a cellular mechanism by which the larger mol wt. glucan mediate their immunomodulatory activities *in vivo* (**fig. 6.4** & (Brown and Gordon 2003)). Furthermore, the retention of Dectin-1 within the cell also explains why glucan phosphate was much better than laminarin at inhibiting cellular responses to zymosan *in vitro* (Brown, Herre et al. 2003). In conclusion, we have shown that Dectin-1 is both a phagocytic and an endocytic receptor that recycles depending on ligand size.

Unresolved, however, is how soluble glucans mediate their effects as we have previously noted that soluble glucans such as glucan phosphate did not induce, but

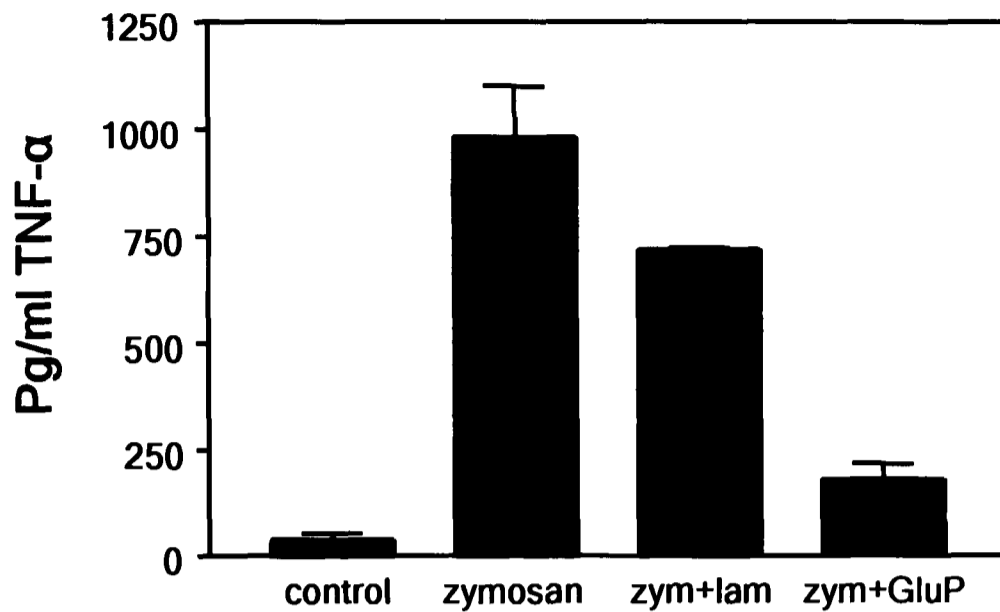


Figure 6.4

Large soluble glucans are more effective inhibitors of Dectin-1 mediated pro-inflammatory cytokine production than small soluble glucans. Thioglycollate-elicited macrophages secrete large amounts of TNF- α in response to incubation with zymosan particles (as previously described). Incubating cells with the small (7.7kDa) glucan, laminarin (lam), prior to the addition of excess zymosan particles has little inhibitory effect on the secretion of TNF- α , whereas production is almost completely abrogated by treatment with the large (156 kDa) glucan polymer, glucan phosphate (GluP).

rather were potent inhibitors of zymosan-induced biological responses in thioglycollate-elicited macrophages (**fig. 5.3.1**), implying that the observed immunomodulatory effects must operate by a different mechanism.

Chapter 7

Final summary, working models and future work

7.1 Original aims

This project was initially based on the observation that zymosan particles bound to Dectin-1 expressing NIH-3T3 cells induced actin polymerisation at sites of particle contact, leading us to hypothesise that Dectin-1 may be a phagocytic receptor. We thus examined this possibility. Sequence analysis of the cytoplasmic tail of Dectin-1 revealed an ITAM-like motif, very similar to the canonical ITAMs of Fc γ Rs, which we hypothesised to be responsible for Dectin-1 mediated phagocytic uptake. Using mutational analysis we examined the role of cytoplasmic tyrosine residues, and expanded the study to include signalling intermediaries linking cytoplasmic tail to actin cytoskeleton. The recognition of zymosan particles and live yeast by Dectin-1, and the observation that this receptor mediates phagocytosis through its ITAM-like motif, led us to suspect that Dectin-1 might also play a role in biological responses to β -glucan rich particles and micro organisms. We therefore examined the role of Dectin-1 in mediating pro-inflammatory cytokine production. Finally, in macrophages, zymosan-containing phagosomes have been shown to traffic to the lysosomal compartment. As some receptors recycle from phagosomes and others traffic to the phagolysosomal compartment still bound to their ligands, we wanted to establish whether internalised Dectin-1 recycled or was degraded along with its ligand.

7.2 Summary of key findings

In host defence, pattern recognition plays an essential role in the discrimination of self from pathogenic non-self (Janeway 1992; Medzhitov 2001; Janeway and Medzhitov 2002). Phagocytosis is essential for anti-microbial defence and its contribution is not

limited to the clearance and killing of pathogens, but extends to the activation of adaptive immunity through production of pro-inflammatory mediators and antigen presentation (Underhill and Ozinsky 2002). Anti-fungal immunity is extremely efficient and operates via recognition, phagocytosis and killing of fungal pathogens by leukocytes. We have shown that Dectin-1 recognises live fungi and fungal derived fungal particles (Brown and Gordon 2001) and that it is highly expressed on various leukocyte populations (Brown, Taylor et al. 2002). We have thus tried to establish whether Dectin-1 contributes to anti-fungal defence by looking at various aspects of receptor biology, illustrated in **figure 7.2**.

7.2.1 Dectin-1 is a phagocytic receptor

Dectin-1 recognises fungal particles, and we found that increased Dectin-1 expression lead to increased binding of zymosan particles. Using confocal microscopy to study Dectin-1 expressed in a poorly phagocytic cell line, we found that actin polymerisation was induced at points of particle contact, and that zymosan particles that appeared to be undergoing internalisation were surrounded by actin rich cups. Expression of Dectin-1 in its appropriate cellular context, in macrophages, markedly enhanced the phagocytic uptake of zymosan particles. Using quantitative flow-cytometry based assays we showed that the majority of zymosan particles bound to Dectin-1 undergo rapid and complete internalisation, in both phagocyte and non-phagocyte cell lines. Dectin-1 was thus identified as a phagocytic pattern recognition receptor for fungal derived ligands. As it has previously been shown that neither CR3 nor MMR are responsible for the recognition of unopsonised zymosan particles by macrophages (Brown, Taylor et al. 2002), Dectin-1 is the first described phagocytic PRR for fungal particles.

Dectin-1 and innate immunity to fungal infections

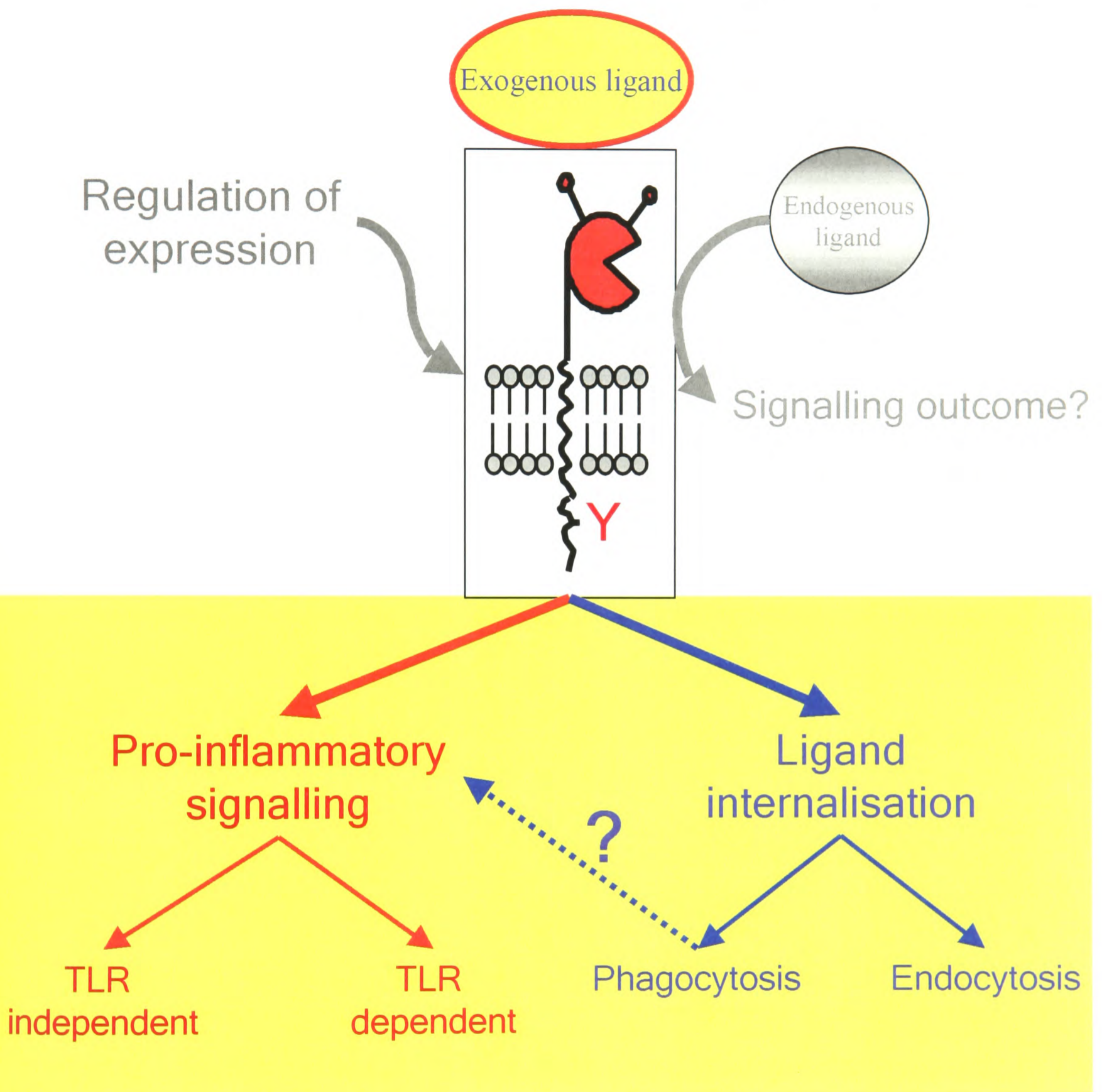


Figure 7.2

Aspects of Dectin-1 function potentially relevant to anti-fungal immunity examined in this thesis. On the yellow background, the functions analysed. The dashed line represents a link between Dectin-1 clustered by phagocytic particles and pro-inflammatory signals. The precise nature of this interaction remains to be defined. In grey other aspects of anti-fungal immunity not studied.

7.2.2 Dectin-1 mediated phagocytosis depends on the membrane proximal tyrosine in the ITAM-like motif

The cytoplasmic tail of Dectin-1 contains an ITAM-like motif very reminiscent of the ITAMs of FcγRs, which are central to phagocytic and activatory signalling by these receptors. We were intrigued to find out whether the tyrosine residues present in the ITAM-like motif of Dectin-1 were responsible for mediating its phagocytic uptake particularly as it has been shown that a perfect ITAM is not required for single chain FcγR mediated phagocytosis (Mitchell, Huang et al. 1994). Using an approach that involved stably expressing recombinant Dectin-1 molecules with cytoplasmic tail mutations in both phagocyte and non-phagocyte cell models, we found that the membrane proximal tyrosine of Dectin-1 was the critical residue mediating phagocytic uptake and that, unlike FcγRs, the membrane distal tyrosine played no role in this process. Despite this difference, the pivotal role of tyrosine residues in mediating phagocytosis suggested that tyrosine phosphorylation was likely required to initiate phagocytosis, and also raised the possibility that the established mediators of classical ITAM phagocytic signalling may be involved in driving the process. This is the first description of a functional tyrosine based phagocytic motif found in a leukocyte PRR.

7.2.3 Dectin-1 mediated phagocytosis uses novel mechanisms of particle internalisation

We next studied the mechanisms involved in relaying signals from Dectin-1 to the actin-cytoskeleton. In particular, we examined the effects of selective inhibitors of molecules involved in the canonical ITAM signalling pathway to the cytoskeleton on Dectin-1 mediated phagocytosis. We concluded that, in a non-phagocyte model, tyrosine phosphorylation was an essential initiator of this process. Furthermore, the mediators of tyrosine phosphorylation as well as subsequent events leading to actin

polymerisation appeared to be the same as those involved in FcγR phagocytosis. These findings suggested that tyrosine based signalling to the actin cytoskeleton may involve common rather than receptor specific mediators. When we, however, examined Dectin-1 in its correct cellular context and compared FcγR and Dectin-1 mediated phagocytosis in macrophages, we found that there were significant differences in the mechanisms of cytoskeletal activation. The most surprising finding was that Syk, a kinase essential for all FcγR mediated cellular effects, appeared to play no role in the process. Furthermore, we found that Dectin-1 mediated phagocytosis was not solely dependent on the Src family kinases, PI3K or the Rho GTPases as is the case for FcγRs. These findings are significant because they show firstly that it is essential to study receptors in the correct cellular context, and secondly because they confirm that Dectin-1 does not share a common pathway for mediating actin polymerisation with the FcγRs, despite this process being initiated by phosphorylation of cytoplasmic tyrosine residues. Importantly, these findings suggest that even receptors that have tyrosine based signalling motifs in their cytoplasmic tails and phagocytic capacities in common, do not necessarily share the same mechanisms for mediating actin polymerisation and that novel signalling processes and mediators might be involved in Dectin-1 mediated phagocytosis.

7.2.4 Dectin-1 mediates biological responses to β-glucans in macrophages

Classical ITAM containing receptors such as the FcγRs mediate not only ligand internalisation, but also cellular activation by means of pro-inflammatory signals emanating from their phosphorylated ITAMs (Daeron 1997). This integration of functions is essential for host defence as clearance and killing of pathogens is often accompanied by appropriate signals to the adaptive immune system such as cytokine

secretion and antigen presentation. This led us to examine whether Dectin-1 could also mediate biological responses to fungal particles by means of zymosan- and live fungi-induced cytokine secretion. We found that Dectin-1 mediated production of the pro-inflammatory cytokine TNF- α in response to fungal particle recognition in both primary macrophages and macrophage cell lines, and that the membrane proximal tyrosine residue in the ITAM-like motif played a central role in cytokine secretion. We also found that fungal particle-induced TNF- α production depended on Dectin-1 co-operating with TLR2 and MyD88. Finally, the interaction between Dectin-1 and the TLRs did not require particle internalisation, indicating that co-operation between these receptors was occurring at the cell surface. This evidence of receptor cooperation between the TLRs and an innate immune PRR helps to clarify how TLRs, that share a common signalling pathway, might be able to discriminate between pathogens.

7.2.5 Dectin-1 does not recycle from phagosomes

Phagocytic receptors can either recycle from phagosomes and be spared from degradation, or traffic with their ligands to phagolysosomes for degradation. Whether a given receptor recycles depends on motifs in the cytoplasmic tail. ITAM containing receptors such as the Fc γ R are both phagocytic and mediate pro-inflammatory signalling. Receptor degradation is a mechanism for limiting signals from such receptors. Similarly, Dectin-1 can mediate both phagocytic uptake and pro-inflammatory responses. We tested the hypothesis that, following phagocytosis, Dectin-1, unlike lectins such as MMR and DEC-205, might not recycle, ultimately resulting in its degradation. Using a particulate ligand-induced receptor internalisation assay, we measured the extent to which Dectin-1 recycles to the cell surface from phagosomes in primary peritoneal macrophages. We found that Dectin-1 does not recycle from

zymosan phagosomes and that new receptor synthesis is necessary for the recovery of cell surface receptor levels. Furthermore, we followed Dectin-1 to lysosomes over 30 minutes by confocal microscopy, confirming that receptor recycling does not occur. Dectin-1 mediated internalisation of particulate ligands that induce pro-inflammatory signals is thus accompanied by transport of the receptor to lysosomes and its degradation. This finding, along with the finding that retention of ligated Dectin-1 on the cell surface strongly enhances inflammatory signalling, might suggest that internalisation and degradation may be a mechanism for regulating such signals.

7.2.6 Dectin-1 mediated endocytosis results in differential, ligand size dependent, intracellular receptor trafficking

Many phagocytic receptors are also endocytic for soluble ligands. Recycling from the endocytic compartment is common for receptors involved in cellular housekeeping, and uncommon for receptors involved in immune defences. We examined the response of Dectin-1 to soluble ligands of various sizes and found that ligand size determined whether Dectin-1 was able to recycle from endosomes. We were particularly intrigued to find that endocytosis of larger glucans that have immunomodulatory properties was not accompanied by receptor recycling, whereas Dectin-1 recycled from the endosomes containing smaller immunologically silent glucans. These observations helped to explain our finding that the larger glucan, glucan phosphate, is a much better inhibitor of both zymosan particle recognition and zymosan particle induced cytokine production than the smaller glucan Laminarin.

Neither glucan phosphate nor laminarin induce pro-inflammatory cytokine production, instead they act as inhibitors of fungal particle induced TNF- α production. So how do soluble glucans mediate their effects? Recently it was shown that glucan phosphate can

stimulate PI3K activity, and pharmacological inhibition of PI3K resulted in the loss of glucan-mediated protection against septic mortality (Williams, Li et al. 2004). Part of the mechanism by which soluble glucans mediate immunomodulation may thus be PI3K mediated damping of excessive inflammatory responses that would otherwise result in cell damage (Li, Ha et al. 2004; Williams, Li et al. 2004). It is nonetheless not yet clear how soluble glucans mediate their wide range of immunological effects, as these molecules both activate the immune system of animal models prior to, but also constrain excessive activation following, septic challenge (Williams, Mueller et al. 1996; Li, Ha et al. 2004; Williams, Li et al. 2004).

7.3 Working models

7.3.1 Dectin-1 mediated signalling

The data presented in chapters 4 and 5 and summarised above can be assembled into a working model describing Dectin-1 mediated phagocytosis and cellular activation, shown schematically in **fig. 7.3.1**. Although Dectin-1 mediated uptake involves some known intracellular signalling molecules in macrophages, the initiating events are novel, and the overall mechanism appears different from any other known phagocytic receptor. These data are a further demonstration of the complexity of phagocytic mechanisms present in macrophages.

Recognition of zymosan by Dectin-1 results in phosphorylation of the membrane proximal tyrosine residue in the cytoplasmic tail in a process that involves the Src family kinases, and potentially other kinases. Subsequent to receptor phosphorylation, and in a process that bypasses p72Syk, PI3K is recruited, either through the actions of early kinases or directly by the clustered, phosphorylated Dectin-1 cytoplasmic tails.

Figure 7.3.1

A model of Dectin-1 mediated signalling events based on the discussion contained in the text. In blue are indicated the signalling mediators we directly examined and in red are potential novel mediators. Solid black lines indicate established interactions, dotted lines indicate potential or suggested interactions present in published literature, red lines indicate proposed interaction partners of novel signalling intermediates. Data compiled from: (Aderem 1992; Allen and Aderem 1996; Chimini and Chavrier 2000; Yamamori, Inanami et al. 2000; Matsui, Adachi et al. 2001; Brown, Taylor et al. 2002; Underhill and Ozinsky 2002; Brown, Herre et al. 2003; Gantner, Simmons et al. 2003).

PKC that binds to the PI3K product PIP3, in the cell membrane, is then recruited and drives both the recruitment of components of the phagosomal phox, leading to ROI production, and, perhaps through the actions of MARCKS and in collaboration with PI3K, membrane fusion and phagosomal closure. The Rho GTPases Rac-1 and Cdc-42 become activated through an unknown process that probably involves early kinases and PI3K, leading to Arp2/3- and Cofilin-mediated cytoskeletal remodelling and phagocytic uptake.

Zymosan phagosomes recruit both TLR-2 and TLR-6 (Underhill, Ozinsky et al. 1999; Ozinsky, Underhill et al. 2000), and subsequent inflammatory signalling requires the adaptor MyD88. MyD88 interaction with IRAK results in TRAF-6 activation (**fig. 1.3.2**) Subsequently I κ B becomes phosphorylated and degraded allowing NF κ B to translocate to the nucleus and initiate transcription of TNF- α and IL-12. The manner in which Dectin-1 interacts with this pathway is unclear, but our findings suggest that the divergence of signalling pathways occurs prior to PI3K activation, and does not involve Syk kinase.

There are many uncertainties in this model. We and others (Gantner, Simmons et al. 2003) have presented strong evidence that Dectin-1 becomes tyrosine phosphorylated in response to receptor ligation. The kinases mediating this process may well be Src-family kinases, yet there must be other, yet to be defined kinases capable of substituting, albeit with lower efficiency. These elements will only be defined when the signalling complexes recruited by clustered Dectin-1 are analysed. As the recruitment of PI3K, downstream of phosphorylated ITAMs, is regulated by Syk, and Dectin-1 mediated phagocytic uptake does not require this kinase, it is not certain how PI3K is

recruited to the phagosome. PI3K plays some role in Dectin-1 mediated phagocytosis, and perhaps the diminished role when compared to FcγR (Allen and Aderem 1996) is due to lack of Syk-mediated activation. PCK certainly plays an important role in phagocytic uptake by several well-studied receptors such as FcγRs and CR3 (Allen and Aderem 1996), and Dectin-1 appears to be no exception. PKC may mediate phagocytic uptake by activating MARCKS, promoting actin-cross linking and providing membrane by focal exocytosis at the point of phagosome formation. PKC has also been shown to directly recruit p47phox, the essential element for the assembly of the phagosomal NADPH oxidase (Reeves, Dekker et al. 1999), and may thus be the mechanism by which Dectin-1 mediates ROI production (Gantner, Simmons et al. 2003). We were surprised to note that Toxin B did not completely block Zymosan phagocytosis when compared to SRBCs and it is unclear how actin polymerisation could occur without the participation of the Rho-GTPases.

7.3.2 Dectin-1 trafficking following internalisation

Figure 7.3.2 shows a working model for particulate and soluble ligand trafficking in Dectin-1 expressing cell populations. Particulate ligands such as fungi and zymosan are internalised by phagocytosis and remain bound to Dectin-1 throughout phagosomal maturation leading to the degradation of both receptor and ligand in the phagolysosomal compartment. Larger glucans such as glucan phosphate are internalised by endocytosis, yet endosomes are not rapidly trafficked to lysosomes. Instead, these large ligands may be sequestered in an endosomal compartment with delayed or limited lysosomal trafficking, possibly allowing for interactions with cytoplasmic components of phagocytes, such as PI3K, known to play a role in the regulation of immune responses (Li, Ha et al. 2004; Williams, Li et al. 2004). Small

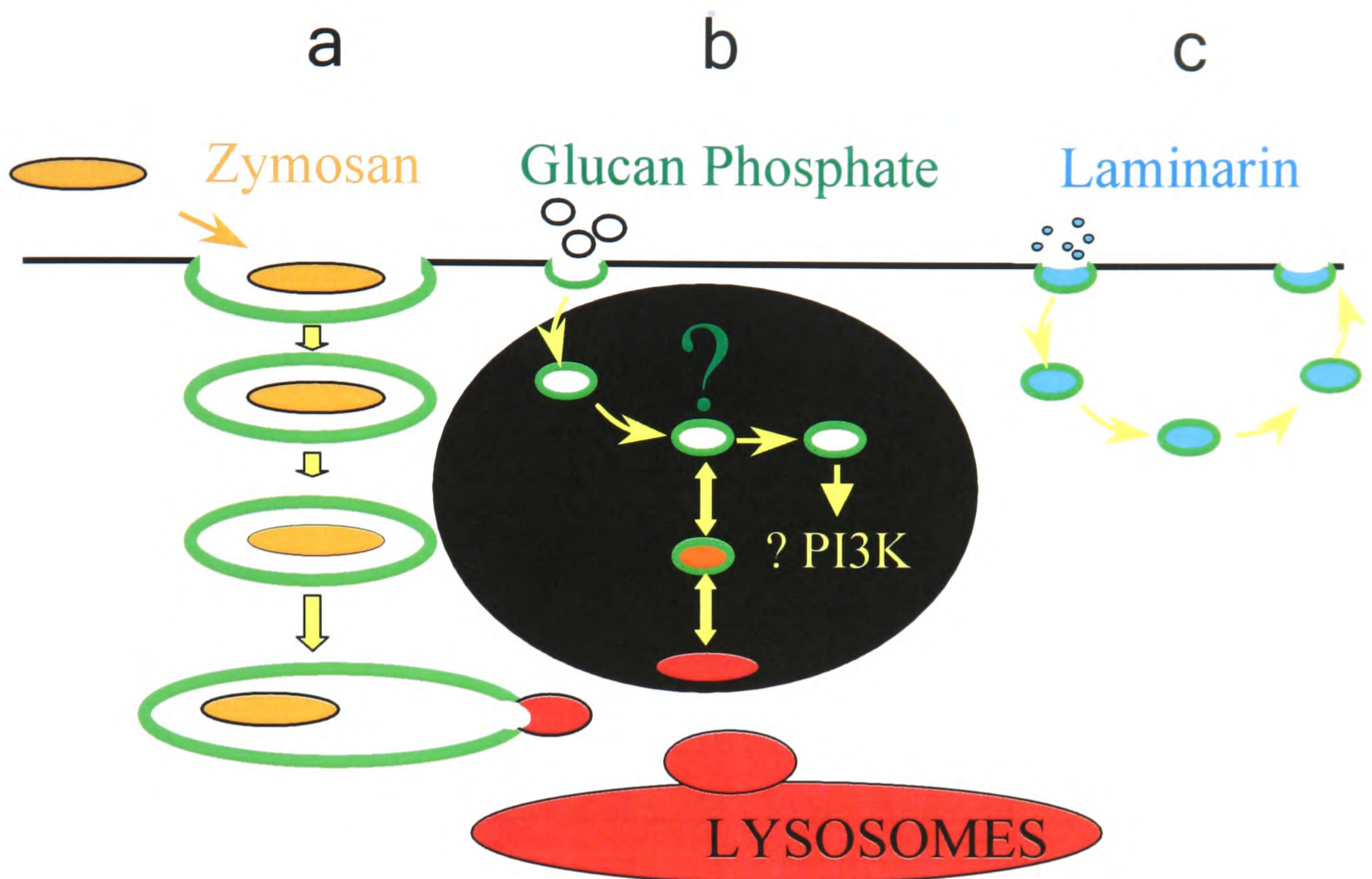


Figure 7.3.2

A working model for Dectin-1 receptor trafficking in response to different ligand sizes. a) Particulate ligands such as fungi and zymosan are internalised by phagocytosis and remain bound to Dectin-1 throughout phagosomal maturation leading to the degradation of both receptor and ligand. b) Larger glucans such as glucan phosphate are endocytosed, yet endosomes are not rapidly trafficked to lysosomes, instead these may be sequestered in an endosomal compartment with delayed lysosomal trafficking, allowing for interactions with components of phagocytes, such as PI3K, that regulate immune responses. c) Small ligands such as Laminarin are rapidly internalised and are released from Dectin-1, allowing the receptor to recycle back to the cell surface without modulating cellular responses.

ligands such as Laminarin are rapidly internalised and are released from Dectin-1, allowing the receptor to recycle back to the cell surface. The lack of intracellular retention may reflect insufficient time for immunomodulatory signalling to occur, or the inability of small clusters of Dectin-1 molecules to engage the appropriate cytoplasmic machinery required to produce immunomodulation. These working models will be further developed in future studies to resolve the outstanding questions.

7.4 Future work

The work described in this thesis forms the basis for a more comprehensive analysis of the role of Dectin-1 in anti-fungal immunity. An important tool not yet available is the genetically deficient mouse. Such animals are in preparation and should be available within a few months and will allow us to define whether Dectin-1 plays an essential role in anti-fungal defences *in vivo*.

There are a number of aspects of Dectin-1 mediated receptor signalling that require further detailed analysis. The significant but not essential role of the Src family kinases and the absence of a role for Syk in Dectin-1 mediated phagocytosis in macrophages require further investigation. We will use phosphorylated cytoplasmic tail constructs to immunoprecipitate cytoplasmic molecules recruited to the cytoplasmic tail. Further analysis will be carried out using antibody and glucan coated magnetic beads to isolate signalling complexes assembled by ligand clustered Dectin-1. Immunoblotting of membrane isolates and proteomic analysis of unknown bands may provide further clues to the mechanism by which Dectin-1 mediates its effects and help to clarify our observations.

Dectin-1 mediated endocytosis of soluble ligands has revealed an unusual pattern of receptor recycling and this, accompanied by evidence that lack of receptor recycling is associated with immunomodulatory effects, makes it important to identify the intracellular trafficking of Dectin-1 and its glucan cargo with higher resolution. We have recently characterised a monoclonal antibody that will recognise fixed Dectin-1, allowing us to study the receptor in fixed primary cells. We have also obtained fluorescinated soluble glucans of various sizes that may allow us to study receptor and cargo separately by confocal microscopy. The use of biotinylated soluble glucans may allow us to pulse cells and extract Dectin-1 containing endosomal membranes for biochemical analysis. We would also like to use RAW cells expressing mutant Dectin-1 molecules to assess whether interference with the cytoplasmic tail has any effect on endocytic uptake and, more importantly, trafficking.

How soluble glucans modulate immune responses *in vivo* remains unknown. In an attempt to identify possible mediators of this process we will use two related approaches. Macrophages cell lines expressing full-length Dectin-1 will be treated with various soluble glucans, and global changes in gene expression analysed by micro array. In parallel, cell lines treated with soluble glucans will be lysed and lysates run on 2D gels. Differentially expressed proteins will be targeted for identification and analysis. A synthesis of these two approaches may provide insights into the immunomodulatory programmes activated by large soluble glucan polymers.

7.5 Closing remarks

In this thesis we sought to analyse the functions of Dectin-1, the recently described PRR for fungal derived β -glucans. A number of novel findings resulted from this work.

Firstly we demonstrated that Dectin-1 is a phagocytic receptor and that this capacity is mediated by a tyrosine based ITAM-like motif. This makes Dectin-1 the first described phagocytic leukocyte expressed receptor for unopsonised fungi and fungal derived particles. We also showed that the mechanisms by which Dectin-1 mediates cytoskeletal activation and actin polymerisation are novel, and not shared with the canonical ITAM containing Fc γ R_s. Further novel findings include the demonstration that Dectin-1 mediates the biological effects of β -glucans in collaboration with the TLRs and that this occurs at the cell surface. Finally we showed that ligand size determines intracellular receptor trafficking and possibly also endosome mediated signalling. Dectin-1 is thus established as both an important fungal pattern recognition receptor and an additional tool with which to study the diversity of signalling processes associated with leukocyte expressed receptors.

Chapter 8

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Appendix

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