Protease Distribution in J774 Macrophages

by

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PREFACE

The experimental work described in this dissertation was carried out in the Department of Biochemistry, School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg from January 2005 to June 2006, under the supervision of Dr Edith Elliott.

These studies represent original work by the author and have not been submitted in any other form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

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Jaclyn McDowall
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ABSTRACT

Cathepsin, matrix metalloproteinase (MMP) enzyme and tissue inhibitor of MMP (TIMP) distribution in J774 mouse macrophages has not been comprehensively studied. The distribution and vesicle regulation, trafficking and release of these is important, possibly suggesting drug targets for the therapeutic regulation of inflammatory disease and phagosomal killing of pathogenic organisms in J774 and other macrophages. Percentage immunofluorescence and ultrastructural enzyme and inhibitor distribution, together with LysoTracker (acidity) and lysosome-associated membrane proteins (LAMPs) colocalisation (both indicating late endosome or "lysosomal" association), western blot estimates of percentage processed- and unprocessed intracellular and secreted enzyme and inhibitor, and vesicle size was used to assign enzyme and inhibitor to "classical" vesicle types. Antibodies against TIMP-1 and TIMP-2 were raised and all antibodies characterised for this purpose.

Together these were used to assign cathepsins H, S, D, B and L to possible secretory vesicles (±20 nm, non-acidic, LAMPs-negative, containing precursor enzymes) and identify at least 6 other endosome-"lysosome-like" vesicles. Cathepsin H appears to be present in classical early endosomes (±100 nm, non-acidic, LAMPs-negative) and cathepsin S in late endosomes (±50 nm, acidic, LAMPs-positive) and possibly "lysosomal" ("hybrid" or digestive organelles) (±150-200 nm, acidic, LAMPs-positive). Both cathepsins H and S, however, seem only reliable markers if used together with additional markers. Cathepsin D appears mainly associated with "lysosomal" ("hybrid" or digestive organelles) (±150-200 nm, acidic, LAMPs-positive), possibly consisting of further subpopulations which requires further investigation e.g. labelling for LAMP-1 and LAMP-2 and cathepsin D. Cathepsins B and L may occur in late endosomes and/or hybrid organelles and "secretory lysosomes" containing cathepsins B, D and L may also exist (±30-50 nm, acidic, LAMPs-positive).

The distribution of MMP-9, TIMP-1 and -2 in vesicles (non-acidic, LAMP-2-negative) that appear novel and distinct from late endosome-"lysosome" vesicles were also demonstrated. In LPS-stimulated cells, the identity of the large (±450 nm), possible recycling endosomes (Rab11-positive, LAMPs-negative), containing colocalised MMP-9 and TIMP-1, needs investigation i.e., requires further verification with triple labelling and EM. Possible cell membrane and recycling endosome localisation of TIMP-2 needs confirmation with labelling of non-permeabilised cells and labelling for MT1-MMP and proMMP-2, respectively.

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ε	extinction coefficient
λ	wavelength
Φ	quantum yield
%C	monomer crosslinking concentration
%T	total monomer concentration
ABTS	2,2'-azino-di(3-ethyl)-benzthiozoline sulfonic acid
ADAM	a disintegrin and metalloprotease domain
ADAM-TS	a disintegrin and metalloprotease domain with thrombospondin type-1 domains
ALG-2	apoptosis-linked gene -2
AMAC-1	alternative macrophage activation-associated chemokine-1
APCs	antigen presenting cells
ARP3	actin-like protein 3
ATPase	adenosine triphosphatase
BCIP	5-bromo-4-chloro-3-indolyl phophate
bFGF	basic fibroblast growth factor
BIP	binding protein
BM-40 (SPARC)	osteonectin
BPI	bactericidal/permeability increasing protein
Brij 35	polyoxyethylene (23) lauryl alcohol
BSA	bovine serum albumin
CABP1	calcium-binding protein 1
CAP 1	adenylyl cyclase-associated protein 1
CAPZ	actin capping protein
Cat	cathepsin
CBB	Coomassie brilliant blue
CD	cluster determinant/cluster of differentiation
C_{H1}	immunoglobulin heavy chain constant region 1
C_{H2}	immunoglobulin heavy chain constant region 2
COPD	chronic obstructive pulmonary disease

COPI	coat protein I
CR	complement receptor
CRAMP	cathelicidin-related antimicrobial peptide
CTGF	connective tissue growth factor
CT-PCPE	C-terminal fragment of procollagen C-terminal proteinase enhancer
CY3	cyanine3
CyCAP	cyclophilin C-associated protein
Da	dalton(s)
DAB	diaminobenzidine/3,3',4,4'-tetraaminobiphenyl
DAMP	3-(2,4-dinitroanilino)-3'-amino-*N*-methyldipropylamine
DEC-205	dendritic epidermal T cell -205
dH_2O	distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMF	dimethylformamide
DNP	2,4-dintrophenol
DPPII	dipeptidyl peptidase II
DTT	dithiothreitol
ECM	extracellular matrix
ECV(s)	endosome carrier vesicle(s)
EDTA	ethylene diamine tetra acetic acid
EEA1	early endosomal antigen 1
EF-TU	elongation factor Tu
EGTA	ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*,*N*',*N*'-tetra acetic acid
ELISA	enzyme linked immunosorbent assay
EM	electron microscope/microscopy
ENA-78	epithelial neutrophil activating peptide
ER	endoplasmic reticulum
ERP	leucine-responsive regulatory protein
F	"free" (i.e soluble)
Fc	fragment crystallisable
FcγRs	receptors binding the Fc region of the indicated Ig molecule (γ for IgG)

FCA	Freund's complete adjuvant
FCS	foetal calf serum
FIA	Freund's incomplete adjuvant
FITC	fluorescein isothiocyanate
fMLP	formylmethionyl-leucyl-phenylalanine
FSG	fish skin gelatin
g	relative centrifugal force
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCAP39	calcium ion- and polyphosphoinositide-regulated actin capping protein
GDI	GDP-dissociation inhibitor
GFP	green fluorescent protein
GILT	IFN-γ-inducible lysosomal thiol reductase
GM-CSF	granulocyte-macrophage colony stimulating factor
GPI	glycosylphosphatidylinositol
GRB2	growth factor receptor bound protein 2
GRO	growth-related oncogene protein
GRP	glycine-rich protein
GTPase	guanosine triphosphatase
h	hour(s)
HBSS	Hank's Balanced Salts
hCAP-18	human cathelicidin protein
Hck	hematopoietic cell kinase
HEPES	*N*-2-hydroxy-piperazine-*N*'-2 ethane sulfonic acid
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
HSC	hemopoietic stem cell
HSP	heat shock protein
ICAM	intercellular adhesion molecule
IFN-γ	interferon-γ
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IgG, IgY, IgA, IgE	immunoglobulins G, Y, A, E
Ii	invariant chain

IL	interleukin
IL-1RA	interleukin-1 receptor antagonist
iNOS	inducible NO synthase
IP-10, -20	IFN-inducible protein-10, -20
ITAMs	immunoreceptor tyrosine-based activation motifs
kDa	kilodalton(s)
l	litre(s)
LAMP(s)	lysosomal-associated membrane protein(s)
LBPs	latex bead containing phagosomes
LE	large, electron-dense, late endosome-like
LIMP II	lysosomal integral membrane protein
LPS	lipopolysaccharide
LT	large, translucent, early endosome-like
LYAAT	lysosomal amino acid transporter
LYCAM	lysozyme C, type M
Lys	lysosome-like i.e. “hybrid” organelle or digestive body
M	membrane-bound
mA	milliAmp
MAC-2	galectin-3
MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage colony-stimulating factor
MDM(s)	monocyte-derived macrophage(s)
MFG-E8	milk fat globule-epidermal growth factor 8
MHC	major histocompatibility complex
min	minute(s)
MIP-1α	macrophage-inflammatory protein 1α
MPO	myeloperoxidase
MMP(s)	matrix metalloproteinase(s)
MOPS	3-(*N*-morpholino)propanesulfonic acid
MPR(s)	mannose-6-phosphate receptor(s)
MPS	mononuclear phagocyte system
MPS1	mitogen-activated protein kinase
M_r	relative molecular mass
MR	mannose receptor

MS-1	MS-1 high-molecular-weight protein
MT-MMP	membrane-type matrix metalloproteinase
MVB(s)	multivesicular body(ies)
MWM	molecular weight marker
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
NC	non-collagenous
ND	not determined
NDK B	nucleoside diphosphate kinase
NGAL	neutrophil gelatinase-associated lipocalin
nm	nanometer (10^{-9} meter)
NO	nitric oxide
NSF	*N*-ethyl-maleimide sensitive fusion protein
O_2^-	superoxide
$^1O_2^\bullet$	singlet oxygen
OCl	hypochlorite
$OH^\bullet$	hydroxyl radical
ORP	oxygen-regulated protein
P21RAS	GTPase HRas precursor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDI	protein disulfide isomerase
PEG	polyethylene glycol
PFA	paraformaldehyde
PGAM	phosphoglycerate mutase
PGE_2	prostaglandin E_2
PHEM	PIPES, HEPES, EGTA and $MgCl_2$ buffer
PIPES	piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)
PMN(s)	polymorphonuclear leukocyte(s)
Ponceau S	3-hydroxy-4-[2-sulfo-4(sulfo-phenylazo)phenylazo]2,7-naphthalene disulfonic acid
PRR(s)	pattern-recognition receptor(s)
PVDF	polyvinyldifluoride

RANTES	regulated upon activation, normal T-cells, expressed and secreted
RAP1B	GTP-binding protein smg p21B
RASI	rheumatoid arthritis synovial inflammation
RECK	reversion-inducing cysteine-rich protein with kazal motifs
RSP	radial spoke protein
RT	room temperature
s	second(s)
S	small, secretory vesicle-like
SL	"secretory lysosome"
SCAMP	secretory carrier membrane protein
SDS	sodium dodecyl sulfate
SNAP	soluble NSF attaching protein
SNAP-23	synaptosome-associated protein-23
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
• SO^-_4	sulfate radical
SR	scavenger receptor
TACE	tumour necrosis factor-α converting enzyme
TACO	tryptophane aspartate containing coat protein
TBS	Tris buffered saline
TCBP	taipoxin-associated calcium binding protein
TCP	T complex polypeptide
TEM	transmission electron microscopy
TGF	transforming growth factor
Th1	T-helper cell type 1
Th2	T-helper cell type 2
TEMED	*N*,*N*,*N*',*N*',-tetramethyl ethylenediamine
TFPI-2	tissue factor pathway inhibitor-2
TIMP(s)	tissue inhibitor of matrix metalloproteinase(s)
TLR	toll-like receptor
TMB	tetramethylbenzidine

TMP	tyrosinase mimetic peptide
TNF	tumour necrosis factor
TRAIL	tumour necrosis factor-related apoptosis inducing ligand
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
TRITC	tetramethyl rhodamine isothiocyanate
Triton X-100	polyoxyethylene (9-10) *p-t*-octyl phenol
UDP	uridine diphosphate
UDPGT	UDP-glucuronosyltransferase
UV	ultraviolet
V	volts
VAMP-2	vesicle-associated membrane protein-2
VAP	virion-associated protein
V-ATPase	vacuolar-ATPase
VDAC	voltage-dependent anion channel
VEGF	vasoactive epidermal growth factor
μm	micrometer (10^{-6} meter)

CHAPTER 1

INTRODUCTION

1.1 Macrophages

The macrophage or activated blood monocyte is an extremely important cell, second to the site of infection after the neutrophils, orchestrating many of the body's responses to infection, via cytokine release (Ross and Auger, 2002). Monocytes/macrophages, however, seem less effective in killing rapidly growing microorganisms (most pathogens) but more effective in killing slow replicating organisms, such as *Mycobacterium tuberculosis* (Heale and Speert, 2002). Certain pathogens, however, have evolved ways of evading killing by the macrophage under certain circumstances and the emerging view now suggests that the major killing event avoided is the fusion of protease-containing vesicles with the phagosome (Anes *et al*., 2006). Which proteases are important in such fusion events, their distribution and regulation remains largely unknown.

The distribution of proteases in macrophages, or protease colocalisation and hence patterns of fusion of proteases with the phagosome, characteristics of the protease-containing vesicle populations (i.e. whether acidic and lysosome-like or non-acidic and whether the proteases are stored active or inactive), is also largely unknown and form the focus of this dissertation. Before what is known about the protease distribution is described, some background about macrophages will be given as macrophage- or activated monocyte phenotypes are strongly influenced by their environment. They also differ phenotypically depending upon their species (e.g. mouse macrophages vary slightly from human macrophages). These facts complicate studies of these cells, potentially invalidating generalisations.

The term 'macrophage' (Greek: big eater) was first used by Elie Metchnikoff more than 100 years ago to describe large mononuclear phagocytic cells (Oppenheim and Leonard, 1989; Ross and Auger, 2002). The most differentiated cells of the mononuclear phagocyte system (MPS) that is comprised of bone marrow monoblasts, promonocytes, blood monocytes, are the tissue and blood macrophages (Ross and Auger, 2002). The monoblast is the least mature cell of this system. Division of this cell results in two promonocytes or precursors of the monocyte. These enter the blood stream, migrate to various organs, and differentiate into

tissue macrophages (Table 1.1) that are widely distributed and show great structural and functional heterogeneity (Roitt, 1997; Ross and Auger, 2002).

Table 1.1 **Distribution and names of monocytes/macrophages in the body.**

Location	Name
Bone marrow	Monoblasts, promonocytes, monocytes, macrophages
Peripheral blood	Monocytes
Lungs	Alveolar macrophages
Liver	Kupffer cells
Bones	Osteoclasts
Kidneys	Glomerular mesangial cells
Skin	Langerhans cells
Spleen	Splenic macrophages/fixed tissue macrophages
Central nervous system	Microglial cells
Connective tissue	Histiocytes
Lymph nodes	Monocytes/macrophages
Thymus	Fixed tissue macrophages
Endocrine organs	Monocytes/macrophages
Peritoneal cavity	Peritoneal macrophages
Lamina propria	Fixed tissue macrophages

(Roitt, 1997; Handel-Fernandez and Lopez, 2000; Ross and Auger, 2002).

Circulating monocytes and tissue macrophages are phylogenetically primitive cells (Varesio *et al*., 2000; Ross and Auger, 2002). During the course of evolution, they have retained certain amoeboid characteristics and functions, including their mobility and capacity for phagocytosis. Their additional capacity for secretion of immunoregulatory cytokines and their receptors enable them to interact with and shape the reactivity of the cells of the immune system.

Phagocytosis describes the engulfment of large particles (van Oss, 1986; May and Machesky, 2001) and constitutes an innate response of the immune system triggered by the engagement of various receptors (Section 1.2). In addition to phagocytosis and cytokine release, that can alter the behaviour of phagocytes and many other cell types, macrophages secrete a variety of other important molecules including cytotoxic radicals of oxygen and nitrogen, enzymes that degrade the extracellular matrix (ECM) and lipid mediators of inflammation (Rabinovitch, 1995) (Section 1.3).

1.2 Phagocytosis

The professional phagocytes including, polymorphonuclear leukocytes (PMNs), monocytes and macrophages are responsible for phagocytosis in mammals. In macrophages, phagocytosis is required for pathogen destruction, antigen presentation, tissue remodelling

and inflammation (Rabinovitch, 1995) and involves the engulfment of particles that are usually larger than 0.5 μm in diameter (Allen and Aderem, 1996; May and Machesky, 2001; Ross and Auger, 2002). It has been shown, however, that particles as small as 0.13-0.26 μm and as large as 3.0 μm have been effectively phagocytosed (Desjardins and Griffiths, 2003).

The core mechanisms of phagocytosis are exceedingly complex but can be broken down into several steps. Firstly, the uptake of the foreign particle is initiated by the interaction of specific receptors on the surface of the macrophage with particular ligands on the surface of the particle (Aderem, 2003). Secondly, signals are produced that stimulate actin-polymerisation under the membrane at the site of contact (Aderem, 2003; Desjardins and Griffiths, 2003). Thirdly, actin-rich pseudopods extend around the particle resulting in its internalisation (Allen and Aderem, 1996; Aderem, 2003). After internalisation is complete, the phagosome matures via a series of fusion and fission events with components of the endocytic pathway (endosomes and lysosomes) as well as other vesicle types. The phagosome thus acquires a variety of proteins including acid hydrolases and proton ATPases, allowing phagosomal acidification and the development of a microbicidal organelle (Mellman, 1992; Desjardins and Griffiths, 2003).

1.2.1 Receptors involved in phagocytosis

Macrophages express a variety of phagocytic receptors that can be divided into two main groups. The first group are the opsonin-dependent receptors. These need the foreign particles to be coated by an opsonin and include the complement receptor type 3, Fc receptors, C1q- and CD14 receptor that bind to particles coated by iC3b, antibodies, C1q and lipopolysaccharide (LPS)-binding proteins, respectively (Peiser *et al*., 2000). The second group are pattern-recognition receptors (PRRs) including the mannose receptor (MR), the MR-like T cell-205 receptor (DEC-205), CD36 and scavenger receptor-A (SR-A), these recognise conserved motifs on pathogens that are not found on higher eukaryotes. These motifs are essential to the biology of the pathogen and, therefore, are not subject to high mutation rates making them good targets for pathogen recognition, binding and uptake (Peiser *et al*., 2000; Aderem, 2003).

1.2.1.1 Fc-receptor-mediated phagocytosis

Fcγ Receptors (FcγRs) for the Fc region of the major opsonin, IgG, were the first macrophage receptors to be identified (Ross and Auger, 2002). Studies have since defined at

least three distinct FcγRs, FcγRI, FcγRII and FcγRIII in macrophages (Shibata *et al*., 1991; Kusner *et al*., 1999). In addition to IgG, IgA and IgE also have cognate receptors, Fcα and Fcε, respectively (May and Machesky, 2001). Binding of IgG to the FcγRs activates a wide variety of antimicrobial responses, including secretion of various antimicrobial agents, cytokine synthesis and secretion, the production of pro-inflammatory lipids such as arachidonic acid and reactive oxygen intermediates (Aderem and Underhill, 1999; Kusner *et al*., 1999). IgG-FcγR interaction may result in phosphorylation of specific receptor tyrosine residues in immunoreceptor tyrosine-based activation motifs (ITAMs) essential for phagocytosis (Greenberg *et al*., 1993). Tyrosine kinase-dependent activation of phospholipase D results and appears to regulate the ingestion of IgG-opsonised particles (Kusner *et al*., 1999). The monomeric GTPase, Rho is subsequently essential for the accumulation of phosphotyrosine and of F-actin around phagocytic cups as well as for Fcγ-receptor-mediated Ca^{2+} signalling. This results in differential vesicle fusion with the phagosome (Hackam *et al*., 1997).

1.2.1.2 Complement-receptor-mediated phagocytosis

Unlike Fc-receptor-mediated phagocytosis, complement receptor-mediated phagocytosis involves minimal membrane disturbance. Complement-opsonised particles appear to "sink" into the phagocyte and internalisation does not normally result in an inflammatory response or respiratory burst (May and Machesky, 2001). Following complement activation C3b containing a thioester group, can covalently bind to hydroxyl or amino groups on the microbial surface. The deposited C3b acts as an opsonin and is recognised by complement receptor (CR)1 (CD35), CR4 (CD11c/CD18), and, after its conversion to iC3b, by CR3 (CD11b/CD18) (May and Machesky, 2001; Ross and Auger, 2002). Phagocytosis of iC3b particles occurs only if the macrophage has been activated, for example, by cytokines. Activation causes a conformational change in the receptor, resulting in the clustering of receptors (necessary for the binding), allowing transduction of the phagocytic signal and the subsequent uptake of the foreign particle (Allen and Aderem, 1996; May and Machesky, 2001).

1.2.2 The phagosome and phagosome maturation

Despite the considerable membrane expanse that is internalised during phagocytosis, no net loss of surface membrane has been detected suggesting that exocytosis of membranes occurs in conjunction with phagocytosis (Hackam *et al*., 2001). Fission of vesicles from the

phagosomal membrane is partially dependent on coat protein I (COPI) which is also involved in budding from endosomes and the Golgi (Botelho *et al*., 2000) and is indirectly required for phagocytosis as it helps to maintain a pool of vesicles which fuse with the plasma membrane and promote the extension of pseudopodia around the particle to be engulfed (Hackam *et al*., 2001). Vesicles, possibly recycling endosomes, bud off the phagosome and appear to fuse with the plasma membrane near the developing phagosome, compensating for lost plasma membrane (Greenberg and Grinstein, 2002).

Recently, Gagnon *et al*. (2002) provided new insight into phagocytosis by showing that when macrophages phagocytose particles, most of the phagosomal membrane is not derived from the plasma membrane but rather from the endoplasmic reticulum (ER). The binding of a foreign particle to the macrophage cell surface initiates the formation of pseudopodia, attaching the particle to the plasma membrane. The ER is subsequently recruited to the surface of the cell where it fuses with the plasma membrane and opens at the site of contact. The particle slides into the ER membrane and the membrane closes, resulting in a phagosome made primarily from ER membrane (Gagnon *et al*., 2002). ER-mediated phagocytosis can be used to explain the presence of ER proteins in isolated latex bead containing phagosomes (LBPs) (Garin *et al*., 2001). It also explains how antigens from phagosomal pathogens can be presented by MHC class I molecules, a process normally associated with the ER, instead of MHC class II molecules normally associated with phagocytosis and MHC class II presentation (Kleijmeer *et al*., 2001).

Irrespective of how the phagosome forms, phagosome maturation occurs via a series of fusion and fission events involving organelles of the endocytic pathway, the early endosomes, late endosomes and finally lysosomes as well as other vesicle populations. This results in the formation of the phagolysosome, a hybrid organelle with bactericidal properties (Desjardins *et al*., 1994a; Desjardins *et al*., 1997; Vieira *et al*., 2002; Griffiths, 2004; Niedergang and Chavrier, 2004). There is also evidence to suggest that when phagosomes fuse with endosomes and lysosomes they do not necessarily form a single organelle (Desjardins, 1995; Duclos *et al*., 2000). Phagosomes may experience a transient and partial fusion with endocytic organelles. This is known as ‘the kiss’, allowing for the transfer of membrane and luminal contents, generating what is often called a “phagolysosome”. Subsequently, a fission event, known as ‘the run’ occurs, preventing the mixing of the two compartments and the two compartments separate (Desjardins, 1995). Soluble *N*-

ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and Rab5 have been shown to be involved in the transient fusion events between phagosomes and early endosomes, deactivation of Rab5 resulting in uncontrolled fusion events and the formation of giant phagosomes (Duclos *et al*., 2000; Niedergang and Chavrier, 2004).

1.3 Microbicidal killing mechanisms

Phagolysosomes possess a variety of degradative properties including a very low pH, hydrolytic enzymes for particle digestion, antimicrobial peptides and the ability to produce oxidative compounds (Table 1.2) (Tjelle *et al*., 2000; Heale and Speert, 2002; Vieira *et al*., 2002). It has been assumed that macrophages are capable of killing phagocytosed microbes by both oxidative and non-oxidative mechanisms as extrapolated from neutrophils (Ross and Auger, 2002).

Table 1.2 **Microbicidal agents produced by macrophages upon ingestion of microorganisms.**

Class of mechanism	Specific Products
Acidification	pH ~ 3.5-4.0, bacteriostatic or bactericidal
Toxic oxygen derived products	O_2^-, H_2O_2, $^1O_2^\bullet$,$OH^\bullet$, OCl
Toxic nitrogen oxides	NO
Antimicrobial peptides	Defensins and cationic proteins
Enzymes	Lysozyme (dissolves cell walls of certain gram-positive bacteria), acid hydrolases (further digest bacteria)

Abbreviations: O_2^-, superoxide; H_2O_2, hydrogen peroxide; $^1O_2^\bullet$, singlet oxygen; $OH^\bullet$, hydroxyl radical; OCl, hypochlorite; NO, nitric oxide. (Janeway *et al*., 2001; Heale and Speert, 2002).

Like neutrophils, macrophages produce, and intracellularly release, reactive oxygen intermediates (Ross and Auger, 2002). The exposure to specific stimuli, results in a greatly enhanced oxygen uptake, especially in neutrophils, and gives rise to what is known as 'respiratory burst' (Babior, 1984; Labro, 2000). During such events cells produce large amounts of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), intermediates that are not directly used in microbial killing but rather in the production of bacteriological oxidised halogens and other oxidising radicals (Babior, 1984). As already mentioned, macrophage microbicidal power varies between different activation states and is generally inferior to that of circulating neutrophils as they posses a less potent respiratory burst in comparison to neutrophils (Labro, 2000; Heale and Speert, 2002). Evidence also suggests that maturation of monocytes into macrophages is accompanied by a loss of microbicidal activity. This appears to be due to a decreased capacity for O_2^-, H_2O_2 and myeloperoxidase (MPO) production (Nakagawara *et al*., 1981; Ross and Auger, 2002). It has also been suggested that

macrophages are unable to produce significant amounts of reactive oxygen intermediates in the phagosome, as they appear to lack the large pool of NADPH-oxidase components found in neutrophils (Johansson *et al*., 1995).

In addition to oxygen-dependent mechanisms, macrophages are also equipped with oxygen-independent killing mechanisms. Many vesicle-associated proteins have been shown to have antimicrobial activity. These include lysozyme, elastase, collagenases, lipases, sulfatases, phosphatases, defensins, polysaccharides, cathepsins and deoxyribonucleases (Heale and Speert, 2002; Selsted and Ouellette, 1995; Ross and Auger, 2002). These substances are delivered to the phagosome as the various compartments of the endocytic pathway fuse sequentially with the phagosome during the process of phagosomal maturation (Vieira *et al*., 2002; Henry *et al*., 2004).

Some years ago cathepsins B and D, released into the phagosome, were shown to possess bactericidal properties, including the ability to effect lysis of lysozyme-resistant *Staphylococcus aureus* and rendering *Acinetobacter* 199A sensitive to lysozyme (Thorne *et al*., 1976). Despite this, until recently (Anes *et al*., 2006; Del Cerro-Vadillo *et al*., 2006), a direct role for macrophage proteases in controlling bacterial infection had not been established and it was thought that the proteases mainly performed protein turnover functions in the phagosome (Heale and Speert, 2002; Rosenberger *et al*., 2004). Murine macrophages have been shown to use a combination of proteases and cationic peptides to limit the growth of intracellular bacterial pathogens (Rosenberger *et al*., 2004). Multiplication of *Salmonella typhimurium* inside infected murine splenic and liver macrophages has also been shown to be controlled using increased levels of reactive oxygen intermediates, increased expression of the murine cathelicidin-related antimicrobial peptide (CRAMP), activated by an intracellular macrophage elastase-like serine proteases (Rosenberger *et al*., 2004). This study demonstrated the importance of antimicrobial peptides in the destruction of macrophage bacterial pathogens and, that independent killing mechanisms in macrophages, reactive oxygen species as well as intracellular proteases, cooperate and complement each other in impairing bacterial growth.

A recent study has also identified cathepsin D as an important non-oxidative bactericidal agent effective against *Listeria monocytogenes* infection in both macrophages and fibroblasts (Del Cerro-Vadillo *et al*., 2006). Acid sphingomyelinase was shown to produce ceramide, a

signaling molecule that activates cathepsin D, and the lack of ceramide, and hence cathepsin D activation in acid sphingomyelinase knockout mice appears to correlate with the survival of *L. monocytogenes*. Phagosomes containing cathepsin D were also listericidal whereas those lacking cathepsin D were not (Heinrich *et al*., 1999; Prada-Delgado *et al*., 2001; Utermohlen *et al*., 2003). Del Cerro-Vadillo *et al*. (2006) also observed that fibroblasts and bone-marrow macrophages from cathepsin D-deficient mice showed enhanced susceptibility to *L. monocytogenes* infection in comparison with wild types. A further finding suggests that Rab5a is also required for the activation of cathepsin D, and cathepsin D targets the main virulence factor listeriolysin O of *L. monocytogenes*, limiting the capacity for survival in the phagosome. The involvement of Rab5a suggests that some vesicles containing precursor (inactive) cathepsin D need to fuse with an early endosomal compartment (Rab5a-positive) in order to become active. This is unexpected as the dogma states that activation of most cathepsins occurs in acidic, late endosomal compartments (Turk *et al*., 2001; Bühling *et al*., 2004).

For the killing of *Mycobacterium smegmatis* in J774 macrophages, Anes *et al*. (2006) showed that J774 cells used dynamic interactions of various compartments and conditions to fight the live bacterium in the phagosome. Anes *et al*. (2006) proposed three different killing phases (Figure 1.1).

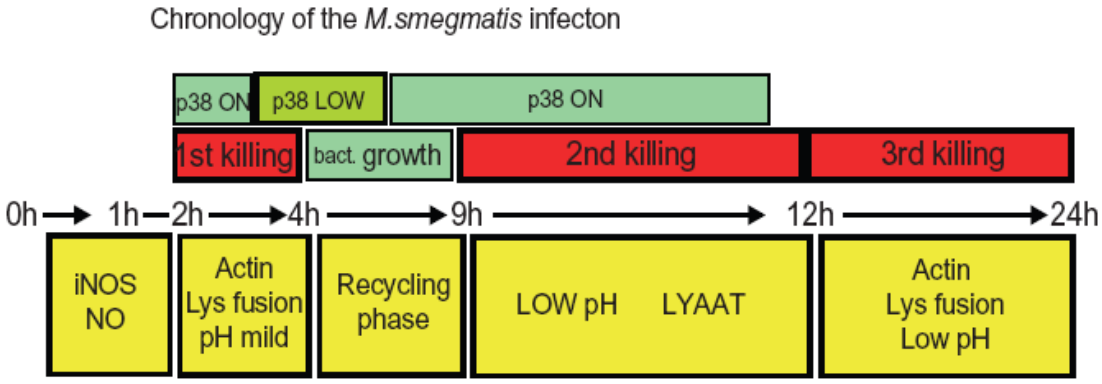

Figure 1.1 **Schematic diagram showing the killing mechanisms used by J774 macrophages to control *Mycobacterium smegmatis* infection.**
The killing of *M. smegmatis* is a dynamic process involving initial periods of killing, followed by bacterial growth and two subsequent killing phases. NO synthesis was the first killing factor and functioned in the first killing phase only. Phagosome actin assembly and fusion with late endocytic compartments occurred during both the first and last killing phases. The recycling of phagosomal content as well as membrane occurred simultaneously with bacterial growth. Phagosomal acidification was prominent in the second and third killing phases. Map kinase p38 was an important regulator in most of the processes except for NO synthesis (Anes *et al*., 2006). Abbreviations: p38, map kinase p38; NO, nitric oxide; iNOS, inducible NO synthase; LYAAT, lysosomal amino acid transporter; Lys, lysosome.

The first phase was characterised by NO synthesis which stopped before the beginning of the second killing phase (Figure 1.1). During the first phase macrophages also utilised their second killing mechanism, the fusion of late endosomes and lysosomes with the phagosome. It was shown that lysosomal enzymes were responsible for some bacterial killing as macrophages preloaded with an inhibitor cocktail for lysosomal enzymes had reduced killing effects. The initial killing stage was followed by a stage of bacterial growth, which interestingly coincided with the recycling of both lysosomal-associated membrane protein-1 (LAMP-1) and the gold content marker (both markers for late endosomes and lysosomes) out of phagosomes containing live bacteria. The second phase of killing occurred between 9 and 12 h after infection, suggesting that fusion of compartments with the phagosome must have occurred prior to the beginning of this stage (Figure 1.1). Interestingly, these compartments appeared to be distinct from the regular late endosomes and lysosomes as the bulk of the gold content and LAMP-1 markers had been recycled out of the phagosome by this time and were subsequently only re-acquired during the third killing stage. The third killing stage was characterised by the fusion of late endosomes and lysosomes with the phagosome and the formation of an acidic phagosomal compartment (Figure 1.1). This study demonstrated that J774 macrophages use several killing mechanisms which, appear to be more successful in combating bacterial infection if used during specific periods as opposed to being utilised continuously during the infection control process. The precise identity of which protease was responsible for killing *M. smegmatis* is still, however, unknown and needs investigation.

1.4 Content of the macrophage

As previously mentioned, macrophages are capable of synthesising a vast number of different products. These include not only enzymes but many other biologically active compounds (Nathan *et al*., 1987; Ross and Auger, 2002). Lysosomal proteases including cathepsins B, D, L, S, H and Z, LAMP-1 and -2, (Claus *et al*., 1998; Garin *et al*., 2001), matrix metalloproteinases (MMPs) -1, -2, -3 and -9, (Goetzl *et al*., 1996) and tissue inhibitor of metalloproteinases (TIMPs), -1 and -2 (Triebel *et al*., 1995) to name a select few, have all been identified in macrophages. Many of these macrophage products can be secreted either into the extracellular environment or into the phagosome and are listed in Table 1.3. It was also obvious at the outset of this study that the vesicle populations of the macrophage in no way resemble the large granule populations of the neutrophils and characterisation of vesicle populations would possibly not be a simple task.

Table 1.3 **Products synthesised and secreted by macrophages.**

Enzymes	**Acrachidonic acid intermediates**
Lysozyme	Cyclooxygenase products:
Lysosomal acid hydrolases:	PGE_2, prostacyclin, thromboxane
lipases, proteases, (deoxy)ribonuclease,	Lipooxygenase products:
phosphatases, glycosidases, sulfatases	Hydroxyeicosotetranic acids, leukotrienes
Neutral proteases:	Platelet-activating factors
collagenase, elastase, myelinase, angiotensin, convertase, plasminogen activator, cytolytic proteinase, lipases, lipoprotein lipase, phospholipase A2, arginase	
Enzyme and cytokine inhibitors	**Coagulation factors**
Protease inhibitors:	Tissue factor
α_2-macroglobulin, α1-antitrypsin inhibitor,	Prothrombin activator
plasminogen activator inhibitor, collagenase inhibitor	Coagulation factors II, VII, IX, X, XIII
Phospholipase inhibitor	Plasminogen activator
IL-1 inhibitors	
Complement components	**Cytokines**
Classical pathway:	IL-1, IL-6, IL-10, IL-12, IL-15, IL-18
C1, C4, C2, C3, C5	TNF-α
Alternative pathway:	Interferon-α and -γ
factor B, factor D, properdin	Platelet-derived growth factors
Active fragments:	Fibroblast growth factor
C3a, C3b, C5a, Bb	Transforming growth factor-β
Inhibitors:	GM-CSF
C3b inactivator, β-1H	M-CSF
	Erythropoietin
	Factor inducing monocytopoieisis
	Angiogenesis factor
	CXC chemokines (IL-8, GRO, ENA-78, IP-10)
Reactive oxygen and nitrogen intermediates	**Others**
O_2^-	Thrombospondin
H_2O_2	Fibronectin
$OH^\bullet$	Lipocortin
NO	Transcobalamin II
Peroxynitrite	Transferrin
	Ferritin
	Haptoglobin
	Glutathione
	Uric acid
	Apolipoprotein
	Neopterin

Abbreviations: PGE_2, prostaglandin E_2; IL, interleukin; TNF-α, tumour necrosis factor-α; GM-CSF, granulocyte-macrophage colony stimulating factor; M-CSF, macrophage colony-stimulating factor; GRO, growth-related oncogene protein; ENA-78, epithelial neutrophil activating peptide; IP-10; IFN, interferon; IFN-inducible protein-10; O_2^-, superoxide; H_2O_2, hydrogen peroxide; $OH^\bullet$, hydroxyl radical, NO, nitric oxide (Nathan, 1987; Ross and Auger, 2002).

Using proteomic analysis of LBPs, which are easily isolated by density-shift methods, Garin *et al*. (2001) identified more than 140 proteins in isolated J774 phagosomes (Table 1.4) and Morrissette *et al*. (1999) generated approximately 150 monoclonal antibodies to phagosomal proteins. It is assumed that as the phagosome is not a pre-existing organelle its protein content arises by fusion with a series of vesicular compartments, including the

various organelles of the endocytic pathway (Tapper, 1996; Jahraus *et al*., 1998). Therefore, the proteins identified in the phagosome by Garin *et al*. (2001) and Morrissette *et al*. (1999) will be vesicle-derived. How many proteins have eluded identification to date is unknown. It is interesting that no MMPs and their inhibitors are mentioned in the phagosome content (Morrissette *et al*., 1999; Garin *et al*., 2001). It is, therefore, possible that these have an exclusively extracellular role as many are known to be involved in cytokine processing (Table 1.8 and Table 1.9) (Somerville *et al*., 2003; Folgueras *et al*., 2004).

The following sections focus on the cathepsins, MMPs and TIMPs found in the macrophage and are, therefore, expected to be present in the vesicular compartments of the macrophage at some point. One of the aims of the present study was also to establish which proteases and proteins are localised in the phagosome and hence could possibly be responsible for killing of microorganisms and phagosome fusion (Table 1.4).

Table 1.4 **Identified phagosomal proteins.**

Protein	Remark	Protein	Remark
14-3-3	Involved in exocytosis through actin interaction.	Acid ceramidase	Lysosomal.
A-X actin	Cytoskeletal protein.	β-actin	Cytoskeletal protein.
γ-actin	Cytoskeletal protein.	Alix	Programmed cell death 6-interacting protein. Implicated in apoptosis, with ALG-2.
Annexin 5	Amount bound to phagosome stays approximately the same as the phagosome matures.	Apolipoprotein D	Transports a variety of ligands in a number of different contexts.
ARP3	Actin-like protein 3. Cytoskeletal protein.	Arylsulfatase B	Lysosomal.
Ash	-	ATP synth. β	Mitochondrial protein.
CABP1	Calcium-binding protein 1. Probable PDI P5 precursor.	Calnexin	Retains incorrectly folded glycoproteins in the ER.
Calreticulin	ER chaperone. Found T-cell lytic granules.	CAP 1	Adenylyl cyclase-associated protein 1. Located on cell membrane.
CAPZ (α-actinin)	F-actin capping protein β subunit isoform 2.	Cathepsin A	Lysosomal protective protein; carboxypeptidase C.
Cathepsin B	Lysosomal cysteine protease.	Cathepsin D	Lysosomal aspartic protease.
Cathepsin L	Lysosomal cysteine protease.	Cathepsin S	Lysosomal cysteine protease.
Cathepsin Z	Lysosomal cysteine protease.	Coronin	Shared homology with TACO.
CyCAP	Cyclophilin C-associated protein. Lysosomal.	Cytochrome P450	ER-membrane bound protein
EEA1	Rab5 effector. Early endosome/phagosome.	Elongation factor 1-a 1	EF-TU.
Endoplasmin	ER protein.	α-enolase	2-phospho-D-glycerate hydro-lyase. Cytoplasmic.
Epididymal secretory protein	Unknown function.	ERP29	Found in the lumen of the ER.

Protein	Remark	Protein	Remark
Ferritin heavy chain	Stores iron in a soluble, non-toxic, readily available form.	Ferritin light chain 1	Stores iron in a soluble, non-toxic, readily available form.
Flotillin	Present in lipid rafts.	Galectin-3	MAC-2, laminin-binding protein. Galactose-specific lectin that binds IgE. Highest levels in activated macrophages. Involved in apoptosis.
GAPDH	Glycolysis.	GILT	IF-γ inducible lysosomal thiol reductase. May be involved in MHC class II-restricted antigen processing.
Glucosylceramidase	β-glucocerebrosidase. Lysosomal. Membrane bound.	β-glucuronidase	Lysosomal.
GRB2	Associates with tyrosine-phosphorylated proteins. Also interacts with Ras in the signalling pathway leading to DNA synthesis.	GRP 78	BIP. An ER chaperone.
β-hexosaminidase α	*N*-acetyl-β-glucosaminidase, β-*N*-acetylhexosaminidase. Lysosomal.	β-hexosaminidase β	*N*-acetyl-β-glucosaminidase, β-*N*-acetylhexosaminidase. Lysosomal.
HSC70T	Heat-shock related protein. Usually an ER or mitochondrial protein.	HSC71	Heat shock cognate protein.
HSP-60	Mitochondrial matrix protein P1. Chaperonin. Interacts with P21RAS. Usually an ER or mitochondrial protein.	HSP-70	Cytoplasmic chaperone.
HSP-70 precursor	Cytoplasmic.	HSP-70 protein2	-
HSP-73	Heat shock cognate 71 kDa protein	HSP-90b (HSP-84)	Molecular chaperone. Has ATPase activity. Cytoplasmic. Interacts with the cytoskeleton as well.
Lactadherin	Milk fat globule-EGF factor 8 (MFG-E8). Antiviral activity.	Lamin B1	Component of the nuclear lamina.
LAMP-1	Lysosome-associated membrane glycoprotein 1. Type I membrane protein.	"LAMP-2, type B"	Lysosome-associated membrane glycoprotein 2.
Legumain	Lysosomal cysteine endopeptidase.	LIMP II	Lysosome membrane protein II. May act as a lysosomal receptor. Type II membrane protein. Belongs to the CD 36 family.
Lysosomal acid lipase/cholesteryl ester hydrolase	Crucial for the intracellular hydrolysis of cholesterol esters and triglycerides. Lysosomal.	Lysosomal membrane glycoprotein-type B	Very similar to LAMP2.
Lysozyme C, type M (LYCAM)	1, 4-β-*N*-acetylmuraminidase C. Bacteriolytic. Enhances the activity of immunoagents.	Macrophage capping protein	Actin capping protein GCAP39; myc basic motif homolog-1.
MHC class Ib mature α chain	Expected to be secreted in soluble form due to absence of exon 5, which encodes the transmembrane domain.	MPS1	Macrophage-specific protein. Upregulated during monocyte to macrophage differentiation.
Mysosin heavy chain-A	Non muscle form.	Napsin	Membrane-anchored aspartyl protease.

Protein	Remark	Protein	Remark
NDK B	Nucleotide diphosphatase kinase B.	NSF	Vesicular-fusion protein. *N*-ethylmaleimide-sensitive factor.
ORP150	Oxygen regulated protein.	Palmitoyl-protein thioesterase	Removes thioester-like fatty acyl groups from modified cysteine residues in proteins or peptides during lysosomal degradation.
PDI (ER-59)	Protein disulfide isomerase.	PDI-(ER-60)	Involved in MHC class I assembly.
PGAM-B	Phosphoglycerate mutase, brain form.	Prohibitin	Present in lipid rafts.
Rab2	Vesicular traffic. Associated with an intermediate compartment between the ER and Golgi apparatus.	Rab3c	Protein transport and vesicular traffic.
Rab5c	Regulates early endocytic/phagocytic trafficking.	Rab7	Regulates late endocytic /phagocytic trafficking.
Rab10	Vesicular traffic and neurotransmitter release.	Rab11b	Involved in membrane recycling.
Rab14	Vesicular traffic and neurotransmitter release.	RAP1B	Involved in initiation of oxidative burst in neutrophils.
RHO GDI α	RHO GDP-dissociation inhibitor 1.	RSP4	40S ribosomal protein SA. Cytoplasmic.
SNAP-α	Soluble NSF attachment protein.	SNAP-γ	Required to prepare intracellular membranes for fusion.
Stomatin	Found in lipid rafts, exposed on the cytoplasmic surface of the membrane.	Syntenin	Localised in early endocytic compartments.
TCP-1α	T-complex protein 1. Molecular chaperone. Known to play a role, *in vitro*, in the folding of actin and tubulin. Cytoplasmic.	TCBP-49	Taipoxin-associated calcium binding protein 49.
Thioreductase peroxidase 2	Cytoplasmic.	TCP-1β	T-complex protein 1. Molecular chaperone. Known to play a role, *in vitro*, in the folding of actin and tubulin. Cytoplasmic.
TMP21	Transmembrane protein. Vesicular protein trafficking. Type I membrane protein. Present in Golgi cisternae.	Ti-225 (ubiquitin C)	Similar to human ubiquitin.
Trimeric G α2	Guanine nucleotide-binding protein. G(I)/G(S)/G(T). Adenylate cyclase-inhibiting Ga protein. Regulatory G-proteins of signalling cascades.	TRAIL	TNF-related apoptosis inducing ligand.
Tropomyosin 5	Cystoskeletal type.	Trimeric Gβ1	Guanine nucleotide-binding protein. G(I)/G(S)/G(T). Adenylate cyclase-inhibiting Ga protein. Regulatory G-proteins of signalling cascades.
UDPGT	UDP-glucuronosyltransferase. ER protein	Tubulin α-6	Microtubule protein.

Protein	Remark	Protein	Remark
v-ATPase α (catalytic subunit)	Involved in phagosome acidification.	VAP33	Vesicle-associated membrane protein, associated protein A. Associated with ER and microtubules.
v-ATPase ∈	Involved in phagosome acidification.	v-ATPase β	Involved in phagosome acidification.
Vimentin	Class III intermediate filaments.	VDAC1	Voltage-dependent anion-selective channel protein 1. Mitochondrial. Also found on the plasma membrane and endosomes.

Abbreviations: see List of Abbreviations and Symbols, pp ix. (Modified from Garin *et al*., 2001).

1.4.1 Cathepsins

Based on their catalytic mechanism, proteases are classified into four families. These include the cysteine-, serine-, aspartic- and metalloproteases (Portnoy *et al*., 1986; Chapman *et al*., 1997; Bühling *et al*., 2004). Proteases may also be classified as either exopeptidases, acting on the terminal ends of a polypeptide chain, or endopeptidases, cleaving internal peptide bonds. The term "cathepsin" was first introduced by Willstätter and Bamann (1929) and means "lysosomal proteolytic enzyme" irrespective of the protease class. This term, therefore, includes the cysteine proteases, cathepsins B, S, L, H, C, K, O, F, V, W and Z, the aspartic proteases, cathepsins D and E, and the serine proteases, cathepsins A and G (Chapman *et al*., 1997; Turk *et al*., 2001; Bühling *et al*., 2004).

Cathepsins belonging to the papain superfamily of cysteine proteases share similar amino acid sequences and folding. They are composed of heavy and light chains connected by disulfide bonds and have a two-domain structure with a V-shaped active site cleft along the domain interface (Turk *et al*., 2001). The left domain is composed of three α-helices and the right, is based on a β-barrel motif with the catalytic Cys25 positioned at the N-terminus of the characteristic α-helix (McGrath, 1999; Turk *et al*., 2001). Cys25 is able to form an ion pair with His159 which is located in the β-barrel domain on the opposite side of the active site (Chapman *et al*., 1997; Turk *et al*., 2001). The cathepsins can be classified as either endopeptidases or exopeptidases and are expressed in many different tissues types throughout the body (Table 1.5).

Cathepsins B, H, L, N, S, T and K have been identified in various compartments of the endosome-lysosome system. Although this pathway will be discussed in detail in Chapter 4, it should be noted that the compartments become increasingly acidic along the pathway.

Table 1.6 indicates the fairly wide pH optimum, of most cathepsins. This suggests that the cathepsins may occur in different vesicular compartments along the endocytic pathway, where the pH for maximal activity exists or may be regulated by pH fluctuation. In order for cathepsins to play a role in the three killing phases in J774 macrophages (Figure 1.1) (Anes *et al*., 2006), and the accompanying changing pH of the maturing phagosome, suggest that cathepsins B, H, S and L may be located in different vesicle populations that may fuse with the phagosome during the first killing phase (mild pH) and subsequently recycle out of the phagosome, or, as they are active at lower pH, fuse with the phagosome prior to the second and third killing phases (low pH) (Figure 1.1).

Table 1.5 **Properties and distribution of cathepsins.**

Cathepsin	Endopeptidase	Exopeptidase		Tissue expression
		Carboxypeptidase	**Aminopeptidase**	
B	+	+		Widespread
L	+			Widespread
S	+			APCs
H	+		+	Widespread
K	+			Osteoclasts, Bronchial epithelium
F	+			Macrophages Widespread?
V	+			Thymic epithelium
W	Unknown	Unknown	Unknown	$CD8^+$ T cells Natural killer cells
C			+	Leukocytes Macrophages
O	Unknown	Unknown	Unknown	Widespread
Z		+		Widespread
D	+			Widespread
E	+			Restricted
G	+			Neutrophils Monocytes

Abbreviation: APCs, antigen presenting cells. (Modified from Riese and Chapman, 2000; Wolters and Chapman, 2000; Turk *et al*., 2001).

Aspartic proteases are different from cysteine or serine proteases in that an activated water molecule, as opposed to an amino acid side chain is the nucleophile that attacks the substrate peptide bond. Cathepsins D and E are aspartic endopeptidases (Table 1.5), and are bi-lobed molecules with the active site situated between the two lobes (James, 2004). The aspartate residues making up the catalytic dyad bind to, and activate, the catalytic water molecule. Catalysis requires a third residue in addition to the two aspartate residues. One of the lobes has an extra β hairpin loop known as the 'flap' that caps the active site. This loop carries Tyr137 and Thr139 which are important residues for specificity. The secreted peptidase

cathepsin D, has four disulfide bonds, whereas, the proform of cathepsin E is a disulfide linked dimer (James, 2004). Table 1.6 reflects the operating pH and pI of most of the cathepsins and cathepsins D and E appear to have a more limited, acidic operating pH range in comparison to that of the cathepsins belonging to the cysteine protease family. This suggests that the active forms of these two proteases are likely to be located in the most acidic vesicular compartments, which probably fuse with the phagosome during the second killing phase mentioned in Section 1.3 (Figure 1.1).

Table 1.6 **Endoproteases found within compartments of the endocytic pathway.**

Name	Catalytic group	Operating pH*	pI
Cathepsin B	Cys	5-6.5	5.4
Cathepsin H	Cys	5.0-6.5	7.1
Cathepsin L	Cys	4.5-6.0	5.8-6.1
Cathepsin N	Cys	3.5	6.2
Cathepsin S	Cys	5.0-7.5	6.3-6.9
Cathepsin T	Cys	6.9	?
Cathepsin K	Cys	6.0-6.5	?
Cathepsin D	Asp	2.8-5.0	5.5-6.5
Cathepsin E	Asp	3-3.5	4.1-4.44
Cathepsin G	Ser	7.5	10

* 'Operating pH' is the pH at which the enzyme is stable, this need not be the optimal pH. (Modified from Pillay *et al*., 2002).

The serine proteases consist of a two-domain structure, with one open-ended, β-barrel in each domain, and the active site situated between the domains. Cathepsin G, the main protease in this class, has a catalytic triad consisting of His, Asp and Ser and is unusual as it lacks the highly conserved 191-220 disulfide region present in other serine proteases (Table 1.5) (Salvesen, 2004). Despite cathepsin G playing a significant role in neutrophil microbial killing, it only appears to be present in small quantities in monocytes and is virtually absent from macrophages (Kargi *et al*., 1990).

Cathepsins are synthesised as inactive, preproenzymes. The prepeptide is removed during passage to the ER resulting in the formation of the proform of the enzyme. The propeptide assists in the proper folding, stability and correct targeting of the enzyme and blocks the active site cleft, thus keeping the protein inactive until activity is required (Chapman *et al*., 1997). Crystallography has shown that the propieces of cathepsins B, L and K occupy the active site cleft and are positioned in the opposite orientation to that of the natural substrates and are, therefore, not digested (Riese and Chapman, 2000). Activation of the enzyme may occur by proteolytic cleavage of the N-terminal propeptide by either intermolecular

autocatalysis or by other proteases (Chapman *et al*., 1997; Turk *et al*., 1997; Turk *et al*., 2001). The activation process is dependent on low pH and the concentration of glycosaminoglycans present, suggesting that active cathepsins are likely to occur in acidic vesicles (Turk *et al*., 2001; Bühling *et al*., 2004). Normally, the activity of proteases with limited proteolytic ability is controlled by simply balancing the amount of active enzyme with an equivalent amount of active inhibitor. The regulation of lysosomal protease activity is, however, a more complicated process and is controlled in a number of different ways, which are summarised in Figure 1.2.

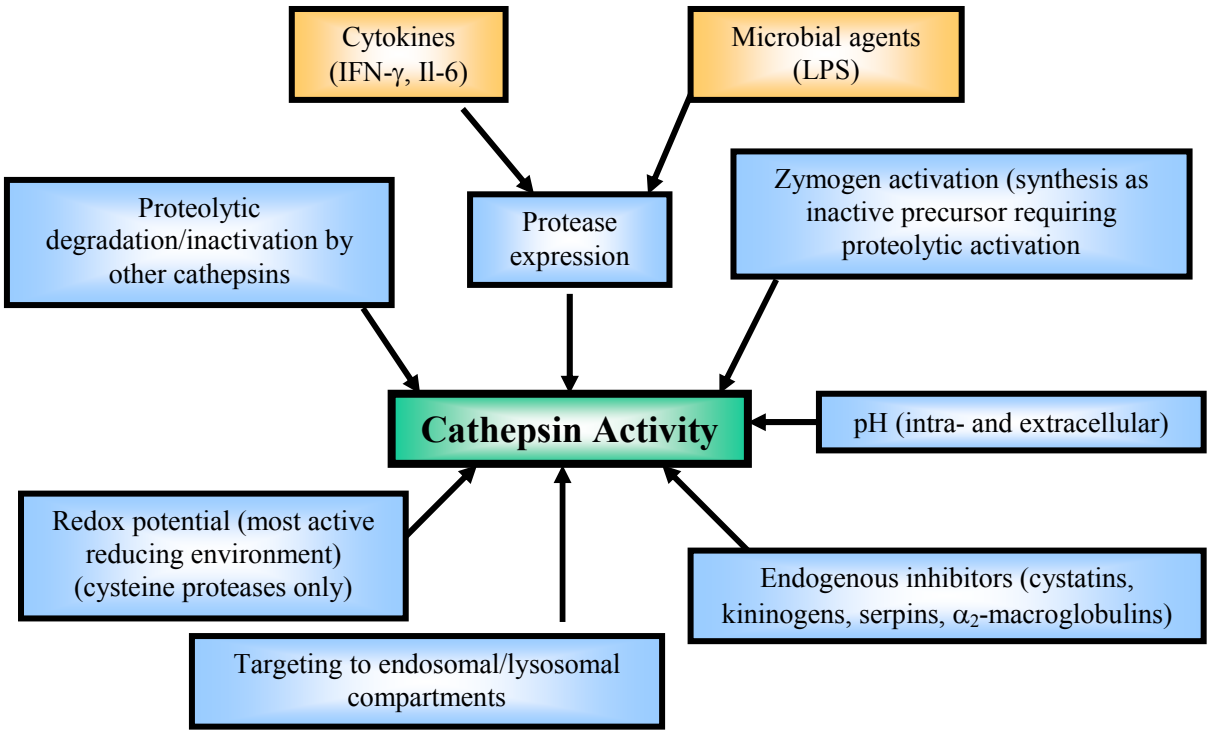

Figure 1.2 **Factors responsible for the regulation of cathepsin activity.**
Certain cathepsins have widespread expression throughout the body, whereas, the expression of others is limited to particular cell types. Cytokines and microbial agents can stimulate macrophages, resulting in enhanced cathepsin expression. Cathepins are synthesised as inactive precursors, which undergo proteolytic activation to produce the mature, active enzyme which can be inactivated or degraded by other cathepsins when neccessary. Cathepsins function optimally at acidic pH and are generally unstable and weakly active at neutral pH. The active site cysteine residue is easily oxidised; therefore, the enzymes are most active in reducing compartments. They possess glycosylation sites and residues allowing for binding to MPRs and the subsequent transport to endosomes and lysosomes. A number of endogenous cathepsin inhibitors are present in cells to control both intra-and extracellularly expressed cathepsins (Chapman *et al*., 1997; Turk *et al*. 2001; Bühling *et al*., 2004).

Cathepsins, were originally considered to be ubiquitously expressed, "housekeeping" proteins responsible for the degradation of unnecessary, abnormal or endocytosed proteins. This view has changed, however, since the discovery of distinct expression patterns for cathepsins and the use of gene knockouts, showing that cathepsins have specific, individual functions which are vital for the normal functioning of many biological processes (Wolters

and Chapman, 2000; Turk *et al*., 2001; Bühling *et al*., 2004). Cathepsins are involved in antigen processing (Shi *et al*., 1999), normal bone and ECM remodelling (Chapman *et al*., 1997; Turk *et al*., 2000; Wolters and Chapman, 2000) and apoptosis (Leist and Jäättelä, 2001; Salvesen, 2001; Roberg *et al*., 2002), for example. Cathepsins are also associated with various pathological conditions such as rheumatoid arthritis (Schurigt *et al*., 2005), osteoarthritis, osteoporosis, neurological disorders (Goto *et al*., 1987), chronic obstructive pulmonary lung disease including asthma, emphysema, idiopathic pulmonary fibrosis (Takahashi *et al*., 1993; Chapman and Shi, 2000; Wolters and Chapman, 2000) and cancer (Turk *et al*., 2000) as well as with inherited genetic diseases such as pycnodysostosis, a disease characterised by osteosclerosis, short stature, acro-osteolyis of the distal phalanges, bone fragility, clavicular dysplasia and skull deformities with delayed suture closure (Gelb *et al*., 1996) and Papillon-Lefèvre syndrome, a disorder characterised by palmoplantar hyperkeratosis and severe early onset periodontitis, resulting in the loss of the primary and secondary dentitions (Hart *et al*., 1999; Toomes *et al*., 1999).

1.4.1.1 Macrophage cathepsins

As mentioned cathepsins A, B, D, H, L, S and Z have been identified in J774 macrophage phagosomes (Garin *et al*., 2001) and isolated vesicles (Claus *et al*., 1998). Diment and Stahl (1985) first identified a cathepsin D-like protease in rabbit alveolar macrophages and later identified this as a (possibly early endosome-) membrane-associated form of cathepsin D, processed to an active form while still attached to the endosomal membrane (Diment *et al*. 1988). Both cathepsins B and D have been associated with inflammation and alveolar macrophages of smokers have larger vesicles containing more cathepsin D compared to those of non-smokers (Chang *et al*., 1989). As cathepsin D is capable of degrading a number of proteins, it is possibly responsible for the structural damage of the lungs associated with cigarette smoking. Increased levels of active cathepsin D and B have also been identified in peritoneal macrophages stimulated *in vivo* with mineral oil and thioglycollate (Lesser *et al*., 1985) and with inflammation of the peritoneal cavity, inflammation also correlating with an increase in the size and number of cytoplasmic vesicles containing cathepsin D and B. *In vitro* studies indicate that macrophages are capable of secreting cathepsin B between the macrophage and ECM, suggesting a role for cathepsin B in the degradation of the ECM (Mørland and Pedersen, 1979) and cathepsins B and D also play degradative roles within the phagosome (Mørland and Pedersen, 1979).

In vitro labelling of macrophages with [^{35}S] methionine and immunoprecipitation with anti-cathepsin L antibodies have indicated that macrophages also synthesise cathepsin L. This is synthesised as a 43 kDa precursor and processed into a 25 kDa mature form through a 34 kDa intermediate (Reilly *et al*., 1989). Claus *et al*. (1998) suggested that both cathepsins B and L may be located in a specialised secretory lysosome or vesicle, as both were secreted upon addition of either chloroquine or bafilomycin A1. Whereas, cathepsin H, α-galactosidase and β-hexosaminidase were not. This is possibly an important fact which should be borne in mind when interpreting vesicle colocalisations.

Cathepsin L was initially considered to be the only elastinolytic cysteine protease present in human alveolar macrophages (Mason *et al*., 1986; Reilly *et al*., 1989; Shi *et al*., 1992). Macrophages of smokers, however, were shown to have greater intracellular, elastinolytic activity than non-smokers, but both had equivalent cathepsin L activities. This suggested the contribution of at least one additional protease (Reilly *et al*., 1991). One such protease was subsequently identified as cathepsin S (Shi *et al*., 1992; Reddy *et al*., 1995). Both cathepsins S and L are now known to be differentially expressed in antigen presenting cells (APCs), however, and appear to be mainly involved in degradation of the invariant chain (Ii) and regulation MHC class II presentation (Shi *et al*., 1999; Hsieh *et al*., 2002; Beers *et al*., 2003). Cathepsin S has also been found to be mainly associated with the late endosome where such processing may take place (Claus *et al*., 1998).

Cathepsin K, on the other hand, discovered in rabbit osteoclasts (Chapman *et al*., 1997), human monocyte-derived macrophages (MDMs) (Punturieri *et al*., 2000) and alveolar macrophages (Shi *et al*., 1995) has subsequently been shown to have the greatest elastinolytic potential of all mammalian elastases (Chapman *et al*., 1997; Punturieri *et al*., 2000). It appears to be up-regulated in inflamed areas, with cigarette smokers having twice the amount of mRNA and more protein than non-smokers (Chapman *et al*., 1997). By using MDMs, cultured under conditions in which the differentiated cells show a tissue-destructive phenotype similar to that seen in chronic inflammatory sites *in vivo*, Punturieri *et al*. (2000) showed that MDMs secrete both processed and inactive cathepsin K along with cathpsins L and S. Up-regulated expression of vacuolar-type H^+-ATPase components for pumping protons out of the cell, simultaneously allows the development of an acidic pericellular

milieu and provides optimal conditions for extracellular activity of cysteine proteases facilitating tissue destruction.

In human neutrophils, cathepsin G is possibly the most important cathepsin and is located in peroxidase-positive azurophil granules (Campbell *et al*., 1989; Hanson *et al*., 1990; Kargi *et al*., 1990). Certain monocyte subsets possess cathepsin G in peroxidase-positive vesicles as well. The amount is approximately 6% of that found in neutrophils and the source of cathepsin G in these cells has been questioned, however, it is expressed at fairly high levels in promonocytic cell lines such as the U-937 cell line (Campbell *et al*., 1989; Kargi *et al*., 1990). In developing promyelocytes, however, cathepsin G mRNA has not been detected after the myelocyte stage of development (Kargi *et al*., 1990). The lack of mRNA in peripheral blood monocytes has suggested that any cathepsin G present after this stage is synthesised and stored in vesicles, prior to the release of the mature cells from the bone marrow (Hanson *et al*., 1990). Alveolar macrophages also possess a limited amount, if any cathepsin G, suggesting that as monocytes differentiate into macrophages they lose some of their serine proteinase activity (Campbell *et al*., 1989; Campbell *et al*., 1991) and, therefore, rely on other proteases such as cathepsins and MMPs to maintain their functionality.

Cathepsin Z has a cathepsin B-like active site and although the actual function of cathepsin Z in macrophages is unknown, it is suspected to function in antigen processing (Riese and Chapman, 2000; Shi *et al*., 2000).

Based on the literature, it appears that several cathepsins could be used as potential markers for vesicle populations within J774 cells. Cathepsin H has been shown to be enriched in the early endosomes of J774 macrophages, while cathepsin S occurs predominantly in late endosomes (Claus *et al*., 1998; Jahraus *et al*., 1998) and could, therefore, be viewed as potential markers for these compartments. It will also be borne in mind that cathepsins B an L may be colocalised or located in special ‘secretory lysosomes’, considering results obtained with chloroquine and bafilomycin (Claus *et al*., 1998). Whereas the cathepsins are usually lysosomal proteases, the MMPs and their inhibitors the TIMPs are not. Some background to these proteases and their inhibitors will be given before the MMPs and TIMPs found in the macrophage are described.

1.4.2. Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases

MMPs, also known as matrixins, are a group of zinc metalloendopeptidases (Nagase and Woessner, 1999; Parks and Shapiro, 2001). There are at least 24 different vertebrate MMPs, with at least 23 being found in humans (Visse and Nagase, 2003; Folgueras *et al*., 2004). Previously, MMPs were classified according to their substrate specificities. With the knowledge of their structural design, a new structurally based classification system has been developed, however (Table 1.7) (Folgueras *et al*., 2004).

Table 1.7 **Matrix metalloproteinases.**

MMP designation*	Alternative name
MMP-1	Collagenase-1
MMP-2	Gelatinase-A
MMP-3	Stromelysin-1
MMP-7	Matrilysin
MMP-8	Collagenase-2, Neutrophil elastase
MMP-9	Gelatinase-B, 92 kDa gelatinase, 92 kDa type IV collagenase
MMP-10	Stromelysin-2, Transin-2
MMP-11	Stromelysin-3
MMP-12	Macrophage metalloelastase
MMP-13	Collagenase-3
MMP-14	MT1-MMP (membrane-type MMP)
MMP-15	MT2-MMP
MMP-16	MT3-MMP
MMP-17	MT4-MMP
MMP-18	Collagenase-4
MMP-19	RASI-1
MMP-20	Enamelysin
MMP-23	CA-MMP
MMP-24	MT5-MMP
MMP-25	Leukolysin, MT6-MMP
MMP-26	Endometase, Matrilysin-2
MMP-28	Epilysin

* Athough in humans there are 23 MMPs, 29 numbers have been used. MMP-4, -5, -6 and -29 are redundant in humans and are not used. After the discovery of MMP-7 (matrilysin), MMP-4, -5 and -6 were identified as being either MMP-2 or MMP-3. MMP-18 corresponds to the collagenase isolated from *Xenopus laevis* for which a mammalian homolog is not known. The human protein that was named MMP-18 is now called MMP-19. Two almost identical human genes located in a segment of chromosome 1 that is duplicated were called MMP-21 and MMP-22 and are now known as MMP-23A and MMP-23B. Abbreviations: MT, membrane type; RASI, rheumatoid arthritis synovial inflammation (modified from Parks and Shapiro, 2001; Somerville *et al*., 2003; Visse and Nagase, 2003).

Most MMPs have two domains, namely a protease domain and an ancillary domain (Figure 1.3). These are joined together by a proline-rich, flexible hinge peptide. The protease domain consists of the signal peptide, the pro-domain and the catalytic domain. The signal peptide directs the secretion of the MMP from the cell. The pro-domain containing the cysteine switch motif PRCGXPD, is responsible for keeping the enzyme in its zymogen or proMMP form, and the catalytic domain contains a conserved zinc-binding region (Parks and

Shapiro, 2001; Somerville *et al*., 2003; Visse and Nagase, 2003). The ancillary domain consists of a hemopexin-vitronectin-like domain. When present (Figure 1.3), this domain influences inhibitor binding and the binding of certain substrates, membrane activation and proteolytic activity (Sternlicht and Werb, 2001). Both MMP-2 and MMP-9 possess three tandem fibronectin type II repeats within the amino terminal of the catalytic domain allowing for gelatin binding (Figure 1.3). In addition, MMP-9 has a type-V collagen-like domain in its hinge region, the function of which is unknown (Somerville *et al*., 2003) (Figure 1.3).

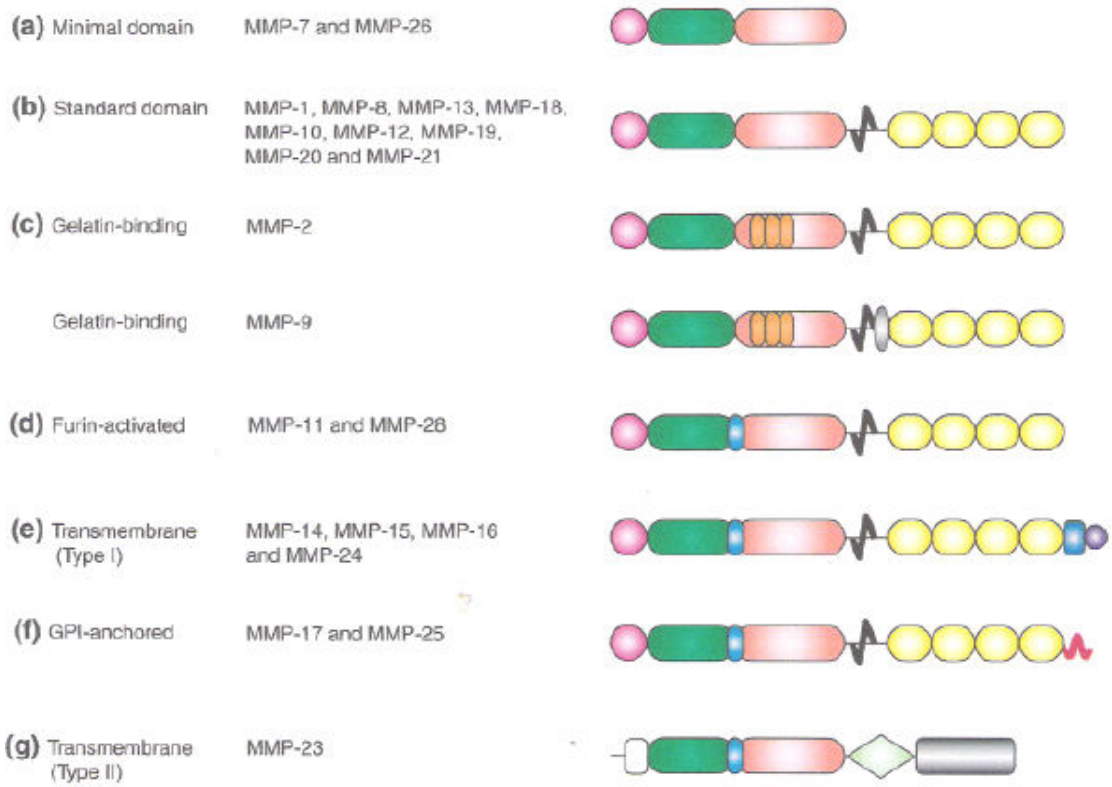

Figure 1.3 **Domain composition and important structural features of the various subtypes of MMPs.**

(Somerville *et al*., 2003).

The membrane-type matrix metalloproteinases (MT-MMPs) (MMP-14, -15, -16, -17, -24 and -25) (Table 1.7) as well as MMP-11, -23 and -28 all possess furin cleavage sites (Figure 1.3), which are cleaved by furin-like serine proteases resulting in the intracellular activation of the proforms of the enzymes (Somerville *et al*., 2003; Folgueras *et al*., 2004). MT-MMPs

are localised at the cell surface either through a carboxy-terminal transmembrane domain or a glycosylphosphatidylinositol (GPI) anchoring domain (Folgueras *et al*., 2004) (Figure 1.3).

The regulation of MMPs is complex and occurs at multiple levels including transcription, zymogen activation and inhibition of enzyme activity (Kerrigan *et al*., 2000; Sternlicht and Werb, 2001; Somerville *et al*., 2003). Synthesised as inactive zymogen forms, the activation of MMPs is brought about by thiol-modifying agents, mercurial compounds, reactive oxygen radicals, denaturing reagents as well as low pH and high temperatures *in vitro*. Activation requires the disruption of the interaction between the sulfydryl group in the pro-domain and the zinc ion of the catalytic site (Nagase and Woessner, 1999) and *in vivo* activation requires the proteolytic removal of the pro-domain. Generally the proteases involved form part of a proteolytic cascade which occurs extracellularly (Folgueras *et al*., 2004).

After activation, MMPs may be inactivated via several mechanisms. Four classes of MMP inhibitor with broad inhibitory activity exist extracellularly and in body fluids. In the tissues the major inhibitors are the TIMPs, these bind the N-terminal domain and block the active site, whereas, a general protease inhibitor, α_2-macroglobulin blocks MMP activity in plasma and in tissue fluids by a 'bait and trap' mechanism (Price *et al*., 2000). A number of recently identified proteins have sequences similar to that of the N-terminal inhibitory domain of TIMPs and inhibit MMPs. These include the C-terminal fragment of procollagen C-terminal proteinase enhancer (CT-PCPE) an inhibitor of MMP-2 (Mott *et al*., 2000), the non-collagenous (NC1) domain of type IV collagen appears to inhibit MMP-2 and -3 (Netzer *et al*., 1998), tissue factor pathway inhibitor-2 (TFPI-2), a serine proteinase inhibitor, can inhibit MMP-1, -2, -9 and -13 (Herman *et al*., 2001), and the membrane-anchored protein RECK (reversion-inducing cysteine-rich protein with kazal motifs) is capable of inhibiting MMP-2, -9 and -14 (Oh *et al*., 2001).

In the past, it was thought that the ECM was a structure used simply for the attachment of cells and for mechanical support and the role of the MMPs was, therefore, to remodel the ECM for its homeostasis and to facilitate cell migration (Somerville *et al*., 2003). Secretion of MMPs has also been associated with the invasive properties of tumour cells as ECM degradation facilitates penetration and infiltration of the cancer cells (Folgueras *et al*., 2004). Degradation of certain ECM components, however, alters cellular behaviour and phenotypes (Table 1.8) (Visse and Nagase, 2003). The functions of MMPs have been shown to go far

beyond ECM degradation. MMPs have a wide variety of target substrates including growth-factor receptors, cell adhesion molecules, chemokines, cytokines, apoptotic ligands and angiogenic factors (Somerville *et al*., 2003; Folgueras *et al*., 2004) (Table 1.8).

Table 1.8 **Biological effects generated by MMPs.**

Responsible MMPs	Substrate cleaved	Biological effect
MMP-1	Type 1 collagen	Keratinocyte migration and re-epithelialisation
MMP-13	Type 1 collagen	Osteoclast activation
MMP-2	Chondroitin sulfate proteolglycan	Neurite outgrowth
MMP-7	Fibronectin	Adipocyte differentiation
MMP-1, -2, -3	Fibronectin	Cell migration
MT1-MMP	CD44	Cell migration
MMP-3	Basement membrane	Mammary epithelial cell apoptosis
MMP-3	Basement membrane	Mammary epithelial alveolar formation
MMP-3	E-cadherin	Epithelial-mesenchymal conversion (mammary epithelial cells)
MMP-2	Not identified	Mesenchymal cell differentiation with inflammatory phenotype
MMP-1	Not identified	Platelet aggregation
MMP-3, -7, -9, -12	Generation of angiostatin-like fragment	Plasminogen
MMPs	Type XVIII	Generation of endostatin-like fragment
MMP-2, -3, -7, -9, -13 (not MMP-1)	BM-40 (SPARC/osteonectin)	Enhanced collagen affinity
MT1-MMP	Type 1 collagen	Kidney tubulogenesis
MMP-3, -13	Perlecan	Release of bFGF
MMP-1, -2, -3	IGFBP-3	Increased bioavailability of IGF1 and cell proliferation
MMPs	IGFBP-5	Increased bioavailability of IGF1 and cell proliferation
MMP-11	IGFBP-1	Increased bioavailability of IGF1 and cell proliferation
MMPs	CTGF	Activation of VEGF
MMP-2, MT1-MMP	Laminin 5γ2 chain	Epithelial cell migration
Collagenase	Type 1 collagen	Apoptosis (amnion epithelial cells)
MMP-1, -3, -9	Processing IL-1β from the precursor	Pro-inflammatory
MMP-9	ICAM-1	Tumour cell resistance
MMP-1, -2, -9	IL-1β degradation	Anti-inflammatory
MMP-1, -2, -3, -13, -14	Monocyte chemoattractant protein-3	Anti-inflammatory
MMP-2, -3, -7	Decorin	Increased bioavailability of TGF-β
MMP-3, -7	E-cadherin	Disrupted cell aggregation and increased cell invasion
MT1-MMP, MT2-MMP, MT3-MMP	Cell surface tissue transglutaminase	Reduced cell adhesion and spreading
MMP-7	Fas ligand	Fas receptor-mediated apoptosis
MMP-9	IL-2Rα	Reduced IL-2 response

Abbreviations: see list of abbreviations and symbols, pp iv. (Modified from Visse and Nagase, 2003).

Genetically altered mouse models as well as human diseases have lead to the identification of surprising biological functions of MMPs (Table 1.9), suggesting that MMPs may possess substrates that still need to be identified. In addition to facilitating invasion and metastasis of cancer cells, MMPs are involved in other pathological conditions such as arthritis, oral pathology and periodontal disease, cardiovascular disease and pulmonary emphysema (Shapiro, 1998; Woessner and Nagase, 2002). As they are inhibited in a 1:1 ratio by their inhibitors the TIMPs, the ratio of these inhibitors to their target MMPs is highly important (Brew *et al*., 2000; Baker *et al*., 2002).

Table 1.9 **Phenotypes of MMP knockout mice.**

Gene	Phenotype
MMP-2	Reduction in angiogenesis and tumour growth; suppression of experimentally induced pancreatic carcinogenesis; delayed mammary gland differentiation; mild growth retardation.
MMP-3	Impaired wound contraction; accelerated arthritis; resistance to contract dermatitis; accelerated mammary adipogenesis.
MMP-7	Inability to repair mucosal epithelial wounds; reduced ability to kill pathogenic bacteria; reduced tumorigenesis; defective prostrate involution; impaired ex vivo herniated disc resorption.
MMP-9	Normal neutrophils extravasation; lack of alveolar bronchiolization in fibrosis; resistant to induced blister formation; persistent contact hypersensitivity response; protection against aortic aneurysm formation; reduced ventricular enlargement and rupture postmyocardial infarction; delayed tumour progression and reduced metastases.
MMP-11	Fewer chemically induced tumours and reduced tumour cell implantation; accelerated and enhanced neointimal formation after vessel injury; suppression of experimentally induced mammary carcinogenesis.
MMP-12	Reduced elastolytic capacity of macrophages; protection against smoking-induced emphysema; reduced ability of macrophages to migrate through matrix.
MMP-14	Severe abnormalities in bone and connective tissue; reduced collagen turnover; impaired endochondral ossification; defective angiogenesis; premature death.
MMP-20	Amelogenesis imperfecta.

(Modified from Parks and Shapiro, 2001; Somerville *et al*., 2003; Folgueras *et al*., 2004).

Four human TIMPs, named TIMP-1, -2, -3 and -4 have been identified. These are expressed by a range of cell types and are present in most tissues and body fluids (Brew *et al*., 2000; Lambert *et al*., 2004). Although they share many general characteristics they do exhibit distinct structural/biochemical features and have varied expression patterns (Table 1.10) (Baker *et al*., 2002).

Table 1.10 **Molecular characteristics of human TIMPs.**

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Molecular weight (kDa)	20.6	21.5	21.6	22.3
Glycosylated molecular weight (kDa)	28.5	-	27	-
N-glycosylation sites	2	0	1	0
Protein localisation	Soluble	Soluble/cell surface	ECM	Soluble/cell surface
ProMMP association	ProMMP-9	ProMMP-2	ProMMP-2 ProMMP-9	ProMMP-2
MMPs poorly inhibited	MMP-14 MMP-15 MMP-16 MMP-24 MMP-19	None	None	None
ADAM inhibition	ADAM 10 (Kuzbanian)	None	ADAM 12 (Meltrin-α) ADAM 17 (TACE) ADAM 19 (Meltrin-β) (ADAM 10) ADAMTS-4 (Aggrecanase-1) ADAMTS-5 (Aggrecanase-2)	None
Expression	Inducible	Constitutive	Inducible	Inducible

Abbreviations: ADAM, a disintegrin and metalloprotease domain; TACE, tumour necrosis factor-α converting enzyme; ADAMTS, a disintegrin and metalloprotease domain with thrombospondin type-1 domains. (Modified from Woessner and Nagase, 2002; Baker *et al*., 2002; Lambert *et al*., 2004).

TIMPs are composed of an N-terminal domain and a C-terminal domain with each domain being stabilised by three disulfide bonds (Brew *et al*., 2000; Baker *et al*., 2002). Natural inhibitors of MMPs, the TIMPs form noncovalent 1:1 stoichiometric enzyme-inhibitor complexes with MMPs. Some TIMPs are capable of binding to the proforms of their target enzymes (e.g. TIMP-1 to proMMP-9, TIMP-2 to proMMP-2, TIMP-3 to proMMP-2 and proMMP-9, and TIMP-4 to pro-MMP-2) and thus regulate the activation process. As proMMP-2 and proMMP-9 are particularly bound by TIMPs this possibly indicates the importance of MMP-2 and MMP-9. Looking at the phenotypic changes in knockout mice (Table 1.9), it would seem that these are particularly important in growth, carcinogenesis and invasion.

TIMP expression is tightly regulated to maintain a suitable balance during ECM degradation and if the balance is not maintained, uncontrolled ECM degradation occurs resulting in diseases such as cancer, arthritis, cardiovascular disease, neurological disorders, tissue

ulceration and fibrosis (Brew *et al*., 2000). In addition to MMP inhibition, TIMPs appear to have a variety of other cellular activities (Table 1.11) including promotion of cell growth, anti-apoptotic activity, steroidogenic activity, anti-angiogenic activity and embryonic activity (Lambert *et al*., 2004).

Table 1.11 **Biological activities of TIMPs.**

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Cell growth promotion	Erythroid precursors Keratinocytes Fibroblasts Epithelial cells Tumour cells	Erythroid precursors Fibroblasts Tumour cells	Growth-retarded, non-transformed cells	Mammary tumour cells
Cell growth inhibition		Endothelial cells Smooth muscle cells Tumour cells	Colon cancer cells	G401 Wilm's tumour cells
Apoptosis	Burkitt's lymphoma cells	Colorectal cancer cells Human T lymphocytes	Smooth muscle cells Tumour cells Epithelial cells	Cardiac fibroblasts
Survival	B cells Hepatic stellate cells Hematopoietic cells Human breast epithelial cells	Melanoma Folliculo-stellate		Tumour cells
Steroidogenesis promotion	Leydig cells Ovarian granulosa cells Ovary Testicular development			
Angiogenesis inhibition	Chick embryo yolk-sac membranes Pancreatic cancer cells	Chick embryo yolk-sac membranes	Human fibrosarcoma HT1080 cells	
Embryogenic promotion	Mouse embryo implantation		Mouse embryo implantation	

(Modified from Baker *et al*., 2002; Lambert *et al*., 2004).

1.4.2.1 Macrophage MMPs and TIMPs

The exact range of MMPs synthesised by the macrophage appears to differ from species to species, as well as from one environment to another, and is greatly affected by the state of activation and differentiation of the macrophages concerned (Gibbs *et al*., 1999a). This complicates any attempt to characterise macrophage vesicles. Human alveolar macrophages are known to produce MMP-1, -2, -3, -9 and a unique metalloelastase known as MMP-12, found only in macrophages (Welgus *et al*., 1990; Campbell *et al*., 1991; Woessner, 1994; Goetzl *et al*., 1996). In addition, human blood monocytes and macrophages produce MMP-

7, whereas, human alveolar macrophages do not (Busiek *et al*., 1992; Filippov *et al*., 2000). MMP-12 has a variety of substrates (Shipley *et al*., 1996) and elimination of the expression of the MMP-12 gene results in macrophage inability to penetrate membranes both *in vivo* and *in vitro*, indicating the importance of this particular MMP in degrading the ECM and in tissue invasion (Shipley *et al*., 1996). MMP-12 knockout mice, however, appeared to be protected from emphysema, whereas, exposure to cigarette smoke lead to the recruitment of inflammatory cells and enlarged alveolar space similar to the lesions that form in humans in the control mice (Hautamaki *et al*., 1997).

Rat alveolar macrophages produce a similar, but not identical MMP range to the human counterpart. They do not appear to produce MMP-1, however, and have very little, if any MMP-3 and MMP-7 (Gibbs *et al*., 1999b).

The cellular differentiation of human mononuclear phagocytes affects the synthesis of neutral proteinases including MMPs and TIMPs both *in vitro* and *in vivo* (Campbell *et al*., 1991). Monocytes contain traces of matrix degradative, neutral, neutrophil serine proteases including human leukocyte elastase and cathepsin G. In neutrophils, these proteases are synthesised by precursor cells and are stored in large peroxidase-positive granules. In contrast, macrophages contain very little if any of these proteases, and rely on their ability to synthesise and secrete substantial quantities of MMPs and TIMPs for regulated ECM degradation. During mononuclear phagocyte differentiation, the cells appear to develop the ability to secrete TIMP-1 prior to proMMP-9 and fully differentiated macrophages are able to secrete both (Welgus *et al*., 1986; Campbell *et al*., 1987). Exposure to LPS and phorbol esters appear to increase the production of MMP-3, MMP-9 and TIMP-1 (Welgus *et al*., 1985; Welgus *et al*., 1990). The effects of LPS will be discussed in greater detail in Chapter 5.

Type-IV collagenases, MMP-2 and -9, are known as gelatinases as they readily digest gelatin (Nagase, 1996). Large amounts of MMP-9 are produced in macrophages (Goetzl *et al*., 1996) but MMP-9 is also present in neutrophils. MMP-2, however, is synthesised in smaller amounts by macrophages and is not found in neutrophils (Hibbs, 1992; Russell *et al*., 2002a). As previously mentioned, MMP-9 is one of the major elastolytic MMPs. MMP-2, however, is more involved in the processing of cell receptors (Levi *et al*., 1996; Russell *et al*., 2002a). In neutrophils, MMP-9 is found in the specific granules. A distinct MMP-9-positive

compartment in macrophages has not yet been identified as the vesicular compartments and the MMP protease complement of vesicles remains virtually unknown. TIMP-1 is able to complex and inhibit both proMMP-9 and MMP-9 itself (Triebel *et al*., 1995; Goetzl *et al*., 1996; Price *et al*., 2000), and complex formation shields the carboxyl-terminus of MMP-9 from MMP-3 thus preventing MMP-3 from activating MMP-9 (Goetzl *et al*., 1996). Price *et al*. (2000) demonstrated that, in neutrophils, TIMP-1 is found in distinct vesicles, largely separate from the neutrophil MMPs proMMP-8 and proMMP-9. Differential release of TIMP-1 and proMMPs may play a role in controlling the extent of extracellular MMP activity. Differential secretion is extremely important as it may provide a drug target for controlling many pathological conditions, especially inflammatory disease. This is currently under investigation in the neutrophil.

Vesicle localisation and regulation is also unknown in macrophages as vesicle characterisation has not been performed to date but is potentially important and more so as Osiewicz *et al*. (1999), showed that mice deficient for TIMP-1 appear to be more resistant to *Pseudomonas aeruginosa* infections, indirectly suggesting that active MMPs are required for immunity. Differential regulation of TIMP-1 and MMPs may, therefore, contribute to immunity by regulation of processing or degradation of cytokines, MMP activity being very important in cytokine processing (Folgueras *et al*., 2004) (Table 1.8). Even though this seemed to be a neutrophil-dependent phenomenon, macrophages may also require active MMPs for fighting bacterial infection and it would thus be useful to know whether the various TIMPs, MMPs and cathepsins are located in a particular vesicle types and whether fusion with the phagosome and release of these vesicles is separately and differentially regulated under specific stimulation. An initial step to establishing which combinations of proteins are present in the macrophage vesicles and whether there are indeed different vesicle populations is required and will assist in future studies of the various macrophage processes, such as antigen presentation and the killing of organisms in the phagosome which seem highly complex (Figure 1.1) (Anes *et al*., 2006).

1.5 Objectives of the current study

At the commencement of this study, the vesicular distribution of cathepsins B, D, H, S and L as well as MMP-9, TIMP-1 and TIMP-2 in J774 macrophages was virtually unknown as few studies have focused on establishing their vesicle distribution. The major aim of this study was to establish the distribution of the above cathepsins, MMPs and TIMPs in the hope of

identifying marker enzymes for different vesicle populations. Antibodies to cathepsins B, D, H, S and L and to MMP-9, TIMP-1 and TIMP-2 raised against antigens from other species were available in our laboratory. For studies on TIMP-1 and TIMP-2, however, it was decided to raise additional antibodies against recombinant human forms of the proteins, as TIMP-1 and -2 are highly conserved blood proteins and hence most antibodies react weakly at best. All antibodies needed to be characterised for species cross-reactivity with the mouse antigens and to be optimised (Chapter 3). These antibodies were subsequently used in immuno-EM and fluorescent microscopy studies of J774 cells, the cells most characterised to date (Claus *et al*., 1998; Jahraus *et al*., 1998).

As cathepsins H and S had previously been identified by activity assays as possible markers for early and late endosomes, respectively (Claus *et al*., 1998; Jahraus *et al*., 1998), colocalisation studies were performed to determine whether cathepsins B, D and L occurred in such vesicular compartments (Chapter 4). As macrophages are known to have highly active and fairly complex endosome-lysosome systems (Rabinowitz *et al*., 1992; Astarie-Dequeker *et al*., 2002), additional markers were used to verify the presence of the cathepsins in classically defined endosome-lysosome systems i.e. low pH compartments, labelling for lysosomal-associated membrane proteins (LAMPs). Colocalisations between the cathepsins and LysoTracker (acidic compartment) (Via *et al*., 1998) and LAMPs and proposed vesicle subpopulations were compared with those identified by Anes *et al*. (2006) (Chapter 4) and markers such as cathepsins S, H and D evaluated as markers for the early endosome (non-acidic, LAMPs-neagtive), late endosome (acidic, LAMPs-positive) and lysosome (most acidic, LAMPs-positive), respectively. Emerging evidence seems to indicate that the final killing of specific pathogens is brought about by the fusion of specific digestive enzymes (proteases) with the phagosome. Knowledge of the complement of proteases in specific vesicles and regulation of their release is potentially important as such information may allow therapeutic strategies to be developed. Knowledge of the distribution of proteases in specific vesicle populations may also reveal new marker enzymes for specific types.

The distribution of MMP-9 and MMP inhibitors (TIMPs) in J774 macrophages was considered next (Chapter 5). It was suspected that, as MMPs and their inhibitors are usually secreted and seem largely to function extracellularly (Campbell *et al*., 1991), they would be located in vesicles belonging to the secretory pathway (non-acidic, LAMPs-negative) as opposed to the endosome-lysosome vesicle system (acidic, LAMPs-positive). Thus, it was

hoped that localisation studies performed on MMP-9, TIMP-1 and TIMP-2 would lead eventually to the identification of vesicle populations distinct from the late endosome-lysosome system and the development of a marker system for this pathway. In this final study, however, the colocalisation between MMP-9 and the MMP inhibitors TIMP-1 and TIMP-2, LAMP-2 and LysoTracker in inactivated macrophages establish whether enzyme and inhibitor are located in different vesicle populations as is the case in neutrophils (Price *et al*., 2000) and exist in organelles other than the early and late endosomes or lysosomes indicated by LAMPs and LysoTracker (Chapter 5). Studies on the localisation of MMPs and the TIMPs are therapeutically important as MMPs are involved in many inflammatory diseases and if protease and inhibitor are separately localised and their release is subject to different regulatory stimuli, such information may allow therapeutic targets to be identified for inflammatory diseases.

As resting macrophages usually synthesise fairly low levels of MMP-9, TIMP-1 and -2 and activation seemed to influence colocalisation of marker enzymes and phenotype, it was decided that the cells should be stimulated with LPS to induce a "classically activated-like" phenotype and up-regulate MMP-9 and TIMP-1 expression (Welgus *et al*., 1985; Campbell *et al*., 1991; Welgus *et al*., 1991). This also allowed stimulated and unstimulated cells to be compared to assess whether activation alters the intracellular localisation of either MMP-9 or TIMP-1 (Chapter 5). Finally, colocalisation studies with LysoTracker and LAMPs were performed to establish whether MMP-9, TIMP-1 and TIMP-2 are present in the endosome-lysosome-like vesicles. Results and implications are discussed in their broader context in Chapter 6. For ease of reading, all common reagents and methods are reported in Chapter 2.

CHAPTER 2

GENERAL MATERIALS AND METHODS

The common biochemical techniques that were employed in this study are described here. Specialised techniques will be described in the relevant chapters.

2.1 Reagents

Dulbecco's modified Eagle's medium (DMEM), Hanks' balanced salts (HBSS), glutamine, trypsin-ethylenediaminetetra-acetic acid (EDTA), sodium bicarbonate, bisacrylamide (*N*,*N*'-methylene-bisacrylamide), Coomassie blue R-250, goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate, rabbit anti-chicken IgG (whole molecule) alkaline phosphatase conjugate, donkey anti-sheep IgG (whole molecule) alkaline phosphatase conjugate, goat anti-mouse IgG (whole molecule) alkaline phosphatase conjugate, rabbit anti-chicken IgG (whole molecule) fluorescein isothiocyanate (FITC) conjugate, donkey anti-sheep IgG (whole molecule) FITC conjugate, goat anti-rabbit IgG (whole molecule) FITC conjugate, goat anti-rat IgG (whole molecule) FITC conjugate, goat anti-rabbit IgG (whole molecule) tetramethyl rhodamine isothiocyanate (TRITC) conjugate, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES), *N*-2-hydroxy-piperazine-*N*'-2 ethane sulfonic acid (HEPES), ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*,*N*',*N*'-tetra acetic acid (EGTA), polyoxyethylene (23) lauryl alcohol (Brij 35 solution), diaminobenzidine/3,3',4,4'-tetraaminobiphenyl (DAB), Freund's complete and Freund's incomplete adjuvants (FCA and FIA), citric acid, bovine serum albumin (BSA), saponin, gelatin (porcine skin) and fish skin gelatin (FSG) were from Sigma (St. Louis, Missouri). Acrylamide, ammonium persulfate, sodium chloride, sodium hydroxide, potassium chloride, Na_2HPO_4, $Na_2HPO_4.H_2O$, sodium azide, glycine, paraformaldehyde (PFA), glutaraldehyde (25% solution), polyoxyethylene (9-10) *p*-*t*-octyl phenol (Triton X-100) and EDTA disodium salt, hydrochloric acid, $CaCl_2.2H_2O$, 2,2'-azino-di(3-ethyl)-benzthiozoline sulfonic acid (ABTS) and H_2O_2 35% (v/v) were from BDH (Poole, England). Sodium dodecyl sulfate (SDS) was from Boehringer Mannheim (Mannheim, Germany) and 2-amino-2-(hydroxymethyl)-1,3-propandiol (Tris) was from MP Biomedicals (Eschwege, Germany). Methanol, glacial acetic acid, KH_2PO_4 and $MgCl_2.6H_2O$ were from Saarchem (Wadeville, South Africa). Glycerol was from AR–Associated Chem. Enterprises (Glenvista, South Africa). Mercaptoethanol and amido black were from Merck Schuchardt OHG (Munich, Germany). Serva blue G was

from Serva (Heidelberg, Germany). Ponceau S was from Searle (High Wycombe, Bucks, United Kingdom), Elite milk powder was from Clover SA (Pty) Ltd (Roodepoort, South Africa). Donkey anti-chicken IgY cyanine3 (CY3) conjugate and rabbit anti-chicken IgY peroxidase conjugate were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, Pennsylvania). Monoclonal antibodies against lysosomal-associated membrane proteins (LAMPs)-1 and 2 were from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, Iowa, USA). SlowFade™ antifade reagent and LysoTracker Red DND-99 were from Molecular Probes (Eugene, USA). 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) were from Roche (Indianapolis, Indiana). Tris, Tween 20, *N*,*N*,*N*',*N*',-tetramethyl ethylenediamine (TEMED), imidazole, zinc sulfate and polyethylene glycol (PEG) 6 kDa were from Merck (Darmstadt, Germany). Dimethylformamide (DMF) was from Fluka (Seelze, Germany). Foetal calf serum (FCS) was from Highveld Biologicals (Johannesburg, South Africa). Hybond-C-extra nitrocelluose membrane was from Amersham Biosciences (Buckinghamshire, England). Whatman No. 1 and No. 4 filter paper were from Whatman International Ltd (Maidstone, England). LR White resin was from London Resin (London, United Kingdom). Ciprobay IV intravenous infusion was from Bayer and Nunc Easy Flasks and Multidishes (24 well) Nuclon™ and Immuno Maxisorp F96 multiwell plates were from Nunc Intermed (Roskilde, Denmark).

2.2 Cell culture

The J774 mouse macrophage cell line was originally derived from a tumour in a female BALB/c mouse and has been shown to possess characteristics typical of macrophages including adherence, receptors for immunoglobulin, antibody-dependent lysis of target cells as well as morphology (Ralph *et al*., 1975a; Ralph *et al*., 1975b; Ralph *et al*., 1977a; Ralph *et al*., 1977b). As previous attempts have been made to characterise the vesicles of the endosome-lysosome system these cells (Claus *et al*., 1998; Jahraus *et al*., 1998; Kuehnel *et al*., 2001), this cell line, was chosen for further characterisation to develope possible marker systems for the vesicles of the J774 macrophage.

2.2.1 Reagents

DMEM (with L-glutamine and 1000 mg/l glucose without sodium bicarbonate) and HBSS (without calcium chloride, magnesium sulfate, phenol red and sodium bicarbonate) were prepared according to the manufacturer's instructions.

1 x Trypsin-EDTA solution. 10 x Trypsin-EDTA (1 ml) was diluted in HBSS (9 ml) and warmed to 37^{o}C just before use.

2.2.2 Procedure

J774 cells were cultured in DMEM, with 10% FCS at 37^{o}C with 5% CO_2 in a Nuaire US Autoflow CO_2 water-jacketed incubator. Upon reaching approximately 70% confluency, cells were washed in HBSS and adherent cells were trypsinised using a minimal volume of 1 x trypsin-EDTA solution. Cells were generally split in a ratio of 1:3. Since excessive granularity of these cells may indicate the presence of an infection, cells were treated with Ciprobay [80 μg/ml] if granularity was observed.

2.3 SDS-PAGE

During gel electrophoresis, an externally applied electric field causes the migration of charged particles through a polymeric gel matrix to either the anode or cathode (Garfin, 1990; Switzer and Garrity, 1999). Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS has become one of the most widely used techniques for the analysis of protein mixtures (Smith, 1984; Bischoff *et al*., 1998). Polyacrylamide gels are formed by the copolymerisation of acrylamide (water soluble monomer) with *N*,*N*'-methylene bisacrylamide (cross-linking agent) (Ninfa and Ballou, 1998). The gel formation mechanism is vinyl addition polymerisation and is catalysed by a free-radical generating system, where ammonium persulfate is the di-sulfate ester of H_2O_2 and readily forms unstable $\bullet SO_4^-$ radicals. TEMED, a tertiary amine reacts with these radicals forming TEMED radicals that subsequently react with the acrylamide, inducing polymerisation (Garfin, 1990; Ninfa and Ballou, 1998). The free-radicals can also be generated by photolysis of a labile compound such as riboflavin, a light sensitive compound that generates free-radicals when irradiated with UV light (Ninfa and Ballou, 1998; Switzer and Garrity, 1999). The average pore size of the gel can be controlled by varying the amount of monomer used or by varying the degree of cross-linking (Ninfa and Ballou, 1998). By convention, gels are characterised by a pair of figures (*%T, %C*), where *%T* is the weight percentage of total monomer (acrylamide and cross-linker, in grams per 100 ml) and *%C* is the proportion of cross-linker (as a percentage of total monomer) in the gel. The practical limits for *%T* are 3-30% and the gel pore size decreases as *%T* increases (Garfin, 1990).

The electrophoretic separation of proteins in polyacrylamide gels is affected by both the charge and size of the protein as well as by the frictional forces they experience during migration. If the size and charge of the proteins compensate for each other it is possible for proteins of different charge and size to move at the same rate and this complicates M_r estimations. SDS, an anionic detergent, overcomes this problem by imposing uniform hydrodynamic and charge characteristics on all proteins (Garfin, 1990; Ninfa and Ballou, 1998). Proteins will on average bind 1.4 ± 0.3 g SDS per gram of protein or about one SDS molecule for every two amino acid residues (Bischoff *et al*., 1998). To allow for complete saturation with SDS, the polypeptide chain should be unfolded or denatured; this is facilitated by heating the protein in the presence of SDS and a reducing agent such as β-mercaptoethanol or DTT which assist in the breaking of the disulfide bonds holding the oligomeric complexes together and internal disulfide bonds that maintain the tertiary structure of polypeptide subunits (Bischoff *et al*., 1998). Each SDS molecule contributes a negative charge and this, combined with high stoichiometric binding, ensures that the SDS-polypeptide complexes carry a high net negative charge and will thus have anodal migration. Additionally, the charge-to-mass ratio will be essentially the same for different proteins, as the SDS coating dominates the charge. Thus the relative electrophoretic mobility of a complex through the polyacrylamide gel is a function of size and thus an indication of the M_r of the protein. In SDS-PAGE, a linear relationship exists between the relative migration of proteins and the log of their respective M_r's. This allows for M_r estimation of proteins but it should be noted that this does not take into account post-translational modifications that may alter the apparent M_r.

2.3.1 Laemmli system

The Laemmli SDS-PAGE system consists of two distinct gels, an upper stacking gel and a lower separating gel (Laemmli, 1970). The gels are cast with different porosities, pH and ionic strength (Garfin, 1990). In this system, the different ionisation states of glycine are responsible for the stacking and separation of proteins in the respective gels. The stacking gel usually (4% *T*) (large pore size), pH 6.8 at 4^{o}C, does not retard the migration of most proteins. At pH 6.8 most of the glycine species exist as zwitterions that carry no charge with only a small fraction being in the anionic state (Ninfa and Ballou, 1998). Laemmli gels are run at constant current and in order for this to be maintained the anionic protein species carry the charge instead of the zwitterions. This results in the stacking of the proteins in a thin band behind highly mobile chloride ions. When the stacked proteins enter the separating gel

which has a higher pH and decreased pore size the glycine becomes anionic. The glycinate ions now move faster than the proteins, with their mobility approaching that of the chloride ions, resulting in the proteins being left behind the chloride and glycinate ions to separate in a constant voltage gradient. The proteins are separated by the sieving effect of the gel according to their M_r's.

2.3.1.1 Reagents

Acrylamide/bisacrylamide monomer stock solution [30% (m/v) acrylamide, 2.7% (m/v) bisacrylamide]. Acrylamide monomer (58.4 g) and *N*, *N*'-methylenebisacrylamide (1.6 g) were dissolved and made up to 200 ml with dH_2O. The solution was filtered through Whatman No. 1 filter paper and stored in an amber bottle at 4°C.

4 x Separating gel buffer [1.5 M Tris-HCl buffer, pH 8.8]. Tris (36.3 g) was dissolved in dH_2O (~ 180 ml), adjusted to pH 8.8 with HCl and made up to 200 ml. The buffer was filtered through Whatman No. 1 filter paper and stored at 4°C.

4 x Stacking gel buffer [500 mM Tris-HCl, buffer pH 6.8]. Tris (12 g) was dissolved in dH_2O (~ 180 ml), adjusted to pH 6.8 with HCl and made up to 200 ml. The buffer was filtered through Whatman No. 1 filter paper and stored at 4°C.

SDS stock solution [10% (m/v) in dH_2O]. SDS (10 g) was dissolved in dH_2O, with gentle heating and made up to 100 ml. The solution was stored at room temperature (RT).

Ammonium persulfate initiator solution [10% (w/v) in dH_2O]. Ammonium persulfate (0.1 g) was dissolved in dH_20 (1 ml) just before use. The solution was kept at 4°C for up to 1 week.

Tank buffer [25 mM Tris-HCl and 192 mM glycine-HCl buffer, 0.1% (m/v) SDS, pH 8.3]. Tris (3 g) and glycine (14.4 g) were dissolved in dH_2O and made up to 1 l. Prior to use SDS stock solution (2.5 ml) was added to 250 ml.

Reducing treatment buffer [125 mM Tris-HCl buffer, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]. 4 x Stacking gel buffer (2.5 ml), SDS stock solution (4 ml), glycerol (2ml) and 2-mercaptoethanol (1 ml) were mixed together and made up to 10 ml with dH_2O.

Non-reducing treatment buffer [125 mM Tris-HCl buffer, 4% (m/v) SDS, 20% (v/v) glycerol, pH 6.8]. 4 x Stacking gel buffer (2.5 ml), SDS stock solution (4 ml), glycerol (2ml) were mixed and made up to 10 ml with dH_2O.

2.3.1.2 Procedure

The SDS-PAGE electrophoresis unit (Hoefer® Mighty Small) was assembled according to manufacturer's instructions. The glass and aluminium plates, plastic combs and spacers were rinsed in dH_2O, 96% ethanol and rinsed in dH_2O and dried. The glass plates, spacers and aluminium plates were assembled in a gel caster according to manufacturer's instructions.

Table 2.1 **Reagent composition and proportions for two Laemmli gels.**

Reagent	**Separating gel (%)**					**Stacking gel (%)**	
	15	**12.5**	**10**	**7.5**	**5.0**	**4.0**	**3.0**
Monomer (ml)	7.5	6.25	4.99	3.75	2.5	0.94	0.71
4 x Separating gel buffer (ml)	3.75	3.75	3.75	3.75	3.75	0	0
4 x Stacking gel buffer (ml)	0	0	0	0	0	1.75	1.75
SDS stock solution (µl)	150	150	150	150	150	70	70
Initiator (µl)	75	75	75	75	75	35	35
dH_2O (ml)	3.5	4.75	5.98	7.25	8.5	4.3	4.53
TEMED (µl)	7.5	7.5	7.5	7.5	7.5	15	15

The acrylamide/bisacrylamide monomer stock solution, gel buffer, SDS and dH_2O were mixed with ammonium persulfate initiator solution and TEMED as indicated for the separating gel (Table 2.1) and the solution loaded into the gel caster, overlaid with dH_2O to exclude oxygen and allowed to polymerise (11 h). After polymerisation the dH_2O was poured out, the stacking gel solution was made up as described in Table 2.1 and layered on top of the polymerised separating gel. Plastic 10 or 15 well combs were inserted into the stacking gel and polymerisation was allowed to occur (30 min). The combs were removed and the gels placed into the electrophoresis units. During electrophoresis the gels were cooled using a circulating water bath (4^{o}C) and the proteins separated (36 mA, unlimited

voltage). Gels were subsequently processed further for either western blotting, zymography or stained for protein.

2.4 Staining of protein in SDS-PAGE gels

Various methods exist for the detection of proteins in polyacrylamide gels including fluorescent staining using terbium chloride (Copeland, 1994), SYPRO Ruby (Steinberg *et al*., 1996; Lopez *et al*., 2000), reversible staining using either imidazole (Fernandez-Patron *et al*., 1995a) or methyl trichloroacetate (Candiano *et al*., 1996) and permanent visible staining techniques such as Coomassie brilliant blue (CBB) staining and silver staining (Merril, 1990). Each of these has their own specific applications as well as advantages and disadvantages in protein visualisation. In this study, proteins in SDS-PAGE gels were detected using either CBB or imidazole-SDS-zinc reverse staining, depending on the objective of the experiment.

2.4.1 Coomassie brilliant blue staining

CBB is a popular staining method and is simple, economical and compatible with further downstream analysis, but, it lacks sensitivity (approximately 50 ng protein/band) and has a low affinity for acidic proteins (Merril, 1990; Fernandez-Patron *et al*., 1995a). CBB R-250 was the first triphenylmethane stain to be introduced. The letter "R" stands for a reddish hue while the number "250" indicates the strength of the dye. This was followed by Coomassie blue G-250, where "G" indicates a greenish hue and subsequently Coomassie violet R-150 (Merril, 1990). CBB has three charged forms that exist at an acidic pH. The red, blue and green forms have absorbance maxima at 470, 590 and 650 nm, respectively with the blue form being responsible for the binding of proteins, resulting in a complex that absorbs light at 594 nm (Zor and Selinger, 1996). An acidic medium is required for CBB staining which allows for an electrostatic attraction between the dye molecules and the protein. Van der Waals' forces, together with the ionic attraction, hold the protein-dye complex together. The dye appears to interact with the basic groups in the polypeptides, as the intensity of the stain increases with increasing numbers of basic amino acid residues (Merril, 1990). During staining, the gel is placed in a dye solution containing acetic acid, methanol and dH_2O. As the acetic acid and methanol fix the proteins within the gel matrix, the dye binds to the proteins within the gel. The gel is destained with a solution of acetic acid and methanol to remove excess dye (Switzer and Garrity, 1999), allowing for the visualisation of dark blue

protein bands. The initial shrinkage of the gel in the first destain solution is overcome by the second destain solution which rehydrates the gel as it contains less methanol and more dH_2O.

2.4.1.1 Reagents

Stain stock solution [1% (m/v) Coomassie blue R-250 in dH_2O]. Coomassie blue R-250 (0.5 g) was dissolved in dH_2O (50 ml) by magnetic stirring for 1 h, RT. The solution was filtered through Whatman No. 1 filter paper and stored at RT.

Staining solution [0.125% (m/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]. Stain stock solution (62.5 ml) was mixed with methanol (250 ml) and acetic acid (50 ml), made up to 500 ml with dH_2O and stored at RT.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (500 ml) was mixed with acetic acid (100 ml) and made up to 1 l with dH_2O. The solution was stored at RT.

Destaining solution II [7% (v/v) acetic acid, 5% (v/v) methanol]. Acetic acid (70 ml) was mixed with methanol (50 ml) and made up to 1 l with dH_2O. The solution was stored at RT.

2.4.1.2 Procedure

After electrophoresis, the gel was placed in a clean plastic container and covered with the staining solution (4 h or overnight). The stain was decanted back into the original container and destain solution I was poured over the gel. This destain solution was changed several times until dark blue protein bands were visible and the gel background was relatively clear. The gel was placed in destaining solution II until fully hydrated, photographed using a VersaDoc 4000 Imager (BioRad, California, USA), analysed using Quantity One software and stored in a sealed plastic bag at 4^oC.

2.4.2 Imidazole-SDS-zinc reversible staining

Imidazole-SDS-zinc reversible staining is reported to be more sensitive than CBB staining (Fernandez-Patron *et al*., 1995a) and can be used on both unstained or previously CBB stained gels. During pre-treatment the proteins are complexed with SDS and appear as transparent bands against a white gel background. The background results from the formation of an insoluble, white imidazole-zinc complex (Fernandez-Patron *et al*., 1995b).

The intensity of background staining is important for the visualisation of the transparent protein bands. When previously stained CBB gels are used the blue protein bands appear superimposed on transparent bands (Fernandez-Patron *et al*., 1995a). Staining appears to be sensitive to both the concentration of the staining reagents as well as glycine and possibly Tris. This problem can be avoided by washing the gels in dH_2O prior to staining and by using a pre-treatment incubation time of 15 min (Fernandez-Patron *et al*., 1995a).

2.4.2.1 Reagents

Imidazole-SDS pre-treatment solution [200 mM imidazole, 0.1% (m/v) SDS in dH_2O]. Imidazole (1.36 g) and SDS stock solution (1 ml) were dissolved in dH_2O (100 ml) and stored at RT.

Zinc sulfate developing solution [200 mM zinc sulfate]. Zinc sulfate (5.75 g) was dissolved in dH_2O (100 ml) and stored at RT.

2.4.2.2 Procedure

After electrophoresis the gels were placed in clean, glass petri dishes and rinsed in dH_2O. Gels were pre-treated in imidazole-SDS pre-treatment solution (15 min), rinsed briefly in dH_2O (30 s) and developed in zinc sulfate developing solution, until the gel background turned intensely white, with transparent protein bands (15-60 s). Development was monitored during manual agitation of the gels over a dark surface and stopped by removal of the developing solution, followed by rapid rinsing with dH_2O (10-15 s). As development continues for a few seconds after the developing solution has been removed, the reaction was best stopped just as the bands of interest were first visualised. Gels were photographed using the epi-white light and dark background of a VersaDoc 4000 Imager (BioRad, California, USA), analysed using Quantity One software and stored in sealed plastic bags at $4^{o}C$.

2.5 Zymography

Zymography allows for the detection of enzyme activity after proteins have been electrophoresed (Bischoff *et al*., 1998). The proteinase substrate such as gelatin, casein or fibrin, is copolymerised with the acrylamide of the separating gel (Kleiner and Stetler-Stevenson, 1994). The concentration of the substrate incorporated into the gel is important for optimal band formation, resolution and detection of enzyme activity (Makowski and Ramsby, 1996). Usually, a non-reducing SDS-PAGE gel is used to ensure the retention of

enzyme activity, but, some enzymes have been successfully renatured in the presence of a chaotropic agent such as urea even after the samples have been heated under reducing SDS-PAGE conditions (Bischoff *et al*., 1998). Following electrophoresis, the SDS bound to the proteinase substrate and protein samples is removed by washing the gels in an unbuffered solution of Triton X-100 (Kleiner and Stetler-Stevenson, 1994; Oliver *et al*., 1997). SDS has a significantly higher critical micelle concentration (CMC) than Triton X-100 [CMC_{SDS} = 8.27 mM; $CMC_{TRITON\ X\text{-}100}$ = 0.24 mM (Sigma Handbook, 1998)] and is, therefore, easily removed by dilution. The gel is subsequently incubated at optimal temperature in an appropriate buffer, which should also contain the relevant inhibitors for other proteinases that degrade the substrate, as well as any additional cofactors that may be required for enzyme activity. Gels may be stained with either CBB or Amido black and enzymatic activity is indicated by the absence of staining in areas where the substrate has been degraded (Kleiner and Stetler-Stevenson, 1994; Oliver *et al*., 1997).

2.5.1 Reagents

Stock gelatin solution [1% (m/v) in 4 x separating gel buffer]. Porcine skin gelatin (0.015 g) was added to 4 x separating gel buffer (1.5 ml). The solution was heated until the gelatin dissolved. The solution was made up fresh each time.

Renaturation solution [2.5% (v/v) Triton X-100 in dH_2O]. Triton X-100 (5 ml) was dissolved in dH_2O to a final volume of 200 ml.

Gelatinase digestion buffer [50 mM Tris-HCl buffer, 200 mM NaCl, 5 mM $CaCl_2.2H_2O$, pH 7.6]. Tris (6.05 g), NaCl (11.70 g) and $CaCl_2.2H_2O$ (0.735 g) were dissolved in dH_2O (950 ml), adjusted to pH 7.6 with HCl and made up to 1 l. The solution was stored at 4^oC.

Amido black staining solution [0.1% (m/v) Amido black in 30% (v/v) methanol and 10% (v/v) acetic acid]. Amido black (0.1 g) was dissolved in methanol: acetic acid: dH_2O in the proportions (30: 10: 60) (100ml) and filtered through Whatman No. 1 filter paper. The filtrate was stored at RT.

Destaining solution [30% (v/v) methanol and 10% (v/v) acetic acid]. Methanol (300 ml) and acetic acid (100 ml) were dissolved in dH_2O (600 ml). The solution was stored at RT.

2.5.2 Procedure

The procedure for SDS-PAGE was modified from that described (Section 2.3.1.2) in that 0.1% gelatin was incorporated into the separating gel to allow for the detection of proteinases (Heussen and Dowdle, 1980). Stock gelatin solution (1.5 ml) was added to 4 x separating gel buffer (2.25 ml) and the rest of the solution for casting a 12.5% gel (Table 2.1) and by pouring the gel as quickly as possible. Gels were allowed to set for 1 h. Samples were prepared in non-reducing treatment buffer (Section 2.3.1.1) and the electrophoresis carried out as described (Section 2.3.1.2).

After electrophoresis the gel was briefly rinsed in dH_2O and renatured in unbuffered renaturation solution (50 ml) (3 x 30 min, RT) with agitation. The gel was subsequently placed in a clean plastic container with pre-warmed digestion buffer (250 ml) and the digestion allowed to proceed for 18 h at 37^{o}C for MMPs. After this time, the gel was washed with dH_2O (3 x 1 min), stained for 1 h in Amido black staining solution, destained in several changes of destaining solution and stored at 4^{o}C until photographed. Gels were photographed using a VersaDoc 4000 Imager (BioRad, California, USA) and analysed using Quantity One software.

2.6 Western blotting

Many techniques have been utilisd for the detection of specific proteins following SDS-PAGE, however, the most widely used is western blotting with antibodies directed against the proteins of interest (Bischoff *et al*., 1998). This technique provides information about antibody specificities and the target antigen such as its molecular weight, its activation state (i.e. proform of the enzyme versus the active form), oligomeric arrangement or post-translational modification.

The choice of immobilisng matrix used for protein transfer is dependent on the subsequent investigations that follow the transfer. Generally, for immunoblotting, proteins are transferred onto a nitrocellulose membrane with a pore size of 0.45 µm (Van Dam, 1994). Nitrocellulose membranes are made by allowing nitric acid-esterified cellulose, solubilisd in an organic solvent mixture, to gel by the evaporation of the solvents. The pore size of the membrane depends on how the temperature and the time of the drying process are regulated (Gershoni and Palade, 1983). Nylon membranes appear to bind certain antigens more strongly than nitrocellulose. The major disadvantage of this membrane type is that blocking

is usually performed at high temperatures which may denature sensitive antigens (Van Dam, 1994). If the protein transfer is followed by protein sequencing, then polyvinyldifluoride (PVDF) membranes are used as they are able to withstand the harsh chemicals used in the sequencing process.

The composition of the transfer buffer should be carefully considered especially if proteins are being transferred to nitrocellulose membranes. Towbin *et al*. (1979) used a transfer buffer containing methanol, which counteracted the swelling of the gel. The incorporation of methanol into transfer buffers has both advantages and disadvantages. It increases the binding capacity of the nitrocellulose membrane for proteins, however, it also decreases the pore size of the gel, removes SDS from the proteins and may lead to fixation of the proteins in the gel, so concentrations greater than 20% should be avoided (Gershoni and Palade, 1983; De Maio, 1994; Van Dam, 1994). The addition of SDS to transfer buffers increases protein transfer, however, this appears to be dependent on the nature of the proteins being transferred (Van Dam, 1994). Transfer buffers containing SDS but lacking methanol have been used successfully in protein transfer (Gershoni and Palade, 1982). As the choice of specific buffers and detergents is determined by the nature of the proteins being transferred, the buffer proposed by Towbin *et al*. (1979) was used with the incorporation of 0.01% SDS for the current study.

The unoccupied binding sites of the membrane are blocked before antibody probing. This is done by incubating the membrane in a solution of an 'inert' protein such as BSA, ovalbumin, haemoglobin or non-fat milk. Non-fat milk is not suitable for blocking when lectins or antibodies recognisng carbohydrate moieties are to be used, as milk contains large amounts of sugar that may prevent binding. Non-ionic detergents such as Tween-20 may also be used as they reduce the binding of proteins to the nitrocellulose, thus reducing the background but, they should not be used at concentrations greater than 0.5% as they may remove proteins from the membrane (De Maio, 1994).

2.6.1 Chromogenic blots

The substrate products used in chromogenic blots should be insoluble, light stable and easily visible. The enzymes most frequently used for western blotting are HRP and alkaline phosphatase (Nadkarni and Linhardt, 1997). Although the most common substrates for HRP are 4-chloro-1-napthol and DAB, tetramethybenzidine (TMB) can also be used (Van Dam,

1994). H_2O_2 needs to be added to both 4-chloro-1-napthol and DAB before use, whereas, TMB requires dioctylsulfosuccinate. DAB is considered to have intermediate sensitivity which can be enhanced by the use of imidazole and divalent metal ions such as cobalt. HRP catalyses the transfer of electrons from DAB, causing the DAB to become oxidised, forming an insoluble brown polymer. It is thought that the imidazole causes the formation of an additional electron transfer site in HRP, increasing its activity (Nadkarni and Linhardt, 1997). Additional metal ions improve DAB polymer formation and cause a colour change in the final product. 4-chloro-1-napthol is less sensitive than DAB but generally produces less background. DAB and 4-chloro-1-napthol can be combined to produce a highly sensitive detection system (Van Dam, 1994). Alkaline phosphatase hydrolyses BCIP forming an intermediate that can dimerise to produce an insoluble indigo precipitate. The reduction of NBT yields an insoluble purple formazan. The combination of BCIP and NBT with alkaline phosphatase yields a dark purple precipitate that is much more sensitive than either substrate alone. During this reaction the NBT is reduced by the two reducing equivalents produced by the dimerisation of BCIP. BCIP/NBT usually produces sharp band resolution with little background. The reaction rate of alkaline phosphatase remains constant during the reaction allowing for the relative sensitivity to be controlled. This is not possible with other enzymes.

2.6.2 'Tank' buffer system

This type of apparatus is fairly simple. An electrophoretic field is generated to transfer proteins from a matrix, such as an acrylamide gel, to an immobilising matrix such as a nitrocellulose membrane. The transfer cassette containing the gel and nitrocellulose is placed in a 'tank' of buffer allowing for the electrophoretic protein transfer. Usually, an efficient cooling system is used in conjunction with this apparatus. Transfers can be carried out overnight as the buffer does not become depleted during the transfer.

2.6.2.1 Reagents

Transfer buffer [25 mM Tris-HCl and 192 mM glycine-HCl buffer, 20% (v/v) methanol, 0.01% (m/v) SDS, pH 8.3]. Tris (6.05 g), glycine (28.8 g) and SDS [2 ml of 10% (w/v) solution] were dissolved in 1.6 l of dH_2O. Methanol (400 ml) was added and the solution stored at –20^{o}C without pH adjustment.

Ponceau S protein staining solution [0.1% (w/v) Ponceau S in 1% (v/v) acetic acid]. Ponceau S (0.1 g) and acetic acid (1 ml) were added to a 100 ml volumetric flask and made up to volume with dH_2O. The solution was stored at RT.

Tris-buffered saline I (TBS I) [20 mM Tris-HCl buffer, 200 mM NaCl, pH 7.4]. Tris (2.42 g) and NaCl (11.7 g) were dissolved in dH_2O (950 ml), adjusted to pH 7.4 with HCl and made up to 1 l. The solution was stored in aliquots at –20°C.

Tris-buffered saline II (TBS II) [50 mM Tris-HCl buffer, 2.5% (m/v) NaCl, 0.3% (m/v) Brij 35, pH 7.4]. Tris (6.05 g) and NaCl (25 g) were dissolved in dH_2O (950 ml) and adjusted to pH 7.4 with HCl. Brij 35 [10 ml of 30% (m/v) solution] was added and the volume made up to 1l with dH_2O. The solution was stored in aliquots at –20°C.

Tris-buffered saline III (TBS III) [50 mM Tris-HCl buffer, 0.9% (m/v) NaCl, pH 8.2]. Tris (3.03 g) and NaCl (4.5 g) were dissolved in dH_2O (450 ml) and adjusted to pH 8.2 with HCl. Brij 35 [10 ml of 30% (m/v) solution] was added and the volume made up to 500 ml with dH_2O. The solution was stored in aliquots at –20°C.

Phosphate buffered saline (PBS). NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (1.44 g) and KH_2PO_4 (0.24 g) were dissolved in dH_2O (800 ml), adjusted to pH 7.4 and made up to 1 l.

Blocking solution [5% (m/v) non-fat milk powder in TBS I]. Elite non-fat milk powder (5 g) was dissolved in TBS I (99 ml) with Brij 35 [1 ml of 30% (m/v) solution] immediately before use.

Antibody diluent [0.5% (m/v) BSA in TBS I, 0.3% (m/v) Brij 35]. BSA (0.5 g) was dissolved in TBS I (99 ml) with Brij 35 [1 ml of 30% (v/v) solution] immediately before use.

Alkaline phosphatase substrate buffer [50 mM Tris-HCl buffer, 5 mM $MgCl_2$, pH 9.5]. Tris (6.05 g) and $MgCl_2.6H_2O$ (1.0 g) were dissolved in dH_2O (980 ml), adjusted to pH 9.5 with HCl and made up to 1 l. The solution was stored at 4°C.

BCIP stock solution. BCIP (150 mg) was dissolved in DMF (3 ml). The solution was stored at –20°C in a foil-covered amber bottle.

NBT stock solution. NBT (300 mg) was dissolved in DMF [70% (v/v)] (2.1 ml) and the solution made up to 3 ml with dH_2O. The solution was stored at –20°C in a foil-covered amber bottle.

Alkaline phosphatase substrate solution [0.015% (m/v) BCIP, 0.03% (m/v) NBT in substrate buffer]. BCIP stock solution (30 µl) and NBT stock solution (30 µl) were dissolved in alkaline phosphatase substrate buffer (10 ml) just before use.

0.2 M Tris buffer, pH 7.6. Tris base (24.2 g) was dissolved in dH_2O (800 ml), adjusted to pH 7.6 with 1M HCl and made up to 1 l.

DAB substrate solution. DAB (2.5 mg) was dissolved and made up to 5 ml in 0.2 M Tris buffer, pH 7.6. Immediately before use, 30% H_2O_2 (15 µl) was added. As DAB is a potential carcinogen, gloves were worn and care was taken to avoid inhalation of DAB powder. All contaminated glassware, spills or waste solutions were decontaminated in a solution of hypochlorite ("Jik").

2.6.2.2 Procedure

Following SDS-PAGE (Section 2.3.1.2) the gel was removed from the electrophoresis unit and submerged in transfer buffer along with 6 sheets of Whatman filter paper and Hybond™-C Extra nitrocellulose hybridization transfer membrane (0.45 µm). The transfer cassette (Bio-Rad Mini Trans-Blot® Electrophoretic Transfer Cell) was assembled and positioned in the electrophoresis tank to ensure that the membrane was closest to the anode. The chamber was filled with transfer buffer and stirred with a magnetic stirrer throughout the run. The transfer was performed at 30 V, 90 mA for 16 h. Subsequently, the gel and nitrocellulose were removed from the cassette and the gel outline marked on the nitrocellulose. Ponceau S was used to stain the nitrocellulose, allowing for visualisation of the M_r markers and determination of the efficiency of the transfer. Light pencil lines were used to mark the positions of the lanes and markers. The stain was removed with dH_2O and a few drops of 1 M NaOH. The nitrocellulose was air dried between sheets of filter paper before probing or alternatively stored between sheets of filter paper in a dessicator at 4°C until required.

The blocking solution was added to the nitrocellulose (1 h, RT) to prevent non-specific adsorption of antibodies. The nitrocellulose was washed in TBS II (3 x 5 min) and incubated

with primary antibody diluted in the antibody diluent solution (2 h, RT). Following washing in TBS II (2 x 5 min), the membrane was incubated with the appropriate enzyme conjugated secondary antibody diluted in the antibody diluent solution (1 h, RT). The membrane was subsequently washed in both TBS II (2 x 5 min) and TBS III (1 x 5 min), immersed in the appropriate substrate solution and developed in the dark until distinct bands were observed. The nitrocellulose was rinsed with dH_2O, dried between filter paper and kept in the dark until photographed with a VersaDoc 4000 Imager (BioRad, California, USA) and analysed using Quantity One software, the percentage of each processing form being visually assessed.

Towards the end of the study, PBS was used instead of the three separate TBS solutions.

2.6.3 'Semi-dry blotting' system

With the 'semi-dry blotting' system a stack of wet filter papers surrounding the gel and nitrocellulose membranes act as the buffer reservoir instead of the tank as in the 'tank' buffer system. The electrodes consist of conductive plates which produce a high-strength electrical field with higher current densities in comparison to the wire electrodes that are used in the 'tank' systems. The plate electrodes are in direct contact with the buffer soaked filter papers maximising the field strength across the gel, allowing for fast and efficient transfers. This system is less expensive than the 'tank' system as a relatively small amount of buffer is required. This small buffer volume limits the time for which the transfer can be carried out as the buffer becomes depleted. It should be noted that small proteins may pass straight through the membrane with this system and, as the voltages are limited by the lack of a cooling system, the transfer of high molecular weight proteins may be difficult (Jacobson, 1994).

2.6.3.1 Reagents

See Section 2.6.2.1.

2.6.3.2 Procedure

Following SDS-PAGE (Section 2.3.1.2) the gel was removed from the electrophoresis unit and submerged in transfer buffer. A mylar mask was prepared by cutting a rectangle 2 mm smaller than the gel on either side and placed on the anode in the base of the apparatus (Hoefer SemiPhor™ Semi-Dry Transfer Unit). Nitrocellulose membrane and six pieces of filter paper were cut slightly smaller than the gel and saturated in transfer buffer. Three

pieces of filter paper were centered in the opening of the mylar mask, with their edges slightly overlapping the cutout on all sides. The nitrocellulose was placed on the filter paper stack and the gel on top of the nitrocellulose. Three pieces of filter paper were placed on top of the gel. The transfer was performed at 26 mA for 2 h. The remainder of the procedure was carried out as previously described (Section 2.6.2.2).

2.7 Electron microscopy and immunogold labelling

The main EM techniques used in this study were transmission electron microscopy (TEM) of ultrathin resin sections and immunogold labelling (De Mey, 1987).

If fine structure immunocytochemistry is to be performed the choice of resin to be used should be carefully considered as it should possess certain characteristics. Firstly, it should easily infiltrate cells/tissues and must harden uniformly without any shrinkage or swelling. Secondly, the resin blocks should be hard but have a degree of plasticity allowing for smooth sectioning. The processes leading up to and including infiltration, polymerisation and sectioning should not prevent the demonstration of antigens by immunocytochemistry nor change the fine structure. The resin should allow for drying of sections without loss of fine structure and sections should also be resistant to radiation by the electron beam (Griffiths, 1993).

Methacrylates or 'acrylic resins' have several advantages over other embedding media used for EM. These include, the rapid penetration of cells/tissues (as a result of the low viscosity and the rapid diffusion of low molecular weight monomers of the resin), the resin is also relatively inexpensive and non-toxic. Disadvantages include absolute hydrophobicity and the requirement for total dehydration of the specimens in organic solvents prior to infiltration. This, together with a requirement for strong heat polymerisation (60^{o}C for 4 days), make methacrylate resins unsuitable for immunocytochemical studies, as such treatment of tissue destroys antigencity.

The resin chosen for the current study was LR White, a hydrophilic resin that has become increasingly popular. Its hydrophilicity makes ultrathin sections permeable to aqueous solutions (immunoreagents), eliminating the need for pre-etching of hydrophobic resin surfaces to introduce hydrophilic groups before labelling. Membrane and cytosol structures can be observed without lipid-stabilising osmium treatment as LR White does not solubilise

lipids. The only problem, however, is that membrane structures are quite difficult to stain reproducibly. As the resin is miscible with a small amount of water, however, infiltration can be performed on partially dehydrated specimens (70% dehydrated) that have greater antigenicity than fully dehydrated samples.

Besides difficulties with staining and contrast, disadvantages include instability in the electron beam, and the adverse effects of oxygen on polymerisation. Final polymerisation of resin in a resin-filled gelatin capsule impermeable to oxygen, solves this problem. Slowly increasing the intensity of the beam also allows resin stabilisation and prevents specimen damage (Griffiths, 1993; Philimonenko *et al*., 2002). Another compromise between the preservation of antigenicity and organelle structure also needs to be made for successful immunocytochemistry. This involves the choice of fixative and fixation protocol.

Glutaraldehyde is the best choice for the preservation of tissue ultrastructure. It is extensively cross-linking, however, and can alter epitopes resulting in loss of antigenicity or prevention of access to the antigen. Formaldehyde, on the other hand, results in better preservation of antigenicity, due to lower cross-linking, but gives poorer ultrastructural preservation. Macrophage ultrastructure also seems difficult to preserve and combination fixatives with approximately 4% PFA or less and low levels of glutaraldehyde (0.5% or less) have been shown to provide an adequate balance between the retention of ultrastructure and antigenicity, however (Griffiths, 1993).

The buffers used during the fixation process are important as well. Both carbonate and cacodylate buffers have been used in the past, but both have pKa's too low for proper fixation. Phosphate buffers are also popular, but they have limited solubility in the presence of divalent cations and generally extract protein during the fixation process. The buffers introduced by Good *et al*. (1966) are considered to be good buffers for fixation and include PIPES, HEPES and MOPS. PIPES buffers in particular result in much less extraction in comparison with phosphate buffers (Griffiths, 1993). The buffer chosen for use in the current study is a combination of PIPES, HEPES, EGTA and MOPS and is described by Santama *et al*. (1998).

Sectioning of specimens requires the use of an ultramicrotome. Glass knives are produced by fracturing glass strips into squares and then right-angled triangles to produce edges that

can cut hard resin. Knives for resin sectioning have a limited lifespan as edges become blunt over time due to the fluid nature of glass. Older knives can be used for the initial trimming of the resin block, whereas ultrathin sectioning requires the use of new knives. The sections are usually collected on formvar coated grids, the formvar acting as a support for the section. Immunolabelling requires that ultrathin sections be incubated with an antibody against the antigen to be located. Subsequently, the section is incubated with another molecule that binds the antigen-antibody complex allowing for detection by the EM (Geuze *et al*., 1981; Slot and Geuze, 1985). Protein A from *Staphylococcus aureus* binds the Fc region of antibodies in a 1:1 stoichiometric ratio. This protein can, therefore be adsorbed to electron-dense, gold colloids and used to localise a particular antigen in a cell section. Protein A has a particularly high affinity for rabbit IgG, so if the primary antibody used is not raised in rabbits, but another species with a lower protein A affinity, a linker antibody (e.g. rabbit anti-chicken IgY) should be incorporated and the protein A gold probe used to detect this antibody. The use of a linker antibody increases the labelling density as more than one rabbit IgG binds per IgY molecule (Griffiths, 1993; Slot and Geuze, 1985).

Immunocytochemistry allows for the cellular location of antigens to be determined and if it is to be performed there are several important points that need to be considered. A well characterized, high-affinity antibody against the antigen should be used. The antibody and electron dense markers should have access to all parts of the cells/tissues. The antigen being localised should possess a large amount of antigenicity after fixation and at the same time the preservation of fine-structure should be adequate (Griffiths, 1993). Control labelling experiments are essential for identifying any potential non-specific, high-affinity interactions with pre-immune sera. Controls include: incubation of the section with pre-immune IgG/IgY and detection with protein A and incubation of the section with protein A gold only. In all cases the pre-immune IgG/IgY and protein A gold probes are used at the same concentration as used in the labelling with the specific antibodies.

2.7.1 Fixation and embedding of J774 cells in LR White resin

2.7.1.1 Reagents

130 mM PIPES, 60 mM HEPES, 20 mM EGTA, 4 mM $MgCl_2$, pH 7.3 (2 x PHEM). PIPES (9 g), HEPES, (2.68 g), EGTA, (1.875 g) and $MgCl_2.6H_2O$ (0.163 g) were dissolved in 180 ml of dH_2O, adjusted to pH 7.3 with NaOH and made up to 200 ml. The solution was aliquotted and stored at -20°C.

65 mM PIPES, 30 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 7.3 (1 x PHEM). 2 x PHEM (50 ml) was diluted with dH_2O (45 ml), adjusted to pH 7.3 if necessary and made up to 100 ml.

PFA stock solution [16% (m/v) in dH_2O]. PFA (16 g) was dissolved in dH_2O (80 ml), heated to 60°C (in a fumehood) and the solution cleared with the dropwise addition of a 1M NaOH solution. After cooling, the volume was made up to 100 ml and aliquots stored at –20°C.

8% PFA in PHEM, pH 7.3. PFA stock solution (25 ml) was added to 2x PHEM (12.5 ml), adjusted to pH 7.3 with 1 M HCl and made up to 50 ml with dH_2O. The solution was stored at –20°C until required.

8% (m/v) PFA, 0.2% (v/v) glutaraldehyde in PHEM, pH 7.3. PFA stock solution (50 ml) and glutaraldehyde [800 µl of 25% (v/v)] were added to 2 x PHEM (25 ml), made up to 90 ml with dH_2O, adjusted to pH 7.3 with 1 M HCl and made up to 100 ml. The solution was stored at –20°C until required.

20 mM Glycine in PHEM, pH 7.3. Glycine (15 mg) was dissolved in 1 x PHEM (10 ml).

10 % (m/v) Gelatin in PHEM, pH 7.3. Microbiological grade gelatin (10 g) was added to 1 x PHEM (100 ml) and dissolved by heating. The volume was made up to 100 ml (if necessary) with dH_2O and the solution chilled rapidly on ice.

2.7.1.2 Procedure

Table 2.2 **Protocol for the fixation and embedding of J774 cells in LR White resin.**

Fixation	Time and Temperature
Fix in 8% (m/v) PFA, 0.2% (v/v) glutaraldehyde in PHEM, pH 7.3 with equal volume of medium.	2 h, RT
Replace with 8% PFA in PHEM, scrape of monolayers, transfer to tubes, centrifuge (700 x g, 10 min), store in 8% PFA.	Overnight, 4^{o}C
Pellet the cells (223 x g, 2 min), remove excess fixative.	
Quench remaining free aldehyde groups with 20 mM glycine in PHEM.	2 x 15 min, RT
Pellet the cells (223 x g, 2 min). Remove excess glycine. Infiltrate with 10 % (m/v) gelatin in PHEM, pH 7.3.	2 h, 37^{o}C
Pellet the cells (223 x g, 2 min). Remove excess glycine and chill pellet rapidly on ice.	
Place a thin layer of buffer over the gelatin-infiltrated pellet to prevent drying out and cut into small blocks (2 x 2 x 2 mm).	
Embedding	
Dehydration 25% ethanol	15 min, RT
Dehydration 50% ethanol	15 min, RT
Dehydration 70% ethanol	1 h, RT
Dehydration 90% ethanol	30 min, RT
Dehydration 100% ethanol	30 min, RT
LR White resin : ethanol (1 : 1)	30 min, RT
LR White resin : ethanol (2 : 1)	30 min, RT
LR White resin	2 x 30 min, RT
LR White resin	1 h, RT
LR White resin	Overnight, RT
Fill gelatin capsules with fresh resin, place cubes in tip of a gelatin capsule.	
Quickly close the capsule and fill completely with syringe. Allow to polymerise.	48 h, 50^{o}C

2.7.2 Glass knife production and preparation of grids

The glass strip was cleaned with detergent and water, dried and positioned on a LKB 7800 glass knife maker modified as described by Moorewood *et al*. (1992) and fractured to produce glass knives. The knife edges were examined and those with a barely visible counter-piece width were selected for ultrathin sectioning. A section collection trough made from aluminium foil tape was attached to the knife and sealed with nail varnish to produce a watertight boat. Ultrathin resin sections (90-110 nm) were cut using an ultramicrotome and sections were collected on formvar coated, copper grids.

2.7.3 Immunolabelling protocol

2.7.3.1 Reagents

10 x PBS stock solution, pH 7.2. NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (0.115 g), KH_2PO_4 (0.2 g) and NaN_3 (0.2 g) were dissolved in dH_2O (90 ml), titrated to pH 7.2 with NaOH and made up to 100 ml. The solution was autoclaved and stored at 4^{o}C.

1 x PBS working solution, pH 7.2. PBS stock solution (1 ml) was diluted with dH_2O (9 ml).

Glutaraldehyde fixative [1% (v/v) in PBS]. Glutaraldehyde [1 ml of a 25% (v/v) stock solution] was diluted to 25 ml with PBS.

BSA-PBS [1% (m/v) in PBS]. BSA (1 g) was dissolved in PBS and made up to 100 ml.

20 mM Glycine-PBS. Glycine (0.15 g) was dissolved in PBS and made up to 100 ml.

FSG-BSA [1% (v/v) FSG, 0.8% (m/v) BSA in 20 mM glycine-PBS]. FSG [1.11 ml of a 45% (v/v) solution] and BSA (0.4 g) were dissolved in glycine-PBS in a final volume of 50 ml. The solution was centrifuged (10 000 x g, 2 h, 4^{o}C) to remove insoluble debris and the supernatant aliquotted and stored at -20^{o}C.

2.7.3.2 Procedure

Immunogold labelling was performed by incubating the grids on droplets of reagent on Parafilm at RT. The steps followed are shown in Table 2.3. After the final staining the grids were air dried and viewed in a Philips CW120 Biotwin TEM at 80-100 kV.

Table 2.3 **Procedure for immunogold labelling of J774 cells.**

Step	Solution	Volume	Incubation Time
1. Blocking	BSA in PBS	20 µl	10 min
2. Blocking and aldehyde quenching	FSG-BSA	20 µl	4 x 1 min
3. Primary antibody	Diluted in FSG-BSA	10 µl	1 h
4. Wash	FSG-BSA	20 µl	5 x 4 min
5. Linker antibody (if required)	Diluted in FSG-BSA	10 µl	1 h
6. Wash	FSG-BSA	20 µl	5 x 4 min
7. Protein-A gold probe	Diluted in FSG-BSA	10 µl	1 h
8. Wash	FSG-BSA	20 µl	5 x 4 min
9. Fixation*	Glutaraldehyde fixative	10 µl	5 min
10. Wash	dH_2O	100 µl	4 x 5 min
11. Staining	Uranyl acetate	Droplet	6 min
	Lead citrate	Droplet	4 min

* If double immunogold labelling is to be performed, steps 1-9 are repeated at this point.

Controls included the substitution of pre-immune antisera at the same concentration as the test.

2.8 Fluorescent microscopy and immunolabelling

One of the most important applications of fluorescent microscopy has been immunofluorescence. This application combines the sensitivity, specificity and spatial resolution of normal fluorescent microscopy with the binding of antibodies to specific antigens within cells or tissues (Taylor and Salmon, 1989). During indirect immunofluorescence the cells are incubated with an unlabelled antibody which complexes with a specific antigen. The antigen-antibody complex is detected by a second antibody that is conjugated to a fluorochrome. Indirect immunofluorescence results in a brighter image than direct immunofluorescence as more than one secondary-conjugated antibody can bind to the unlabelled primary antibody (Karp, 1999).

The choice of fluorochrome is dependent on several factors. Two fundamental properties of fluorescence that need to be considered are the extinction coefficient (ε) and the quantum yield (Φ). The ε is an indication of the probability of absorption and optimal values for this parameter are 30 000-100 000 $cm^{-1}M^{-1}$ (Kerr and Loomes, 1994). The Φ measures the total photon emission over the fluorescence spectral profile and optimal values for this parameter are 0.05-1.0 (Taylor and Salmon, 1989). The fluorescence intensity per dye molecule is proportional to the product of ε and Φ. The choice of fluorochrome is also dependent on the light source and the detection system of the microscope (Tanke, 1998). The most common light sources used in fluorescence microscopy are high-pressure vapour lamps filled with either mercury or xenon gas. The mercury lamp is the most versatile as it has strong emission peaks in the near ultraviolet, violet, green and yellow parts of the spectrum and can thus excite a wide range of fluorochromes. Lasers are used in confocal laser scanning microscopy. Fluorochromes selected for double immunolabelling studies should have minimal spectral overlap.

The detection of intracellular antigens by indirect immunofluorescence requires the permeabilisation of cell membranes to allow the penetration of the antibodies. Saponin, a detergent-like molecule, acts by solubilising cholesterol and can thus permeabilise membranes without totally destroying them (Jacob *et al*., 1991). Permeabilisation with saponin has a number of advantages. It is a fast and simple method, it does not alter the expression of membrane bound antigens and it permeabilises both cytoplasmic and nuclear membranes (Jacob *et al*., 1991).

Two problems associated with fluorescent microscopy are photobleaching and quenching (Tanke, 1998). Photobleaching or fading refers to the loss of fluorescence during high-intensity excitation and is caused by photodecomposition of the fluorochrome or by the production of heat as energy is absorbed. This destruction of the excited fluorochrome becomes the factor limiting fluorescence detectability (Tanke, 1998). Photobleaching can be reduced by increasing the detection sensitivity, allowing the excitation intensity to be reduced. Detection sensitivity can be increased by using low-light detection devices such as CCD cameras or by using objectives with high numerical apertures. Photobleaching can also be significantly reduced by using antifade reagents during mounting (Longin *et al*., 1993; Ono *et al*., 2001). These reagents usually contain compounds that scavenge the oxygen radicals that result from the process of fluorescence; these radicals if not removed may react with the fluorochrome producing a product with less fluorescence (Tanke, 1998).

2.8.1 Immunolabelling protocol

2.8.1.1 Reagents

PBS, pH 7.4 [8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2.6H_2O$, pH 7.4]. Na_2HPO_4 (1.144 g) and KH_2PO_4 (0.2 g) were first dissolved in dH_2O (200 ml). NaCl (7.999 g), KCl (0.1998 g), $CaCl_2.2H_2O$ (0.147 g) and $MgCl_2.6H_2O$ (0.1016 g) were added and the solution made up to 1 l with dH_2O. The solution was filtered through Whatman No. 1 filter paper and stored at 4^oC.

PFA stock solution [16% (m/v) in dH_2O]. PFA (16 g) was dissolved in dH_2O (80 ml), heated to 60^oC (in a fumehood) and the solution cleared with the dropwise addition of a 1M NaOH solution. After cooling, the volume was made up to 100 ml and aliquots stored at $–20^oC$.

3.7% (m/v) PFA in PBS, pH 7.4. PFA stock solution (6.0 ml) was added to PBS, pH 7.4 (20 ml). The solution was made up just before use.

Saponin-PBS [0.1% (m/v) in PBS, pH 7.4]. Saponin (0.17 g) was dissolved in PBS (170 ml). The solution was filtered through Whatman No. 1 filter paper and was made up just before use.

BSA-PBS [1% (m/v) in PBS, pH 7.4]. BSA (0.13 g) was dissolved in PBS, pH 7.4 (13 ml). The solution was made up just before use.

BSA-Saponin-PBS [1% (m/v) in saponin-PBS, pH 7.4]. BSA (0.13 g) was dissolved in saponin-PBS, pH 7.4 (13 ml). The solution was made up just before use.

2.8.1.2 Procedure

A round, glass coverslip (12 mm diameter) was placed into each well of a 24 well plate, DMEM supplemented with 10% FCS (500 µl/well) added and the coverslips allowed to condition overnight. J774 macrophages were cultured and trypsinized (Section 2.2.2) and resuspended in approximately 12 ml DMEM supplemented with 10% FCS. The medium from each well was removed, J774 cell suspension (500 µl/well) added and the cells grown to approximately 70% confluency. Cells were fixed with 3.7% PFA in PBS (400 µl/well, 10 min, RT), washed with PBS (400 µl/well, 3 x, RT) and non-specific binding sites blocked by incubating the cells in BSA-PBS (400 µl/well, 45 min, RT). Cells were incubated in primary antibody diluted in saponin-PBS (150 µl/well, 1 h, RT) and washed in saponin-PBS (400 µl/well, 6 x, RT) and blocked again with BSA-saponin-PBS (400 µl/well, 1 h, RT). This was followed by incubation in secondary antibody diluted in saponin-PBS (150 µl/well, 1 h, RT) and as fluorescent probes are light sensitive, the plate was wrapped in foil from this point onwards. The cells were washed in saponin-PBS (400 µl/well, 6 x), post-fixed with 3.7% PFA (400 µl/well, 10 min) and washed finally in saponin-PBS (400 µl/well, 3 x). If double immunolabelling was to be performed the labelling procedure was repeated at this point. Controls included the substitution of pre-immune sera at the same level as the test and performing each labelling individually and in the opposite order (labelling for second antigen first) in repeat experiments. Coverslips was removed from the wells, dipped several times in dH_2O and air dried (RT). *SlowFade*™(anti-fade reagent) (1 µl) or Mowiol (4 µl) were applied to microscope slides and the coverslips mounted and sealed with clear nail varnish. Labelling was viewed using either an Olympus epifluorescent microscope and F-View CCD camera or a Zeiss 510 Meta confocal microscope and images analysed using ImageJ software. Images of colocalisation using colour (i.e. red and green images merged to form yellow) are highly influenced by display settings and intensity, and, therefore, colocalisation based on colour analysis alone is problematic and can lead to incorrect conclusions. Grey scale images (i.e. black and white) on the other hand, are not affected by display settings and

should be assessed when visually judging the following colocalisation images, as the yellow colour (indicating the degree of colocalisation in the composite image) varies depending on settings and printer type. The percentage colocalisation was determined manually by counting the number of vesicles in an average of at least 3 representative cells and reporting average vesicle colocalisation as a percentage.

CHAPTER 3

PRODUCTION AND CHARACTERISATION OF ANTIBODIES

3.1 Introduction

The primary aim of this study was to use immunofluorescence microscopy and gold labelling to establish firstly, the distribution of two classes of proteases, the cathepsins and MMPs as well as the tissue inhibitors of MMPs, TIMP-1 and -2 and secondly, a marker system for the vesicular compartments of J774 macrophages. Isolation and enzymatic activity assays of the content of organelles of the endosome-lysosome system from J774 macrophages suggest that cathepsin H and cathepsin S may be used as markers for the early and late endosomes, respectively (Claus *et al*., 1998; Jahraus *et al*., 1998), but this remains unverified using microscopy techniques.

Various antibodies have been raised against cathepsins (B, D, H, S and L) and MMP-9 (by past and present members of our research group). These have been mainly generated against isolated whole protein- or recombinant- or peptide sequences of human proteins. In chickens these include, anti-human liver cathepsin B (Elliott, 1993), -TIMP-1 (Clulow, M., unpublished data), -cathepsin S (raised against NVNHGVL peptide coupled to BSA using glutaraldehyde) (Morrison, L., unpublished data), -MMP-9 (Price *et al*., 2000). In rabbits, anti-cathepsin H (Coetzer, 1992) and -cathepsin L (Pike, 1990). A few have been raised against isolated animal proteins. These include a chicken anti-porcine cathepsin D antibody (Fortgens *et al*., 1997; Elliott *et al*., 1995) which will be used and an antibody against TIMP-2 was supplied by a collaborator, Dr Linda Troeberg (Imperial College, London). None, however, have been generated against mouse proteins and the cross-reactivity with mouse proteins was unknown at the beginning of this study.

Most commercially and other available antibodies against MMP-9 and the TIMPs seem to produce extremely variable or no results (Holten-Andersen *et al*., 2002). Raising antibodies against MMPs and the TIMPs is a difficult task as MMPs for example, share several common domains (Section 1.4.2, Figure 1.3) and together with TIMP-1 and -2 occur in the blood and are highly conserved across species (Holten-Andersen *et al*., 2002). Recombinant human TIMP-1 and TIMP-2 also available, as part of a collaboration with Dr Linda Troeberg (Imperial College, London) were, therefore, used to raise antibodies. It was reasoned that

TIMP-containing vesicles would possibly be distinct from those containing MMPs (Price *et al*., 2000) and also from cathepsins. If the antibodies raised in chickens against the collaboratively available recombinant TIMP-1 and -2 proved more reactive than the anti-human TIMP-1 (whole protein) antibodies previously raised in chickens (Clulow, M., unpublished data) and the sheep anti-human recombinant TIMP-2 (Dr L. Troeberg, Imperial College, London), these would be applied in this study.

In order to check both the recognition of the mouse antigen and specificity of these antibodies, a crude homogenate of the J774 cell line was used for western blotting characterisation studies. In such studies it was reasoned that both specific and non-specific cross-reactivity would be identified. For the recombinant antigens SDS-PAGE should be used to check for the presence of the required antigens and their apparent purity. The homology of proteins in laboratory animals chosen for production should be as low as possible and, therefore, the laboratory animals should be selected on this basis. Various adjuvants, inoculation protocols and antibody purification procedures are available and options will, therefore, be covered before the protocols chosen for the current study are described.

In this chapter, the production of antibodies in chickens against TIMP-1 and TIMP-2 (Section 3.4) and secondly, the characterisation of antibodies against cathepsins B, D, H, S and L, MMP-9, TIMP-1 and TIMP-2 to be used in the localisation studies is reported (Sections 3.6 and 3.7). Further information that can be gained from western blotting assuming that antibody recognition is equivalent for each form of an enzyme is the approximate ratio of mature to immature enzyme. This may be useful as the available antisera recognise all forms of the target enzyme (i.e. both immature and mature forms).

3.2 SDS-PAGE of recombinant TIMP-1 and TIMP-2

TIMP-1 has two N-glycosylation sites and depending on the degree of glycosylation may have a molecular weight ranging from 28.5 to 34 kDa (Caterina *et al*., 1998; Lambert *et al*., 2004). The human recombinant TIMP-1 used to raise antibodies was expressed as a 28 kDa glycosylated form. On the other hand, TIMP-2 lacks N-glycosylation sites and has a molecular weight of approximately 22 kDa. The supplied human recombinant TIMP-2 protein was reported to have a molecular weight of 22.8 kDa.

3.2.1 Reagents

Mature human recombinant TIMP-1 (glycosylated, 28.5 kDa) and TIMP-2 (22.8 kDa, non-glycosylated) proteins (sequences P01033 and P16035 available at http://us.expasy.org) expressed using pCEP4 vectors in HEK293, purified by ion exchange, affinity chromatography and gel filtration were supplied by Dr Linda Troeberg (Imperial College, London).

Reagents for SDS-PAGE and CBB staining were prepared according to Sections 2.3.1.1 and 2.4.1.1, respectively.

3.2.2 Procedure

Preparation of human recombinant TIMP-1 and TIMP-2 samples for SDS-PAGE analysis

TIMP-1 [1mg/ml] (1 µl) was combined with reducing treatment buffer (30 µl) and boiled for 90 s. Approximately 0.67 µg of TIMP-1 was loaded on the gel.

TIMP-2 [1.2 mg/ml] (1 µl) was combined with reducing treatment buffer (30 µl) and boiled for 90 s. Approximately 0.8 µg of TIMP-2 was loaded on the gel.

SDS-PAGE was performed to assess the purity of the human recombinant TIMP-1 and TIMP-2 proteins prior to immunisation (Section 2.3.1.2).

3.2.3 Results

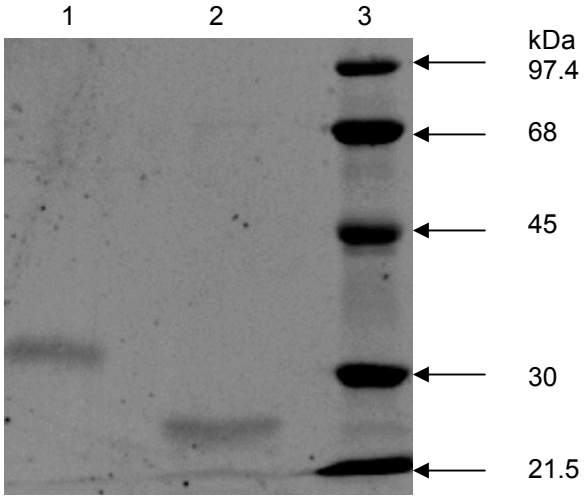

Figure 3.1 **Reducing SDS-PAGE of human recombinant TIMP-1 and TIMP-2 to assess protein purity.** Human recombinant TIMP-1 (lane 1, 0.67 µg), human recombinant TIMP-2 (lane 2, 0.8 µg) and molecular weight marker (lane 3, 5 µl). Both samples were prepared in reducing treatment buffer (30µl), combined with bromophenol blue [5 µl, 0.1% (m/v) in dH_2O], separated on a 12.5% Laemmli gel and stained with CBB.

Both recombinant TIMP samples gave a single band, indicating apparent purity. A TIMP-1 band of approximately 32 kDa (Figure 3.1, lane 1) which is slightly higher than the 28.5 kDa published value for the glycosylated form was observed (Woessner and Nagase, 2002). A band of approximately 24 kDa was detected for the recombinant human TIMP-2 (Figure 3.1, lane 2), a non-glycosylated inhibitor for which a molecular weight of 22.8 kDa has been reported (Troeberg, personal communication). A value of 24 kDa seen in this study is, therefore, reasonable.

3.3 Choice of laboratory animal for antibody production

For raising laboratory antibodies (in most countries) rabbits are the usual choice. This is especially true for immunocytochemistry purposes where protein A-gold probes are to be used, as protein A from *S. aureus* binds C_{H1} and C_{H2} domains of the Fc region of rabbit IgG better than IgG from most species (Harlow and Lane, 1999). Blood collection from rabbits, however, is difficult and exsanguination may often be required for a good final antibody yield.

Laying hens, on the other hand, produce antibodies that are transferred to the egg yolks (IgY) to provide the embryo with protection until it has a fully developed immune system. Chickens, therefore, have a number of advantages for antibody production. Collection of eggs, instead of invasive bleeding, is simpler and ethically preferable. From an evolutionary perspective, chickens should have a greater phylogenetic difference for most species to be studied and hence target antigens should have less sequence homology (Narat, 2003). Lastly, the yields of IgY from egg yolks are generally, considerably higher than IgG from other experimental animals (Losch *et al*., 1986). Although protein A does not bind to IgY and not many anti-IgY detection systems are available, this can be overcome by using a rabbit anti-IgY linker antibody (Harlow and Lane, 1999; Griffiths, 1993). The choice of animal in which to raise antibodies against a specific antigen, however, is best made after sequence alignments of the antigen in various species are performed, and the choice made on the basis of the greatest sequence variation to ensure the injected antigen has maximum antigencity. For this reason, 'BLAST 2 Sequences' alignments were perfomed on human, chicken, mouse, rabbit and sheep proteins.

3.3.1 Procedure

Sequence alignments of human TIMP-1 and TIMP-2 using 'Blast 2 Sequences' were performed in chickens, mice, rabbits and sheep to establish sequence homology, the potential antigenicity of human TIMPs in these species and hence the optimal choice of animal to be used in the current study.

'BLAST 2 Sequences'(http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) is a BLAST-based tool, utilising the algorithm for pairwise protein-protein sequence comparisons allowing for the alignment of two protein sequences that are known to be homologous. The sequences for TIMP-1 and -2 from humans, mice and rabbits were obtained from Entres protein database (http://www.ncbi.nlm.nih.gov) and included the following, human TIMP-1 (P01033), human TIMP-2 (P16035), mouse TIMP-1 (P12032), mouse TIMP-2 (P25785), rabbit TIMP-1 (P20614) and rabbit TIMP-2 (Q9TRS7). Chickens appear to lack TIMP-1 and, therefore sequences for TIMP-2 (O42146) and TIMP-3 (P26652) were obtained for comparison and only the sheep TIMP-1 sequence was available (P50122).

3.2.2 Results

Table 3.1 **TIMP sequence homology in human, chicken, mouse, rabbit and sheep species.**

	Chicken TIMP-2	Chicken TIMP-3	Mouse TIMP-1	Mouse TIMP-2	Rabbit TIMP-1	Rabbit TIMP-2	Sheep TIMP-1
Human TIMP-1	43%	40%	74%	-	83%	-	85%
Human TIMP-2	81%	44%	-	98%	-	94%	-

Human TIMP-1 showed the most sequence homology with sheep TIMP-1 (85%), followed by rabbit TIMP-1 (83%) and mouse TIMP-1 (74%), suggesting that antibodies against human TIMP-1 may cross-react with mouse antigens (Table 3.1). Chicken TIMP-2 and -3 (no chicken TIMP-1) appear to share only 43% and 40% sequence homology with human TIMP-1 and 81% and 44% with human TIMP-2 (Table 1.3). Human TIMP-2 showed the most overall sequence homology overall with rabbit-, mouse- and chicken TIMP-2, with the homology being highest to mouse (98%) and rabbit TIMP-2 (94%) and lowest to chicken TIMP-2 (81%) and -3 (44%) (Table 3.1). Sequence alignments, therefore, indicate that both human TIMP-1 and -2 would have the greatest antigenicity in chickens. As human TIMP-1 differs significantly from chicken TIMP-2 and -3, a greater immune response to human TIMP-1 in the chicken as opposed to TIMP-2 would be anticipated. As human TIMP-1 and

-2 shared a fairly high sequence homology with their mouse counterparts, antibodies raised against human TIMP-1 and -2 would be anticipated to cross-react with their corresponding mouse antigens in the J774 cell line.

3.4 Production of TIMP-1 and TIMP-2 antibodies in chickens

Adjuvants are usually used to stimulate the immune response and allow for the gradual release of antigen from the inoculation site ensuring maximal antigen exposure to MHC class II APCs in the peripheral tissues (Warren *et al*., 1986; Roitt, 1997). Efficient uptake of antigens by APCs is necessary for an optimal antibody response in the lymph nodes and is greatly influenced by the size and form of the antigen as well as the site of inoculation. Molecules of pathogenic origin are usually included in the adjuvant as they improve the non-specific immune response by producing a 'depot' effect. This is a slow leak of antigen into the tissue, resulting in continual stimulation of the immune system by encouragement of infiltration of inflammatory APCs (Warren *et al*., 1986). Pathogen products also stimulate lymphokine production and B-cell proliferation. Heat killed *Mycobacterium tuberculosis*, *M. tuberculosis*-derived muramyl peptide (Ellous *et al*., 1974) and the lipid A portion of LPS (Johnson *et al*., 1956) are well suited for inducing a good non-specific immune response. One of the most commonly used adjuvants for primary inoculations, Freund's complete adjuvant (FCA) is comprised of killed or attenuated *M. tuberculosis* and mineral oil (Freund and McDermott, 1942; Freund, 1956). This, followed by booster injections of antigen emulsified in FIA (FCA without *M. tuberculosis*) provide continual stimulation of the immune system and induce the IgM to IgG class switch. Additional booster injections improve antibody titre and avidity (Harlow and Lane, 1999). For this reason this adjuvant was chosen for the current study.

A number of antibody isolation methods exist. These include affinity chromatography (Ey *et al*., 1978; Gurvich and Drislikh, 1964), ion exchange chromatography (Bokovsky and Kennett, 1987) and precipitation (Russ *et al*., 1983). PEG 6 kDa precipitation has been extensively used for the rapid, inexpensive and high yield isolation of IgY from egg yolk (Polson *et al*., 1985). Steric differential exclusion of proteins into the extrapolymer space is effected by removing the hydration shells from the proteins. By adding increasing amounts of PEG various proteins exceed their solubility limit and precipitate out of solution, the PEG remaining in the supernatant associated with the aqueous phase (Dennison, 1999). This isolation method has been used successfully in our laboratory for many years (Pike *et al*.,

1992; Fortgens *et al*., 1997; Price *et al*., 2000) and for this reason, was chosen for the current study.

3.4.1 Reagents

Recombinant human TIMP-1 and TIMP-2 (Section 3.2), FCA and FIA.

3.4.2 Procedure

Antibodies to both human recombinant TIMP-1 and TIMP-2 were raised using one chicken for each antigen, as only limited amounts of the antigens were available. Each antigen was triturated through a 26-gauge needle with adjuvant [1:1 (v/v)] until no dispersion occurred when a drop of the emulsion was added to water. All immunisations were administered by intramuscular injection at a single site in each of the breast muscles (Table 3.2). Unfortunately, as only limited amounts of antigen were available, only a single boost could be administered at week 2.

Table 3.2 **Immunisation protocol for the production of antibodies against human recombinant TIMP-1 and TIMP-2 proteins in chickens.**

Week	Adjuvant	Antigen amount	
		TIMP-1	**TIMP-2**
0	FCA	~ 30 µg	~ 30 µg
2	FIA	~ 30 µg	~ 30 µg

3.5 ELISA assessment of immune response using diluted egg yolk extracts and IgY isolation

Immunoassays are based on the specific interaction between an antibody and antigen and provide quantitative information about the concentration of either the antibody or antigen in unknown samples (Johnstone and Thorpe, 1987). One of the most commonly used immunoassays is the enzyme-linked immunosorbent assay (ELISA). An ELISA utilises an enzyme chemically conjugated to either the antibody or antigen to allow detection of specific recognition and immune complex formation on a solid surface. One of the simplest and most frequently used ELISAs for the detection of antibodies is known as the three layer system. In this system, target antigen coated on to polystyrene microtitre plates is allowed to react with suitably diluted primary antibody to be assessed. Excess antibody is washed away and the binding of primary antibody is assayed using an appropriate detection system. This system usually consists of an enzyme conjugated secondary antibody capable of recognising the primary antibody bound to the immobilised antigen. The enzyme is allowed to react with

a chromogenic substrate and the coloured product measured spectrophotometrically. As ELISA yields quantitative results it complements western blotting (Section 2.6) which provides qualitative information about antibody specificity.

The progress of the chickens' immune response to the immunogens was followed by ELISA, using diluted egg yolk (crude, unpurified preparations containing antibodies), as diluted egg yolks give results comparable to those obtained using isolated IgY and offer a satisfactory method for determining which eggs should be selected for isolation of optimal levels of specific IgY (Coetzer, 1992).

3.5.1 Reagents

Phosphate buffer [100 mM sodium phosphate buffer, 0.02% (w/v) NaN_3, pH 7.6]. $NaH_2PO_4.H_2O$ (13.8 g) and NaN_3 (0.2 g) were dissolved in dH_2O (950 ml). The pH was adjusted to 7.6 with NaOH and the solution was made up to 1 l with dH_2O.

PBS, pH 7.4. NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in dH_2O (800 ml), adjusted to pH 7.4 with HCl and made up to 1 l.

BSA in PBS (BSA-PBS) [0.5% (m/v) in PBS]. BSA (0.5 g) was dissolved in PBS and made up to 100 ml.

Tween 20 in PBS (Tween-PBS) [0.1% (v/v) in PBS]. Tween 20 (1 ml) was diluted to 1 l in PBS.

Substrate buffer [150 mM citrate-phosphate buffer, pH 5.0]. Na_2HPO_4 (2.84 g) and citric acid (2.29 g) were each dissolved in dH_2O and made up to 100 ml. The citric acid solution was titrated against the Na_2HPO_4 (50 ml) solution to pH 5.0.

Substrate solution [0.05% (m/v) ABTS and 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer]. ABTS (7.5 mg) and H_2O_2 (7.5 µl) were dissolved in citrate-phosphate buffer, pH 5.0 (15 ml), for one ELISA plate.

0.1% (m/v) Sodium azide in 150 mM citrate-phosphate buffer, pH 5.0. For each ELISA plate, NaN_3 (15 mg) was dissolved in citrate-phosphate buffer (15 ml).

3.5.2 Procedure

Wells of microtitre plates were coated with human recombinant antigen [150 µl/well, 1 µg/ml in PBS, 16 h, RT], blocked with BSA-PBS (200 µl/well, 1 h, 37^{o}C) and washed with Tween-PBS (3 x). Egg yolk extracts were diluted with two volumes of phosphate buffer and used as the primary antibody [100 µl/well, 1:20 (v/v) in BSA-PBS and serially diluted twofold thereafter to 1: 10 240 (v/v), 1 h, 37^{o}C]. Excess antibody was washed out with Tween-PBS (3 x) and a suitable dilution of rabbit anti-chicken IgY-HRP conjugate in BSA-PBS was added (120 µl/well, 30 min, 37^{o}C). Excess antibody was washed out with Tween-PBS (3 x). The substrate solution was added and incubated in the dark for optimal colour development (150 µl/well, 10-20 min, RT) and the enzyme reaction stopped by the addition of NaN_3 [50 µl/well, 0.1% (m/v) in citrate-phosphate buffer]. Absorbances were read at 405 nm in a Bio-Tek EL312 Microplate Bio-kinetics reader. For the controls, either the blocking solution, primary antibody or secondary antibody was omitted to assess the efficiency of the blocking or the specificities of the primary/secondary antibodies. Titration curves were constructed by plotting -log IgY dilution versus absorbance to assess the immune response and determine which eggs should be selected for IgY isolation.

Selected egg yolks were separated from the egg whites, carefully washed in a stream of water, the yolk sacs punctured and the yolk volume measured. Two volumes of phosphate buffer were added and mixed gently by inversion after sealing the measuring cylinder with Parafilm. Crushed PEG 6 kDa was added [3.5% (m/v)] and dissolved by stirring. The precipitated vitellin fraction (containing lipoproteins) was pelleted by centrifugation (4420 x g, 30 min, RT) and the contaminating lipids removed by filtering the supernatant fluid through cottonwool placed in the neck of a funnel. PEG [8.5% (m/v)] was added to the clear filtrate to bring the final volume to 12% (m/v). The solution was mixed, centrifuged (12 000 x g, 10 min, RT) and the pellet dissolved in phosphate buffer, in a volume equal to that of the initial egg yolk volume. PEG was added [12% (m/v)], mixed and the solution centrifuged (12 000 x g, 10 min, RT). The supernatant fluid was discarded and the IgY pellet dissolved in phosphate buffer equal to 1/6th of the original egg yolk volume. A 1:50 dilution of IgY in phosphate buffer was prepared and the IgY in the indiluted solution was calculated using the IgY extinction coefficient ($E^{1mg/ml}_{280nm}$ = 1.25) and the equation [IgY] = [(A_{280}/1.25) x 50] (mg/ml) (Goldring and Coetzer, 2003).

3.5.3 Results

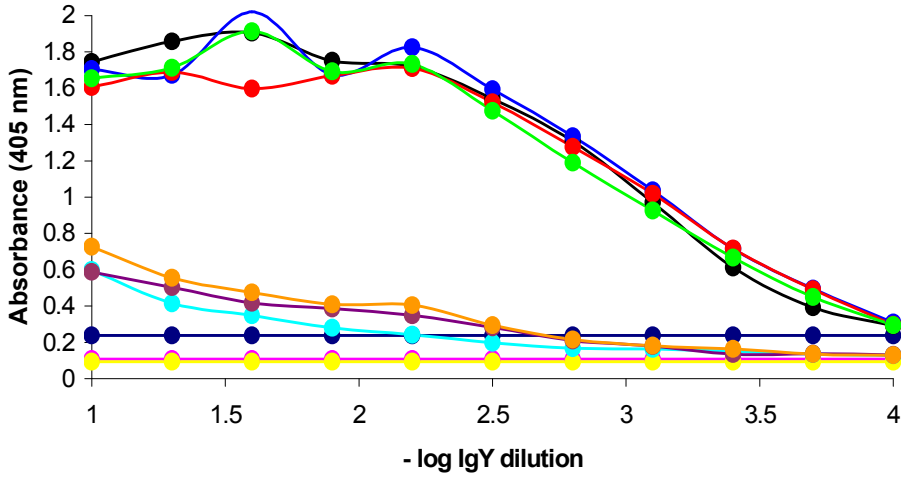

Figure 3.2 **ELISA of the progress of immunisation of a chicken with human recombinant TIMP-1.** TIMP-1 coated [1 µg/ml in PBS], incubated with egg yolk extracts (weeks 2-11) [diluted in two volumes of phosphate buffer, subsequently diluted 1:20 (v/v) in BSA-PBS and serially diluted twofold thereafter to 1:10 240 (v/v)], detected with rabbit anti-chicken IgY-HRP [1:5000] and developed in substrate solution [0.05% (m/v) ABTS, 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer]. Diluted egg yolks, pre-immune (•), week 2 (•), week 4 (•), week 6 (•), week 8 (•), week 10 (•), week 11 (•). No coat control (•), no primary antibody (•), no secondary antibody (•).

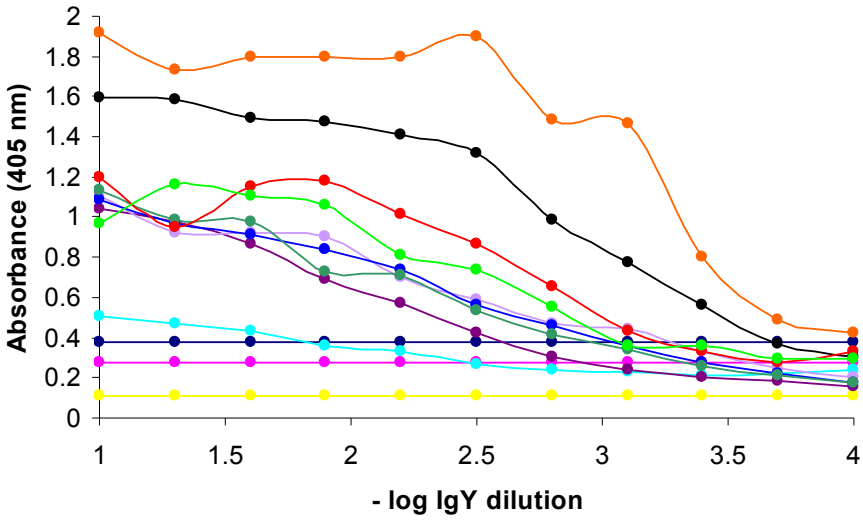

Figure 3.3 **ELISA of the progress of immunisation of a chicken with human recombinant TIMP-2.** TIMP-2 coated [1 µg/ml in PBS], incubated with egg yolks (weeks 2-11) [diluted in two volumes of phosphate buffer, subsequently diluted 1:20 (v/v) in BSA-PBS and serially diluted twofold thereafter to 1:10 240 (v/v)], detected with rabbit anti-chicken IgY-HRP [1:5000] and developed in substrate solution [0.05% (m/v) ABTS, 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer]. Diluted egg yolks, pre-immune (•), week 2 (•), week 4 (•), week 6 (•), week 7 (•), week 8 (•), week 9 (•), week 10 (•), week 11 (•). No coat control (•), no primary antibody (•), no secondary antibody (•).

The chicken immunised with human recombinant TIMP-1 demonstrated a slightly enhanced immune response for weeks 2 and 4 in comparison with the pre-immune control (Figure 3.2). Week 6-11 showed a much greater and sustained similar response (Figure 3.2) suggesting that the best eggs for chicken anti-TIMP-1 antibody isolation were those laid at this time,

especially during weeks 6-8. Eggs from week 8 were, therefore, selected for isolation and yielded 32.42 mg/ml IgY. In comparison, the immune response from the chicken immunised with human recombinant TIMP-2 was not generally as high overall, though a seemingly higher response was seen at week 4 (Figure 3.3). This response decreased thereafter, most markedly after week 6 (Figure 3.3), suggesting that the best eggs from which to isolate chicken anti-TIMP-2 antibodies were those laid in week 4. Eggs from this week were selected for isolation and 21.48 mg/ml IgY obtained.

3.6 Western blot characterisation of chicken anti-TIMP-1 and anti-TIMP-2 antibodies

The TIMPs 1-4 differ slightly in molecular weight, with molecular weights of 28-34 kDa being published for glycosylated TIMP-1 which has two glycosylation sites (Table 1.10). Differences in molecular weight seen may, therefore, be due to differences in glycosylation (Caterina *et al*., 1998). Non-glycosylated TIMP-1 has a molecular weight of approximately 20 kDa, similar to TIMP-2 (21 kDa) which has no glycosylation sites and hence a less variable molecular weight (Woessner and Nagase, 2002). TIMP-1 and TIMP-2 also share some sequence homology (Lambert *et al*., 2004) which may give rise to cross-reactivity. Possibly the best way of checking not only recognition of the antibodies for their target antigens, but also for any cross-reactivity with mouse TIMP-1 and TIMP-2 is western blotting using crude homogenates of J774 macrophages as the cell line contains both TIMP-1 and TIMP-2 and any non-specfic cross-reactivity would be with other proteins of the J774 macrophage.

3.6.1 Reagents

IgY preparations of anti-TIMP-1 (week 8) and anti-TIMP-2 (week 4) were isolated as described in Section 3.5.2.

Pre-immune IgY preparations were isolated from the same hens as used to raise anti-TIMP-1 and anti-TIMP-2 antibodies (Section 3.5.2).

The rabbit anti-chicken IgG (whole molecule) alkaline phosphatase conjugate used for western blotting cross-reacts with IgY.

Reagents for the culture of J774 cells, SDS-PAGE, staining of gels and western blotting were prepared according to Sections 2.2.1, 2.3.1.1, 2.4.2.1 and 2.6.2.1, respectively.

3.6.2 Procedure

Insufficient target immunogen was available so such antigens could not blotted, therefore, crude J774 macrophage homogenates were used instead to check cross-reactivity with mouse proteins. As part of a collaboration, however, Dr Linda Troeberg (Imperial College, London) tested the chicken anti-TIMP-1 and anti-TIMP-2 antibodies against recombinant human TIMP-1 and -2, and the results will be included.

Preparation of serum-containing and serum-free, crude J774 macrophage homogenates and supernatants

J774 macrophages were cultured in serum-containing medium until 70% confluent (Section 2.2.2). For serum-free macrophages, cells were additionally grown overnight in serum-free medium. Cells were scraped off the culture flask into serum-containing or serum-free culture medium, respectively using a rubber policeman, poured into a 15 ml centrifuge tube, centrifuged (460 x g, 3 min), the supernatant removed and stored at -20^{o}C before analysis. The cell pellets were either resuspended in serum-containing or serum-free medium (depending on which medium was used for culture) (500 µl) or resuspended directly in reducing treatment buffer (500 µl). Both homogenate preparations were stored at -20^{o}C before analysis. Homogenate and supernatant samples were thawed, combined with an equal volume of reducing treatment buffer and boiled for 90 s when required for SDS-PAGE with 15 µl of homogenate and 10 µl of supernatant samples being loaded on a 12.5% Laemmli gel.

Control samples of serum-containing medium (DMEM and FCS) and serum-free medium (DMEM without FCS) were stored at -20^{o}C, thawed, combined with an equal volume of reducing treatment buffer and boiled for 90 s when required for SDS-PAGE, with 10 µl of each sample being loaded on a 12.5% Laemmli gel.

SDS-PAGE and imidazole-SDS-zinc reversible staining were carried out according to Sections 2.3.1.2 and 2.4.2.2.

Serum-free J774 homogenate and supernatant samples were prepared as above for western blotting. Serum-free J774 homogenate (10 µl) and supernatant (10 µl) were separated on a 12.5% Laemmli gel, blotted and probed with chicken anti-TIMP-1 [20 µg/ml], chicken anti-TIMP-2 [40 µg/ml] and rabbit anti-chicken IgY HRP [1:5000] according to Section 2.6.2.2.

3.6.3 Results

Reducing SDS-PAGE showed that both serum-containing- (Figure 3.4, B, lane 3) and serum-free J774 supernatants (Figure 3.4, B, lane 4) had fewer bands than the serum-containing (Figure 3.4, B, lane 1) and serum-free J774 homogenates (Figure 3.4, B, lane 2). Comparison of the serum-containing J774 supernatant sample (Figure 3.4, B, lane 3) with serum- containing medium control (Figure 3.4, B, lane 5) indicate that some bands but not all are from the medium, suggesting that the remainder are possibly secreted proteins.

Of the two methods of J774 homogenate preparation investigated (Figure 3.4, A and B), samples treated with reducing treatment buffer immediately after isolation and subsequently frozen and thawed (Figure 3.4, A), showed a more distinct banding pattern than those first frozen in medium before preparation for SDS-PAGE analysis (Figure 3.4, B, lanes 1 and 2), with greater smeering possibly being related to an increase in denaturation by freezing in media as opposed to in reducing treatment buffer. Cells cultured in serum-containing medium (Figure 3.4, B, lane 1), showed a prominent band at 68 kDa (albumin) and appeared to have more bands in comparison with cells cultured overnight in serum-free medium (Figure 3.4, B, lane 2). The serum-free medium control (DMEM without FCS) (Figure 3.4, B, lane 6) appeared to show a band of approximately 68 kDa which seems to correspond to the albumin present in the serum-containing medium control (Figure 3.4, B, lane 5), similarly a band of approximately 68 kDa appears to be present in the empty lane (Figure 3.4, B, lane between lanes 4 and 5) suggesting that the DMEM and FCS sample loaded in lane 5 may have contaminated the serum-free control and the empty lane. To prevent this contamination in future, the delay between loading different samples should be kept to a minimum.

Based on the above results, the immediate treatment of the cell pellet with reducing treatment buffer, followed by freezing and thawing appeared to be the optimal method for J774 homogenate preparation and was used for subsequent western blotting.

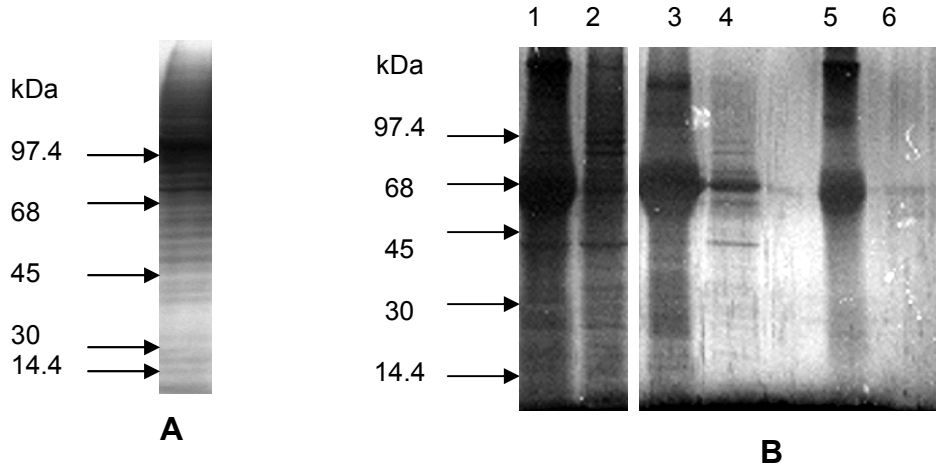

Figure 3.4 **Reducing SDS-PAGE separation of J774 macrophage homogenates and supernatants.**
A, serum-free J774 homogenate prepared directly in reducing treatment buffer (15 µl) and B, serum-containing J774 homogenate frozen first in serum-containing medium (lane 1, 15 µl), serum-free J774 homogenate frozen first in serum-free medium (lane 2, 15 µl), serum-containing J774 supernatant (lane 3, 10 µl), serum-free J774 supernatant (lane 4, 10 µl), DMEM and FCS (lane 5, 10 µl), DMEM without FCS (lane 6, 10 µl). All samples were diluted [1:1] with reducing treatment buffer boiled for 90 s, combined with bromophenol blue [5 µl, 0.1% (m/v) in dH_2O] and separated on a 12.5% (v/v) Laemmli gel. A and B, stained with imidazole-SDS-zinc reverse staining.

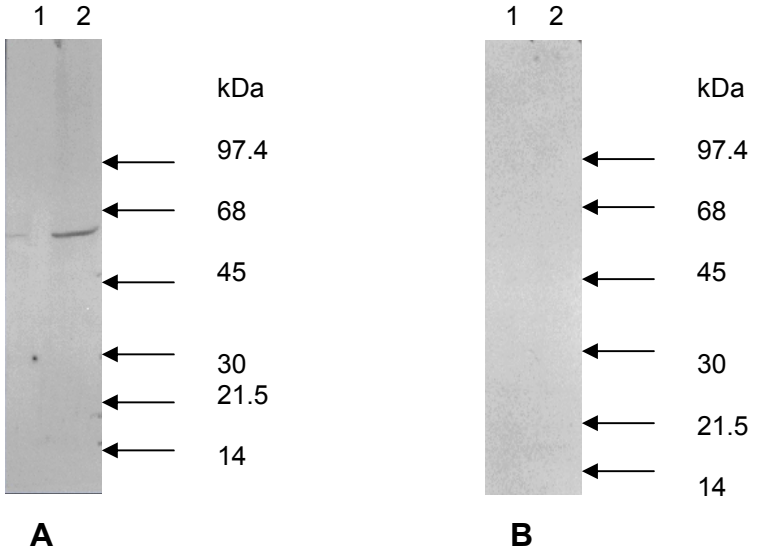

Figure 3.5 **Characterisation of chicken anti-TIMP-1 using J774 macrophage homogenates and supernatants.**
A and B, serum-free J774 supernatant (lane 1, 10 µl) and serum-free J774 homogenate (lane 2, 10 µl) were probed with chicken anti-TIMP-1 [20 µg/ml (A)], pre-immune IgY [20 µg/ml (B)], detected using rabbit anti-chicken IgY-HRP [1: 5000 (A and B)] and developed using DAB substrate solution after separation on a 12.5% Laemmli gel and blotting on to nitrocellulose.

Western blots of serum-free J774 homogenate and supernatant using the chicken anti-TIMP-1 antibody detected a band of approximately 61 kDa in the J774 homogenate (Figure 3.5, A, lane 2) and a less distinct band of the same molecular weight was detected in the supernatant (Figure 3.5, A, lane 1). No bands were detected in the pre-immune control (Figure 3.5, B). The molecular weight of 61 kDa is much higher than expected for the TIMP-1 monomer (28-

30 kDa) (Murphy and Willenbrock, 1995; Gomez *et al*., 1997). High molecular weight forms of TIMP-1 of approximately 66 kDa have been detected in neutrophil homogenates (Price *et al*., 2000), in mouse bone calvaria (Nagayama *et al*., 1984) and secreted by human umbilical vein endothelial cells treated with nafoxidine (De Lorenzo *et al*., 2000). A 62 kDa form of TIMP-1 was identified in parotid saliva (Drouin *et al*., 1988) and high molecular weight aggregates of 56 and 70 kDa have been observed in human plasma (Cawston *et al*., 1986). Polymeric forms of TIMP-1 (28-120 kDa), resistant to reduction have also been identified by western blotting of sputum samples (Sorsa *et al*., 1994). These forms may exist *in vivo* or may be isolation artifacts generated during homogenisation by enzymes such as PDI present in macrophages (Table 1.4) or due to glycosylation and concentration effects (Murzin, 1993; Gomez *et al*., 1997; Hasegawa *et al*., 2003). Recognition off different epitopes exposed at various times during blotting has also been demonstrated (Holten-Andersen *et al*., 2002) but high molecular weight bands were not visible in the original sample (Figure 3.1).

The chicken anti-TIMP-2 antibody detected bands of approximately 72 and 69 kDa in the J774 homogenate (Figure 3.6, A, lane 1). The 69 kDa also appeared in the pre-immune control (prepared from the same hen in which antibodies were raised) and may, therefore, be the product of specific binding of background antibodies present in the hen prior to immunisation (Figure 3.6, B, lane 1). The chicken anti-TIMP-2 antibodies should, therefore, be affinity purified to remove contaminating background antibodies. The band of 72 kDa is higher than the expected for TIMP-2 (21 kDa). It has, however, been reported that TIMP-2 appears to migrate in two positions in reducing SDS-PAGE gels, one form migrates in the 72 kDa position and the other migrates at the expected molecular weight of 21 kDa. It is suspected that the 72 kDa form of TIMP-2 is actually a relatively stable, reduction resistant complex with a processed form of MMP-2 (Zeng and Millis, 1994). This suggests most of the TIMP-2 is complexed with MMP-2 and very little, if any TIMP-2 occurs in its free form in the cell homogenate.

Blots performed by Dr Linda Troeberg (Imperial College, London) against human recombinant TIMP-1 and TIMP-2 showed that both chicken anti-TIMP-1 (Figure 3.7, lanes 1-6) and anti-TIMP-2 (Figure 3.7, lanes 7-13) recognised low molecular weight forms of human TIMP-1 and TIMP-2, respectively.

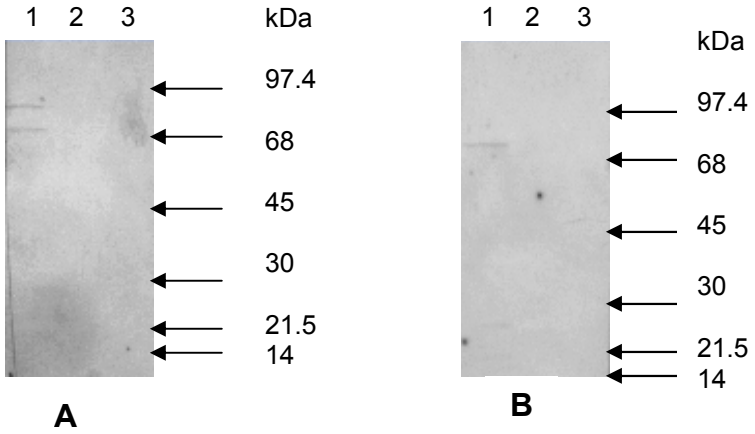

Figure 3.6 **Characterisation of chicken anti-TIMP-2 using J774 macrophage homogenates and supernatants.**

A and B, serum-free J774 homogenate (lane 1, 10 µl), serum-free J774 supernatant (lane 2, 10 µl), DMEM without FCS (lane 3, 10 µl), were probed with chicken anti-TIMP-2 [40 µg/ml (A)], pre-immune IgY [40 µg/ml (B)], detected with rabbit anti-chicken IgY-HRP [1: 5000 (A and B)] and developed using DAB substrate solution after separation on a 12.5% (v/v) Laemmli gel and blotting on to nitrocellulose.

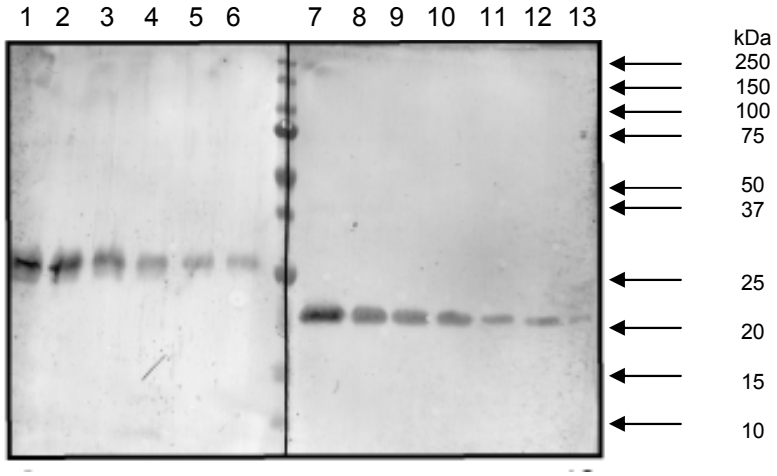

Figure 3.7 **Characterisation of chicken anti-TIMP-1 and anti-TIMP-2 using human recombinant TIMP-1 and TIMP-2.**

Human recombinant TIMP-1 100 ng (lane1), 50 ng (lane 2), 25 ng (lane 3), 12 ng (lane 4), 6 ng (lane 5), 3 ng (lane 6) and human recombinant TIMP-2 100 ng (lane7), 50 ng (lane 8), 25 ng (lane 9), 12 ng (lane 10), 6 ng (lane 11), 3 ng (lane 12), 1.5 ng (lane 13), were probed with chicken anti-TIMP-1 [65 µg/ml (lanes 1-6)], chicken anti-TIMP-2 [43 µg/ml (lanes 7-13)], detected and developed with a rabbit anti-chicken IgY-alkaline phosphatase system after separation on a 12.5% (v/v) Laemmli gel and blotting on to nitrocellulose.

3.7 Characterisation of other antibodies used in the study

Cathepsin B (Mørland and Pedersen, 1979; Muno *et al*., 1990; Lah *et al*., 1995; Reddy *et al*., 1995), cathepsin D (Diment and Stahl, 1985; Diment *et al*., 1988), cathepsin H (Muno *et al*., 1990; Portnoy *et al*., 1986; Claus *et al*., 1998), cathepsin S (Shi *et al*., 1992; Claus *et al*., 1998; Jahraus *et al*., 1998) and cathepsin L (Reilly *et al*., 1989; Reddy *et al*., 1995) have been reported in various macrophage types.

Early studies suggested that only the proforms of certain cathepsins are secreted by monocytes/macrophages (Portnoy *et al*., 1986; Reilly *et al*., 1989). Both thioglycolate-elicited mouse peritoneal macrophages and J774 mouse macrophages secrete a 36 kDa form of procathepsin L (Portnoy *et al*., 1986), whereas, human alveolar macrophages secrete a 43 kDa precursor form (Reilly *et al*., 1989). Monocytes allowed to mature under specific culture conditions form MDMs with a highly proteolytic phenotype. These cells secrete the proforms of cathepsin B (45 kDa), cathepsin L (43 kDa), cathepsin S (37 kDa) as well as the mature, single chain forms of cathepsin B (31 kDa), cathepsin L (34 kDa) and cathepsin S (25 kDa) (Reddy *et al*., 1995). Both the pro- and mature forms of cathepsins K and L appear to be secreted within 5 and 3 culture days, respectively, whereas, the proforms of cathepsin B and S were detected after 3 culture days, with mature forms being detected after 5 days for cathepsins B and L and 12 days for cathepsin S (Punturieri *et al*., 2000). The variation in secretion between the types of cathepsin as well as between the different forms suggests that these proteins are packaged and secreted from different intracellular compartments. Although both forms of cathepsin L were secreted, cathepsin L was preferentially secreted as mature enzyme (Punturieri *et al*., 2000). Active cathepsin H has been identified in both mouse macrophages (Muno *et al*., 1990; Claus *et al*., 1998) and human monocytes (Greiner *et al*., 2003). Fully differentiated peripheral blood monocytes show more cathepsin H activity than immature monocytes (Greiner *et al*., 2003). MDMs are also capable of secreting both pro- and mature forms of cathepsin D, with the proform being secreted in the first 5 days of culture (Punturieri *et al*., 2000).

Before immunolocalisation studies on these antigens in macrophages were carried out the cross-reactivity of chicken anti-human liver cathepsin B (Elliott, 1993), chicken anti-porcine cathepsin D (Fortgens *et al*., 1997; Elliott *et al*., 1995), chicken anti-cathepsin S (Morrison, L., unpublished), rabbit anti-cathepsin H (Coetzer, 1992), rabbit anti-cathepsin L (Pike, 1990), chicken anti-human MMP-9 (Price *et al*., 2000) and chicken anti-human TIMP-1 (Clulow, M., unpublished) with mouse antigens was checked. Sheep anti-human TIMP-2 serum supplied by Dr Linda Troeberg (Imperial College, London) was previously checked for cross-reactivity with mouse antigens and was, therefore, not repeated.

3.7.1 Reagents

Antibodies were kind gifts from current or former members of our research group in the Department of Biochemistry, University of KwaZulu-Natal, Pietermaritzburg. Chicken anti-human liver cathepsin B was from Dr E. Elliott, chicken anti-porcine cathepsin D was from Dr P. Fortgens, chicken anti-cathepsin S was from Miss L. Morrison, rabbit anti-cathepsin H was from Prof T. H. T. Coetzer, rabbit anti-cathepsin L was from Dr R. Pike, chicken anti-human MMP-9 was from Dr B. Price and chicken anti-human TIMP-1 was from Miss M. Clulow.

Pre-immune sera were not from the same animals used to raise the antibodies but were from pooled samples.

Reagents for the culture of J774 cells, SDS-PAGE, CBB staining and western blotting were prepared according to Sections 2.2.1, 2.3.1.1, 2.4.1.1 and 2.6.2.1, respectively.

10 x PBS. Na_2HPO_4 (2.6 g), $NaH_2PO_4 \cdot H_2O$ (0.36 g) and NaCl (16.4 g) were dissolved in a final volume of 200 ml dd.H_2O without pH adjustment. The solution was autoclaved (121°C, 15 min) and stored in aliquots at –20°C.

Percoll [63% (v/v) in PBS]. 63 parts 100% Percoll (density 1.13 g/ml) were diluted with 7 parts 10 x PBS and 30 parts 1 x PBS just before use, and kept on ice.

Percoll [72% (v/v) in PBS]. 72 parts 100% Percoll (density 1.13 g/ml) were diluted with 8 parts 10 x PBS and 20 parts 1 x PBS just before use, and kept on ice.

3.7.2 Procedure

Preparation of crude monocyte homogenates

Venous blood was drawn from a healthy, non–smoking volunteer into a centrifuge tube containing citrate–phosphate–dextrose anticoagulant (7 ml) to a final volume of 50 ml. Percoll [63% (v/v)] (15 ml) was added to a sterile 50 ml conical centrifuge tube and carefully underlaid with Percoll [72% (v/v)] (15 ml), taking care not to disturb the discontinuous gradient with air bubbles. Anticoagulated whole blood (15 ml) was slowly layered on top of the pre–cooled Percoll gradient and centrifuged (500 x g, 30 min, RT). The plasma and mononuclear cell layer (from which the monocytes were obtained) were removed, aspirated, the cells resuspended in physiological saline (~1–5 ml) and stored at -20^{o}C (Boyum, 1976).

When required, blood monocytes were thawed and centrifuged (17 203 x g, 12 min, RT). The pellet was sonicated (30 min, RT) and for SDS-PAGE was combined with equal volumes of reducing treatment buffer and boiled for 90 s. A sample of human plasma was similarly treated and used as the control.

Crude serum-containing J774 macrophage homogenates were initially frozen in medium before preparation in reducing treatment buffer for SDS-PAGE analysis (Section 3.6.2). SDS-PAGE and CBB staining were performed according to Sections 2.3.1.2 and 2.4.1.2. Serum-containing J774 homogenates (12 µl) and supernatants (10 µl), human monocyte homogenate (5 µl) and plasma (10 µl) were separated on 12.5% Laemmli gels (Section 2.3.1.2), transferred to nitrocellulose and probed with chicken anti-human liver cathepsin B [20 µg/ml or 35 µg/ml], chicken anti-cathepsin D [20 µg/ml or 40 µg/ml], rabbit anti-cathepsin H [10 µg/ml or 20 µg/ml], rabbit anti-cathepsin L [5 µg/ml or 10 µg/ml], chicken anti-cathepsin S [10 µg/ml or 20 µg/ml], chicken anti-MMP-9 [30 µg/ml], chicken anti-human TIMP-1 [10 µg/ml], rabbit anti-chicken IgG-alkaline phosphatase [1:100 000], goat anti-rabbit IgG-alkaline phosphatase [1: 30 000] according to Section 2.6.2.2.

3.7.3 Results

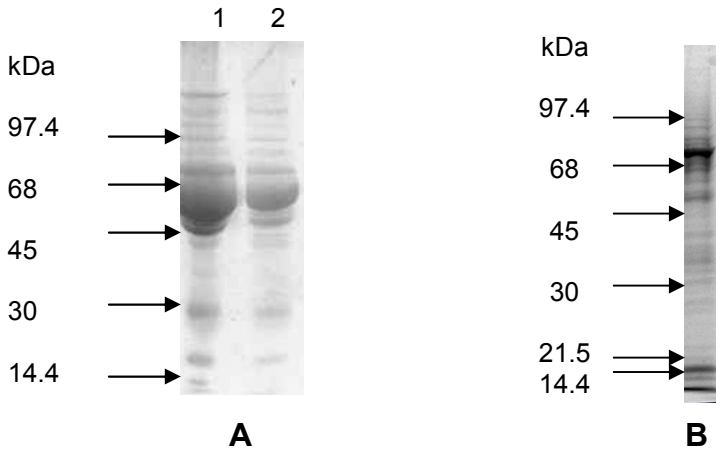

Figure 3.8 **Reducing SDS-PAGE separation of human monocyte and J774 mouse macrophage homogenates.**
A, human plasma (lane 1, 2 µl), human monocyte homogenate (lane 2, 2 µl) and B, serum-free J774 homogenate prepared immediately in reducing treatment buffer (15 µl), diluted [1:1] with reducing treatment buffer, combined with bromophenol blue [5 µl, 0.1% (m/v) in dH_2O] and separated on a 12.5% (v/v) Laemmli gel. A and B, stained with CBB.

Reducing SDS-PAGE showed a number of bands present in the serum-free J774 homogenate (Figure 3.8, B) which are not found in the human plasma and monocyte homogenate (Figure 3.8, A, lanes 1 and 2, respectively). Certain bands in the human monocyte homogenate

(Figure 3.8, A, lane 2) may be due to the presence of plasma in the monocyte homogenate (Figure 3.8, A, lane 1).

The antibodies to cathepsins B, D, H, L and S targeted only bands of the anticipated molecular weights in crude monocyte and J774 cell homogenates. Bands of approximately 39 kDa in the J774 mouse macrophage homogenate (Figure 3.9, A, lane 4) and 44 kDa in the human monocyte homogenate (Figure 3.9, B, lane 4) identified with the IgY anti-human liver cathepsin B antibody seem to correspond to the 40 kDa molecular weight of glycosylated human procathepsin B (Chan *et al*., 1986) with variations in targeted bands possibly representing differences in glycosylation patterns. A 20 kDa band in the J774 homgenate (Figure 3.9, A, lane 4) may correspond to the 22 kDa heavy chain of human cathepsin B (Kirschke *et al*., 1998) and a band of approximately 31 kDa in the homogenate of human monocytes (Figure 3.9, B, lane 4) may correspond to the single chain of human cathepsin B (25 kDa) (Kirschke *et al*., 1998). No detectable secreted cathepsin B was observed in the J774 supernatant (Figure 3.9, A, lane 3), however, the 44 kDa proform of cathepsin B appeared to be detected in the human plasma (Figure 3.9, B, lane 3). Assuming that the IgY anti-human liver cathepsin B antibody detects both pro- and mature forms to the same extent and from the intensity of the targeted bands it appears that antibody detected equivalent amounts of both pro- (approximately 50%) and mature (approximately 50%) cathepsin B in J774 macrophages (Figure 3.9, A, lane 4).

The 56 and 16 kDa bands in the J774 homogenate (Figure 3.10, A, lane 4) and 58 and 15 kDa bands in the human plasma (Figure 3.10, B, lane 3) revealed with the chicken anti-cathepsin D antibody appear to correspond to the 53 kDa precursor and 15 kDa light chain of cathepsin D previously observed in rabbit alveolar macrophages (Diment *et al*., 1988). In the J774 homogenate (Figure 3.10, A, lane 4), cathepsin D appears predominantly (approximately 80%) as a light chain as opposed to the precursor form in the monocyte homogenate (Figure 3.10, B, lane 4). This could indicate that the light chain contains most of the antigenic epitopes or that the heavy chain (35 kDa) has been degraded (Takahashi and Tang, 1983; Campaine *et al*., 1995).

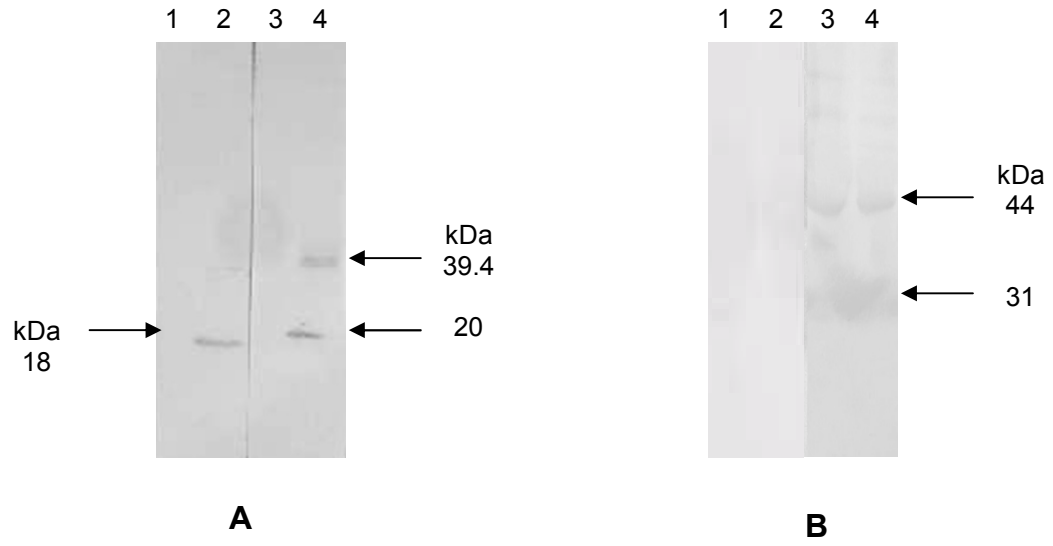

Figure 3.9 **Detection of cathepsin B in J774 macrophage and human monocyte homogenates.**
A, serum-containing J774 homogenate (lanes 2 and 4, 12 µl) and supernatant (lanes 1 and 3, 10 µl) and
B, human monocyte homogenate (lanes 2 and 4, 5 µl), human plasma (lanes 1 and 3, 10 µl) were probed with chicken pre-immune IgY [20 µg/ml (A, lanes 1 and 2) or 35 µg/ml (B, lanes 1 and 2)], chicken anti-human liver cathepsin B [20 µg/ml (A, lanes 3 and 4) or 35 µg/ml (B, lanes 3 and 4)], detected with rabbit anti-chicken IgG (whole molecule)-alkaline phosphatase [1:100 000 (A and B)] and developed in alkaline phosphatase substrate buffer after separation on a 12.5% (v/v) Laemmli gel and blotting on to nitrocellulose.

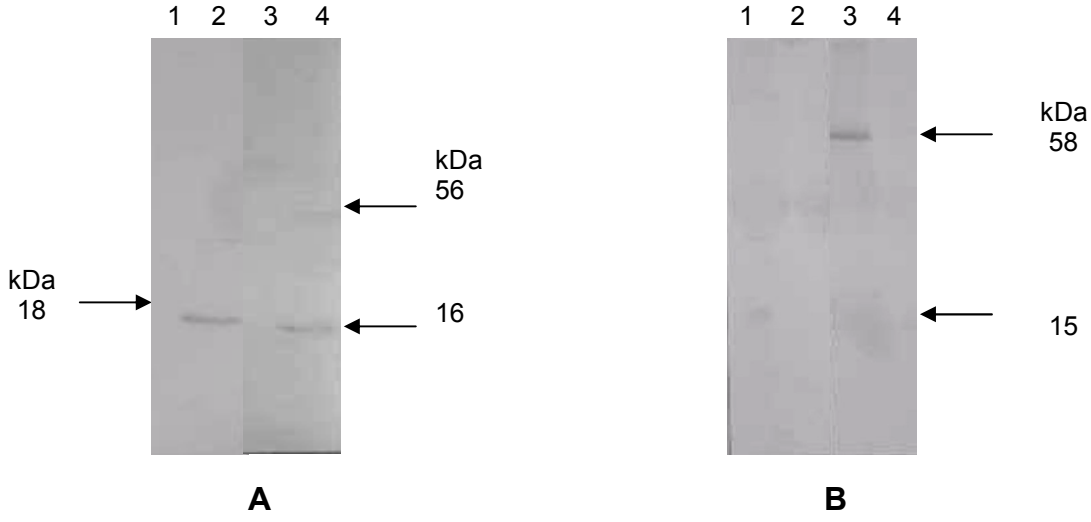

Figure 3.10 **Detection of cathepsin D in J774 macrophage and human monocyte homogenates.**
A, serum-containing J774 homogenate (lanes 2 and 4, 12 µl) and supernatant (lanes 1 and 3, 10 µl) and
B, human monocyte homogenate (lanes 2 and 4, 5 µl), human plasma (lanes 1 and 3, 10 µl) were probed with chicken pre-immune IgY [20 µg/ml (A, lanes 1 and 2) or 40 µg/ml (B, Lanes 1 and 2)], chicken anti-cathepsin D [20 µg/ml (A, lanes 3 and 4) or 40 µg/ml (B, lanes 3 and 4)], detected with rabbit anti-chicken IgG (whole molecule)-alkaline phosphatase [1:100 000 (A and B)] and developed using alkaline phosphatase substrate buffer after separation on a 12.5% (v/v) Laemmli gel and blotting on to nitrocellulose.

When the J774 and human monocyte homogenates as well as human plasma were probed with rabbit anti-cathepsin H antibodies, bands of approximately 36 kDa (Figure 3.11, A, lane 3) and 37 kDa (Figure 3.11, B, lanes 3 and 4), respectively, were seen. These are approximately the molecular weight reported for human procathepsin H (41 kDa) (Kirschke

et al., 1998). An 18 kDa (Figure 3.11, A, lane 3), close to the molecular weight (21 kDa) of the heavy chain reported for cathepsin H (Portnoy *et al*., 1986) was also observed in the J774 homogenate. Assuming that the rabbit anti-cathepsin H antibody detects both pro- and mature cathepsin H equally, there appears to be approximately 50% pro- and approximately 50% mature cathepsin H in J774 macrophages. The light chain band (5 kDa) (Kirschke *et al*., 1998) seems to have run off the lower edge of the blot (Figure 3.11, A, lane 3).

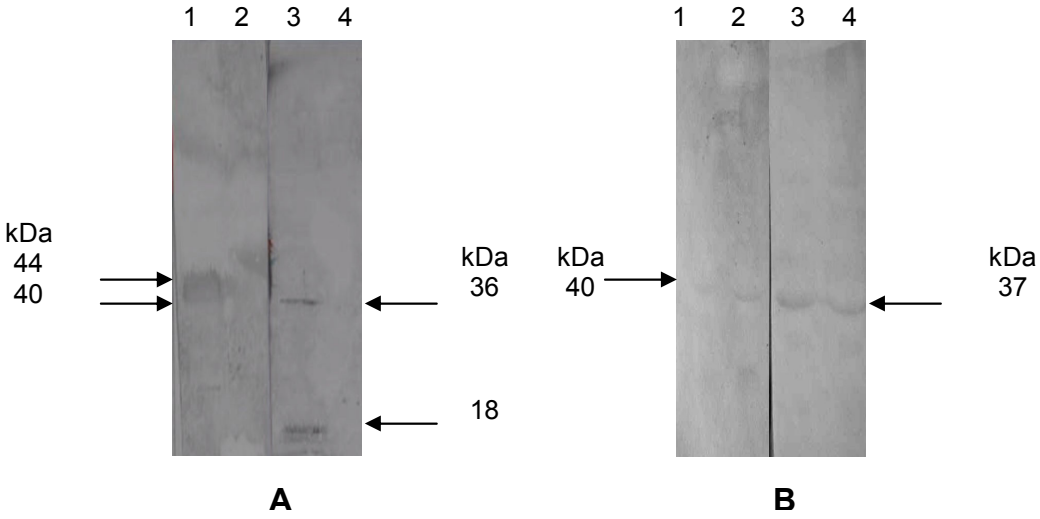

Figure 3.11 **Detection of cathepsin H in J774 macrophage and human monocyte homogenates.**
A, serum-containing J774 homogenate (lanes 1 and 3, 10 µl) and supernatant (lanes 2 and 4, 12 µl) and
B, human monocyte homogenate (lanes 2 and 4, 5 µl), human plasma (lanes 1 and 3, 10 µl) were probed with rabbit pre-immune IgG [10 µg/ml (A, lanes 1 and 2) or 20 µg/ml (B, lanes 1 and 2)], rabbit anti-cathepsin H [10 µg/ml (A, lanes 3 and 4) or 20 µg/ml (B, lanes 3 and 4)], detected with goat anti-rabbit IgG (whole molecule)-alkaline phosphatase [1:30 000 (A and B)] and developed in alkaline phosphatase substrate buffer after separation on a 12.5% (v/v) Laemmli gel and blotting on to nitrocellulose.

The various forms of cathepsin L have molecular weights similar to those of cathepsin H (Kirschke *et al*., 1998). Bands of approximately 37 kDa in the J774 homogenate (Figure 3.12, A, lane 3) and in the human plasma and monocyte homogenate (Figure 3.12, B, lanes 3 and 4) correspond to the reported molecular weight of procathepsin L (Kirschke *et al*., 1998). The 66 kDa bands observed in the human plasma and monocyte homogenate (Figure 3.12, B, lanes 3 and 4) may represent a dimeric form of cathepsin L. Detectable active cathepsin L (28 kDa) does not appear to be present in monocyte homogenate (Figure 3.12, B), however, a band of approximately 19 kDa in the J774 homogenate (Figure 3.12, A, lane 3) may correspond to the heavy chain of cathepsin L. From the blot it appears that equivalent amounts of pro- (approximately 50%) and mature (approximately 50%) cathepsin L are present in the J774 homogenate (Figure 3.12, A, lane 3).

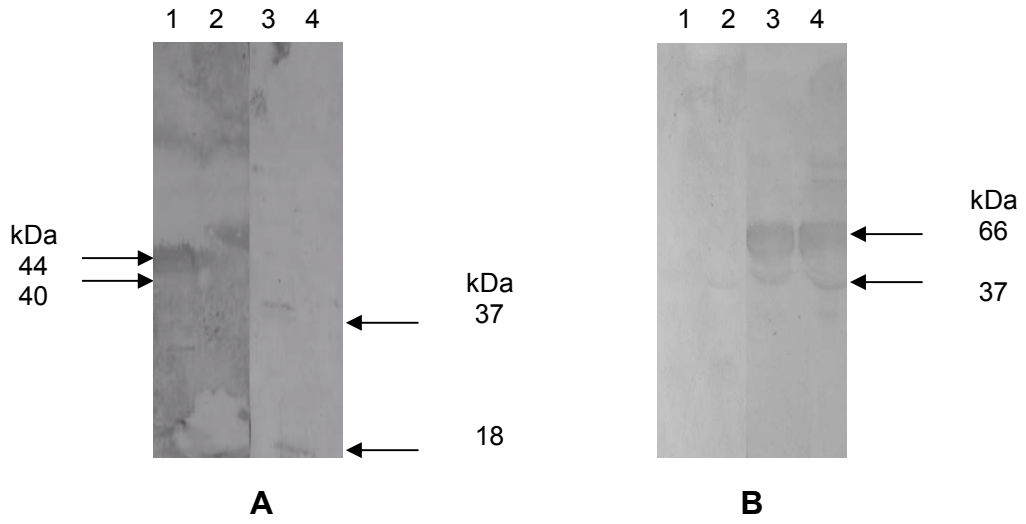

Figure 3.12 **Detection of cathepsin L in J774 macrophage and human monocyte homogenates.**
A, serum-containing J774 homogenate (lanes 1 and 3, 10 µl) and supernatant (lanes 2 and 4, 12 µl) and B, human monocyte homogenate (lanes 2 and 4, 5 µl), human plasma (lanes 1 and 3, 10 µl) were probed with rabbit pre-immune IgG [5 µg/ml (A, lanes 1 and 2) or 10 µg/ml (B, lanes 1 and 2)], rabbit anti-cathepsin L [5 µg/ml (A, lanes 3 and 4) or 10 µg/ml (B, lanes 3 and 4)], detected with goat anti-rabbit IgG (whole molecule)-alkaline phosphatase [1:30 000 (A and B)] and developed in alkaline phosphatase substrate buffer after separation on a 12.5% (v/v) Laemmli gel and blotting on to nitrocellulose.

Two bands of cathepsin S of approximately 39 kDa and 25 kDa corresponding possibly to pro- (37 kDa) and mature cathepsin S (24 kDa) were observed in the monocyte homogenate (Figure 3.13, B, lanes 3 and 4), whereas, a 34 kDa band (Figure 3.13, A, lane 3), possibly corresponding to procathepsin S was detected in the J774 homogenate (Kirschke *et al*., 1998) and no detectable mature cathepsin S was seen in the J774 homogenate, this is in agreement with Punturieri *et al*. (2000) only detected significant amounts of mature cathepsin S in MDM lysates after 12 days of culture. It is, therefore, possible that only undetectable amounts of mature cathepsin S were present as cells were not cultured for 12 days.

A summary of the percentage of precursor to mature cathepsin present in J774 homogenates is given in Table 3.3.

Table 3.3 **Summary of western blot data showing percentage occurrence of the precursor and mature forms of cathepsins H, S, D, B and L in J774 macrophages.**

	Precursor form (%)	**Mature form (%)**
Cat H	50	50
Cat S	90	10
Cat D	20	80
Cat B	50	50
Cat L	50	50

Abbreviation: Cat, cathepsin.

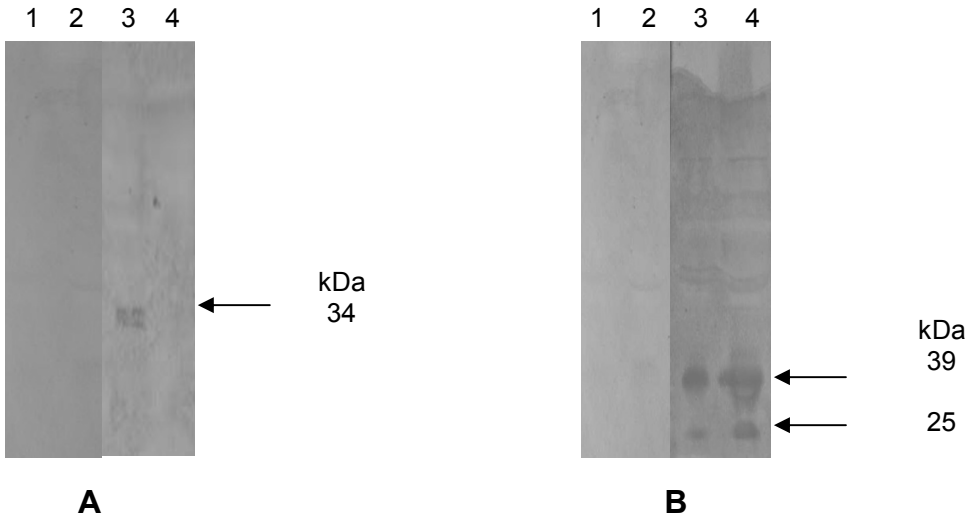

Figure 3.13 **Detection of cathepsin S in J774 macrophage and human monocyte homogenates.**
A, serum-containing J774 homogenate (lanes 1 and 3, 10 µl) and supernatant (lanes 2 and 4, 12 µl) and B, human monocyte homogenate (lanes 2 and 4, 5 µl), human plasma (lanes 1 and 3, 10 µl) were probed with chicken pre-immune IgY [10 µg/ml (A, lanes 1 and 2) or 20 µg/ml (B, lanes 1 and 2)], chicken anti-cathepsin S [10 µg/ml (A, lanes 3 and 4) or 20 µg/ml (B, lanes 3 and 4)], detected with rabbit anti-chicken IgG (whole molecule)-alkaline phosphatase [1:100 000 (A and B)] and developed alkaline phosphatase substrate buffer after separation on a 12.5% (v/v) Laemmli gel and blotting on to nitrocellulose.

The antibodies against MMP-9 and TIMP-1 appeared to target antigens of the anticipated molecular weight. The chicken anti-MMP-9 antibody detected bands of approximately 121, 70 and 65 kDa (Figure 3.14, lanes 3 and 4) in the monocyte homogenate. The high molecular weight form could be a heterodimer of MMP-9 (Goldman *et al*., 2003), however, a 120 kDa complex of MMP-9 bound to neutrophil gelatinase-B associated lipocalin (NGAL) has been identified under reducing SDS-PAGE conditions in neutrophils (Owen *et al*., 2003). NGAL or an NGAL equivalent does not seem to be present in macrophages, at least not according to phagosomal analyses (Table 1.4) but PDI does (Garin *et al*., 2001). Macrophage PDI involved in disulfide crosslinking, may be responsible for the high M_r complexes seen in TIMPs, though glycosylation has been reported to be a major contributor (Hasegawa *et al*., 2003). The 70 and 65 kDa bands are probably processed active forms of MMP-9 as bands of 74 and 65 kDa have been reported (Woessner and Nagase, 2002). Interestingly, the chicken anti-MMP-9 did not detect either pro- or mature MMP-9 in the J774 homogenate (results not shown). It is known, however, that mouse macrophages appear to have less MMP-9 than human macrophages (Filippov *et al*., 2003), therefore, the levels of MMP-9 in the J774 cells may have been below the detection limit. The chicken anti-human TIMP-1 antibody detected a band of approximately 22 kDa in the J774 homogenate which may correspond to a 20.6 kDa unglycosylated form of TIMP-1 (Figure 3.15) (Woessner and Nagase, 2002).

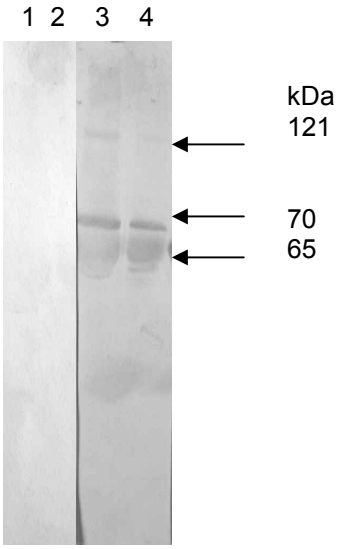

Figure 3.14 **Detection of MMP-9 in human monocyte homogenate.**
Human monocyte homogenate (lanes 1 and 3, 5 μl), human plasma (lanes 2 and 4, 10 μl) were probed with chicken pre-immune IgY [30 μg/ml (lanes 1 and 2)], chicken anti-MMP-9 [30 μg/ml (lanes 3 and 4)], detected with rabbit anti-chicken IgG (whole molecule)-alkaline phosphatase [1:100 000] and developed in alkaline phosphatase substrate buffer after separation on a 12.5% (v/v) Laemmli gel and blotting on to nitrocellulose.

The pooled pre-immune preparations, however, used at the same level showed non-specific bands at 44 and 40 kDa in the J774 homogenate using the rabbit pre-immune (Figure 3.11, A, lane 1 and Figure 3.12, A, lane 1) and 18 kDa using the chicken pre-immune (Figure 3.9, A, lane 2 and Figure 3.10, A, lane 2), respectively. The 18 kDa band was not seen in the pre-immune IgY preparation from chickens used to make anti-TIMP-1 and anti-TIMP-2 antibodies (Figures 3.5, B and 3.6, B) used at similar levels on similar homogenates and may, therefore, just represent background antibodies present at low levels in this particular pre-immune IgY preparation.

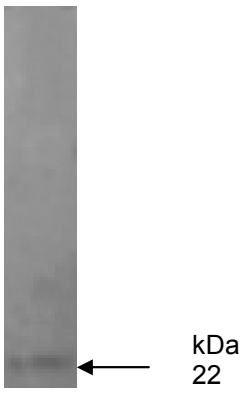

Figure 3.15 **Detection of TIMP-1 in J774 macrophage homogenate.**
Serum-containing J774 macrophage homogenate were separated on a 12.5% Laemmli gel, blotted on to nitrocellulose, probed with chicken anti-human TIMP-1 [10 μg/ml] detected with rabbit anti-chicken IgG (whole molecule)-alkaline phosphatase [1:100 000] and developed using BCIP, 0.015% (m/v), NBT, 0.03% (m/v) in alkaline phosphatase substrate buffer.

3.8 Discussion

High molecular weight forms of TIMPs (56, 62, 66, 56, 70 and polymeric forms 28-120 kDa) have been targeted in many studies and their presence has been ignored (Nagayama *et al*., 1984; Cawston *et al*., 1986; Drouin *et al*., 1988; Sorsa *et al*., 1994; De Lorenzo *et al*., 2000; Price *et al*., 2000). Various antibody preparations have also shown variable recognition for different antigenic regions in TIMP-1 (Holten-Andersen *et al*., 2002). As the newly produced antibodies against TIMP-1 and -2 do not detect reported and recognised forms of TIMP-1 and -2, these antibodies will not be used in this study and rather a chicken anti-TIMP-1 antibody previously made against human TIMP-1 (Clulow, M., unpublished) and a sheep anti-recombinant human TIMP-2 (Dr Linda Troeberg, Imperial College, London) targeting the correct molecular weights will be used. To verify that the high molecular weight bands are TIMP-1 and TIMP-2 western ligand blots (Price *et al*., 2000) and sequencing may be performed to verify the identification of such bands.

The current western blotting of J774 mouse macrophages showed pro- (39 and 44 kDa) and a smaller processed form (20 kDa) of cathepsin B. Greiner *et al*. (2003), however, reported only a 32 kDa form of cathepsin B in human monocytes and it is possible that only the precursor is present as the lower band may actually be the same molecular weight as the non-specific band detected by the pre-immune IgY. Western blotting also showed only the proform of cathepsin L in human monocyte homogenates, whereas, both pro- and mature forms were present in J774 macrophages. Only the proform of cathepsin S was seen in J774 homogenates, though both pro- and mature forms were present in human monocytes. If cathepsin S occurs only in its precursor form and is considered a marker for the late endosome (Jahraus *et al*., 1998), the presence of an immature form would be most unanticipated, as cathepsins are usually processed in the acidic environment of the late endosome (Kirschke *et al*., 1998).

Whereas both the pro- and mature forms of cathepsins B and H where detected in the J774 and human monocyte homogenates, only mature cathepsin D was detected in J774 cells. For immunolabelling studies this could have important consequences. Polyclonal antibody preparations recognise all forms of cathepsins and if different vesicle populations contain either pro- or mature cathepsins, it would be impossible to distinguish these different populations using such antibodies. As cathepsin D is mainly known as a marker for the acidic digestive body i.e. the lysosome and/or possibly the late endosome, where cathepsin D should be in its active form in both cases, and as J774 cells seem to contain almost

exclusively mature cathepsin D, the cathepsin D antibody could form one of the most reliable markers. Cathepsin B and H antibodies, for example, would be labelling both precursor and mature forms of the enzyme, here demonstrated to be present in the J774 cell, and hence acidic (containing mature enzyme) and non-acidic secretory vesicles (containing immature enzyme) could not be distinguished. This would complicate the use of these antigens as marker antigens for distinguishing various endocytic populations without the use of additional markers or probes for e.g. pH. Where only a mature or immature form of the enzyme is present, as apparently the case with cathespsin D (mature form), polyclonal antibodies are more useful, as they could be used reliably without additional probes.

From these blots, assuming equal recognition of all bands, it is interesting to note the differences in cathepsin content between human monocyte/macrophages and J774 cells. It would seem, that J774 mouse macrophages and human blood monocytes contain equal amounts of pro- and mature cathepsin B, blood monocytes have more pro- than mature cathepsin D, whereas, J774 macrophages appear to be the reverse. Similarly, cathepsins H and L appear to have equal mounts of both pro- and mature in J774 macrophages, while monocytes have only the proform. The blood monocytes have equal amounts of pro- and mature cathepsin S, but J774 macrophages have only procathepsin S.

The specificity and cross-reactivity of cathepsin, MMP-9 and TIMP-1 antibodies with mouse antigens seems to have been established and, therefore, were used in immunolabelling studies. The most important findings, besides the establishment of the variable suitability of the available antibodies for immunolabelling studies, seems to be that there do appear to be differences between the molecular weight and ratio of forms of cathepsin (precursor and mature) in J774 macrophages and human blood monocytes, verifying previous statements about the phenotypic differences between monocyte/macrophages from different species.

CHAPTER 4

J774 MOUSE MACROPHAGE VESICLE POPULATIONS

4.1 Introduction

Neutrophils (PMNs) and mononuclear phagocytes, or their activated counterparts, macrophages, are considered first and second line defence phagocytes of the innate immune system, respectively (van Oss, 1986; Garin *et al*., 2001; Ross and Auger, 2002). Both differentiate from a single myeloblast precursor that expresses key enzymes, such as cathepsins B and D, MPO and elastase (Tapper, 1996).

Upon promyeloblast differentiation and progression to the promyelocye, myelocyte and finally to the PMN, cathepsin G and MPO continue to be expressed but cathepsin B and D expression ceases. Mature myelocytes or PMNs, therefore, contain cathepsin G and MPO but little cathepsin B or D. Upon synthesis, PMN expressed proteins are packaged into large granules via a non-sorting process known as "targeting by timing", particular proteins expressed at a specific time being packaged into granules of specific morphology (Tapper, 1996; Gullberg *et al*., 1997; Borregaard and Cowland, 1997; Faurschou and Borregaard, 2003).

On the other hand, during differentiation of the promonoblast, to give rise to promonocytes, monocytes and finally macrophages, expression of cathepsins B and D persists but the expression of elastase and MPO is terminated (Tapper, 1996; Gullberg *et al*., 1997; Borregaard and Cowland, 1997; Faurschou and Borregaard, 2003). Monocytes and their activated macrophage counterparts, therefore, contain cathepsin B and D but little elastase or MPO (Campbell *et al*., 1989; Ross and Auger, 2002). As promonocytes differentiate to monocytes, distinctive granule populations seem to be lost and smaller vesicular- and endosome-lysosome type populations seem to predominate. Many more cathepsins are also expressed (Schmid *et al*., 2002; Rivera-Marrero *et al*., 2004). These enzymes have been described in various cell types as having a distinctive trafficking and processing pattern. This involves precursor trafficking from the Golgi to the late endosome via the MPR for activation and subsequent targeting to "lysosomal" storage organelles (Chapman *et al*., 1997). This is unlike the "targeting by timing" of proteases seen in the PMN which utilises

no sorting mechanism (Borregaard and Cowland, 1997; Faurschou and Borregaard, 2003). The markers associated with this trafficking and other markers and properties associated with the endosome-lysosome-like system of most cell types (Clague, 1998; Pillay *et al*., 2002) may, therefore, potentially be useful in describing the organelles present in the macrophage (Claus *et al*., 1998; Anes *et al*., 2006). It is now beginning to become evident that many of the classical markers for organelles previously designated "lysosomal" due to the presence of LAMPs, acidity or mature lysosomal enzymes and the lack of the 215 kDa MPR, may require re-evaluation as subpopulations within these previous groupings have been identified (Anes *et al*., 2006). For this reason, effort will be made to highlight these possibly oversimplified classifications by indicating such as "classical" assignations in the explanations given below.

In classical descriptions of endosome-lysosome systems (Figure 4.1), small particles, solutes, transmembrane proteins and membrane-bound ligands are incorporated into vesicles derived from the plasma membrane (Vieira *et al*., 2002), or, in the case of the macrophage this is most often membrane from the ER (Gagnon *et al*., 2002). Vesicles and their cargo are classically targeted to the early or tubulovesicular, sorting endosomes which label with Rab5 and EEA1 (Ghosh *et al*., 1994). Classically described as mildly acidic (pH of 6.3-6.5), and usually poor in proteases, these organelles have been reported to sort and recycle membrane and contain receptors such as the transferrin receptor to the plasma membrane, recycling endosomes or to the late endosome to be degraded (Pillay *et al*., 2002; Vieira *et al*., 2002). Generally closely associated with microtubules, recycling endosomes have been described as less acidic (pH 6.5) than lysosomes (pH <5.5) and Rab11-positive (Vieira *et al*., 2002).

Sorting in sorting endosomes is also classically agreed to occur via either of two model systems. In the shuttle vesicle model, vesicles carrying endocytosed material and specific membrane components shuttle between pre-existing, stable early endosomes, endosome carrier vesicles (ECVs) and multivesicular bodies (MVBs) or late endosomes (Griffiths, 1996a; Clague, 1998; Pillay *et al*., 2002). In the maturation model, on the other hand, sorting endosomes are transient organelles capable of maturing into ECVs, MVBs or late endosomes via fusion and fission events (Murphy *et al*., 1991; Vieira *et al*., 2002). The phagosome, into which larger particles are captured, is the only organelle that can definitively be seen to "mature". In this case, only recently has it been revealed that there is a gradual pH change from near neutral to acidity. This is accompanied by the acquisition of lysosomal enzymes

which is in turn associated with the transient association of markers for the early endosome (Rab5), late endosome (Rab7) and finally most of the markers of the late endosome and lysosome (Desjardins *et al*., 1994a; Desjardins *et al*., 1995; Desjardins *et al*., 1997; Pillay *et al*., 2002). Since the phagosome is not a focus of the current study, however, further details of phagosome maturation will not be included here. Most of the studies on the early and late endosomes, recycling endosomes, and lysosomes, as well as the phagosome, have been considerably facilitated by the ability to distinguish organelles in the endosome-lysosome system by filling the pathway and following endocytic traffic in pulse-chase studies. This has allowed the separation, and hence identification and purification of marker receptors such as Rab5 or EEA1 and the late endosomal Rab7 marker (Meresse *et al*., 1995) as well as the major 215 kDa MPR involved in trafficking of cathepsin enzymes (Griffiths, 1996a; Pillay *et al*., 2002; Vieira *et al*., 2002).

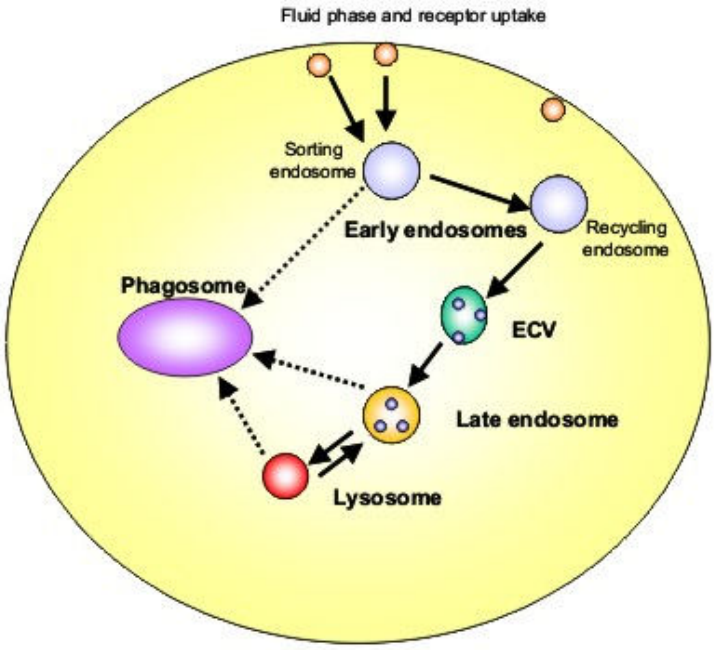

Figure 4.1 **Model of the endocytic pathway.**
Organisation of the endocytic pathway showing most important organelles, the early endosome, late endosome and lysosome. Solid arrows between compartments indicate vesicular trafficking or direct fusion. The phagosome is an additional compartment of the endocytic pathway and broken arrows indicate the compartments that interact with the phagosome and deliver proteolytic content to the phagosome, aiding in the process of phagosomal maturation (modified from Pillay *et al*., 2002; Gruenberg and van der Goot, 2006).

Identification of the classical acidic multivesicular or multilamellar late endosomes (pH 5.5) on the other hand was considerably facilitated using two approaches. These involved following the endocytic traffic for sorting and degradation, and the distinctive trafficking and processing pattern of cathepsins. The unique trafficking of cathepsins was revealed initially by studies on lysosomal enzyme storage and secretion diseases, such as I cell and Hurler's

diseases (Kornfeld, 1986). These diseases showed the importance of ER and Golgi processing, i.e. the addition of the high mannose-6-phosphate tag to lysosomal cathepsins, and the presence of 215 kDa MPR for the correct trafficking of the cathepsins between the Golgi and the late endosome for activation (Kornfeld, 1986; Turk *et al*., 2000; Wolters and Chapman, 2000; Barrett *et al*., 2004). These studies also showed that deficiency in either results in secretion of procathepsins i.e. default secretion via a "secretory" pathway not to be confused with the "regulated secretory" pathway which contains late endosome-processed cathepsins (Brown *et al*., 1986). Such a "regulated secretory" vesicle is also classically known as a "secretory lysosome" or a lysosome capable of regulated exocytosis (Griffiths, 1996b). Though later studies showed an additional 46 kDa receptor also responsible for lysosomal targeting of these proteins (Hoflack and Kornfeld, 1985; Riese and Chapman, 2000; Wolters and Chapman, 2000), studies on the 215 kDa receptor revealed the late endosome as the major sorting and processing organelle for enzymes such as the cathepsins and receptors such as the MPR and the necessity for an acid pH to perform the sorting and recycling role (Brown *et al*., 1986).

At the acidic pH of the classical late endosome (identified due to the presence of the 215 kDa MPR) the MPR releases the MPR-bound proenzyme and weakens the interaction between the propeptide and the catalytic region. This allows activation of the cathepsins (Riese and Chapman, 2000; Turk *et al*., 2000; Barrett *et al*., 2004). Activated lysosomal enzymes may be subsequently trafficked to a "lysosomal" population where they are stored (Griffiths, 1996a). On the other hand, the late endosome has been described as containing 20% of the total cellular hydrolytic enzymes, at any one time, and to be the main site for protein turnover (Griffiths, 1996a; Tjelle *et al*., 1996). It is also described to be associated with Rab7, Rab9, lyosbisphosphatidic acid, MPRs and LAMPs markers (Griffiths, 1996a; Pillay *et al*., 2002; Vieira *et al*., 2002). Whereas LAMP-1 and LAMP-2 are reportedly located on the limiting membranes of late endosomes and lysosomes, LAMP-3 appears predominantly associated with the internal multivesicular membranes of the late endosome and the outer membranes of specialised secretory organelles (Kobayashi *et al*., 2000). Not all late endosomes may be LAMPs-positive, however. This may be possible as lysosomal enzyme storage vesicles or "lysosomes" have been shown to fuse with the late endosomes forming a hybrid, acidic organelle only when proteolysis is required (Figure 4.2). Such a fusion may bring with it both the content and markers of the lysosomes i.e. LAMPs markers as well as proteolytic enzymes (Bright *et al*., 1997; Luzio *et al*., 2000), the MPRs recycle back to the

Golgi (Brown *et al*., 1986) before the proteolytic enzymes and LAMPs fuse. Therefore, hybrid organelles between late endosomes and lysosomes would appear to be "lysosomal" (MPR-negative, proteolytic enzymes-positive and LAMPs-positive after such an event). On the other hand, fission and reformation of individual organelles may result in loss of the LAMPs marker and enzymes when proteolysis is no longer required (Bright *et al*., 1997; Luzio *et al*., 2000) potentially giving rise to a LAMP-negative-, acidic organelle at some point, such as the lysosomal population described by Bright *et al*. (1997) (Figure 4.2). A summary of the markers most often used for identification of the late endosomes and lysosomes is given in Table 4.1.

Table 4.1 **Summary of major markers for late endosomes and "lysosomes".**

	Rab7	MPR	LAMPs	pH	Active enzymes	Active cathepsin D
Late endosome	+	+	+/-	6.5	+ (site of activation)	?
Lysosome	-	-	+	<5.5	+ (site of storage of active)[#]	+ [##]

[#] Small electron-dense organelle containing activated enzymes. Initially named a primary lysosome and described as acidic by de Duve, (1983), but subsequently as a lysosomal enzyme "storage organelle" by Griffiths, (1996a) but not necessarily acidic or cathepsin D-containing (Butor *et al*., 1995).
[##] Larger electron-dense organelle containing active cathepsin D and fused endocytic cargo previously described by de Duve, (1983) as a secondary lysosome but now considered to be a "hybrid organelle" or digestive body formed by fusion of the late endosome and lysosome (Figure 4.2) (Modified from de Duve, 1983; Brown *et al*., 1986; Berg *et al*., 1995; Griffiths, 1996a, Bright *et al*., 1997).

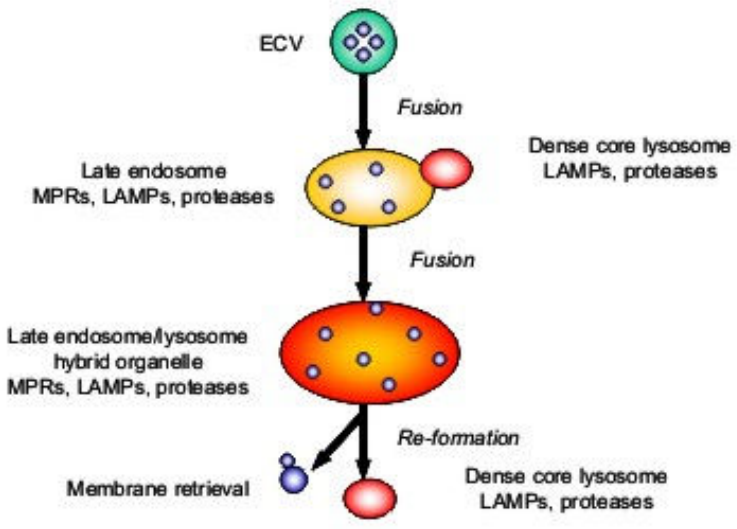

Figure 4.2 **Model of dense core lysosome-late endosome fusion and re-formation of lysosomes**.
ECVs derived from early endosomes, fuse with late endosomes and deliver their contents. The late endosomes, positive for MPRs and LAMPs contain about 20% of the total hydrolase pool. Dense core lysosomes, rich in hydrolytic enzymes fuse with late endosomes resulting in hybrid organelles rich in proteolytic enzymes. Lysosomes reform after the selective retrieval of membranes, post proteolytic events (modified from Bright *et al*., 1997).

"Lysosomes", classically the most acidic (pH < 5.5), small, electron-dense (dense core), MPR-negative organelles have been described as containing the bulk of activated and stored, but not necessarily active, hydrolytic enzymes (Table 4.1). The literature also often does not distinguish if the lysosome-like organelle to which reference is made is the small electron-dense organelle, previously known as a primary lysosome [as it contained no fused endocytic cargo (de Duve, 1983)] this will be called a "storage lysosome" (but may be acidic or non-acidic) (Griffiths, 1996a) from the late endosome and cathepsin D-labelling, LAMPs-positive, acidic body judged to be a "hybrid organelle" formed by fusion of the small electron-dense organelle with the late endosome also known as a "secondary lysosome" or "digestive body" (de Duve, 1983) (Table 4.1 and Figure 4.2). It is also not absolutely clear that the "hybrid organelle" is not different from the cathepsin D-labelled "digestive body" described by de Duve, (1983) (Table 4.1). This complicates reporting and interpretation of the literature. When a lack of certainty exists describing such populations, quotation marks (i.e. "lysosome") will be used. If it is the "hybrid organelle" to which reference is made, this term will be substituted. Both the "hybrid organelles" and "digestive bodies" as well as "storage lysosomes" are usually located in the perinuclear area of the cell and label with LAMPs (Griffiths, 1996a; Pillay *et al*., 2002) (Figure 4.2) (Table 4.1), however. Though usually perinuclear, these "lysosomes" may redistribute towards the cell edge, if the cytoplasmic pH becomes acidic (Heuser, 1989) and may be tubular, depending upon the state of polymerisation of microtubules (Swanson *et al*., 1987; Knapp and Swanson, 1990), or may be released in reponse to various agonists via regulated exocytosis (Andrews, 2000).

Regulated exocytosis of lysosome-like populations ("secretory lysosomes") may be induced by increased levels of free, intracellular Ca^{2+}, especially in cells of a haemopoietic lineage (Griffiths, 1996b; Stinchcombe and Griffiths, 1999). This seems a temperature and ATP-dependent process regulated by synaptotagmin VII (Rodríguez *et al*., 1997; Andrews, 2000; Martinez *et al*., 2000). Claus *et al*. (1998) identified only two functionally distinct, dense "lysosomal compartments" in J774 macrophages. One was secreted in the presence of the acidotropic drugs, chloroquine and bafilomycin A1 (and hence possibly acidic) and contained primarily cathepsins B and L as well as furin. The non-secreted compartment contained dipeptidyl peptidase II (DPPII), β-glucuronidase and β-hexosaminidase. Addition of acidotropic drugs did not affect the secretion of the latter compartment. This compartment could, therefore, be non-acidic. The morphology of "secretory lysosomes" is

also reported to be a combination of the multilamellar structures of conventional late endosomes and the dense cores of secretory granules (Blott and Griffiths, 2002) and could in fact be late endosomes and classical lysosomes. Rabinowitz *et al*. (1992) similarly described two compartments in mouse macrophages as tubular elements and small vesicles in which poorly degradable endocytic or phagocytic material accumulated. These, in fact could even be "residual bodies" of the autophagic pathway (Eskelinen *et al*., 2002). Without additional markers morphological descriptions seem to be of limited used in distinguishing numbers of vesicles.

Early studies on human bone marrow promonocytes and blood monocytes also suggested the existence of at least two distinct vesicle populations (Nichols *et al*., 1971; Stachura, 1989). One type was shown to contain acid phosphatase, aryl sulfatase and peroxidase. The content of the second type remained unknown (Ross and Auger, 2002). Two ultrastructurally distinct vesicle populations were subsequently described in rabbit alveolar macrophages (Cohn and Wiener, 1963). These were thought to be lysosomal or secretory but were shown, using enzyme marker assays (Peters *et al*., 1972; Peters, 1976), to consist of three vesicle populations (Lowrie *et al*., 1979) (Table 4.2).

Table 4.2 **Identification of three vesicle types in rabbit alveolar macrophages.**

	Vesicle Types		
Vesicle Contents	**Type A**	**Type B**	**Type C**
Lysozyme	✓	?	
N-acetyl-β-glucosaminidase		✓	
β-galactosidase		✓	
β-glucuronidase		✓	
Cathepsin D			✓
Acid phosphatase		✓	✓

✓ present in vesicle population, ? may be present. (Lowrie *et al*., 1979).

Lysozyme was shown to be a marker for one of these populations (type A-vesicles). Cathepsin D, the major protease present in lysosomes and frequently used as a marker for lysosomes (Conner, 2004), was shown to be a marker for another (type C-vesicles). A third vesicle (type B-vesicles) was identified by the presence of *N*-acetyl-β-glucosaminidase, β-galactosidase, β-glucuronidase or by the absence of cathepsin D (Lowrie *et al*., 1979). These possibly, respectively, represented the acidic- and two non-acidic- or slightly acidic vesicles subsequently described by Anes *et al*. (2006).

In human macrophages, Astarie-Dequeker *et al*. (2002) also described two of the three "endosomal" compartments, later described by Anes *et al*. (2006), a lysosome-like compartment positive for LAMP-3 (CD63) and another Src-family protein tyrosine kinase, hematopoietic cell kinase (Hck)-positive "lysosomal" compartment mobilised in a receptor-mediated, microtubule-independent way. Studies on the J774 phagosome by Anes *et al*. (2006) increased the number of identifiable "lysosomal" vesicle populations to four, if the late endosome is included, all populations being capable of fusing with late stage *Mycobacterium smegmatis* phagosomes (Figure 4.3). Three "lysosomal" populations may have been revealed if labelling for the 215 kDa MPR was included, i.e. to exclude the late endosome, each "lysosomal" population having at least one feature of a classical lysosome, i.e. the acidity of at least that of the late endosome, and either the positivity for LAMPs or content of active lysosomal enzymes and a lack of labelling for MPRs (Figure 4.3). Labelling for MPRs was not, however, carried out (Anes *et al*., 2006)

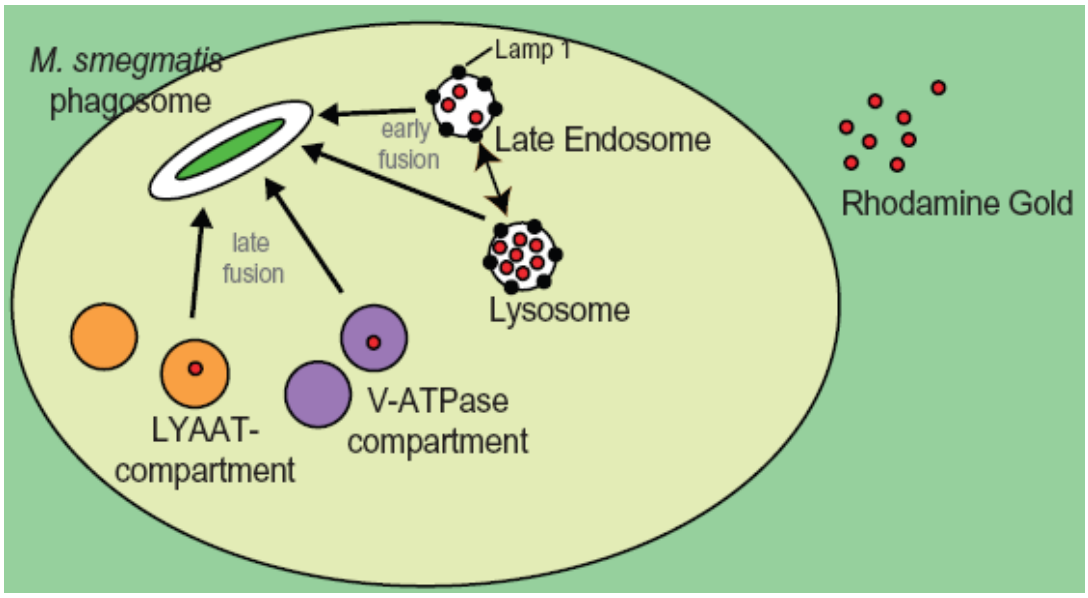

Figure 4.3 **Schematic model of the four major vesicular compartments capable of fusing with the phagosome during *M. smegmatis* infection**

At least four distinct vesicle populations fuse with the macrophage phagosome. LAMP positive late endosomes and lysosomes fuse initially, followed by vesicles enriched in either V-ATPase structures or LYAAT. Although all four compartments are distinct they can be considered as late endocytic vesicles as they appear to be accessible to rhodamine gold (Anes *et al*., 2006).

One population described by Anes *et al.* (2006) was enriched with V-ATPase and was the most acidic organelle identified and LAMP-1-negative. Another was enriched with LYAAT, a recently discovered membrane lysosomal exporter protein exporting apolar amino acids from lysosomes (Sagne *et al*., 2001; Boll *et al*., 2002) (Figure 4.3). This vesicle strongly colocalised with Hck, was LAMP-1-negative and weakly labelled with LysoTracker (i.e.

non-acidic) (Anes *et al*., 2006) (Figure 4.3). Another poopulation was LAMP-positive and acidic (possibly a hybrid organelle). The last was a LAMP-1-positive organelle which was largely non-acidic (only 2% colocalisation with LysoTracker) (Figure 4.3). Anes *et al*. (2006) did not seem to consider the early endosomal vesicle population (LAMP-negative, slightly acidic) which would be difficult to distinguish from the Hck/LYAAT vesicle (LAMP-negative, moderately acidic). The 5 possible populations identified could, therefore, either be largely acidic and LAMP-1-negative, largely weakly acidic and LAMP-1-negative (potential early endosomes and Hck/LYAAT vesicles), acidic and LAMP-1-positive or largely non-acidic and LAMP-1-positive (Figure 4.3). The late endosomal population was, however, defined by pulse-chase uptake of gold particles and no labelling was performed for MPRs. The possibility that one of the 5 populations described was also a late endosome, therefore, cannot be conclusively excluded.

4.2 Localisation of cathepsins in J774 macrophages using both immunogold and fluorescent labelling

It was hoped that, with the establishment of the cathepsin enzyme distribution [especially with respect to cathepsin H, as an early endosome marker, and cathepsin S, as a late endosome marker (Claus *et al*., 1998; Jahraus *et al*., 1998)] in relation to LysoTracker, it would be posssible to identify equivalents of the V-ATPase-compartment and other populations described by Anes *et al*. (2006). LysoTracker labelling (i.e. acidity) of vesicles should also be an indicator that cathepsins should be in their activated or processed form. LAMP-1 (and possibly LAMP-2), it was hoped, would assist in identifying similar sub-populations to those identified by Anes *et al*. (2006) which classically would be associated with active enzymes (Table 4.1). The approximate percentage of pro- and mature enzyme, it was hoped, would be moderately accurately determined from western blotting (Chapter 3). It was anticipated that at least 5 populations would be revealed, either largely acidic and LAMP-1-negative, largely weakly acidic and LAMP-1-negative (early endosomes or Hck/LYAAT vesicles), acidic and LAMP-1-positive and largely non-acidic and LAMP-1-positive. An additional equivalent of the late endosomal population (acidic, LAMPs-positive) could possibly be defined by the presence of cathepsin S or cathepsin D, differentiating this body from the “hybrid organelle” or “digestive body”.

The cathepsins have a wide range of optimal operating pH’s (Section 1.4.1, Table 1.6) and are thus well suited to the endosome-lysosome system where the pH ranges from less than

5.5 to 6.5 (Berg *et al*., 1995). The distribution of proteases and differences in pH, particularly in late endosomes, where activation and inactivation of specific proteins as well as antigen processing occurs, may also be used to control proteolysis e.g. for restricted cleavage of the invariant chain and limited antigen processing for MHC class II antigen presentation (Lennon-Duménil *et al*., 2002a).

Diment *et al*. (1988) also described a 46 kDa, partially membrane-bound endosomal form of cathepsin D, and both cathepsins D and B have been implicated in the macrophage early endosomal degradation of the A chain of the plant toxin ricin in this compartment of almost neutral pH (Blum *et al*., 1991). Although cathepsins D and B are known to have largely acidic pH optima it has been suggested that the local conditions in early endosomes may affect the activity of particular enzymes (Pillay *et al*., 2002). *In vitro* studies have also shown different buffer systems to have marked affects on the activity and observed pH optimum of cathepsins B and L, both cathepsins being shown to have significant activity at physiological pH in certain buffers (Dehrmann *et al*., 1996). The membrane-bound form of cathepsin D may also have a higher pH optimum than the soluble form present in lysosomes. Cathepsin B also has both exo- and endopeptidase activity, with the endopeptidase having a higher pH optimum (Blum *et al*., 1991; Linebaugh *et al*., 1999). The presence of cathepsin B in a compartment with a higher pH than considered optimal for the exopeptidase activity, therefore, may favour the endopeptidase activity.

In the macrophage phagosome, a process of maturation, during which sequential fusion with early endosomes, late endosomes and lysosomes, appears to be required for the acquisition of various proteolytic enzymes, and for the phagosome to perform degradative functions (Desjardins *et al*., 1997) (Figure 4.1). The sequential delivery of proteases to the phagosome of J774 mouse macrophages emphasises their heterogenous distribution along the endocytic pathway (Garin *et al*., 2001; Lennon-Duménil *et al*., 2002a). Cathepsins B and Z appear to be amongst the first to be delivered to the phagosome. The activity of cathepsin B in the phagosome increases with time, suggesting that whilst cathepsin Z may be located in early endosomes, cathepsin B may be distributed throughout the endocytic pathway. Cathepsins L and D appear to be located in the late endosomes and lysosomal compartments. Finally cathepsin S seems mainly associated with late endosomes (Claus *et al*., 1998; Lennon-Duménil *et al*., 2002a), whereas, cathepsin H occurs primarily in the early endosome (Jahraus *et al*., 1998). Apart from these studies very little work has focussed on the

distribution of cathepsins in macrophages. Whether these proteases are restricted to the endocytic compartments or whether they may occur in "secretory lysosomes" or other vesicles is unknown.

The strategy adopted in this study was first to establish the ultrastructural and immunofluorescent distribution of cathepsins in J774 mouse macrophages using single labelling, while optimising labelling and checking adequate preservation of ultrastructure and tissue immunogenicity. This was followed subsequently by double labelling to establish cathepsin colocalisations, once again, considering cathepsin H as an early endosome marker (Jahraus *et al*., 1998) and cathepsin S as a late endosome marker (Claus *et al*., 1998; Lennon-Duménil *et al*., 2002a). Finally, LysoTracker was used to verify whether the cathepsins were associated with the (acidic) late endosomes, "lysosomes", and the equivalent of the most acidic (or V-ATPase-positive) compartment seen in the Anes *et al*. (2006) study and whether a less acidic LAMP-1-positive compartment and a non-acidic, LAMP-1-negative compartment (possibly a LYAAT-positive compartment) could be identified. Further colocalisation studies were carried out with anti-LAMP-1 and anti-LAMP-2 to see whether any difference in cathepsin colocalisation in "lysosomal" subpopulations could be discerned.

For fluorescent labelling of J774 macrophages where preservation of ultrastructure is less crucial, PFA was used and the presence and preservation of cathepsins B, D, H, S and L antigenicity was first verified. Ultrastructural preservation is important for EM and a combination fixative (PFA and glutaraldehyde) was, therefore, used in preliminary immunogold labellings. Double labelling colocalisation studies were subsequently performed**.**

4.2.1 Reagents

Reagents for culture, fixation and embedding, immunolabelling of ultrathin sections and for fluorescent immunolabelling of J774 cells for cathepsins B, D, H, S and L were prepared according to Sections 2.2.1, 2.7.1.1, 2.7.3.1 and 2.8.1.1, respectively.

The anti-cathepsin antibodies used were as previously described (Section 3.7.1). Goat anti-rabbit IgG FITC, donkey anti-chicken IgG CY3 and rabbit anti-chicken IgG FITC secondary antibodies were employed.

4.2.2 Procedure

J774 cells were cultured, fixed and embedded in LR White resin according to the procedure in Sections 2.2.2 and 2.7.1.2. Protein A gold labelling on the ultrathin sections was performed with rabbit anti-cathepsin H [20 μg/ml], chicken anti-cathepsin S [20 μg/ml], chicken anti-cathepsin D [50 μg/ml], chicken anti-cathepsin B [10 μg/ml] and rabbit anti-cathepsin L [20 μg/ml] according to Section 2.7.3.2. Cathepsins in vesicle populations were judged to be membrane-bound if located around the vesicle periphery and lumenal or "free" if observed within the vesicle itself.

Fluorescent immunolabelling was performed with rabbit anti-cathepsin H [100 μg/ml (epifluorescence) or 20 μg/ml (confocal)], chicken anti-cathepsin S [20 μg/ml], chicken anti-cathepsin D [100 μg/ml (epifluorescence) or 50 μg/ml (confocal)], chicken anti-cathepsin B [20 μg/ml], rabbit anti-cathepsin L [20 μg/ml], goat anti-rabbit IgG FITC [5 μg/ml or 2 μg/ml], donkey anti-chicken IgG CY3 [2 μg/ml] and rabbit anti-chicken IgG FITC [10 μg/ml] according to Section 2.8.1.2. Pre-immune controls were performed in all cases to check labelling specificity. Double labelling controls were also performed omitting or substituting antibodies with pre-immunes and changing the order of the labelling to check non-specificity of all components of the labelling system. As macrophages are fairly small and round and normal epifluorescence microscopy focal planes are fairly thick it may, therefore, be difficult to unequivocally determine colocalisation using conventional epifluorescence. Confocal microscopy was, therefore, also used where possible, as it allows for the viewing of thin "optical slices" to exclude apparent colocalisation due to superimposition of two differently labelled vesicles within the viewed focal plane. Labelling was, therefore, viewed using either an Olympus epifluorescent microscope and F-View CCD camera or a Zeiss 510 Meta confocal microscope and images analysed visually using ImageJ software. Images of colocalisation using colour (i.e. red and green images merged to form yellow) are highly influenced by display settings and intensity, and, therefore, colocalisation based on colour analysis alone is problematic and can lead to incorrect conclusions. Grey scale images (i.e. black and white) on the other hand, are not affected by display settings and should be assessed when visually judging the following colocalisation images, as the yellow colour (indicating the degree of colocalisation in the composite image) varies depending on settings and printer type. In confocal images, the number of colocalising vesicles in at least 3 representative cells was assessed manually, reported as a percentage and checked with reference to the epifluorescent image.

4.2.3 Results

No non-specific protein A gold- or fluorescent labelling was observed in controls and labelling was assumed to be specific (results not shown). Ultrastructural preservation was extremely variable as is evident in micrographs labelled for cathepsin H (Figure 4.4, C) and cathepsin B (Figure 4.7, C), where labelled vesicles (±100 nm) are only slightly electron-dense and are not easily discerned (Figure 4.4, C) or preservation is moderately acceptable (Figure 4.7, C).

Early endosomes are usually fairly large (±100 nm), quite electron-translucent vesicles with an associated tubular network (Ghosh *et al*., 1994). Neither tubular network nor any vesicle definition is evident in the cathepsin H-labelled sections (Figure 4.4, C). The majority of labelled vesicles are possibly early endosomes, similar in size (±100 nm) but more electron-dense or secretory vesicles i.e. small (±20 nm) and electron-translucent containing procathepsin H (Figure 4.4, C), in approximately equal proportions as indicated by blots (Figure 3.11, A, lane 4). Labelling in the epifluorescent image (Figure 4.4, A) rather than the confocal image (Figure 4.4, B1 and B3) appears more distinct and with larger organelles [more like early endosomes (±100 nm) than small (±20 nm) secretory vesicles]. Labelling also appears membrane bound (Figure 4.4, C). Cathepsin H labelling, however, seemed variable but mainly spread throughout the cell (Figure 4.4, A and B).

Cathepsin S labelling was also performed with a peptide antibody (Figure 4.5, A, B and C) but appears to give denser, more definite labelling at the EM level, with labelling apparently located near or in tubulovesicular areas in the cell, possibly associated with the ER (Figure 4.5, C). The more electron-dense organelles are possibly late endosomes (±50 nm) but the vesicles associated with the ER (±20 nm) would be anticipated to contain newly synthesised precursor cathepsin S, which, according to western blots, should represent the content of the majority (approximately 90% pro-) of vesicles (Figure 3.13, A, lane 3).

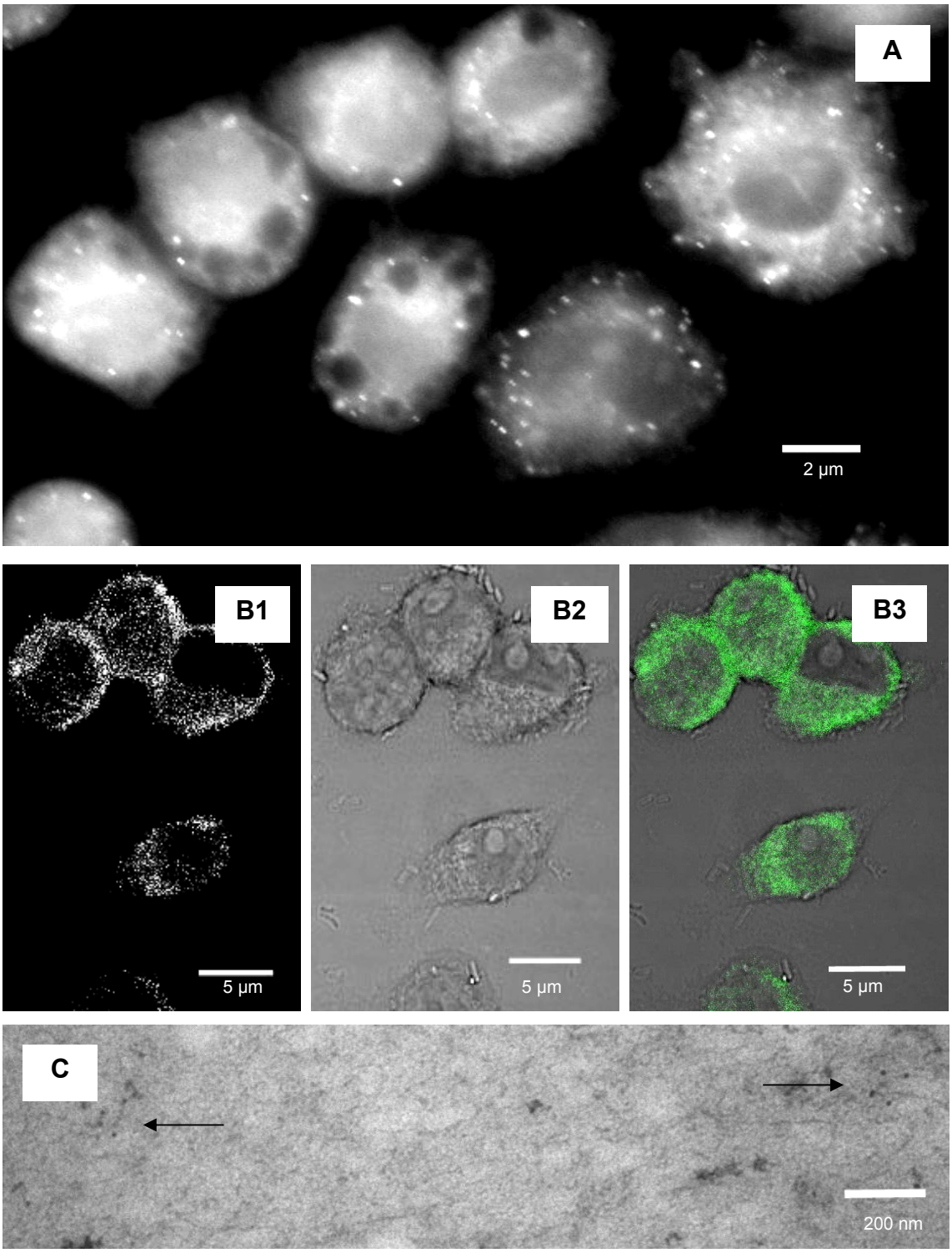

Figure 4.4 **Fluorescent and protein A gold labelling of cathepsin H in J774 macrophages.**
Rabbit anti-cathepsin H [100 µg/ml (A) or 20 µg/ml (B1)] and goat anti-rabbit IgG FITC [5 µg/ml (A) or 2 µg/ml (B1)] applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed with an Olympus epifluorescent microscope (A) or a Zeiss Meta 510 confocal microscope (B). FITC filter (A and B1), DIC image (B2), composite image (B3). Bars = 2 µm (A) or 5 µm (B) .
Rabbit anti-cathepsin H [20 µg/ml (C)] and protein-A gold probe (10 nm) used on LR White sections viewed using a Philips CW120 Biotwin TEM (80-100 kV). Cathepsin H labelling seen in slightly electron-dense vesicles (arrows), some possibly membrane-associated. Bar = 200 nm.

Ultrastructural preservation and staining seems fairly good, but many vesicles seem swollen and not all membraneous structures are equally well preserved (Figure 4.5, C). Cathepsin S labelling seems either located within the vesicle (approximately 34%) and apparently soluble, or membrane-bound (approximately 66%) (Figure 4.5, C). Immunofluorescence labelling for cathepsin S (Figure 4.5, A and B) does not appear to be distributed as peripherally as the cathepsin D labelling (Figure 4.6, A and B) but seems a little more extensively spread throughout the cell than cathepsin H (Figure 4.4, A and B). Such a distribution of cathepsin S may concur with both a late endosomal distribution and the high perinuclear ER and Golgi-association indicated by the predominant levels of precursor enzyme (approximately 90%) reflected in blots (Figure 3.13, A, lane 3). From the size of vesicles present in images of cathepsin S labelling seen in immunofluorescence micrographs, vesicles seem to be largely secretory (±20 nm), containing approximately 66% membrane-bound cathepsin S (precursor), and very few active cathepsin S containing-late endosomes (±50 nm) (Figure 4.5, C and Table 4.3).

Immunofluorescence labelling of cathepsin D, on the other hand, shows labelling in vesicles that are larger than most other organelles (±150-200 nm) except the early endosomes (±100 nm) (Figure 4.6, A). These are seen in the cytoplasm and towards the cell periphery, with some polarised cathepsin D distribution (Figure 4.6, A and B). Cathepsin D is also seen in the pseudopodia of activated cells (Figure 4.6, B, cell on top left), suggesting possible secretion or involvement in invasion. Though cathepsin D is traditionally regarded as a "lysosomal marker", it is usually associated with a digestive body often called an endosome-lysosome "hybrid organelle" (size ±150-200 nm) (Griffiths, 1996a) (Table 4.1) like those in Figure 4.6, A, B and C.

Cathepsin D has also been suggested to occur in macrophage early endosomes in (Diment *et al.*, 1988). This may also account for a peripheral distribution of cathepsin D (Figure 4.6, A and B). If this is the case, however, cathepsin D would be anticipated to colocalise with cathepsin H (Jahraus *et al*., 1998). On the other hand, most of the labelled vesicles should contain mature cathepsin D (western blots indicate approximately 80% mature enzyme, Figure 3.10, A, lane 4). This would mean that vesicles may also be late endosomal (±50 nm) or hybrid endosome-lysosomal organelles or digestive bodies (±150-200 nm) (Figure 4.6, A and B). From their size, however, the majority of mature cathepsin D-labelled organelles would seem to be hybrid or digestive bodies (±150-200 nm). Cathepsin D also seems, to a

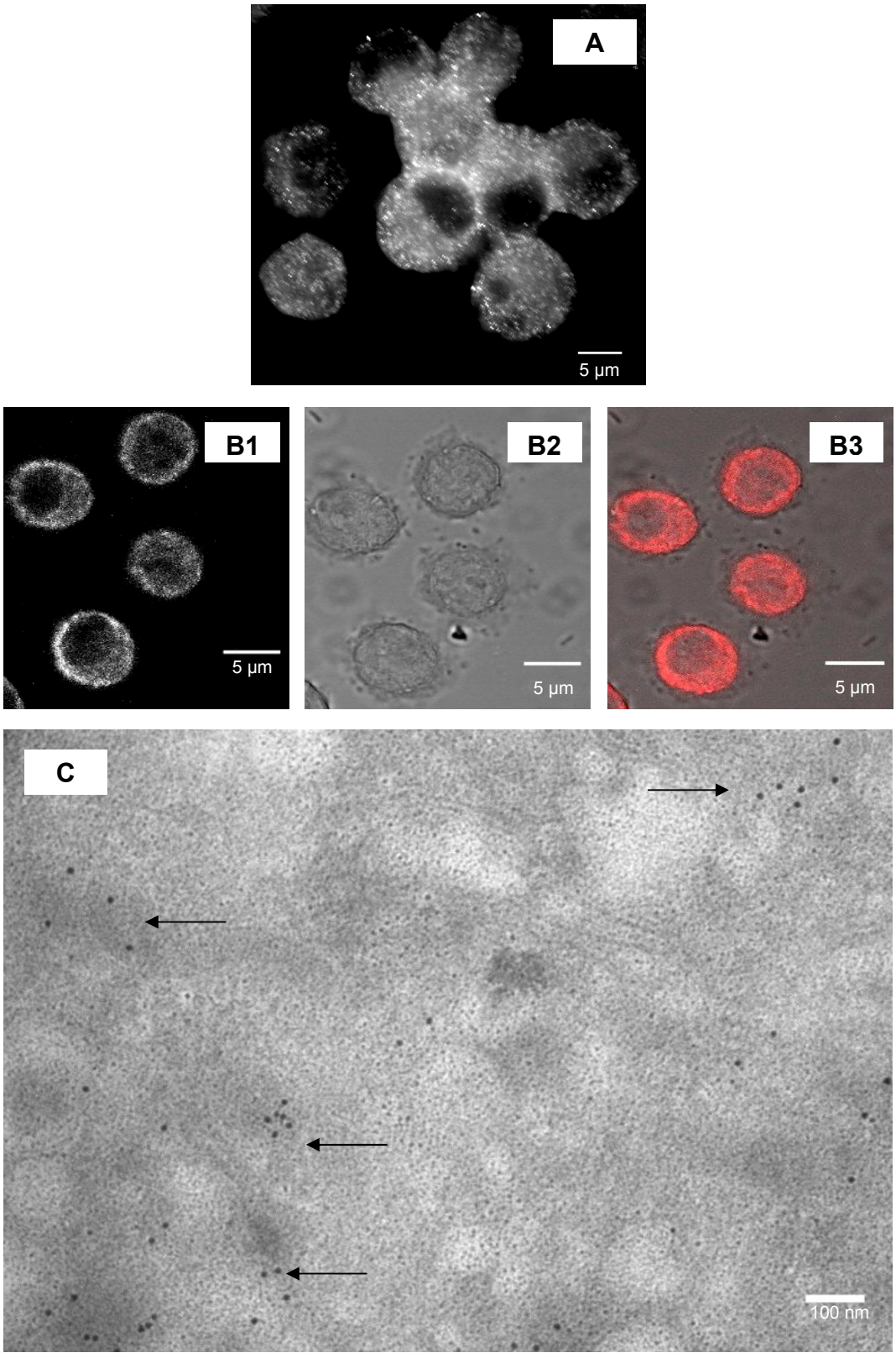

Figure 4.5 **Fluorescent and protein A gold labelling of cathepsin S in J774 macrophages.**
Chicken anti-cathepsin S [20 µg/ml (A and B1)] and donkey anti-chicken IgG CY3 [2 µg/ml (A and B1)] applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed with an Olympus epifluorescent microscope (A) or a Zeiss Meta 510 confocal microscope (B). CY3 filter (A and B1), DIC image (B2), composite image (B3). Bars = 5 µm.
Chicken anti-cathepsin S [20 µg/ml (C)], a rabbit anti-chicken linker antibody [50 µg/ml (C)] and protein-A gold probe (10 nm) used on LR White sections viewed using a Philips CW120 Biotwin TEM (80-100 kV). Cathepsin S detected in tubulovesicular areas (arrows), the majority membrane-bound. Bar = 100 nm.

small extent, membrane-associated (Figures 4.6, C and Figure 4.10, C, 66% free and 34% membrane-bound) (Table 4.3), suggesting a presence in early endosomes (Diment *et al*., 1988).

Cathepsin B on the other hand, appears to localise to electron-dense vesicles (±50 and 100 nm, respectively) resembling late endosomes or "hybrid" organelles on the basis of size (usually containing processed cathepsins). Small vesicles (±20 nm) close to membraneous systems resembling ER where precursor enzyme would occur were also observed (Figure 4.7, C). Such a distribution seems to fit with the 50:50 precursor:mature cathepsin B content of J774 cells evident in blots (Figure 3.9, A, lane 4). In Figure 4.7, C, once again, a variable ultrastructural preservation is evident, this time in a single section, with organelles and structures towards the left-hand-side of the micrograph becoming less well preserved. Labelling also seems to indicate a 60% membrane-association (possibly precursor enzyme) as gold labels seems to be located around the periphery of labelled vesicles (Figure 4.7, C). In approximately 40% of vesicles cathepsin B appeared lumenally distributed and soluble or "free" (Figure 4.7, C and other micrographs not shown) (Table 4.3).

Cathepsin L labelling with the anti-cathepsin L peptide antibody is sparse (Figure 4.8, A and B), with labelling being largely confined to small vesicles (±20 nm) and membraneous ER-like structures positioned towards the perinuclear area of the cell and scattered sparsely through the cell (Figure 4.8, C). This labelling pattern and distribution was anticipated, since western blots indicated that J774 cells contain approximately 50% precursor and approximately 50% mature cathepsin L (Figure 3.12, A, lane 3) consistent with ER/Golgi vesicles containing precursor enzyme and larger vesicles (±50 and 100 nm) possibly representing late endosomes and "hybrid" organelles, respectively, containing active enzyme (Figure 4.8, A, B and C). The majority of cathepsin L (70%) appears membrane-bound (Figure 4.8, C) (Table 4.3).

It should be noted that bacillus-like structures seen in many of the micrographs are artefacts introduced in the mounting of the labelled cells on coverslips (Figure 4.4, B2 and B3; 4.5, B2 and B3; 4.8, A2 and A3; 4.9, B3 and B4; 4.10, B3;

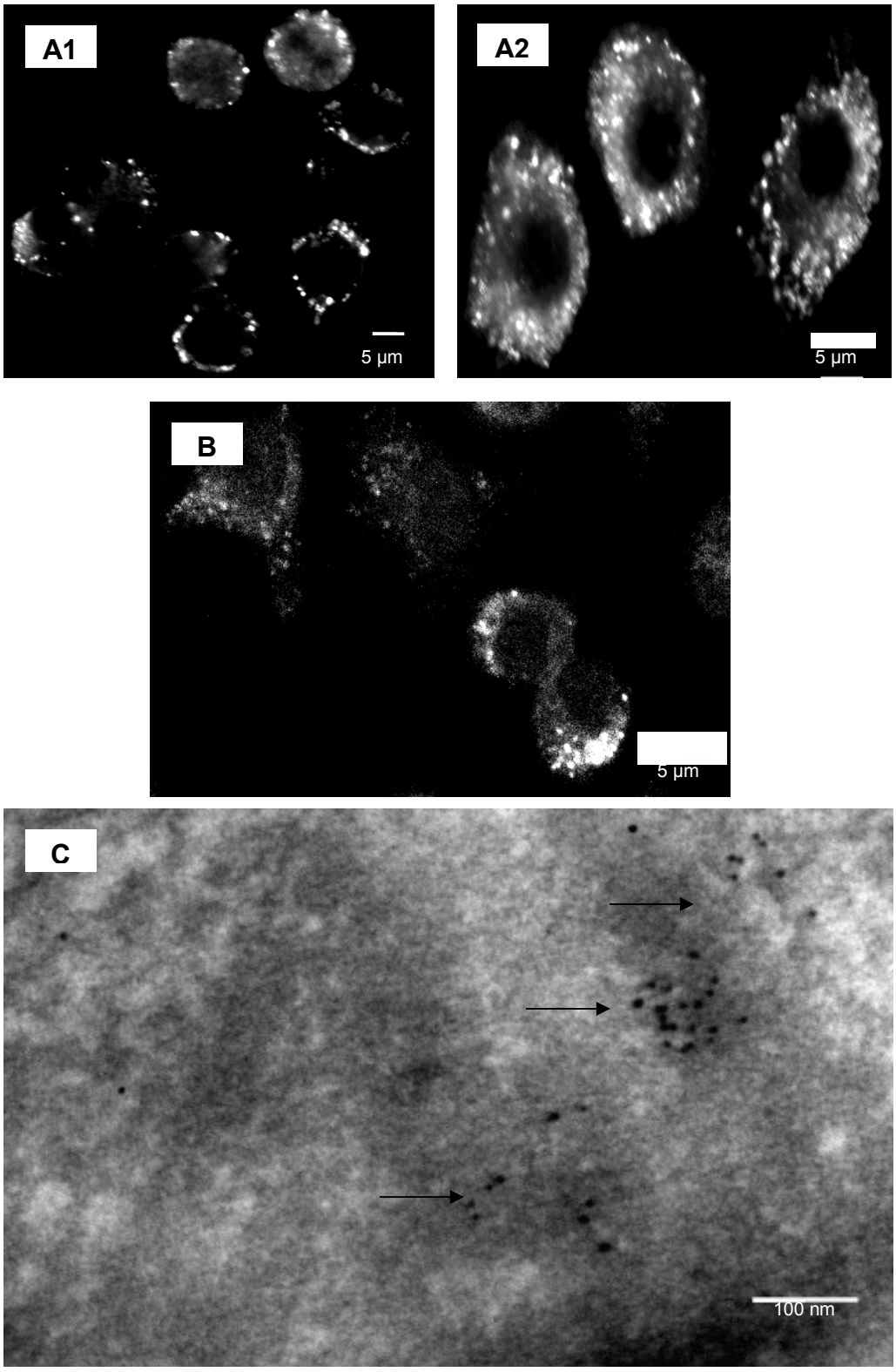

Figure 4.6 F**luorescent and protein A gold labelling of cathepsin D in J774 macrophages.**
Chicken anti-cathepsin D [100 µg/ml (A1) or 200 µg/ml (A2 and B)] and donkey anti-chicken IgG CY3 [2 µg/ml (A1 and A2)] or rabbit anti-chicken FITC IgG [10 µg/ml (B)] applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed with an Olympus epifluorescent microscope (A) or Zeiss Meta 510 confocal microscope (B). Bars = 5 µm.
Chicken anti-cathepsin D [10 µg/ml (C)], a rabbit anti-chicken linker antibody [50 µg/ml (C)] and protein-A gold probe (10 nm) were used on LR White sections which were viewed using a Philips CW120 Biotwin TEM (80-100 kV).Cathepsin D in electron-dense vesicles (arrows). Bar = 100 nm.

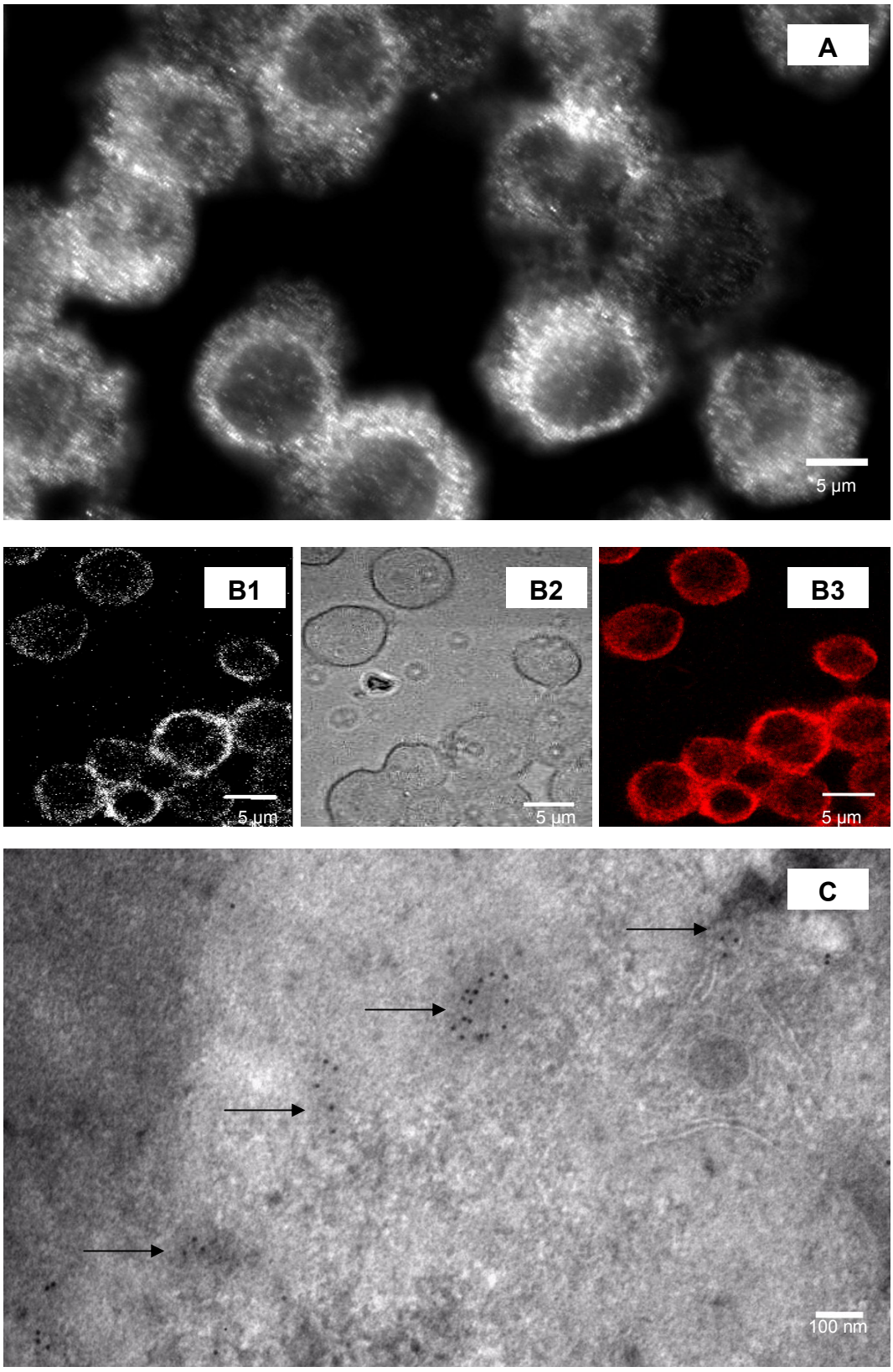

Figure 4.7 **Fluorescent and protein A gold labelling of cathepsin B in J774 macrophages.**
Chicken anti-human liver cathepsin B [20 µg/ml (A and B1)] and donkey anti-chicken IgG CY3 [2 µg/ml (A and B1)] applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed with an Olympus epifluorescent microscope (A) or Zeiss Meta 510 confocal microscope (B). CY3 filter (A and B1), DIC image (B2), composite image (B3). Bars = 5 µm.
Chicken anti-human liver cathepsin B [10 µg/ml (C)], a rabbit anti-chicken linker antibody [50 µg/ml (C)] and protein-A gold probe (10 nm) used on LR White sections viewed using a Philips CW120 Biotwin TEM (80-100 kV). Cathepsin B in electron-dense vesicles, possibly membrane-bound (arrows) and close to membraneous systems. Bar = 100 nm.

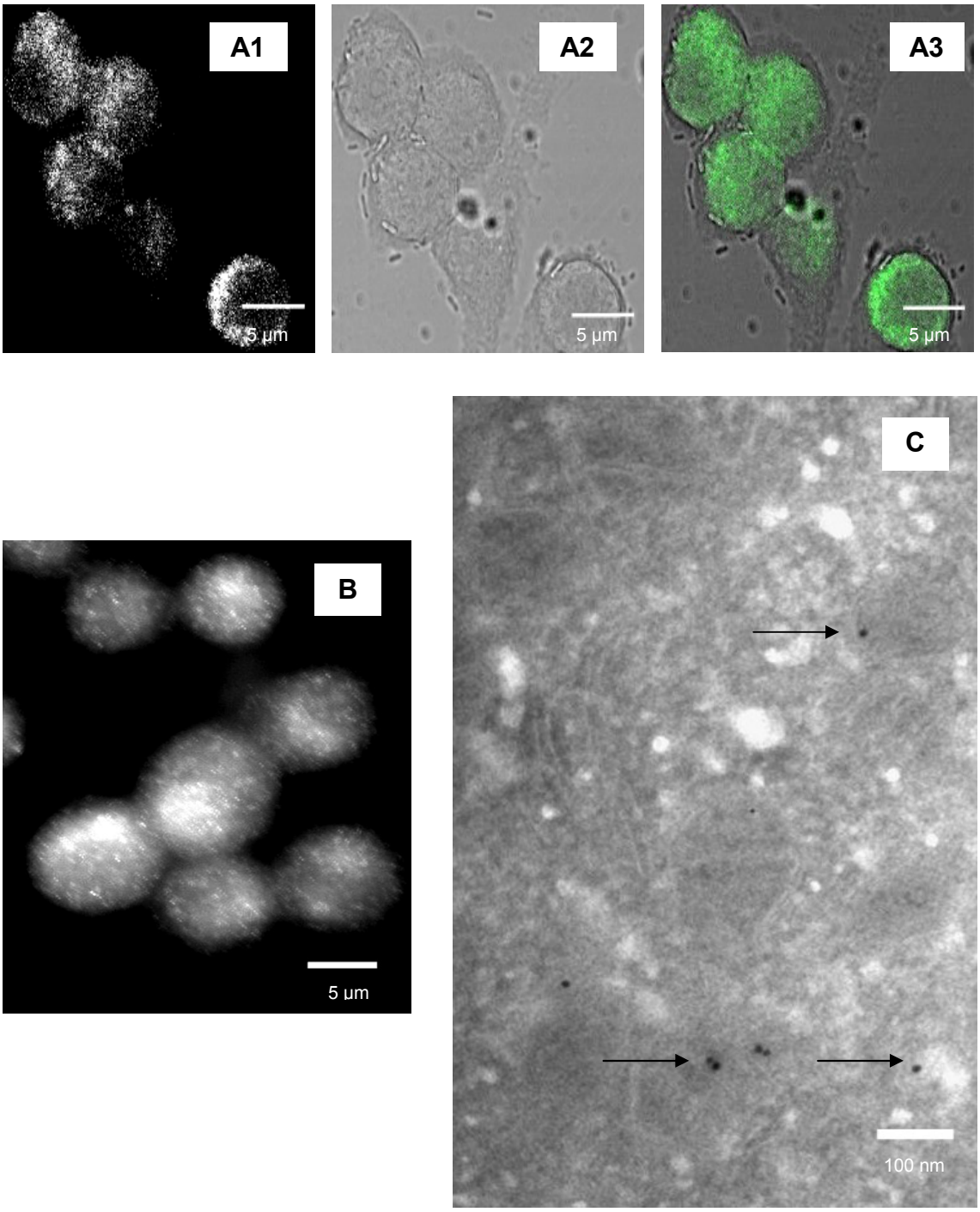

Figure 4.8 **Fluorescent and protein A gold labelling of cathepsin L in J774 macrophages.**
Rabbit anti-cathepsin L [20 µg/ml (A1 and B)] and goat anti-rabbit IgG FITC [5 µg/ml (A1 and B)] applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed with an Olympus epifluorescent microscope (B) or a Zeiss Meta 510 confocal microscope (A). FITC filter (A1 and B), DIC image (A2), composite image (A3). Bars = 5 µm.
Rabbit anti-cathepsin L [20 µg/ml (C)] and protein-A gold probe (10 nm) used on LR White sections viewed using a Philips CW120 Biotwin TEM (80-100 kV). Cathepsin L detected in small vesicles, possibly membrane-bound (arrows). Bar = 100 nm.

Summary

Table 4.3 **Summary western blot data, apparent vesicle morphology and enzyme distribution.**

	Precursor form (%)	Mature form (%)	Vesicle type	M (%)	F (%)
Cat H	50	50	S/LT	50	50
Cat S	90	10	>S/LE/Lys	66	34
Cat D	20	80	LE/Lys	34	66
Cat B	50	50	S/>LE	66	34
Cat L	50	50	>S/LE	66	34

Abbreviations: Cat, cathepsin; LT, large (±100 nm) electron-translucent, early endosome-like; S, small (±20 nm) secretory vesicle-like; LE, large (±50 nm), electron-dense, late endosome-like; Lys, lysosome-like, hybrid, digestive organelles (±150-200 nm); M, membrane-bound; F, free; >, mostly.

Results (Table 4.3) seem to indicate the presence of at least 4 vesicle types, small (±20 nm) electron-translucent secretory-like (possibly containing membrane-bound, precursor cathepsins especially cathepsins H, S, B and L), early endosomal-type vesicles containing mature cathepsin H, large (±100 nm). Cathepsins D, L, S and B appear to be present in late endosomes (±50 nm) and large "hybrid" organelles (±150-200 nm) contain cathepsins D, B and L. Vesicle types could not, at this stage, be accurately assigned, however, without at least probes indicating acidity (LysoTracker) or some ultrastructural detail.

4.3 Colocalisation of cathepsins in J774 macrophages

Cathepsin precursor forms should be found only in the ER, Golgi and any secretory vesicles either transporting precursor from the ER and Golgi to the late endosome for activation or being secreted from the cell (approximately 10% of traffic) (Pillay *et al*., 2002).

The polyclonal cathepsin antisera used in the current study detect both the proforms (associated with ER, Golgi and non-acidic vesicular compartments) and the mature, active enzymes (late endosomes, lysosomes and other acidic vesicular compartments). Therefore, the presence and percentage of precursor or mature forms of the cathepsins, it was hoped, could possibly be predicted from western blot data and by morphological identification of the containing compartment or vesicle. This assignment, it was hoped, would subsequently be confirmed using labelling for LAMP-1 and -2 and the demonstration of a low pH, all usually associated with the classical late endosome and lysosome which should contain mature enzyme (Fukuda *et al*., 1991).

Both double immunogold and fluorescent labelling were also performed to check that cathepsins H and S do not colocalise and to assess whether other cathepsins i.e. cathepsins B and L, colocalise with the markers for early endosomes (±100, cathepsin H), late endosomes (±50 nm, cathepsin S) or "lysosomes", hybrid organelles or digestive bodies (±150-200 nm, cathepsin D) or if additional vesicle populations were present.

4.3.1 Reagents

Reagents for culture, fixation and embedding, immunolabelling of ultrathin sections and fluorescent immunolabelling of J774 cells for cathepsins B, D, H, S and L were prepared according to Sections 2.2.1, 2.7.1.1, 2.7.3.1 and 2.8.1.1, respectively.

The anti-cathepsin and secondary fluorescent antibodies used, were as previously described (Section 3.7.1 and 4.2.1).

4.3.2 Procedure

J774 cells were cultured, fixed and embedded in LR White resin according to the procedure in Sections 2.2.2 and 2.7.1.2. Double protein A gold labelling on the ultrathin sections was performed with chicken anti-cathepsin S [15 μg/ml] and rabbit anti-cathepsin H [50 μg/ml], chicken anti-cathepsin D [10 μg/ml] and chicken anti-cathepsin S [10 μg/ml], chicken anti-cathepsin B [10 μg/ml] and chicken anti-cathepsin D [15 μg/ml], chicken anti-cathepsin B [10 μg/ml] and rabbit anti-cathepsin L [20 μg/ml] according to Section 2.7.3.2. Fluorescent immunolabelling was performed with chicken anti-cathepsin S [15 μg/ml or 50 μg/ml] and and rabbit anti-cathepsin H [100 μg/ml or 80 μg/ml], chicken anti-cathepsin D [200 μg/ml] and chicken anti-cathepsin S [20 μg/ml or 50 μg/ml], chicken anti-cathepsin D [100 μg/ml or 200 μg/ml] and chicken anti-cathepsin B [10 μg/ml or 50 μg/ml], chicken anti-cathepsin D [200 μg/ml] and rabbit anti-cathepsin L [50 μg/ml], chicken anti-cathepsin B [15 μg/ml or 50 μg/ml] and rabbit anti-cathepsin L [15 μg/ml or 50 μg/ml], chicken anti-cathepsin S [50 μg/ml] and chicken anti-cathepsin B [50 μg/ml], chicken anti-cathepsin S [20 μg/ml or 50 μg/ml] and rabbit anti-cathepsin L [20 μg/ml or 50 μg/ml], donkey anti-chicken IgG CY3 [2 μg/ml or 1 μg/ml], goat anti-rabbit IgG FITC [5 μg/ml or 3 μg/ml or 2 μg/ml], rabbit anti-chicken IgG FITC [10 μg/ml or 6 μg/ml] according to Section 2.8.1.2. Labelling was viewed using either an Olympus epifluorescent microscope and F-View CCD camera or a Zeiss 510 Meta confocal microscope and images analysed using ImageJ software and manually as previously described (Sections 2.8.1.2 and 4.2.2).

As the polyclonal anti-mature cathepsin antisera used cannot distinguish between precursor and mature forms of the enzyme, cathepsins associated with membraneous, ER/Golgi-like structures were assumed to be precursor and inactive. Those associated with vesicles may represent either mature active enzyme in acidic compartments or precursor in secretory vesicles in non-acidic vesicles. Due to generally poor ultrastructural preservation achieved, the knowledge that most of the cathepsin S and cathepsin D, would label mainly precursor or mature cathepsin, respectively, labelling for cathepsins S and B and cathepsins S and L were performed using only fluorescence microscopy.

When both antibodies used in the colocalisation experiment were from the same host, a fixation step between labelling fixation steps and the relevant controls were performed to eliminate any possible cross-reactivity between primary and secondary labelling systems (Section 2.7.1.2 and 2.8.1.2, respectively).

4.3.3 Results

Labelling showed no non-specificity (results not shown). Cathepsin H and cathepsin S labelling does not appear to colocalise to any extent (less than 20%, Figure 4.9, A, B and C), except in perinuclear areas where the synthesis and processing may occur concurrently (e.g. in the ER and Golgi) or where cells come into close contact (Figure 4.9, A and B). This seems to be most clearly demonstrated in epifluorescence labelling results (Figure 4.9, A). Confocal immunofluorescence labelling seems, however, to indicate that some peripheral polarised labelling colocalisation may also occur (Figure 4.9, B).

Protein A gold labelling confirms both cathepsin S and cathepsin H were separately located (Figure 4.9, C). Cathepsin S is associated with tubulovesicular areas (Figure 4.9, C) as was previously observed with the single labelling (Figure 4.5, C). Labelling for the potential early endosome marker (cathepsin H) was anticipated to be more peripherally located than labelling for the potential late endosome marker (cathepsin S), but in some places in the cell this seems to be the opposite (Figure 4.9, A and B). Western blots showed that approximately 50% cathepsin H and approximately 90% cathepsin S in the J774 cells are of the precursor form (Figure 3.11, A, lane 3 and Figure 3.13, A, lane 3, respectively). Labelling, however, indicates that neither enzyme colocalises to any extent, even if in their respective secretory (±20 nm, precursor enzyme-containing) vesicle populations (Figure 4.9,

A, B and C). Though ultrastructural preservation is once again poor, larger slightly electron-dense vesicles possibly represent late endosomes (±50 nm) and the balance of small electron-translucent (±20 nm) or electron-dense (±30-50 nm) vesicles possibly representing secretory vesicles or "secretory lysosomal" populations, respectively (Figure 4.9, C).

It is difficult to say whether confocal microscopy or conventional epifluorescence gives more labelling information, as different levels of antibody were used (Figure 4.9, A and B). Confocal labelling did reveal some peripheral colocalisation, however. The thinner "optical slice" being examined prevents such information from being obscured in conventional fluorescence microscopy (Figure 4.9, A - epifluorescence compared to Figure 4.9, B - confocal). From EM labelling results, however, it would seem that cathepsin H and cathepsin S labelling may only assist in assigning cathepsins to either the early or late endosomes or to secretory vesicles if fair ultrastructural detail is preserved (Figure 4.9, C). Once again vesicular structures seem more swollen than other membraneous organelles such as the ER (Figure 4.9, C).

Immunofluorescent labelling for cathepsin S and D seemed to indicate that these cathepsins are largely non-colocalised (approximately 25% colocalised) except in a few large structures which possibly represent hybrid endosome-lysosome organelles (±150-200 nm) (Figure 4.10, A and B) and in perinuclear areas which may represent the areas of synthesis and processing, the ER and Golgi (Figure 4.10, A and B). Western blotting data indicate that cathepsin D is mainly mature (Figure 3.10, A, lane 4) and cathepsin S immature (Figure 3.13, A, lane 3). Since cathepsin D is considered mainly a marker for the "lysosome" (Connor, 2004) or digestive body of the cell, the number (usually relatively few) and size of colocalising vesicles, possibly confirms that such vesicles are what are known as "late endosome-lysosome hybrid organelles". These organelles are especially obvious in confocal micrographs where an optical slice of approximately 1-2 μm is recorded (Figure 4.10, B) and seem to be most concentrated in regions of cell-cell contact (Figure 4.10, A). Protein A gold labelling seems to confirm a lysosome-like digestive body localisation as it reveals colocalisation of cathepsins S and D in large (±150-200 nm) electron-dense, non-multivesicular vesicles (Figure 4.10, C, red arrows). Cathepsin S (Figure 4.10, C, black arrows) and cathepsin D (Figure 4.10, C, white arrows) are also seen located separately in small vesicles (±20 nm) that are possibly secretory (containing precursor enzymes).

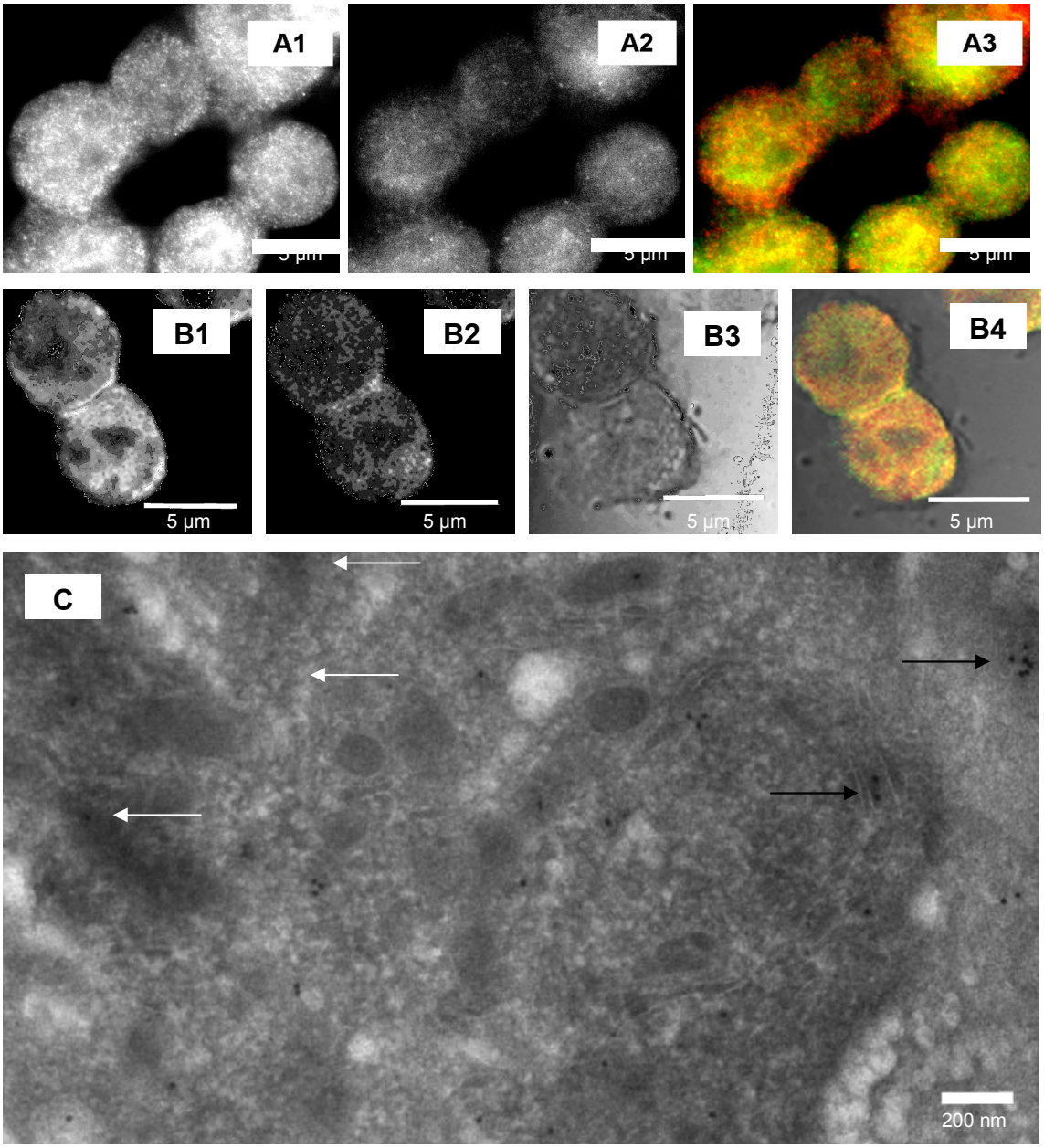

Figure 4.9 **Fluorescent and protein A gold labelling of cathepsins S and H in J774 macrophages.**
Chicken anti-cathepsin S [15 µg/ml (A1)] or rabbit anti-cathepsin H [100 µg/ml (B1)] and donkey anti-chicken IgG CY3 [2 µg/ml (A1)] or goat anti-rabbit IgG FITC [2 µg/ml (B1)], post-fixed (3.7% PFA) and probed with rabbit anti-cathepsin H [80 µg/ml (A2)] or chicken anti-cathepsin S [50 µg/ml (B2)] and either goat anti-rabbit FITC [2 µg/ml (A2)] or donkey anti-chicken CY3 [2 µg/ml (B2)], applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using an Olympus epifluorescent microscope (A) a Zeiss 510 Meta confocal microscope (B). CY3 filter (A1 and B2), FITC filter (A2 and B1), DIC image (B3), composite image (A3 and B4). Bars = 5 µm.
Rabbit anti-cathepsin H [18 µg/ml (C)], chicken anti-cathepsin S [15 µg/ml (C)], a rabbit anti-chicken linker antibody [50 µg/ml (C)] and protein-A gold probe for cathepsin H (10 nm) and for cathepsin S (15 nm) used on LR White sections viewed using a Philips CW120 Biotwin TEM (80-100 kV). Cathepsin H (white arrows), cathepsin S (black arrows) did not appear to colocalise. Bar = 200 nm.

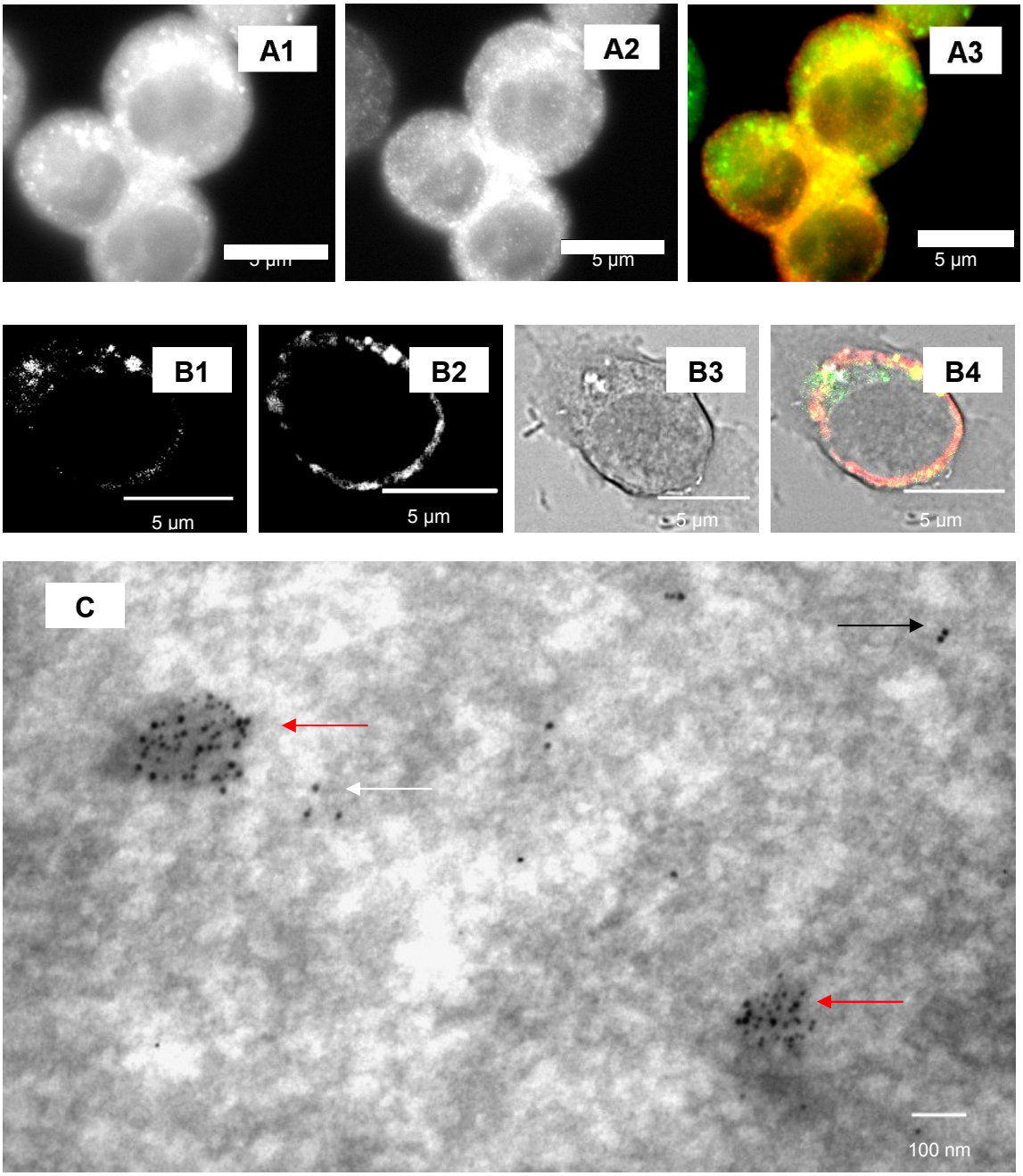

Figure 4.10 **Fluorescent and protein A gold labelling of cathepsins D and S in J774 macrophages.**
Chicken anti-cathepsin D [200 µg/ml (A1 and B1)] and rabbit anti-chicken FITC [10 µg/ml (A1 and B1)], post-fixed (3.7% PFA) and probed with chicken anti-cathepsin S [20 µg/ml (A2) or 50 µg/ml (B2)] and donkey anti-chicken IgG CY3 [2 µg/ml (A2 and B2)], applied to cells on coverslips, initially fixed with 3.7% PFA, and permeabilised with saponin. Coverslips viewed using an Olympus epifluorescent microscope (A) or a Zeiss 510 Meta confocal microscope. FITC filter (A1 and B1), CY3 filter (A2 and B2), DIC image (B3), composite image (A3 and B4). Bars = 5 µm.
Chicken anti-cathepsin D [10 µg/ml (C)], chicken anti-cathepsin S [10 µg/ml (C)], a rabbit anti-chicken linker antibody [50 µg/ml (C)] and protein-A gold probe for cathepsin D (10 nm) and for cathepsin S (15 nm) used on sections viewed using a Philips CW120 Biotwin TEM (80-100 kV). Cathepsin S and D colocalised in certain areas (red arrows). However, cathepsin S (black arrows) and cathepsin D (white arrows) still appeared separately. Bar = 100 nm.

Cathepsins B and D, like cathepsins S and D, also appear to colocalise (approximately 30%) in a few electron-dense, "lysosomal" vesicles possibly late endosomes (±50 nm) (Figure 4.11, C, black arrows), though there are also vesicles in which either cathepsin B or cathepsin D are separately located (Fig 4.11, C, white arrows). Vesicular labelling for cathepsin B appears to be slightly more peripherally located than labelling for cathepsin D (Figure 4.11, B). Some of the peripheral vesicles not colocalised with cathepsin D may represent procathepsin B-labelled organelles, as indicated by blots (approximately 50% precursor and 50% mature, Figure 3.9, A, lane 4). Certain regions showing colocalisation between cathepsins B and D appear in peripheral invadopodia-like protrusions, suggesting possible secretion of electron-dense vesicular compartments such as "secretory lysosomes" (±30-50 nm) to assist invasion (Figure 4.11, C, black arrows, upper left-hand-side).

Fluorescent immunolabelling confirmed colocalisation between cathepsins B and D in a few vesicles towards the cell periphery (Figure 4.11, A and B). There also appear to be vesicular compartments that label only for cathepsin D or cathepsin B, with fewer labelling for cathepsin D than B (Figure 4.11, A, B and C). At least half of the non-colocalised cathepsin B-labelled organelles are small (±20 nm) and electron-translucent and may contain newly synthesised precursor cathepsin B, in which case it is possible to speculate that they are secretory vesicles (Figure 4.11, C). The rest, according to western blots (Figure 3.9, A, lane 4), should contain mature active cathepsin B. As these do not colocalise with cathepsin D and their morphology is not definitive in electron micrographs (Figure 4.11, C), it is difficult to classify these vesicles without further markers or probes.

Cathepsins D and L colocalise in approximately 40% of labelled vesicle populations (Figure 4.12, A), cathepsin D appearing to be present in fairly large (±150-200 nm), vesicular compartments (Figure 4.12, A1) in non-activated macrophages (judged non-activated due to round morphology). In activated macrophages (judged activated due to elongated morphology), however, there is an almost total lack of colocalisation, approximately less than 20% indicating colocalising vesicles may have been secreted, and the remaining cathepsin L is possibly of the precursor form (Figure 4.12, B1), and remaining cathepsin D is active, occurring mainly in hybrid organelles. The activated cells mentioned above were not activated on purpose. During the culture process the addition of trypsin-EDTA appeared on

occasion to activate cells, resulting in the loss of the round morphology and formation of el

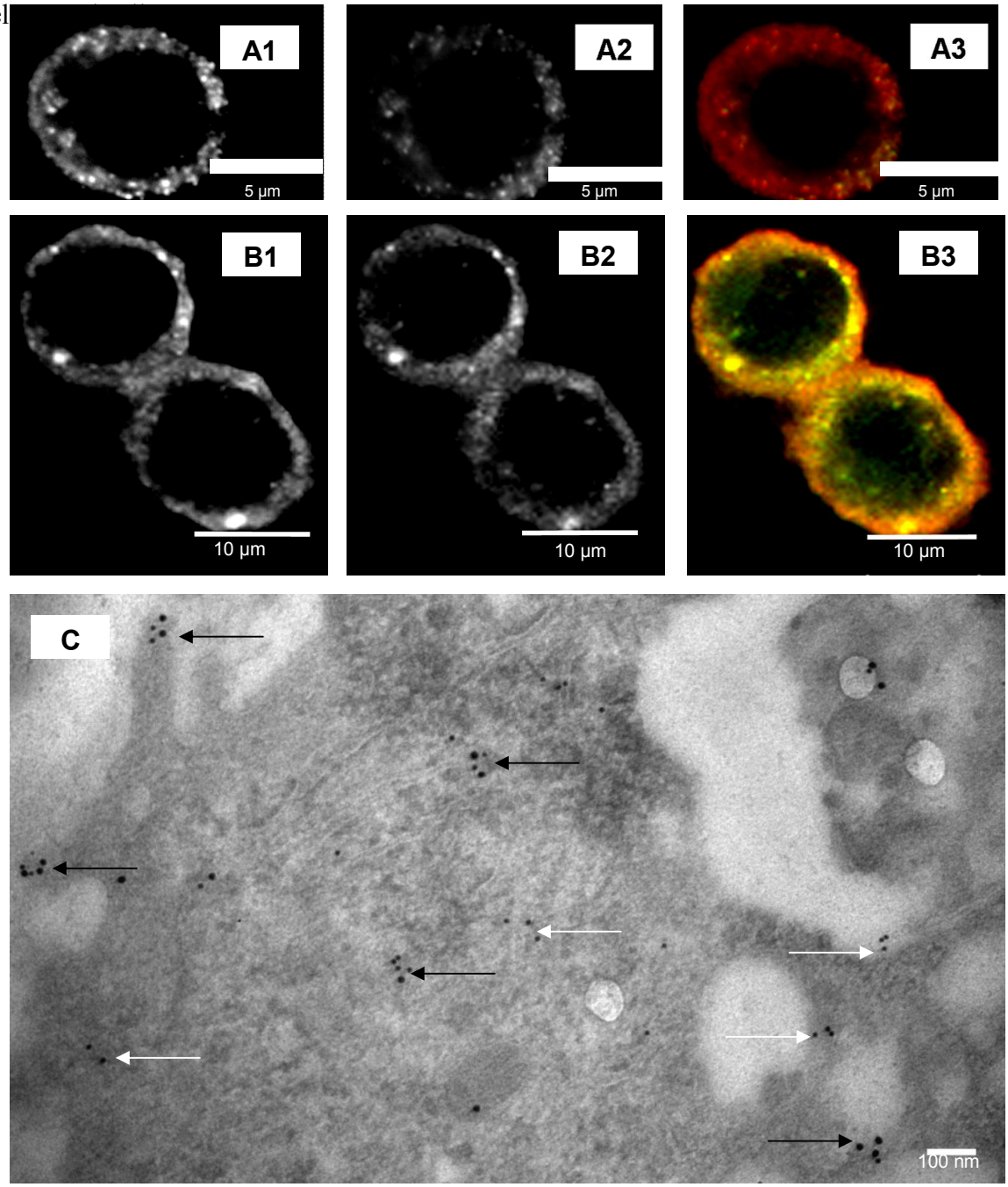

Figure 4.11 **Fluorescent and protein A gold labelling of cathepsins D and B in J774 macrophages.**
Chicken anti-cathepsin D [100 µg/ml (A2) or 200 µg/ml (B2)] and rabbit anti-chicken IgG FITC [10 µg/ml (A2 and B2)], post-fixed (3.7% PFA) and probed with chicken anti-cathepsin B [15 µg/ml (A1) or 50 µg/ml (B1) and donkey anti-chicken IgG CY3 [2 µg/ml (A1 and B1), applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using an Olympus epifluorescent microscope (A) or a Zeiss 510 Meta confocal microscope (B). CY3 filter (A1 and B1), FITC filter (A2 and B2), composite image (A3 and B3). Bars = 5 µm (A) or 10 µm (B).
Chicken anti-human liver cathepsin B [10 µg/ml (C)] and chicken anti-cathepsin D [15 µg/ml (C)] and a rabbit anti-chicken linker antibody [50 µg/ml (C)] and protein-A gold probe for cathepsin B (10 nm) and for cathepsin D (15 nm) used on LR White sections viewed using a Philips CW120 Biotwin TEM (80-100 kV). Cathepsin B and D in vesicles (black arrows) or only cathepsin B (10 nm) or cathepsin D (15 nm) (white arrows). Bar = 100 nm.

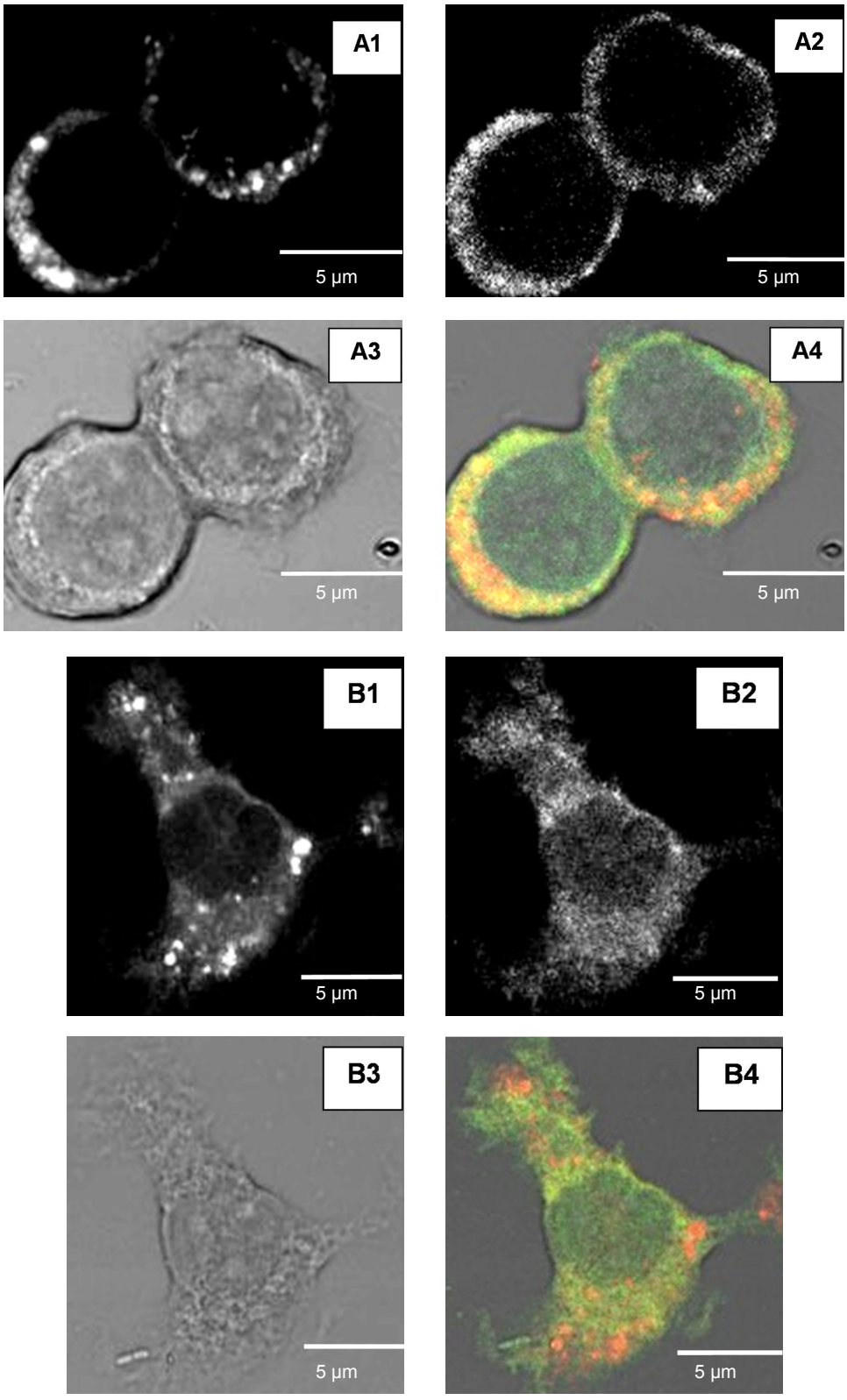

Figure 4.12 **Fluorescent labelling of cathepsins D and L in J774 macrophages.**
Chicken anti-cathepsin D [200 µg/ml (A1 and B1)] and donkey anti-chicken IgG CY3 [2 µg/ml (A1 and B1)], post-fixed (3.7% PFA), probed with rabbit anti-cathepsin L [50 µg/ml (A2 and B2)] and goat anti-rabbit IgG FITC [2 µg/ml (A2 and B2)], applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using a Zeiss 510 Meta confocal microscope. CY3 filter (A1 and B1), FITC filter (A2 and B2), DIC images (A3 and B3), composite images (A4 and B4). Bars = 5 µm.

Few compartments show colocalisation between cathepsins B and L, according to fluorescence microscopy (approximately 25% colocalisation) (Figure 4.13, A and B). Regions showing colocalisation often occur between cells in contact (Figure 4.13, B) or towards one cell edge (Figure 4.13, A). The latter observation seems confirmed by EM where cathepsin B and L colocalisation seemed to occur in relatively small vesicles (±30-50 nm) possibly "secretory lysosomes" mainly towards the cell periphery (Figure 4.13, C, red arrows). Cathepsin B (Figure 4.13, C, white arrows) and cathepsin L (Figure 4.13, C, black arrows) were also observed individually in small (±20 nm) vesicles, reminiscent of secretory vesicles which may contain newly synthesised proforms of the proteases in different vesicle populations. Vesicular swelling and generally poor ultrastructure made EM confirmation of results less useful (Figure 4.13, C).

Labelling colocalisation for cathepsins S and B was difficult to interpret without EM clarification of the labelling pattern, however. It seems to indicate a large amount of colocalisation (approximately 70%) of which approximately 50% occurs in small vesicle (±20 nm) populations reminiscent of secretory-type vesicles which usually contain newly synthesised procathepsins and colocalisation in very few ±50 nm vesicles resembling late endosomes (Figure 4.14, A). This would seem to support blot data which indicates the major form of cathepsin S in J774 cells is the precursor (Figure 3.13, A, lane 3) and that this is also, to some extent true for cathepsin B (approximately 50% immature) (Figure 3.9, A, lane 4). Colocalisation would also seem to indicate that the enzymes are co-expressed and co-packaged (Figure 4.14, A3).

In labelling for cathepsins S and L only minor numbers of small (±20 nm) vesicles show apparent colocalisation (approximately 25%) in non-activated cells (Figure 4.15, A). Cathepsin S appears to be distributed throughout the cells and especially peripherally, whereas, cathepsin L labelling is more centrally located (Figure 4.15, A2 and A3, respectively). Here, too, the compartments labelled seem small (±20 nm) supporting blot predictions that most of the cathepsin S and approximately 50% of cathepsin L of J774 cells may be in the precursor form in secretory vesicles (Figure 3.13, A, lane 3 and Figure 3.12, A lane 3, respectively). In activated cells (elongated), however, (Figure 4.15, B) a higher percentage of colocalisation seems apparent (approximately 70%), indicating how cell activation may affect results.

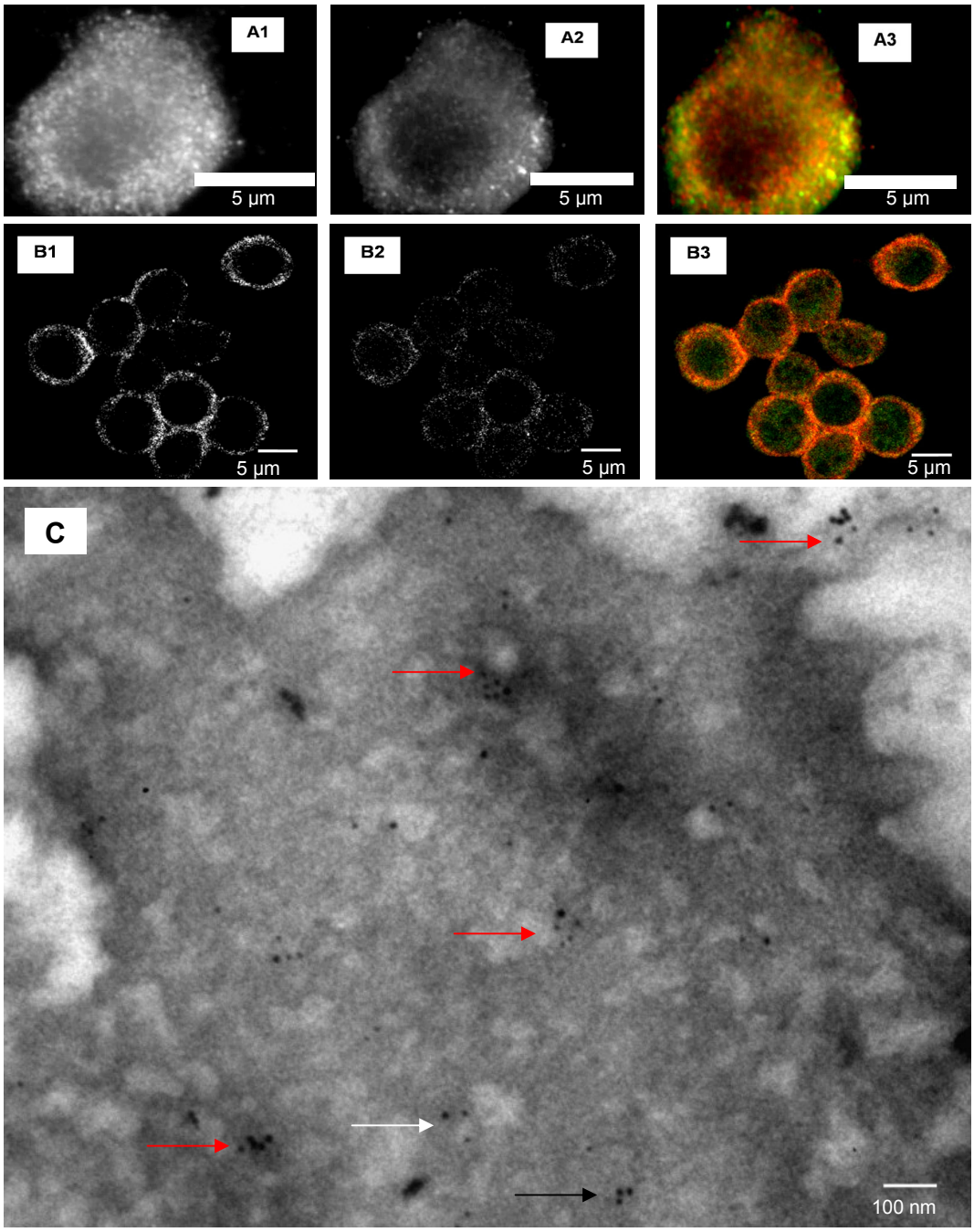

Figure 4.13 **Fluorescent and protein A gold labelling of cathepsins B and L in J774 macrophages.**
Chicken anti-cathepsin B [15 µg/ml (A1) or 50 µg/ml (B1)] and donkey anti-chicken IgG CY3 [2 µg/ml (A1) or 1 µg/ml (B1)], post-fixed (3.7% PFA), probed with rabbit anti-cathepsin L [15 µg/ml (A2) or 50 µg/ml (B2)] and goat anti-rabbit IgG FITC [5 µg/ml (A2) or 2 µg/ml (B2)], applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using an Olympus epifluorescent microscope (A) or a Zeiss 510 Meta confocal microscope (B). CY3 filter (A1 and B1), FITC filter (A2 and B2), composite images (A3 and B3). Bars = 5 µm.
Chicken anti-human liver cathepsin B [10 µg/ml (C)], rabbit anti-cathepsin L [20 µg/ml (C)], a rabbit anti-chicken linker antibody [50 µg/ml (C)] and protein-A gold probe for cathepsin B (10 nm) and for cathepsin L (15 nm) used on LR White sections viewed using a Philips CW120 Biotwin TEM (80-100 kV). Cathepsin B and L colocalised in certain vesicles (red arrows). Cathepsin B (white arrows) and cathepsin L (black arrows) still appeared separately. Bar = 100 nm.

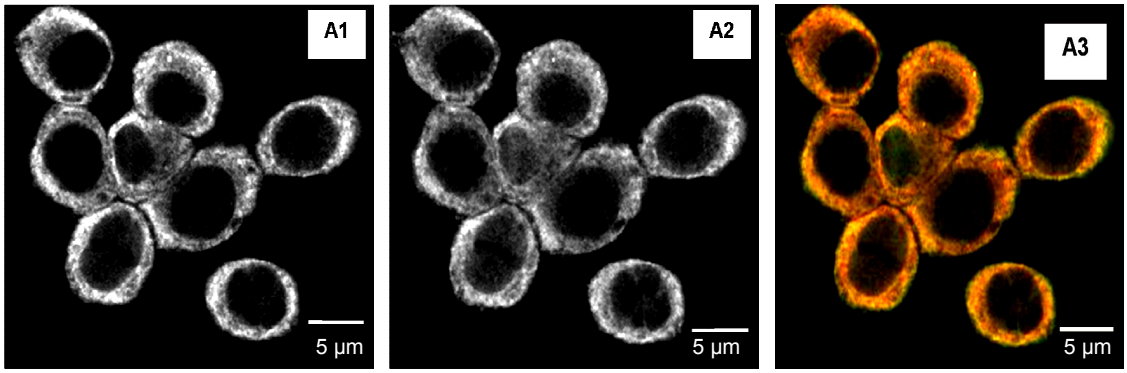

Figure 4.14 **Fluorescent labelling of cathepsins S and B in J774 macrophages.**
Chicken anti-cathepsin S [50 µg/ml (A1)] and donkey anti-chicken IgG CY3 [1 µg/ml (A1)], post-fixed (3.7% PFA), probed with chicken anti-cathepsin B [50 µg/ml (A2)] and rabbit anti-chicken IgG FITC [10 µg/ml (A2)], applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using a Zeiss 510 Meta confocal microscope. FITC filter (A1), CY3 filter (A2), composite image (A3). Bars = 5 µm.

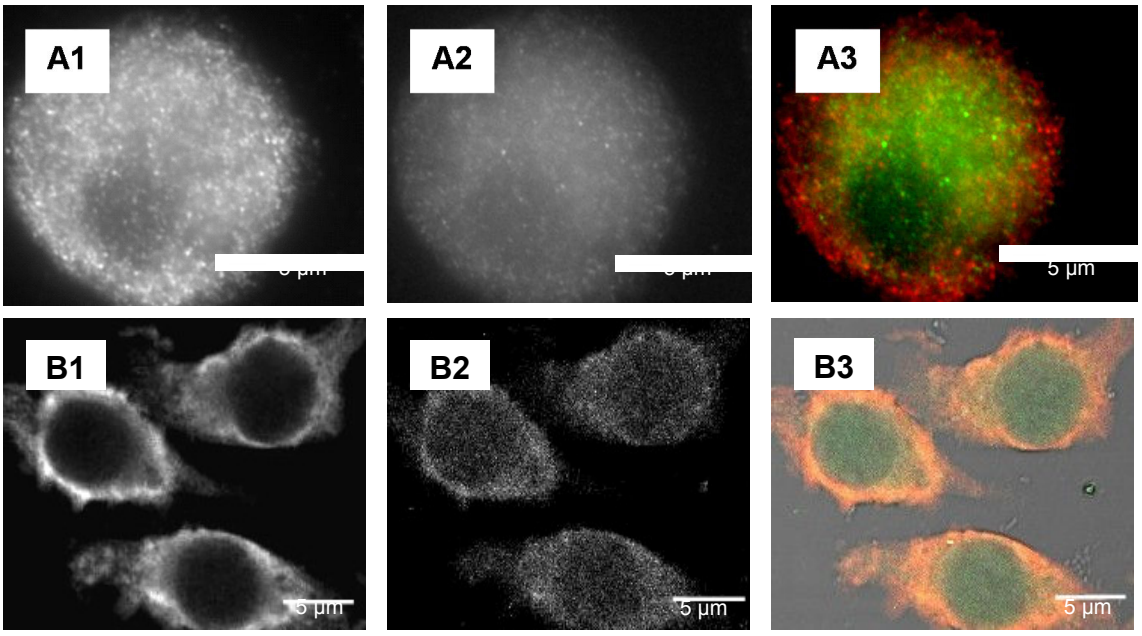

Figure 4.15 **Fluorescent labelling of cathepsins S and L in J774 macrophages.**
Chicken anti-cathepsin S [20 µg/ml (A1)] or 50 µg/ml (B1)] and donkey anti-chicken IgG CY3 [2 µg/ml (A1) or 1 µg/ml (B1)], post-fixed (3.7% PFA), probed with rabbit anti-cathepsin L [20 µg/ml (A2) or 50 µg/ml (B2) and goat anti-rabbit IgG FITC [5 µg/ml (A2) or 2 µg/ml (B2)], applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using an Olympus epifluorescent microscope (A) or a Zeiss 510 Meta confocal microscope (B). CY3 filter (A1 and B1), FITC filter (A2 and B2), composite images (A3 and B3). Bars = 5 µm.

Summary

In summary, the data represented in Table 4.4 seem to indicate the possible presence of at least 2 major vesicle populations labelled for cathepsin H (vesicle 1A and B). One population appears secretory, containing either membrane-bound or free procathepsin H (±20 nm, vesicle 1B). The other population is possibly an early endosomal population containing active cathepsin H (Claus *et al*., 1998) (±100 nm, vesicle 1A). Without labelling for other cathepsins to check colocalisation with cathepsin H and further markers or probes, it is difficult to know whether cathepsin H would be a good marker for an early endosomal population (provisional early endosomal vesicle 1A and a secretory vesicle population 1B).

Precursor cathepsins B and S could be mainly colocalised and membrane-bound (Table 4.4). The mature cathepsin S (approximately 10%) may be largely colocalised with mature cathepsin B in late endosomes (±50 nm) and there seem to be relatively few late endosomes. Colocalisation would best be checked at the EM level and with labelling for precursor and mature cathepsins and a marker, such as the 215 kDa MPR, for the late endosome (provisionally classified in this study as a late endosome, vesicle 2).

There seems more active cathepsin B (approximately 40% more) than active cathepsin S [the total active cathepsin S appears to be approximately 10%, whereas, cathepsin B is approximately 50%, Table 4.4]. It is highly likely that the 30% of active cathepsin B and 40% of mature cathepsin L colocalise with mature cathepsin D, generally found in classical "lysosomal" or "hybrid" organelles, [i.e. most acidic and cathepsin D labelling (de Duve, 1983)] or in an acidic, digestive organelle other than the late endosome (if cathepsin S is a marker for the late endosome) (Table 4.4) (provisional hybrid, digestive vesicle 3, distinct from the late endosome).

If secretory vesicles were included, 4 vesicle types (an early endosomal-, late endosomal-, a digestive and secretory vesicle type) are evident by this stage. These vesicle populations could be assigned with more certainty to known vesicle population groups if antisera recognising precursor and mature cathepsins and hence the enzymes in late endosomes/lysosomes and precursor enzymes of secretory vesicles could be identified. It also seemed necessary, at this stage, to include a second marker for the late endosome or lysosomal population. Without the availability of antisera against the precursor enzyme, or

Table 4.4 **Summary of colocalisation, western blot data and apparent vesicular distribution of cathepsins.**

	Colocalisation (%) and potential vesicle type					Precursor form (%)	Mature form (%)	Vesicle type	M (%)	F (%)
	Cat H	**Cat S**	**Cat D**	**Cat B**	**Cat L**					
Cat H	ND	<20	ND	ND	ND	50	50	LT/S	50	50
Cat S	<20	ND	25 (Lys)	70 (LE/S)	25 (S)	90	10	>S/LE/Lys	66	34
Cat D	ND	25 (Lys)	ND	30 (LE/Lys/SL)	40 (Lys)	20	80	LE/Lys	34	66
Cat B	ND	70 (LE/S)	30 (LE/Lys/SL)	ND	25 (SL)	50	50	S/>LE/Lys	66	34
Cat L	ND	25 (S)	40 (Lys)	25 (SL)	ND	50	50	>S/LE/Lys	66	34

Abbreviations: Cat, cathepsin, ND, not determined, LT, large (±100 nm) electron-translucent, early endosome-like, S, small (±20 nm) secretory vesicle-like, LE, large (±50 nm), electron-dense, late endosome-like; Lys, lysosome-like, hybrid, digestive organelles (±150-200 nm); SL, "secretory lysosome"; M, membrane-bound, F, free, >, mostly.

LYAAT and V-ATPase antibodies (to differentiate early endosomes from the Hck/LYAAT vesicle), LysoTracker seemed the obvious choice as a marker for acidic compartments including late endosomes, classical lysosomes and possibly other vesicles such as the V-ATPase-rich compartment described by Anes *et al*. (2006). LAMP-1 and -2 also seemed appropriate for identifying both the classical late endosomes and lysosomes (Fukuda *et al*., 1991).

4.4 Localisation of cathepsins in LAMP-1 and LAMP-2 positive compartments

Several highly N-glycosylated proteins are present in "lysosomal" membranes and are known as LAMP-1, LAMP-2 and LAMP-3 (Eskelinen *et al*., 2003). LAMP-1 and LAMP-2 are evolutionarily related and share great structural similarity (Fukuda *et al*., 1991), however, they appear to be differentially regulated. LAMP-1 appears to be constitutively expressed (Amos *et al*., 1990), whereas, LAMP-2 expression varies with cell type and with the developmental stage of the cell (Hatem *et al*., 1995; Hua *et al*., 1998).

At steady state, most LAMPs are localised to the limiting membranes of both classical late endosomes and lysosomes (Fukuda *et al*., 1991) and have, therefore, been used as markers for these compartments in many studies. Small amounts have, however, been detected in classical early endosomal membranes, the plasma membrane as well as in the limiting membrane of autophagic vacuoles (Eskelinen *et al*., 2002; Eskelinen *et al*., 2003). LAMP surface expression appears to occur in certain cell types including cytotoxic T lymphocytes and highly metastatic tumour cells as well as under certain conditions such as the activation of platelets and blood monocytes (Kannan *et al*., 1996; Eskelinen *et al*., 2003). In contrast to LAMP-1 and -2, LAMP-3 appears to be predominantly located in multivesicular late endosomes and is associated with the internal membranes of these compartments which are rich in lyosbisphosphatidic acid (Kobayashi *et al*., 2000). Interestingly, LAMP-3 appears to be shared by both endocytic compartments as well as specialised secretory organelles. These include the Weibel-Palade bodies of endothelial cells (Kobayashi *et al*., 2000), azurophil granules of neutrophils (Dahlgren *et al*., 1995) and the α-granules of platelets (Eskelinen *et al*., 2003). Unexpectedly, LAMP-1 and LAMP-2 are not located in the azurophil granules of neutrophils but appear to be present in peroxidase-negative specific granules and secretory vesicles (Dahlgren *et al*., 1995).

The functional significance of LAMPs has been unknown for many years. It was originally thought that they served only as structural elements for lysosomal membranes (Furuta *et al*., 1999; Furuta *et al*., 2001). LAMPs have high carbohydrate content and it was thought that these complex carbohydrates maintained the stability of the 'lysosomal' membrane by protecting them from various hydrolytic enzymes (Fukuda *et al*., 1991). Recent studies, however, have demonstrated a number of specific functions for LAMPs. Mice deficient in LAMP-1 appear to be viable and fertile, and the various properties of lysosomes such as the processing of enzymes, enzyme activity, pH as well as morphology and subcellular distribution remain normal. An up-regulation of LAMP-2 was observed in certain tissues and it appears that increased levels of LAMP-2 are required to compensate for the lack of LAMP-1 (Andrejewski *et al*., 1999). LAMP-2 deficient mice, however, show severe symptoms. Approximately 50% of the mice die 20-40 days post partum and are smaller in size. There is also significant accumulation of autophagic vacuoles in their liver, muscle and heart (Tanaka *et al*., 2000). LAMP-2 deficiency is also the main defect in Danon disease, which is characterised by fatal cardiomyopathy, mental retardation and mild skeletal myopathy with an accumulation of autophagic vacuoles in both the skeletal and cardiac muscle. It has been suggested that LAMP-2 deficiency results in impaired recycling of the 46 kDa MPR and the subsequent mistargeting of specific lysosomal enzymes. The accumulation of autophagic vacuoles is, therefore, due to impaired lysosomal degradation (Eskelinen *et al*., 2002).

LAMP-3 is a tetraspanin and has been shown to act as a "molecular facilitator" by enhancing the formation and stability of signaling complexes. It also appears to be involved in cell activation and mediator release (Mahmudi-Azer *et al*., 2002). Recently, it has been demonstrated that LAMP-3 appears to act as a cell surface binding partner for TIMP-1. In MCF10A human breast epithelial cells, TIMP-1 associates with integrin β1 in a LAMP-3-dependent manner, regulating signaling pathways involved in cell survival and polarisation (Jung *et al*., 2006). This is extremely interesting as TIMPs have long been known to be involved in signaling but the mechanism has always been unclear.

LAMP-1, LAMP-2 and LysoTracker were used in the current study as markers for classical late endosomal and lysosomal compartments, as they have both been identified in classical late endosomes and lysosomes of various cell types including macrophages (Desjardins *et al*., 1994a; Jahraus *et al*., 1998 Eskelinen *et al*., 2002; Sun-Wada *et al*., 2003; Anes *et al*.,

2006). Double immunolabelling for cathepsins and LAMPs or cathepsins and LysoTracker were performed to verify the possible presence of cathepsins in classical late endosomal/lysosomal-like digestive compartments as indicated towards the end of Section 4.1. The definitions of "lysosomal" populations are no longer adequate and as mentioned at the end of Section 4.1, marker variations, identifying 5 organelles seem evident (Table 4.5).

Table 4.5 **Classification of J774 macrophage endosome-lysosome vesicle populations based on pH and the presence of LAMPs.**

Labelling patterns of anticipated 5 vesicle types	LAMP-1	LAMP-2	Acidic (LysoTracker-positive)
Early endosome	Negative	Negative	No
Late endosome	Positive	Positive	Yes
Lysosome-like[+]	Positive[#]	Positive[$]	Yes
V-ATPase vesicle*	Negative	Negative	Yes
LYAAT vesicle*	Negative	Negative	Moderately

([+] hybrid organelle or digestive body; [#] Gough and Fambrough, 1997; Falcon-Perez *et al*., 2005; [$] Eskelinen *et al*., 2002; *Anes *et al*., 2006)

Late endosomes are classically acidic and may label for LAMPs upon fusion with LAMP-positive lysosomal populations, when lysosomal enzymes are required for digestion of endocytosed products (Gough and Fambrough, 1997; Falcon-Perez *et al*., 2005; Eskelinen *et al*., 2002). Other times, it is always possible that late endosomes may be LAMPs-negative and transiently non-acidic or less acidic than usual. This may complicate vesicle classification. Whether late endosomes are ever of neutral or less acidic pH has never also been established. Here we, therefore, used LAMP-1 and -2, LysoTracker as the markers for a digestive body most like de Duve's primary and secondary lysosomes (de Duve, 1983) and the Griffith's "hybrid organelle" (Griffiths, 1996a), and labelling for various cathepsins, especially cathepsin H for the early endosome (Claus *et al*., 1998) and cathepsin S for the late endosome (Jahraus *et al*., 1998) to attempt to classify the J774 macrophage vesicle populations. The anticipated vesicle populations with labelling patterns are indicated in Table 4.5. Very weak colocalisation was considered negative.

4.4.1 Reagents

Reagents for the culture of J774 cells, reducing SDS-PAGE, western blotting and fluorescent immunolabelling were prepared according to Sections 2.2.1, 2.3.1.1, 2.6.2.1 and 2.8.1.1, respectively.

Rat anti-mouse LAMP-1 (1D4B) and rat anti-mouse LAMP-2 (ABL-93) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, Iowa, USA) and goat anti-rat IgG-alkaline phosphatase conjugate was from Sigma.

The anti-cathepsin and secondary fluorescent antibodies used, were as previously described (Section 3.7.1 and 4.2.1). Goat anti-rat IgG FITC and goat anti-rabbit IgG TRITC were also used.

4.4.2 Procedure

Serum-containing and serum-free J774 mouse macrophage homogenates and supernatants were prepared (Section 3.6.2), separated on a 12.5% (v/v) Laemmli gel (Section 2.3.1.2), transferred to nitrocellulose and probed with rat anti-LAMP-1 [1:800], rat anti-LAMP-2 [1:1000] and goat anti-rat IgG-alkaline phosphatase [1:30 000] according to Section 2.6.2.2.

J774 cells were cultured (Section 2.2.2) and fluorescent immunolabelling performed with rat anti-mouse LAMP-1 [1:300 or 1:800 or 1:900], rat anti-mouse LAMP-2 [1:900 or 1:1200], chicken anti-cathepsin D [100 µg/ml or 200 µg/ml], chicken anti-cathepsin B [50 µg/ml], rabbit anti-cathepsin H [100 µg/ml], chicken anti-cathepsin S [100 µg/ml] and rabbit anti-cathepsin L [20 µg/ml]. Goat anti-rat IgG FITC [3 µg/ml or 2µg/ml or 1 µg/ml], donkey anti-chicken IgG CY3 [1.5 µg/ml or 2 µg/ml], goat anti-rabbit IgG TRITC [18 µg/ml or 11 µg/ml] were used as detection antibodies according to Section 2.8.1.2. In some instances, only the Olympus epifluorescent microscope was used for viewing immunolabellings as the Zeiss 510 Meta confocal was only on loan for 3 months and soon became unavailable. Images were analysed using ImageJ software. The percentage colocalisation was determined as described in Section 4.2.2. As the polyclonal anti-mature cathepsin antisera used cannot distinguish between precursor and mature forms of the enzyme, cathepsins associated with LAMPs-positive and/or acidic organelles will assumed to be mature and active.

4.4.3 Results

The commercial LAMP-1 and LAMP-2 antibodies characterised using crude J774 mouse macrophage homogenates revealed bands of approximately 109 kDa and 113 kDa corresponding to LAMP-1 and LAMP-2, respectively (Figure 4.16, lanes 2 and 4, Figure 4.17, lanes 2 and 4), confirming the specificity and suitability of these antisera for immunolabelling studies on the J774 cell line.

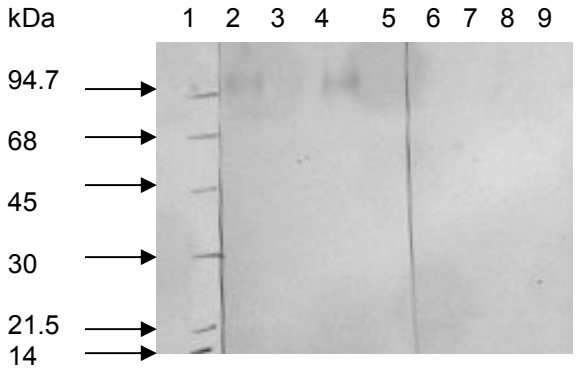

Figure 4.16 **Detection of LAMP-1 in J774 homogenates and supernatants.**
MWM (lane 1, 5µl), serum-containing J774 homogenate (lanes 2 and 6, 10 µl) and supernatant (lanes 3 and 7, 10 µl), serum-free J774 homogenate (lanes 4 and 8, 10 µl) and supernatant (lanes 5 and 9, 10 µl) were probed with rat anti-LAMP-1 [1:800] (lanes 2-5), rat IgG [1:800] (lanes 6-9), detected with goat anti-rat IgG (whole molecule)-alkaline phosphatase [1: 30 000] and developed in alkaline phosphatase substrate solution after separation on a 12.5% (v/v) Laemmli gel and blotting on to nitrocellulose.
MWM = phosphorylase b, 97.4 kDa, BSA, 68 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 30 kDa, soybean trypsin inhibitor, 21.5 kDa and lysozyme, 14 kDa.

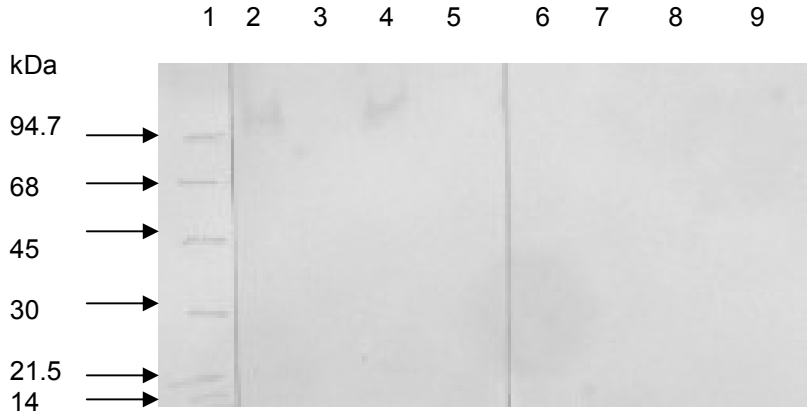

Figure 4.17 **Detection of LAMP-2 in J774 homogenates and supernatants.**
MWM (lane 1, 5µl), serum-containing J774 homogenate (lanes 2 and 6, 10 µl) and supernatant (lanes 3 and 7, 10 µl), serum-free J774 homogenate (lanes 4 and 8, 10 µl) and supernatant (lanes 5 and 9, 10 µl) were probed with rat anti-LAMP-2 [1:1000] (lanes 2-5), rat IgG [1:1000] (lanes 6-9), detected with goat anti-rat IgG (whole molecule)-alkaline phosphatase [1: 30 000] and developed in alkaline phosphatase substrate solution after separation on a 12.5% (v/v) Laemmli gel and blotting on to nitrocellulose.
MWM = phosphorylase b, 97.4 kDa, BSA, 68 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 30 kDa, soybean trypsin inhibitor, 21.5 kDa and lysozyme, 14 kDa).

A large number of LAMP-1- (Figure 4.18, A1) and LAMP-2-positive (Figure 4.18, A2) compartments were observed in J774 mouse macrophages.

As anticipated little colocalisation was evident between cathepsin H and LAMP-1 (Figure 4.19, A) and LAMP-2 (Figure 4.19, B) (less than 25% colocalisation). Cathepsin H-labelled vesicles (±100 nm, possibly early endosomes) were, therefore, classified as LAMP-1- and/or -2-negative (Table 4.6). Vesicles showing no colocalisation between cathepsin H and either LAMP-1 or LAMP-2 may represent either newly synthesised precursor enzyme

[approximately 50% of all cathepsin H present, small (±20 nm) secretory vesicles] or possibly the presence of mature cathepsin H (in approximately 50% of all cathepsin H-labelled organelles) in LAMP-negative early endosomes (±100 nm). Claus *et al*. (1998) reported that approximately 70% of active cathepsin H in J774 macrophages was located in early endosomes, with only 10% and 20% occurring in late endosomes and "lysosomes", respectively. These values almost agree with our labelling results and similarly, our blot data (Figure 3.11, A, lane 3) almost concurs with these figures, as approximately 50% of both precursor and mature cathepsin H was observed in J774 homogenates. Careful scrutiny of fluorescent images indicates limited colocalisation of cathepsin H and LAMPs as [less than 25%, previously indicated by Claus *et al*. (1998)] seems associated with late endosomes (±50 nm) and lysosome-like or digestive organelles (±100-200 nm). This colocalisation may represent cathepsin H undergoing activation in LAMP-positive, "late endosome-lysosome hybrid organelles" (Figure 4.19, A and B, arrows). Cathepsin H, therefore, mainly seems to occur in early endosomes and secretory vesicles as indicated in the Summary at the end of Section 4.3.

Some colocalisation was observed between cathepsin S and LAMP-1 (Figure 4.20, A). Only approximately 10% of the total cellular cathepsin S seems to be active according to western blots (Figure 3.13, A, lane 3), therefore, of the 45% colocalising with LAMP-1 only approximately 10% could colocalise with active enzyme. (The percentage colocalisation with LAMP-2 is unknown). LAMP-1 association with a vesicle containing precursor enzyme was unanticipated as LAMP-positive organelles are generally associated with enzyme processing or processed enzymes. According to the dogma such organelles should be acidic and contain active cathepsins (Table 4.1). Anes *et al*. (2006), however, identified an unanticipated "lysosomal" subpopulation (largely non-acidic and LAMP-1-positive). [Classically "lysosomal" populations labelling for LAMPs, are acidic (pH of ± <5.5), containing processed lysosomal cathepsins and lacking in the 215 kDa MPR (Table 4.1)]. It is always possible that LAMPs-positive, non-acidic, MPR-negative vesicles containing unprocessed enzymes (Table 4.1) may exist, however. Enzymes may be required initially in precursor form and after slow acidification and processing of enzymes has occurred, such an organelle may secrete "mature" enzymes. Punturieri *et al*. (2000) demonstrated such a scenario as mature cathepsin S, as well as cathepsins K and B, were only shown to be released from MDMs some days after induction of an inflammatory process. Enzymes may, therefore, be stored as precursors for some time before activation and release, or may

initially be released as precursors and subsequently as active enzymes after a slow acidifying or maturation process. Whether there are two enzyme processing sites, a late endosome and another acidic organelle (i.e. LAMPs-positive, acidic, MPR-negative and containing mature lysosomal enzymes) (Table 4.1) or whether the late endosome "matures" into such an organelle is unknown. The latter scenario seems a possibility from the current cathepsin S-labelling results. Cathepsin S, however, seems located mainly in secretory and late endosomal vesicle populations.

Mature cathepsin D, present in 80% of vesicles, on the other hand, colocalised in a 50:50 ratio with LAMP-1 and LAMP-2 (Figure 4.21, A and C, respectively, i.e. within 50% of all mature cathepsin D-containing vesicles, possibly ±30-50 nm, "secretory lysosomes", ±50 nm late endosomes and ±150-200 nm hybrid organelles). LAMP-1 and -2 may colocalise with each other within the same 50% of active cathepsin D-labelled vesicles of all vesicle populations or may colocalise minimally at 20% (i.e. 2 x 30% of mature cathepsin D-labelled vesicles associated with one label and 20% associated with both labels, contributing to a total of 80% active enzyme) or some proportion between these two extremes i.e. 20-50% colocalisation between LAMP-1 and -2. With good EM ultrastructure and triple labelling for cathepsin D (precursor and mature), LAMP-1 and -2 it may be possible to identify the different LAMP labelling populations.

Activation (elongated cell morphology) seemed to increase the level of colocalisation of cathepsin D with LAMP-1 to almost 100% of the ±50 nm late endosome-like vesicles containing the mature cathepsin (Figure 4.21, B) (LAMP-2 colocalisation could not be assessed as a similarly activated cell could not be found and triple labelling was not performed). The increase in association with LAMP-1 (up to approximately 100%) upon activation (Figure 4.21, B) illustrates how dynamic and variable localisations may be with activation.

Colocalisation seems to occur mainly in specific areas of the cell and such organelles seem to be relatively large (±50 nm) and greater than 100 nm (±150-200 nm) (Figure 4.21, A and B). These are possibly late endosomes and "hybrid" organelles or digestive bodies, therefore, agreeing with previous Summary observations (Summary end of Section 4.3 and Table 4.4).

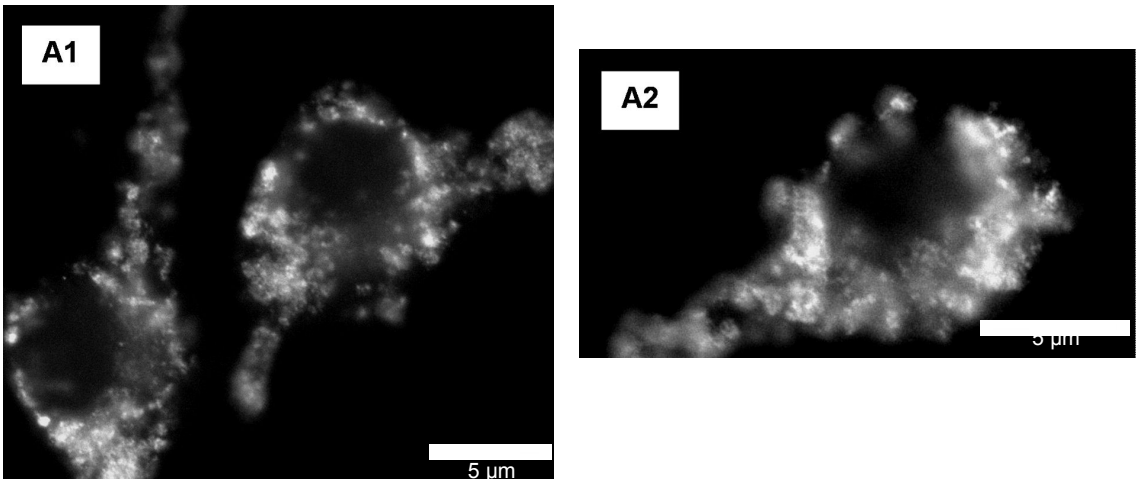

Figure 4.18 **Fluorescent labelling of LAMP-1 and LAMP-2 in J774 macrophages.**
Rat anti-mouse LAMP-1 [1:900 (A1)] or rat anti-mouse LAMP-1 [1:1200 (A2)] and goat anti-rat FITC [3 µg/ml (A1 and A2)] applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed with an Olympus epifluorescent microscope. Bars = 5 µm.

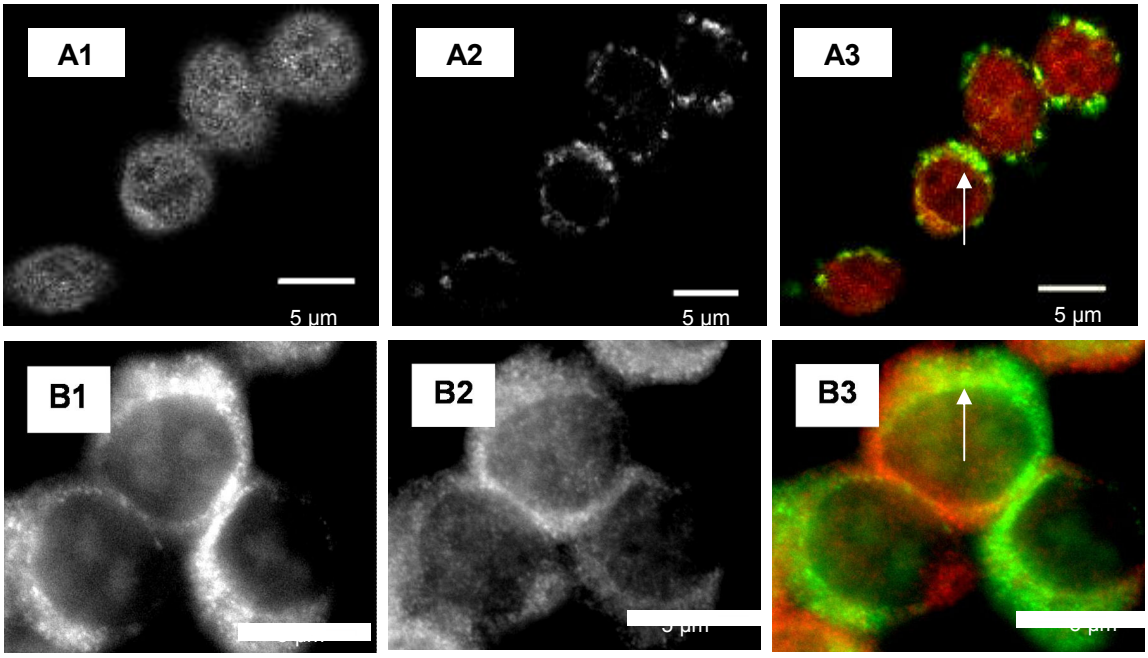

Figure 4.19 **Fluorescent labelling of LAMP-1 or LAMP-2 and cathepsin H in J774 macrophages.**
Rabbit anti-cathepsin H [100 µg/ml (A1)] and goat anti-rabbit TRITC [18 µg/ml (A1 and B1)], post-fixed (3.7% PFA), probed with rat anti-mouse LAMP-1 [1:900 (A2)] or rat anti-mouse LAMP-2 [1:900 (B2)] and goat anti-rat FITC [2 µg/ml (A2)] or goat anti-rat FITC [1 µg/ml (B2)], applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using a Zeiss 510 Meta confocal microscope (A) or an Olympus epifluorescent microscope (B). TRITC filter (A1 and B1), FITC filter (A2 and B2), composite images (A3 and B3). Less than approximately 25% between cathepsin H and LAMP-1 or -2 (arrows). Bars = 5 µm.

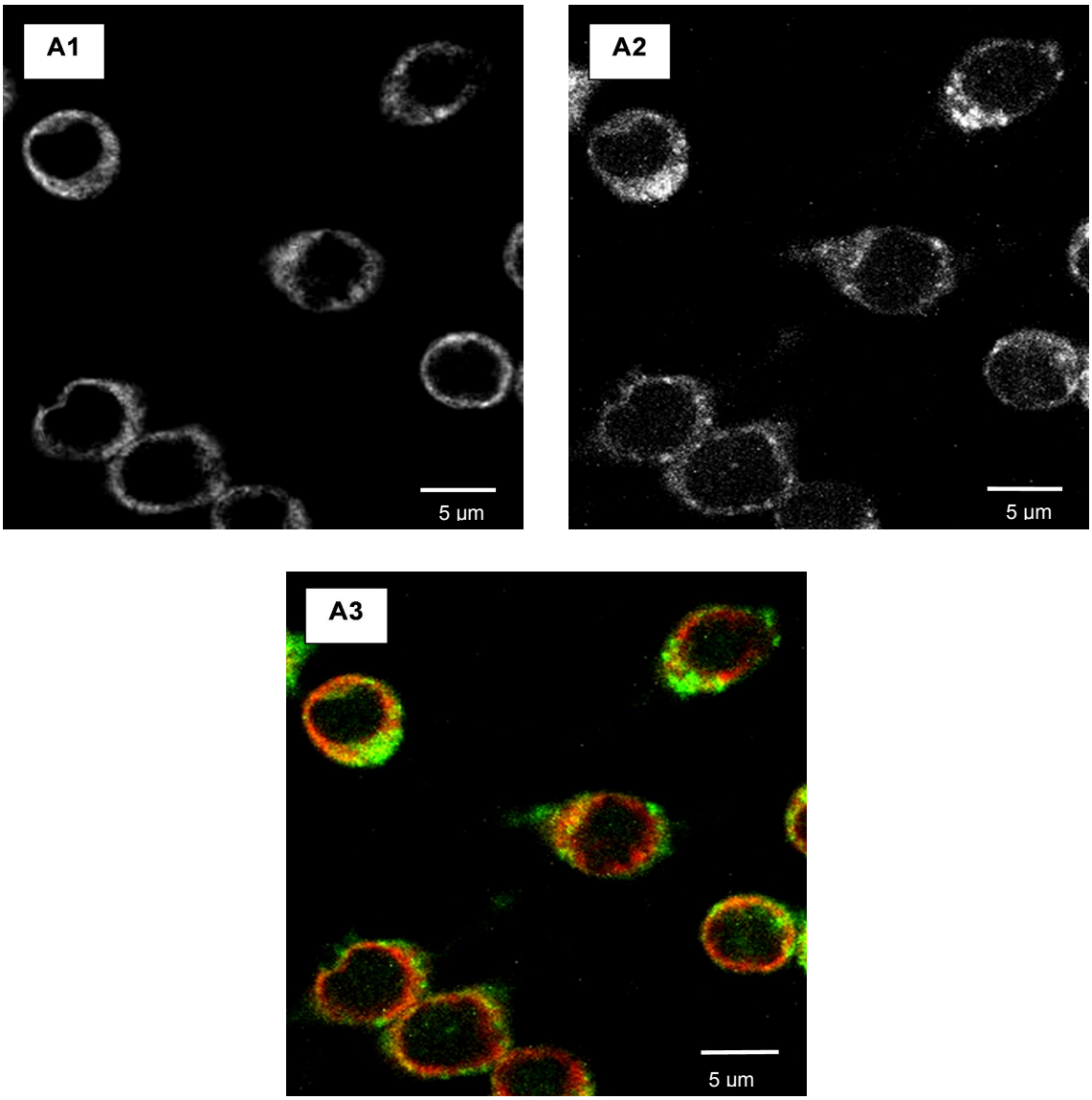

Figure 4.20 **Fluorescent labelling of LAMP-1 and cathepsin S in J774 macrophages.**
Chicken anti-cathepsin S [50 µg/ml (A1)] and donkey anti-chicken CY3 [2 µg/ml (A1)], post-fixed (3.7% PFA), probed with rat anti-mouse LAMP-1 [1:900 (A2)] and goat anti-rat FITC [2 µg/ml (A2)] applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using a Zeiss 510 Meta confocal microscope. CY3 filter (A1), FITC filter (A2), composite image (A3). Bars = 5 µm.

The minimum percentage of cathepsin D possibly not associated with LAMPs may be approximately 20%, as 20% of the cathepsin D-labelled vesicles may contain immature cathepsin D according to western blots (Figure 3.10, A, lane 3). These vesicles are possibly precursor-containing secretory vesicles (Table 4.4).

Limited colocalisation was observed between cathepsin B and LAMP-1 and LAMP-2 (in approximately 15% and 30% of total cathepsin B-labelled vesicles, Figure 4.22, A and B, respectively), with colocalisation occurring in relatively small vesicles (±30-50 nm) and a few larger (±150-200 nm) possibly "hybrid" organelles. This suggests that the antibody is largely either detecting procathepsin B or mature cathepsin B in vesicles other than late endosomes (±50 nm), possibly the digestive body or hybrid organelle and this seems to be borne out by Figure 4.22, A and B. This also seems possible as blots reflect that about 50% of the cathepsin B present in J774 cells is in the precursor form (Figure 3.9, A, lane 4) and approximately 50% mature (Figure 3.9, A, lane 4). It would seem that LAMP-2 may also colocalise more with cathepsin B (approximately 30%) than LAMP-1 (approximately 15%) (Figure 4.22, B compared to A). A small proportion (approximately 15%) of mature cathepsin B- and LAMP-1-labelled vesicles and approximately 30% of mature cathepsin B- and LAMP-2-labelled vesicles may colocalise in any of the above-mentioned vesicles. Alternatively LAMP-1 and -2 may largely label separate mature enzyme-containing, acidic populations. Overlapping colocalisation for LAMP-1 and -2 and mature cathepsin B may, therefore, occur in a maximum of 15% of mature cathepsin B-containing vesicles, with 15% non-colocalised. If only mature cathepsin colocalises with LAMPs, cathepsin B-labelled vesicles may be classified mainly as LAMP-2-positive. At least 5% of the mature enzyme may occur in a LAMP-1- and -2-negative organelle, however.

Lastly, cathepsin L showed only slight colocalisation with LAMP-1 and LAMP-2 (approximately 30% and 15%, Figure 4.23, A and B, in ±150-200 nm possibly hybrid organelles, ±50 nm late endosomes and ±30-50 nm secretory lysosomes, respectively). The LAMPs distribution seems to be different and opposite to that of cathepsin B and LAMPs labelling. Blots indicating that 50% of cathepsin L may occur in the mature form (Figure 3.12, A, lane 3) and colocalisation between cathepsin L and LAMP-1 and -2 in ±150 nm and ±100 nm vesicles, respectively may, therefore, represent cathepsin L in acidic compartments other than late endosomes i.e. hybrid or digestive organelles. If LAMPs labels are associated only with mature enzyme, only 30% and 15% of the total 50% cathepsin L may, therefore,

be either LAMP-1- and/or LAMP-2-associated, respectively. Non-colocalised vesicles (approximately 55% of ±20 nm cathepsin L-labelled vesicles) could possibly contain newly synthesised procathepsin L. This may mean that at least 15% of the mature enzyme may be separately located in a LAMP-1-positive, LAMP-2-negative organelle population and possibly at least 15% may be colocalised (Table 4.6). Colocalisation seems to occur mainly in relatively large (greater than ±100 nm) vesicles (Figure 4.23, B) which may possibly be hybrid organelles or digestive bodies. Lastly, like for cathepsin B, at least 5% of active enzyme is also possibly found in a LAMP-1- and LAMP-2-negative compartment indicating the presence of LAMP-1/LAMP-2-labelled subpopulations. LAMP-2 labelled cells seem to be slightly activated (elongated morphology) (Figure 4.23, B), however, and activation could have affected localisation patterns observed for LAMP-2.

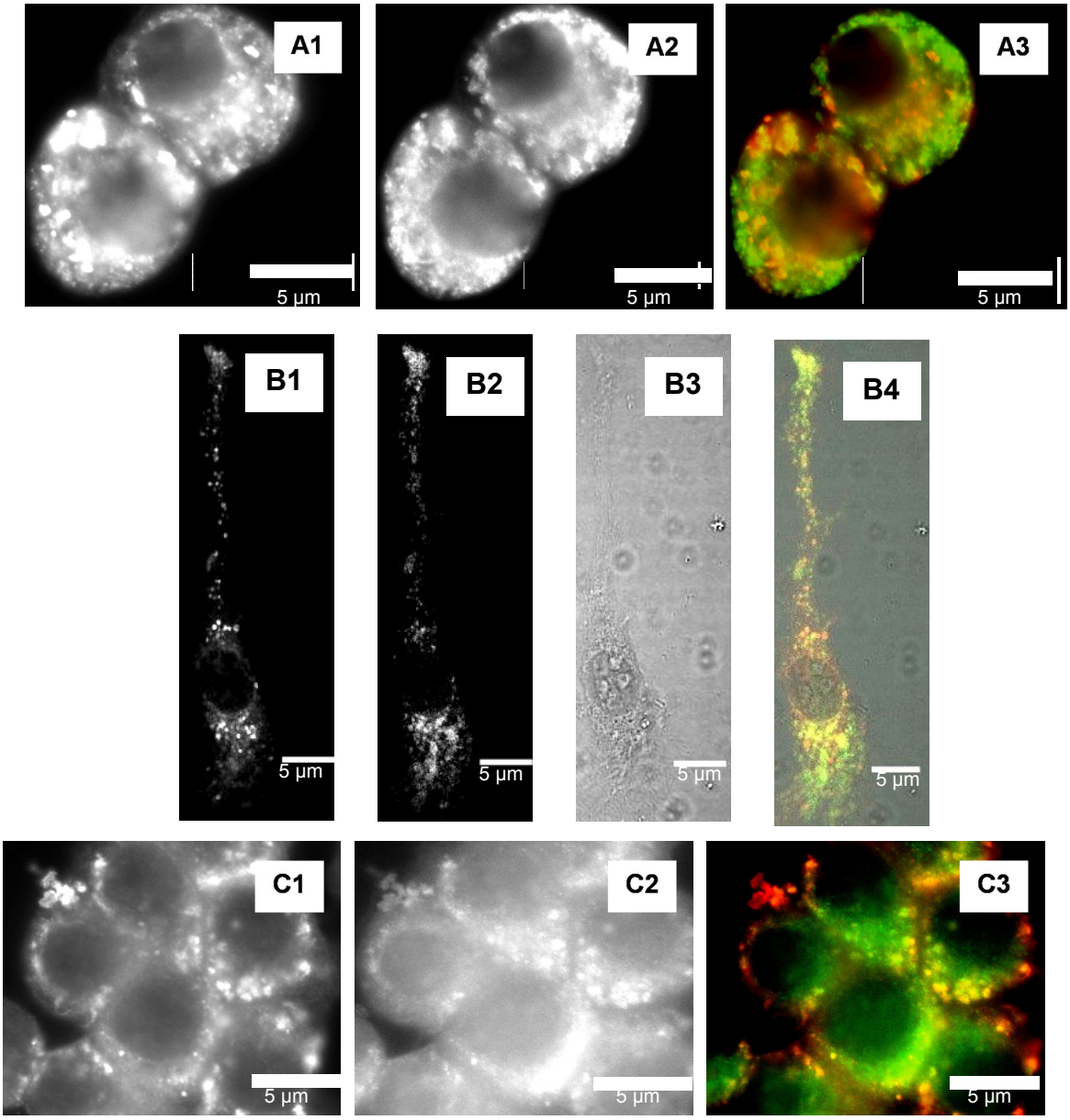

Figure 4.21 **Fluorescent labelling of LAMP-1 or LAMP-2 and cathepsin D in J774 macrophages.** Chicken anti-cathepsin D [100 µg/ml (A1) or 200 µg/ml (B1 and C1)] and donkey anti-chicken CY3 [2 µg/ml (A1, B1 and C1)], post-fixed (3.7% PFA), probed with rat anti-mouse LAMP-1 [1:300 (A2) or 1:800 (B2)] or rat anti-mouse LAMP-2 [1:900 (C2)] and goat anti-rat FITC [3 µg/ml (A2 and B2) or 1 µg/ml (C2)] applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using an Olympus epifluorescent microscope (A and C) or a Zeiss 510 Meta confocal microscope (B). CY3 filter (A1, B1 and C1), FITC filter (A2, B2 and C2), composite images (A3 and C3), DIC image (B3), composite images (A3, B4 and C3). Bars = 5 µm.

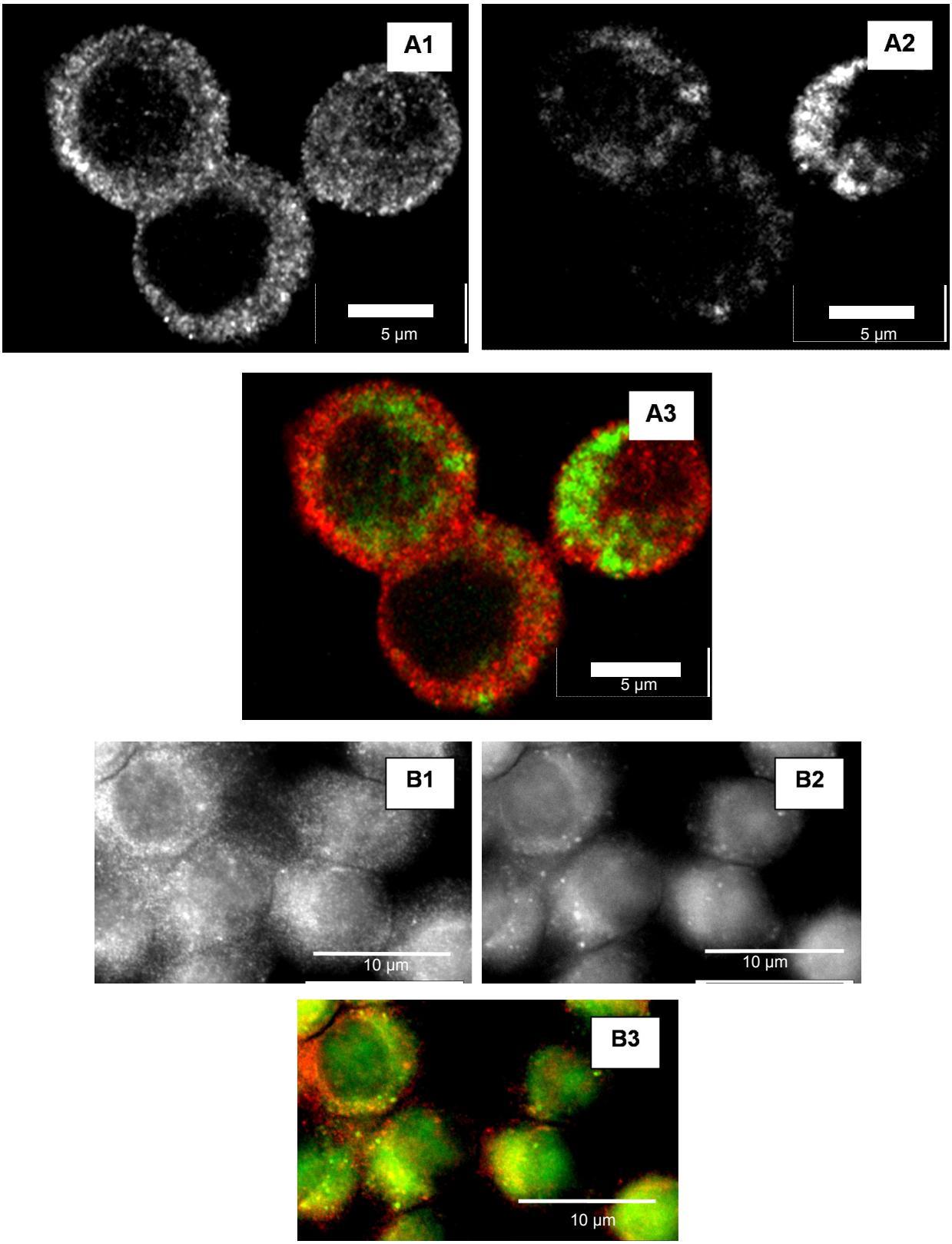

Figure 4.22 **Fluorescent labelling of LAMP-1 or LAMP-2 and cathepsin B in J774 macrophages.** Chicken anti-cathepsin B [50 µg/ml (A1 and B1)] and donkey anti-chicken CY3 [1 µg/ml (A1) or 2 µg/ml (B1)], post-fixed (3.7% PFA), probed with rat anti-mouse LAMP-1 [1:900 (A2)] or rat anti-mouse LAMP-2 [1:900 (B2)] and goat anti-rat FITC [2 µg/ml (A2)] or 1 µg/ml (B2)] applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using a Zeiss 510 Meta confocal microscope (A) or an Olympus epifluorescent microscope (B). CY3 filter (A1 and B1), FITC filter (A2 and B2), composite images (A3 and B3). Bars = 5 µm (A) or 10 µm (B).

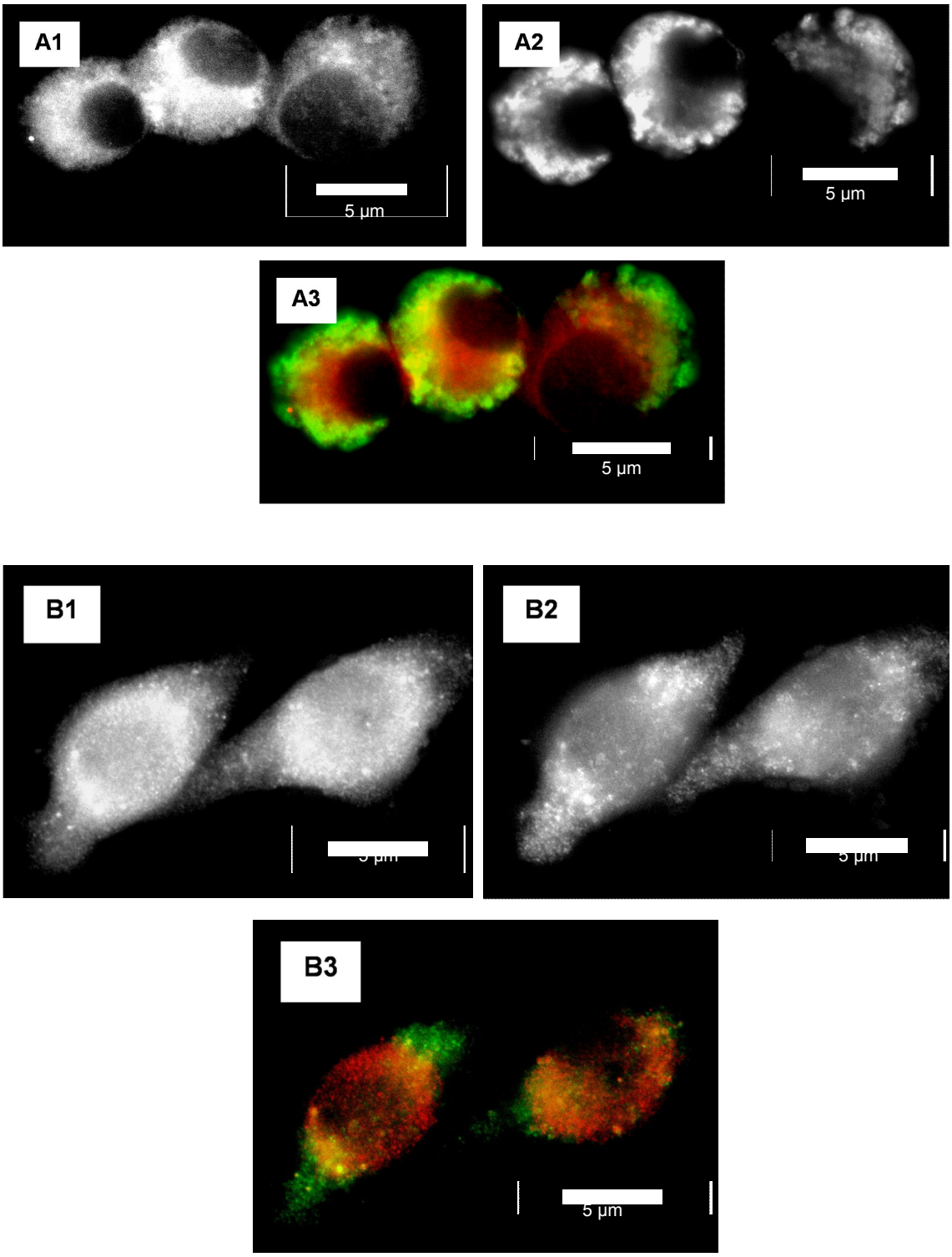

Figure 4.23 **Fluorescent labelling of LAMP-1 or LAMP-2 and cathepsin L in J774 macrophages.** Rabbit anti-cathepsin L [20 µg/ml (A1) or 40 µg/ml (B1)] and goat anti-rabbit TRITC [11 µg/ml (A1) or 18 µg/ml (B1)], post-fixed (3.7% PFA), probed with rat anti-mouse LAMP-1 [1:300 (A2)] or rat anti-mouse LAMP-2 [1:9002 (B2)] and goat anti-rat FITC [3 µg/ml (A2) or 1 µg/ml (B2)] applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using an Olympus epifluorescent microscope. TRITC filter (A1 and B1), FITC filter (A2 and B2), composite images (A3 and B3). Bars = 5 µm.

Summary

Taken together, these results suggest that there seems to be some heterogeneity in distribution between LAMP-1 and LAMP-2 compartments, with cathepsins D, S and L apparently exhibiting the highest association with LAMP-1 (50, 45 and 30%, respectively) and cathepsin B showing a greater association with LAMP-2 (30%) than LAMP-1 and cathepsin L the opposite (Table 4.6).

Five vesicle labelling patterns for LAMPs were predicted from the results of Anes *et al.* (2006) as indicated in Table 4.5, i.e. three different LAMPs-negative populations [early endosomes (EE), V-ATPase and LYAAT) and two LAMPs-positive populations [lysosome-like (Lyso), late endosomes (LE)].

In this study a potential early endosome (cathepsin H-labelling, ±100 nm organelle) was also detected. The cathepsin H-labelling early endosome (±100 nm) appears to be LAMP-1- and LAMP-2-negative as anticipated (Table 4.5). Thus cathepsin H-labelling populations may include an early endosome population (±100 nm, vesicle 1A) and a secretory population (±20 nm, vesicle 1B) which cannot be distinguished unless labelling with further markers for the early endosome (EEA1) or precursor enzyme is carried out (secretory vesicles also LAMP-1- and -2-negative but contain proenzyme and are negative for EEA1). On the basis of size (±100 nm vs ±20 nm) it would appear that approximately 50% of the vesicles are early endosomes and the remaining 50% secretory vesicles as indicated by blots (Table 4.6).

The late endosome (cathepsin S-labelling ±50 nm organelle), a second population (vesicle 2A) would appear to label with LAMP-1 (LAMP-positive) as anticipated (Tables 4.1 and 4.5). A minor ±20 nm, LAMP-1-positive population labelling for precursor cathepsin S is possibly also present (vesicle 2B). Colocalisation with LAMP-2 still needs to be checked and may identify a subgroup in this population.

The classical lysosome-like or hybrid late endosome-lysosome-like vesicle (±100-200 nm) containing active cathepsin D may consist of at least two different populations, possibly labelling for LAMP-1 or LAMP-2 (vesicles 3A and B) or some vesicles may be both LAMP-1- and -2-positive (vesicle 3C). A minor LAMP-1- or -2-negative, mature cathepsin D-positive population may also exist (vesicle 3D). This would make a total of at least 7 possible vesicle populations or 6 if secretory vesicles not included by Anes *et al*. (2006) are

Table 4.6 **Summary of colocalisation between LAMPs and cathepsins, western blot data and apparent vesicular distribution of these proteins.**

	Colocalisation (%) and potential vesicle type					Precursor form (%)	Mature form (%)	Vesicle type	M (%)	F (%)
	Cat H	**Cat S**	**Cat D**	**Cat B**	**Cat L**					
LAMP-1	<25	45 (LE/Lys)	50 (LE/Lys)	15 (LE/Lys)	30 (LE/Lys)	-	-	LE/Lys/SL	-	-
LAMP-2	<25	ND	50	30	15	-	-	LE/Lys/SL	-	-
Cat H	ND	<20	ND	ND	ND	50	50	LT	50	50
Cat S	<20	ND	25 (Lys)	70 (LE/S)	25 (S)	90	10	>S/LE/Lys	66	34
Cat D	ND	25 (Lys)	ND	30 (LE/Lys/SL)	40 (Lys)	20	80	LE/Lys	34	66
Cat B	ND	70 (LE/S)	30 (LE/Lys/SL)	ND	25 (SL)	50	50	S/>LE/Lys	66	34
Cat L	ND	25 (S)	40 (Lys)	25 (SL)	ND	50	50	>S/LE/Lys	66	34

Abbreviations: Cat, cathepsin, ND, not determined, LT, large (±100 nm) electron-translucent, early endosome-like, S, small (±20 nm) secretory vesicle-like, LE, large (±50 nm), electron-dense, late endosome-like; Lys, lysosome-like, hybrid, digestive organelles (±150-200 nm); SL, "secretory lysosome"; M, membrane-bound, F, free, >, mostly.

not counted. Additional "secretory lysosomes" may also exist containing cathepsins B, D and L.

Anes *et al*. (2006) labelled only with LAMP-1. The opposite distribution of cathepsins B and L in LAMP-1 and -2 labelling populations, and the possibility that both cathepsins show some LAMP-1-negative labelling like the early endosome may indicate that these populations are the three LAMP-1-negative organelles described by Anes *et al.* (2006). The only way to establish whether these populations could be compared with the Anes *et al.* (2006) populations (Table 4.5) was now to check whether or not these are acidic by labelling with LysoTracker.

4.5 Localisation of cathepsins in acidic compartments

Cellular compartments with low internal pH selectively accumulate weakly basic amines. DAMP (3-(2, 4-dinitroanilino)-3'-amino-*N*-methyldipropylamine), one such weak base, has been used in conjunction with anti-DNP antibodies. These cross-react with a component of DAMP and allow the measurement of the acidity of low pH compartments, with labelling increasing in proportion to the accumulating weak base. Fluorescent probes such as neutral red and acridine orange have also been used in the identification of acidic compartments but appear to lack specificity. Fluorescent LysoTracker probes**,** on the other hand, are acidotropic and can be used for labelling and tracing acidic cellular compartments in live cells and have a number of advantages.

These can be used in both short- and long-term tracking experiments. They are highly selective for acidic compartments and can be used effectively at nanomolar concentrations. LysoTracker Red DND-99 consists of a weak base conjugated to a red fluorophore. The weak base is partially protonated at neutral pH and hence freely permeates cell membranes. Upon protonation in acidic compartments LysoTracker is trapped and can be fixed in these organelles using aldehyde fixation (Via et al., 1998). With excitation and emission maxima at 577 and 592 nm, LysoTracker Red DND-99 can also be used in colocalisation studies with FITC, GFP or Oregan Green fluorescent probes. It has previously been used in the identification of trypanosome lysosomal compartments (Magez et al., 1997), the identification of endosomes and lysosomes in neuronal cells (White and Kacsmarek, 1997), in studies of the mobility of MHC class-II carrying vesicles (Wubbolts *et al*., 1996) and in phagosome maturation studies (Via *et al*., 1998; Harrison *et al*., 2003). LysoTracker has also

been shown to classically occupy both Rab7- and LAMP-1-positive compartments (potential late endosomes and lysosomes), in both bone marrow-derived macrophages and J774 mouse macrophages but does not appear to occupy Rab5-positive compartments in bone marrow-derived macrophages (potential early endosomes) (Via *et al*., 1998). These results suggest that LysoTracker may be used as a selective marker for the identification of classical acidic late endosome and lysosomal compartments of macrophages. Thus, LysoTracker was used in the current study to determine whether cathepsins B, D, H, S and L are associated with acidic compartments.

4.5.1 Reagents

Reagents for culture and fluorescent immunolabelling of J774 cells for cathepsins and LysoTracker were prepared according to Sections 2.2.1 and 2.8.1.1 respectively.

LysoTracker Red (Molecular Probes) was diluted in DMEM [1:20 000] with 10% FCS.

The anti-cathepsin and secondary fluorescent antibodies were as previously described (Section 3.7.1 and 4.2.1).

4.5.2 Procedure

J774 cells were cultured according to Section 2.2.2. Prior to fluorescent immunolabelling the cells were incubated in medium containing LysoTracker [1:20 000], (30 min, 37^{o}C). Fluorescent immunolabelling was subsequently performed with chicken anti-cathepsin D [200 µg/ml], chicken anti-cathepsin B [20 µg/ml], rabbit anti-cathepsin H [100 µg/ml], chicken anti-cathepsin S [20 µg/ml], rabbit anti-cathepsin L [20 µg/ml], rabbit anti-chicken IgG FITC [10 µg/ml] and goat anti-rabbit IgG FITC [5 µg/ml] according to Section 2.8.1.2. Only the Olympus epifluorescent microscope was used for viewing as the Zeiss 510 Meta confocal was no longer available and images analysed using ImageJ software. As per Section 4.2.2, the average percentage colocalisation was determined manually and percentage colocalisations below approximately 25% will be considered negative (i.e. no colocalisation) and above approximately 25%, positive.

4.5.3 Results

As anticipated from previous results (Section 4.4.3), cathepsin H showed no significant colocalisation with LysoTracker (Figure 4.24, A1, A2 and A3), i.e. labelling occurs in a non-acidic organelle. Labelling also seems to concur with early endosomal (±100 nm) association i.e. a lack ofcolocalisation with LAMP-1 and -2 and (Figure 4.19 and Figure 4.24). If cathepsin H-labelling vesicles contain approximately 50% precursor enzymes and 50% mature enzyme (as suggested by blots, Figure 3.11, A, lane 3), three populations i.e. LAMP-1 and/or -2-negative and LysoTracker-negative (i.e. non-acidic/low acidity) vesicles resembling the classical description of the early endosome (±100 nm, containing active enzymes) or secretory vesicles (±20 nm, containing precursor enzymes) (vesicles 1A and B, similar to Summary of results Section 4.4.3) may exist. Approximately 50% of cathepsin H may, therefore, be contained in LAMPs-negative, non-acidic organelles previously described by Anes *et al*. (2006). [LAMPs-negative, non-acidic, possibly two of the three non-acidic LAMPs-negative organelles described by Anes *et al*. (2006) (one containing precursor and one containing mature enzyme) (Table 4.7).

The relatively high colocalisation between cathepsin S and LysoTracker (approximately 40%) in ±50 nm vesicles and the presence of approximately 90% immature cathepsin S (Figure 4.25, A1, A2 and A3), suggests that more cathepsin S should be in the mature form than is indicated by blots (Figure 3.13, A, lane 3). The partial colocalisation between cathepsin S and LAMP-1 (approximately 45%) also suggests that some of the vesicles (approximately 40%) are likely to represent late endosomes (LAMPs-positive, acidic and containing mature cathepsin S, if cathepsin S is a marker for the late endosome) (vesicle 2A). The rest of the cathepsin S-labelling vesicles would represent LAMP-1-positive, non-acidic vesicles possibly containing precursor enzyme (vesicle 2B) (Table 4.7) or LAMP-1-negative, non-acidic vesicles possibly contribute a third LAMPs-negative, non-acidic population (vesicle 3C) described by Anes *et al*. (2006). The size of the cathepsin S-labelled organelles, however, seem to indicate that most are late endosomes (±50 nm), which may not yet be acidic (Figure 4.25) as previously speculated (Section 4.4.3).

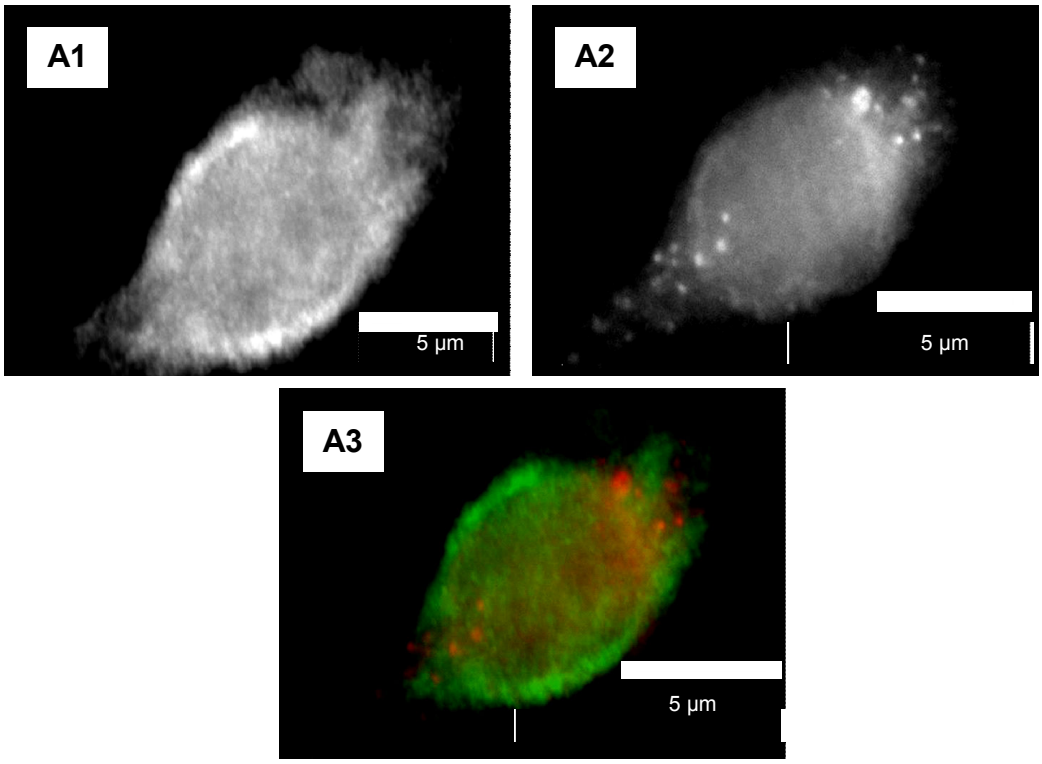

Figure 4.24 **LysoTracker and labelling of cathepsin H in J774 macrophages.**
Cells grown on coverslips were incubated in DMEM with LysoTracker [1:20 000 (A1)] (30 min, 37°C), initially fixed with 3.7% PFA, permeabilised with saponin and probed with rabbit anti-cathepsin H [100 µg/ml (A2)], goat anti-rabbit FITC [5 µg/ml (A2)]. Coverslips viewed using an Olympus epifluorescent microscope. FITC filter (A1), LysoTracker filter (A2), composite image (A3). Bars = 5 µm.

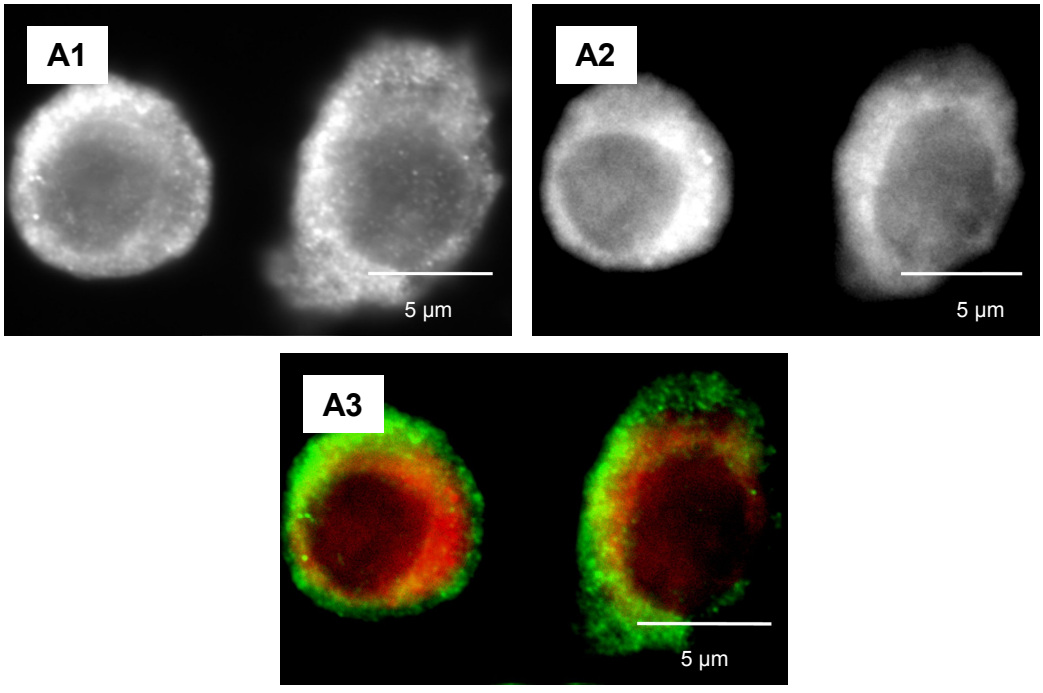

Figure 4.25 **LysoTracker and labelling of cathepsin S in J774 macrophages.**
Cells grown on coverslips were incubated in DMEM with LysoTracker [1:20 000 (A1)] (30 min, 37°C), initially fixed with 3.7% PFA, permeabilised with saponin and probed with chicken anti-cathepsin S [20 µg/ml (A2)], rabbit anti-chicken FITC [10 µg/ml (A2)]. Coverslips viewed using an Olympus epifluorescent microscope. FITC filter (A1), LysoTracker filter (A2), composite image (A3). Bars = 5 µm.

Of all the cathepsins, cathepsin D showed the most extensive colocalisation with LysoTracker (Figure 4.26, A1, A2 and A3, 90%). The vesicular compartments showing colocalisation were generally large (±100 nm or greater), like classical hybrid late endosomes-lysosomes, or ±30-50 nm or ±50 nm, secretory lysosomes or late endosomes, respectively, located towards the centre of the cell, generally to one side. Cathepsin D-containing vesicles, therefore, appear to be LAMP-1 and/or -2-positive and LysoTracker-positive (i.e. acidic) (Tables 4.5 and 4.7) and most like the classical secretory lysosome or late endosome-lysosome hybrid organelle. These vesicle populations, LAMP-1 and/or -2-positive, acidic, are possibly 3 vesicle populations i.e. vesicles 3A, B and C, and the LAMPs-negative secretory vesicles (vesicle 3D) give rise to the 4th population as indicated in the Summary of results in Section 4.4.3 and Table 4.6.

Partial colocalisation was observed also between cathepsin B and LysoTracker in (approximately 40% of all cathepsin B-labelled ±50 nm possibly late endosomes and ±150-200 nm "hybrid organelles"). Within the remaining 60% of cathepsin B-labelled vesicles, approximately 10% active enzyme is found in ±100-150 nm vesicles and 50% inactive enzyme in ±20 nm vesicles in which cathepsin B was not associated with acidic compartments (Figure 4.27, A1, A2 and A3). The 50% inactive enzyme, as suggested by blots (Figure 3.9, A, lane 4), is most likely to be in secretory vesicles (±20 nm) containing newly synthesised proforms. If mature cathepsin B is only contained in acidic vesicles, these vesicles may, therefore, be partially LAMP-2-positive (Table 4.6) and partially LAMP-1-positive (15% of total cathepsin B) and LysoTracker-positive (i.e. 40% of cathepsin B-labelled organelles acidic). As such these may be the first of the LAMP-positive acidic vesicles labelling more for LAMP-2 than LAMP-1, with some non-acidic vesicles (5-10%) appearing LAMP-positive (Table 4.7). These vesicle populations may, therefore, represent 3 possible subgroups (late endosomes, hybrid organelles and LAMP-positive, non-acidic vesicles).

Cathepsin L also colocalises (approximately 40%) with LysoTracker in relatively large vesicles (±50 nm, possibly late endosomes) and larger vesicles (±100 nm or greater, possibly hybrid organelles) (Figure 4.28 and Table 4.6). This cathepsin L is most likely to be associated with acidic hybrid organelles (one type of vesicle). From blot data and Table 4.6, cathepsin L populations consist of approximately 50% mature and 50% immature enzyme [it is most likely that most of the 50% mature enzyme is associated with LysoTracker within the

40% colocalised with LysoTracker and 5-10% of the vesicles may contain mature enzyme and be LysoTracker-negative (a second type of vesicle). Cathepsin L-labelled vesicles may, therefore, be LAMP-1 and/or -2-positive (30 and 15%, respectively), suggesting that some of the first vesicle group (LAMP-1- and -2-positive) are likely to represent hybrid organelles or digestive bodies (±150-200 nm) (Table 4.7) but 2 other subgroups (i.e. either LAMP-1-positive or LAMP-2-positive) and a secretory vesicle (±20 nm) population containing immature enzyme and may give rise to total of 6 cathepsin L-labelling vesicle populations including the secretory vesicles.

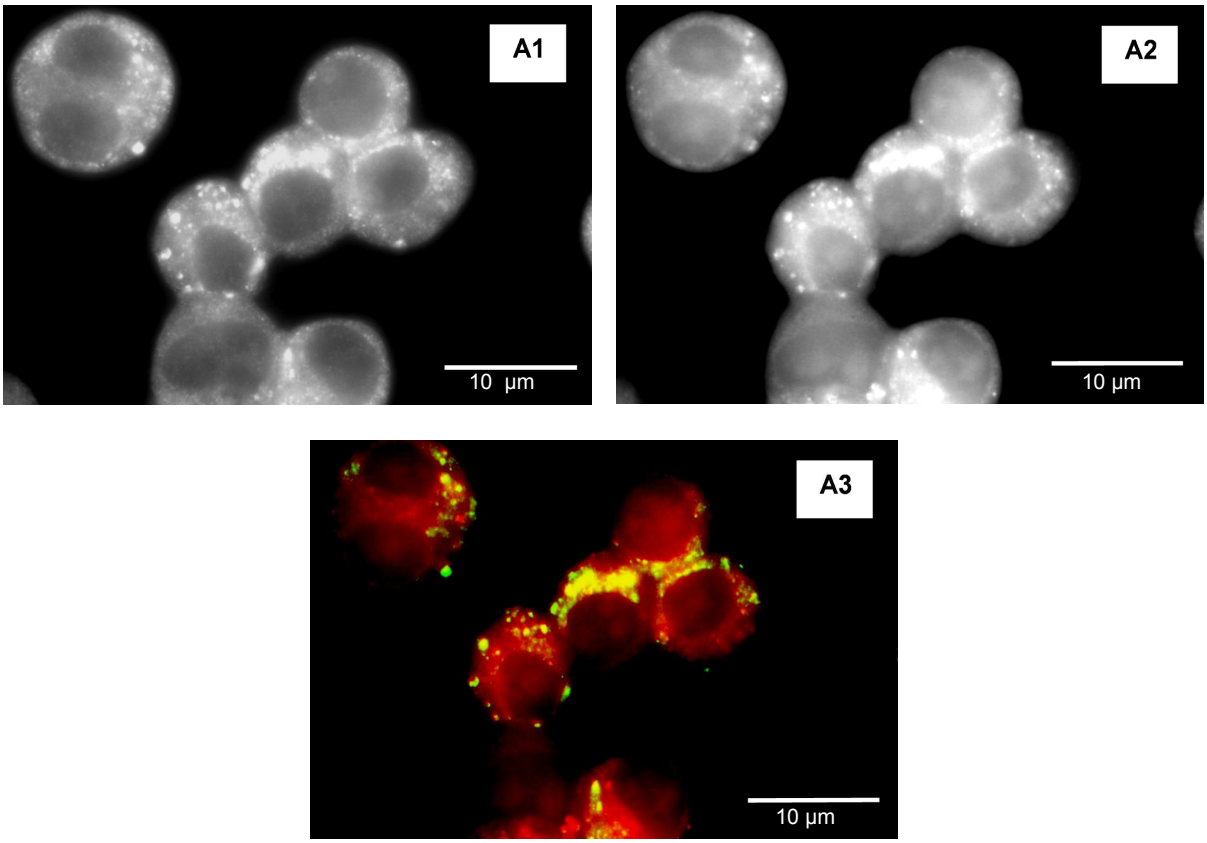

Figure 4.26 **LysoTracker and labelling of cathepsin D in J774 macrophages.**
Cells grown on coverslips were incubated in DMEM with LysoTracker [1:20 000 (A2)] (30 min, 37°C), initially fixed with 3.7% PFA, permeabilised with saponin and probed with chicken anti-cathepsin D [200 µg/ml (A1)], rabbit anti-chicken FITC [10 µg/ml (A1)]. Coverslips viewed using an Olympus epifluorescent microscope. FITC filter (A1), LysoTracker filter (A2), composite image (A3). Bars = 10 µm.

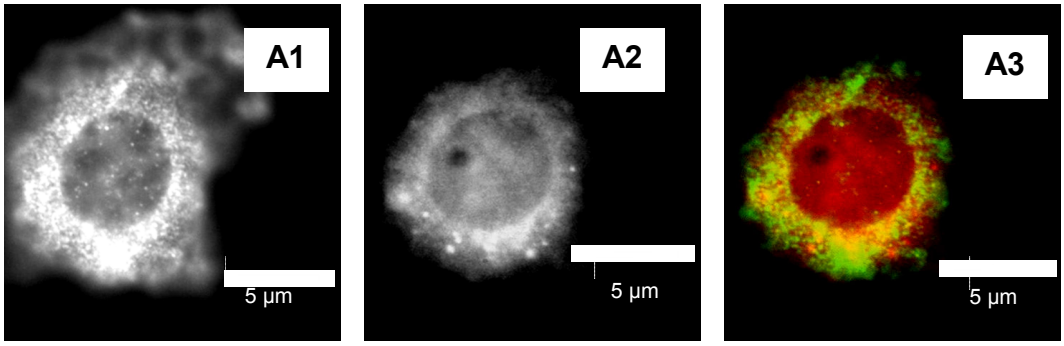

Figure 4.27 **LysoTracker and labelling of cathepsin B in J774 macrophages.**
Cells grown on coverslips were incubated in DMEM with LysoTracker [1:20 000 (A2)] (30 min, 37°C), initially fixed with 3.7% PFA, permeabilised with saponin and probed with chicken anti-cathepsin B [20 µg/ml (A1)], rabbit anti-chicken FITC [10 µg/ml (A1)]. Coverslips viewed using an Olympus epifluorescent microscope. FITC filter (A1), LysoTracker filter (A2), composite image (A3). Bars = 5 µm.

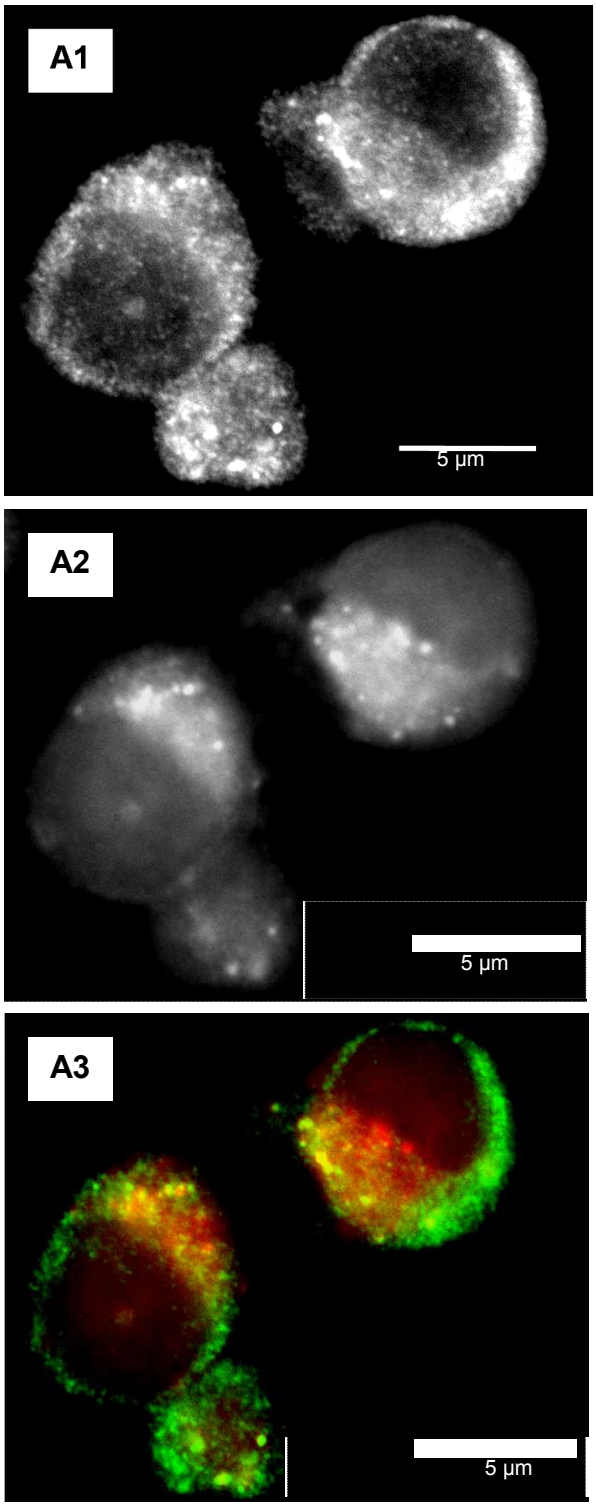

Figure 4.28 **LysoTracker and labelling of cathepsin L in J774 macrophages.**
Cells grown on coverslips were incubated in DMEM with LysoTracker [1:20 000 (A2)] (30 min, 37°C), initially fixed with 3.7% PFA, permeabilised with saponin and probed with rabbit anti-cathepsin L [20 µg/ml (A1)], goat anti-rabbit FITC [5 µg/ml (A1)]. Coverslips viewed using an Olympus epifluorescent microscope. FITC filter (A1), LysoTracker filter (A2), composite image (A3). Bars = 5 µm.

Summary

The cathepsin H-labelling organelles (±100 nm), seem to be LAMPs-negative and non-acidic in accordance with the classical definition of an early endosome, and largely cathepsin S-negative, assuming mature cathepsin S is taken as a marker for the late endosome (±50 nm), non-colocalisation with this marker would be in accordance with an early endosome marker. Some membrane-association of the precursor in small (±20 nm), non-acidic, LAMPs-negative secretory vesicles is also present. [Vesicle 1A, ±100 nm, non-acidic, LAMPs-negative, cathepsin H (mature) and vesicle 1B, ±20 nm, non-acidic, LAMPs-negative, cathepsin H (immature)]. Thus cathepsin H may possibly be a good marker for early endosomes if the currently indistinguishable population containing immature enzyme could be distinguished (vesicle 1B). Colocalisation labellings for other cathepsins, however, need to be performed to futher verify cathepsin H as a suitable marker for the early endosome. From sizes of organelles, however, cathepsin H seems to be contained in either early endosomes or secretory type vesicles.

Cathepsin S seems mainly contained in late endosomes or hybrid bodies and secretory vesicles (in some secretory vesicles it seems to colocalise with cathepsins B and L. Blots seem to give an underestimate of the mature cathepsin S content of macrophages (approximately 10%). The approximate 40% colocalisation of LAMP-1 and LysoTracker with cathepsin S indicates that approximately 40% of cathepsin S should be mature enzyme (Table 4.7). This may, however, be due to slow conversion of the enzyme to its mature form in the late endosome. A lack of colocalisation with cathepsin H and colocalisation with LAMP-1 and LysoTracker (acidic nature) suggests that cathepsin S may be a good marker for the late endosome (vesicle 2A, ±50 nm) only if verified with a second marker for the late endosome e.g. MPR. As a LAMP-1-positive, non-acidic, precursor enzyme-containing vesicle also seems to exist (vesicle 2B) (Table 4.6 and end of Section 4.3.3).

The organelles labelling for mature cathepsin D are most like the classical hybrid organelle or a separate, distinct digestive body (±150-200 nm), secretory lysosome (±30-50 nm) and the late endosome (±50 nm) i.e. LAMPs-positive, acidic and usually containing active cathepsin D. The hybrid organelle, however, may consist either of two acidic populations labelling for different LAMPs, the LAMP-2 organelle possibly being the most uniformly acidic and colocalising largely with cathepsin B (vesicle 3A), while the LAMP-1 labelling organelle may be less uniformly acidic and may contain some cathepsin L (vesicle 3B).

Further organelles such as secretory lysosomes (±30-50 nm) may show labelling for both LAMP-1 and -2 and either or both cathepsin B and cathepsin L (3 additional populations could, therefore, exist) (Table 4.6).

At least 6 vesicle populations or "endosome-lysosome-like" organelles have been shown (much as predicted in Table 4.5 and as seen by Anes *et al*. (2006). However, subpopulations of vesicles 2 and 3 may exist, thereby defining 7 or more vesicle populations. This is assuming such populations do not overlap (i.e. possibly consisting of early endosomes, late endosomes, hybrid or digestive organelles, secretory lysosomes, secretory vesicles, LAMP-negative, acidic and mature enzyme-containing vesicles and LAMP-positive, non-acidic and immature enzyme-containing vesicle).

Table 4.7 **Summary of colocalisation between LysoTracker, LAMPs and cathepsins, western blot data and apparent vesicular distribution of these proteins.**

	Colocalisation (%) and potential vesicle type					Precursor form (%)	Mature form (%)	Vesicle type	M (%)	F (%)
	Cat H	**Cat S**	**Cat D**	**Cat B**	**Cat L**					
Lyso-Tracker	0	40 (LE/Lys)	90 (LE/Lys/SL)	40 (LE/Lys/SL)	40 (LE/Lys/SL)	-	-	LE/Lys/SL	-	-
LAMP-1	<25	45 (LE/Lys)	50 (LE/Lys)	15 (LE/Lys)	30 (LE/Lys)	-	-	LE/Lys/SL	-	-
LAMP-2	<25	ND	50	30	15	-	-	LE/Lys/SL	-	-
Cat H	ND	<20	ND	ND	ND	50	50	LT	50	50
Cat S	<20	ND	25 (Lys)	70 (LE/S)	25 (S)	90*	10*	>S/LE/Lys	66	34
Cat D	ND	25 (Lys)	ND	30 (LE/Lys/SL)	40 (Lys)	20	80	LE/Lys	34	66
Cat B	ND	70 (LE/S)	30 (LE/Lys/SL)	ND	25 (SL)	50	50	S/>LE/Lys	66	34
Cat L	ND	25 (S)	40 (Lys)	25 (SL)	ND	50	50	>S/LE/Lys	66	34

* Possibly incorrect. Abbreviations: Cat, cathepsin, ND, not determined, LT, large (±100 nm) electron-translucent, early endosome-like, S, small (±20 nm) secretory vesicle-like, LE, large (±50 nm), electron-dense, late endosome-like; Lys, lysosome-like, hybrid, digestive organelles (±150-200 nm); SL, "secretory lysosome"; M, membrane-bound, F, free, >, mostly.

4.6 Discussion

Active enzymes are the most relevant in many processes such as invasion, microbial killing and inflammation. Even immature enzymes may be secreted and become important after extracellular activation, however (Reddy *et al*., 1995; Punturieri *et al*., 2000). This study seems to indicate that this may be the case especially with cathepsin S. Inability to confirm and distinguish precursor from mature enzyme was, however, a drawback in this study. The necessity to perform double labelling to distinguish the mature from the immature form of enzyme and hence indirectly confirm organelle identity and establish markers for vesicle populations requires the purchase of additional anti-precursor sequence peptide antibodies or monoclonal antibodies recognising only the precursor sequence. The possible alternate use of ultrastructural detail of containing organelles to judge the form of the enzyme (Kirschke *et al*., 1998; Wolters and Chapman, 2000; Pillay *et al*., 2002) was, however, chosen in this study and proved marginally useful for this purpose except for giving some indication of membrane-bound-, possibly precursor form, or unbound ("free"), possibly mature forms, relative size of organelle and electron-density. Size of organelles, it was realised, could also be roughly estimated from fluorescent micrographs, and this proved useful.

Organelle ultrastructure was, unfortunately, variable and none of the finer structural details were obvious. Tubular structures resembling ER, usually containing immature enzyme, were preserved to some degree. Lysosomes may also be tubular, however, and have been identified in human monocytes, bone marrow-derived macrophages, J774 and phorbol ester-treated peritoneal macrophages (Swanson *et al*., 1987; Knapp and Swanson, 1990). These were not obvious in the current study. Though fixation did not preserve optimal ultrastructural detail, all antigens seemed to survive fixation almost equally, an important primary consideration when labelling multiple antigens. Western blot data also proved a limited guide for the assignment of the proportion of the various protease forms present in both J774 macrophages and human monocytes.

During this study certain assumptions and deductions were made. These include the following assumptions:

I. that the percentage of precursor and mature enzyme was as indicated in blots.

II. A. if the vesicle is acidic, cathepsins present may not be in the precursor form.

B. if the vesicle is acidic, cathepsins present are mature and active.

C. if the vesicle is non-acidic, cathepsins may be mature but not active.

III. LAMP-1 and -2 classically occur in late endosomes (±50 nm) and "lysosomes", including hybrid organelles, and should, therefore, be associated with mature cathepsins.

IV. that vesicle size was an indication of possible organelle identity.

Deductions:

IV. since cathepsin D was mainly found to be active (approximately 80%) and localised in acidic vesicles, all vesicles where cathepsins H, S, B and L colocalise with cathepsin D are acidic organelles and will be active, except in the case of approximately 20% of cathepsin D-labelled vesicles which contain precursor enzyme.

V. cathepsin S seems to occur mostly in the precursor form (in possibly small secretory vesicles), with only approximately 10-20% being active and mature, i.e. present in an acidic, late endosome (±50 nm). At least 10% will, therefore, occur in cathepsin D-positive, hybrid or digestive type vesicles.

VI. approximately 50% of cathepsin H occurs in early endosomes (±100 nm) and the remaining 50% in precursor form in secretory vesicles (±20 nm).

VII (from III) cathepsin S has approximately 45% association with LAMP-1, suggesting either that the amount of mature cathepsin S originally indicated by blots (approximately 10%) is likely to be inaccurate (24 kDa mature cathepsin S band may have run off gel) or LAMP markers may label organelles containing precursor enzymes. Cathepsin S colocalises (approximately 25%) with cathepsin D and could occur in a few late endosomal (±50 nm) compartments and hybrid organelles (±150-200 nm).

VIII. if LAMP-1 (approximately 50%) and -2 (approximately 50%) are predominantly associated with the mature cathepsin D-containing compartments (approximately 80%).

A. up to 50% of the mature cathepsin D-containing vesicles may be LAMP-1 and -2-positive and the remaining mature vesicles (up to 80%) may be LAMP-2-positive, with 30% non-colocalised and 20% non-colocalised with either LAMPs.
B. approximately 50% of the vesicles may contain active cathepsins and be both LAMP-1- and -2-positive and the remaining mature cathepsin D-labelled vesicles LAMPs-negative.

IX. approximately 2/3 of cathepsins S, B and L appear to be membrane-bound and 1/3 "free", whereas, cathepsin D seems to be the opposite. Therefore, it would seem precursor cathepsins appear to be membrane-bound.

Just about all of these assumptions and deductions could be validated by performing labelling for the precursor and mature enzymes or a third relevant marker i.e. triple labelling. Though the lack of triple labelling and labelling for precursor requires some speculation to predict numbers of vesicle populations, as overlapping populations cannot be distinguished, at least 7 different vesicle types including the classical early endosomes (cathepsin H), classical late endosomes (cathepsin S), at least 3 lysosome-like populations (possibly late endosome-lysosome hybrid organelles or digestive bodies), "secretory lysosomes" and secretory populations may be inferred from the above assumptions and deductions.

Cathepsin B shows a significant association with cathepsin S (approximately 70%), of the colocalising cathepsins some proportion may be precursor but some mature and active, and possibly present in late endosomes. At least 10% of cathepsin S appears to colocalise with cathepsin D (acidic vesicle). It is, therefore, possible that cathepsins B, S and D may occur in the same LAMP-1-positive vesicle population, which could be late endosomal or possibly a late endosome-lysosome hybrid organelle. The percentages of precursor and mature cathepsins needs to be verified with time course studies over a few days. It appears that cathepsin L may also be located in a secretory population, together with cathepsins S, B and possibly D and a secretory lysosome with cathepsin B. Such colocalisations should be checked by triple labelling and labelling for precursor enzyme, particularly for cathepsin S where labelling for both forms is required to validate this cathepsin as a late endosomal

marker and also to establish whether LAMPs labels ever associate with non-acidic, cathepsin-containing organelles.

Double labelling for the precursor and current antisera (detecting all forms), and either the use of endocytic tracer molecules, or double labelling with an early endosomal marker such as EEA1 (Ghosh *et al*., 1994) is also required, to verify cathepsin H as an early endosome marker.

As indicated in Table 4.6, at least 3 subpopulations of cathepsin D-positive vesicles may exist (late endosomes, hybrid organelles and secretory lysosomes). Triple labelling studies using localising cathepsin D, LAMP-1 and -2 simultaneously are needed to confirm this. However, cathepsin D labelling seems to be a fairly reliable marker for acidic vesicles containing active enzymes.

If cathepsin S is predominantly associated with late endosomes in J774 macrophages as previously reported (Claus *et al*., 1998; Jahraus *et al*., 1998) and cathepsin D is predominantly lysosomal, vesicles showing colocalisation may represent hybrid organelles (Bright *et al*., 1997; Luzio *et al*., 2000). Supporting cathepsin S as a marker for the late endosomal compartment and cathepsin H for the early endosome (Claus *et al*., 1998; Jahraus *et al*., 1998), double labelling showed an apparent lack of colocalisation between these two cathepsins. Unfortunately, LAMP-2 labelling was not performed as an interesting relationship between cathespin B and S seems to be revealed by the colocalisations seen in Tables 4.5 and 4.6. The two cathepsins colocalise to the largest extent of all the cathepsins (approximately 70%, Table 4.4). This apparent colocalisation should be checked at the EM level, to check membrane- and vesicle association and organelle ultrastructure, if ultrastructural definition is improved.

We have shown some cathepsin D and B colocalisation in late endosomes and secretory lysosomes, in agreement with Jahraus *et al*. (1998), who reported cathepsins D and B in fairly high concentration in both the late endosomes and "lysosomes" of J774 macrophages. Due to the lack of vesicle markers, however, Jahraus *et al*. (1998) could not distinguish the various "lysosomal" populations as indicated in this study. Colocalisation may also occur in hybrid organelles, formed by fusion between late endosomes and lysosomes (Bright *et al*., 1997; Luzio *et al*., 2000). On the other hand, the colocalisation in organelles near to the cell

periphery suggests some organelles may be "secretory lysosomes" as indicated in this study. MDMs are known to secrete both the pro- and mature forms of both cathepsin B (Reddy *et al*., 1995) and cathepsin D (Punturieri *et al*., 2000). As the antibodies used in this study detect both forms of these enzymes, however, it is not possible to distinguish which forms are localised in these organelles. "Secretory lysosomes" are thought to be acidic and hence should contain mature cathepsin. In the current study, western blot data suggested the presence of mainly mature cathepsin D and significant amounts of mature cathepsin B. Colocalising cathepsins are, therefore, highly likely to be in the mature form. But should be checked using antibodies recognising precursor and mature enzyme at the EM level.

Both EM and fluorescent microscopy showed that cathepsins B and L colocalise in compartments which at times were situated close to the periphery of the cells, suggesting possible secretion of these enzymes. Claus *et al*. (1998) also suggest that cathepsins B and L may be localised to a special type of "secretory lysosome", as in the presence of bafilomycin and chloroquine the bulk of cathepsins B and L appeared to be secreted as opposed to being delivered to newly synthesised phagosomes. The form of enzyme present (i.e. mature or immature) would assist in making such an identification but this study seems to confirm the colocalisation of these enzymes in secretory lysosome-like organelles.

Partial colocalisation was observed between cathepsin L and cathepsin S (possibly in secretory vesicles) as well as with cathepsin D in some late endosome-lysosome hybrid organelles as previously suggested in the literature (Lennon-Duménil *et al*., 2002a). Cathepsin L, like cathepsin D, has generally been considered to be a lysosomal enzyme (Kirschke, 2004). Processing usually results in a single chain form found in both classical late endosomes, and a two-chain form found predominantly in lysosomes (Ishidoh *et al*., 1998). In the current study, however, cathepsins S (a proposed late endosome marker) and cathepsin L share limited colocalisation (approximately 25%), possibly in secretory vesicles.

Cathepsins S and L appear to play important roles in the degradation of the MHC class II invariant chain and thus in peptide loading by macrophages (Shi *et al*., 1999; Wolters and Chapman, 2000). The p41 isomer of the invariant chain is known to act as both an inhibitor of cathepsin L, by binding to the active site, and a chaperone for cathepsin L, helping to preserve a pool of undegraded mature enzyme in late endosomes (Lennon-Duménil *et al*. 2001; Lennon-Duménil *et al*., 2002b). It has also been suggested that the cathepsin L/p41

complex may be packaged in "secretory lysosomes" and discharged extracellularly (Lennon-Duménil *et al*. 2001), as, unlike mature cathepsin L, this complex is stable at neutral pH (Ogrinc *et al*., 1993). This may also preserve a similar pool of undegraded cathespin L extracellularly. As the major portion of cathepsin S described here and by Punturieri *et al*. (2000) may only "mature" over time when required it is possible that all data may only reflect a highly dynamic situation and may be unreliable due to variability over time and that the cathepsin S and L colocalised enzymes judged to be in secretory vesicles may be or become secretory lysosomes or late endosomes upon acidification.

It has been suggested that macrophages possess a number of lysosomal subpopulations that differ in pH and the presence of LAMPs (Astarie-Dequeker *et al*. 1999; Astarie-Dequeker *et al*., 2002; Anes *et al*., 2006). The current localisation studies between cathepsins and LAMPs/LysoTracker seem to support this hypothesis (summary of results in Table 4.6).

Based on the findings of this study it would seem it can provisionally be stated that only cathepsin D seems a reliable marker for acidic, lysosome-like subpopulations. (The identity of this population should now be established with an independent marker such as gold uptake). Cathepsin H may also possibly be considered as a marker for the early endosome in J774 mouse macrophages only if confirmed reliable by another early endosomal marker and only if mature or immature cathepsin H dominates in this vesicle. The activation state of the macrophage (cathepsin D has a seemingly increased association with LAMP-positive vesicles in activated cells). The evidence, however, suggests that in J774 macrophages cathepsin S is primarily associated with the late endosome and cathepsin D with a "digestive lysosome", secretory lysosome and late endosome. The study also suggests that some cathepsin B and L and possibly cathepsins B and D may be located in "secretory lysosomes". As cathepsins are processed from inactive proforms to mature, active forms along the endocytic pathway, it will be necessary in future studies to use antibodies that specifically target either the pro- or mature form of the proteases, making it possible to differentiate between the precursor and mature, active enzymes, thus making colocalisation studies more definitive as one can differentiate between sites of synthesis and storage. Other EM fixation methods should also be investigated as improved ultrastructural preservation would aid in the identification of organelles thus verifying the probable forms of the cathepsins.

There also seems possible merit in performing LAMP-1 and LAMP-2 colocalisations, as well cathepsin labelling (i.e. triple labelling) as to there may be some difference in cathepsin distribution in such cells. This almost seems evident from the fluorescent micrographs, where cathepsins D and B, and a lesser extent, S, seemed more associated with LAMP-2, and slightly larger vesicles, and cathepsin L, and a minor extent cathepsin H, with LAMP-1. These studies would also best be confirmed using EM and improved ultrastructural preservation. This may lead to the identification of compartments that label for one or other specific LAMP and give an indication of why LAMP-2 is more important than LAMP-1 for survival of knockouts. This was omitted in the current study as only one anti-rat fluorescent conjugate was available. An additional secondary antibody should now be purchased.

Astarie-Dequeker *et al*. (2002) and more recently Anes *et al*. (2006) suggest that both Hck and LYAAT may be used as markers for LAMP-negative, "secretory lysosomes" in both human macrophages and J774 cells. Such studies now need to be performed.

The focus on the lysosome-endosome pathway and cathepsin distribution was due to an interest in the microbicidal killing mechanisms involving the cathepsins, their separate regulation and ultimate fusion with the phagosome. Although not directly involved in killing, but more involved in reaching the site of infection and in signalling, the MMPs and their inhibitors are also extremely important. The ratio of inhibitor to active enzyme released may result in the activation or inhibition of cytokines, and hence regulate the entire innate and adaptive immune system. The regulation of release and localisation of MMP to inhibitor is, therefore, extremely important and forms part of the current study. Though the primary question being asked is the distribution of MMP-9 in relation to TIMP-1 and TIMP-2, inhibitors, apparently in themselves involved in signalling (Somerville *et al*., 2003; Folgueras *et al*., 2004), the pattern of distribution in relation to the LAMP-positive and acidic compartments is also important as these antigens could potentially also be used to distinguish subpopulations of lysosomes in the J774. One of the problems seen during the study of the cathepsins, however, has been the fact that the activation state seems to influence protease distribution. Macrophage activation is also known to up-regulate certain MMPs. For this reason, it was decided in the next study, to perform localisations on both activated and non-activated J774 macrophages.

CHAPTER 5

DISTRIBUTION OF MMP-9, TIMP-1 AND -2 IN UNSTIMULATED AND LPS-STIMULATED J774 MOUSE MACROPHAGES

5.1 Introduction

The effect of macrophage activation became evident during initial macrophage vesicle characterisation studies. In the past, "activated" macrophages were defined as cells capable of secreting various inflammatory mediators and destroying intracellular pathogens. However, it is now becoming increasingly apparent that activated macrophages consist of subpopulations which have different physiologies and distinct functions (Gordon, 2003; Mosser, 2003). Initially, it appeared that macrophages had two different activation states, and the cells were known as either "classically" or "alternatively" activated macrophages (Stein *et al*., 1992). "Classically" activated macrophages are effector cells in T-helper cell type 1 (Th1) cellular immune responses and are involved in ECM degradation, the promotion of inflammation and apoptosis (Duffield, 2003; Mosser, 2003) (Table 5.1). In comparison "alternatively" activated macrophages are involved in immunosuppression, ECM construction, cell proliferation and angiogenesis (Gordon, 2003) (Table 5.1).

Table 5.1 Comparison of activated murine macrophage subpopulations.

	Classical	**Alternative**	**Type II**
Activating signals	IFN-γ, TNF	IL-4, glucocorticoids	IgG complexes, TLR ligation
Secretory products	↑ TNF, ↑ IL-12, IL-1, IL-6	↑ IL-1RA, IL-10	↑ IL-10, TNF, IL-6
Biological markers	↑ MHC class II, ↑ CD86, ↓ MR	↑ MR, ↑ Scavenger receptor, ↑ CD23, ↓ CD14, CD163, MS-1	↑ MHC class II, ↑ CD86 (unique markers not yet available)
Killer molecules	NO, O_2^-	None	*NO, O_2^-
Chemokine production	IP-10, MIP-1α, MCP-1	AMAC-1	Unknown

* Although these cells are able to make NO and O_2^- in direct response to activating stimuli, the production of high levels of IL-10 by type II activated macrophages inhibits neighbouring cells from responding to IFN-γ activation and the production of reactive nitrogen intermediates. Abbreviations: IFN-γ, interferon-γ; TNF, tumour necrosis factor; MHC, Major histocompatibilty complex; MR, mannose receptor; MS-1, MS-1-high molecular-weight protein; IP-10, IFN-inducible protein 10; AMAC-1, alternative macrophage activation-associated CC-chemokine-1; MIP-1α, macrophage inflammatory protein-1α; MCP-1, monocyte chemoattractant protein-1; IL-1RA, interleukin-1 receptor antagonist; TLR, toll-like receptor (modified from Mosser, 2003).

Recently, it has been established that macrophages exposed to classical activating signals and IgG immune complexes have an activation state that varies from the "classical" activation state (Mosser, 2003). These macrophages produce large quantities of IL-10 and, therefore, inhibit the acute inflammatory response to bacterial endotoxin and have been named type II-activated macrophages as they are able to induce T helper cell type 2 (Th2) responses (Mosser, 2003) (Table 5.1).

Activated macrophages have a wide range of functions (Figure 5.1). Essentially, macrophages migrate to areas of inflammation where they are responsible for the destruction of pathogens. Interestingly, activated macrophages do not have enhanced phagocytic capabilities and they express reduced levels of MR and Fc receptors in comparison to resting macrophages (Mosser, 2003). Activated macrophages do, however, have improved microbial killing and degradative properties. The improved killing results from an increased production of both reactive oxygen species and induction of iNOS (Mosser, 2003). Although all activated macrophages function in innate and adaptive immunity, the "classically" activated cells can cause tissue injury and chronic inflammation, whereas, "alternatively" activated cells appear to regulate inflammation and allow for wound healing (Duffield, 2003; Mosser, 2003).

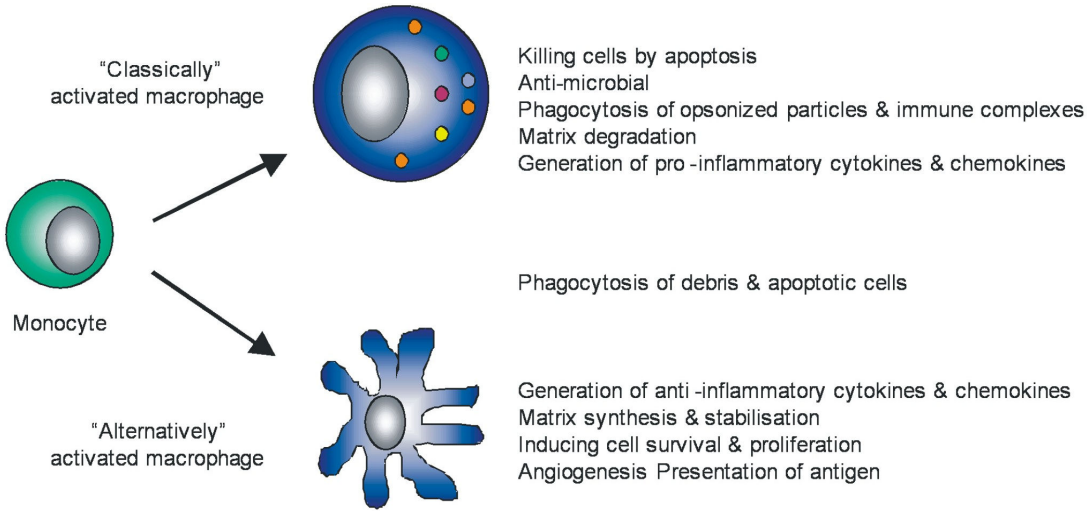

Figure 5.1 **Functions of activated macrophages.**
Monocytes can differentiate into either "classically" or "alternatively" activated macrophages. The respective functions of each of these distinct phenotypes are shown. Overlapping functions are also included (modified from Duffield, 2003).

To become "classically" activated, macrophages require two activation signals. The first requisite signal is IFN-γ, which is responsible only for the priming of the macrophage and does not activate the cell. The second signal is either TNF itself or an inducer of TNF. The second signal may result from TLR ligation, which induces the macrophage to produce TNF. Thus macrophages exposed to IFN-γ and either microbes or microbial products such as LPS become "classically" activated (Mosser, 2003). The biological activities of LPS are due to the lipid A moiety and LPS activation in monocytes requires both the LPS-binding protein and CD14. The LPS-binding protein binds to the lipid A region which subsequently delivers the LPS to CD14. It has been proposed that unidentified transducer proteins probably initiate the intracellular signals (Sweet and Hume, 1996; Amura *et al*., 1998). LPS activates several signal transduction pathways in macrophages. The mechanisms of these pathways, however, remain largely unknown (Amura *et al*., 1998). Classical activation results in the secretion of a variety of chemokines including IL-8, IFN-inducible protein-20 (IP-20), macrophage-inflammatory protein (MIP)-1α and -1β and RANTES (regulated upon activation, normal T-cells, expressed and secreted) which act as chemoattractants for neutrophils, immature dendritic cells, natural killer cells and T-lymphocytes (Luster *et al*., 2002). Pro-inflammatory cytokines are released including, IL-1β, IL-6 and TNF-α and the subsequent secretion of Fas ligand and the production of NO. Additionally, "classically" activated macrophages secrete several proteases including MMP-1, -2, -7, -9 and -12 (Gibbs *et al*., 1999a; Gibbs *et al*., 1999b; Chizzolini *et al*., 2000).

Human monocytes usually appear to have a limited capacity for the synthesis and secretion of MMPs and TIMPs. As they mature into macrophages, however, their capacity for MMP secretion increases and continues to increase upon activation (Owen and Campbell, 1999; Owen, 2005). Several substances are known to increase MMP biosynthesis, these include LPS (Shapiro *et al*., 1991), zymosan, (Pierce *et al*., 1996), diethyl maleate, GM-CSF and, more recently, surfactant protein D appears capable of selectively increasing the production of certain MMPs *in vitro* (Trask *et al*., 2001).

Stimulants or activating molecules seem to have different effects on different macrophage subtypes. Human macrophages are known to express a variety of MMPs (Chapter 1, Section 1.4.2.1), whereas, mouse macrophages predominantly produce MMP-12 and lower levels of MMP-9 (Filippov *et al*., 2003). Human alveolar macrophages, but not peripheral blood monocytes, show an increased production of MMP-9 after exposure to LPS (Welgus *et al*.,

1990; Campbell *et al*., 1991) with some stimulation of secretion of TIMP-1 but to a lesser extent than MMP-9 (Welgus *et al*., 1985). In contrast to TIMP-1, TIMP-2 secretion may be decreased by stimulants such as LPS (Shapiro *et al*., 1992; Lacraz *et al*., 1995). The effects of only LPS on J774 macrophages do not seem to have been described, though it is known that mRNA specific for TNF-α, IFN-β and iNOS is increased and biologically active TNF-α, IFN-β and NO also become secreted (Fujihara *et al*., 1994). As the major aim of this part of the study was to investigate the localisations of MMP-9, TIMP-1 and -2, and, as LPS has been shown to up-regulate the production of MMP-9 and TIMP-1, it was decided for the purposes of the current study, to LPS-activate J774 macrophages. This, it was thought, would allow comparative studies on LPS-stimulated and unstimulated cells and determination of whether the activation state of the cells has any effect on the localisation of MMP-9, and TIMP-1 and -2.

MMP-9 is responsible for connective tissue turnover during both physiological and pathological processes (Somerville *et al*., 2003; Folgueras *et al*., 2004). This protease has a wide range of substrates including ECM components such as denatured collagen, type IV, V and IX collagen, elastin as well as basement membrane constituents. MMP-9 is also able to cleave non-matrix proteins such as IL-1β precursor and IL-8 resulting in their activation. Cleavage of serpins (both protease inhibitors and signaling molecules) and mature IL-1β (cytokine) by MMP-9, on the other hand results in inactivation (Owen *et al*., 2003; Visse and Nagase, 2003). MMP-9, therefore, plays significant roles in the remodeling and repair of the ECM and contributes to the regulation of the inflammatory response. Uncontrolled expression and synthesis of MMP-9 has, however, been associated with various pathological conditions including lung diseases such as COPD, asthma and idiopathic pulmonary fibrosis (Atkinson and Senior, 2003), as well as many other diseases associated with different organs, including tumour invasiveness and metastasis (Owen *et al*., 2003). Despite these important roles, the intracellular localisation of MMP-9 in macrophages remains unknown.

MMP activity is controlled at four different levels. The first level of control is via the regulation of gene expression. In rodent macrophages, MMP-9 secretion is up-regulated by protein kinase C. MMP-9 secretion is, however, stimulated by LPS, and is down-regulated by inhibition of tyrosine kinases and not by inhibition of protein kinase A (Tremblay *et al*., 1994; Xie *et al*., 1994; Friedland *et al*., 2002). At a second level, MMPs are secreted as inactive, precursor forms. MMP-9 can be activated *in vitro* by organo mercurials. I*n vivo,*

however, MMP-9 appears to be activated via a proteolytic cascade and regulation in such as case may be used to control activation. The pro-domain can be cleaved by MMP-2 or -3 or may be removed in an acidic environment (e.g. low levels of HCl) (Atkinson and Senior, 2003; Woessner and Nagase, 2002). At the last level, the activity of MMPs is controlled by TIMPs and other inhibitors. The predominant inhibitor in the bloodstream is α_2-macroglobulin, whereas, the TIMPs are considered to be the most important inhibitors in the tissues (Atkinson and Senior, 2003; Price *et al*., 2000). Although all TIMPs have an affinity for MMP-9, the enzyme is usually secreted as a non-covalent complex with TIMP-1, [proMMP-9 released from active PMNs is, however, not complexed to TIMP-1, but is released in three different forms including a 92 kDa monomer, a 200 kDa homodimer and a 120 kDa covalent complex with NGAL (Owen *et al*., 2003)]. TIMP-1 binds MMP-9 and other MMPs but has the specific ability to bind to both the carboxy-terminal of the proform and the catalytic domain of only MMP-9 active enzyme (Goldberg *et al*., 1992). The secretion of an MMP-9-TIMP-1 complex may suggest TIMP-1 and MMP-9 intracellular colocalisation. Lastly, MMP secretion by monocytes appears to be regulated by the extracellular environment including matrix components (Galt *et al*., 2001), T-lymphocytes (Ferrari-Lacraz *et al*., 2001) and a variety of cytokines (Goetzl *et al*., 1996).

In most cell types, MMPs are synthesised and subsequently secreted by the cells as opposed to being stored. Neutrophils, however, appear to be an exception as both MMP-8 and MMP-9 are stored in the secondary (specific) and tertiary granules, respectively (Dewald *et al*., 1982; Murphy and Docherty, 1992; Plesner *et al*., 1994). Pro-inflammatory mediators appear to be capable of inducing the expression of MMP-9 on the cell surface of neutrophils (Owen *et al*., 2003) and in contrast to soluble MMP-9, membrane-bound MMP-9 appears to be resistant to inhibition by both TIMP-1 and TIMP-2 (Owen *et al*., 2003). A more recent study revealed that, in resting neutrophils, proMMP-9 is complexed with the $\alpha_M\beta_2$ integrin (Stefanidakis *et al*., 2004). This complex appears to form in the gelatinase granules and cellular activation results in translocation of the complex to the cell membrane (Stefanidakis *et al*., 2004). In neutrophils, TIMP-1 occurs mainly in distinct, oval vesicles which are larger than the azurophilic granules (Price *et al*., 2000). These vesicles lack markers for the various known granule/vesicle populations. Minor colocalisation was observed between TIMP-1 and proMMP-9, however, the purpose of such a colocalisation still seems unclear.

In most types of biological tissues, on the other hand, TIMP-2 appears complexed to MMP-2 (Goldberg *et al*., 1989; Wilhelm *et al*., 1989). TIMP-2 has often been associated with the cell membranes of various cell types. It appears that TIMP-2 is involved in the activation of proMMP-2. The simultaneous binding of TIMP-2 to active MT1-MMP and to proMMP-2 results in the formation of a ternary complex on the cell membrane that allows proMMP-2 activation by a free, adjacent MT1-MMP molecule (Bernando and Fridman, 2003; Emonard *et al*., 2004).

Although many studies have dealt with the expression and secretion of MMPs and TIMPs by both monocytes and macrophages, the intracellular localisations of these proteins in these cell types remains unknown. It is unlikely that macrophage MMP-9 is located in storage granules equivalent to those previously described in neutrophils by our research group (Price *et al*., 2000). It is more likely that MMP-9, TIMP-1 and -2 are located in a type of secretory vesicle as MMP/TIMP synthesis and secretion in macrophages appears to occur only when required. MMP-2 and -9 seem to be very important in basement membrane invasion, inflammation, ECM degradation and the processing of cytokines and other proteins (Owen and Campbell, 1999; Price *et al*., 2000; Parks and Shapiro, 2001; Atkinson and Senior, 2003; Visse and Nagase, 2003; Folgueras *et al*., 2004) (Table 1.8 and Table 1.9). MMP-2 has mostly been associated with TIMP-2 in activational complexes (Goldberg *et al*., 1989; Wilhelm *et al*., 1989). A lack of access to MMP-2 antibodies, but to MMP-9-, TIMP-1- and -2 antibodies, however, lead to exploratory studies on the distribution of MMP-9, TIMP-1 and -2. It was also suspected that these do not colocalise, though some coloicalisation should be evident if MMP-9 and TIMP-1 are secreted as a complex, but this was not tested as yet. The localisation of MMP-9, TIMP-1 and TIMP-2 in J774 macrophages using mainly fluorescent microscopy was, therefore, initially performed. LPS was subsequently used to stimulate the cells and the effects of stimulation on the immunofluorescent labelling patterns monitored. Subsequently, LysoTracker was used to investigate the acidity of MMP-9- or TIMP-1- or -2-labelled vesicles. Due to a shortage of time, however, the apparent importance of LAMP-2 relative to LAMP-1, determined by knockout mice (Tanaka *et al*., 2000) (Section 4.4) and the association of LAMP-2 with biogenesis of the "lysosome" and autophagy (Eskelinen *et al*., 2002) (Section 4.4), double labelling for LAMP-2 (and not LAMP-1), MMP-9 and TIMP-1 was performed to verify whether the MMP-9- or TIMP-1-containing compartments were LAMP-2-positive.

5.2 Localisation of MMP-9, TIMP-1 and TIMP-2 in unstimulated and LPS-stimulated J774 macrophages.

As monocytes differentiate into macrophages, there is a substantial increase in the synthesis and secretion of both MMPs and TIMPs (Campbell *et al*., 1991). Their intracellular localisations, however, remain unknown. It is not known whether they are stored in vesicle populations or are rapidly secreted after synthesis. Fluorescent labelling was, therefore, performed to establish the possible colocalisation of MMP-9, TIMP-1 and -2 in J774 macrophages. It was suspected that TIMP-2 may also be located on the macrophage cell membrane. As cell membrane permeabilisation is used to fluorescently label internal vesicles, however, such a localisation may be missed. Although "classical" activation is only fully induced by treatment with both LPS and IFN-γ, LPS alone induces macrophage production of reactive oxygen species and nitric oxide as well as the secretion of TNF-α and IL-1β (Trask *et al*., 2001). As LPS is also known to up-regulate MMP-9/TIMP-1 synthesis and secretion in certain types of macrophages (Welgus *et al*., 1990; Campbell *et al*., 1991), J774 cells were stimulated with LPS to investigate the effect of activation on the localisation of MMP-9, TIMP-1 and -2. Western blotting performed on human monocyte homogenates (Section 3.7.3) indicated the presence of both pro- and mature MMP-9, yet neither form was detected in unstimulated J774 macrophage homogenates, this may, however, be due to the low almost undetectable levels of MMP-9 in unstimulated mouse macrophages (Filippov *et al*., 2003). Zymography was, therefore, performed to investigate the presence of MMP-9 in unstimulated and LPS-stimulated J774 homogenates as it is more sensitive than western blotting.

5.2.1 Reagents

LPS stock solution [2 mg/ml]. LPS (0.002 g) was dissolved in dH_2O (1 ml), sterile filtered and stored at 4^oC.

Reagents for the culture of J774 cells, SDS-PAGE and zymography of both unstimulated and stimulated J774 cells were prepared according to Sections 2.2.1, 2.3.1.1 and 2.5.1, respectively.

Reagents for fixation and embedding, immunolabelling of ultrathin sections and for fluorescent immunolabelling of unstimulated and stimulated J774 cells for MMP-9, TIMP-1 and TIMP-2 were prepared according to Sections 2.2.1, 2.7.1.1, 2.7.3.1 and 2.8.1.1, respectively.

Antibodies were kind gifts from former members of our research group in the Department of Biochemistry, University of Natal, Pietermaritzburg. Chicken anti-human MMP-9 was provided Dr B. Price and chicken anti-human TIMP-1 by Miss M. Clulow. The sheep anti-human recombinant TIMP-2 was supplied by Dr L. Troeberg a member of Dr Nagase's group at Imperial College, London. Secondary antibodies used were a donkey anti-chicken IgG CY3, a rabbit anti-chicken IgG FITC (both cross-react with chicken IgY) and a donkey anti-sheep IgG FITC.

5.2.2 Procedure

J774 cells were cultured according to the procedure in Section 2.2.2 and stimulated when required by incubation in culture medium to which LPS was added to a final concentration of 10 μg/ml (overnight, 37^{o}C) (Pierce *et al*., 1996). Serum-containing J774 homogenate and supernatant samples were prepared for zymography in non-reducing treatment buffer according to Section 3.6.2 and zymography carried out according to Section 2.6.2. For an inhibitor control EDTA-Na_2 (0.05% m/v) was added to the digestion buffer (100 ml).

Unstimulated and LPS-stimulated J774 cells were fixed and embedded in LR White resin according to Section 2.7.1.2. Due to time constraints Protein A immunogold labelling on ultrathin sections was performed only for MMP-9 according to Section 2.7.3.2 and a chicken anti-human MMP-9 [10 or 15 μg/ml] and a rabbit anti-chicken IgY linker antibody [50 μg/ml] were used.

The major part of this study using fluorescent immunolabelling on unstimulated and LPS-stimulated J774 cells was carried out according to Section 2.8.1.2 with chicken anti-human MMP-9 [50 or 100 μg/ml (single and double labelling)], chicken anti-human TIMP-1 [20 or 50 μg/ml (single labelling) and 50 or 100 or 200 μg/ml (double labelling)], sheep anti-human recombinant TIMP-2 [1:25 (single labelling) and 1:50 (double labelling)], donkey anti-chicken IgY-CY3 [1 or 2 μg/ml], rabbit anti-chicken IgY-FITC [6 or 9 μg/ml] and donkey anti-sheep IgY-FITC [3 or 17 or 25 μg/ml]. Labelling was viewed using an Olympus

epifluorescent microscope and F-View CCD camera and images analysed using ImageJ software as previously described (Chapter 4, Section 4.2.2).

When both primary antibodies used in the colocalisation experiments were from the same host, the relevant controls were performed to eliminate any possible cross-reactivity, i.e. the second primary antibody was omitted to ensure that the secondary labelling system did not cross-react with the primary system (Section 4.3.2).

5.2.3 Results

Gelatin zymography used to investigate the effects of LPS stimulation on MMP-9 produced by J774 macrophages indicates that unstimulated J774 homogenates show gelatinolytic activity (Figure 5.2, lane 2). This is, therefore, in agreement with the negative MMP-9 result in the western blot of unstimulated J774 homogenates (Section 3.7.3, results not shown). The supernatant of unstimulated cells, however, showed four bands of gelatinolytic activity (Figure 5.2, lane 3). Two of the bands were approximately 67 kDa and 62 kDa. The 67 kDa band appears in all of the samples excluding the unstimulated homogenate and including the DMEM and FCS control, and is possibly present in sera used in the medium (Figure 5.2, lane 2) it seems present at a higher concentration, in stimulated cells, however (Figure 5.2, lanes 4-8). The remaining two bands of approximately 91 kDa and 87 kDa seem to be secreted before stimulation but appear in both homogenate and supernatant after stimulation, and bands become more prominent as the concentration of LPS used increases (Figure 5.2, lanes 4-7). It is, therefore, likely that the 91 kDa band seen (Figure 5.2, lanes 4-7) corresponds to proMMP-9 (Woessner and Nagase, 2002) and the 87 kDa band (Figure 5.2, lanes 4-7) a slightly processed form of this protein (Sang *et al*., 1995). The EDTA-Na_2 control showed no gelatinolytic bands (results not shown) indicating that bands seen are infact due to metallo enzymes.

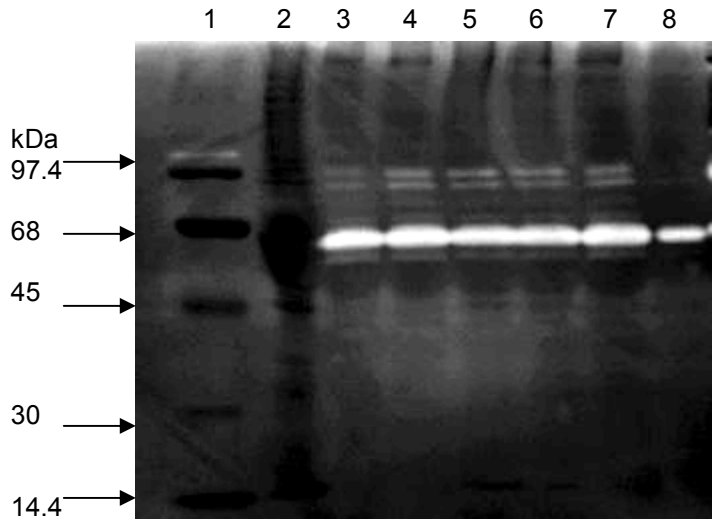

Figure 5.2 **Detection of MMP-9 in unstimulated or LPS-stimulated J774 macrophages.**
MWM (lane 1, 5 µl), unstimulated, serum-containing J774 homogenate (lane 2, 12 µl) and supernatant (lane 3, 10 µl), LPS activated [10 µg/ml] serum-containing J774 homogenate (lane 4, 12 µl) and supernatant (lane 5, 12 µl), LPS activated [100 µg/ml] serum-containing J774 homogenate (lane 6, 12 µl) and supernatant (lane 7, 12 µl), DMEM and FCS control (lane 8, 12 µl). All samples were diluted 1:1 with non-reducing treatment buffer, combined with bromophenol blue [5 µl, 0.1% (m/v) in dH_2O], separated on a 12.5% Laemmli gel containing gelatin [1% (m/v) in separating gel buffer] and stained with Amido black staining solution.
MWM = phosphorylase b, 97.4 kDa, BSA, 68 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 30 kDa, lysozyme, 14.4 kDa.

No non-specific protein A gold- or fluorescent labelling was observed in controls and labelling was assumed to be specific (results not shown). EM studies on both unstimulated and stimulated J774 cells showed that MMP-9 is located in slightly electron-dense compartments (±100 nm, possibly early endosomes) and greater in some instances (±100-300 nm, possibly "hybrid" organelles) (Figure 5.3, B1, C1 and Figure 5.3, B2, C2). MMP-9 was oftem closely associated with membraneous, ER-like structures and is seems membrane-bound (Figure 5.3, B1). Although the fluorescent microscopy showed MMP-9-containing compartments throughout the cytoplasm (Figure 5.3, A1), a significant fraction of MMP-9 labelled vesicles (ranging in size from ± 55-80 nm, resembling "secretory lysosomes") were also observed towards the cell periphery of unstimulated cells (Figure 5.3, A1 and C1). In stimulated cells, however, MMP-9 labelling seemed sparser (Figure 5.3, A2) but was still also observed at the cell edge suggesting possible secretion (Figure 5.3, B2).

TIMP-1-containing vesicles (ranging in size from ±100-400 nm) in unstimulated cells (Figure 5.4, A1) seem fewer and appeared to be positioned more centrally than MMP-9-containing vesicles (Figure 5.3, A1). LPS-stimulated J774 cells showed more intense

labelling for TIMP-1 (Figure 5.4, A2) than unstimulated cells (Figure 5.4, A1), even though a lower primary antibody concentration 20 μg/ml was used for the stimulated cells as opposed to 50 μg/ml for the unstimulated cells. TIMP-1-positive compartments of stimulated cells were more numerous and smaller (±100 nm) and prominent in the perinuclear region and were distributed throughout the cytoplasm, with some appearing to have a cell membrane-association (Figure 5.4, A2).

In TIMP-2 labellings the opposite seems to occur. In unstimulated cells, TIMP-2 appears to occur in vesicular compartments often closely associated with the cell membrane and appears polarised to a particular side of the macrophage or where cells are in contact (Figure 5.4, B1). Unlike with TIMP-1, no significant difference in TIMP-2 labelling was observed in the stimulated cells (Figure 5.4, B2) in comparison with unstimulated ones (Figure 5.4, B1). Although the majority of TIMP-2 labelling in stimulated cells was associated with the cell membrane (Figure 5.4, B2) and less TIMP-2 seemed to be present in the stimulated cells (Figure 5.4, B2) compared to unstimulated cells (Figure 5.4, B1).

It would seem from vesicle sizes seen here that TIMP-1 and TIMP-2, and MMP-9 initially secreted, may be being taken up via early endocytic organelles (±100 nm) to hybrid organelles (±150-200 nm).

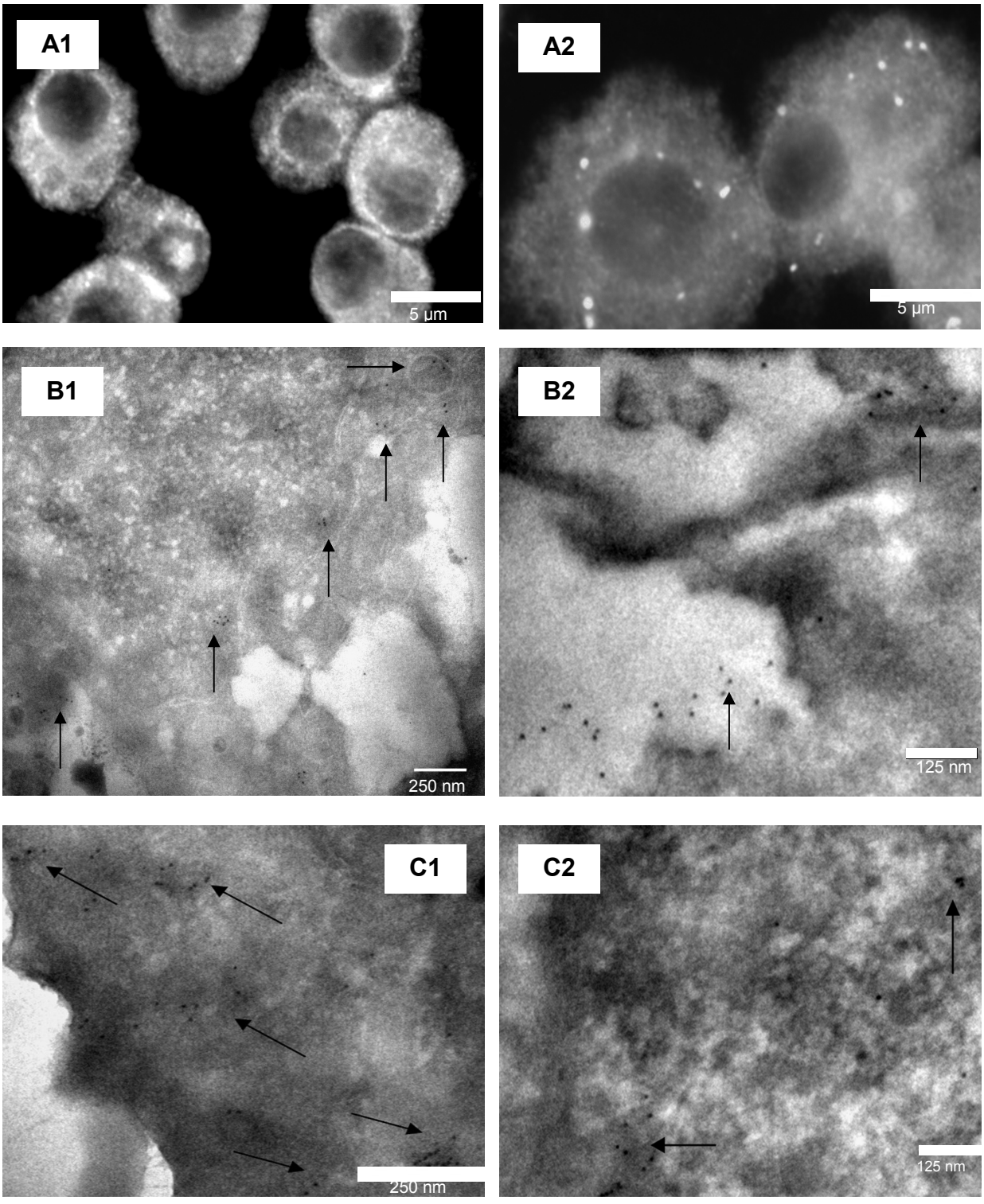

Figure 5.3 **Localisation of MMP-9 in unstimulated and LPS-stimulated J774 macrophages.**
Chicken anti-MMP-9 [50 µg/ml (A1) or 100 µg/ml (A2)] and donkey anti-chicken IgG CY3 [2 µg/ml (A1) or 1 µg/ml (A2)] applied to unstimulated (A1) or cells stimulated with LPS [10 µg/ml] (A2) on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed with an Olympus epifluorescent microscope. Bars = 5 µm (A1 and A2).
Chicken anti-MMP-9 [10 µg/ml (B1 and C1) or 15 µg/ml (B2 and C2)], a rabbit anti-chicken linker antibody [50 µg/ml (B1, B2, C1 and C2)] and protein-A gold probe (10 nm) used on LR White sections of unstimulated (B1 and C1) or cells stimulated with LPS [10 µg/ml] (B2 and C2). Sections viewed using a Philips CW120 Biotwin TEM (80-100 kV). MMP-9 detected in slightly electron-dense vesicles within the cell and towards the cell periphery (arrows). Bars = 250 nm (B1 and C1) or 125 nm (B2 and C2).

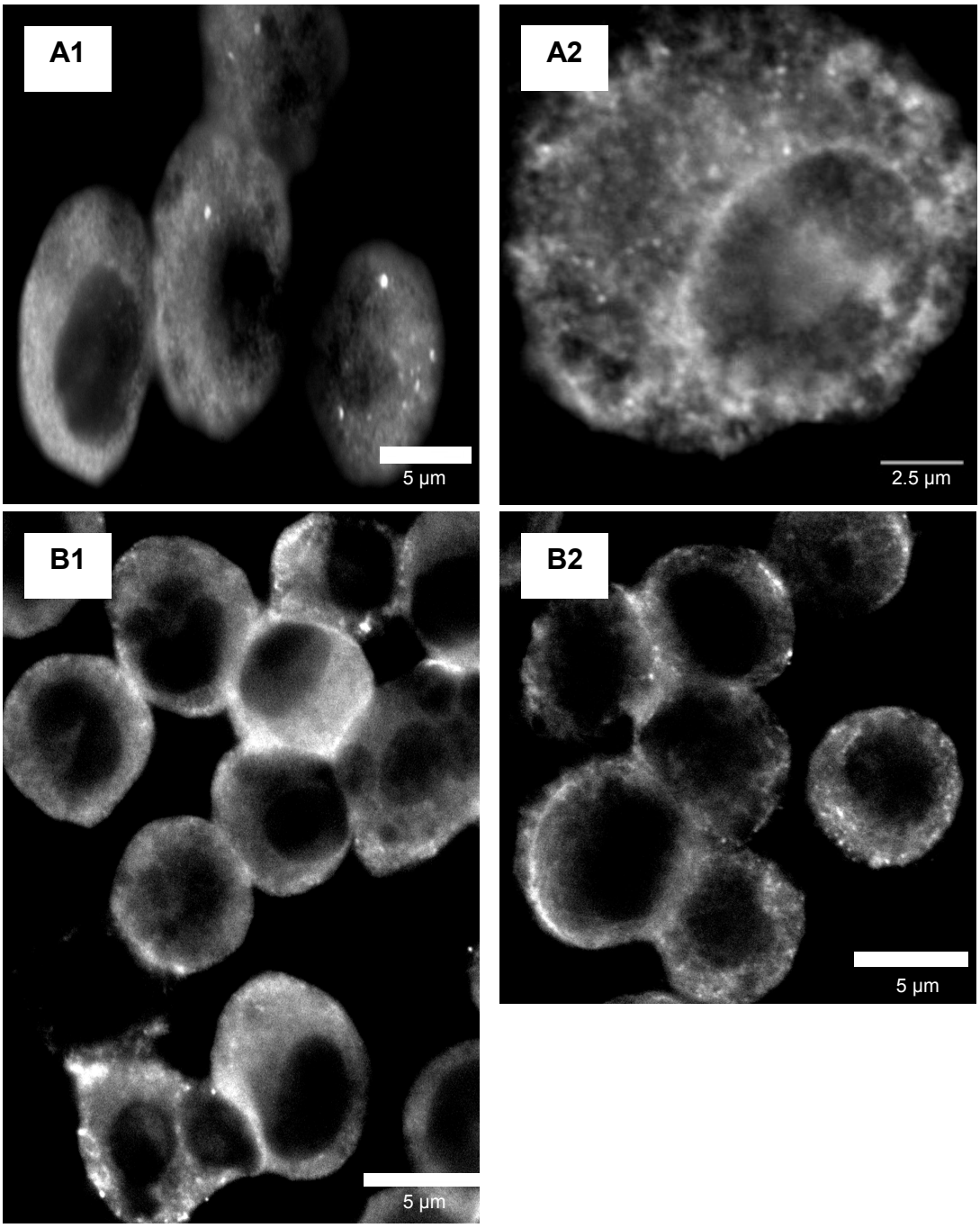

Figure 5.4 **Localisation of TIMP-1 or TIMP-2 in unstimulated and LPS-stimulated J774 macrophages.** Chicken anti-TIMP-1 [50 µg/ml (A1) or 20 µg/ml (A2)], rabbit anti-chicken IgG FITC [9 µg/ml (A1) or 6 µg/ml (A2)] and sheep anti-TIMP-2 serum [1:25 (B1 and B2)], donkey anti-sheep IgG FITC [25 µg/ml (B1 and B2)] applied to unstimulated (A1 and B1) or cells stimulated with LPS [10 µg/ml] (A2 and B2) on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed with an Olympus epifluorescent microscope. Bars = 5 µm (A1, B1 and B2) or 2.5 µm (A2).

Though labelling was, at best, sub-optimal, colocalisation studies revealed that MMP-9 and TIMP-1 appear to be largely located in separate compartments except in areas (ER and Golgi) where their synthesis would possibly occur (Figure 5.5, A). In stimulated cells MMP-9-positive compartments also appear to be more peripherally distributed (Figure 5.5, A1) than TIMP-1-positive compartments (Figure 5.5, A2). Double labelling of stimulated J774 cells, however, showed partial (approximately 30%) colocalisation between MMP-9 and TIMP-1 in large (±450-1000 nm, possibly "hybrid" organelles) (Figure 5.5, B1, B2 and B3), contrasting with the lack of colocalisation observed in the unstimulated cells (Figure 5.5, A1, A2 and A3). The compartments showing colocalisation in stimulated cells seemed predominantly located in the perinuclear region and seem considerably larger (±500-1000 nm) than the single vesicles containing the individual antigens (±100-200 nm) (Figure 5.5, B1, B2 and B3).

TIMP-1 (Figure 5.6, A1) and TIMP-2 (Figure 5.6, A2) were predominantly located in separate vesicles in unstimulated cells, although some perinuclear colocalisation (approximately 25%) was observed, in large vesicles (±400 nm), however. Similarly, double labelling of TIMP-1 (Figure 5.6, B1 and C1) and TIMP-2 (Figure 5.6, B2 and C2) in LPS-stimulated cells showed almost no colocalisation. TIMP-1 labelled vesicles seem to occur close to the nucleus in unstimulated cells (Figure 5.6, A1) and show more colocalisation with TIMP-2 than stimulated cells (Figure 5.6, B and C). The greater the activation (i.e. more elongated morphology) of the cell the more spread out the TIMP-1 and -2 vesicles appear and less colocalisation is observed (compare Figure 5.6, B, more activated with Figure 5.6, C, less activated).

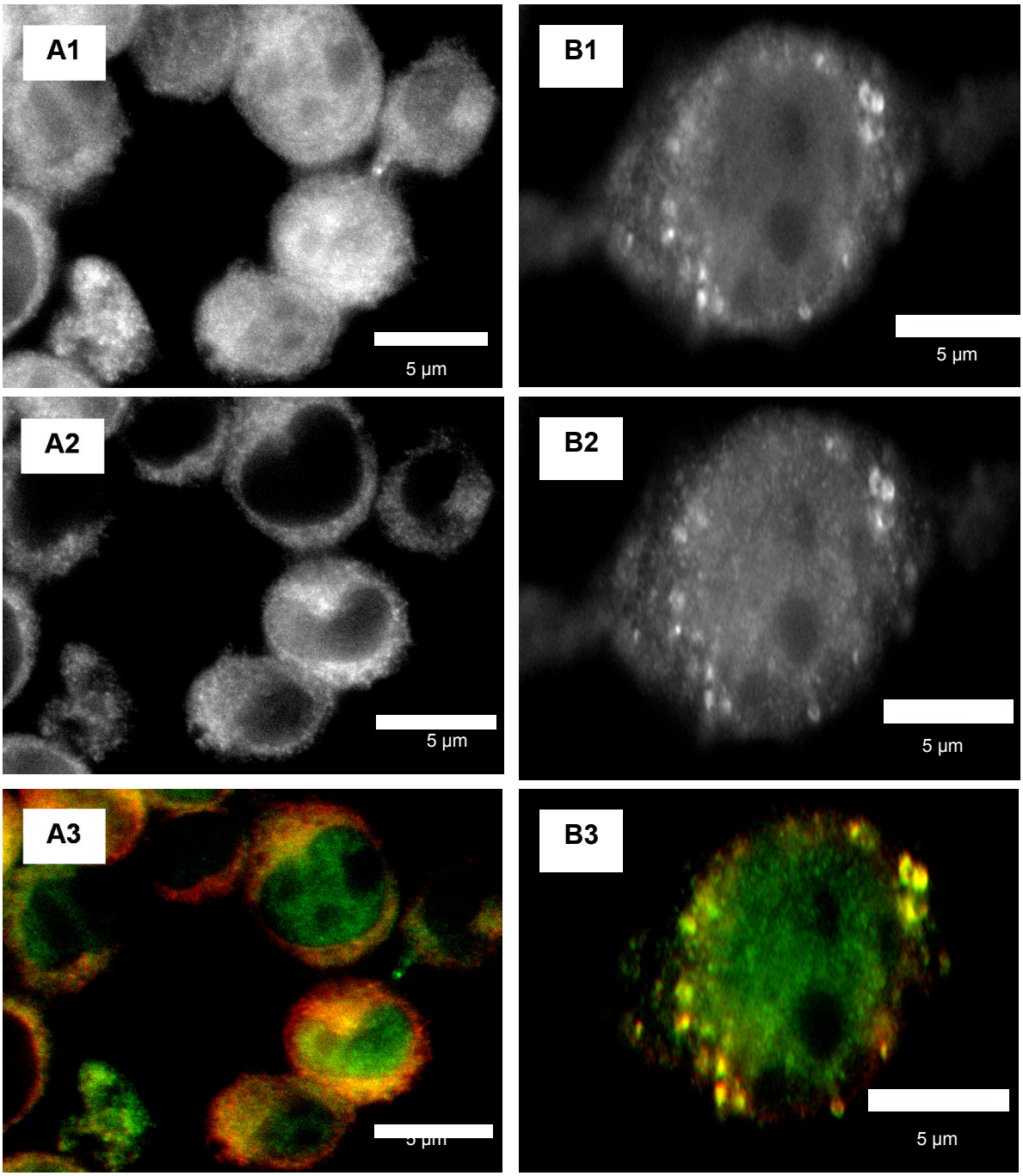

Figure 5.5 **Colocalisation of MMP-9 and TIMP-1 in unstimulated and LPS-stimulated J774 macrophages.**

Chicken anti-MMP-9 [100 µg/ml (A1) or 50 µg/ml (B1)] and donkey anti-chicken CY3 [2 µg/ml (A1 and B1)], post-fixed (3.7% PFA), probed with chicken anti-TIMP-1 [200 µg/ml (A2) or 100 µg/ml (B2)] and rabbit anti-chicken IgG FITC [9 µg/ml (A2 and B2) applied to unstimulated (A) or cells stimulated with LPS [10 µg/ml] (B), on Coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using an Olympus epifluorescent microscope. CY3 filter (A1 and B1), FITC filter (A2 and B2), composite images (A3 and B3). Bars = 5 µm.

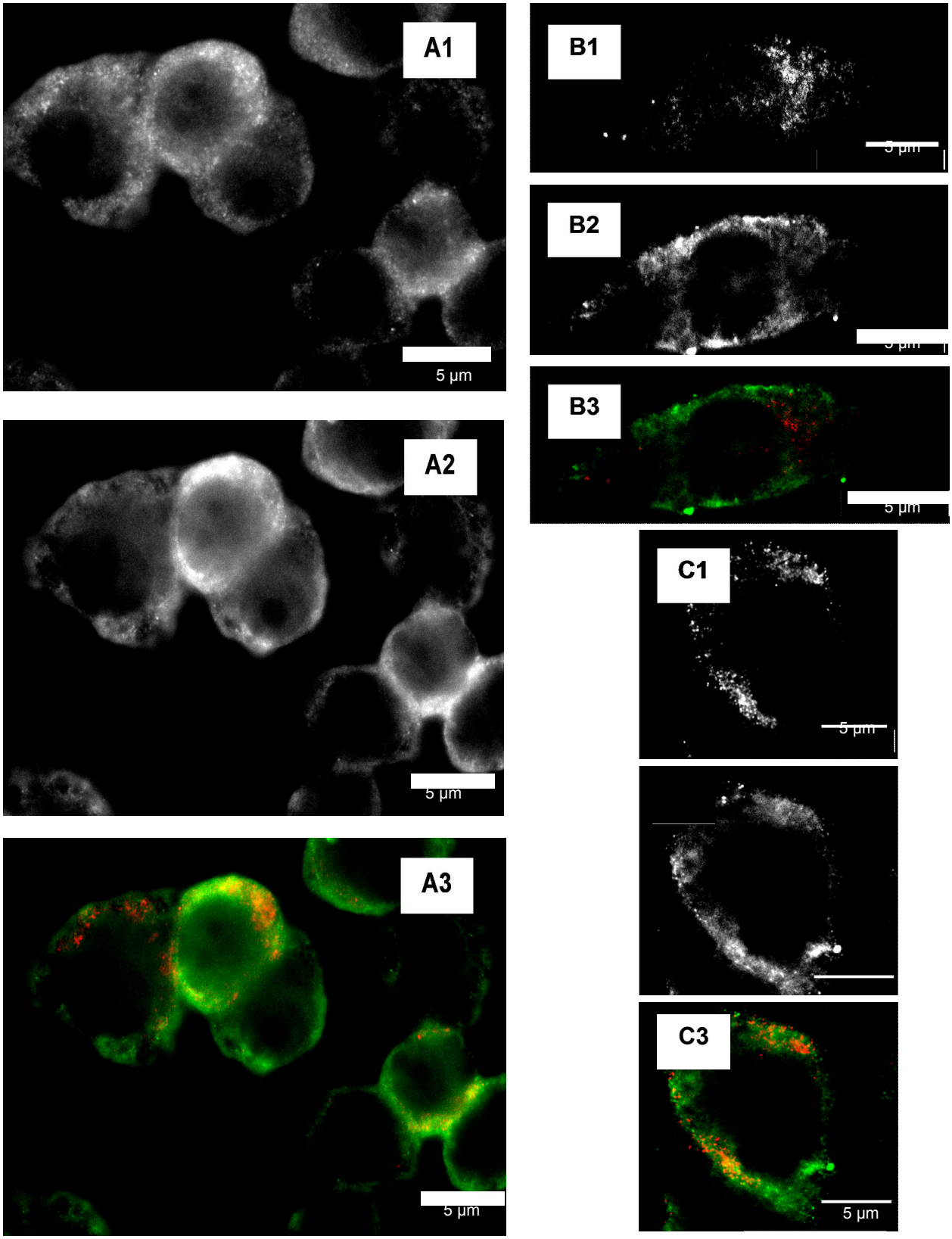

Figure 5.6 **Colocalisation of TIMP-1 and TIMP-2 in unstimulated and LPS-stimulated J774 macrophages.**
Chicken anti-TIMP-1 [50 µg/ml (A1 and B1) or 100 µg/ml (C1)] and donkey anti-chicken CY3 [2 µg/ml (A1, B1 and C1)], post-fixed (3.7% PFA), probed with sheep anti-TIMP-2 serum [1:50 (A2 and B2)] or 1:25 (C2)] and donkey anti-sheep IgG FITC [25 µg/ml (A2 and C2) or 17 µg/ml (B2)], applied to unstimulated (A) or cells stimulated with LPS [10 µg/ml] (B and C), on Coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using an Olympus epifluorescent microscope. CY3 filter (A1, B1 and C1), FITC filter (A2, B2 and C2), composite images (A3, B3 and C3). Bars = 5 µm.

5.3 Localisation of MMP-9 and TIMP-1 in comparison to LAMP-2 and acidity of MMP-9-, TIMP-1- and -2-containing compartments in unstimulated J774 macrophages

In contrast to cathepsins, MMP-9, TIMP-1 and TIMP-2, generally active at approximately neutral pH, according to the literature, are usually secreted by macrophages and function predominantly extracellularly (Okada *et al*., 1995). It, therefore, seemed unlikely that they would be located in acidic compartments and, as the late endocytic compartments (late endosomes and "lysosomes" or "hybrid" organelles) are generally acidic (Griffiths, 1996a) it also seemed unlikely that MMP-9, TIMP-1 and TIMP-2 would colocalise with LAMP-1 or -2 [markers for late endosomes and "lysosomes" (Section 4.4)]. To verify this prediction studies using LysoTracker to identify acidic vesicles and labelling for MMP-9, TIMP-1 and -2 were performed on J774 macrophages. Due to time constraints all experiments were carried out on unstimulated cells only. Due to the apparent greater importance of LAMP-2 [determined from knockout mice studies (Tanaka *et al*., 2000) (Chapter 4, section 4.4)], only colocalisation between LAMP-2 and MMP-9 and TIMP-1 was investigated.

5.3.1 Reagents

LysoTracker Red (Molecular Probes) was diluted in DMEM [1: 20 000] with 10% FCS.

Reagents for the culture and fluorescent immunolabelling of J774 cells for MMP-9, TIMP-1, TIMP-2 and LAMP-2 were prepared according to Sections 2.2.1 and 2.8.1.1.

MMP-9, TIMP-1 and -2 and secondary fluorescent antibodies used were as previously described (Section 5.2.1) and a goat anti-rat IgG FITC antibody was also used. Rat anti-mouse LAMP-2 (ABL-93) was obtained from the Developmental studies Hybridoma Bank (University of Iowa, Iowa City, Iowa, USA)

5.3.2 Procedure

For LysoTracker studies, unstimulated J774 cells were cultured according to the procedure in Section 2.2.2. Prior to fluorescent immunolabelling cells were incubated in medium containing LysoTracker [1:20 000], (30 min, 37^{o}C). Fluorescent immunolabelling was subsequently carried out according to Section 2.8.1.2 using chicken anti-human MMP-9 [50 μg/ml], chicken anti-human TIMP-1 [50 μg/ml], sheep anti-human recombinant TIMP-2 [1:25], rabbit anti-chicken IgG FITC [9 μg/ml] and donkey anti-sheep IgG FITC [17 μg/ml].

For LAMP-2 double labelling, unstimulated J774 cells were cultured and fluorescent immunolabelling carried out according to Sections 2.2.3 and 2.8.1.2 with rat anti-mouse LAMP-2 [1:900], chicken anti-human MMP-9 [50 μg/ml], chicken anti-human recombinant TIMP-1 [100 μg/ml], goat anti-rat IgG FITC [0.8 μg/ml] and donkey anti-chicken IgG CY3 [2 μg/ml]. TIMP-2 labelling was not performed as no more sheep anti-TIMP-2 serum was available.

All labelling was viewed using an Olympus epifluorescent microscope and F-View CCD camera and images analysed using ImageJ software as previously described (Chapter 4, section 4.2.2).

5.2.3 Results

In unstimulated J774 cells, with LysoTracker and MMP-9 labelling, no colocalisation was in labelled (±100-300 nm) vesicles (Figure 5.7, A1-A3). Minor colocalisation (approximately 10-20%) between MMP-9 and LAMP-2 (Figure 5.7, B1-B3) was, however, observed. This suggests that MMP-9 is generally not associated with a "lysosome-like" compartment as anticipated (not LAMPs- and LysoTracker-positive). A perinuclear colocalisation of MMP-9 and LAMP-2, however, in the absence of acidity (LysoTracker labelling), suggests colocalisation in the ER and Golgi region and concurrent synthesis. Results contradict earlier conclusions, which, on the basis of size, speculated that the large vesicles observed were hybrid/digestive bodies (acidic and LAMPs-positive). Besides a possible early endosomal identity (LAMPs-negative, non-acidic), vesicles appear to be distinct and novel, different from those identified in the cathepsin immunolabelling studies (Chapter 4). These cannot be identified without e.g. filling the endocytic/hybrid-/digestive body with gold.

Though largely LysoTracker- and LAMP-2 negative, certain TIMP-1-labelled vesicles (±150 nm), showed minor colocalisation (less than 10%) with LysoTracker (Figure 5.8, A1-A3) and LAMP-2 (Figure 5.8, B1-B3). Unlike the vesicles labelled for MMP-9, these seem to be "lysosome-like", hybrid organelles, on the basis of their size (±150 nm) and positivity for LAMPs and LysoTracker. These vesicles possibly arise after initial secretion of TIMP-1 and subsequent endocytic uptake and fusion with a hybrid digestive organelle.

TIMP-2 (Figure 5.9, A1-A3), unlike TIMP-1 (Figure 5.8, A1-A3), showed no association with LysoTracker, so is not found in an acidic body.

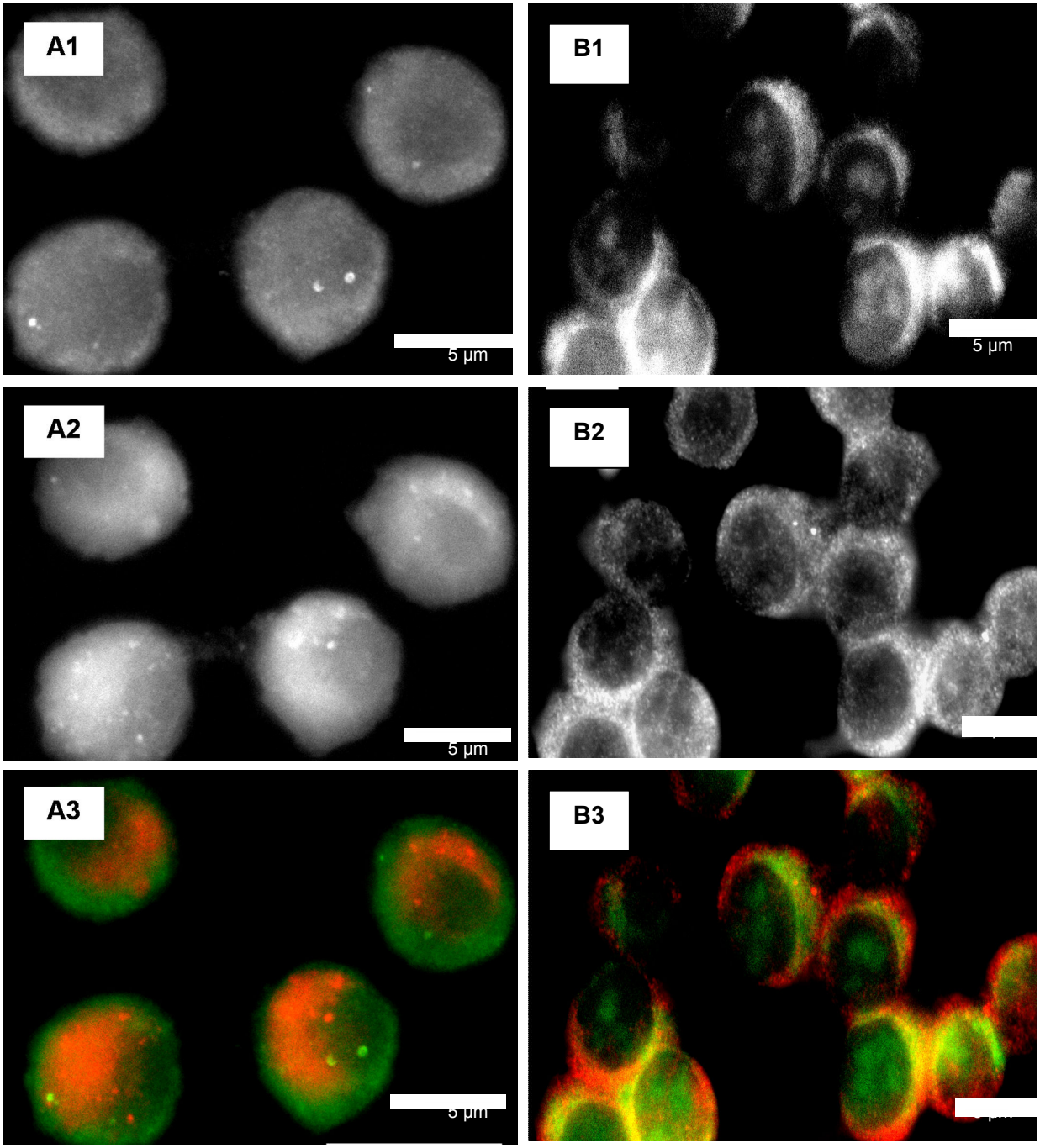

Figure 5.7 **LysoTracker and labelling of LAMP-2 and MMP-9 in unstimulated J774 macrophages.**
Cells grown on coverslips were incubated in DMEM with LysoTracker [1:20 000 (A2)] (30 min, 37°C), initially fixed with 3.7% PFA, permeabilised with saponin and probed with chicken anti-MMP-9 [50 µg/ml (A1)], rabbit anti-chicken FITC [9 µg/ml (A1)]. Coverslips viewed using an Olympus epifluorescent microscope. FITC filter (A1), LysoTracker filter (A2), composite image (A3). Bars = 5 µm.
Rat anti-mouse LAMP-2 [1:900 (B1)] and goat anti-rat IgG FITC [1:1200 (B1)], post-fixed (3.7% PFA), probed with chicken anti-MMP-9 [50 µg/ml (B2)] and donkey anti-chicken IgG CY3 [1:1000 (B2)], applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using an Olympus epifluorescent microscope. FITC filter (B1), CY3 filter (B2), composite image (B3). Bars = 5 µm.

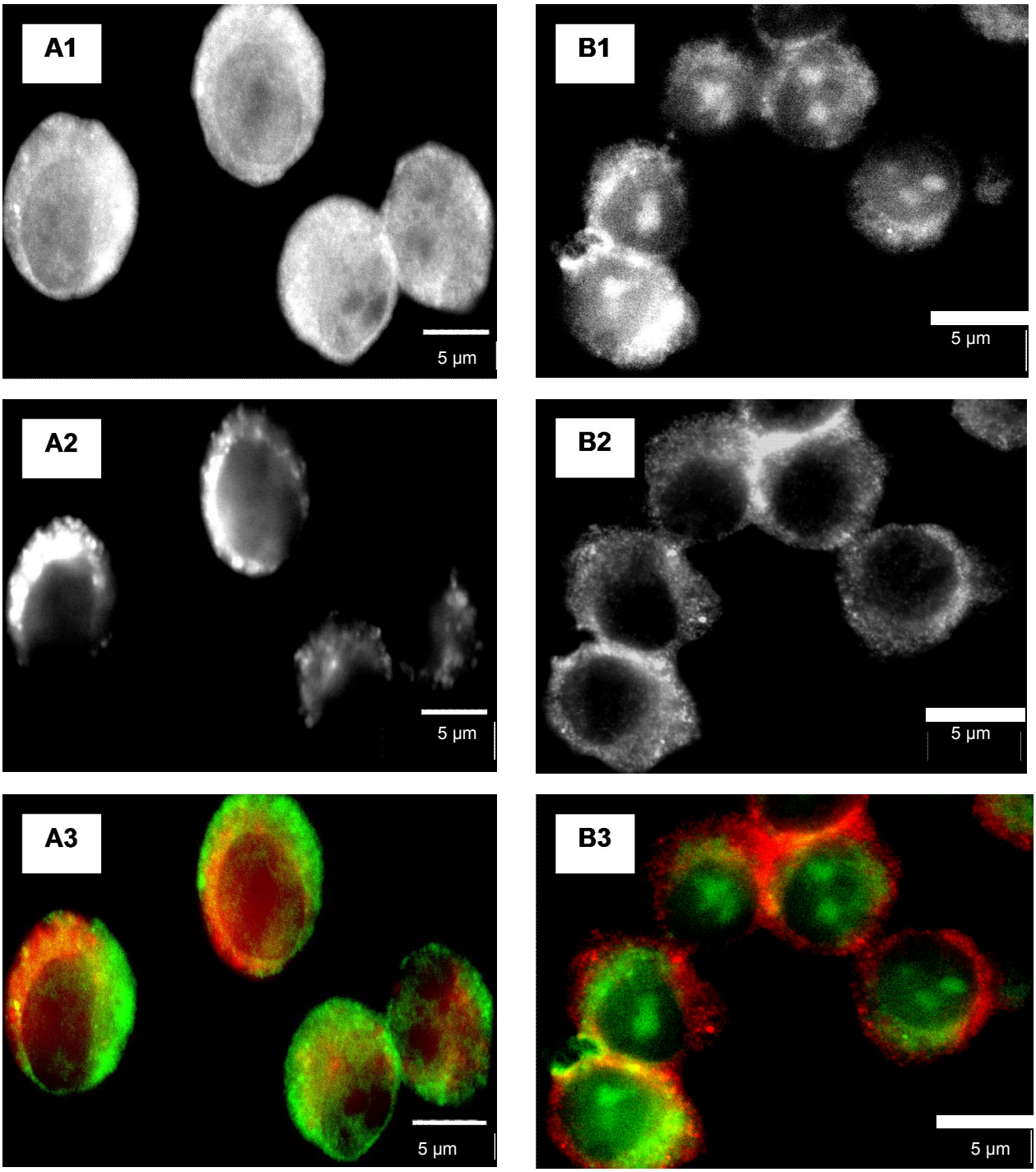

Figure 5.8 **LysoTracker and labelling of LAMP-2 and TIMP-1 in unstimulated J774 macrophages.**
Cells grown on coverslips were incubated in DMEM with LysoTracker [1:20 000 (A2)] (30 min, 37°C), initially fixed with 3.7% PFA, permeabilised with saponin and probed with chicken anti-TIMP-1 [100 µg/ml (A1)], rabbit anti-chicken IgG FITC [9 µg/ml (A1)]. Coverslips viewed using an Olympus epifluorescent microscope. FITC filter (A1), LysoTracker filter (A2), composite image (A3). Bars = 5 µm.
Rat anti-mouse LAMP-2 [1:900 (B1)] and goat anti-rat IgG FITC [0.8 µg/ml (B1)], post-fixed (3.7% PFA), probed with chicken anti-TIMP-1 [100 µg/ml (B2)] and donkey anti-chicken IgG CY3 [2 µg/ml (B2)], applied to cells on coverslips, initially fixed with 3.7% PFA and permeabilised with saponin. Coverslips viewed using an Olympus epifluorescent microscope. FITC filter (B1), CY3 filter (B2), composite image (B3). Bars = 5 µm.

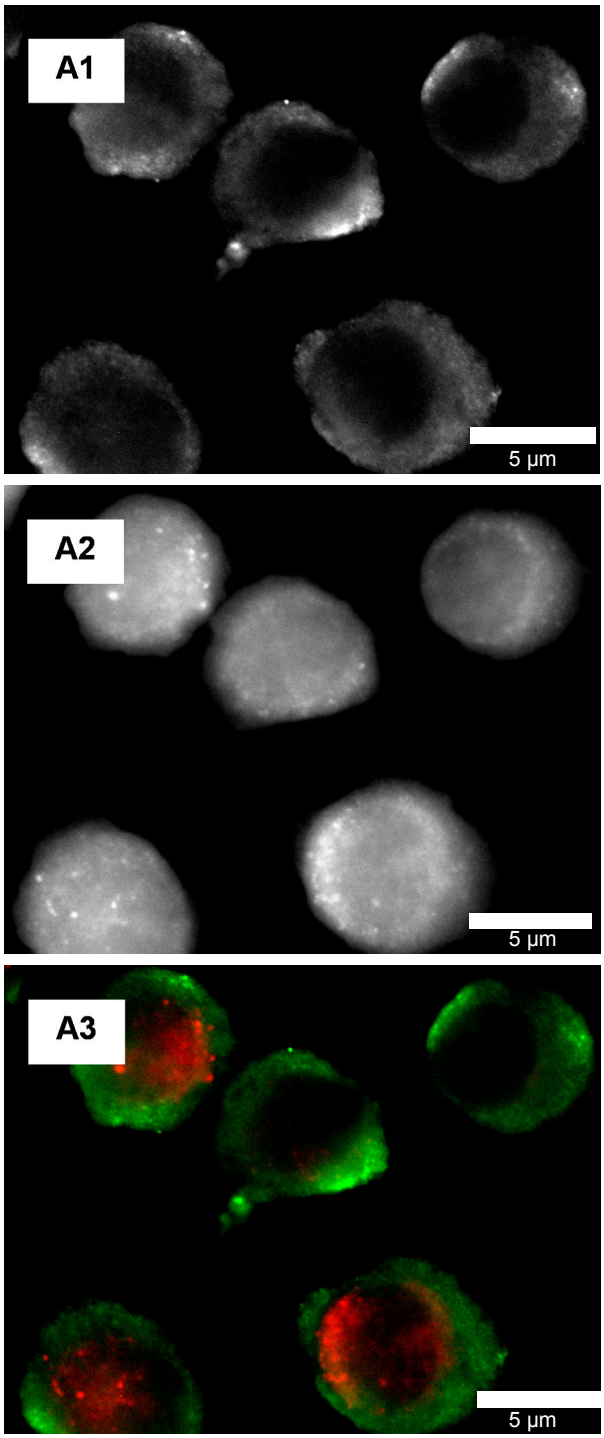

Figure 5.9 **LysoTracker and labelling of TIMP-2 in J774 macrophages.**
Cells grown on coverslips were incubated in DMEM with LysoTracker [1:20 000 (A2)] (30 min, 37°C), initially fixed with 3.7% PFA, permeabilised with saponin and probed with sheep anti-TIMP-2 serum [1:25 (A1)], donkey anti-sheep IgG FITC [17 µg/ml (A1)]. Coverslips viewed using an Olympus epifluorescent microscope. FITC filter (A1), LysoTracker filter (A2), composite image (A3). Bars = 5 µm.

5.4 Discussion

MMPs are highly important in ECM degradation and the processing of cytokines (Folgueras *et al*., 2004). Investigations into the separate localisation of MMPs and their inhibitors, the TIMPs, and their potential differential regulation of release is potentially important for the control of diseases or processes involving the MMPs, i.e. inflammatory disease, tumour invasion, cancer and in fighting infection. It has been shown in neutrophils that, the bulk of MMP-9 is located in the electron-dense, peroxidase-negative gelatinase granules (Kjeldsen *et al*., 1993). Here MMP-9 is stored in an inactive state and only activated after degranulation (Gullberg *et al*., 1997). The current study revealed that, in J774 macrophages, MMP-9 and TIMP-1 appear to be located in separate, novel vesicles distributed throughout the cytoplasm and prominent towards the cell membrane.

In addition to being regulated by cellular differentiation, it appears that, in macrophages, MMP and TIMP synthesis and secretion is regulated by external stimuli such as LPS (Welgus *et al*., 1990). LPS was seen to induce secretion of MMP-9 in this study. Another major effect seemed to be the induction of fusion of TIMP-1- and MMP-9-containing vesicles. It has always been thought that the site of MMP activation is extracellular, post secretion, MMP-9 being activated by secreted MMP-2 (Fridman *et al*., 1995) and MMP-3 (Goldberg *et al*., 1992). The colocalisation of MMP-9 and TIMP-1 in certain large vesicles, post LPS-stimulation, prior to the appearance of mature MMP, however, may indicate that TIMP-1 has some role in either stabilising- or assisting in the intracellular activation of MMP-9 (Price *et al*., 2000).

Price *et al*. (2000) identified a TIMP-1-containing vesicle that was distinct from the various known neutrophil granule/vesicle populations and also a minor subpopulation in which MMP-9 colocalised with TIMP-1. It appears that in J774 macrophages, TIMP-1 and -2 are similarly largely separate, MMP-9 colocalising with TIMP-1 in minor vesicle populations only after LPS stimulation. This may enable TIMP-1 to finely control extracellular proteolytic activity (Price *et al*., 2000) or, under LPS stimulation that causes macrophages to exhibit a more degradative phenotype, partial colocalisation may enhance the proteolytic activity of MMP-9, stabilising and protecting MMP-9 against degradation by elastase and MMP-2 (Price *et al*., 2000), making the surrounding tissue more vulnerable to degradation by MMP-9. MMP-9-TIMP-1 complex formation may, thereby, produce an almost inactivatable complex (i.e. an enzyme/inhibitor complex which alternates between the active and inactive

state as the partially-bound inhibitor moves into and out of the active site, a complex to which no other inhibitors can bind, and a complex stabilised and protected by the bound inhibitor). This would contribute to a damaging degradative phenotype such as is found in inflammatory diseases. Membrane-association has, also been shown to make MMP-9 uninhibitable by TIMP-1 (Owen *et al*., 2003) and it would appear that most of the MMP-9 visualised by EM in this study seems to be membrane-associated.

In most J774 macrophages, in this study, TIMP-2 also appears to be predominantly associated with- or near the cell membrane. This needs verification with labelling of non-permeablised cells and EM. Surface-association may be due to a role in the activation of proMMP-2, a process requiring both MT1-MMP and TIMP-2 (Hernandez-Barrantes *et al*., 2000; Toth *et al*., 2000). TIMP-2 is capable of inhibiting both MT1-MMP and MMP-2 by binding via its inhibitory N-terminal domain to the active sites of MT1-MMP and MMP-2. TIMP-2 is, however, also able to bind to the hemopexin domain of MMP-2 via its C-terminal domain, thus forming a non-covalent complex (Bernardo and Fridman, 2003). This results in the formation of a ternary complex in the cell membrane, allowing activation of proMMP-2 by an adjacent MT1-MMP molecule. The apparent surface location of TIMP-2 in J774 macrophages may, therefore, be an indication of the possible site of formation of the ternary proMMP-2-MT1-MMP-TIMP-2 complex in the macrophage cell membrane, and could be confirmed by triple labelling.

Unlike TIMP-1, LPS-stimulation produced no visible differences in the TIMP-2/MMP-9 separate localisation. This was anticipated as stimulants such as LPS have generally been shown not to affect the localization or production of TIMP-2 **(**Shapiro *et al*., 1992; Lacraz *et al*., 1995). In contrast, TIMP-1 synthesis is usually increased after LPS-stimulation suggesting that different mechanisms of regulation of expression of TIMP-1 and TIMP-2 must exist.

As previously mentioned cathepsins are primarily located in the endosome-lysosome system. Labelling for LysoTracker and LAMP-2 used to establish whether the compartments in which MMP-9, TIMP-1 and -2 occurred were part of the endosome-lysosome system, indicate that most compartments positive for MMP-9 and TIMP-2 are of a relatively high pH (LysoTracker-negative) and seemed to lack labelling for LAMP-2. Although the ultrastructural preservation of the EM sections was poor and possible

multilamellar/multivesicular morphology could not be seen, the results suggest that the MMP-9 vesicles are generally not late endosomal or "lysosomal" compartments. TIMP-1 did, however, appear to show some colocalisation with LysoTracker and LAMP-2, suggesting that at times, TIMP-1 may be found in late endocytic and/or "hybrid" digestive organelles. It has been suggested that some MMPs and TIMP-1 are involved in fighting certain bacterial infections in neutrophils (Osiewicz *et al*., 1999) and possibly macrophage phagosomes, and may aid in immunity and fighting infection in some way. Whether this unanticipated localisation of only TIMP-1, and not TIMP-2, in a digestive body has any significance in this regard remains unknown and possibly should be investigated.

Taken together the above results suggest that MMP-9 and TIMP-1 are located in separate possibly novel vesicular compartments, almost certainly different from those occupied by the "lysosomal" enzymes the cathepsins. It is likely that the MMP-9-containing vesicle is a secretory compartment that is used for the temporary storage and subsequent transportation of the newly synthesised protein to the site of secretion. Hence, this compartment may not be equivalent to the gelatinase granule of the neutrophil.

Both macrophages and neutrophils appear to have separately- and colocalised MMP-9- and TIMP-1-containing vesicles subpopulations. Whether these compartments have equivalent roles i.e. in the activation or regulation of MMP-9 activity still needs to be verified. Although TIMP-1 and TIMP-2 are predominantly not associated with late endosomes and "lysosomes" it also appears that J774 macrophages may internalise initially secreted or possibly surface associated TIMPs via an endosome-lysosome-like pathway, possibly via the recycling endosomal pathway (Remacle *et al*., 2003; Remacle *et al*., 2005). This pathway has been shown to be used to internalise activated MT-1-MMP and direct it towards the invasive front of an invading cell (Remacle *et al*., 2003). The large, novel organelle containing MMP-9 and TIMP-1, identified in this study may be such a recycling endosome. This possibility should be investigated by labelling for Rab11, a marker for such an organelle.

CHAPTER 6

DISCUSSION

Macrophages participate in both innate and acquired immunity, defending the host against microbial pathogens (Ross and Auger, 2002). They function in tissue remodelling (Rabinovitch, 1995) and the inflammatory response; produce a wide variety of cytokines and kill intracellular pathogens, functioning in the acquired immunity in processing and presenting microbial peptides to T-cells (Newman, 1999; Heale and Speert, 2002). Certain pathogens have, however, developed ways of avoiding destruction within the macrophage phagosome by preventing the fusion of protease containing vesicles with the phagosome (Anes *et al*., 2006). Although proteases, such as cathepsins B and D, are now known to play direct roles in controlling bacterial and fungal infections (Thorne *et al*., 1976; Rosenberger *et al*., 2004; Del Cerro-Vadillo *et al*., 2006), the proteases involved in phagosomal killing of various organisms, and the vesicular distribution of such proteases in macrophages, is essentially unknown.

Fusion and fission events with organelles of the endosome-lysosome system and possibly with other vesicle populations, acquiring e.g. proteases and proton-ATPases (Desjardins *et al*., 1994a; Desjardins *et al*., 1997) results in the formation of a phagolysosome, an organelle with powerful microbicidal properties (Desjardins and Griffiths, 2003). Although it appears that in macrophages, the phagosome fuses sequentially with early endosomes, late endosomes and finally "lysosomes". These compartments remain virtually uncharacterised in terms of their protease content and a reliable marker system does not exist for the endosome-lysosome system. Cathepsins are predominantly located in the endosome-lysosome system, whereas MMPs and their inhibitors are primarily in the secretory pathway and hence could possibly be used for the development of marker systems for both the endosome-lysosome system and the secretory pathway. Thus, in the current study, an attempt was made to assess the distribution of cathepsins B, D, H, S and L as well as MMP-9, TIMP-1 and -2 in the vesicles of J774 macrophages.

Based on activity assays, Claus *et al*. (1998) and Jahraus *et al*. (1998) proposed that cathepsins H and S could be used as markers for the early and late endosomes, respectively,

in J774 macrophages and cathepsin D has been previously associated with lysosomes of many different cell types including macrophages (Conner, 2004). The current study indicates that cathepsins H and S seem to be located in separate compartments, possibly the early and late endosomes of J774 macrophages (Chapter 4). However, cathepsin H antisera, in detecting both pro- and mature forms of the protease, cannot be used reliably as a marker for the early endosome without the use of an additional marker, such as EEA1. Significant amounts of procathepsin S in comparison to mature cathepsin S, (a suggested marker for the late endosome) (Jahraus *et al*., 1998) were observed in the current study. The amount of mature enzyme present appears to be dependent on the duration of activation (Punturieri *et al*., 2000), as significant amounts of mature cathepsin S has been shown to appear in MDMs only after approximately 12 days of activation (Punturieri *et al*., 2000). This suggests the use of cathepsin S as a reliable marker for the late endosome is questionable.

In comparison to cathepsins H and S, mature cathepsin D appears to be predominantly associated with acidic compartments, some of which are likely to be LAMPs-positive, implying that these compartments are probably "classical" lysosomes or "hybrid"/digestive bodies. As a result of the dynamic nature of the endosome-lysosome system it is difficult to assign cathepsins to specific vesicles within this system, particularly when immunolabelling studies are performed with antisera that detect both newly synthesised and mature cathepsins. The dynamic formation of hybrid organelles with mixed luminal content also makes the system more complex (Bright *et al*., 1997; Luzio *et al*., 2000). *In vitro* studies of J774 macrophages have shown transient fusion events even between early and late endosomes (Jahraus *et al*., 1998), a phenomenon that does not occur in other cells. It is also becoming increasingly apparent that "lysosomes" ("hybrid/digestive organelles) appear to consist of a number of subpopulations with varied membrane and luminal content, thus making the vesicle characterisation of this system extremely complex. It seems that perhaps at least two subpopulations of cathepsin D-labelled "hybrid" or "digestive" bodies may be revealed by performing triple labelling with LAMP-1, -2 and cathepsin D, together with LysoTracker. This possibility should now be explored. Due to the importance of LAMP-2 shown in knock-out mice (Tanaka *et al*., 2000), the "digestive" organelle labelling for LAMP-2 may be one of the most important vesicle populations in the cell.

Anes *et al*. (2006) demonstrated that most of the detectable V-ATPase in J774 macrophages seemed to be located in vesicles distinct from late endosomes and lysosomes and which

appear to fuse with the phagosome prior to the second killing stage. Some of the acidic vesicles lacking cathepsins/LAMPs observed in the current study may be equivalent to the V-ATPase-rich compartments. The current study also suggested that some mature cathepsin D, for example, may be located in an acidic, LAMPs-negative "lysosomal" subpopulation which could be equivalent to the V-ATPase vesicle identified by Anes *et al*. (2006). A Hck-positive, lysosomal population, distinct from LAMP-3-positive lysosomes was recently identified in human macrophages (Astarie-Dequeker *et al*., 2002). Despite the lack of LAMPs this compartment exhibited other lysosomal characteristics and appeared to be mobilised under a receptor-regulated, microtubule-independent process, suggesting that it may function as a "secretory lysosome". In addition to this lysosomal population, LYAAT-rich vesicles, distinct from the V-ATPase-rich vesicles as well as from late endosomes and "lysosomes" have also been identified (Anes *et al*., 2006). Interestingly, LYAAT was shown to strongly colocalise with Hck in J774 macrophages (Anes *et al*., 2006). The results from the current study, suggest that cathepsins B and D, cathepsins B and L and possibly cathepsins D and L may be located in lysosome-like secretory vesicles. As Hck and LYAAT appear to colocalise with each other (Anes *et al*., 2006), triple labelling studies could be used to establish whether the Hck/LYAAT-positive lysosomes contain either of the cathepsin pairs mentioned above. As LYAAT-positive and V-ATPase-rich compartments fuse with the phagosome prior to the second killing phase (Chapter 4) any colocalisation between Hck/LYAAT and cathepsins may implicate these cathepsins in the killing process taking place within the low pH phagosome.

In addition to the cathepsins, V-ATPase, Hck and LYAAT, there are several other proteins that could be used as potential markers for the various vesicular compartments of the J774 macrophage. These include annexins, syntaxins and Rab-GTPases. Annexins are involved in signal transduction, cell transformation, ion-channel formation, apoptosis and membrane-membrane and membrane-cytoskeletal interactions (Diakonova *et al*., 1997). In J774 macrophages, annexins I, II and III appear to be located in the early endosome, whereas, annexin V is associated with both early and late endosomes (Diakonova *et al*. 1997). Annexins I, II, III, IV, V, VI, VII and XI have been observed in the phagosomes (Desjardins *et al*., 1994b; Diakonova *et al*., 1997; Pittis *et al*., 2003). The amounts of annexin I, II, III and V found in phagosomes isolated at different stages of maturation remained constant, whereas, the amount of annexins IV and VI increased during phagosome maturation (Desjardins *et al*., 1994b; Diakonova *et al*., 1997), suggesting that annexins IV and VI are

located in vesicular compartments distinct from early and late endosomes and may, therefore, be used to identify additional vesicle populations. As Hck/LYAAT appear to be located in vesicles distinct from late endosomes and lysosomes, it would be interesting to investigate whether annexins IV and VI occur in these vesicles or perhaps represent two additional vesicle populations.

Syntaxin 13 has been localised to a recycling endosome (transferrin receptor-positive), whereas, syntaxin 7 is found in late endosomes and lysosomes (Collins *et al*., 2002). Hackam *et al*. (1996) have demonstrated the presence of syntaxins 2, 3 and 4 in both J774 and human macrophages, with syntaxins 3 and 4 being present on both plasma membrane and intracellular vesicles of unknown origin. These proteins, therefore, also have the potential to serve as markers for vesicle populations.

Rab5, initially considered to be an early endosomal marker and Rab7 a marker for late endosomes (Griffiths, 1996a), are not useful for distinguishing between the early and late endosomes of J774 macrophages as Rab5 and Rab7 occur in both the early and late endosomes (Jahraus *et al*., 1998). Rab11 identified in the post-Golgi membranes of secretory cells and a recycling compartment in mouse macrophages promotes phagocytosis (Cox *et al*., 2000) and has a much more interesting role in focussing MT1-MMP and TIMP-2 to the invasive front where the enzymes are required for invasion (Remacle *et al*., 2003; Remacle *et al*., 2005). The current study showed that MMP-9, TIMP-1 and TIMP-2 are generally not located in acidic, LAMP-2-positive compartments (non late endosome-lysosome system) but may be present in recycling endosomes (Rab11-positive, LAMP-negative). This is possibly the novel body (±450 nm) identified in the current study (containing MMP-9 and TIMP-1) and may be the very body in which MT-1-MMP is internalised and recycled to the invasion front, as previously described by Remacle *et al*. (2003). This should now be investigated using labelling for Rab11, MMP-9 and TIMP-1. The novel body containing MMP-9 and TIMP-1 was not a phagosome as the J774 cultures were not contaminated and TIMP-1 and MMPs have not previously been identified in macrophage phagosomes (Garin *et al*., 2001). Their occurance in a phagosome is also unlikely as NADPH-oxidases are capable of inactivating MMPs (Kassim *et al*., 2005).

Interestingly, an increase in colocalisation between MMP-9 and TIMP-1 was observed after stimulation with LPS, though the identity of such a body should be investigated and the role

of an MMP-9-TIMP-1 complex should be investigated. It has previously been suggested that a proMMP-9-TIMP-1 heterodimeric complex may exist (Triebel *et al*., 1995). The binding of TIMP-1 to proMMP-9 at a site distinct from its inhibitory domain and the extracellular release of the complex, may result in a brief period of MMP-9 activity followed by inhibition, stabilising the enzyme and perhaps extending the duration of activity and giving the macrophage some control over the degree of extracellular proteolytic degradation.

It has previously also been suggested that the activation of proMMP-2 occurs at the cell surface and requires both TIMP-2 and MT1-MMP (Hernandez-Barrantes *et al*., 2000; Toth *et al*., 2000). A ternary complex is formed from the binding of TIMP-2 and MT1-MMP to proMMP-2, resulting in the activation of proMMP-2 by an adjacent MT1-MMP molecule (Bernado and Fridman, 2003). The association of TIMP-2, MT1-MMP and proMMP-2 in a single complex with triple labelling may be an indication of this activation process and may also identify the recycling endosome (responsible for targeting the complex to the invasion front) (Remacle *et al*., 2003; Remacle *et al*., 2005) and could be verified by investigating the possible colocalisation between TIMP-2, MT1-MMP and proMMP-2.

Macrophage proteases have generally been thought to be primarily involved in degradation. Recent studies have, however, established that proteases may play a direct role in bacterial killing (Rosenberger *et al*., 2004). As previously mentioned, pathogens such as *Mycobacterium tuberculosis* are capable of surviving within the phagosome, by preventing the fusion of protease containing vesicles with the phagosome. It is, therefore, essential that the different vesicle populations are identified and characterised based on their protease distribution, if we are to understand how the various proteases are capable of contributing to the killing of intracellular pathogens. Cathepsins appear to be primarily located in the endosome-lysosome system in macrophages in at least 7 different vesicle populations. In contrast, MMPs and their inhibitors are generally secreted and function extracellularly (Finlay *et al*., 1997) and are, therefore, expected to be located in vesicles of the secretory pathway. The presence of separate vesicles containing TIMP-1 and TIMP-2 is consistent with Shapiro *et al*. (1992) who demonstrated that the biosynthetic regulation of macrophage TIMP-2 appears to be opposite to that of TIMP-1. The mechanisms governing the biosynthetic regulation of the macrophage MMPs, including MMP-9, -2, -3 and -12 remain unknown.

Minor colocalisation between TIMP-1 and LAMP-2 suggests that previously secreted or possibly cell membrane-associated TIMP-1 may be internalised via the endosome-lysosome pathway and be distributed in late endosomes and "hybrid" organelles possibly for degradation, though the importance of LAMP-2 and many functions of TIMP-1 (Baker *et al*., 2002; Lambert *et al*., 2004) makes this explanation too simple and other possible explanations should be sought.

The current study has produced some answers to the distribution of cathepsins B, D, H, S and L as well as MMP-9, TIMP-1 and TIMP-2 in J774 macrophages and has also provided antibodies necessary for the continuation of this work. This study forms the basis for further characterisation of the J774 macrophage vesicle populations as well as for the development of potential marker systems for the vesicular compartments of both the endosome-lysosome system and the secretory pathway of J774 mouse macrophages.

The work in this study demonstrated that in J774 mouse macrophages, cathepsins H, S, D, B and L occur in secretory vesicles (±20 nm, non-acidic, LAMPs-negative), containing precursor enzymes and at least 6 other endosome-"lysosome" vesicles. Cathepsin H appears to be present in early endosomes (±100 nm, non-acidic, LAMPs-negative) and cathepsin S in late endosomes (±50 nm, acidic, LAMPs-positive) and possibly "lysosomal" ("hybrid" or digestive organelles) (±150-200 nm, acidic, LAMPs-positive). Both cathepsins H and S, however, are only reliable markers for the early and late endosomes, repectively if used with additional markers. Cathepsin D appears mainly associated with "lysosomal" ("hybrid" or digestive organelles) (±150-200 nm, acidic, LAMPs-positive), possibly consisting of further subpopulations which requires more investigation e.g. triple labelling for LAMP-1 and LAMP-2 and cathepsin D. Cathepsins B and L may occur in late endosomes and/or hybrid organelles and "secretory lysosomes" containing cathepsins B, D and L may also exist (±30-50 nm, acidic, LAMPs-positive).

The distribution of MMP-9, TIMP-1 and -2 in vesicles (non-acidic, LAMP-2-negative) which generally appear novel and distinct from late endosome-"lysosome" vesicles was also demonstrated. Colocalised MMP-9 and TIMP-1 in large (±450 nm) vesicles of LPS-stimulated cells, in possible recycling endosomes (Rab11-positive, LAMPs-negative), requires further verification with triple labelling and EM. Possible cell membrane and

recycling endosome localisation of TIMP-2 needs confirmation with labelling of non-permeabilised cells and labelling for MT1-MMP and proMMP-2, respectively.

REFERENCES

Aderem, A. and D. M. Underhill. (1999) Mechanisms of phagocytosis in macrophages. *Annu*. *Rev*. *Immunol*. **17**, 593-623.

Aderem, A. (2003) Phagocytosis and the inflammatory response. *J*. *Infect*. *Dis*. **187** (Suppl. 2), S340-S345.

Allen, L-A. H. and A. Aderem. (1996) Mechanisms of phagocytosis. *Curr*. *Opin*. *Immunol*. **8**, 36-40.

Amos, B. and R. Lotan. (1990) Modulation of lysosomal-associated membrane glycoproteins during retinoic acid-induced embryonal carcinoma cell differentiation. *J*. *Biol*. *Chem*. **31**, 19192-19198.

Amura, C. R., Kamei, T., Ito, N., Soares, M. J. and D. C. Morrison. (1998) Differential regulation of lipopolysaccharide (LPS) activation pathways in mouse macrophages by LPS-binding proteins. *J*. *Immunol*. **161**, 2552-2560.

Andrejewski, N., Punnonen, E-L., Guhde, G., Tanaka, Y., Lüllmann-Rauch, R., Hartmann, D., von Figura, K. and P. Saftig. (1999) Normal lysosomal morphology and function in LAMP-1 deficient mice. *J*. *Biol*. *Chem*. **274**, 12692-12701.

Andrews, N. W. (2000) Regulated secretion of conventional lysosomes. *Trends Cell Biol*. **10**, 316-321.

Anes, E., Peyron, P., Staali, L., Kress, H., Hagedorn, M., Maridonneau-Parini, I., Skinner, M. A., Wildeman, A. G., Kalamidas, S. A., Kuehnel, M. and G. Griffiths. (2006) Dynamic life and death interactions between *Mycobacterium smegmatis* and J774 macrophages. *Cell*. *Microbiol*. **8**, 939-960.

Astarie-Dequeker, C., N'Diaye, E-N., Le Cabec, V., Rittig, M. G., Prandi, J., and I. Maridonneau-Parini. (1999) The mannose receptor mediates uptake of pathogenic and nonpathogenic mycobacteria and bypasses bactericidal responses in human macrophages. *Infect*. *Immun*. **67**, 469-477.

Astarie-Dequeker, C., Carreno, S., Cougoule, C. and I. Maridonneau-Parini. (2002) The protein tyrosine kinase Hck is located on lysosomal vesicles that are physically and functionally distinct from CD63-positive lysosomes in human macrophages. *J*. *Cell Sci*. **115**, 81-89.

Atkinson, J. J. and R. M. Senior. (2003) Matrix metalloproteinase-9 in lung remodeling. *Am*. *J*. *Respir*. *Cell Mol*. *Biol*. **28**, 12-24.

Babior, B. M. (1984) The respiratory burst of phagocytes. *J. Clin. Invest.* **73**, 599-601.

Baker, A. H., Edwards, D. R. and G. Murphy. (2002) Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J. Cell Sci.* **115**, 3719-3727.

Barret, A. J., Rawlings, N. D. and J. F. Woessner. (2004) Handbook of Proteolytic Enzymes, 2nd Edition, Elsevier Academic Press, London.

Beers, C., Honey, K., Fink, S., Forbush, K. and A. Rudensky. (2003) Differential regulation of cathepsin S and cathepsin L in interferon γ-treated macrophages. *J. Exp. Med.* **197**, 169-179.

Berg, T., Gjøen, T. and O. Bakke. (1995) Physiological functions of endosomal proteolysis. *Biochem. J.* **307**, 313-326.

Bernando, M. M. and R. Fridman. (2003) TIMP-2 (tissue inhibitor of metalloproteinase-2) regulated MMP-2 (matrix metalloproteinase-2) activity in the extracellular environment after pro-MMP-2 activation by MT1 (membrane type-1)-MMP. *Biochem. J.* **374**, 739-745.

Bischoff, K. M., Shi, L. and P. J. Kennelly. (1998) The detection of enzyme activity following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* **260**, 1-17.

Blott, E. J. and G. M. Griffiths. (2002) Secretory lysosomes. *Nat. Rev. Mol. Cell Biol.* **3**, 122-131.

Blum, J. S., Fiani, M. L. and P. D. Stahl. (1991) Proteolytic cleavage of ricin A chain in endosomal vesicles. Evidence for the action of endosomal protease at both neutral and acidic pH. *J. Biol. Chem.* **266**, 22091-22095.

Bokovsky, J. and R. H. Kennett. (1987) Simple and rapid purification of monoclonal antibodies from cell culture supernatants and ascites fluid by hydroxylapatite chromatography on analytical and preparative scales. *Hybridoma* **6**, 219-228.

Boll, M., Foltz, M., Rubio-Aliaga, I., Kottra, G. and H. Daniel. (2002) Functional characterization of two novel mammaliam electrogenic proton-dependent amino acid cotransporters. *J. Biol. Chem.* **277**, 22966-22973.

Borregaard, N. and J. B. Cowland. (1997) Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89**, 3503-3521.

Botelho, R. J., Hackam, D. J., Schreiber, A. D. and S. Grinstein. (2000) Role of COPI in phagosome maturation. *J. Cell Biol.* **275**, 15717-15727.

Boyum, A. (1976) Isolation of lymphocytes, granulocytes and macrophages. *Scand*. *J*. *Immunol*. (Supp. 5) **5**, 9-15.

Brew, K., Dinakarpandian, D. and H. Nagase. (2000) Tissue inhibitors of matrix metalloproteinases: evolution, structure and function. *Biochim*. *Biophys*. *Acta* **1477**, 267-283.

Bright, N. A., Reaves, B. J., Mullock, B. M. and J. P. Luzio. (1997) Dense core lysosomes fuse with late endosomes and are re-formed from the resultant hybrid organelles. *J*. *Cell Sci*. **110**, 2027-2040.

Brown, W. J., Goodhouse, J. and M. G. Farquhar. (1986) Mannose-6-phophate receptors for lysosomal enzymes cycle between the Golgi complex and endosomes. *J*. *Cell Biol*. 103, 1235-1247.

Bühling, F., Waldburg, N., Resenauer, A., Heimburg, A., Golpon, H. and T. Welte. (2004) Lysosomal cysteine proteases in the lung: role in protein processing and immunoregulation. *Eur*. *Respir*. *J*. **23**, 620-628.

Busiek, D. F., Ross, F. P., McDonnell, S., Murphy, G., Matrisian, L. M. and H. G. Welgus. (1992) The matrix metalloprotease matrilysin (PUMP) is expressed on developing human mononuclear phagocytes. *J*. *Biol*. *Chem*. **267**, 9087-9092.

Butor, C., Griffiths, G., Aronson, N. N. and A. Vark. (1995) Co-localization of hydrolytic enzymes with widely disparate pH optima: implications for the regulation of lysosomal pH. *J*. *Cell Sci*. **108**, 2213-2219.

Campaine, A., Schein, J. D., Tabb, J. S., Mohan, P. S. and R. A. Nixon. (1995) Limited proteolytic processing of the mature form of cathepsin D in human and mouse brain: post-mortem stability of enzyme structure and activity. *Neurochem*. *Int*. **27**, 385-396.

Campbell, E. J., Cury, J. D., Lazarus, C. J. and H. G. Welgus. (1987) Monocyte procollagenase and tissue inhibitor of metalloproteinases. *J*. *Biol*. *Chem*. **262**, 15862-15868.

Campbell, E. J., Silverman, E. K. and M. A. Campbell. (1989) Elastase and cathepsin G of human monocytes quantification of cellular content, release in response to stimuli, and heterogeneity in elastase-mediated proteolytic activity. *J*. *Immunol*. **143**, 2961-2968.

Campbell, E. J., Cury, J. D., Shapiro, S. D., Goldberg, G. I. and H. G. Welgus. (1991) Neutral proteinases of human mononuclear phagocytes. Cellular differentiation markedly alters cell phenotype for serine proteinases, metalloproteinases and tissue inhibitor of metalloproteinases. *J*. *Immunol*. **146**, 1286-1293.

Candiano, G., Porotto, M., Lanciotti, M. and G. M. Ghiggeri. (1996) Negative staining of proteins in polyacrylamide gels with methyl trichloroacetate. *Anal*. *Biochem*. **243**, 245-248.

Caterina, N. C., Windsor, L. J., Bodden, M. K., Yermovsky, A. E., Taylor, K. B., Birkedal-Hansen, H. and J. A. Engler. (1998) Glycosylation and NH_2-terminal domain mutants of the tissue inhibitor of metalloproteinases-1 (TIMP-1). *Biochim*. *Biophys*. *Acta* **1388**, 21-34.

Cawston, T. E., Noble, D. N., Murphy, G., Smith, A. J., Woodley, C. and B. Hazleman. (1986) Rapid purification of tissue inhibitor of metalloproteinases from human plasma and identification as a γ-serum protein. *Biochem*. *J*. **238**, 677-682.

Chan, S. J., Segundo, B. S., McCormick, M. B. and D. F. Steiner. (1986) Nucleotide and predicted amino acid sequences of cloned human and mouse procathepsin B cDNAs. *Proc*. *Natl*. *Acad*. *Sci*. *USA* **83**, 7721-7725.

Chang, J. C., Yoo, O. H. and M. Lesser. (1989) Cathepsin D activity is increased in alveolar macrophages and bronchoalveolar lavage fluid of smokers. *Am*. *Rev*. *Respir*. *Dis*. **140**, 958-960.

Chapman, H. A., Munger, J. S. and G. P. Shi. (1994) Role of thiol proteases in tissue injury. *Am*. *J*. *Resp*. *Crit*. *Care Med*. **150**, S155-S159.

Chapman, H. A., Riese, R. J. and G-P. Shi. (1997) Emerging roles for cysteine proteases in human biology. *Annu*. *Rev*. *Physio*l. **59**, 63-88.

Chapman, H. A. Jr. and G-P. Shi. (2000) Protease injury in the development of COPD: Thomas A. Neff lecture. *Chest* **117**, 295-299.

Chizzolini, C., Rezzonico, R., De Luca, C., Burger, D. and J-M. Dayer. (2000) Th2 cell membrane factors in association with IL-4 enhance matrix metalloproteinase-1 (MMP-1) while decreasing MMP-9 production by granulocyte-macrophage colony-stimulating factor-differentiated human monocytes. *J*. *Immunol*. **164**, 5952-5960.

Clague, M. J. (1998) Molecular aspects of the endocytic pathway. *Biochem*. *J*. **336**, 271-282.

Claus, V., Jahraus, A., Tjelle, T., Berg, T., Kirschke, H., Faulstich, H. and G.Griffiths. (1998) Lysosomal enzyme trafficking between phagosomes, endosomes and lysosomes in J774 cells: enrichment of cathepsin H in early endosomes. *J*. *Biol*. *Chem*. **273**, 9842-9851.

Coetzer, T. H. T. (1992) Type IV collagenase and cathepsins L and H: proteinases involved in tumour invasion. MSc Thesis, University of Natal.

Cohn, Z. A. and E. Wiener. (1963) The particulate hydrolases of macrophages. I. Comparative enzymology, isolation, and properties. *J*. *Exp*. *Med*. **118**, 991-1008.

Collins, R. F., Shreiber, A. D., Grinstein, S. and W. S. Trimble. (2002) Syntaxins 13 and 7 function at distinct steps during phagocytosis. *J*. *Immunol*. **169**, 3250-3256.

Conner, G. E. (2004) Cathepsin D. In: Handbook of Proteolytic Enzymes, 2nd Edition, Volume 1, (Barrett, A. J., Rawlings, N. D. and J. F. Woessner Eds.), Elsevier Academic Press, London, pp 43-52.

Copeland, R. A. (1994) Reverse fluorescence staining of proteins in polyacrylamide gels using terbium chloride. *Anal*. *Biochem*. **220**, 218-219.

Cox, D., Lee, D. J., Dale, B. M., Calafat, J. and S. Greenberg. (2000) A Rab11-containing rapidly recycling compartment in macrophages that promotes phagocytosis. *Proc*. *Natl*. *Acad*. *Sci*. *USA* **97**, 680-685.

Dahlgren, C., Carlsson, Sven R., Karlsson, A., Lundqvist, H.and C. Sjolin. (1995) The lysosomal membrane glycoproteins Lamp-1 and Lamp-2 are present in mobilizable organelles, but are absent from the azurophil granules of human neutrophils. *Biochem*. *J*. **311**, 667-674.

de Duve, C. (1983) Lysosomes revisited. *Eur*. *J*. *Biochem*. **137**, 391-397.

Dehrmann, F. M., Elliott, E. and C. Dennison. (1996) Reductive activation markedly increases the stability of cathepsins B and L to extracellular ionic conditions. *Biol*. *Chem*. *Hoppe Seyler*. **377**, 391-394.

Del Cerro-Vadillo, E., Madrazo-Toca, F., Carrasco-Marin, E., Fernandez-Prieto, L., Beck, C., Leyva-Cobian, F., Saftig, P. and C. Alvarez-Dominguez. (2006) Cutting edge: a novel nonoxidative phagosomal mechanism exerted by cathepsin-D controls *Listeria monocytogenes* intracellular growth. *J*. *Immunol*. **176**, 1321-1325.

De Lorenzo, M. S., Alonso, D. F. and D. E. Gomez. (2000) Nafoxidine modulates the expression of matrix-metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinases-1 (TIMP-1) in endothelial cells. *Anticancer Res*. **20**, 395-400.

De Maio, A. (1994) Protein blotting and immunoblotting using nitrocellulose membranes. In: Protein Blotting a Practical Approach, (Dunbar, B. S., Ed.), Oxford University Press, Oxford, pp 11-32.

De Mey, J. (1987) The preparation and use of gold probes. In: Immunocytochemistry Modern Methods and Applications, (Polak, J. M. and S. Van Noorden, Eds.), John Wright & Sons Ltd., Bristol, pp 115-145.

Dennison, C. (1999) A Guide to Protein Isolation, Kluwer Academic Publishers, Dordrecht.

Desjardins, M., Huber, L. A., Parton R. G. and G. Griffiths. (1994a) Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. *J*. *Cell Biol*. **124**, 677-688.

Desjardins, M., Celis, J. E., van Meer, G., Dieplinger, H., Jahraus, A., Griffiths, G. and L. A. Huber. (1994b) Molecular characterization of phagosomes. J. Biol. Chem. **269**, 32194-32200.

Desjardins, M. (1995) Biogenesis of phagolysosomes: the 'kiss and run' hypothesis. *Trends Cell Biol*. **5**, 183-186.

Desjardins, M., Nzala, N., Corsini R., and C. Rondeau. (1997) Maturation of phagosomes is accompanied by changes in their fusion properties and size-selective acquisition of solute materials from endosomes. *J. Cell Sci*. **110**, 2303-2314.

Desjardins, M. and G. Griffiths. (2003) Phagocytosis: latex leads the way. *Curr*. *Opin*. *Cell Biol*. **15**, 498-503.

Dewald, B., Bretz, U. and M. Baggiolini. (1982) Release of gelatinase from a novel secretory compartment of human neutrophils. *J. Clin*. *Invest*. **70**, 518-525.

Diakanova, M., Gerke, V., Ernst, J., Liautard, J-P, van der Vusse, G. and G. Griffiths. (1997) Localization of five annexins in J774 macrophages and on isolated phagosomes**.** *J. Cell Sci*. **110**, 1199-1213.

Diment, S. and P. Stahl. (1985) Macrophage endosomes contain proteases which degrade endocytosed protein ligands. *J. Biol*. *Chem*. **260**, 15311-15317.

Diment, S., Leech, M. S and P. D. Stahl. (1988) Cathepsin D is membrane-associated in macrophage endosomes. *J. Biol*. *Chem*. **263**, 6901-6907.

Drouin, L., Overall, C. M. and J. Sodek. (1988) Identification of matrix metalloendoproteinase inhibitor (TIMP) in human parotid and submandibular saliva: partial purification and characterization. *J. Periodont. Res*. **23**, 370-377.

Duclos, S., Diez, R., Garin, J., Papadopoulou, B., Descoyeaux, A., Stenmark, H. and M. Desjardins. (2000) Rab5 regulates the kiss and run fusion between phagosomes and endosomes and the acquisition of phagosome leishmanicidal properties in RAW 264.7 macrophages. *J. Cell Sci*. **113**, 3531-3541.

Duffield, J. S. (2003) The inflammatory macrophage: a story of Jekyll and Hyde. *Clin*. *Sci*. **104**, 27-38.

Elliott, E. (1993) A study of proteinases of invasive cells using cryoultramicrotomy and immunogold labelling. PhD Thesis, University of Natal.

Elliott, E., Dennison, C., Fortgens, P. H. and J. Travis. (1995) Paraformaldehyde fixation of neutrophils for immunolabeling of granule antigens in cryoultrasections. *J. Histochem. Cytochem*. **43**, 1019-1025.

Ellouz, F., Adam, A., Ciorbaru, R. and E. Lederer. (1974) Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. *Biochem. Biophys. Res. Commun*. **59**, 1317-1325.

Emonard, H., Bellon, G., Troeberg, L., Berton, A., Robinet, A., Henriet, P., Marbaix, E., Kirkegaard, K., Patthy, L., Eekhout, Y., Nagase, H., Hornebeck, W. and P. J. Courtoy. (2004) Low density lipoprotein receptor-related protein mediates endocytic clearance of pro-MMP-2-TIMP-2 complex through a thrombospondin-independent mechanism. *J. Biol. Chem*. **279**, 54944-54951.

Eskelinen, E-L., Illert, A. L., Tanaka, Y., Schwarzmann, G., Blanz, J., von Figura, K. and P. Saftig. (2002) Role of LAMP-2 in lysosome biogenesis and autophagy. *Mol. Biol. Cell* **13**, 3355-3368.

Eskelinen, E-L., Tanaka, Y. and P. Saftig. (2003) At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends Cell Biol*. **13**, 137-145.

Ey, P. L., Prowse, S. J. and C. R. Jenkin. (1978) Isolation of pure IgG1, IgG2a, IgG2b immunoglobulins from mouse serum using protein A-Sepharose. *Biochemistry* **15**, 429-436.

Falcon-Perez, J. M., Nazarian, R., Sabatti, C., and E. C. Dell'Angelica. (2005) Distribution and dynamics of Lamp1-containing endocytic organelles in fibroblasts deficient in BLOC-3. *J. Cell Sci*. **118**, 5243-5255.

Faurschou, M. and N. Borregaard. (2003) Neutrophil granules and secretory vesicles in inflammation. *Microb. Infect*. **5**, 1317-1327.

Fernandez-Patron, C., Hardy, E., Sosa, A., Seoane, J. and L. Castellanos. (1995a) Double staining of Coomassie-Blue-stained polyacrylamide gels by imidazole-sodium dodecyl sulfate-zinc reverse staining: sensitive detection of Coomassie Blue-undetected proteins. *Anal. Biochem*. **224**, 263-269.

Fernandez-Patron, C., Calero, M., Collazo, P. R., Garcia, J. R., Madrazo, J., Musacchio, A., Soriano, F., Estrada, R., Frank, R., Castellanos-Serra, L. R. and E. Mendez. (1995b) Protein reverse staining: high efficiency microanalysis of unmodified proteins detected on electrophoresis gels. *Anal. Biochem*. **224**, 203-211.

Ferrari-Lacraz, S., Nicod, L. P., Chicheportiche, R., Welgus, H. G. and J-M, Dayer. (2001) Human lung tissue macrophages, but not alveolar macrophages, express matrix metalloproteinases after direct contact with activated T lymphocytes. *Am. J. Respir. Cell Mol. Biol*. **24**, 442-451.

Filippov, S., Caras, I., Murray, R., Matrisian, L. M., Chapman, H. A., Shapiro, S. and S. J. Weiss. (2003) Matrilysin-dependent elastolysis by human macrophages. *J. Exp. Med.* **198**, 925-935.

Finlay, G. A., O'Driscoll, L. R., Russell, K. J., D'Arcy, E. M., Masterson, J. B., FitzGerald, M. X. and C. M. O'Connor. (1997) Matrix metalloproteinase expression and production by alveolar macrophages in emphysema. *Am. J. Respir. Crit. Care Med.* **156**, 240-247.

Folgueras, A. R., Pendás, A. M., Sánchez, L. M. and C. López-Otín. (2004) Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. *Int. J. Dev. Biol.* **48**, 411-424.

Fortgens, P. H. Dennison, C. and E. Elliott. (1997) Anti-cathepsin D chicken IgY antibodies characterization, cross-species reactivity and application in immunogold labelling of human splenic neutrophils and fibroblasts. *Immunopharmacology* **36**, 305-311.

Freund, J. (1956) The mode of action of immunological adjuvants. *Adv. Tuberc. Res.* **7**, 130-148.

Freund, J. and K. McDermott. (1942) Sensitisation to horse serum by means of adjuvants. *Proc. Soc. Exp. Biol. Med.* **49**, 548-553.

Fridman, R., Toth, M., Pena, D. and S. Mobashery. (1995) Activation of progelatinase B (MMP-9) by gelatinase A (MMP-2). *Cancer Res.* **55**, 2548-2555.

Friedland, J. S., Shaw, T. C., Price, N. M. and J. M. Dayer. (2002) Differential regulation of MMP-1/9 and TIMP-1 secretion in human monocytic cells in response to *Mycobacterium tuberculosis*. *Matrix Biol.* **21**, 103-110.

Fujihara, M., Ito, N., Pace, J. L., Watanabe, Y., Russell, S. W. and T. Suzuki. (1994) Role of endogenous interferon-beta in lipopolysaccharide-triggered activation of the inducible nitric-oxide synthase gene in a mouse macrophage cell line, J774. *J. Biol. Chem.* **269**, 12773-12778.

Fukuda, M. (1991) Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking. *J. Biol. Chem.* **266**, 21327-21330.

Furuta, K., Yang, X-L., Chen, J-S., Hamilton, S. R. and J. T. August. (1999) Differential expression of the lysosome-associated membrane proteins in normal human tissues. *Arch. Biochem. Biophys.* **365**, 75-82.

Furuta, K., Ikeda, M., Nakayama, Y., Nakamura, K., Tanaka, M., Hamasaki, N., Himeno, M., Hamilton, S. R. and J. T. August. (2001) Expression of lysosome-associated membrane proteins in human colorectal neoplasms and inflammatory diseases. *Am. J. Pathol.* **159**, 449-455.

Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P. H., Steele-Mortimer, O., Paiement, J. Bergeron, J. J. and M. Desjardins. (2002) Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell* **110**, 119-131.

Galt, S. W., Lindemann, S., Medd, D., Allen, L. L., Kraiss, L. W., Harris, E. S., Prescott, S. M., McIntyre, T. M., Weyrich, A. S. and G. A. Zimmerman. (2001) Differential regulation of matrix metalloproteinase-9 by monocytes adherent to collagen and platelets. *Circ*. *Res*. **89**, 509-516.

Garfin, D. E. (1990) One-dimensional gel electrophoresis. In: Guide to Protein Purification. Methods in Enzymology vol. 182 (Deutscher, M. P. Ed.), Academic Press, San Diego, pp 425-441.

Garin, J., Diez, R., Kieffer, S., Dermine, J-F., Duclos, S., Gagnon, E., Sadoul, R., Rondeau, C. and M. Desjardins. (2001) The phagosome proteosome: insight into phagosome functions. *J*. *Cell Biol*. **152**, 165-180.

Gelb, B. D., Shi, G. P., Chapman, H. A. and R. J. Desnick. (1996) Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. *Science* **273**, 1236-1238.

Gershoni, J. M. and G. E. Palade. (1983) Protein Blotting: Principles and Applications. *Anal*. *Biochem*. **131**, 1-15.

Geuze, H. J., Slot, J. W., van der Ley, P. A. and R. C. T. Scheffer. (1981) Use of colloidal gold particles in double-labeling immunoelectron microscopy of ultrathin frozen tissue sections. *J*. *Cell Biol*. **89**, 653-665.

Ghosh, R. N., Gelman, D. L. and F. R. Maxfield. (1994) Quantification of low density lipoprotein and transferring in endocytic sorting in Hep2 cells using confocal microscopy. *J*. *Cell Sci*. **107**, 2177-2189.

Gibbs, D. F., Warner, R. L., Weiss, S. J., Johnson, K. J. and J. Varani. (1999a) Characterization of matrix metalloproteinases produced by rat alveolar macrophages. *Am*. *J*. *Respir*. *Cell Mol*. *Biol*. **20**, 1136-1144.

Gibbs, D. F., Shanley, T. P., Warner, R. L., Murphy, H. S., Varani, J. and K. J. Johnson. (1999b) Role of matrix metalloproteinases in models of macrophage-dependent acute lung injury. *Am*. *J*. *Respir*. *Cell Mol*. *Biol*. **20**, 1145-1154.

Goetzl, E. J., Banda, M. J. and D. Leppert. (1996) Matrix metalloproteinases in immunity. *J*. *Immunol*. **156**, 1-4.

Goldberg, G. I., Marmer, B. L., Grant, G. A., Eisen, A. Z., Wilhelm, S. and C. He. (1989) Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteases designated TIMP-2. *Proc*. *Natl*. *Acad*. *Sci USA* **86**, 8207-8211.

Goldberg, G. I., Srongin, A., Collier, I. E., Genrich, L. T. and B. L. Marmer. (1992) Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. *J. Biol. Chem.* **267**, 4583-4591.

Goldman, S., Weiss, A., Eyali, V. and E. Shalev. (2003) Differential activity of the gelatinases (matrix metalloproteinases 2 and 9) in the fetal membrane and decidua, associated labour. *Mol. Hum. Reprod.* **9**, 367-373.

Goldring, J. P. D. and T. H. T. Coetzer. (2003) Isolation of chicken immunoglobulins (IgY from egg yolk. *Biochem. Mol. Biol. Educ.* **31**, 185-187.

Gomez, D. E., Alonso, D. F., Yoshiji, H. and U. P. Thorgeirsson. (1997) Tissue inhibitors of metalloproteinases: Structure, regulation and biological functions. *Eur. J. Cell Biol.* **74**, 111-123.

Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S. and R. M. M. Singh. (1966) Hydrogen ion buffers for biological research. *Biochem. J.* **5**, 467-477.

Gordon, S. (2003) Alternative activation of macrophages. *Nature Rev. Immunol.* **3**, 23-35.

Goto, I., Shinno, N. and Y. Kuroiwa. (1986) Proteolytic enzyme activities of macrophages and lymphocytes in neurological diseases. *J. Neurol.* **233**, 373-375.

Gough, N. R. and D. M. Fambrough. (1997) Different steady state subcellular distributions of the three splice variants of lysosome-associated membrane protein LAMP-2 are determined largely by the COOH-terminal amino acid residue. *J. Cell Biol.* **137**, 1161-1169.

Greenberg, S., Chang, P. and S. C. Silverstein. (1993) Tyrosine phosphorylation is required for Fc receptor-mediated phagocytosis in mouse macrophages. *J. Exp. Med.* **177**, 529-534.

Greenberg, S. and S. Grinstein. (2002) Phagocytosis and innate immunity. *Curr. Opin. Immunol.* **14**, 136-145.

Greiner, A., Lautwein, A., Overkleeft, H. S., Weber, E. and C. Driessen. (2003) Activity and subcellular distribution of cathepsins in primary human monocytes. *J. Leukoc. Biol.* **73**, 235-242.

Griffiths, G. (1993) Fine Structure Immunocytochemistry, Springer-Verlag, Berlin.

Griffiths, G. (1996a) On vesicles and membrane compartments. *Protoplasma* **195**, 37-58.

Griffiths, G. M. (1996b) Secretory lysosomes - a special mechanism of regulated secretion in haemopoietic cells. *Trends Cell. Biol.* **9**, 329-332.

Griffiths, G. (2004) On phagosome individuality and membrane signalling networks. *Trends Cell Biol*. **14**, 343-351.

Gullberg, U., Andersson, E., Garwicz, D., Lindmark, A. and I. Olsson. (1997) Biosynthesis, processing and sorting of neutrophils proteins: insight into neutrophils granule development. *Eur*. *J*. *Haematol*. **58**, 137-153.

Gurvich, A. E. and G. I. Drizlikh. (1964) Use of antibodies on an insoluble support for specific detection of radioactive antigens. *Nature* **5**, 648-649.

Hackam, D. J., Rotstein, O. D., Bennett, M. K., Klip, A., Grinstein S. and M. F. Manolson. (1996) Characterization and subcellular localization of target membrane soluble NSF attachment protein receptors (t-SNAREs) in macrophages. Syntaxins 2, 3 and 4 are present on phagosomal membranes. *J*. *Immunol*. **156**, 4377-4383.

Hackam, D. J., Rotstein, O. D., Schreiber, A., Zhang, W. and S. Grinstein. (1997) Rho is required for the initiation of calcium signalling and phagocytosis by Fcγ receptors in macrophages. *J*. *Exp*. *Med*. **186**, 955-966.

Hackam, D. J., Botelho, R. J., Sjolin, C., Rotstein, O. D., Robinson, J. M., Schreiber, A. D. and S. Grinstein. (2001) Indirect role for COPI in the completion of Fcγ receptor-mediated phagocytosis. *J*. *Biol*. *Chem*. **276**, 18200-18208.

Handel-Fernandez, M. E. and D. M. Lopez. (2000) Isolation of macrophages from tissues, fluids, and immune response sites. In: Macrophages (Paulnock, D. M. Ed.), Oxford University Press, Oxford, 1-30.

Hanson, R. D., Connolly, N. L., Burnett, D., Campbell, E. J., Senior, R. M. and T. J. Ley. (1990) Developmental regulation of the human cathepsin G gene in myelomonocytic cells. *J*. *Biol*. *Chem*. **265**, 1524-1530.

Harlow, E. and D. Lane. (1999) Using Antibodies A Laboratory Manual, Cold Spring Harbour Laboratory Press, New York.

Harrison, R. E., Bucci, C., Vieira, O. V., Schroer, T. A. and S. Grinstein. (2003) Phagosomes fuse with late endosomes and/or lysosomes by extension of membrane protrusions along microtubules: role of Rab7 and RILP. *Mol*. *Cell Biol*. **23**, 6494-6506.

Hart, T. C., Hart, P. C., Bowden, D. W., Michalec, M. D., Callison, S. A., Walker, S. J., Zhang, Y. and E. Firatli. (1999) Mutations of the cathepsin C gene are responsible for Papillon-Lefèvre syndrome. *J*. *Med*. *Genet*. **36**, 881-887.

Hasegawa, G., Suwa, M., Ichikawa, Y., Ohtsuka, T., Kumagai, S., Kikuchi, M. and Y. Sato. (2003) A novel function of tissue-type transglutaminase: protein disulphide isomerase. *Biochem*. *J*. **373**, 793-803.

Hatem, C. L., Gough, N. R. and D. M. Fambrough. (1995) Multiple mRNAs encode the avian lysosomal membrane protein LAMP-2, resulting in alternative transmembrane and cytoplasmic domains. *J. Cell Sci*. **108**, 2093-2100.

Hautamaki, R. D., Kobayashi, D. K., Senior, R. M. and S. D. Shapiro. (1997) Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* **277**, 2002-2004.

Hawkes, S. P., Li, H. and G. T. Taniguchi. (2001) Zymography and reverse zymography for detection of MMPs and TIMPs. In: Matrix Metalloproteinase Protocols, Methods in Molecular Biology, (Clark, I. M. Ed.), Humana Press Inc., New Jersey, 399-410.

Heale, J-P. and D. P. Speert. (2002) Macrophages in bacterial infection. In: The Macrophage, 2nd Edition, (Burke, B. and C. E. Lewis Eds.), Oxford University Press, Oxford, pp 210-252.

Heinrich, M., Wickel, M., Schneider-Brachert, W., Sandberg, C., Gahr, J., Schwandner, R., Weber, T., Brunner, J., Kronke, M. and S. Schutze. (1999) Cathepsin D targeted by acid sphingomyelinase-derived ceramide. *EMBO*. *J*. **18**, 5252-5263.

Henry, R. M., Hoppe, A. D., Joshi, N. and J. A. Swanson. (2004) The uniformity of phagosome maturation in macrophages. *J. Cell Biol*. **164**, 185-194.

Herman, M. P., Sukhova, G. K., Kisiel, W., Foster, D., Kehry, M. R., Libby, P. and U. Schonbeck. (2001) Tissue factor pathway inhibitor-2 is a novel inhibitor of matrix metalloproteinases with implications for atherosclerosis. *J. Clin*. *Invest*. **107**, 1117-1126.

Hernandez-Barrantes, S., Toth, M., Bernardo, M. M., Yurkova, M., Gervasi, D.C., Raz, Y. Sang, Q-X. A. and R. Fridman. (2000) Binding of active (57 kDa) membrane type 1-matrix metalloproteinase (MT1-MMP) to tissue inhibitor of metalloproteinase (TIMP)-2 regulates MT1-MMP processing and pro-MMP-2 activation. *J. Biol*. *Chem*. **275**, 12080-12089.

Heuser, J. (1989) Changes in lysosome shape and distribution correlated with changes in cytoplasmic pH. *J. Cell Biol*. **108**, 855-864.

Heussen, C. and E. B. Dowdle. (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and coploymerized substrates. *Anal*. *Biochem*. **102**, 196-202.

Hibbs, M. S. (1992) Expression of 92 kDa phagocyte gelatinase by inflammatory and connective tissue cells. In: Matrix Metalloproteinases and Inhibitors: Proceedings of the Matrix Metalloproteinase Conference, (Birkendal-Hansen, H., Werb, Z., Welgus, H. and H. Van Wart Eds.), Gustaf Fischer Verlag, New York, pp 51-57.

Hoflack, B., Fujimoto, K. and S. Kornfeld. (1987) The interactions of phosphorylated oligosaccharides and lysosomal enzymes with bovine liver cation-independent mannose 6-phosphate receptor. *J. Biol. Chem.* **262**, 123-129.

Holten-Andersen, M. N., Brunner, N., Maimonis, P., Jensen, V., Murphy, G.and T. Piironen. (2002) Characterization of monoclonal antibodies to tissue inhibitor of metalloproteinases-1. *J. Clin. Ligand Assay* **25**, 87-90.

Hsieh, C-S., deRoos, P., Honey, K., Beers, C. and A. Y. Rudensky. (2002) A role for cathepsin L and cathepsin S in peptide generation for MHC class II presentation. *J. Immunol.* **168**, 2618-2625.

Hua, C. T., Hopwood, J. J., Carlsson, S. R., Harris, R. J. and P. J. Meikle. (1998) Evaluation of the lysosome-associated membrane protein LAMP-2 as a marker for lysosomal storage disorders. *Clin. Chem.* **44**, 2094-2102.

Ishidoh, K., Saido, T. C., Kawashima, S., Hirose, M., Watanabe, S., Sato, N. and E. Kominami. (1998) Multiple processing of procathepsin L to cathepsin L *in vivo*. *Biochem. Biophys. Res. Commun.* **252**, 202-207.

Jacob, M. C., Favre, M. and J-C. Bensa. (1991) Membrane cell permeabilisation with saponin and multiparametric analysis by flow cytometry. *Cytometry* **12**, 550-558.

Jacobson, G. (1994) Protein blotting using semi-dry electrophoretic transfer equipement. In: Protein Blotting a Practical Approach, (Dunbar, B. S. Ed.), Oxford University Press, Oxford, 53-70.

Jahraus, A., Tjelle, T. E., Berg, T., Habermann, A., Storrie, B., Ullrich, O. and G. Griffiths. (1998) *In vitro* fusion of phagosomes with different endocytic organelles from J774 macrophages. *J. Biol. Chem.* **273**, 30379-30390.

James, M. N. G. (2004) Introduction: aspartic peptidases and their clans. In: Handbook of Proteolytic Enzymes, 2nd Edition, Volume 1, (Barrett, A. J., Rawlings, N. D. and J. F. Woessner Eds.), Elsevier Academic Press, London, pp 3-19.

Janeway, C. A., Travis, P., Walport, M. and J. D. Copra. (2001) Immunobiology, the Immune System in Health and Disease, 5th Edition, Garland Publishing, New York.

Johansson, A., Jesatis, A. J., Lundqvist, H., Magnusson, K-E., Sjölin, C., Karlsson, A. and C. Dahlgren. (1995) Different subcellular localization of cytochrome b and the dormant NADPH-oxidase in neutrophils and macrophages: effect on the production of reactive oxygen species during phagocytosis. *Cell. Immunol.* **161**, 61-71.

Johnson, A. G., Gaines, S. and M. Landy. (1956) Studies on the O antigen of Salmonella typhosa V. Enhancement of antibody response by the purified lipopolysaccharide. *J. Exp. Med*. **103**, 225-246.

Johnstone, A. and R. Thorpe. (1987) Immunoassays. In: Immunochemistry in Practice, Blackwell Scientific Publications, London, pp 241-258.

Jung, K-K., Liu, X-W., Chirco, R., Fridman, R.and H-R. C. Kim. (2006) Identification of CD63 as a tissue inhibitor of metalloproteinase-1 interacting cell surface protein. *EMBO J.* **25**, 3934-3942.

Kannan, K., Stewart, R. M., Bounds, W., Carlsson, Sven R., Fukuda, M., Betzing, K. W. and R. F. Holcombe. (1996) Lysosome-associated membrane proteins h-LAMP-1 (CD107a) and h-LAMP-2 (CD107b) are activation-dependent cell surface glycoproteins in human peripheral blood mononuclear cells which mediate cell adhesion to vascular endothelium. *Cell. Immunol*. **171**, 10-19.

Kargi, H. A., Campbell, E. J. and C. Kuhn. (1990) Elastase and cathepsin G of human monocytes: heterogeneity and subcellular localization to peroxidase-positive granules. *J. Histochem. Cytochem*. **38**, 1179-1186.

Karp, G. (1999) Cell and Molecular Biology Concepts and Experiments, 2nd Edition, John Wiley & Sons, Inc., New York.

Kassim, S. Y., Fu, X., Liles, W. C., Shapiro, S. D., Parks, W. C. and J. W. Heinecke. (2005) NADPH oxidase restrains the matrix metalloproteinase activity of macrophages. *J. Biol. Chem*. **280**, 30201-30205.

Kerr, M. A. and L. M. Loomes. (1994) Derivatization of antibodies and antigen: fluorescent labeling, biotinylation, immunogold derivatives. In: Immunochemistry Labfax, (Kerr, M. A and R. Thorpe Eds.), Bios Scientific Publishers, Oxford, 157-174.

Kerrigan, J. J., Mansell, J. P. and J. R. Sandy (2000) Matrix turnover. *J. Orthod*. **27**, 227-233.

Kirschke, H., Barrett, A. J. and N. D Rawlings. (1998) Lysosomal Cysteine Proteinases, 2nd Edition, Oxford University Press, Oxford, pp 2-23.

Kirschke, H. (2004) Cathepsin L. In: Handbook of Proteolytic Enzymes, 2nd Edition, Volume 2, (Barrett, A. J., Rawlings, N. D. and J. F. Woessner Eds.), Elsevier Academic Press, London, pp 1097-1102.

Kjeldsen, L., Bainton, D. F., Sengeløv, H. and N. Borregaard. (1993) Structural and functional heterogeneity among peroxidase-negative gelatinase granules in human

neutrophils: identification of a distinct gelatinase containing granule subset by combined immunocytochemistry and subcellular fractionation. *Blood* **82**, 3183-3191.

Kleijmeer, M. K., Escola, J. M., UyteHaag, F. G., Jakobson, E., Griffith, J. M., Osterhaus, A. D., Stoorvogel, W., Melief, C. J., Rabouille, C. and H. J. Geuze. (2001) Antigen loading of MHC class I molecules in the endocytic tract. *Traffic* **2**, 124-137.

Kleiner, D. E. and W. G. Stetler-Stevenson. (1994) Quantitative zymography: detection of picogram quantities of gelatinases. *Anal*. *Biochem*. **218**, 325-329.

Knapp, P. E. and J. A. Swanson. (1990) Plasticity of the tubular lysosomal compartment in macrophages. *J. Cell Sci*. **95**, 433-439.

Kobayashi, T., Vischer, U. M., Rosnoblet, C., Lebrand, C., Lindsay, M., Parton, R. G., Kruithof, E. K. and J. Gruenberg. (2000) The tetraspanin CD63/lamp3 cycles between endocytic and secretory compartments in human endothelial cells. *Mol*. *Biol*. *Cell* **11**, 1829-1843.

Kornfeld, S. (1986) Trafficking of lysosomal enzymes in normal states. *J*. *Clin*. *Invest*. **77**, 1-6.

Kuehnel, M. P., Goethe, R., Habermann, A., Mueller, E., Rohde, M., Griffiths, G. and P. Valentin-Weigand. (2001) Characterization of the intracellular survival of *Mycobacterium avium* ssp. *paratuberculosis*: phagosomal pH and fusogenicity in J774 macrophages compared with other mycobacteria. *Cell*. *Microbiol*. **3**, 551-566.

Kusner, D. J., Hall, C. F. and S. Jackson. (1999) Fcγ receptor-mediated activation of phospholipase D regulates macrophage phagocytosis of IgG-opsonized particles. *J. Immunol*. **162**, 2266-2274.

Labro, M-T. (2000) Interference of antibacterial agents with phagocyte functions: immunomodulation or "immuno-fairy tales?" *Clin*. *Microbiol*. *Rev*. **13**, 615-650.

Lacraz, S., Nicod, L. P., Chicheportiche, R., Welgus, H. G. and J-M. Dayer. (1995) IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. *J. Clin*. *Invest*. **96**, 2304-2310.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lah, T. T., Hawley, M., Rock, K. L. and A. L. Goldberg. (1995) Gamma-interferon causes a selective induction of the lysosomal proteases, cathepsins B and L, in macrophages. *FEBS Lett*. **363**, 85-89.

Lambert, E., Dassé, E., Haye, B., and E. Petitfrère. (2004) TIMPS as multifacial proteins. *Crit*. *Rev*. *Oncol*. *Hematol*. **49**, 187-198.

Leist, M. and M. Jäätelä. (2001) Triggering apoptosis by cathepsins. *Cell Death*. *Differ*. **8**, 324-326.

Lennon-Duménil, A-M., Roberts, R. A., Valentijn, K., Driessen, C., Overkleefdt, H. S., Erickson, A., Peters, P. J., Bikoff, E., Ploegh, H. L. and P. Wolf-Bryant. (2001) The p41 isoform of invariant chain is a chaperone for cathepsin L. *EMBO J*. **20**, 4055-4064.

Lennon-Duménil, A-M., Bakker, A. H., Maehr, R., Fiebiger, E., Overkleeft, H. S., Rosemblatt, M., Ploegh, H. L. and C. Lagaudriére-Gesbert. (2002a) Analysis of protease activity in live antigen-presenting cells shows regulation of the phagosomal proteolytic contents during dendritic cell activation. *J*. *Exp*. *Med*. **196**, 529-539.

Lennon-Duménil, A-M., Bakker, A. H., Wolf-Bryant, P., Ploegh, H. L. and C. Lagaudrière-Gesbert. (2002b) A closer look at proteolysis and MHC-class-II-restricted antigen presentation. *Curr*. *Opin*. *Immunol*. **14**, 15-21.

Lesser, M., Chang, J. C. and M. Orlowski. (1985) Cathepsin B and D activity in stimulated peritoneal macrophages. *Mol*. *Cell Biochem*. **69**, 67-73.

Linebaugh, B. E., Sameni, M., Day, N. A., Sloane, B. F. and D. Keppler. (1999) Exocytosis of active cathepsin B: enzyme activity at pH 7.0, inhibition and molecular mass. *Eur*. *J*. *Biochem*. **264**, 100-109.

Longin, A., Souchier, C., FFrench, M. and P-A., Bryon. (1993) Comparison of anti-fading agents used in fluorescence microscopy: image analysis and laser confocal microscopy study. *J*. *Histochem*. *Cytochem*. **41**, 1833-1840.

Lopez, M. F., Berggren, K., Chernokalskaya, E., Lazarev, A., Robinson, M. and W. F. Patton. (2000) A comparison of silver stain and SYPRO Ruby Protein Gel Stain with respect to protein detection in two-dimensional gels and indentification by peptide mass profiling. *Electrophoresis* **21**, 3673-3683.

Losch, U., Schranner, I. and R. Wanke. (1986) The chicken egg, an antibody source. *J*. *Vet*. *Med*. *B*. **33**, 609-619.

Lowrie, D. B., Andrew, P. W. and T. J. Peters. (1979) Analytical subcellular fractionation of alveolar macrophages from normal and BCG-vaccinated rabbits with particular reference to heterogeneity of hydrolase-containing granules. *Biochem*. *J*. **178**, 761-767.

Luster, A. D. (2002) The role of chemokines in linking innate and adaptive immunity. *Curr*. *Opin*. *Immunol*. **14**, 129-135.

Luzio, J. P., Rous, B. A., Bright, N. A., Pryor, P. R., Mullock, B. M. and R. C. Piper. (2000) Lysosome-endosome fusion and lysosome biogenesis. *J. Cell Sci*. **113**, 1515-1524.

Magez, S., Geuskens, M., Beschin, A., del Favero, H., Verschueren, H., Lucas,R., Pays, E. and P. de Baetselier. (1997) Specific uptake of tumor necrosis factor-alpha is involved in growth control of *Trypanosoma brucei*. *J. Cell Biol*. **137**, 715-727.

Mahmudi-Azer, S., Downey, G. P. and R. Moqbel. (2002) Translocation of the tetraspanin CD63 in association with human eosinophil mediator release. *Blood* **99**, 4039-4047.

Makowski G. S. and M. L. Ramsby. (1996) Calibrating gelatin zymograms with human gelatinase standards. *Anal*. *Biochem*. **236**, 353-356.

Martinez, I., Chakrabarti, S., Hellevik, T., Morehead, J., Fowler, K. and N. W. Andrews. (2000) Synaptotagmin VII regulates Ca^{2+}-dependent exocytosis of lysosomes in fibroblasts. *J. Cell Biol*. **148**, 1141-1149.

Mason, R. W., Johnson, B., Barret, A. J. and H. A. Chapman. (1986) The N-terminal amino acid sequences of the heavy and light chains of human cathepsin L. *Biochem*. *J*. **240**, 373-377.

May, R. C. and L. M. Machesky. (2001) Phagocytosis and the actin cytoskeleton. *J. Cell Sci*. **114**, 1061-1077.

McGrath, M. E. (1999) The lysosomal cysteine proteases. *Annu*. *Rev*. *Biophys*. *Biomol*. *Struct*. **28**, 181-204.

Mellman, I. (1992) The importance of being acid: the role of acidification in intracellular membrane traffic. *J. Exp*. *Biol*. **172**, 39-45.

Meresse, S., Gorvel, J. P. and P. Chavrier. (1995) The rab7 GTPase resides on a vesicular compartment connected to lysosomes. *J. Cell Sci*. 11, 3349-3358.

Merril, C. R. (1990) Gel staining techniques. In: Guide to Protein Purification Techniques. Methods in Enzymology vol. 182, (Deutscher, M. P. Ed.), Academic Press, New York, pp 477-488.

Moorewood, C. R., Elliott, E., Dennison, C. and A. G. Bruton. (1992) Further modification of the LKB 7800 series knifemaker for improved reproducibility in breaking 'cryo' knives. *J. Microsc*. **168**, 111-114.

Mørland, B. and A. Pedersen. (1979) Cathepsin B activity in stimulated mouse peritoneal macrophages. *Lab*. *Invest*. **41**, 379-384.

Morrissette, N. S., Gold, E. S., Guo, J., Hamerman, J. A., Ozinsky, Adrian, Bedian, V. and A. A. Aderem. (1999) Isolation and characterization of monoclonal antibodies directed against novel components of macrophage phagosomes. *J. Cell Sci*. **112**, 4705-4713.

Mosser, D. M. (2003) The many faces of macrophage activation. *J. Leukoc. Biol*. **73**, 209-212.

Mott, J. D., Thomas, C. L., Rosenbach, M. T., Takahara, K., Greenspan, D. S. and M. J. Banda. (2000) Post-translational proteolytic processing of procollagen C-terminal proteinase enhancer releases a metalloproteinase inhibitor. *J. Biol. Chem*. **275**, 1384-1390.

Muno, D., Sutoh, N., Watanabe, T., Uchiyama, Y. and E. Kominami. (1990) Effect of metabolic alterations on the density and the contents of cathepsins B, H and L of lysosomes in rat macrophages. *Eur. J. Biochem*. **191**, 91-98.

Murphy, R. F. (1991) Maturation models for endosome and lysosome biogenesis. *Trends Cell Biol*. **1**, 77-82.

Murphy, G. and A. J. Docherty. (1992) The matrix metalloproteinases and their inhibitors. *Am. J. Respir. Cell Mol. Biol*. 7, 120-125.

Murphy, G. and F. Willenbrock. (1995) Tissue inhibitors of matrix metalloproteinases. In: Proteolytic Enzymes: Aspartic and Metallo-peptidases. Methods in Enzymology, Vol 248, (Barret, A. J. Ed.), Academic press, San Diego, pp 496-510.

Murzin, A. G. (1993) OB (oligonucleotide/oligosaccharide binding)-fold: common structural and functional solution for non-homologous sequences. *EMBO J*. **12**, 861-867.

Nadkarni, V. D. and R. J. Linhardt. (1997) Enhancement of diaminobenzidine colorimetric signal in immunoblotting. *BioTechniques* **23**, 385-388.

Nagase, H. (1996) Matrix metalloproteinases. In: Zinc Metalloproteases in Health and Disease, (Hooper, N. M. Ed.), Taylor & Francis, London, pp 153-204.

Nagase, H. and J. F. Woessner. (1999) Matrix metalloproteinases. *J. Biol. Chem*. **274**, 21491-21494.

Nagayama, M., Sakamoto, S. and M. Sakamoto. (1984) Mouse bone collagenase inhibitor: purification and partial characterization of the inhibitor from mouse calvaria cultures. *Arch. Biochem. Biophys*. **228**, 653-659.

Nakagawara, A., Nathan, C. F. and Z. A. Cohn. (1981) Hydrogen peroxide metabolism in human monocytes during differentiation *in vitro*. *J. Clin. Invest*. **68**, 1243-1252.

Narat, M. (2003) Production of antibodies in chickens. *Food Technol*. *Biotechnol*. **41**, 259-267.

Nathan, C. F. (1987) Secretory products of macrophages. *J. Clin*. *Invest*. **79**, 319-326.

Netzer, K. O., Suzuki, K., Itoh, Y., Hudson, B. G. and R. G. Khalifah. (1998) Comparative analysis of the noncollagenous NC1 domain of type IV collagen: identification of structural features important for assembly, function, and pathogenesis. *Protein Sci*. **7**, 1340-1351.

Newman, S. L. (1999) Macrophages in host defense against *Histoplasma capsulatum*. *Trends Microbiol*. **7**, 67-71.

Nichols, B. A., Bainton D. F. and M. G. Farquhar. (1971) Differentiation of monocytes. Origin, nature and fate of their azurophil granules. *J. Cell Biol*. **50**, 498-515.

Niedergang, F. and P. Chavrier. (2004) Signaling and membrane dynamics during phagocytosis: many roads lead to the phagos(R)ome. *Curr*. *Opin*. *Cell Biol*. **16**, 422-428.

Ninfa, A. J. and D. P. Ballou. (1998) Gel electrophoresis of proteins. In: Fundamental Laboratory Approaches for Biochemistry and Biotechnology. Fitzgerald Science Press, Inc., Maryland, pp 125-146.

Ogrinc, T., Dolenc, I., Ritonja, A. and V. Turk. (1993) Purification of the complex of cathepsin L and the MHC class II-associated invariant chain fragment from the human kidney. *FEBS Lett*. **336**, 555-559.

Oh, J., Takahashi, R., Kondo, S., Mizoguchi, A., Adachi, E., Sasahara, R. M., Nishimura, S., Imamura, Y., Kitayama, H., Alexander, D. B., Ide, C., Horan, T. P., Arakawa, T., Yoshida, H., Nishikawa, S-I., Itoh, S., Takahashi, C. and M. Noda. (2001) The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. *Cell* **107**, 789-800.

Okada, Y., Naka, K., Kawamura, K., Matsumoto, T., Nakanishi, I., Fujimoto, N., Sato, H. and M. Seiki. (1995) Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase = gelatinase B) in osteoclasts: implications for bone resorption. *Lab*. *Invest*. **72**, 311-322.

Oliver, G. W., Leferson, J. D., Stetler-Stevenson, W. G. and D. E. Kleiner. (1997) Quantitative reverse zymography: analysis of picogram amounts of metalloproteinase inhibitors using gelatinase A and B reverse zymograms. *Anal. Biochem*. **244**, 161-166.

Ono, M., Murakami, T., Kudo, A., Isshiki, M., Sawada, H. and A. Segawa. (2001) Quantitative comparison of anti-fading mounting media for confocal laser scanning microscopy. *J*. *Histochem*. *Cytochem*. **49**, 305-311.

Oppenheim, J. J. and E. J. Leonard (1989) Human Monocytes (Zemabala, M. and G. L. Asherson Eds.), Academic Press, London, pp 1-6.

Osiewicz, K., McGarry, M. and P. D. Soloway. (1999) Hyper-resistance to infection in TIMP-1 deficient mice is neutrophil dependent but not immune cell autonomous. *Ann*. NY *Acad*. *Sci*. **878**, 494-496.

Owen, C. A. (2005) Proteinases and oxidants as targets in the treatment of chronic obstructive pulmonary disease. *Proc*. *Am*. *Thorac*. *Soc*. **2**, 373-385.

Owen, C. A. and E. J. Campbell. (1999) The cell biology of leukocyte-mediated proteolysis. *J*. *Leukoc*. *Biol*. **65**, 137-150.

Owen, C. A., Hu, Z., Barrick, B. and S. D. Shapiro. (2003) Inducible expression of tissue inhibitor of metalloproteinases-resistant matrix metalloproteinase-9 on the cell surface of neutrophils. *Am*. *J*. *Respir*. *Cell Mol*. *Biol*. **29**, 283-294.

Parks, W. C. and S. D. Shapiro. (2001) Matrix metalloproteinases in lung biology. *Respir*. *Res*. **2**, 10-19.

Peiser, L., Gough, P. J., Darley, E. and S. Gordon. (2000) Characterization of macrophage antigens and receptors by immunochemistry and fluorescent analysis: expression, endocytosis, and phagocytosis. In: Macrophages (Paulnock, D. M. Ed.), Oxford University Press, Oxford, pp 61-91.

Peters, T. J. (1976) Analytical subcellular fractionation of jejunal biopsy specimens: methodology and characterization of the organelles in normal tissue. *Clin*. *Sci*. *Mol*. *Med*. **51**, 557-574.

Peters, T. J., Müller, M. and C. de Duve. (1972) Lysosomes of the arterial wall. Isolation and subcellular fractionation of cells from normal rabbit aorta. *J*. *Exp*. *Med*. **136**, 1117-1139.

Philimonenko, V. V., Janacek, J. and P. Hozak. (2003) LR White is preferable to Unicryl for immunogold detection of fixation-sensitive nuclear antigens. *Eur*. *J*. *Histochem*. **46**, 359-364.

Pierce, R. A., Sandefur, S., Doyle, G. A. R. and H. G. Welgus. (1996) Monocytic cell type-specific transcriptional induction of collagenase. *J*. *Clin*. *Invest*. **97**, 1890-1899.

Pike, R. N., Coetzer, T. H. T. and C. Dennison. (1992) Proteolytically active complexes of cathepsin L and a cysteine proteinase inhibitor; purification and demonstration of their formation *in vitro*. *Arch*. *Biochem*. *Biophys*. **294**, 623-629.

Pike, R. N. (1990) A study of the proteinase, cathepsin L, in the context of tumour invasion. MSc Thesis, University of Natal.

Pillay, C. S., Elliott. E. and C. Dennison. (2002) Endolysosomal proteolysis and its regulation. *Biochem*. *J*. **363**, 417-429.

Pittis, M. G., Muzzolini, L., Giulianini, P. G. and R. C. Garcia. (2003) Mycobacteria-containing phagosomes associate less annexins I, VI, VII and XI, but not II, concomitantly with a diminished phagolysosomal fusion. *Eur*. *J. Cell Biol*. **82**, 9-17.

Plesner, T., Ploug, M., Ellis, V., Rønne, E., Høyer-Hansen, G., Wittrup, M., Pedersen, T. L., Tscherning, T., Danø, K. and N. E. Hansen. (1994) The receptor for urokinase-type plasminogen activator and urokinase is translocated from two distinct intracellular compartments to the plasma membrane on stimulation of human neutrophils. *Blood* **83**, 808-815.

Polson, A., Coetzer, T., Kruger, J., von Maltzen, E. and K. J. van der Merwe. (1985) Improvement in the isolation of IgY from the yolks of eggs laid by immunised hens. *Immunol*. *Invest*. **14**, 323-327.

Portnoy, D. A., Erickson, A. H., Kochan, J., Ravetch, J. V. and J. C. Unkeless. (1986) Cloning and characterization of a mouse cysteine proteinase. *J*. *Biol*. *Chem*. **261**, 14697-14703.

Prada-Delgado, A., Carrasco-Marin, E., Bokoch, G. M. and C. Alvarez-Dominguez. (2001) Interferon-gamma listericidal action is mediated by novel Rab5a functions at the phagosomal environment. *J*. *Biol*. *Chem*. **276**, 19059-19065.

Price, B., Dennison, C., Tschesche, H. and E. Elliott. (2000) Neutrophil tissue inhibitor of matrix metalloproteinases-1 occurs in novel vesicles that do not fuse with the phagosome. *J*. *Biol*. *Chem*. **275**, 28308-28315.

Punturieri, A., Filippov, S., Allen, E., Caras, I., Murray, R., Reddy, V. and S. J. Weiss. (2000) Regulation of elastinolytic cysteine proteinase activity in normal and cathepsin K-deficient human macrophages. *J*. *Exp*. *Med*. **192**, 789-799.

Rabinovitch, M. (1995) Professional and non-professional phagocytes: an introduction. *Trends Cell Biol*. **5**, 85-87.

Rabinowitz, S., Horstmann, H., Gordon, S. and G. Griffiths. (1992) Immunocytochemical characterization of the endocytic and phagolysosomal compartments in peritoneal macrophages. *J*. *Cell Biol*. **116**, 95-112.

Ralph, P. and I. Nakoinz. (1975a) Phagocytosis and cytolysis by a macrophage tumour and its cloned cell line. *Nature* **257**, 393-394.

Ralph, P., Prichard, J. and M. Cohn. (1975b) Reticulum cell sarcoma: and effector cell in antibody-dependent cell-mediated immunity. *J*. *Immunol*. **114**, 898-905.

Ralph, P. and I. Nakoinz. (1977a) Direct toxic effects of immunopotentiators on monocytic, myelomonocytic, and histiocytic or macrophage tumor cells in culture. *Cancer Res*. **37**, 546-550.

Ralph, P. and I. Nakoinz. (1977b) Antibody-dependent killing of erythrocyte and tumour targets by macrophage-related cell lines: enhancement by PPD and LPS. *J. Immunol*. **119**, 950-954.

Reddy, V. Y., Zhang, Q-Y. and S. J. Weiss. (1995) Pericellular mobilization of the tissue-destructive cysteine proteinases, cathepsins B, L, and S, by human monocyte-derived macrophages. *Proc*. *Natl*. *Acad*. *Sci*. *USA* **92**, 3849-3853.

Reilly, J. J., Mason, R. W., Chen, P., Joseph, L. J., Sukhatme, V. P., Yee, R. and H. A. Chapman. (1989) Synthesis and processing of cathepsin L, an elastase, by human alveolar macrophages. *Biochem*. *J*. **257**, 493-498.

Reilly, J. J. Jr., Sailor, L. Z., Chen, P., Mason, R. W. and H. A. Chapman. (1991) Cigarette smoking induces an elastolytic cysteine proteinase in macrophages distinct from cathepsin L. *Am*. *J*. *Physiol*. **261**, L41-L48.

Remacle, A., Murphy, G. and C. Roghi. (2003) Membrane type-1 matrix metalloproteinase (MT1-MMP) is internalised by two different pathways and is recycled to the cell surface. *J. Cell Sci*. **116**, 3905-3916.

Remacle, A. G., Rozanov, D. V., Baciu, P. C., Chekanov, A. V., Golubkov, V. S. and A. Y. Strongin. (2005) The transmembrane domain is essential for microtubular trafficking of membrane type-1 matrix metalloproteinase (MT1-MMP). *J. Cell Sci*. **118**, 4975-4984.

Riese, R. J. and H. A. Chapman. (2000) Cathepsins and compartmentalization in antigen presentation. *Curr*. *Opin*. *Immunol*. **12**, 107-113.

Rivera-Marrero, C. A., Stewart, J., Shafer, W. M. and J. Roman. (2004) The down-regulation of cathepsin G in THP-1 monocytes after infection with *Mycobacterium tuberculosis* is associated with increased intracellular survival of bacilli. *Infect*. *Immun*. **72**, 5712-5721.

Roberg, K., Kagedal, K. and K. Ollinger. (2002) Microinjection of cathepsin D induces caspase-dependent apoptosis in fibroblasts. *Am*. *J*. *Pathol*. **161**, 89-96.

Roitt, I. (1997) Roitt's Essential Immunology, 9th Edition, Blackwell Science, London, pp 6-8.

Rosenberger, C. M., Gallo, R. L. and B. B. Finlay. (2004) Interplay between antibacterial effectors: a macrophage antimicrobial peptide impairs intracellular *Salmonella* replication. *Proc*. *Natl*. *Acad*. *Sci*. *USA* **101**, 2422-2427.

Ross, J. A. and M. J. Auger. (2002) The biology of the macrophage. In: The Macrophage 2nd Edition (Burke, B. and C. E. Lewis Eds.), Oxford University Press, Oxford, pp 1-72.

Russ, C., Callegaro, I., Lanza, B. and S. Ferrone. (1983) Purification of IgG monoclonal antibodies by caprylic acid precipitation. *J. Immunol. Methods* **65**, 269-271.

Russell, R. E. K., Culpitt, S. V., DeMatos, C., Donnelly, L., Smith, M., Wiggins, J. and P. J. Barnes. (2002a) Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am. J. Respir. Cell Mol. Biol.* **26**, 602-609.

Russell, R. E. K., Thorley, A., Culpitt, S. V., Dodd, S., Donnelly, L. E., Demattos, C., Fitzgerald, M. and P. J. Barnes. (2002b) Alveolar macrophage-mediated elastolysis: roles of matrix metalloproteinases, cysteine, and serine proteases. *Am. J. Physiol. Lung Cell Mol. Physiol.* **283**, 867-873.

Sagne, C., Agulhorn, C., Ravassard, P., Darmon, M., Hamon, M., Mestikawy, S. E., Gasnier, B. and B. Giros. (2001) Identification and characterization of a lysosomal transporter for small neutral amino acids. *Proc. Natl. Acad. Sci. USA* **98**, 7206-7211.

Salvesen, G. S. (2001) A lysosomal protease enters the death scene. *J. Clin. Invest.* **107**, 21-22.

Salvesen, G. S. (2004) Cathepsin G. In: Handbook of Proteolytic Enzymes, 2nd Edition, Volume 1, (Barrett, A. J., Rawlings, N. D. and J. F. Woessner Eds.), Elsevier Academic Press, London, 1524-1526.

Sang, Q. X., Birkedal-Hansen, H. and H. E. Van Wart. (1995) Proteolytic and non-proteolytic activation of human neutrophil progelatinase B. *Biochim. Biophys. Acta.* **1251**, 99-108.

Santama, N., Krijnse-Locker, J., Griffiths, G., Noda, Y., Hirokawa, N. and C. G. Dotti. (1998) KIF2β, a new kinesin superfamily protein in non-neuronal cells, is associated with lysosomes and may be implicated in their centrifugal translocation. *EMBO. J.* **17**, 5855-5867.

Schmid, H., Sauerbrei, R., Schwarz, G., Weber, E., Kalbacher, H. and C. Driessen. (2002) Modulation of the endosomal and lysosomal distribution of cathepsins B, L and S in human monocytes/macrophages. *Biol. Chem.* **383**, 1277-1283.

Schurigt, U., Stopfel, N., Hückel, M., Pfirschke, C., Wiederanders, B. and R. Bräuer. (2005) Local expression of matrix metalloproteinases, cathepsins, and their inhibitors during the development of murine antigen-induced arthritis. *Arthritis Res. Ther.* **7**, R174-R188.

Selsted, M. E. and A. J. Ouellette. (1995) Defensins in granules of phagocytic and non-phagocytic cells. *Trends Cell Biol*. **5**, 114-119.

Shapiro, S. D., Campbell, E. J., Kobayashi, D. K. and H. G. Welgus. (1991) Dexamethasone selectively modulates basal and lipopolysaccharide-induced metalloporteinase and tissue inhibitor of metallproteinase production by human alveolar macrophages. *J. Immunol*. **146**, 2724-2729.

Shapiro, S. D., Kobayashi, D. K. and H. G. Welgus. (1992) Identification of TIMP-2 in human alveolar macrophages. *J. Biol. Chem*. **267**, 13890-13894.

Shapiro, S. D. (1998) Matrix metalloproteinase degradation of extracellular matrix: biological consequences. *Curr. Opin. Cell Biol*. **10**, 602-608.

Shi, G-P., Munger, J. S., Meara, J. P., Rich, D. H. and H. A. Chapman. (1992) Molecular cloning and expression of human alveolar macrophage cathepsin S, an elastinolytic cysteine protease. *J. Biol. Chem*. **267**, 7258-7262.

Shi, G-P., Chapman, H. A., Bhairi, S. M., DeLeeuw, C., Reddy, V. Y. and S. J. Weiss (1995) Molecular cloning of human cathepsin O, a novel endoproteinase and homologue of rabbit OC-2. *FEBS Lett*. **357**, 129-134.

Shi, G-P., Villadangos, J. A., Dranoff, G., Small, C., Gu, L., Haley, K. J., Riese, R., Ploegh, H. L. and H. A. Chapman. (1999) Cathepsin S required for normal MHC class II peptide loading and germinal center development. *Immunity* **10**, 197-206.

Shi, G-P., Bryant, R. A. R., Riese, R., Verhelst, S., Driessen, C., Li, Z., Bromme, D., Ploegh, H. L. and H. A. Chapman. (2000) Role for cathepsin F in invariant chain processing and major histocompatibility complex class II peptide loading by macrophages. *J. Exp. Med*. **191**, 1177-1185.

Shibata, Y., Abiko, Y. and H. Takiguchi. (1991) Megakaryocytes, Platelets, Macrophages, and Eosinophils (Harris, J. R. Ed.), Plenum Press, New York, pp 209-236.

Shipley, J. M., Wesselschmidt, R. L., Kobayashi, D. K., Ley, T. J., and S. D. Shapiro. (1996) Metalloelastase is required for macrophage-mediated proteolysis and matrix invasion in mice. *Proc. Natl. Acad. Sci. USA* **93**, 3942-3946.

Slot, J. W. and H. J. Geuze. (1985) A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol*. **38**, 87-93.

Smith, B. J. (1984) SDS polyacrylamide gel electrophoresis of proteins. In: Methods in Molecular Biology, Volume 32 Proteins (Walker, J. M. Ed.), The Humana Press Inc., New Jersey, pp 41-55.

Somerville, R. P. T., Oblander, S. A. and S. S. Apte. (2003) Matrix metalloproteinases: old dogs with new tricks. *Genome Biology* **4**, 216.1-216.11.

Sorsa, T., Ding, Y., Salo, T., Lauhio, A., Teronen, O., Ingman, T., Ohtani, H., Andoh, N., Takeha, S. and Y. T. Konttinen. (1994) Effects of tetracyclines on neutrophil, gingival, and salivary collagenases. *Ann. N. Y. Acad. Sci.* **732**, 112-131.

Stachura, J. (1989) Cytochemistry of monocytes and macrophages. In: Human Monocytes, (Zembala, M. and G. L. Asherson Eds.), Academic Press, London, 27-36.

Stefanidakis, M., Ruohtula, T., Borregaard, N., Gahmberg, C. G. and E. Koivunen. (2004) Intracellular and cell surface localization of a complex between $\alpha_M\beta_2$ integrin and promatrix metalloproteinase-9 progelatinase in neutrophils. *J. Immunol.* **172**, 7060-7068.

Stein, M., Keshav, S., Harris, N. and S. Gordon. (1992) Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J. Exp. Med.* **176**, 287-292.

Steinberg, T. H., Haugland, R. P. and V. L. Singer. (1996) Applications of SYPRO orange and SYPRO red protein gel stains. *Anal. Biochem.* **239**, 238-245.

Sternlicht, M. D. and Z. Werb. (2001) How matrix metalloproteinases regulate cell behaviour. *Annu. Rev. Cell Dev. Biol.* **17**, 463-516.

Stinchcombe, J. C. and G. M. Griffiths. (1999) Regulated secretion from hemopoietic cells. *J. Cell Biol.* **147**, 1-5.

Sun-Wada, G-H., Wada, Y. and M. Futai. (2003) Lysosome and lysosome-related organelles responsible for specialized functions in higher organisms, with special emphasis on vacuolar-type proton ATPase. *Cell Struct. Funct.* **28**, 455-463.

Sukhova, G. K., Zhang, Y., Pan, J. H., Wada, Y., Yamamoto, T., Naito, M., Kodama, T., Tsimikas, S., Witztum, J. L., Lu, M. L., Sakara, Y., Chin, M. T., Libby, P. and G. P. Shi. (2003) Deficiency of cathepsin S reduces atherosclerosis in LDL receptor-deficient mice. *J. Clin. Invest.* **111**, 897-906.

Swanson, J., Bushnell, A. and S. C. Silverstein. (1987) Tubular lysosome morphology and distribution within macrophages depend on the integrity of cytoplasmic microtubules. *Proc. Natl. Acad. Sci. USA* **84**, 1921-1925.

Sweet, M. J. and D. A. Hume. (1996) Endotoxin signal transduction in macrophages. *J. Leukoc. Biol.* **60**, 8-26.

Switzer, R. L. and L. F. Garrity. (1999) Electrophoresis. In: Experimental Biochemistry, W. H. Freeman and Company, New York, pp 61-77.

Takahashi, T. and J. Tang. (1983) Amino acid sequence of porcine spleen cathepsin D light chain. *J. Biol. Chem.* **258**, 6435-6443.

Takahashi, H., Isihdoh, K., Muno, D., Ohwada, A., Nukiwa, T., Kominami, E. and S. Kira. (1993) Cathepsin L activity is increased in alveolar macrophages and bronchoalveolar lavage fluid of smokers. *Am. Rev. Respir. Dis.* **147**, 1562-1568.

Tanaka, Y., Guhde, G., Suter, A., Eskelinen, E-L. and P. S. Hart. (2000) Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2 deficient mice. *Nature* **406**, 902-906.

Tanke, H. J. (1998) Fluorescence microscopy. In: Cell Biology A Laboratory Handbook, Volume 3, (Celis, J. E., Ed.), Academic Press, San Diego, pp 64-69.

Tapper, H. (1996) The secretion of preformed granules by macrophages and neutrophils. *J. Leukoc. Biol.* **59**, 613-622.

Taylor, D. L. and Salmon, E. D. (1989) Basic fluorescence microscopy. In: Fluorescence Microscopy of Living Cells in Culture, Part A, Fluorescent Analogs, Labeling Cells, and Basic Microscopy, Methods in Cell Biology 29, Academic Press, San Diego, 207-237.

Thorne, K. J. I., Oliver, R. C. and A. J. Barrett. (1976) Lysis and killing of bacteria by lysosomal proteinases. *Infect. Immun.* **14**, 555-563.

Tjelle, T. E., Brech, A., Juvet, L. K., Griffiths, G. and T. Berg. (1996) Isolation and characterization of early endosomes, late endosomes and terminal lysosomes: their role in protein degradation. *J. Cell Sci.* **109**, 2905-2914.

Tjelle, T. E., Lovdal, T. and T. Berg. (2000) Phagosome dynamics and function. *BioEssays* **22**, 255-263.

Toomes, C., James, J., Wood, A. J., Wu, C. L., McCormick, D., Lench, N., Hewitt, C., Moynihan, L., Roberts, E., Woods, C. G., Markham, A., Wong, M.m Widmer, R., Ghaffar, K. A., Pemberton, M., Hussein, I. R., Temtamy, S. A., Davies, R., Read, A. P., Sloan, P., Dixon, M. J., and N. S. Thakker. (1999) Loss-of-function mutations in the cathepsin C gene result in periodontal disease and palmoplantar keratosis. *Nat. Genet.* **23**, 421-424.

Toth, M., Bernardo, M. M., Gervasi, D. C., Soloway, P. D., Wang, Z., Bigg, H. F., Overall, C. M., DeClerck, Y. A., Tschesche, H., Cher, M. L., Brown, S., Mobashery, S. and R. Fridman. (2000) Tissue inhibitor of metalloproteinase (TIMP)-2 acts synergistically with synthetic matrix metalloproteinase (MMP) inhibitors but not with TIMP-4 to enhance the (membrane type 1)-MMP-dependent activation of pro-MMP-2. *J. Biol. Chem.* **275**, 41415-41423.

Towbin, H., Staehlin, T., and J. Gordon. (1979) Electrophoresis transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

Trask, B. C., Malone, M. J., Lum, E. H., Welgus, H. G., Crouch, E. C. and S. D. Shapiro. (2001) Induction of Macrophage Matrix Metalloproteinase Biosynthesis by Surfactant Protein D. *J. Biol. Chem.* **276**, 37846-37852.

Tremblay, P., Houde, M., Arbour, N., Rochefort, D., Masure, S., Mandeville, R., Opdenakker, G. and D. Oth. (1995) Differential effects of PKC inhibitors on gelatinase B and interleukin 6 production in the mouse macrophage. *Cytokine* **7**, 130-136.

Triebel, S., Bläser, J., Gote, T., Günter, P., Schüren, E., Schmitt, M and H. Tschesche. (1995) Evidence for the tissue inhibitor of metalloproteinases-1 (TIMP-1) in human polymorphonuclear leukocytes. *Eur. J. Biochem.* **231**, 714-719.

Turk, B., Turk, V. and D. Turk. (1997) Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. *Biol. Chem.* **378**, 141-150.

Turk, B., Turk, D. and V. Turk. (2000) Lysosomal cysteine proteases: more than scavengers. *Biochim. Biophys. Acta.* **1477**, 98-111.

Turk, V., Turk, B. and D. Turk. (2001) Lysosomal cysteine proteases: facts and opportunities. *EMBO J.* **20**, 4629-4633.

Utermohlen, O., Karow, U., Lohler, J. and M. Kronke. (2003) Severe impairment in early host defense against *Listeria monocytogenes* in mice deficient in acid sphingomyelinase. *J. Immunol.* **170**, 2621-2628.

Van Dam, A. (1994) Transfer and blocking conditions in immunoblotting. In: Protein Transfer a Practical Approach, (Dunbar, B. S. Ed.), Oxford University Press, Oxford, 73-85.

Van Oss, C. J. (1986) Phagocytosis: an overview. In: Immunochemical Techniques Part J – Phagocytosis and Cell-mediated Cytotoxicity, Methods in Enzymology vol. 132 (DiSabato, G. and J. Everse, Eds.), Academic Press, Orlando, pp 3-15.

Varesio, L., Bosco, M. C., Carta, L. and T. Musso (2000) Analysis of macrophage lytic functions. In : Macrophages (Paulnock, D. M. Ed.), Oxford University Press, Oxford, 127-155.

Via, L. E., Deretic, D., Ulmer, R. J., Hibler, N. S., Huber, L. A. and V. Deretic. (1997) Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. *J. Biol. Chem.* **272**, 13326-13331.

Via, L. E., Fratti, R. A., Mcfalone, M., Pagán-Ramos, E., Deretic, D. and V. Deretic. (1998) Effects of cytokines on mycobacterial phagosome maturation. *J. Cell Sci*. **111**, 897-905.

Vieira, O. V., Botelho, R. J. and S. Grinstein. (2002) Phagosome maturation: aging gracefully. *Biochem*. *J*. **366**, 689-704.

Visse, R. and H. Nagase. (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ*. *Res*. **92**, 827-839.

Warren, H. S., Vogel, F. R. and L. A. Chedid. (1986) Current status of immunological adjuvants. *Annu*. *Rev*. *Immunol*. **4**, 369-388.

Welgus, H. G., Campbell, E. J., Bar-Shavit, Z., Senior, R. M. and S. L. Teitelbaum. (1985) Human alveolar macrophages produce a fibroblast-like collagenase and collagenase inhibitor. *J. Clin*. *Invest*. **76**, 219-224.

Welgus, H. G., Connolly, N. L. and R. M. Senior. (1986) 12-o-tetradecanoyl-phorbol-13-acetate-differentiated U937 cells express a macrophage-like profile of neutral proteinases. *J. Clin*. *Invest*. **77**, 1675-1681.

Welgus, H. G., Campbell, E. J., Cury, J. D., Eisen, A. Z., Senior, R. M., Wilheim, S. M. and G. I. Goldberg. (1990) Neutral metalloproteinases produced by human mononuclear phagocytes. Enzyme profile, regulation, and expression during cellular development. *J. Clin*. *Invest*. **86**, 1496-1502.

White, B. H. and L. K. Kaczmarek. (1997) Identification of a vesicular pool of calcium channels in the bag cell neurons of *Aplysia californica*. *J. Neurosci*. **17**, 1582-1595.

Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A. and G. I. Goldberg. (1989) SV 40-transfromed human lung fibroblasts secrete a 92 kDa type IV collagenase which is identical to that secreted by normal macrophages. *J. Biol*. *Chem*. **264**, 17213-17221.

Willstätter, R. and E. Bamann. (1929) Uber die proteasen der magenschleimhaut. *Hoppe-Seyler's Z. Physiol*. *Chem*. **180**, 127-143.

Woessner, J. F., Jr. (1994) The family of matrix metalloproteinases. *Ann*. *N. Y. Acad*. *Sci*. **732**, 11-21.

Woessner, J. F. and H. Nagase. (2002) Matrix Metalloproteinases and TIMPs, (Sheterline, P. Ed.), Oxford University Press, Oxford.

Wolters, P. J. and H. A. Chapman. (2000) Importance of lysosomal cysteine proteases in lung tissue. *Respir*. *Res*. **1**, 170 – 177.

Wubbolts, R., Fernandez-Borja, M., Oomen, L., Verwoerd, D., Janssen, H., Calafat, J., Tulp, A., Dusseljee, S. and J. Neefjes. (1996) Direct vesicular transport of MHC class II molecules from lysosomal structures to the cell surface. *J. Cell Biol*. **135**, 611-622.

Xie, B., Dong, Z. and I. J. Fidler. (1994) Regulatory mechanisms for the expression of type IV collagenases/gelatinases in murine macrophages. *J. Immunol*. **152**, 3637-3644.

Zeng, G. and A. J. Millis. (1994) Expression of 72-kDa gelatinase and TIMP-2 in early and late passage human fibroblasts. *Exp*. *Cell Res*. **213**, 148-155.

Zheng, T., Zhu, Z., Wang, Z., Homer, R. J., Ma, B., Riese, R. J. Jr., Chapman, H. A. Jr., Shapiro, S. D. and J. A. Elias. (2000) Inducible targeting of IL-13 to the adult lung causes matrix metalloproteinase- and cathepsin-dependent emphysema. *J. Clin*. *Invest*. **106**, 1081-1093.

Zor, T. and Z. Selinger. (1996) Linearisation of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Anal*. *Biochem*. **236**, 302-308.

Zuo, F., Kaminski, N., Eugui, E., Allard, J., Yakhini, Z., Ben-Dor, A., Lollini, L., Morris, D., Kim, Y., DeLustro, B., Sheppard, D., Pardo, A., Selman, M. and R. Heller. (2002) Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans. *Proc*. *Natl*. *Acad*. *Sci*. *USA* **99**, 6292-6297.

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