Regulation of SNARE proteins in macrophages by colony stimulating factor-1

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Submitted in total fulfilment of the requirements of the degree of Doctor of Philosophy

October 2007

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Abstract

Macrophages serve key roles in host defence by initiating inflammatory responses to infection and/or injury. They contribute to innate immunity by secreting a range of pro-inflammatory cytokines (e.g. TNF and IL-6) upon activation as well as by phagocytosing pathogens and dead cells, which is necessary for the resolution of inflammation and effective wound repair. Macrophages also contribute to adaptive immunity by functioning as antigen presenting cells.

Colony stimulating factor 1 (CSF-1) is the major growth factor governing the differentiation, proliferation and survival of macrophages. Although not as well appreciated, CSF-1 also regulates some of the immune functions of macrophages, such as cytokine secretion and phagocytosis. However, the mechanisms by which CSF-1 governs the immune functions of macrophages are poorly understood. Cytokine secretion, phagocytosis and antigen presentation involve various vesicle trafficking and membrane fusion events, processes in which SNARE proteins play vital roles. Thus, the hypothesis tested in this thesis was that CSF-1 modulates the immune functions of macrophages by regulating the expression and/or activity of SNARE proteins that regulate endocytic and exocytic processes.

In this study, the endosomal SNARE protein syntaxin 7 was identified, via microarray analysis, as a CSF-1 inducible gene in primary mouse macrophages. Syntaxin 7 has previously been detected in phagosomal membranes in macrophages. Furthermore, syntaxin 7 has recently been implicated in the secretion of cytokines (e.g. TNF) from macrophages by forming a novel complex with syntaxin 6, Vti1b and VAMP3. These prior studies suggest that syntaxin 7 is likely to serve a central role in mediating the immune functions of macrophages.

Bioinformatic analysis of the promoter region of the syntaxin 7 gene identified binding sites for several transcription factors that may explain the ability of CSF-1 to regulate syntaxin 7 gene expression. Analysis of web-available microarray data suggested that syntaxin 7 was highly expressed in macrophages. In addition to regulating syntaxin 7 expression, CSF-1 also induced the rapid phosphorylation of syntaxin 7. Interestingly, syntaxin 7 was phosphorylated on serine residues rather

than tyrosine, as is commonly seen with CSF-1 signalling. The CSF-1-induced phosphorylation of syntaxin 7 was primarily dependent on the activity of phosphatidylinositol-3 kinase (PI3-K). Pharmacological inhibitors of protein kinases that signal downstream of PI3-K, such as Akt and protein kinase C (PKC), suppressed the CSF-1-induced phosphorylation of syntaxin 7. Significantly, CSF-1 enhanced the assembly of an endosomal SNARE complex comprised of syntaxin 7, syntaxin 8, Vti1b and VAMP8, whereas inhibition of PI3-K activity reduced the levels of this SNARE complex. Confocal microscopy-based studies demonstrated the presence of syntaxin 7 in early and late endosomes. This finding suggests syntaxin 7 may have functional roles in both early and late endocytic pathways in macrophages. CSF-1 did not appear to influence the subcellular localisation of syntaxin 7, nor did it affect its co-localisation with syntaxin 8 or Vti1b. Thus, CSF-1 may regulate the assembly rather than the localisation of endosomal SNARE complexes in macrophages.

Both PI3-K and PKC have previously been shown to be important for vesicle trafficking and membrane fusion in various cell types. The ability to PI-3K and PKC to regulate syntaxin 7 phosphorylation, and hence the formation of endosomal SNARE complexes, may help to explain their importance for membrane fusion events. The findings presented here should provide the basis for further studies on the fundamental roles played by SNARE proteins in mediating the immune functions of macrophages.

Declaration

This is to certify that:

(i) the thesis comprises only my original work towards the PhD,

(ii) due acknowledgement has been made in the text to all other material used

(iii) the thesis is less than 100,000 words in length, exclusive of tables, figures and bibliographies.

Adrian Achuthan
October 2007

Acknowledgements

I am extremely thankful to my supervisor Dr Glen Scholz for his guidance and support throughout the course of my PhD candidature. Glen has meticulously reviewed my thesis, moulded my ideas and readily imparted his invaluable knowledge to me. I am also grateful to my co-supervisors Professor John Hamilton and Dr Matthew Sweet for their intellectual insights and guidance in writing my thesis. In particular, I acknowledge Dr Matthew Sweet for performing microarray experiments.

I would like to extend my gratitude to all members of the Hamilton group, past and present, whose technical expertise and support greatly enhanced my learning experience. In particular, I would like to thank Paul Masendycz and for satisfying all my technical queries in a timely manner, Andrew Fleetwood for his expertise on real-time PCR experiments and ELISA assays, Hang Dinh, Alice Holloway and Dean Hewish for flow cytometry analysis, Thao Nguyen and Lena Cross for their mass spectrometry expertise, John Roiniotis and Mark Bailey for their expertise in gel filtration chromatography. I am thankful to Jenny Davis for her assistance with supplying experimental animals.

I am also grateful to Associate professor Heung-Chin Cheng (Bio21, Melbourne) for his expertise on phospho amino acid analysis and Professor Paul Gleeson and Fiona Houghton (Bio21, Melbourne) for their expertise on confocal microscopy. With deep appreciation, I acknowledge Professor David James (Garvan Institute, Sydney) for his insightful ideas and kind encouragement.

Many thanks to members of the Department of Medicine for making it a friendly and enjoyable learning environment. In particular I wish to thank my fellow students, whose vivacity and support made my journey a pleasurable experience. In particular, thanks to Dominic De Nardo for always lending a helping hand. I would also like to acknowledge the University of Melbourne for providing the Melbourne Research Scholarship and the Cooperative Research Centre for Chronic Inflammatory Diseases for supporting my research.

Finally I extend my sincere thanks to my family and friends, whose loving support and understanding made this work possible.

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Abbreviations

AP-1	activating protein-1
APC	adaptor protein complex
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
Ca^{2+}	calcium
CAGE	Cap-analysis gene expression
CaMK II	Ca^{2+}/calmodulin kinase II
cAMP	3'5'-cyclic adenosine monophosphate
CCL	CC chemokine ligand
CCV	clathrin-coated vesicle
CD	cluster of differentiation
Cdc2	cell-division cycle 2-like kinase
cDNA	complementary deoxyribonucleic acid
CIP	calf intestinal alkaline phosphatase
CKII	casein kinase II
CLR	C-type lectin receptor
CpG	cytosine phosphate guanine
CR	complement receptor
CRD	carbohydrate recognition domain
CSF-1	colony-stimulating factor 1
CSF-1R	colony-stimulating factor 1 receptor
C-terminal	carboxyl-terminal
CXCL	CXC chemokine ligand
DAG	diacylglycerol
DAPK	death-associated protein kinase
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECR	evolutionarily conserved region
EDTA	ethylenediaminetetraacetic acid

EE	early endosome
EEA1	early endosome antigen 1
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
Fc	fragment, crystallizable
Fcγ R	Fc gamma receptor
FCS	foetal calf serum
FRET	fluorescence resonance energy transfer
GDI	GDP-disassociation inhibitor
GDP	guanine diphosphate
GFC	gel filtration column chromatography
GFP	green fluorescent protein
Glut	glucose transporter
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
GPI	glycosylphosphatidyl-inositol
GSK3	glycogen synthase kinase 3
GTP	guanine triphosphate
GTPase	guanine triphosphatase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	hydrogen peroxide
h	hour(s)
HRP	horseradish peroxide
IEF	isoelectric focussing
IFN	interferon
IFNAR	interferon alpha receptor
Ig	immunoglobulin
IKK	I kappa B kinase
IL	interleukin
IP	immunoprecipitation
IRAK	interleukin-1 receptor associated kinases
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motifs
JNK	jun N-terminal kinase
kDa	kilo Daltons

LAMP-1	lysosomal-associated membrane protein 1
LDL	low density lipoprotein
LE	late endosome
LPS	lipopolysaccharide
LY	lysosome
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemoattractant-1
MEGA	molecular evolutionary genetics analysis
MEK	mitogen activated protein kinase or extracellular signal-regulated protein kinase (MAPK/ERK) kinase
MHC	major histocompatibility complex
min	minute
MIP-1	macrophage-inflammatory protein-1
MKK	mitogen-activated protein kinase kinase kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NEMO	NF-κB essential modulator
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor kappa B
NIK	NF-κB-inducing kinase
NO^-	nitric oxide anion
NSF	N-ethylmaleimide -sensitive factor
N-terminal	amino-terminal
O_2^-	superoxide anion
PAMPs	pathogen associated molecular patterns
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDK	phosphoinositide-dependent kinase
PH	pleckstrin homology
PI3-K	phosphoinositol 3-kinase
PI(4,5)P2	phosphatidylinositol-4,5-bisphosphate
PI(3,4,5)P3	phosphatidylinositol-3,4,5-trisphosphate
PKA	protein kinase A or cAMP-dependent protein kinase
PKC	protein kinase C

PLC	phospholipase C
PM	plasmamembrane
PMA	phorbol-12-myristate-13-acetate
PVDF	polyvinylidene difluoride
Q	glutamine
Q-PCR	quantitative polymerase chain reaction
R	arginine
RA	rheumatoid arthritis
RANTES	regulated on activation, normal T expressed and secreted
Rb	retinoblastoma
RIP	TNF receptor-interacting protein
RNA	ribonucleic acid
RNase	ribonuclease
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPM	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse-transcription polymerase chain reaction
SAGE	serial analysis of gene expression
SCR	short consensus repeats
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	serine
SH	Src homology
SHP	Src homology 2-containing tyrosine phosphatase
SLAP-2	Src-like adaptor protein 2
SLE	systemic lupus erythematosus
SM	Sec1/Munc18
SNAP	soluble NSF-attachment protein
SNARE	soluble NSF activating protein receptor
Sos	Son of Sevenless
SPD	platelet storage pool deficiency
SR	scavenger receptor
SV	synaptic vesicle
t	time
TAK1	TGF-activated kinase 1

TANK	TRAF-associated NF-κB activator
TBK1	TANK binding kinase 1
TC	transcription cluster
TFBS	transcription factor binding site
TGN	*trans*-Golgi network
Thr	Threonine
TIR	Toll-interleukin 1 receptor
TIRAP	Toll-interleukin 1 receptor domain-containing adaptor protein
TLC	thin layer chromatography
TLR	Toll-like receptor
TMB	tetramethyl benzine
TMD	transmembrane domain
TNF	tumour necrosis factor
TRAF	TNF-receptor associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFN-beta
TSS	transcription start site
t-SNARE	target membrane-SNARE
VAMP	vesicle associated membrane protein
VCAM-1	vascular cellular adhesion molecule-1
v-SNARE	vesicle-SNARE
Vti1b	vesicle transport through interaction with t-SNAREs 1B
WB	Western blotting

INTRODUCTION

1.1 Macrophages, Inflammation and Host Defence

1.1.1 Overview of Macrophages

Inflammation is the initial host response to infection and/or injury. Macrophages are a key component of the inflammatory process as they function as sentinels to detect pathogens (e.g. bacteria, viruses and fungi) [1, 2]. Once activated, macrophages secrete a range of inflammatory mediators (e.g. inflammatory cytokines and chemokines) that are crucial for effective innate immunity (Figure 1.1). They also phagocytose pathogens and dead cells, which is important for host defence as well as wound healing. In addition to their central role in innate immunity, macrophages contribute to adaptive immunity through the production of cytokines and the presentation of antigens to T-cells [3].

Macrophages are equipped with a wide array of receptors that enable them to recognise pathogens based on the presence of so-called *pathogen-associated molecular patterns* (PAMPs), such as peptidylglycan, lipopolysaccharide (LPS), flagellin, bacterial DNA and viral RNA [4]. The recognition of PAMPs by macrophages, for example by Toll-like receptors, triggers the activation of intracellular signalling pathways that eventually lead to the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1) and IL-6, as well as chemokines (e.g. CCL2, CCL3 and CCL5), which act to recruit other immune cells to the site of infection and help coordinate the inflammatory reaction [5, 6]. Macrophages also release cytotoxic, reactive oxygen and nitrogen species that are important for pathogen killing [7, 8]. Phagocytic receptors (e.g. Fc gamma receptors) recognise integral surface components of pathogens or foreign particles that are coated with opsonins (e.g. IgG antibodies) [9]. These phagocytic receptors mediate the engulfment of pathogens (or particles) via the formation of phagosomes. Once internalised, the phagosomes undergo various maturation events before finally fusing with lysosomes to form phagolysosomes [10-12]. It is within phagolysosomes that pathogens are killed and degraded. Pathogen-derived peptides are subsequently presented by major histocompatibility complex II (MHC II) molecules to $CD4^+$ T-cells [13]. Endogenous antigens, on the other hand, are proteolytically processed in the endoplasmic reticulum (ER), where they are loaded onto MHC I molecules and then presented to $CD8^+$ T-cells [14]. Interestingly,

studies have also shown that some extracellular antigens can be taken up by macrophages via macropinocytosis for presentation to $CD4^+$ T-cells [15, 16].

1.1.2 Cytokines, Chemokines and Type I Interferons

1.1.2.1 Regulation of the Production of Cytokines, Chemokines and Type I Interferons by Toll-like Receptors

The secretion of cytokines, such as TNF, IL-6, IL-12 and interferon-β (IFNβ), and chemokines (e.g. CCL2 and CCL5) by macrophages is important for effective host defence against infection. Toll-like receptors (TLRs) are the principal signalling molecules through which infections are detected [17]. To date, ten human (TLR1-10) and twelve mouse (TLR1-9 and TLR11-13) TLRs have been identified. Each TLR recognises a restricted subset of molecules produced by microbes, known as PAMPs [18, 19]. For example, plasma membrane localised TLR4 recognises LPS, a conserved component of the cell wall of Gram-negative bacteria, whereas TLR9 must traffic from the ER to endosomes/lysosomes to detect bacterial DNA [20].

Depending on the TLR involved, different downstream signalling pathways are activated. Prototypical TLR signalling networks are those that are used by TLR4, which has two major signalling branches: one branch leads to the production of inflammatory cytokines, while the other branch primarily leads to the production of IFNβ and the maturation of dendritic cells [21, 22]. These two signalling branches are referred to as the *MyD88-dependent* and *MyD88-independent* pathways, respectively (Figure 1.2) [23, 24]. The binding of TLR ligand results in receptor hetero- or homodimerisation and, in the case of the MyD88-dependent pathway, recruitment of the adaptor proteins MyD88 and TIRAP (also known as Mal) to the activated TLR complex [25]. This is followed by the binding of the Interleukin-1 receptor associated kinases IRAK-1 and IRAK-4 to MyD88. Phosphorylation of IRAK-1 by IRAK-4 leads to IRAK-1 activation and its release from the receptor complex whereupon it binds to TNF receptor-associated factor-6 (TRAF6), an E3 ubiquitin ligase [26]. This interaction results in the activation of the protein kinase TAK1, which ultimately leads to the activation of additional protein kinases, including IκB kinases. Phosphorylation of the NF-κB inhibitory protein IκB by IκB kinases triggers its degradation by the proteasome, leading to the activation and nuclear translocation of the transcription factor NF-κB and expression of target

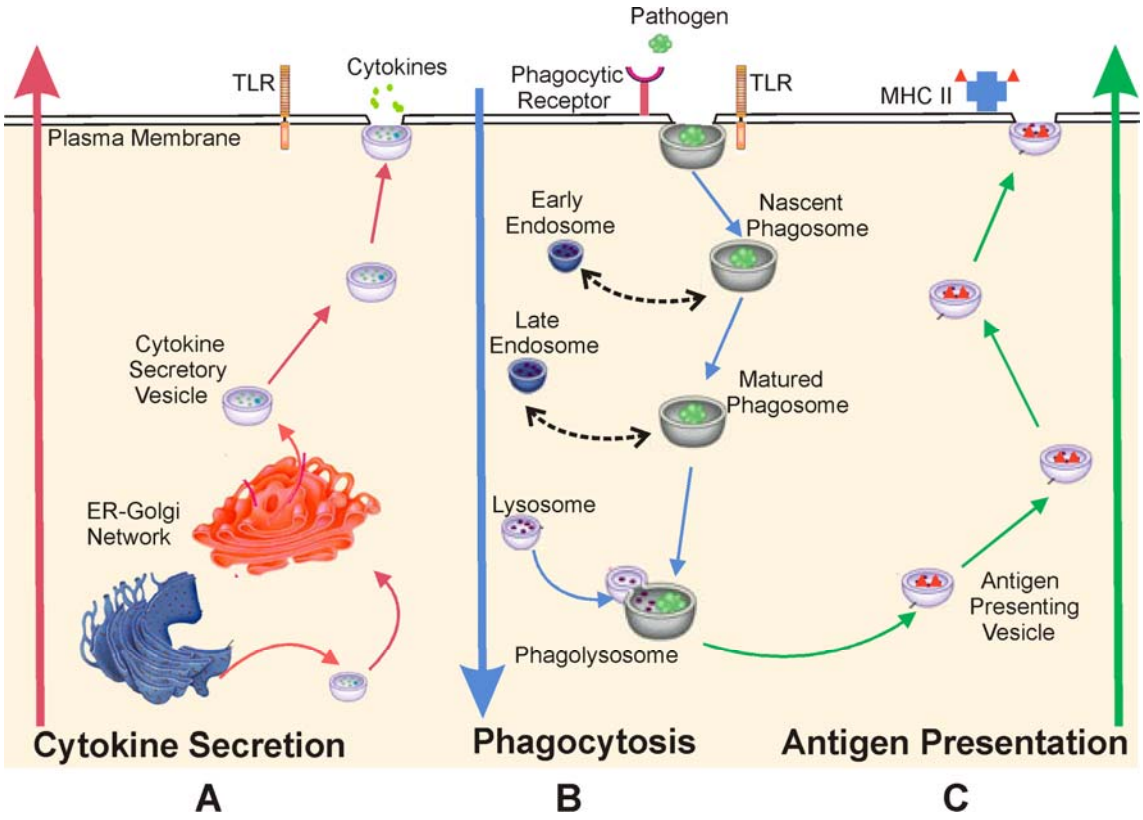


Figure 1.1: Macrophage immune functions.

A, The recognition of pathogens by macrophages is largely mediated by Toll-like receptors (TLRs), which trigger the activation of a number of signalling pathways that eventually result in the secretion of various inflammatory cytokines. The trafficking of newly synthesised cytokines from the ER-Golgi network to the cell surface in vesicles involves a number of membrane fusion events, with the cytokine-containing vesicles finally fusing with the plasma membrane in order to facilitate cytokine release. **B**, Phagocytic receptors mediate the engulfment of pathogens by forming a nascent phagosome that continually interacts with vesicles of the endocytic pathway during its maturation. Mature phagosomes finally fuse with lysosomes to form phagolysosomes, the compartment in which phagocytosed pathogens are killed and degraded. **C**, Pathogen-derived antigens are then trafficked in vesicles to the cell surface where they are presented by MHC class II molecules to T-cells.

genes (e.g. TNF, IL-6, IL-12 and CCL5) [27]. TAK-1 also activates p38 MAP kinase and JNK (via MKK3 and MKK6), which results in the activation of the transcription factor complex AP-1 [28]. *MyD88-independent* signalling is mediated by the TLR adaptor proteins TRIF and TRAM (Figure 1.2) [29, 30]. TRIF has been shown to bind TRAF6 directly, thereby allowing IRAK-independent activation of NF-κB and MAP kinases by TAK1; it has also been shown to activate NF-κB via its interaction with RIP1 (serine/threonine protein kinase receptor-interacting protein-1) [31]. Furthermore, the binding of TRIF to TRAF3 results in the activation of atypical IκB kinases and TBK1, which in turn phosphorylate, and thereby activate, interferon regulatory factor-3 (IRF-3) to induce the expression of type I interferons [32, 33].

1.1.2.2 The Roles of Cytokines, Chemokines and Type I Interferons

TNF is a key cytokine in inflammatory responses [34]. It increases vascular permeability in order to enhance the extravasation of immune cells, such as macrophages and neutrophils, into sites of infection. In addition, low levels of TNF have been shown to maintain homeostasis and enhance the remodelling of injured tissues [35]. Similar to TNF, IL-1α and IL-1β also play important roles in the inflammatory response by up-regulating the expression of cell adhesion molecules (e.g. ICAM-1 and E-selectin) on endothelial cells [36, 37]; it also promotes vasodilation and thereby enhances the extravasation of immune cells. In addition to its role in innate immunity, IL-1β is also involved in adaptive immunity by up-regulating the expression of signalling lymphocyte activation molecule, which contributes to enhanced antigen presentation by dendritic cells [38]. IL-1β is also involved in the maturation and clonal expansion of B-cells [39, 40]. IL-6 is another pro-inflammatory cytokine that is secreted by macrophages in response to PAMP recognition by TLRs. IL-6 recruits immune cells (e.g. macrophages and neutrophils) to the site of infection and promotes the proliferation of B-cells [41, 42]. IL-12 is another pro-inflammatory cytokine that is secreted by activated macrophages and plays crucial roles in host defence, such as promoting the differentiation of naive $CD4^+$ T-lymphocytes into T-helper 1 cells [43, 44]. Furthermore, IL-12 has been shown to stimulate TNF and IFNγ production by T-cells and NK cells [45]. In contrast to these pro-inflammatory cytokines, IL-10 is an anti-inflammatory cytokine produced by macrophages and suppresses the production of pro-inflammatory cytokines, such as IL-6 and TNF, by suppressing NF-κB activation [46, 47].

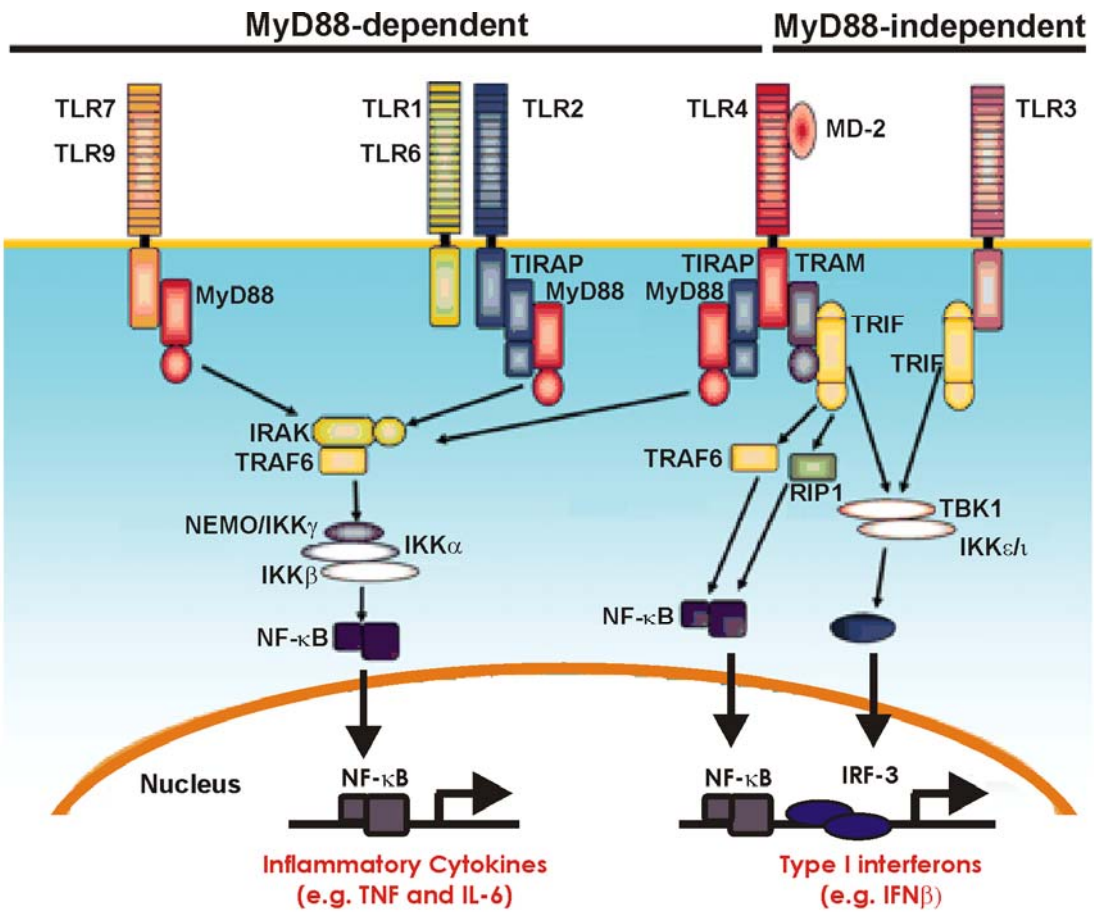


Figure 1.2: Toll-like receptor signalling pathways.

TLR signalling is initiated from the cytoplasmic Toll-interleukin 1 receptor (TIR) domain of TLRs and is transduced via the TIR domain-containing adaptor proteins MyD88, TIRAP, TRIF and TRAM. In *MyD88-dependent* signalling, MyD88 recruits IRAK-1 and -4 to activated TLRs. IRAK-1 in turn activates TRAF6, which then activates the IKK complex (IKKα, IKKβ and NEMO/IKKγ) leading to the nuclear translocation of NF-κB and subsequently to the induction of inflammatory cytokine gene expression. TIRAP is also involved in the *MyD88-dependent* signalling pathways used by TLR2 and TLR4. TRIF and TRAM, on the other hand, mediate *MyD88-independent* signalling. The non-typical IKK complex (IKKι/IKKε and TBK1) mediates IRF-3 activation, while TRAF6 and RIP1 mediate NF-κB activation. Nuclear translocation of these two transcription factors leads to the expression of type I interferons. Adapted from Takeda *et al.* [48].

Chemokines (e.g. CCL2 and CXCL8) are small cytokines (ranging from 8 - 10 kDa) that act as chemoattractants to promote the recruitment of immune cells to sites of infection and/or injury. For example, the chemokines CCL2 (also known as MCP-1), CCL3 (MIP-1α) and CCL5 (RANTES) induce monocytes to leave the bloodstream and enter the surrounding tissues where they differentiate into macrophages, whereas CXCL8 (IL-8) induces the migration of neutrophils into tissue [49-51].

Type I interferons (IFNα and IFNβ) are also secreted by macrophages in response to the activation of TLRs (e.g. TLR3 and TLR7) [52]. These interferons initiate signalling through the IFN receptors IFNAR1 and IFNAR2 to activate the anti-viral/bacterial responses of macrophages [53]. The importance of type I interferons for host defence is apparent from animal model studies in which mice lacking type I interferons died from bacteremia due to their inability to control infection by group B streptococci, pneumococci and *E. coli*; wild-type littermate control mice were resistant to the same bacteria [53]. Furthermore, in the absence of IFNα/β signalling, macrophages produced reduced levels of TNF, IFNγ and nitric oxide. In addition, IFNAR1-deficient mice showed an attenuated sepsis-induced hyperinflammatory response [54].

1.1.3 Phagocytosis

1.1.3.1 Overview of Phagocytosis

The ability of macrophages to engulf particles (e.g. pathogens and apoptotic cells) is fundamental to their roles in innate immunity, and is in fact, a phenotype that defines this cell lineage. Rabinovitch and colleagues [55] originally described phagocytic cells as being 'professional' or 'non-professional' based on their phagocytic capacity and efficiency. Professional phagocytic cells (e.g. macrophages and dendritic cells) possess a wide range of phagocytic receptors that increase both phagocytic rate and particle range. By contrast, non-professional phagocytic cells (e.g. endothelial cells) have a limited phagocytic ability due to the absence of specialised phagocytic receptors [55]. Therefore, macrophages are sometimes referred to as *professional phagocytic cells*.

The phagocytic receptors of macrophages can be divided into either opsonin-dependent or opsonin-independent receptors. For example, Fc-gamma receptors (e.g. FcγR) and complement receptors (e.g. CR3) recognise opsonised pathogens [56, 57]. Immunoglobulin G (IgG) and complement fragments C3b and C3bi are the major opsonins found in serum [58]. The second group of phagocytic receptors bind integral surface components of foreign particles in an opsonin-independent manner and includes scavenger receptors and C-type lectin receptors [59].

Several studies have shown that TLR signalling and phagocytic pathways are interconnected. For example, during the phagocytosis of zymosan, a component of the cell wall of fungi that activates macrophages via TLRs, both TLR2 and TLR6 are recruited to zymosan-containing phagosomes [60, 61]. It has also been shown that the activation of Fcγ receptors on macrophages can modulate TLR signalling. Fcγ receptor ligation results in reduced IL-12 production and increased IL-10 production following LPS stimulation of macrophages [62, 63]. In addition, TLR-mediated MyD88-dependent activation of p38 MAP kinase is required for phagosome maturation [64, 65]. Furthermore, LPS-induced TLR signalling up-regulates the expression of the scavenger family receptors MARCO and SR-A[66].

1.1.3.2 Fcγ Receptor-Mediated Phagocytosis

FcγR-mediated phagocytosis occurs upon the engagement of particles that are coated with IgG antibodies [67-69]. Specifically, FcγRs recognise and bind to opsonised foreign pathogens or particles via the Fc domain of IgG antibodies [70]. The different members of the FcγR family vary in their abilities to bind IgG or IgG-opsonised complexes. There are three groups of FcγRs, namely FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). Each receptor has multiple isoforms encoded by different genes (Figure 1.3) [71]. FcγRI and FcγRII have three isoforms (i.e. FcγRIA, FcγRIB and FcγRIC; FcγRIIA, FcγRIIB and FcγRIIC), whereas FcγRIII has two isoforms (FcγRIIIA and FcγRIIIB). FcγRs typically contain two to three extracellular Ig-like domains, a transmembrane domain and a short intracellular domain [72]. Structural differences in the Ig-like domains amongst the FcγRs allow them to bind a range of immune complexes. For example, FcγRIA exhibits a high affinity for monomeric IgG, while FcγRII and FcγRIII show a weaker affinity of monomeric IgG and hence bind multimeric immune complexes [73]. The

extracellular ligand binding domain of FcγRI contains two to three Ig-like domains; FcγRIA has three extracellular Ig-like domains, while FcγRIB and FcγRIC have two extracellular domains [74]. Unlike FcγRIA, the extracellular domain of FcγRII contains only two Ig-like domains and binds multimeric IgG complexes [74]. The Ig-like domains of FcγRIII are highly homologous to those of FcγRII. FcγRIIIA and FcγRIIIB differ in only nine amino acids and have a high affinity for IgG-containing immune complexes [72]. FcγRIIIB is the only member of the FcγR family that lacks a cytoplasmic domain and is anchored to the outer leaflet of the lipid bilayer by a glycosylphosphatidyl-inositol (GPI) anchor [75].

The recognition of the Fc region of IgG by FcγRs triggers a number of responses including tyrosine phosphorylation of cellular proteins, which plays a key role during FcγR-mediated phagocytosis [76, 77]. The cytoplasmic domains of FcγRs contain two tyrosine residues located in YXXL motifs, referred to as immunoreceptor tyrosine-based activation motifs (i.e. ITAM motifs), which undergo phosphorylation in response to ligand-induced cross-linking of FcγRs [78, 79]. Studies in the monkey kidney cell line COS-1 have revealed that mutation of either tyrosine residue in the ITAM motifs inhibits phagocytosis [80-82]. The protein tyrosine kinase(s) involved in this key regulatory phosphorylation event is thought to be a Src-family kinase [83, 84]. *In vitro* studies have revealed that the ITAM motifs in FcγRIIA/C can be phosphorylated by the Src-family kinases c-Src, Fyn, Lck and Lyn [81, 85, 86]. The phosphorylation of the ITAM motifs in FcγRs allows the tyrosine kinase Syk to bind via its two tandem Src-homology 2 (SH2) domains [87]. This binding results in the activation of Syk, which in turn triggers the activation of a number of signalling pathways leading to transcription factor activation (e.g. NFAT), cytoskeletal rearrangement, and the release of inflammatory mediators such as TNF [83, 88-90]. Although the precise mechanism by which FcγRs induce the formation of phagosomes is not known, phosphatidylinositol 3-kinase (PI3-K), Rho family GTPases and protein kinase C (PKC) are involved [91, 92]. The protein kinases Akt and p70 S6-kinase, both of whom act downstream of PI3-K, are also involved in FcγR-mediated phagocytosis in macrophages [93].

Although FcγRs are among the best-studied phagocytic receptors there are other Fc receptors that recognise different classes of antibodies. For example, FcαRI (CD89) binds IgA, while FcεR (CD23) interacts with IgE [94, 95]. FcαRI is

composed of two Ig-like domains, a transmembrane region and a cytoplasmic tail [94] and transiently interacts with monomeric IgA but has a much higher avidity for polymeric IgA complexes [96]. The structural organisation of FcεRs is similar to that of FcαRI [97]; both Fcα and Fcε receptors mediate downstream signalling via ITAM motif phosphorylation upon binding to IgA and IgE, respectively [98].

1.1.3.3 Complement Receptor-Mediated Phagocytosis

The complement system is an important soluble component of innate immunity. It plays a key role in host defence by promoting inflammation, phagocytosis and the direct lysis of pathogens [99]. Complement proteins (e.g. C3b and C3bi), which are present in serum, opsonise bacteria thereby allowing them to be phagocytosed via complement receptors (CRs). C3 convertase is an enzyme that catalyses the proteolytic cleavage of C3 into C3a and C3b [100], which act as opsonins or themselves become convertases [101]. The complement system contains a number of receptors that play different roles in host defence (Figure 1.4). Both CR1 (CD35) and CR2 (CD21) contain short consensus repeat (SCR) domains in their extracellular regions [102] and mediate the engulfment of pathogens that are opsonised with C3b, C3bi and C4b [103]. CR3 (CD11b/CD18) and CR4 (CD11c/CD18) belong to the β_2 integrin family [104, 105] and bind C3bi and act to recruit other inflammatory cells (e.g. neutrophils) to sites of infection [106]. The CR-mediated engulfment of pathogens requires cytoskeletal rearrangement and membrane fusion, suggesting that proteins involved in these processes are likely to play a key role in CR-mediated phagocytosis and hence host defence [107].

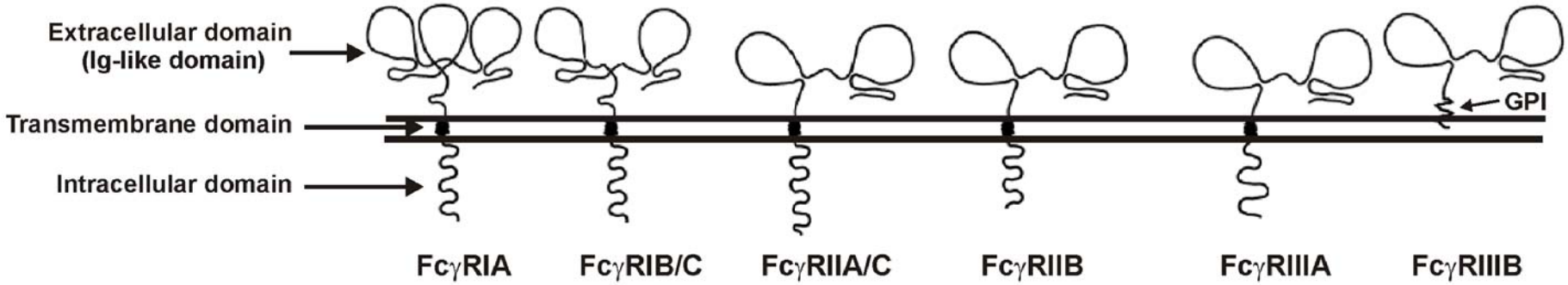


Figure 1.3: Structural organisation of Fc receptors.

In general, Fc receptors consist of three domains: an extracellular Ig-like domain, a transmembrane domain, and an intracellular domain that contains an immunoreceptor tyrosine-based activation (ITAM) motif. FcRIIIB is a notable exception, containing neither a transmembrane nor an intracellular domain; it is anchored to the cell membrane via a glycosylated phosphatidylinositol (GPI) linker. FcRIA contains three Ig-like domains and thus has a high affinity for IgG monomers, whereas the other members of the FcR family contain two Ig-like domains and have relatively higher affinities for IgG complexes. Adapted from Fossati *et al.* [99].

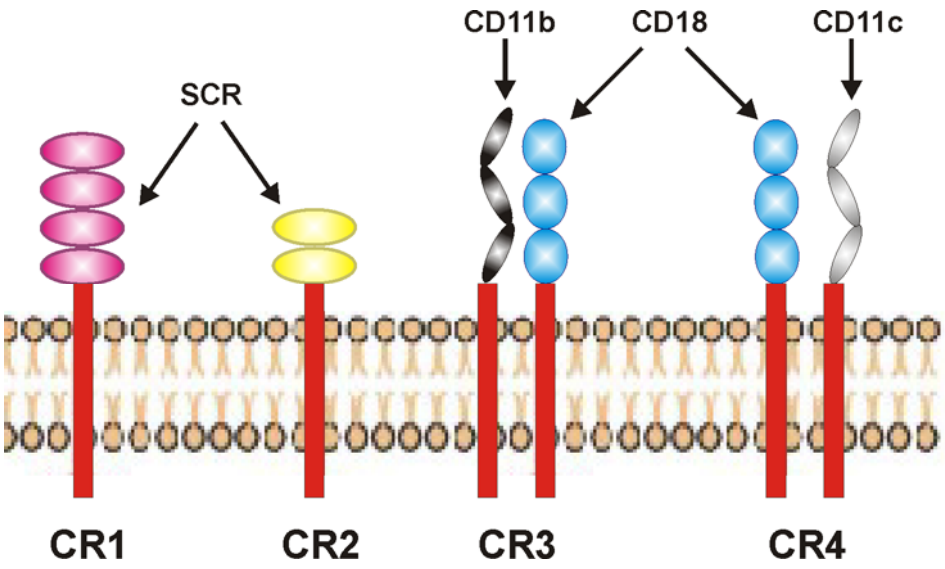


Figure 1.4: Structural organisation of complement receptors.

The complement receptors CR1 and CR2 consist of short consensus repeats (SCR) of 30 and 15 amino acids, respectively, and recognise pathogens that are coated with C3b, C3bi and C4b. CR3 and CR4 belong to the β_2 integrin family and consist of CD11b and CD11c, respectively, along with CD18. They bind to C3bi and play an important role in recruiting inflammatory cells (e.g. neutrophils) to sites of infection.

1.1.3.4 Scavenger Receptor-Mediated Phagocytosis

Scavenger receptors (SRs) facilitate the internalisation of pathogens, apoptotic cells and chemically modified proteins (e.g. acetylated and oxidised low density lipoproteins) [108]. SRs are categorised into three classes based on their structural organisation and the ligands they recognise (Figure 1.5) [109]. Class A SRs, which include SR-AI, SR-AII and MARCO, are homotrimeric glycoproteins [110]. Although SR-A1 differs from SR-AII by having a long C-terminal domain, they both exhibit similar ligand specificities (e.g. polyanionic ligands) [111]. MARCO, on the other hand, differs from both in terms of its structure (it lacks an α-helical coiled coil domain) and ligand specificity (binds acetylated LDL) [112]. Class B SRs include SR-B1 and CD36 [113, 114]. CD36 is a 88 kDa glycoprotein, whereas SR-B1 is 57 kDa in size [109]. Both receptors bind phosphatidylserine and modified proteins, such as acetylated LDL, and oxidised LDL [115]. Although class C SRs have been studied the least, they reportedly bind acetylated LDL, polyinosinic acid and microbial polysaccharide β-glucan [116].

1.1.3.5 C-Type Lectin Receptor-Mediated Phagocytosis

C-type lectin receptors (CLRs), such as dectin-1 and the mannose receptor, are calcium-dependent carbohydrate-binding receptors that contain one or more carbohydrate recognition (CR) domains (Figure 1.6) [117]. The mannose receptor contains eight CR domains, whereas dectin-1 has a single CR domain [118]. Dectin-1 contains an ITAM motif in its intracellular domain which propagates downstream signalling upon ligand binding. CLRs have diverse immune roles including the detection of pathogens (by virtue of PAMPs) and the binding of glycosylated ligands to mediate cell-cell adhesion and the clearance of aberrant cells (e.g. tumour cells and apoptotic cells) during immune responses [119].

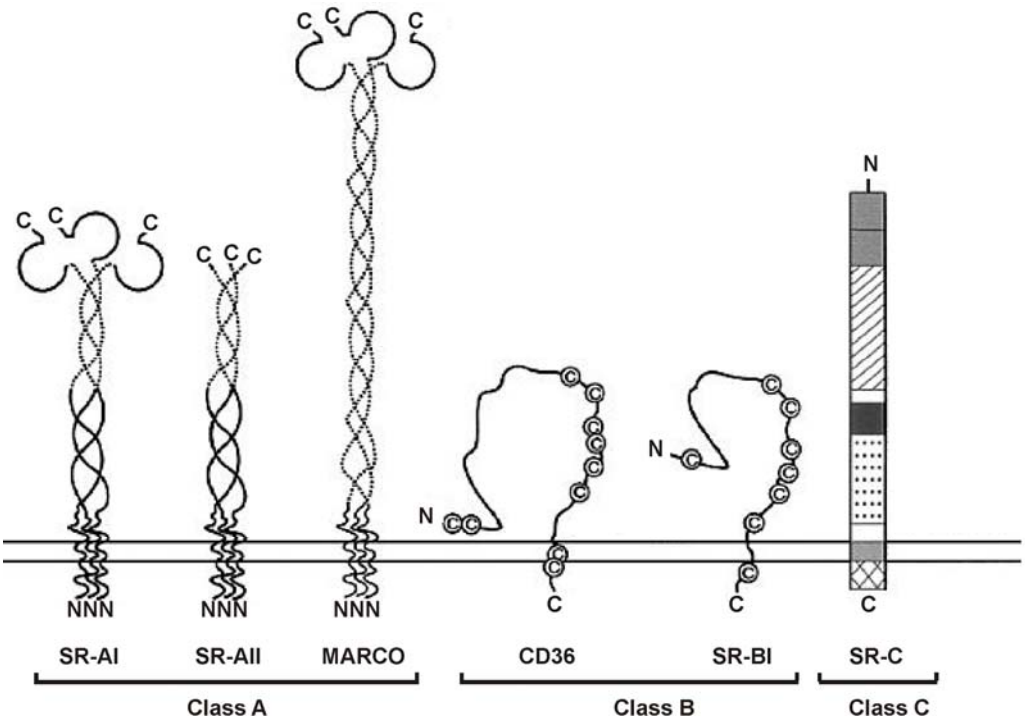


Figure 1.5: Structural organisation of scavenger receptors

Scavenger receptors are grouped into three classes (A, B and C) based on their structure and ligand specificity. Class A scavenger receptors are composed of three homotrimeric glycoproteins. SR-A1 and SR-AII recognise polyanionic ligands (e.g. dextran sulfate and fucoidin), while MARCO binds to acetylated LDL. Class B scavenger receptors CD36 and SR-B1 are glycoproteins of 88 and 57 kDa, respectively, and recognise phosphatidylserine and modified proteins (e.g. oxidised LDL). Finally, SR-C binds acetylated LDL, polyinosinic acid and microbial polysaccharide β-glucan. Adapted from Pearson *et al.* [120].

The mannose receptor recognises both endogenous and exogenous ligands bearing mannose, fucose, N-acetyl glucosamine and sulphated sugars via its CR domain [118]. Dectin-1 recognises β glucans (e.g. β-1,3 and β-1,6 glucans) from fungi [121, 122]. During the phagocytosis of zymosan by macrophages, dectin-1 traffics to the phagolysosome where it is degraded, whereas during the endocytosis of soluble ligands (e.g. laminarin and glucan phosphate) dectin-1 is recycled back to the cell surface [123]. The mannose receptor can recognise a number of pathogens, including *Mycobacterium tuberculosis*, *Pneumocystis carinii*, *Candidia albicans* and the human immunodeficiency virus [124].

1.1.3.6 Membrane Dynamics during Phagocytosis and Phagosome Maturation

The membrane used to form nascent phagosomes is derived from both the invagination of the plasma membrane and stimulus-dependent exocytosis near the site of phagosome formation [125]. Recent studies have shown that recycling endosomes are delivered focally to the plasma membrane near the forming phagosome and that they fuse with the phagosomal membrane prior to the sealing of the nascent phagosome [126-128]. The focal delivery of vesicles to the nascent phagosome membrane suggests a specific role for this phenomenon in phagocytosis, in addition to compensating for membrane lost from the cell surface as a consequence of phagocytosis. It has recently been shown that TNF-containing vesicles fuse with recycling endosomes, which then release TNF at the site of phagocytic cup formation in the RAW 264.7 mouse macrophage cell line [126].

In addition to recycling endosomes, the ER has also been shown to donate membrane for phagosome formation [125, 129, 130]. During the formation and elongation of pseudopodia around the particle to be engulfed, parts of the ER are recruited to the cell surface at the site of phagocytic cup formation where they then fuse with the plasma membrane [125, 131]. In addition to its role in phagocytosing extracellular pathogens, ER-mediated phagocytosis is also used by macrophages to phagocytose intracellular pathogens, including *Legionella pneumophila*, *Leishmania donovani* and *Salmonella typhimurium* [132, 133].

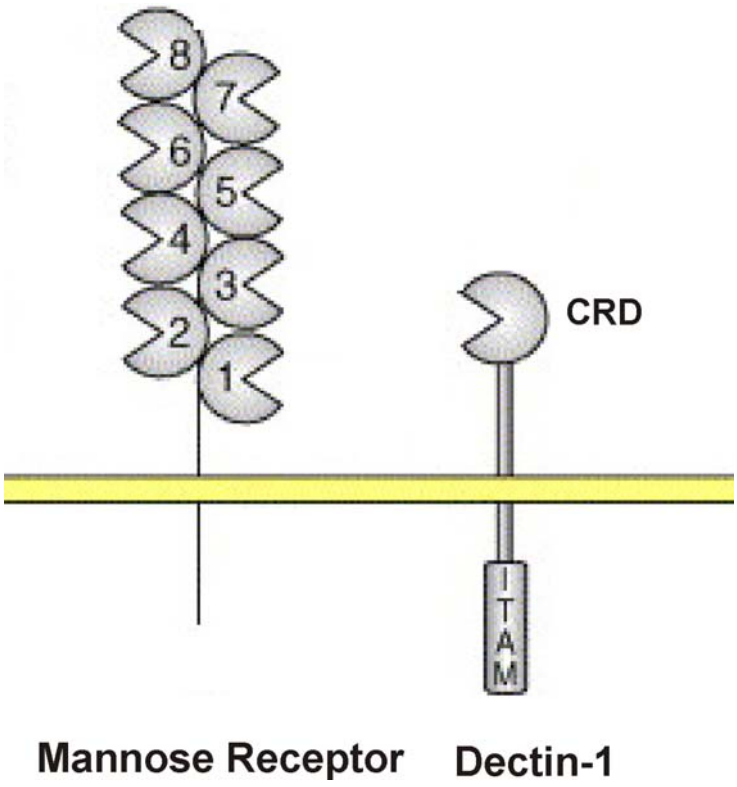


Figure 1.6: Structural organisation of C-type lectin receptors

The C-type lectin family of phagocytic receptors contain a carbohydrate recognition (CR) domain that facilitates ligand recognition. The mannose receptor contains eight CR domains, while dectin-1 has one CR domain. In addition, dectin-1 contains an ITAM motif that allows it to transduce signals upon ligand binding. Adapted from McGreal *et al.* [119].

Phosphatidylinositol-3 kinase (PI3-K) and protein kinase C (PKC) have central roles in coordinating the recruitment and fusion of recycling endosomes with nascent phagocytic cups during phagocytosis [132]. The recognition of pathogens by phagocytic receptors (e.g. Fcγ and complement receptors) initiates a cascade of events that results in the focal and rapid accumulation of phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] in the phagosomal membrane. This is accompanied by the recruitment of PI3-K to the nascent phagosome and the localised production of phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3]. As PI(3,4,5)P3 is produced, PI(4,5)P2 is depleted from the phagocytic cup because of the dual action of PI3-K and phospholipase Cγ (PLCγ) [134]. The cleavage of PI(4,5)P2 by PLCγ generates diacylglycerol and inositol-1,4,5-trisphosphate, intracellular second messengers that activate members of the PKC family and trigger the release of intracellular stores of calcium, respectively [135, 136].

Following their internalisation, phagosomes undergo a number of fission and fusion events with endosomes that modifies both the composition of their membrane and their contents [137]. This process is referred to as phagosome maturation and it closely resembles the endocytic pathway (Figure 1.7). In fact, interactions between phagosomes and endosomes commence soon after the formation of the nascent phagosome. Phagosomes fuse initially with early endosomes, followed by late endosomes and ultimately with lysosomes [138, 139]. The final fusion between phagosomes and lysosomes leads to the formation of phagolysosomes (Figure 1.2). The endocytic pathway initiates from early endosomes and terminates at lysosomes [140, 141]. Endocytosed macromolecules (e.g. receptors for colony stimulating factor-1 and transferrin, as well as their respective ligands) are initially found in small vesicles that are targeted to early endosomes. It is within early endosomes that endocytosed macromolecules are ‘sorted’ for delivery to the appropriate cellular compartment; hence, early endosomes are also referred to as sorting endosomes [140, 141]. For example, the endocytosed transferrin receptor is sorted for delivery back to the cell surface [142], whereas the internalised CSF-1 receptor is sorted for delivery to lysosomes for degradation [143, 144].

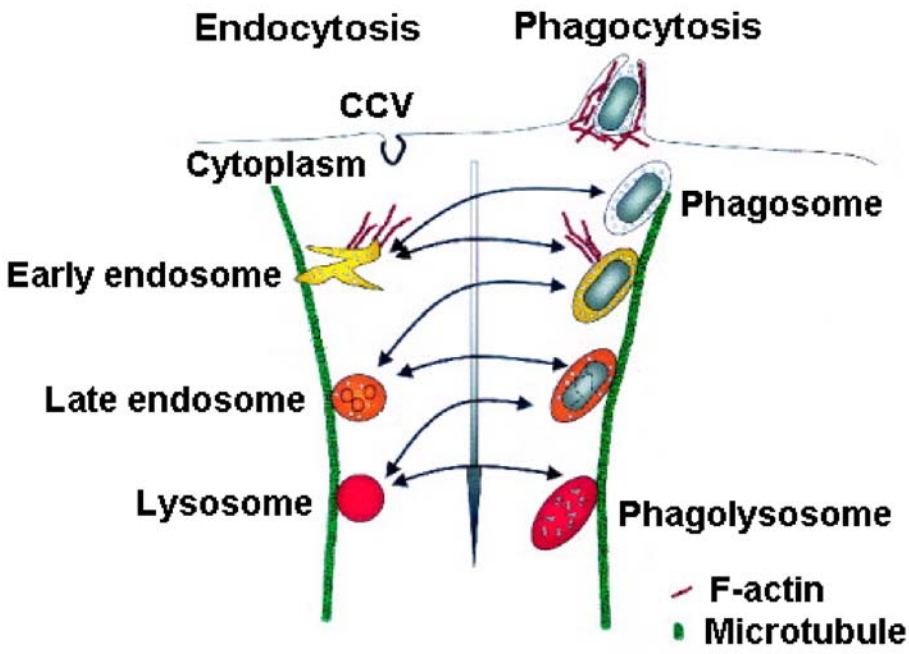


Figure 1.7: Endosome progression and phagosome maturation.

Phagosome maturation is mediated by the transient interaction of nascent phagosomes with vesicles of the endocytic pathway. Nascent phagosomes initially interact with early endosomes and following maturation, then interact with late endosomes. Such interactions are indicated by connection arrows. During these interactions, endosomes and phagosomes can exchange their contents; thus nascent and mature phagosomes can be defined by their contents. For example, nascent phagosomes display early endosome markers, whereas mature phagosomes display late endosome markers. Finally, mature phagosomes fuse with lysosomes to form phagolysosomes, where foreign pathogens are degraded. CCV, clathrin-coated vesicle. Adapted from Vieira *et al*. [137].

Early endosomes are defined by the presence of the Rab protein Rab5 and early endosome antigen 1 (EEA1), as well as by their slightly acidic (pH ~6.0) environment [145-147]. From early endosomes, internalised molecules are either directed towards recycling endosomes, which are distinguished by a more neutral pH (~6.5) and the presence of Rab11, or to late endosomes [148]. Late endosomes are comparatively more acidic than early endosomes (pH ~5.5) and can be identified by their multivesicular nature and the presence of Rab7, Rab9, the mannose-6-phosphate receptor and lysosomal-associated membrane proteins (e.g. LAMP-1) [140, 149]. Eventually, the endocytic pathway ends with the fusion of late endosomes with lysosomes, where the endocytosed particles are degraded. The degradation of phagocytosed particles is carried out by a number of hydrolytic enzymes, such as cathepsin D, alanine aminopeptidase and metalloproteinases [150]. Although there is still some controversy over the process of phagosome-endocytic organelle fusion, the findings so far suggests that the molecular mechanisms driving and regulating membrane fusion and fission along the endocytic pathway are likely to be major contributors to phagosome maturation. Because Rab and SNARE (**S**oluble **N**SF (N-ethylmaleimide-sensitive factor) **a**ctivating protein **re**ceptor) family proteins (see section 1.3) are key regulators of membrane fusion events, they are likely to play important roles in governing phagosome maturation [137]. The roles of Rab and SNARE proteins in vesicle trafficking and membrane fusion are discussed in section 1.3.

1.1.4 Reactive Oxygen Species

The generation of reactive oxygen/nitrogen species (ROS/RNS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide anion (NO^-), is crucial for the killing of pathogens [151, 152]. The importance of superoxide anions, which are generated as a product of the NADPH oxidase complex [153], for pathogen killing is illustrated by the fact that genetic defects in the NADPH oxidase complex results in chronic granulomatous disease and patients with this disease suffer from life-threatening infections [154]. For example, the NADPH oxidase complex plays a major role in the rapid killing of *S.typhimurium* [155]. In addition to directly killing pathogens, ROS has also been shown to act as a second messenger in NF-κB signalling pathways that control the expression of a number of inflammatory cytokines (e.g. TNF, IL-1α, IL-1β and IL-6) [156, 157]. Recently it was

shown that IL-1β stimulation of NF-κB is partially regulated by ROS-mediated activation of NF-κB-inducing kinase (NIK) and subsequent NIK-mediated phosphorylation of IKKα, which leads to nuclear the translocation of NF-κB [157].

1.1.5 Pinocytosis

Pinocytosis is defined as the uptake of fluid-phase solutes (e.g. acetylated LDL, and antigens) from the external environment by cells [158]. Pinocytosis can be classified as either micropinocytosis (pinosome <1 μm in diameter) or macropinocytosis (pinosome 1-5 μm in diameter). Micropinocytosis appears to take place in cells that are not equipped to process large volumes of fluid-phase solutes [159]. For example, the uptake of thyroglobulin by thyrocytes takes place via micropinocytosis [154]. In contrast, immune cells, such as macrophages and dendritic cells, are capable of processing larger volumes of fluid-phase solutes and thus use macropinocytosis [160, 161]. Membrane ruffling is a pre-requisite for macropinocytosis and occurs in response to external signals, such as CSF-1 [162]. Similar to phagocytosis, Rho family GTPases trigger a signalling cascade involving PI3-K that results in the actin-driven formation of membrane protrusions [163]. Macropinosomes are formed when these protrusions collapse onto solutes and then fuse with the plasma membrane. The newly formed macropinosome interacts with the early endosome compartment and eventually fuses with lysosomes, at which stage they acquire late endosome/lysosome markers, such as Rab7 and lysosomal glycoprotein A [164, 165].

1.1.6 Antigen Presentation

Macrophages and dendritic cells play major roles in adaptive immunity by presenting antigenic peptides, mounted on MHC molecules on the cell surface, to T-cells [166]. Both macrophages and dendritic cells are derived from myeloid progenitor cells [167]. Endogenous antigens are generated within the cell as a consequence of normal cell metabolism or intracellular viral or bacterial infection [168]. These antigens are processed into peptides by the ubiquitin-proteasome degradation pathway [169]. The peptides are then trafficked to the endoplasmic reticulum where they are mounted onto MHC I molecules. The MHC I-peptide complex is trafficked to the cell surface for presentation of the peptide to $CD8^+$ T-

cells [132]. On the other hand, exogenous antigens, such as those derived from phagocytosed pathogens, are trafficked to lysosomes where they are degraded into peptides [13]. These processed peptides can either be loaded onto MHC II molecules in phagosomes or be trafficked to MHC II molecule-rich compartments (endosome-like structures) for antigen loading [170, 171]. Peptides mounted onto MHC II molecules are then trafficked to the cell surface for presentation to $CD4^+$ T-cells [172, 173].

Although the presentation of antigens to T-cells has been studied extensively, how antigens are trafficked from phagolysosomes and from the ER to the surface of antigen presenting cells remains largely unknown. However, it is likely that the proteins that mediate vesicle trafficking and membrane fusion, such as SNARE proteins, are crucial for efficient antigen presentation.

1.2 Regulation of Macrophage Development and Functions by CSF-1

1.2.1 Regulation of Macrophage Differentiation, Proliferation and Survival by CSF-1

Colony stimulating factor 1 (CSF-1; also known as M-CSF) is the major growth factor that governs the development of mature macrophage-lineage cells (e.g. macrophages, dendritic cells and osteoclasts) [174]. It is required for the proliferation and differentiation of pluripotent haematopoietic stem cells in the bone marrow into macrophage-lineage cells [175, 176].

1.2.2 Modulation of the Immune Functions of Macrophages by CSF-1

In addition to governing the development of macrophages, CSF-1 also modulates some of the immune functions of macrophages including cytokine secretion and phagocytosis. For example, inhibition of the CSF-1 receptor with the pharmacological agent CYC10268 decreased cytokine production (TNF, IL-6 and IL-12) by LPS-activated, mouse bone marrow-derived macrophages [177, 178]. Furthermore, CSF-1 has been shown to enhance TNF production in RAW 264.7

macrophage cells, mouse bone marrow-derived macrophages and in human peripheral blood monocytes [177, 179, 180]. The mechanisms responsible for such effects are unknown but may involve transcriptional synergy; signalling via CSF-1 receptor-activated kinases (see below) is known to regulate various transcription factors (e.g. Ets-1, Ets-2 and PU.1) [181]. As well as governing the expression of cytokines, these transcription factors may also modulate the expression of the proteins that mediate cytokine secretion.

The phagocytic activity of macrophages is similarly modulated by CSF-1. Alveolar macrophages from rabbits exhibited enhanced phagocytosis of *Penicillium marneffei* and *Aspergillus fumigatus* in the presence of CSF-1 [182] [183]. In another study, CSF-1 was shown to enhance the phagocytosis of *Trichosporon asahii* by mouse peritoneal macrophages [180]. The phagocytosis of abnormal protein aggregates, such as amyloid-β, by mouse microglia is likewise enhanced by CSF-1 [184]. Notably, CSF-1 has also been shown to stimulate macropinosome-mediated pinocytosis by mouse bone marrow-derived macrophages [185] and ROS production in human peripheral blood monocytes [186]. These varied functions of macrophages are crucial for effective host defence and cellular homeostasis. However, the molecular mechanisms by which CSF-1 governs these biological processes are not understood. Given that vesicle trafficking and membrane fusion events are fundamental to each of these processes, CSF-1 could potentially modulate the immune functions of macrophages by regulating the proteins involved in vesicle trafficking and membrane fusion.

1.2.3 CSF-1 Receptor Signalling

1.2.3.1 Structural Features of the CSF-1 Receptor

The biological effects of CSF-1 on target cells (e.g. inducing the proliferation and differentiation of myeloid progenitor cells, priming pro-inflammatory cytokine production, and enhancing the phagocytic activity of macrophages) are mediated via its receptor, a type III receptor protein tyrosine kinase which is encoded by the c-*fms* gene [187]. The CSF-1 receptor is structurally related to the receptors for Flt3 ligand, stem cell factor and platelet-derived growth factor [188]. The CSF-1 receptor consists of three main components: (i) an extensively glycosylated

extracellular domain with five Ig-like domains that bind CSF-1, (ii) a transmembrane domain, and (iii) an intracellular tyrosine kinase domain (Figure 1.8) [189, 190].

The binding of CSF-1 to the N-terminal three Ig-like domains of the CSF-1 receptor induces homodimerization of the receptor, which in turn results in the activation of the tyrosine kinase domain and auto-phosphorylation of the receptor dimer complex. Phosphorylation of specific tyrosine residues in the receptor creates docking sites for various signalling proteins containing SH2 domains (Figure 1.8). It is the binding of these SH2 domain-containing proteins to the receptor that results in the activation of highly complex signalling cascades. The CSF-1 receptor-activated signalling pathways lead to the transcriptional regulation of genes that play important roles in the immune functions of macrophages as well as in maintaining macrophage homeostasis [189, 191, 192].

1.2.3.2 Signalling Pathways Downstream of the CSF-1 Receptor

To date, seven tyrosine residues in the mouse CSF-1 receptor have been identified as major sites of auto-phosphorylation (Figure 1.8). These tyrosine residues are located at position 559 in the juxtamembrane region, positions 697, 706, and 721 in the kinase-insert domain, positions 807 and 921 in the main kinase domain, and position 973 in the C-terminal tail [193, 194]. Of the aforementioned tyrosine residues, four have been shown to act as binding sites for various SH2 domain-containing proteins (i.e. Tyr-559, Tyr-697, Tyr-721 and Tyr-973); similar interactions have not been demonstrated for Tyr-706, Tyr-807 or Tyr-921.

Tyrosine 559 of the CSF-1 receptor has been characterised as a docking site for Src-family kinases (e.g. Hck and Lyn), which modulate the organisation of the actin cytoskeleton by phosphorylating various substrates (e.g. Shc, STAT3, paxillin and myosin XVIIIA) [195, 196]. Furthermore, Hck and Fgr have been shown to be important for β_2 integrin-mediated cell adhesion [197], whereas Lyn may suppress macrophage proliferation by negatively regulating CSF-1 signalling [198].

Another major CSF-1 receptor signalling pathway features Tyr-697, which mediates the binding of the adaptor protein Grb2 via its SH2 domain. Sos (son of sevenless), a guanine nucleotide-releasing factor, binds to Grb2 through its SH3 domain (Figure 1.8) [194]. Sos catalyses the conversion of inactive Ras-GDP to active

Ras-GTP. Ras-GTP induces activation of Raf1, which activates MAP kinase kinase that leads to the activation of ERK1/2 [199]. Activation of ERK1/2 promotes macrophage differentiation by activating transcription factors such as ELK1, ATF-2 and c-JUN, which induce transcription of genes involved in cell activation, proliferation and transformation (e.g. c-fos, c-myc and D-type cyclins) [195, 196]. Mona is another adaptor protein that has been shown to bind to phosphorylated Tyr-697 [200]. Similar to Grb2, Mona is linked to the differentiation of macrophages by activating the ERK1/2 signalling pathway [201].

Gab family proteins are also involved in signalling by the CSF-1 receptor. Gab2 and Gab3 are tyrosine phosphorylated and bind to the tyrosine phosphatase SHP-2 upon activation of the CSF-1 receptor; this interaction has been shown to promote macrophage differentiation [202, 203]. There is also evidence that Gab2 participates in both the Ras-ERK1/2 and Akt pathways by binding to Grb2 and to the p85 subunit of PI3-K [202, 203]. Notably, FcγR-mediated phagocytosis is severely impaired in bone marrow-derived macrophages from Gab2-deficient mice [204].

A key signalling pathway downstream of the activated CSF-1 receptor is the PI3-K pathway. The direct binding of the p85 regulatory subunit of PI3-K to phosphorylated Tyr-721 results in the phosphorylation of the 85 kDa subunit and activation of the 110 kDa catalytic subunit of PI3-K [205]. Downstream effects of PI3-K activation include the activation of Akt (also known as protein kinase B), PKC and p70 S6 kinase [206]. The PI3-K-mediated activation of Akt (via the production of phosphatidylinositol 3-phosphates) is important for cell survival. Akt promotes cell survival by suppressing the activity of pro-apoptotic proteins (e.g. Bad and Bax) and the fork-head DNA transcription factors DAF-16 and FKHR [207, 208]. Notably, activation of PI3-K has also been shown to be important for phagocytosis [162]. The Tyr-721 site has been shown to be a binding site for phospholipase C-γ2 (PLC-γ2), which is required for macrophage differentiation [209].

Tyr-973 serves as a binding site for the E3 ubiquitin ligase c-Cbl [210, 211]. The binding of c-Cbl to the activated CSF-1 receptor leads to the multi-ubiquitination of the receptor and its degradation (see below) [143, 212-214]. Src-like adaptor protein 2 (SLAP-2) has also recently been shown to bind to c-Cbl and may contribute to the down regulation of signalling by the CSF-1 receptor [215, 216].

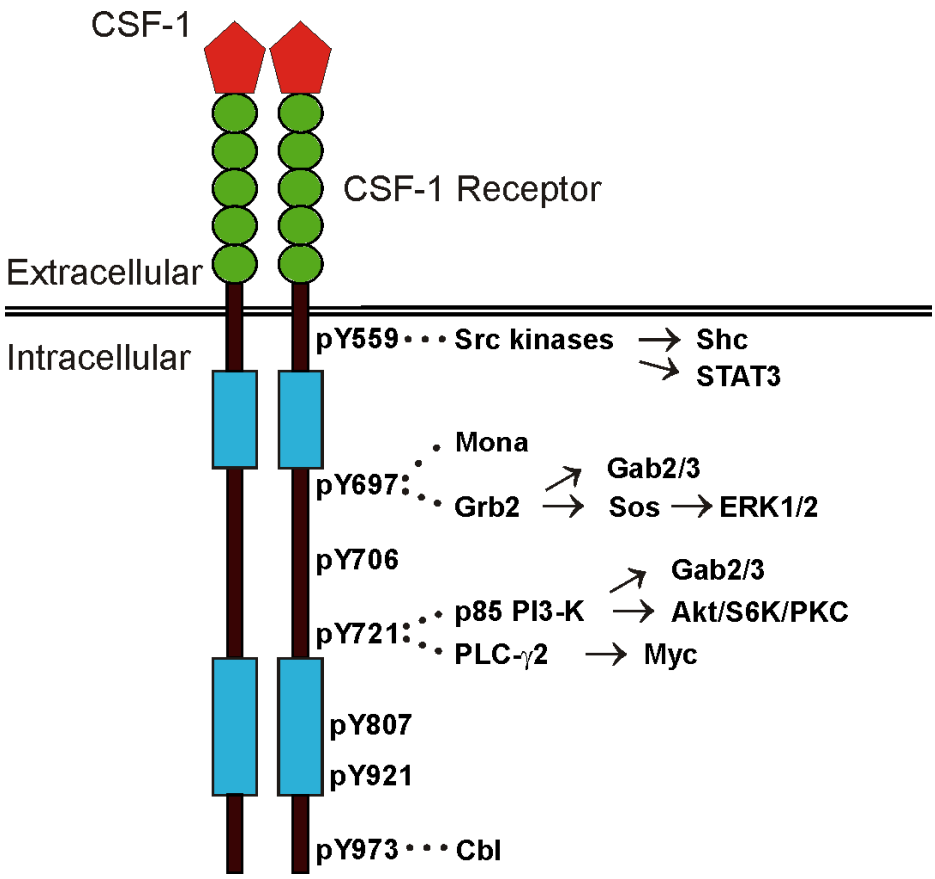


Figure 1.8: CSF-1 receptor signalling pathways.

The binding of CSF-1 to its receptor c-Fms triggers the homodimerization and auto-phosphorylation of the receptor. Phosphorylated tyrosine residues in the CSF-1 receptor function to recruit a number of SH2 domain-containing signalling molecules, including kinases such as Src-family kinases and PI3-K, as well as adaptor proteins (e.g. Grb2 and Mona), to the receptor complex. These signalling molecules activate additional downstream kinases (e.g. ERK1/2, Akt and PKC) or bind to additional signalling proteins (e.g. Shc), thereby amplifying the signal transduction cascade. Red pentagons represent CSF-1, while green circles represent the Ig-like domains of the CSF-1 receptor. Blue rectangles indicate kinase domains.

1.2.3.3 Termination of Signalling by the CSF-1 Receptor

The termination of CSF-1 receptor signalling is important for macrophage homeostasis. Indeed, dysregulated signalling by the CSF-1 receptor has been implicated in acute myeloid leukaemia and myeloproliferative syndromes in humans [211, 217]. The so-called *moth-eaten viable* mice are the result of point mutations in the SH2 domain-containing tyrosine phosphatase 1 (SHP-1) that impair its phosphatase activity [218]. SHP-1 negatively regulates CSF-1 receptor signalling by dephosphorylating tyrosine residues of the receptor [219]. Thus, CSF-1 stimulation of macrophages from *moth-eaten viable* mice results in prolonged hyperphosphorylation of the CSF-1 receptor, leading to increased activation of downstream signalling molecules and a corresponding increase in macrophage proliferation [220].

The rapid internalisation and lysosomal degradation of the activated CSF-1 receptor is also important for the termination of CSF-1 signalling. As mentioned above, c-Cbl binds to the activated CSF-1 receptor (via Tyr-973) and facilitates the multi-ubiquitination of the receptor. This leads to the endocytosis of the CSF-1 receptor and its trafficking to lysosomes for degradation [143, 212].

1.3 Vesicle Trafficking and Membrane Fusion

1.3.1 The Importance of Vesicle Trafficking and Membrane Fusion for Macrophage Functions

As mentioned earlier, the effector functions of macrophages are largely dependent on vesicle trafficking and membrane fusion events. For example, inflammatory cytokines (e.g. TNF) are trafficked in vesicles from the *trans*-Golgi network to the plasma membrane where the vesicles fuse with the plasma membrane to allow cytokine secretion [126]. Similarly, the phagocytosis of pathogens and their transport to lysosomes involves a number of vesicle trafficking and membrane fusion events. Peptides derived from phagocytosed pathogens can then be mounted on MHC molecules and trafficked in vesicles to the cell surface for presentation to T-cells. Notably though, how these various vesicle trafficking and

membrane fusion events are controlled remains a largely unexplored area of macrophage biology.

1.3.2 Rab Proteins and Vesicle Trafficking & Membrane Fusion

Rab proteins are small GTP-binding proteins that are crucial for ***vesicle tethering***, an event that describes the physical attachment of vesicles to target membranes prior to the fusion of the two membranes. Thus, Rab proteins govern the rate at which membrane fusion proceeds [221].

1.3.2.1 Regulation of Rab Proteins

The activity of Rab proteins is primarily regulated by the binding of GTP, followed by its hydrolysis to GDP [222]. Rab proteins are typically maintained in an inactive GDP-bound state in the cytosol via the actions of the protein GDP-disassociation inhibitor (GDI) [223]. Rab-GDP is delivered by GDI to the donor membrane, where Rab exchanges GDP for GTP and is incorporated into the transport vesicle. The hydrolysis of the bound GTP facilitates the trafficking of the vesicle to the target membrane; Rab-GDP is then extracted from the membrane by GDI and recycled to the donor membrane following membrane fusion [223].

1.3.2.2 Regulation of Endocytic Processes by Rab Proteins

A number of Rab proteins are involved in endocytic pathways including Rab5, Rab11 and Rab7, which associate predominantly with early, recycling endosomes and late endosomes/lysosomes, respectively [149, 224]. Rab5 has a role in the tethering and fusion of vesicles with early endosomes and the homotypic fusion of early endosomes [224, 225] as well as in the migration of early endosomes along microtubules [226]. Rab7 plays an important role in regulating the late endocytic pathway by controlling the transition of early endosomes to late endosomes [224, 227, 228]. Some studies have also shown that Rab7 associates with lysosomes and controls their aggregation and fusion with late endosomes [224]. Rab11 is localised to recycling endosomes in macrophages and plays a role in phagosome biogenesis [229].

Although a number of Rab proteins have been found on phagosomal membranes in macrophages, including Rab5, Rab7 and Rab11 [139, 230], few functional studies have been performed to establish their importance for phagocytosis. By analogy with endosomes, the early stages of phagosome maturation are thought to be controlled by Rab5 [231, 232]. Studies in macrophages have demonstrated an essential role for Rab5 in the fusion of isolated early endosomes with purified nascent phagosomes [231, 233, 234]. Moreover, Rab5 has been shown to associate with phagosomes containing *M. tuberculosis* [229]. As is the case for endocytosis, much less is known about the role of Rab proteins during the later stages of phagosome maturation. However, it has been shown that Rab7 is acquired by phagosomes during the progression of phagosomes from the early to the late endosomal stages of maturation [231, 234, 235]. A recent study found that *M.tuberculosis* blocks phagosome maturation, at least in part, by retaining Rab5 and Rab22a in the phagosome membrane. Rab22a is a Rab GTPase that modulates the tethering of vesicles that are going to fuse recycling endosomes [236]. The recruitment and maintenance of Rab22a in the phagosomal membrane prevents the acquisition of Rab7, which is required for phagosome maturation [229]. Similarly, *Listeria monocytogenes* survives in macrophages by retaining the inactive form of Rab5 (i.e. Rab5-GDP) in the phagosome membrane, thereby inhibiting the acquisition of Rab7 and arresting phagosome maturation [237]. During phagocytosis, recycling endosomes supply the extra membrane that is required for phagosome formation [132]. Rab11, which is localised to recycling endosomes, has been shown to play an important role in FcγR-mediated phagocytosis in RAW 264.7 macrophages [229, 238]. Expression of a constitutively active Rab11 led to an increased efflux of the transferrin receptor and the enhanced phagocytosis of IgG-coated beads [239]. Conversely, expression of dominant negative mutant forms of Rab11 decreased the rate of transferrin efflux and impaired FcγR-mediated phagocytosis [229].

1.3.2.3 Regulation of Exocytic Processes by Rab Proteins

In addition to their roles in phagosome maturation, Rab proteins have recently been shown to regulate TNF secretion in RAW 264.7 macrophages [126]. Notably, the secretion of TNF by LPS-activated RAW 264.7 macrophages mirrored the increase

in Rab11 expression [240]. Furthermore, expression of a constitutively active form of Rab11 in RAW 264.7 macrophages enhanced the secretion of TNF; conversely, expression of a dominant negative Rab11 mutant hindered the cell surface delivery of TNF [126].

Rab proteins have also been shown to be involved in antigen presentation. In macrophages, endosome-like compartments expressing Rab5 have been shown to process peptides for loading onto MHC II molecules [241]. Overexpression of Rab7 in B-cells enhances antigen presentation by MHC II molecules [242].

1.3.3 SNARE Proteins and Vesicle Trafficking & Membrane Fusion

1.3.3.1 Overview of SNARE proteins

SNARE proteins comprise a superfamily of small, mostly membrane-anchored proteins that share a motif of 60-70 amino acids referred to as the 'SNARE' motif. They are expressed very early in development and play important roles in ***vesicle tethering*** and ***membrane fusion*** [243]. Although some SNAREs exhibit tissue specific expression, most are widely expressed [244]. The ability of SNARE proteins to form core SNARE complexes is integral in determining the specificity of intracellular membrane fusion and/or serving as the minimal machinery necessary for membrane fusion. Different SNAREs are localised to distinct membrane compartments and are only competent to form complexes with their cognate SNARE partners [245]. SNAREs resident on vesicles are therefore referred to as v-SNAREs, while t-SNAREs reside on target membranes (Figure 1.9). SNARE proteins are also classified based on the amino acid they donate during the assembly of SNARE complexes. Thus, SNARE proteins can be classified as: Qa-, Qb-, Qc-, and Qbc-SNARES or as R-SNAREs (this nomenclature is discussed in section 1.3.3.3) (see Table 1).

1.3.3.2 Structural Organisation of SNARE proteins

All SNARE proteins share a common SNARE motif that is evolutionarily conserved. SNARE proteins typically contain a domain N-terminal to their SNARE domain and

a C-terminal hydrophobic transmembrane domain (TMD) (Figure 1.10). The majority of SNARE proteins are anchored to membranes via their hydrophobic C-terminal TMD. However, some SNARE proteins (e.g. syntaxin 11 and SNAP-25) do not possess a hydrophobic TMD but are anchored to cellular membranes via the palmitoylation of cysteine residues [246, 247]. The SNARE protein Ykt6, on the other hand, is anchored to membranes via the prenylation of C-terminal cysteine residues [248, 249].

1.3.3.3 SNARE Complex Assembly and Membrane Fusion

During the assembly of SNARE complexes, four SNARE motifs form an extended, coil-coil-like helical bundle with the membrane anchor domains extending at the end of the complex (Figure 1.11) [250-252]. In the centre of the bundle, the helices are connected by layers of hydrophobic amino acids, with the exception of a hydrophilic central layer. This central position is called the "0" layer of the SNARE motif and is formed by three conserved glutamine (Q) residues and one conserved arginine (R) residue. In general, glutamines are donated by t-SNAREs, whereas the arginine residue is donated by the v-SNARE. This leads to another nomenclature for SNARE proteins based on the amino acid donated by the SNARE protein to the ionic central layer. Generally, t-SNAREs correspond to Q-SNAREs and v-SNAREs correspond to R-SNAREs. (This amino acid-base nomenclature is used hereafter).

The assembly of *trans*-SNARE complexes leads first to a tight connection between the two adjacent membranes and then drives lipid mixing and the subsequent opening of a fusion pore [253]. The formation of a core SNARE complex takes place in a 'zipper-like' fashion, proceeding in the N- to C-terminal direction (Figure 1.12) [254, 255]. The energy expended during the formation of the *trans*-SNARE complex helps overcome the energy barrier associated with the fusion of cellular membranes. However, *trans*-SNARE complexes may be intrinsically unable to completely assemble in the face of the energy barrier created by the repulsion of the two membranes. In this scenario, the 'zippering' together of the SNARE proteins would come to a pause and external energy would be required to enable 'zippering' to proceed to completion and facilitate the fusion of the two membranes. This extra energy can be provided by extrinsic factors; for example, calcium-loaded synaptotagmin is thought to regulate neuronal SNARE-mediated fusion [255, 256]. In this case, fusion does not take place immediately following the assembly of a

functional *trans*-SNARE complex, instead the protein complexin binds to the assembled SNARE complex and delays membrane fusion by acting as a clamp [257-259]. Complexin inserts as n anti-parallel α helix into a groove in the four helical bundle of the neuronal SNARE complex [260, 261]. Upon an increase in the concentration of intracellular free Ca^{2+}, synaptotagmin releases complexin from the SNARE complex and mediates the final membrane fusion step (Figure 1.12) [258, 259].

Following membrane fusion, the *cis*-SNARE complex needs to be disassembled and the individual SNARE proteins recycled for subsequent fusion events. The disassembly of *cis*-SNARE complexes requires the concerted actions of the evolutionarily conserved proteins ATPase NSF and soluble NSF-attachment protein (α-SNAP) [262]. During the disassembly process, α-SNAP binds to the *cis*-SNARE complex in an anti-parallel orientation, while NSF mediates the hydrolysis of ATP that results in the 'regeneration' of the individual SNARE proteins (Figure 1.13) [263].

Crystallographic studies of both neuronal and late endosomal SNARE complexes have revealed that their ionic central layers are conserved and most of the variations occurred at the surface of the complexes (Figure 1.11 & 14) [264]. Although both core SNARE complexes (i.e. the assembled SNARE motifs alone) exhibited structural similarity, they varied significantly in their amino acid sequences. Late endosomal SNARE complexes consist of four proteins: syntaxin 7 (Qa-SNARE), Vti1b (Vps10p tail interactor 1b; Qb-SNARE), syntaxin 8 (Qc-SNARE), and VAMP8 (Vesicle adhesion membrane protein 8, also known as endobrevin; R-SNARE). Biochemical studies of late endosomal SNARE complexes have revealed a marked similarity to that of the neuronal SNARE complex consisting of syntaxin 1A, SNAP-25 and VAMP2 [264, 265]. Both of these complexes contain one member of each participating SNARE protein (i.e. the complex consists of SNARE monomers) and are resistant to dissolution by sodium dodecyl sulphate. The constituents of both complexes are largely disordered before complex formation but assume an α-helical structure upon complex formation, and both complexes can be disassembled by ATPase NSF in the presence of α-SNAP [265].

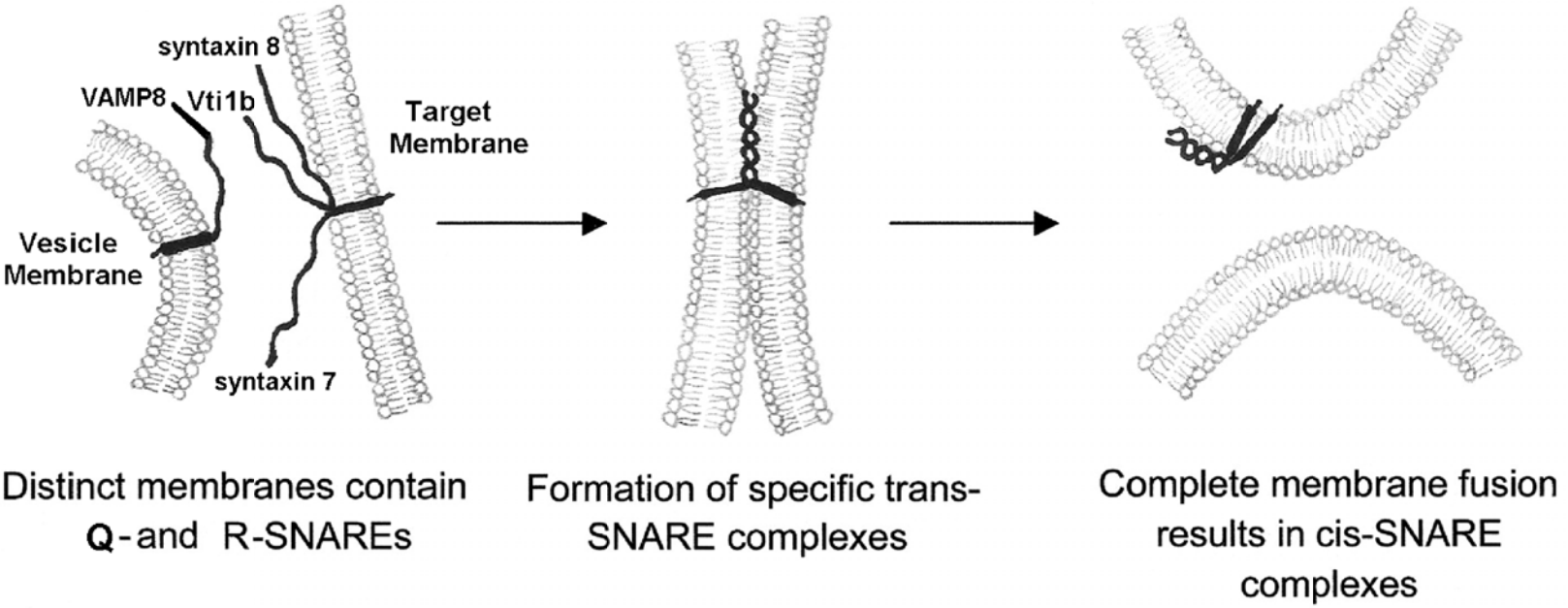


Figure 1.9: SNARE protein-mediated membrane fusion.

The v-SNARE VAMP8 and the t-SNAREs syntaxin 7, syntaxin 8 and Vti1b form a *trans*-SNARE complex to mediate fusion between two membranes. Following membrane fusion a *cis*-SNARE complex forms, which is then disassembled and the SNARE proteins recycled for further rounds of fusion. Adapted from Duman *et al*. [251].

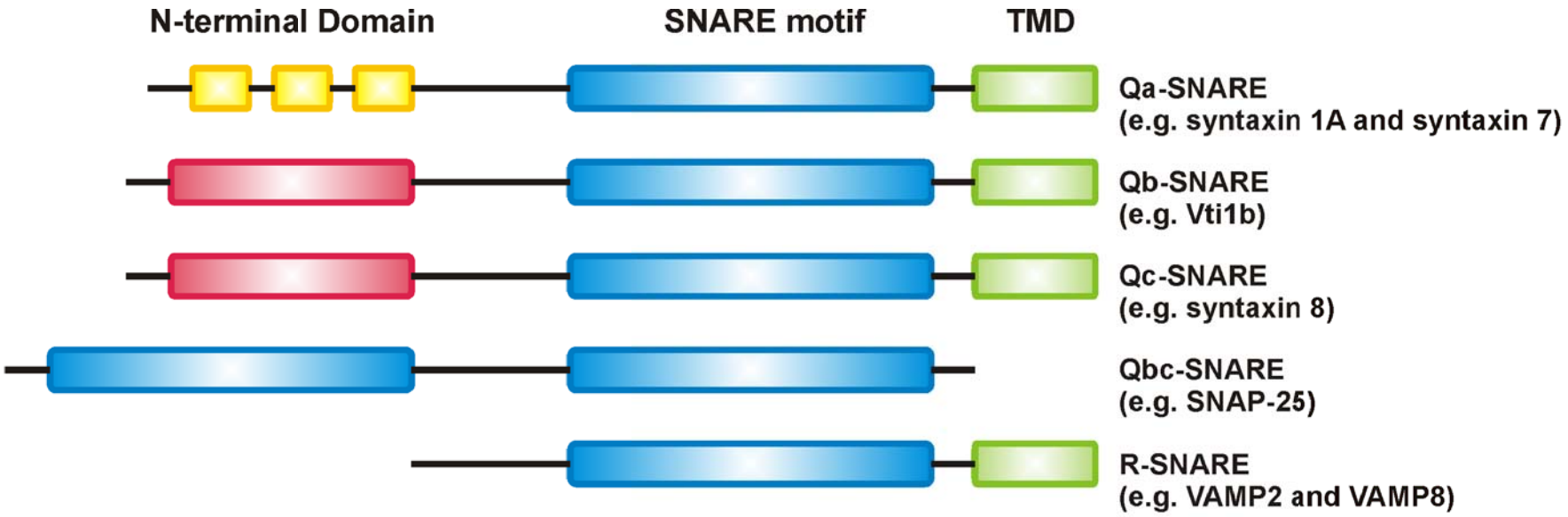


Figure 1.10: Structural organisation of SNARE proteins.

All SNARE proteins share an evolutionarily conserved SNARE motif, which is approximately 60 amino acids in length. Qa-SNARE proteins contain an N-terminal domain that consists of three helical domains (Ha, Hb and Hc - also known as the Habc domain). In general, Qa-SNARE proteins are anchored to membranes via a C-terminal transmembrane domain (TMD). Both Qb- and Qc-SNARE proteins are structurally similar and contain a single N-terminal helical domain. Generally, these SNARE proteins are also anchored to membranes via their C-terminal TMD. In contrast, Qbc-SNARE proteins are anchored to membranes via palmitoylation of cysteine residues in the linker region between their tandem SNARE motifs. Finally, R-SNARE proteins lack an N-terminal domain and are anchored to membranes via their C-terminal TMD.

Table 1: Proposed Functions of SNARE proteins.

SNARE	Function	Ref.
Qa-SNARE		
Syntaxin 1	Fusion between SV and PM	[245]
Syntaxin 2	Role in mouse sperm acrosome reaction	[266]
Syntaxin 3	Involved in neurite outgrowth	[267]
Syntaxin 4	Granule exocytosis in mast cells; Glut4 translocation	[268, 269]
Syntaxin 5	Transport from ER to Golgi; transport from RE to TGN	[270, 271]
Syntaxin 7	Fusion among LEs; fusion between LE and LY	[272]
Syntaxin 11	Role in regulating cellular cytotoxicity	[273]
Syntaxin 13	Fusion between EE and RE	[274]
Syntaxin 16	Transport from EE to TGN; fusion among EEs	[275, 276]
Syntaxin 17	Role in smooth ER membrane dynamics	[277]
Syntaxin 18	ER-mediated phagocytosis	[131]
Qb-SNARE		
Vti1a	Transport from EE to the TGN; fusion among EEs	[278, 279]
Vti1b	Fusion among LEs; fusion between LE and LY	[240]
GS27	ER to Golgi transport	[280]
GS28	Transport from EE/RE to TGN	[281]
Qc-SNARE		
Syntaxin 6	Transport from EE to TGN; fusion among EEs	[282, 283]
Syntaxin 8	Fusion among LEs; fusion between LE and LY	[284]
Syntaxin 10	Localised to TGN	[285]
GS15	Transport from EE/ER to TGN	[281]
Bet1	ER to Golgi transport	[286]
SLT1	Retrograde transport from Golgi to ER	[287]
Qbc-SNARE		
SNAP-23	Granule exocytosis in mast cells	[269]
SNAP-25	Fusion between SV and PM	[288]
SNAP-29	Modulates synaptic transmission	[289]
SNAP-47	Enriched in SV	[290]
R-SNARE		
VAMP1	Fusion between SV and PM	[291]
VAMP2	Neurotransmitter exocytosis	[292]
VAMP3	Fusion between RE and EE and between RE and PM	[126, 282]
VAMP4	Transport from EE to TGN; fusion among EEs	[293]
VAMP5	Localised to PM	[294]
VAMP7	Fusion between LE and LY; phagosome maturation	[295, 296]
VAMP8	Fusion among LEs; Granule exocytosis in mast cells	[269, 296]
Ykt6	EE/RE to TGN	[281]
SEC22b	Fusion between ER and phagosomes	[129]
Q-SNARE (unclassified)		
D12	Fusion between ER and phagosomes	[131]

EE, early endosome ER, endoplasmic reticulum LE, late endosome
LY, lysosome PM, plasma membrane RE, recycling endosome
SV, synaptic vesicle TGN, *trans*-Golgi network

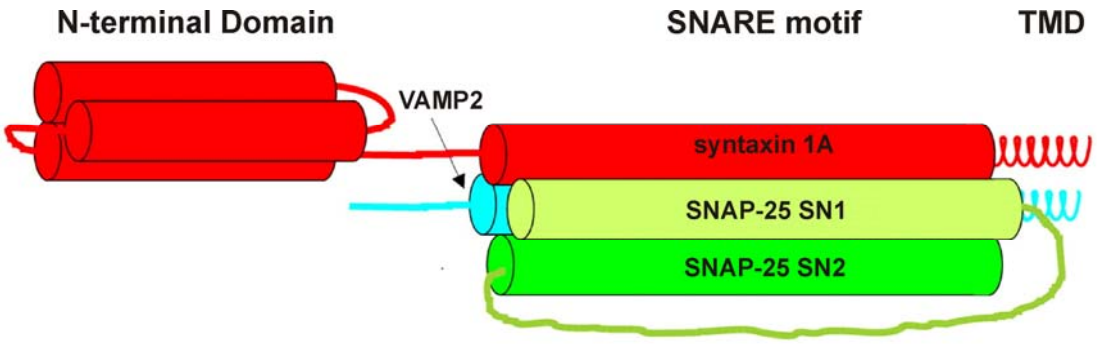


Figure 1.11: Structural organisation of a neuronal SNARE complex.

A cartoon illustrating a neuronal SNARE complex consisting of three SNARE proteins: syntaxin 1A (Qa-SNARE), SNAP-25 (Qbc-SNARE) and VAMP2 (R-SNARE). Syntaxin 1A and VAMP2 are anchored to membranes by their transmembrane domain (TMD), whereas SNAP-25 is attached to membranes via palmitoylation of cysteine residues in the linker region between its two SNARE motifs (i.e. SN1 and SN2). Assembly of the SNARE complex is regulated by phosphorylation of syntaxin 1A and/or SNAP-25, as well as by an interaction between the Habc domain and the SNARE motif of Syntaxin 1A. Adapted from Antonin *et al.* [265].

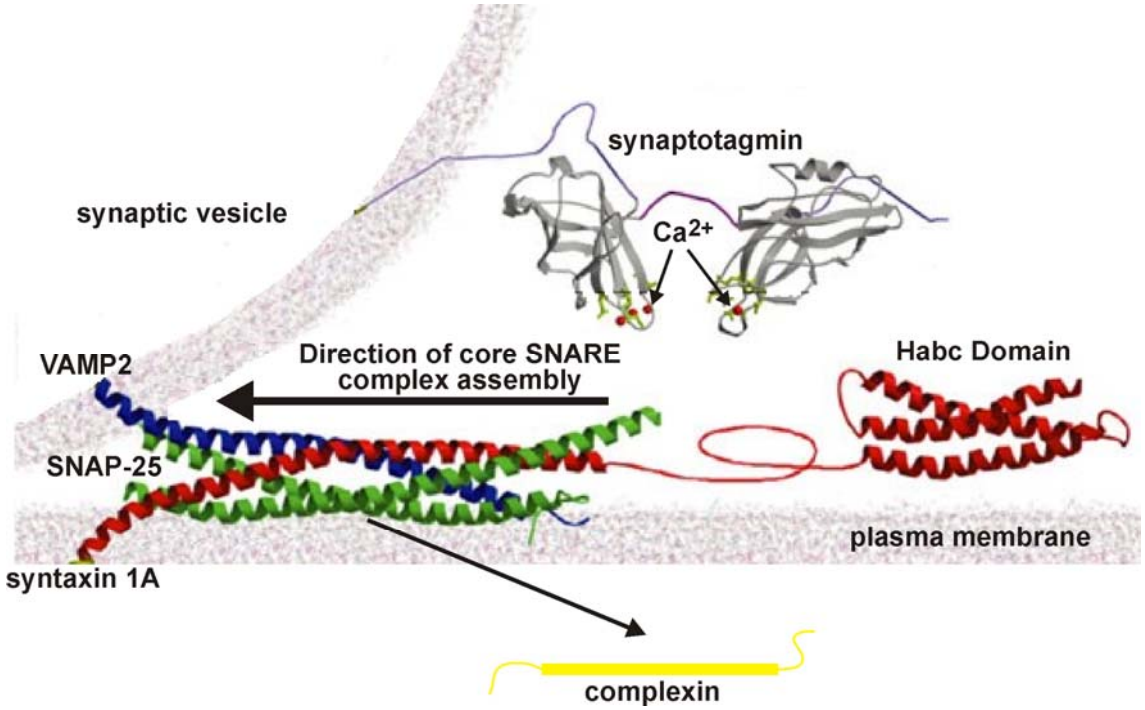


Figure 1.12: Ca^{2+} triggered 'zippering' of neuronal SNARE proteins

Complexin is a SNARE complex-binding protein that inhibits the final fusion step. Upon an influx of Ca^{2+} into neuronal cells, another SNARE complex binding protein synaptotagmin binds to Ca^{2+} and displaces complexin from the assembled neuronal SNARE complex. This allows the 'zippering' of the neuronal SNARE complex to proceed in an N- to C-terminal direction to bring the synaptic vesicle and the plasma membrane into close proximity, overcoming the associated energy barrier and thereby allowing membrane fusion to occur. Diagram adapted from Chapman *et al*. [297].

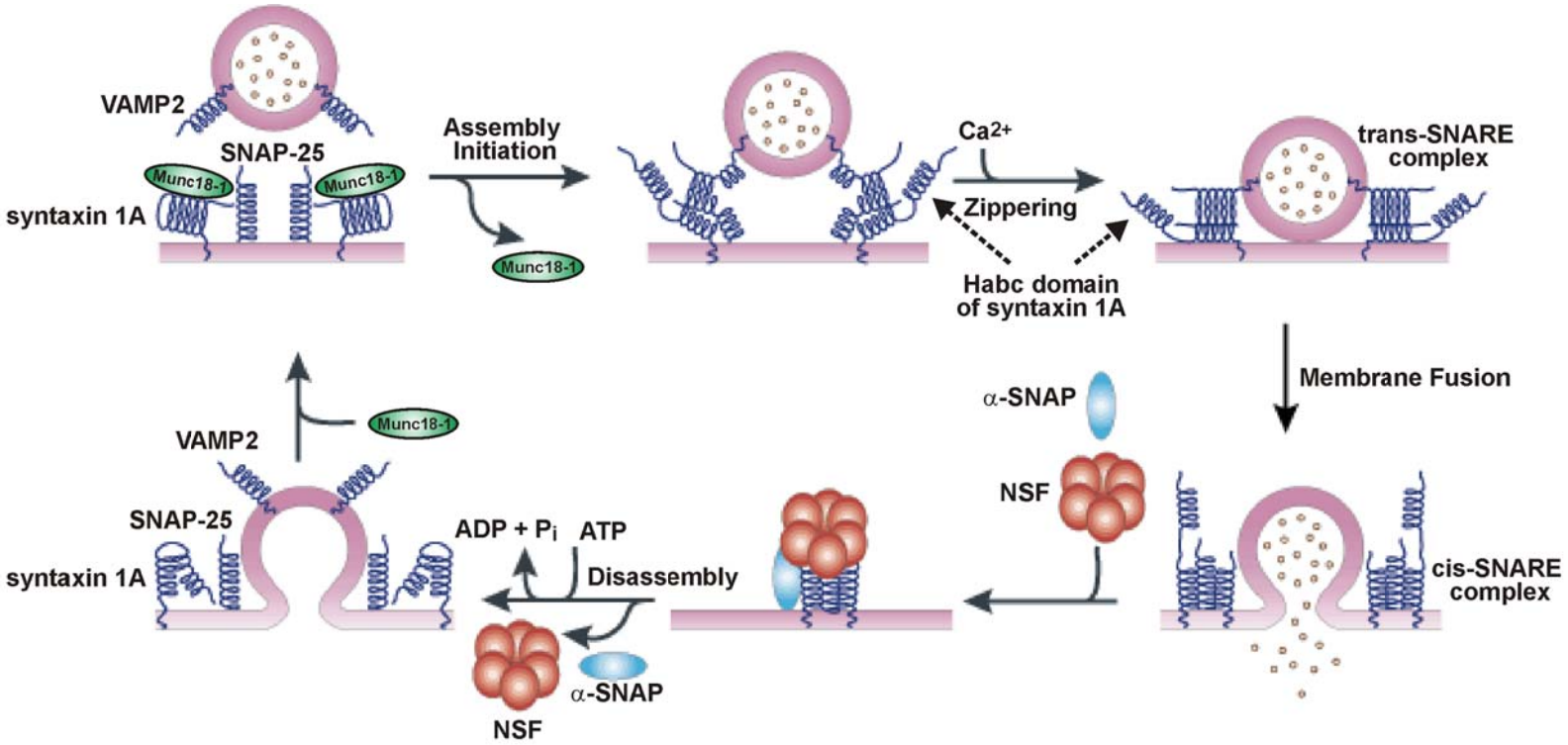


Figure 1.13: Assembly and disassembly of SNARE complexes.

In this model, the Qa-SNARE syntaxin 1A exists in a 'closed' conformation, where the Habc domain interacts with the SNARE motif to prevent SNARE complex assembly. Munc18-1 binds to syntaxin 1A in this 'closed' conformation. Phosphorylation of syntaxin 1A triggers the dissociation of Munc18-1 and thus allows the assembly of a *trans*-SNARE complex consisting of syntaxin 1A, SNAP-25 and VAMP2 to be initiated. The final step in SNARE complex assembly is triggered by external factors, such as in influx in Ca^{2+}, which leads to the 'zippering' of the complex from the N- to C-terminal direction to form a functional *trans*-SNARE complex. Membrane fusion results in the conversion of the *trans*-SNARE complex into a *cis*-SNARE complex, which is subsequently disassembled by NSF ATPase and -SNAP. Diagram adapted from Chen *et al.* [299].

Among neuronal SNARE proteins, syntaxin 1A is the only Q-SNARE that contains an N-terminal Habc domain, whereas all three Q-SNAREs of the late endosomal complex (syntaxin 7, Vti1b and syntaxin 8) have Habc domains (Figure 1.14). The N-terminal Habc domains of syntaxin 7, Vit1b and syntaxin 8 have high α-helical contents that form a three-helix bundle [298]. However, the Habc domain of syntaxin 7 appears to have a different function to that of the Habc domains of Vti1b and syntaxin 8. Similar to the Habc domain of syntaxin 1A, the Habc domain of syntaxin 7 interacts with the SNARE domain, thus forcing syntaxin 7 to adopt a 'closed' conformation which retards the assembly of late endosomal SNARE complexes [298]. Syntaxin 7 is the first syntaxin reported to adopt such a 'closed' conformation that does not function at the plasma membrane [298]; Vit1b and syntaxin 8 were not shown to adopt a similar 'closed' conformation [299].

The number of *trans*-SNARE complexes that are required for the formation of a membrane fusion pore, through which contents are exchanged (e.g. cytokines), is currently unknown as different experimental approaches have arrived at different conclusions [300, 301]. Cooperation between at least three neuronal *trans*-SNARE complexes (each composed of syntaxin 1A, SNAP-25 and VAMP2) have been calculated to be required for the exocytosis of neurotransmitters (e.g. acetylcholine, dopamine and serotonin) by PC12 cells, a rat neuronal cell line [302]. By contrast, studies on the release of norepinephrine from PC12 cells suggested that five to eight syntaxin 1A molecules were required for fusion pore formation [303]. Other estimates, based on the inhibition of synaptic SNARE function by botulinum neurotoxins, have suggested that 10-15 *trans*-SNARE complexes were required for neurotransmitter release [304, 305]. Notably, it has recently been shown that endosomal SNARE complexes can undergo multimerisation [306]. Despite the uncertainty regarding the number of *trans*-SNARE complexes required for the formation of a membrane fusion pore, the cooperative action of non-covalently linked SNARE complexes is crucial for membrane fusion.

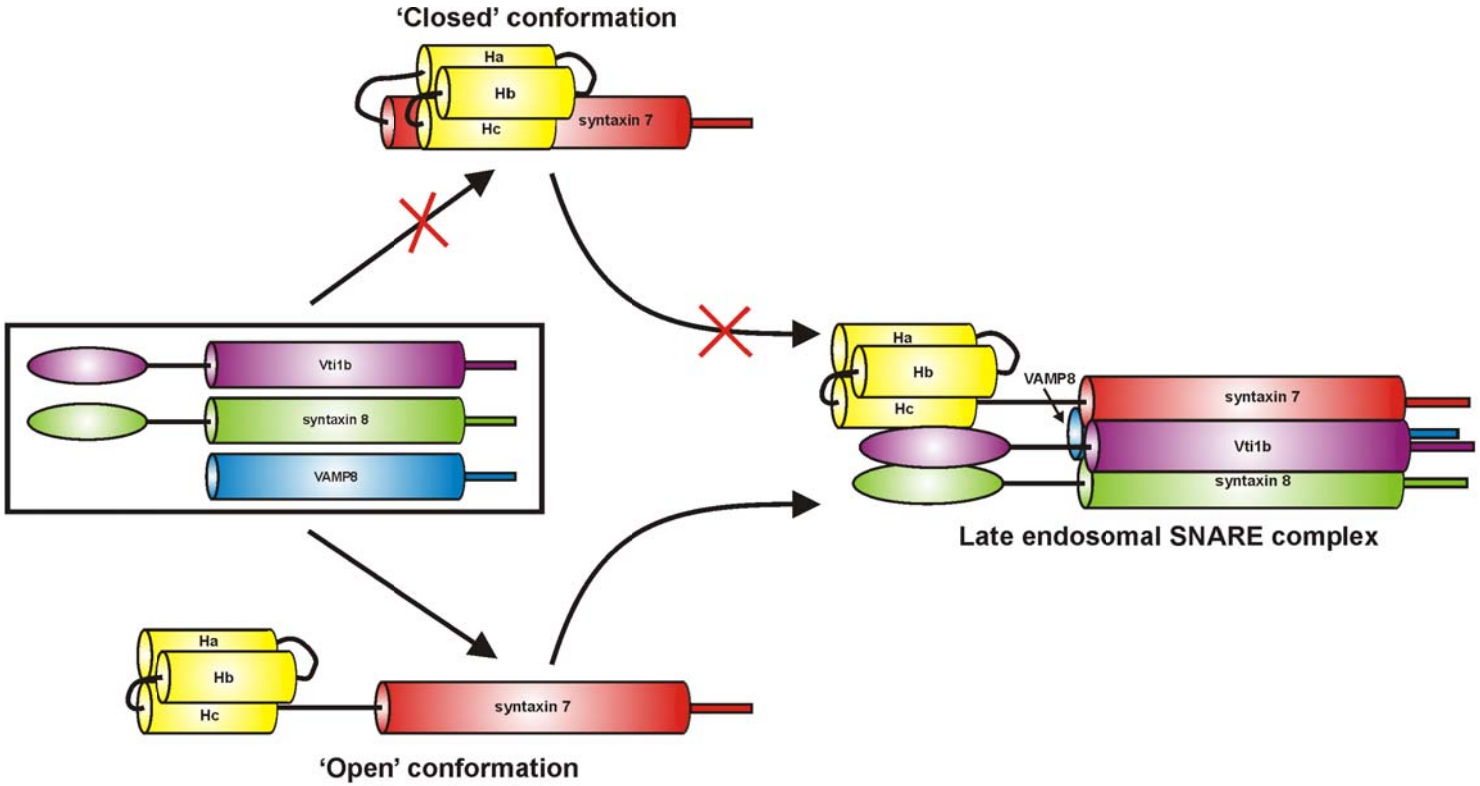


Figure 1.14: Assembly of a late endosomal SNARE complex.

The best studied late endosomal SNARE complex consists of four SNARE proteins: syntaxin 7 (Qa-SNARE), Vti1b (Qb-SNARE), syntaxin 8 (Qc-SNARE) and VAMP8 (R-SNARE). All four proteins are anchored to membranes via their C-terminal transmembrane domain (TMD). VAMP8 lacks an N-terminal domain. In the steady-state, the Habc domain of syntaxin 7 is likely to interact with the SNARE motif of syntaxin 7, thereby preventing late endosomal SNARE complex assembly. Adapted from Antonin *et al.* [286].

1.3.3.4 Regulation of the Assembly of SNARE Complexes by Phosphorylation

Although various mechanisms exist to regulate the assembly of SNARE complexes (e.g. Sec1/Munc18 proteins), the phosphorylation of SNARE proteins is emerging as a major mechanism for regulating SNARE complex assembly. The neuronal SNARE proteins (e.g. syntaxin 1A, SNAP-25 and VAMP2) that mediate the fusion of synaptic vesicles at the pre-synaptic plasma membrane in neuronal cells (e.g. endocrine and PC12 cells) have been studied most extensively and thus provide a paradigm for the regulation of SNARE complex assembly. SNAP-25 has been shown to be phosphorylated at Thr-138 and Ser-187 by PKC in response to the opiate drug morphine [307]. Notably, Ser-187 is located in the C-terminal SNARE motif (SN2) of SNAP-25 (Figure 1.11) and its phosphorylation hinders the interaction of SNAP-25 with syntaxin 1A and VAMP2, thus inhibiting *trans*-SNARE complex formation [308]. No such negative regulation appears to be associated with the PKC-mediated phosphorylation of Thr-138, which is located in the linker region connecting both SNARE motifs [307].

Similar to SNAP-25, phosphorylation of syntaxin 1A has also been shown to regulate neuronal SNARE complex formation [309, 310]. In the steady-state, the Habc domain of syntaxin 1A interacts with the SNARE motif to prevent interactions with other neuronal SNARE proteins (e.g. SNAP-25 and VAMP2) [304, 305]. In response to an 'activation signal' (e.g. an increase in intracellular free Ca^{2+}), syntaxin 1A is phosphorylated at Ser-14 by casein kinase II (CKII) resulting in the release of the Habc domain and the freeing of the SNARE motif for interactions with other SNARE proteins [311, 312]. The CKII-mediated phosphorylation of syntaxin 1A also enhances its interaction with synaptotagmin, which is required for the final step in membrane fusion [311]. Furthermore, synaptotagmin itself is phosphorylated by Ca^{2+}/calmodulin-dependent kinase II; this phosphorylation event is necessary for the maximal binding of synaptotagmin to syntaxin 1A (Figure 1.12) [313].

Syntaxin 4, a Qa-SNARE that has been shown to be involved in cytokine secretion by macrophages [282], is phosphorylated by protein kinase A (PKA) and CKII *in vitro*. Moreover, the binding of syntaxin 4 to SNAP-23 (a Qbc-SNARE) is decreased upon its phosphorylation by PKA [314]. The importance of these

phosphorylation events is illustrated by the finding that the PKA-dependent failure of syntaxin 4/SNAP-23/VAMP2 complexes to form resulted in the impaired release of amylase by rat parotid acinar cells [315]. Additionally, activation of PKC in thrombin-stimulated platelets resulted in a decrease in the interaction of syntaxin 4 with SNAP-23 and hindered the exocytosis of serotonin [316].

SNAP-23, a non-neuronal homologue of SNAP-25, has been shown to be phosphorylated by PKC at a number of sites in platelets and mast cells (e.g. Ser-23, Thr-24, Ser-95, Ser-120 and Ser-161) [317, 318]. Mutation of both Ser-23 and Thr-24 in SNAP-23 to phosphomimetic aspartate residues inhibited the binding of SNAP-23 to syntaxin 4; however, mutation of Ser-95 and Ser-120 had little effect on binding [307].

1.3.3.5 Regulation of the Assembly of SNARE Complexes by SM Proteins

SM (Sec1/Munc18) proteins are a group of cytosolic proteins that interact directly with members of the syntaxin sub-family of SNARE proteins (e.g. syntaxin 1A) [319]. They are peripherally associated with membranes, in part through their interactions with syntaxins. SM proteins are thought to act in a fashion similar to molecular chaperones, organising syntaxins into conformations that are competent to interact with other SNARE proteins [320].

Munc18-1 interacts with the ‘closed’ conformation of syntaxin 1A in order to maintain it in a conformation that inhibits the formation of undesirable SNARE complexes [321]. In syntaxin 1A, the Habc domain and SNARE motif are separated by a highly flexible linker region of approximately 40 amino acids [312]. The introduction of mutations into the linker region resulted in the reduced binding of Munc18-1 to syntaxin 1A [312]. Moreover, deletion of the Habc domain accelerated (up to 3,000-fold) the *in vitro* assembly of SNARE complexes of recombinant forms of syntaxin 1A, SNAP-25 and VAMP2 [309, 322]. Similarly, the *in vitro* fusion of neuronal SNARE protein-bearing liposomes was accelerated by the removal of the Habc domain from syntaxin 1A [223]. Phosphorylation of syntaxin 1A appears to be one of the ‘triggers’ for the release of Munc18-1 [309], the other being phosphorylation of Munc18-1 on Ser-313 by PKC [323, 324]. Structural analysis of syntaxin 1A and Munc18-1 has suggested that the inhibition was caused by

electrostatic repulsion between the phosphate groups on Munc18-1 and the predominantly negatively charged linker region between the Habc domain and SNARE motif in syntaxin 1A [313]. The fact that Munc18-1-deficient mice die immediately after birth due to a complete loss of synaptic exocytosis indicates the importance of Munc18-1 for regulating the correct assembly of SNARE complexes [325].

Munc18-3, another member of the SM family, has been shown to bind to syntaxin 4 in macrophages [326]. Significantly, a SNARE complex consisting of syntaxin 4, SNAP-23 and VAMP3 has recently been reported to be important for cytokine secretion and phagocytosis in macrophages [282, 327]. The binding of Munc18-3 prevents syntaxin 4 from interacting with its partner SNARE proteins, SNAP-23 and VAMP3 [328]. Munc18-3 has also been shown to interact with syntaxin 4 in adipocytes and to regulate the translocation of the glucose transporter Glut4 to the cell surface in response to insulin [282, 329, 330].

1.3.3.6 Intracellular Targeting of SNARE Proteins

The correct subcellular localisation of SNARE proteins is critical to their functions in regulating membrane fusion events. The ability of SNARE proteins to reach the correct destination is potentially mediated by adaptor protein complexes. Adaptor protein complexes (APC-1 through APC-4) are involved in the selection of cargo and the budding of vesicles from donor membranes [331]. They interact with sorting signals in the cytosolic domains of membrane-associated proteins, thereby sorting the appropriate cargo into the budding vesicle [332]. A role for adaptor protein complexes in the targeting of SNARE proteins has been proposed because several SNARE proteins (e.g. syntaxins 4, 6, 7, 8 and 13 as well as VAMPs 4 and 7) contain di-leucine or tyrosine-based motifs that have been shown in other membrane-associated proteins to directly mediate their interaction with adaptor protein complexes [333]. For example, deletion of the tyrosine motif in syntaxin 6, a *trans*-Golgi network SNARE protein, leads to its accumulation at the plasma membrane [283, 334]. Similarly, di-leucine motifs in both syntaxin 7 and 8 have been shown to be critical for their intracellular localisations [335]. In the case of VAMP4, transfection studies in rat kidney cells revealed that wild-type VAMP4 co-localised with APC-1 in the *trans*-Golgi network region, whereas a VAMP4 mutant lacking the di-leucine motif was mis-localised to the periphery of the cells [333].

Although the transmembrane domains of SNARE proteins are important for their anchoring to membranes, the cytoplasmic domains of SNARE proteins have been suggested to contain the information necessary for their specific targeting to membranes [336]. For example, in the PC12 neuronal cell line, chimeric SNARE proteins consisting of the cytoplasmic domain of syntaxin 1A (a plasma membrane-localised SNARE protein) fused to the transmembrane domains of the *trans*-Golgi network/endosomal SNARE proteins syntaxin 6, syntaxin 7 and syntaxin 8 accumulated at the plasma membrane in rat liver cells [336]. In contrast, when the cytoplasmic domains of syntaxin 6, syntaxin 7 and syntaxin 8 were fused to the transmembrane domain of syntaxin 1A, the chimeric SNARE proteins remained localised to *trans*-Golgi network/endosomal membranes [336, 337]. Similarly, truncation of the transmembrane domain of syntaxin 3 resulted in the accumulation of the normally plasma membrane-localised SNARE protein in the Golgi [338].

SM proteins may also contribute to the regulation of SNARE protein subcellular localisation, possibly by functioning as 'trafficking chaperones' [338]. As mentioned earlier, the 'open' conformation of syntaxin 1A is able to form *trans*-SNARE complexes with SNAP-25 and VAMP2, whereas Munc18-1 maintains syntaxin 1A in a SNARE complex-incompatible 'closed' conformation. It has been suggested that Munc18-1 may facilitate the correct intracellular trafficking of syntaxin 1A by maintaining it in a 'protected' conformation that prevents its participation in the formation of unwanted SNARE complexes [338]. For example, experiments in human salivary gland HSY cells showed that GFP-tagged syntaxin 4 required the co-expression of Munc18-3 for its delivery to the plasma membrane [339].

SNAP family proteins (e.g. SNAP-25) do not contain hydrophobic membrane-anchoring transmembrane domains; instead they are attached to cellular membranes via palmitoylation of cysteine residues. Each alpha helix of SNAP-25 possesses a cysteine-rich domain consisting of four cysteine residues, whereas SNAP-23 contains a total of five cysteine residues in its cysteine-rich domain [340]. Palmitoylation of SNAP-25 is highly sensitive to single cysteine substitutions and mutation of any of the four cysteine residues within the cysteine-rich domain of SNAP-25 caused a dramatic decrease in palmitoylation. Experiments in COS-7 cells have shown that palmitoylation of SNAP-23 and SNAP-25 plays an essential role in their targeting to the plasma membrane as mutants lacking the cysteine-rich domains are mis-localised to the cytosol [338].

1.4 The Roles of SNARE Proteins in Macrophages

Endocytic and exocytic processes are central to the effector functions of macrophages. Because these processes involve a number of vesicle trafficking and membrane fusion events, SNARE proteins are likely to have fundamental and crucial roles in macrophages. Indeed, studies have started to emerge which indicate that SNARE proteins have key roles in cytokine secretion and phagocytosis by macrophages [126, 282]. They are also likely to be important for the presentation of antigens to T-cells.

1.4.1 SNARE Proteins and Cytokine Secretion

Syntaxin 7, syntaxin 6 and Vti1b have been shown to be involved in the trafficking of TNF from the Golgi to recycling endosomes in RAW 264.7 macrophages [240, 282]. Recently, IL-6 trafficking has also been shown to utilise the same SNARE proteins in RAW 264.7 macrophages [341]. Interestingly, these cytokines are segregated within recycling endosomes and independently secreted, with TNF released at the site of phagocytosis, but not IL-6. In order to release TNF, VAMP3 (a recycling endosome-localised R-SNARE) forms a *trans*-SNARE complex with plasma membrane-localised syntaxin 4 and SNAP-23 [126]. Significantly, syntaxin 6, Vti1b and VAMP3 expression was up-regulated by LPS in RAW 264.7 macrophages [126, 240]. Furthermore, the treatment of RAW 264.7 macrophages with interferon-gamma (IFNγ) prior to LPS activation resulted in the increased expression of syntaxin 6, Vti1b and VAMP3 [126, 240]. This was a particularly interesting finding as IFNγ is able to prime macrophages for heightened cytokine release upon activation with LPS [126, 240]. Thus, the priming effects of IFNγ on macrophages may be explained in part by its ability to regulate SNARE protein expression in macrophages. The roles of SNARE proteins in the secretion of other cytokines, as well as chemokines, remain to be established.

1.4.2 SNARE Proteins and Phagocytosis

SNARE proteins have been shown to be involved at different stages of phagocytosis. For example, the endosomal SNARE proteins VAMP3 and VAMP7

were recruited to sites directly underneath the points at which particles attached the plasma membrane of RAW 264.7 macrophages during the early stages of phagocytosis [131]. Bone marrow-derived macrophages from VAMP3-deficient mice exhibited a delay in phagocytosing zymosan particles, however, phagosome maturation was not impaired as assessed by acquisition of LAMP-1 [342].

Syntaxin 7 and 13 are Qa-SNARE proteins that regulate endocytic membrane transport. Syntaxin 13 is involved in membrane fusion between recycling and early endosomes [343]. Both syntaxin 7 and syntaxin 13 are required at distinct steps of phagosome maturation where they govern the fusion of phagosomes with either early/late endosomes or lysosomes, respectively [127]. Interestingly, Syntaxin 18 is involved in ER-mediated phagocytosis in J774 macrophages [131]. This Q-SNARE has been proposed to play a role in vesicular transport between the ER and the Golgi [129].

The R-SNARE VAMP8 has been shown to form a complex with syntaxin 7, Vti1b and syntaxin 8 to mediate membrane fusion of late endosomes in alveolar macrophages [344]. Subcellular localisation studies revealed that syntaxin 7 co-localised to mature phagosomes in RAW 264.7 macrophages [127]. Interestingly, both cytokine secretion and phagocytosis pathways have been linked at the plasma membrane; newly synthesised cytokines are trafficked to recycling endosomes, which fuse with the plasma membrane at the site of phagocytosis [126].

1.5 Pathological Conditions Arising from Macrophage Dysfunction

Macrophage dysfunction, or their subversion by pathogens, contributes to the pathogenesis of a number of highly debilitating, and even life-threatening, diseases. For example, the excessive and/or uncontrolled release of pro-inflammatory cytokines (e.g. TNF and IL-6) by macrophages contributes to several pathological conditions including rheumatoid arthritis (RA), inflammatory bowel disease, chronic obstructive pulmonary disease, atherosclerosis and septic shock [345-347]. During uncontrolled bacterial infections, the systemic release of pro-inflammatory cytokines, such as TNF and IL-6, causes vasodilation and enhanced capillary permeability, potentially leading to hypotension [348]. The decreased tissue perfusion and oxygen delivery accompanying this can cause shock, multiple organ failure and, in extreme cases, death. Recent studies indicate that up to 40% of the deaths that occur in Intensive Care Units in Australian hospitals are due to septic shock (Australian Institute of Health and Welfare).

TNF, along with IL-1α and IL-1β, also plays a crucial role in the pathogenesis of RA [349]. It is estimated that 1-2% of the Australian population suffers from RA. RA not only affects the quality-of-life for the individuals concerned but it also places a huge financial burden on the health care system (ABS 2001, National Health Survey). Direct medical costs and indirect costs relating to work disability due to RA are estimated to be more than $10 billion per annum. TNF, IL-1α and IL-1β are produced in excessive amounts in the affected joints of RA patients, thereby maintaining inflammation. The recruitment of other immune cells to the site of inflammation, as well as the proliferation of synovial fibroblasts, eventually leads to a state of chronic inflammation. The release of a number of matrix-degrading enzymes (e.g. collagenase and matrix metalloproteinases) by macrophages at sites of inflammation in joints results in the gradual destruction of the joints [350]. Thus, in order to maintain a fine balance between protection and destruction, the production and secretion of pro-inflammatory cytokines by macrophages needs to be tightly controlled. The regulation of SNARE proteins, and the subsequent membrane fusion events, may hold the key for controlling cytokine secretion.

A number of diseases have been linked to FcγR-mediated phagocytosis [351]. For example, one form of glomerulonephritis develops due to the formation and

deposition of immune complexes around glomeruli in reaction to foreign and/or self-antigens that are captured in glomerular structures [352]. A polymorphism in the transmembrane domain of FcγRIIB in patients with systemic lupus erythematosus results in dysregulated FcγR signalling [99, 353]. FcγRs are also involved in the formation of systemic immune complexes that are characteristic of other autoimmune conditions, such as autoimmune haemolytic anaemia and autoimmune neutropenia [354]. The crucial role of SNARE proteins in membrane fusion and their presence in phagosomal membranes suggest that SNARE proteins have critical roles in phagosome biogenesis. Hence, defective SNARE proteins may be responsible for dysregulated phagocytosis and the resulting pathologies.

Some pathogenic bacteria use phagocytosis as an opportunity to enter macrophages and then use various mechanisms to subvert the microbicidal properties of macrophages to survive as intracellular pathogens. This subversion of macrophages is vital to the pathogenicity of the bacteria as it provides a cellular niche in which to hide from the immune system. *Salmonella typhimurium*, which causes gastroenteritis in humans, invades macrophages and can cause either immediate cell death or establish an intracellular niche within phagosomes [355, 356]. Its survival in phagosomes (so-called *Salmonella-containing vacuoles*) depends on the secretion of virulence factors (e.g. Nramp1, PhoP and PhoQ) that prevent the phagosome from maturing and fusing with lysosomes [357-359]. Nramp1 prevents Salmonella-containing vacuoles from interacting with early endocytic compartments, while the PhoP-PhoQ virulence system prevents their interaction with late endocytic compartments. *S. typhimurium* also secretes various enzymes, such as superoxide dismutases (e.g. sodCI and sodCII) and glucose 6-phosphate dehydrogenase to protect itself from pathogen-killing reactive oxygen species produced by activated macrophages via the NADPH oxidase complex [360].

Similar to *S.typhimurium*, the survival of *M.tuberculosis* in macrophages depends on its capacity to modify the nascent phagosome so that it fails to interact with late endosomes and lysosomes. *M.tuberculosis*-containing phagosomes have fewer or less active H^+-ATPase molecules and do not possess lysosomal hydrolases such as cathepsin D [361]. *M.tuberculosis* secretes lipoglucans, including the phosphatidylinositol analogue, lipoarabinomannan, that blocks the trafficking of vesicles containing the H^+-ATPase and lysosomal constituents from the *trans*-Golgi

network to phagosomes [362, 363]. *L.monocytogenes*, a food-borne pathogen of humans, causes a group of disorders known as listeriosis. The manifestations of listeriosis include septicaemia, meningitis, encephalitis and intrauterine or cervical infections [364, 365]. *L.monocytogenes* survives in macrophages by manipulating intracellular signalling. Elevated levels of intracellular free Ca^{2+}, in combination with diacylglycerol, are necessary for the translocation of PKC to early endosomes [366]. *L.monocytogenes* is able to delay the increase in intracellular free Ca^{2+} in order to enhance its internalisation at sites of phagocytosis. As the phagosomes mature (i.e. become more acidified), *L.monocytogenes* secretes proteins, such as listeriolysin O, that create pores in the phagosome membrane through which the bacteria escape into the cytosol [367]. As the survival of pathogens is dependent on inhibiting phagosome maturation, which involves a number of membrane fusion events, SNARE proteins may play a role in the subversion of macrophages by pathogens.

No diseases to date have been shown to be due to SNARE defects in macrophages. However, a number of diseases have been linked to defective membrane fusion arising from alterations in SNARE proteins and/or their regulation in other immune/haematopoietic cells. For example, a genetic variant of VAMP8, which resulted in altered platelet degranulation, has been linked with early-onset myocardial infarction [368]. Mutations in Munc13-4 result in familial hemophagocytic lymphohistiocytosis, an autosomal recessive disorder characterised by a defect in NK cell function [369]. Recently, mutations in another SNARE protein, namely syntaxin 11, have also been linked to this disease [370]. Hermansky-Pudlak syndrome consists of a group of genetic disorders, which share the clinical findings of oculocutaneous albinism, platelet storage pool deficiency (SPD), and ceroid lipofuscinosis. Pallidin, a protein linked to SPD, interacts with syntaxin 13 and a mutation in pallidin resulted in decreased levels of syntaxin 13 [371, 372]. A recent study has implicated a number of Golgi-associated SNAREs, including syntaxin 6, GS27 and GS28, in monocrotaline-induced pulmonary hypertension [373]. Mutations in SNARE binding partners also result in diseases. For example, arthrogryposis-renal dysfunction-cholestasis syndrome, an autosomal recessive disorder, is caused by mutations in VPS33B, a SNARE binding partner [374, 375]. These genetic studies highlight the importance of SNARE proteins and SNARE regulatory proteins for normal cell functions.

1.6 Hypothesis and Aims

The critical involvement of CSF-1 in governing the differentiation, proliferation and survival macrophages is well established and largely understood [175, 176, 191, 376]. Whilst the ability of CSF-1 to modulate the effector functions of macrophages (e.g. cytokine secretion, phagocytosis and macropinocytosis) has also been established, the molecular basis for their modulation by CSF-1 is poorly understood.

The ***hypothesis*** tested in this thesis was that CSF-1 modulates the immune functions of macrophages (e.g. cytokine secretion and phagocytosis) by regulating the expression and/or activity of proteins (e.g. SNARE proteins) involved in exocytosis and endocytosis.

The specific aims of this thesis were:

Aim 1: To identify proteins involved in exocytosis and endocytosis (e.g. SNARE proteins) whose expression was regulated by CSF-1 and to establish the basis for their transcriptional control by CSF-1.

Aim 2: To investigate the post-translational regulation of the SNARE protein syntaxin 7 by CSF-1.

Aim 3: To investigate the effects of CSF-1 on the formation and subcellular localisation of endosomal SNARE complexes in macrophages.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

Dulbecco's Modified Eagle Medium, Penicillin/Streptomycin, GlutaMAX-1™, pre-cast SDS-polyacrylamide (Tris-Glycine) gels, BenchMark™ pre-stained SDS-PAGE protein molecular mass standards, SuperScript III reverse transcriptase and foetal calf serum were purchased from Invitrogen (Carlsbad, CA, USA). Tissue culture plasticware was supplied by Becton Dickinson (Franklin Lakes, NJ, USA). Pre-cast Criterion SDS-PAGE gels and isoelectric focussing strips (pH 7-10) were obtained from Bio-Rad (Hercules, CA, USA). LY294002 (PI3-kinase inhibitor), Akt1/2i (Akt inhibitor), rapamycin (p70S6 kinase inhibitor), SB203580 (p38 MAP kinase inhibitor), GF109203X (protein kinase C inhibitor) and PP2 (Src-family kinase inhibitor) were purchased from Calbiochem (San Diego, CA, USA), whilst SP600125 (JNK inhibitor) and UO126 (MEK inhibitor) were obtained from Alexis Biochemicals (San Diego, CA, USA) and Upstate Biotechnology (Billerica, MA, USA), respectively. Protein G-Sepharose, enhanced chemiluminescence (ECL) reagents and [^{32}P]-orthophosphate were from Amersham Biosciences (Buckinghamshire, UK). *Complete*™ EDTA-free protease inhibitors were purchased from Roche Diagnostics (Basel, Switzerland). Phorbol myristate acetate, 8-Br-cAMP, paraformaldehyde and Ponceau S were supplied by Sigma Chemicals (St. Louis, MO, USA), while Fuji X-ray film was from Hanimex (Brookvale, NSW, Australia). DNA restriction enzymes, calf intestinal alkaline phosphatase (CIP) and T4 DNA ligase were from BioLabs (Ipswich, MA, USA) and *Pfu* DNA polymerase from Promega (Madison, WI, USA). RNeasy® and QIAGEN® Plasmid purification kits were obtained from QIAGEN (Hilden, Germany), while the UltraClean™ 15 DNA purification kit was from Mo Bio Laboratories, Inc. (Carlsbad, CA, USA). Microcon centrifugal filter devices and polyvinylidene fluoride membranes were from Millipore (Billerica, MA, USA). GEL/MOUNT™ aqueous mounting medium was from ProSciTech (Kirwan, QLD, Australia). Mouse syntaxin 7, syntaxin 8, Vti1b, VAMP8 and 18S rRNA TaqMan Real-Time PCR probe sets were from Applied Biosystems (Foster City, CA, USA). Ultra pure bacterial lipopolysaccharide was purchased from Invivogen (San Diego, CA, USA). Dimethylsulphoxide was purchased from Merck (Whitehouse Station, NJ, USA).

2.1.2 Antibodies

The mouse monoclonal anti-syntaxin 8 and anti-Vti1b antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA), while the rabbit polyclonal anti-syntaxin 7 and anti-VAMP8 antibodies were generous gifts from Dr. David James (Garvan Institute, Sydney, Australia). The rabbit polyclonal anti-EEA1, anti-LAMP1, anti-actin and anti-ERK2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the mouse monoclonal anti-phosphotyrosine 4G10 antibody obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). The rabbit polyclonal anti-phospho-ERK1/2, anti-phospho-Akt and anti-Akt and antibodies were from Cell Signalling (Danvers, MA, USA). AlexaFluor®-594 goat anti-mouse IgG and AlexaFluor®-488 goat anti-rabbit IgG were purchased from Molecular Probes (Carlsbad, CA, USA). ELISA detection antibodies for mouse TNF, IL-6 and IL-10 were purchased from BD Biosciences (Franklin Lakes, NJ, USA).

2.1.3 Growth factors and cytokines

Recombinant, human colony stimulating factor-1 (CSF-1) was a generous gift from Chiron (Emeryville, CA, USA), while recombinant mouse granulocyte/macrophage-colony stimulating factor (GM-CSF) was from Apollo Cytokine Research (Alexandria, NSW, Australia).

2.2 Methods

2.2.1 Preparation of Bone Marrow-Derived Macrophages

The use of mice in this study was approved by the Melbourne Health Animal Ethics Committee (Animal Ethics Projects 2003.038 and 2006.049). Eight week-old, female C57BL/6 mice were sacrificed by asphyxiation with CO_2 and their femurs removed under aseptic conditions. Bone marrow cells were flushed from the femurs into Dulbecco's Modified Eagle Medium (DMEM) using a syringe fitted with a 23-gauge needle and collected by centrifugation at 350 x *g* for 5 min at 4°C. The cells were resuspended at a concentration of 1 x 10^6 cells per ml in DMEM supplemented with 15% heat-inactivated foetal calf serum (FCS), CSF-1 (2,500

U/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and 2 mM GlutaMax-1™ (subsequently referred to as 'macrophage medium'). Approximately 4 x 10^7 cells were seeded per T175 tissue culture flask and cultured for 3 days at 37°C in a humidified atmosphere of 5% CO_2. During this time, stromal cells (e.g. fibroblasts) and activated macrophages adhered to the flask surface, while early lineage macrophage progenitor cells remained in suspension. These cells were collected by centrifugation at 350 x *g* for 7 min at 4°C and then seeded (at a concentration of 1.4 x 10^6 cells per dish) into 10 cm tissue culture dishes. The cells were cultured for an additional 4 days, during which time they differentiated further and became approximately 80-90% confluent. The cells were made quiescent prior to stimulation with various stimuli (e.g. CSF-1 and phorbol myristate acetate) by culturing them in the absence of CSF-1 for 16 h.

2.2.2 Transfection of RAW 264.7 cells

RAW 264.7 cells were maintained in DMEM supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM GlutaMax-1™ (referred to as 10%-DMEM hereafter). The cells were passaged every second or third day, depending on experimental requirements, and transiently transfected by electroporation. Briefly, the cells were seeded at a density of 8 x 10^6 per 10 cm dish. Approximately 16 h later, the cells were harvested from the dishes by flushing with ice-cold DMEM using a syringe fitted with an 18-gauge needle. The cells were centrifuged at 350 x *g* for 5 min at 4°C and resuspended to a concentration of 25 x 10^6 cells/ml in 10%-DMEM supplemented with 10 mM HEPES (pH 7.4). Aliquots of 5 x 10^6 cells (in 0.2 ml) and 10 μg of plasmid were transferred into 0.4 cm GenePulser® cuvettes and electroporated at 280 V and 960 μF with a GenePulser® electroporation system from Bio-Rad (Hercules, CA, USA). The cells were then transferred to tubes containing 10 ml of 10%-DMEM and centrifuged at 175 x *g* for 5 min at 4°C to remove dead cells/cell debris. The cell pellet was gently resuspended in 10%-DMEM and the cells seeded in 10 cm tissue culture dishes.

2.2.3 Cell Lysis

Tissue culture medium was first aspirated and the cells washed twice with ice-cold phosphate-buffered saline (PBS). The cells were then lysed on ice with NP-40 lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate, 20 mM sodium fluoride, 10 mM β-glycerol phosphate and *Complete*™ protease inhibitors) for 30 min. The lysates were centrifuged at 4°C for 10 min at 13,000 x RPM to remove cell debris and the supernatant was retained. The protein content of the cell lysates was determined using a protein assay kit from Bio-Rad. The cell lysates were either used immediately or stored at -70°C.

2.2.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Protein samples (e.g. cell lysates) were prepared in SDS-PAGE sample buffer and heated at 95°C for 5 min. The samples were then loaded onto precast 10% or 14% SDS-PAGE (Tris-Glycine) gels and electrophoresed at 100 V. Proteins were transferred from the SDS-PAGE gels to polyvinylidene fluoride (PVDF) membranes at 500 mA for 90 min using the Towbin transfer buffer system [377]. The membranes were then stained with Ponceau S (0.1% Ponceau S in 5% acetic acid) to confirm equal transfer of proteins. The membranes were blocked in 3% bovine serum albumin (BSA) in TBST (Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature, followed by overnight incubation at 4°C with the appropriate primary antibody diluted in 1% BSA in TBST. The membranes were washed three times for 15 min each with TBST and then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Again, the membranes were washed with TBST before immunoreactive proteins were visualised by incubating the membranes with enhanced chemiluminescence (ECL) reagents for 1 min and then exposing the membranes to X-ray film. Prior to the reprobing of membranes with additional antibodies, the membranes were incubated at 55°C for 30 min in Western blot stripping buffer (50 mM Tris-HCl [pH 7.0], 2% SDS, 100 mM β-mercaptoethanol) to remove bound antibodies. The stripped membranes were then extensively washed for 1-2 h with TBST and re-blocked with 3% BSA in TBST.

2.2.5 Two-Dimensional SDS-PAGE

Isoelectric focussing (IEF) strips (pH 7-10; linear, 11 cm) were passively rehydrated overnight at room temperature in 8 M urea, 0.5% Triton X-100, 0.5% Pharmalytes and 10 mM dithiothreitol. Cell lysates were concentrated using Microcon centrifugal filter devices and 400 μg of the cell lysates diluted in 9 M urea, 50 mM dithiothreitol, 0.2% Pharmalytes, 4% CHAPS and 0.002% bromophenol blue. The samples were then allowed to absorb into the isoelectric focussing strips before being subjected to IEF at 20°C for 30,000 Vh using a PROTEAN IEF cell (Bio-Rad) and a rapid ramp protocol [378]. Focused strips were stored at 20°C overnight prior to resolution in the second dimension. The IEF strips were equilibrated in 0.5 M Tris-HCl (pH 8.8), 6 M urea, 2% SDS, 20% glycerol and 2% DTT for 15 min and then loaded onto 10% Criterion pre-cast gels (Bio-Rad) and electrophoresed at 100 V for 2 h. Proteins were transferred to PVDF membranes as described earlier for Western blotting.

2.2.6 Immunoprecipitation Assays

Cell lysates were pre-cleared of proteins that non-specifically bind to Protein G-Sepharose by adding 50 μl of 25% slurry of Protein G-Sepharose to each aliquot of cell lysate containing 0.5-1 mg of protein (in 1.0 ml of NP-40 lysis buffer) and incubating for 1 h at 4°C with mixing. The mixtures were then centrifuged at 13,000 x RPM at 4°C for 5 min and the cleared supernatants retained. The appropriate antibody (typically 1 μg per immunoprecipitation assay) was added to the supernatant and incubated on ice for 3-4 h. Following this incubation, 20 μl of 25% slurry of Protein G-Sepharose was added and the samples incubated at 4°C for 1 h with mixing. The samples were centrifuged at 5,000 x RPM for 1 min and the captured immune complexes washed four times with ice-cold NP-40 lysis buffer. Following the final wash, 20 μl of SDS-PAGE sample buffer was added and the samples heated at 95°C for 5 min. The samples were subsequently analysed by Western blotting following their electrophoresis on either 10% or 14% pre-cast SDS-PAGE gels.

2.2.7 Flow Cytometric Analysis

Flow cytometric analysis was performed with a FACSCalibur™ machine and CellQuest™ software (Becton Dickinson, San Jose, CA, USA). A total of 5,000 cells (i.e. events) were analysed for each sample. Gates were set such that 0.5% of RAW 264.7 cells which had been electroporated in the absence of plasmid were detected in the lower right quadrant of the FL-1 channel (i.e. GFP signal). Transfection efficiencies of RAW 264.7 cells electroporated with the pEGFP-syntaxin 7 and the empty parental vector pEGFP-C1 were then measured. GFP-positive cells (i.e. transfected cells) were purified using a BD *FACSVantage SE* FACS Sorter and reseeded into tissue culture dishes for further experimentation.

2.2.8 Confocal Microscopy

Bone marrow-derived macrophages were grown on glass coverslips until approximately 50% confluent. The cells were fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature and then quenched with 50 mM glycine in PBS. The cells were permeablised with 0.1% saponin in blocking buffer (2% BSA and 1% rabbit serum in PBS) for 30 min and then stained with the required primary antibodies (diluted in blocking buffer) for 1 h at room temperature. The cells were washed thrice for 5 min each in PBS and then incubated with AlexaFluor®-conjugated secondary antibodies in blocking buffer for a further 1 h at room temperature. The cells were again washed thrice in PBS and the coverslips mounted onto glass slides using GEL/MOUNT™ aqueous mounting medium. The slides were stored in the dark at -20°C prior to analysis. Images were captured using a Leica™ TCS SP2 laser scanning spectral confocal microscope (Leica, Heidelberg, Germany), equipped with three lasers: an Argon laser (488 nm excitation), a Helium-Neon green laser (543 nm excitation) and a Helium-Neon red laser (633 nm excitation). Images were collected independently for multi-colour labelling: (i) for AlexaFluor®-488 staining, excitation was at 488 nm (Argon laser) and emission detected between 500 and 530 nm, (ii) for AlexaFluor®-594 staining, excitation was at 543 nm (Helium-Neon green laser) and emission detected between 610 and 635 nm. Images were captured using classical scan mode xy plane through a 100 x oil immersion objective lens and acquired with LCSLite software (Leica) at a resolution of 512 x 512 pixels.

2.2.9 Metabolic Labelling of Bone Marrow-Derived Macrophages

Bone marrow-derived macrophages were seeded at a concentration of 1 x 10^6 per 6 cm dish and cultured in complete macrophage medium for 24 h and then deprived of CSF-1 for 10 h. The medium was replaced with 2 ml of phosphate-free DMEM supplemented with 10 mM HEPES (pH 7.4) and 3 mCi of [^{32}P]-orthophosphate and the cells cultured for a further 6 h. The cells were either stimulated with CSF-1 for 15 min or left unstimulated before being lysed (as described above).

2.2.10 Phosphorylation Analysis of Metabolically Labelled Proteins

Syntaxin 7 was immunoprecipitated from lysates of [^{32}P]-orthophosphate-labelled macrophages, subjected to one-dimensional SDS-PAGE on 10% SDS-PAGE gels and transferred to PVDF membranes as described above. Metabolically-labelled proteins were visualised by subjecting the PVDF membranes to autoradiography and [^{32}P]-labelled syntaxin 7 bands excised. The PVDF membrane pieces were washed in 100% methanol and then incubated in 6 M hydrochloric acid for 90 min at 110°C to hydrolyse [^{32}P]-labelled syntaxin 7 into its constituent amino acids. The acid hydrolysates were washed four times with Milli-Q water in a Speedvac concentrator and then finally dissolved in electrophoresis buffer (2.2% formic acid and 7.8% acetic acid) containing 100 μg/ml each phosphoserine, phosphothreonine and phosphotyrosine. The samples were spotted onto 20 cm x 20 cm microcrystalline cellulose glass-backed thin layer chromatography (TLC) plates. The TLC plates were wetted with electrophoresis buffer and subjected to high voltage electrophoresis (1.5 kV) for 75 min [379]. Following electrophoresis, the plates were allowed to air dry and the phosphoamino acid standards visualised by spraying with 0.25% ninhydrin in acetone. The TLC plates were then subjected to autoradiography to detect [^{32}P]-labelled phosphoserine, phosphothreonine and/or phosphotyrosine.

2.2.11 Gel Filtration Column Chromatography

Following clarification by centrifugation and filtration through 0.2 μm syringe filters, a 0.2 ml aliquot of each cell lysate (~400 μg of total protein) was applied to a Superose-6 column (HR 10/30, GE Healthcare) fitted to an Agilent 1100 series HPLC system that had been equilibrated with column buffer (20 mM Hepes [pH 7.4], 100 mM NaCl, 10 mM NaF, 2 mM EGTA and 10% glycerol). Elution was performed at a flow rate of 0.2 ml/min and fractions collected each min. The column was calibrated with thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa) and equine myoglobin (17 kDa). Aliquots of the column fractions were subjected to Western blotting as described above.

2.2.12 Real-Time Polymerase Chain Reaction

Total RNA was extracted from bone marrow-derived macrophages using RNeasy® mini kits as per the manufacturer's instructions. Two μg of total RNA, in a 20 μl reaction mix containing 100 ng random primer, 10 mM deoxyribonucleotide triphosphates (dNTPs), 5 mM dithiothreitol (DTT) and 40 U of RNaseOUT was reverse transcribed using SuperScript III reverse transcriptase. The reaction mixture was incubated for 5 min at 25°C, 60 min at 50°C, 15 min at 55°C and finally for 15 min at 70°C. Quantitative PCR was then performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) and pre-developed TaqMan probe/primer combinations for mouse syntaxin 7, syntaxin 8, Vti1b, VAMP8 and 18S rRNA. All samples were assayed in triplicate. Threshold cycle numbers were transformed using the $\Delta\Delta$CT method (an approximation of transcription based on the change in threshold values for control versus target gene) according to the manufacturer's instructions and results expressed relative to 18S rRNA. Data were plotted using GraphPad Prism® software.

2.2.13 Construction of Expression Vectors

A mammalian expression vector encoding mouse syntaxin 7 (i.e. pcDNA3-syntaxin 7) was a generous gift from Dr. David James (Garvan Institute, Sydney, Australia). The vector pEGFP-syntaxin 7, which encodes a fusion protein in which green fluorescent protein (GFP) is fused to the N-terminus of syntaxin 7, was created as follows. The syntaxin 7-encoding sequence from pcDNA3-sytaxin 7 was amplified by PCR using the primer pair: 5'-TC GAATTC TCT TAC ACT CCG GGG ATT GGT GGG GAC-3' (forward primer contains an EcoRI restriction site) and 5'-AT CCCGGG TCA GCC TTT CAG TCC CCA TAC GAT GAG-3' (reverse primer contains an XmaI restriction site) and *Pfu* high fidelity DNA polymerase. The PCR thermal cycling conditions were as follows: 2 min at 94°C; 1 min at 94°C, 45 s at 65°C, 1 min at 72°C (30 cycles); 10 min at 72°C. The PCR reaction was subjected to electrophoresis on a 1% agarose gel and the PCR product visualised by eithidium bromide staining. The syntaxin 7-encoding PCR product was excised from the gel and purified using an UltraClean 15 DNA Purification kit as per the manufacturer's instructions. The purified PCR product and the expression vector pEGFP-C1 (BD Biosciences) were double digested with EcoRI and XmaI for 2 h at 37°C. The digested vector was subsequently dephosphorylated by incubating it with 10 U of calf intestinal alkaline phosphatase for 30 min at 37°C. Following electrophoresis on a 1% agarose gel, the digested PCR product and vector were excised and purified with an UltraClean kit. The vector and PCR product were ligated (at a molar ratio of 1:4) with T4 DNA ligase overnight at room temperature. The ligation reactions were then used to transform chemically-competent DH5α *E.coli* via a 90 s heat-shock in a 42°C water bath. The bacteria were plated onto LB/agar plates containing 30 μg/ml kanamycin and incubated overnight at 37°C. Miniprep DNA was prepared from individual bacterial colonies and the plasmids screened for the presence of the syntaxin 7-encoding PCR insert by digesting the plasmid DNA with EcoRI and XmaI. Large scale plasmid preparations were prepared from positive clones using QIAGEN Plasmid Maxi kits. The expression vector (i.e. pEGFP-syntaxin 7) was subjected to DNA sequencing (Micromon DNA sequencing service, Monash University) to ensure the absence of mutations.

2.2.14 Enzyme-Linked ImmunoSorbent Assays

The levels of TNF, IL-6 and IL-10 in culture supernatants from RAW 264.7 cells were measured by Enzyme-Linked ImmunoSorbent Assays (ELISA) as per the manufacturer's instructions (BD Pharmingen, Franklin Lakes, NJ, USA). Briefly, 96-well plates (Nunc) were coated overnight at 4°C with 1 μg/ml of rat monoclonal anti-mouse TNF, IL-6 or IL-10 antibodies. The next day, the wells were blocked with 5% BSA in PBS for 2 h at room temperature, followed by washing with PBS. Dilutions of the cell culture supernatants, as well as murine TNF, IL-6 and IL-10 standards, were added to the appropriate wells and the plates incubated overnight at 4°C. The plates were washed twice with PBS and then rabbit anti-mouse TNF, IL-6 and IL-10 polyclonal antibodies were added to the required wells and incubated overnight at 4°C. Again the plates were washed with PBS and a goat anti-rabbit IgG-HRP antibody (Pierce, Rockford, IL, USA) added to each well and the plates incubated at room temperature for 2 h. ImmunoPure Tetramethyl benzidine (TMB) substrate solution (Pierce) was added to the wells and colour development allowed to proceed until it was terminated by the addition of 0.5 M H_2SO_4. The absorbance of each well was measured at 450 nm using a Microplate reader (Bio-Rad, CA, USA).

REGULATION OF THE EXPRESSION OF LATE ENDOSOMAL SNARE PROTEINS BY CSF-1

3.1 Introduction

Prior studies have demonstrated that CSF-1 modulates a number of the immune functions of macrophages, such as cytokine secretion, pinocytosis and phagocytosis (see Chapter 1). For example, the levels of certain pro-inflammatory cytokines (e.g. TNF and IL-6) secreted by mouse bone marrow-derived macrophages in response to LPS stimulation were significantly decreased in the absence of CSF-1 [177]. The amplifying effect of CSF-1 on inflammatory cytokine production was not due to a general effect on cell growth, because CSF-1 suppressed cytokine production when CpG DNA was the stimulus [178]. CSF-1 stimulation of mouse bone marrow-derived macrophages also enhanced solute intake as evidenced by their increased accumulation of Lucifer Yellow, a probe commonly used to measure the endocytosis of solutes [380]. In addition, CSF-1 increased the phagocytosis of the fungi *Aspergillus fumigatus* by alveolar macrophages [183]. Similarly, CSF-1 enhanced the phagocytosis of *Penicillium marneffei* by rabbit pulmonary alveolar macrophages [182] and *Leishmania major* by bone marrow-derived macrophages [381]. Each of these immune functions of macrophages involves a number of exocytic and endocytic pathways that consist of various vesicle trafficking and membrane docking and fusion events.

In this thesis, I hypothesised that CSF-1 may regulate the effector functions of mature macrophages (e.g. cytokine secretion and phagocytosis) by governing the expression and/or activity (e.g. due to post-translational modification) of the proteins that are directly involved in mediating the membrane docking and fusion events necessary for exocytic and endocytic processes. As discussed in Chapter 1, SNARE proteins play crucial roles in vesicle docking and membrane fusion during exocytic and endocytic processes [245, 262, 382]. For example, secretion of the cytokines TNF and IL-6 by LPS-activated macrophages occurs in a SNARE-dependent manner [126, 240]. More specifically, the mRNA and protein expression levels of the SNAREs involved in the trafficking of these inflammatory cytokines to the plasma membrane for release (e.g. syntaxin 6, Vti1b and VAMP3) were up-regulated following LPS stimulation [126, 240, 341]. Additionally, the expression level of VAMP3 in macrophages was up-regulated by IFNγ [126, 240], a cytokine that enhances the release of inflammatory cytokines by activated macrophages [383]. In this chapter I have investigated the ability, as well as the underlying

mechanisms, of CSF-1 to regulate the expression of proteins (e.g. SNARE proteins) that mediate membrane docking and fusion events in macrophages.

3.2 Results

3.2.1 Identification of CSF-1-regulated genes by microarray analysis

In order to identify CSF-1-regulated genes that may potentially have a role in mediating the immune functions of macrophages, microarray experiments were conducted in collaboration with Dr. Matthew Sweet from the Institute for Molecular Bioscience at the University of Queensland. DNA microarrays are a high throughput tool that allows the expression of thousands of genes to be investigated in a single experiment. Mouse RIKEN cDNA arrays, containing approximately 22,000 genes, were used in these experiments. Total RNA was extracted from BALB/c mouse bone marrow-derived macrophages that were deprived of CSF-1 for 18 h and then either stimulated with CSF-1 for 4 or 24 h, or left unstimulated. The RNA was reverse transcribed into cDNA and then labelled with two different fluorophors, Cy3 and Cy5, in order to separate differentially expressed samples. The labelled cDNA was hybridised overnight to glass slides spotted with the RIKEN cDNA clone collection. Slides were then washed and scanned with an Agilent Scanner using green SH-YAG (532 nm) and red HeNe (633 nm) lasers. Data was extracted using Digital Genome (MolecularWare, CA, USA) and analysed with Genespring (Agilent, Santa Clara, CA, USA). Gene lists, in which gene expression was regulated more than 2-fold by CSF-1, were then generated.

Because CSF-1-regulated genes that are involved in either endocytosis or exocytosis events may regulate phagocytosis and/or cytokine secretion by macrophages, genes were selected from the microarray experiment based on: (i) their known or potential roles in endocytic and/or exocytic pathways and (ii) the change in gene expression level was at least 2-fold. A two-fold change in gene expression is widely used in microarray experiments to confidently define genes whose expression has changed in response to a particular stimulus [384, 385].

The microarray analysis identified approximately 180 CSF-1-regulated genes in BALB/c mouse bone marrow-derived macrophages. The expression levels of 149 genes were up-regulated by CSF-1, whilst the expression of the remaining 31 genes was repressed by CSF-1. Eight CSF-1-regulated genes were selected for further analysis based on their known functional roles in vesicle trafficking and membrane fusion; these genes are tabulated in Table 3.1.

Expression of annexin A1, which has previously been shown to regulate the phagocytosis of apoptotic neutrophils by differentiated human THP-1 monocytes [386], was induced ~5-fold in response to CSF-1 stimulation (Table 3.1). Adaptor protein complex 3 (APC3) recognises proteins based on tyrosine and leucine motifs and has been shown to sort proteins to late endosomes and lysosomes in fibroblasts [387]. The PH domain-containing family A protein has a role in cytoskeletal reorganisation [388], while the Vasodialator-stimulated phosphoprotein (VASP) has been shown to play a role in reorganising actin around phagosomes in RAW 264.7 mouse macrophages [389]. Calmodulin was another gene that was found to be up-regulated. Calmodulin is a Ca^{2+} binding protein that can activate Ca^{2+}/calmodulin dependent kinase II (CaMKII). This was potentially interestingly as inhibition of CaMKII is implicated in the subversion of macrophages by *Mycobacterium tuberculosis* [390]. Syntaxin 7, a SNARE protein, was found to be up-regulated ~2.5-fold by CSF-1. This particular SNARE protein has been shown previously to mediate membrane fusion between late endosomes and lysosomes [127, 272, 391]. In addition, syntaxin 7 partially localises to mature phagosomes in RAW 264.7 macrophages cells [127]. Moreover, recent studies indicate that it forms an integral part of a SNARE complex that mediates TNF trafficking from the Golgi to recycling endosomes in macrophages [126, 240, 327]. In contrast to syntaxin 7, the mRNA levels of Ykt6, which is also a SNARE protein, were repressed ~2.5-fold by CSF-1. Ykt6 mediates trafficking between recycling endosomes and the Golgi network [281]. Like Ykt6, the expression levels of the gene for cathepsin B, a lysosomal enzyme, were also found to be repressed (~2.5-fold). Given that syntaxin 7 has been implicated in cytokine secretion and phagocytosis by macrophages, and that its expression was up-regulated by CSF-1, I focused on this gene for further investigation.

Table 3.1: CSF-1-regulated genes and their functions

Gene/Protein	Function	Fold change in gene expression	GenBank accession number
Annexin A1	Regulation of the phagocytosis of apoptotic cells	4.6	NP_034860
Adaptor protein complex 3	Sorting of proteins to late endosomes	3.2	NP_033811
PH domain containing family A protein	Role in reorganisation of the actin cytoskeleton	3.2	NP_112547
Vasodialator-stimulated phosphoprotein	Role in reorganising actin around phagosomes	2.7	NP_033525
Calmodulin	Calcium binding protein; activates Ca^{2+}/calmodulin dependent kinase II	2.6	NP_031615
Syntaxin 7	SNARE protein; mediates membrane fusion between late endosomes and lysosomes	2.3	NP_058077
Ykt6	SNARE protein; mediates recycling endosome to Golgi network transport	-2.3	NP_062635
Cathepsin B	Lysosomal hydrolytic enzyme	-2.3	AAH06656

3.2.2 CSF-1 regulates the mRNA levels of late endosomal SNAREs

In order to confirm the microarray data, syntaxin 7 mRNA levels in mouse bone marrow-derived macrophages were measured by quantitative real-time PCR. As shown in Figure 3.1A, syntaxin 7 mRNA levels were up-regulated as early as 2 h after CSF-1 stimulation and maximally induced (~4-fold increase) by 8 h post-CSF-1 stimulation. Syntaxin 7 mRNA levels declined slightly thereafter but remained elevated above basal levels for at least a further 16 h (Figure 3.1A).

Syntaxin 7 is known to form a late endosomal SNARE complex with three other SNARE proteins, namely syntaxin 8, Vti1b and VAMP8, to mediate membrane fusion between late endosomes [264, 265]. Furthermore it forms a SNARE complex with syntaxin 8, Vti1b and VAMP7 to mediate membrane fusion between late endosome and lysosomes [296]. Therefore, it was important to determine if the expression levels of the other members of this SNARE complex were likewise regulated by CSF-1 in bone marrow-derived macrophages. The effects of CSF-1 on Vti1b gene expression were similar to those on syntaxin 7 mRNA expression; Vti1b mRNA levels were up-regulated as early as 2 h post-CSF-1 stimulation and maximally induced (~3.5-fold increase) around 8 h following CSF-1 stimulation (Figure 3.1B). Although Vti1b mRNA levels started to decline thereafter, they were still elevated (~2-fold) 24 h post-CSF-1 stimulation. In contrast to syntaxin 7 and Vti1b, CSF-1 only modestly up-regulated syntaxin 8 and VAMP8 mRNA levels, although the peaks of mRNA induction by CSF-1 coincided with those of syntaxin 7 and Vti1b (Figure 3.1C-D). Thus, although the extents to which the four late endosomal SNARE genes were up-regulated by CSF-1 differed, the kinetics of their up-regulation were similar.

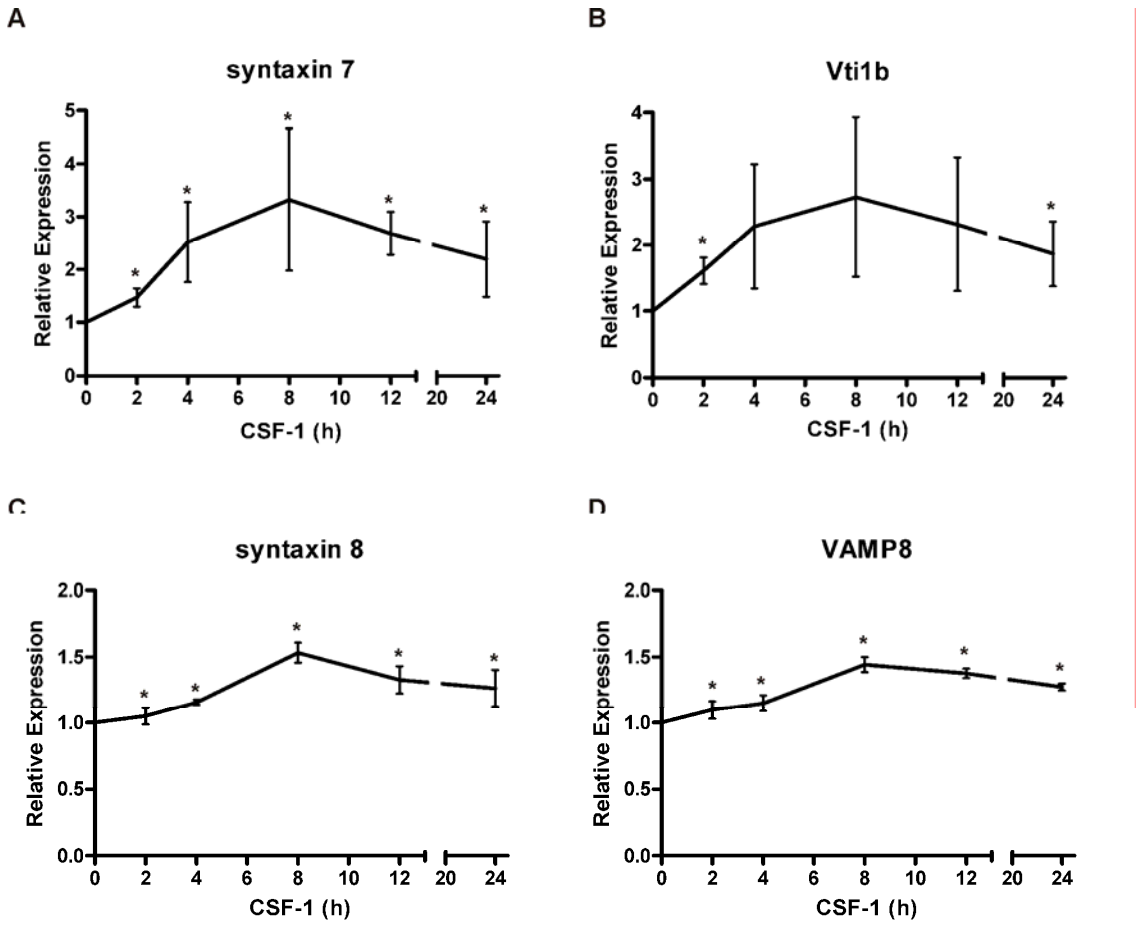


Figure 3.1: Up-regulation of the mRNA levels of late endosomal SNARE proteins by CSF-1

Bone marrow-derived macrophages were deprived of CSF-1 for 16 h before being stimulated with CSF-1 for the indicated periods of time. Total RNA was extracted and reverse transcribed into cDNA, which was then subjected to quantitative real-time PCR. 18S ribosomal RNA was used as the internal control. ***A-D***, mRNA expression levels of the indicated SNAREs are given relative to their expression at t = 0 h (i.e. no CSF-1), which was given an arbitrary value of 1.0. The mRNA levels at each time point were measured in triplicate and the results represent the mean ± SEM of values from three independent experiments. * $p < 0.05$, expression of mRNA levels of SNARE proteins in boned marrow-derived macrophages from non CSF-1-treated versus CSF-1-treated macrophages (i.e. t = 0 Vs. t = 2 , 4, 8, 12 or 24) by Student's *t*-test.

3.2.3 CSF-1 regulates the protein expression levels of late endosomal SNAREs

In order to determine if the CSF-1-induced up-regulation of the mRNA levels of the late endosomal SNAREs led to increased SNARE protein expression, cellular lysates of bone marrow-derived macrophages were subjected to Western blot analysis. As shown in Figure 3.2, the stimulation of bone marrow-derived macrophages with CSF-1 resulted in a modest up-regulation of syntaxin 7 protein levels by 4 h post CSF-1 stimulation, with a maximal increase (~1.5 to 2-fold) occurring around 12 h post-CSF-1 stimulation (Figure 3.2). Interestingly, the fold increase in syntaxin 7 protein levels was less than the observed increase in syntaxin 7 mRNA levels, which was ~4-fold by 12 h post-CSF-1 stimulation (Figure 3.1). A small decline in syntaxin 7 expression was observed by 24 h post-CSF-1 stimulation (Figure 3.2). By contrast, an increase in Vti1b protein expression was detected as early as 2 h post-CSF-1 stimulation, coinciding with its kinetics of induction at the mRNA level (Figure 3.1B and 3.2). Maximum Vti1b protein expression was achieved by 8 h post-CSF-1 stimulation and it remained at this elevated level for at least a further 4 h before declining at the 24 h post-CSF-1 stimulation time-point (Figure 3.2). Notably, the extent of the increase in Vti1b protein expression coincided with the ~3.5-fold increase in its mRNA levels in response to CSF-1 stimulation (Figures 3.1B and 3.2). The protein expression levels of syntaxin 8 did not change in response to CSF-1 (Figure 3.2). Even though VAMP8 mRNA levels appeared to be only slightly induced by CSF-1, its expression at the protein level increased ~4-fold by 24 h post CSF-1 stimulation (Figure 3.2). An increase in VAMP8 protein levels was first detected after 4 h of CSF-1 stimulation and continued to increase for at least a further 20 h (Figure 3.2). Although maximal up-regulation of syntaxin 7 and Vti1b protein expression was achieved 12 h post-CSF-1 stimulation, maximal VAMP8 protein expression did not occur until at least 24 h after CSF-1 stimulation. Despite some subtle differences in the magnitude and the time course of the responses between mRNA and protein expression, CSF-1 clearly up-regulated the expression of several members of the late endosomal SNARE complex, most notably syntaxin 7, Vti1b and VAMP8.

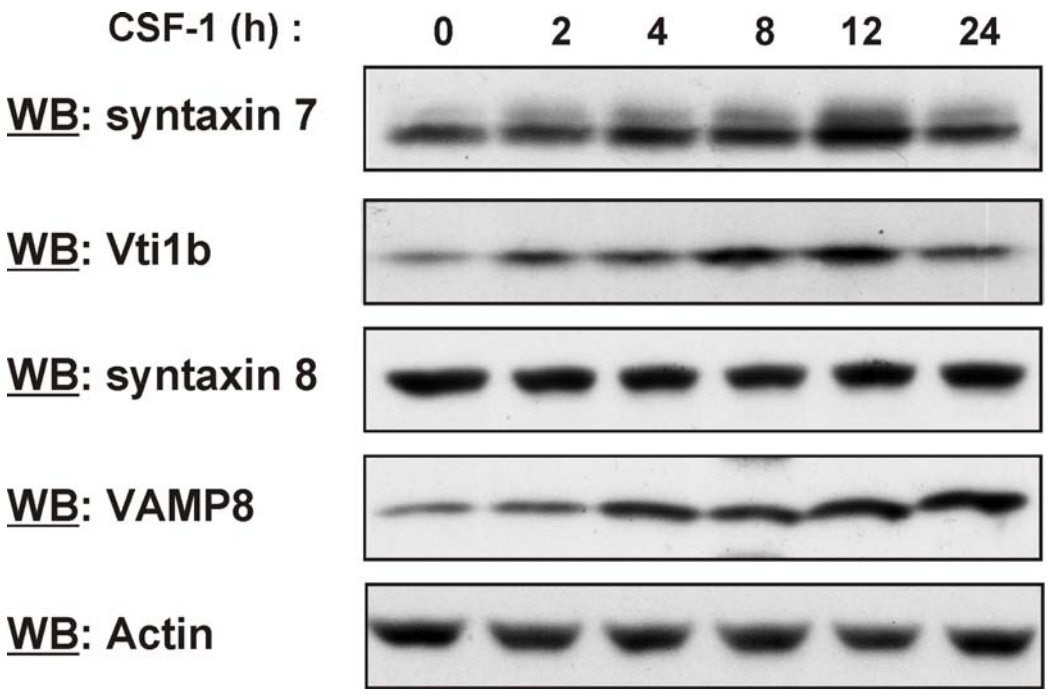


Figure 3.2: Regulation of late endosomal SNARE protein expression by CSF-1.

Bone marrow-derived macrophages were deprived of CSF-1 for 16 h before being stimulated with CSF-1 for the indicated periods of time. The cells were then lysed and aliquots of the cell lysates subjected to Western blotting with anti-syntaxin 7, anti-Vti1b, anti-syntaxin 8 and anti-VAMP8 antibodies. The membrane was stripped and reprobed with an anti-actin antibody (~42 kDa) to establish that equal amounts of total protein were loaded at each time point (n = 3). Molecular masses: syntaxin 7 ~37 kDa; Vti1b: ~27 kDa; syntaxin 8: ~27 kDa; VAMP8: ~13 kDa; Actin ~42 kDa.

Interestingly, CSF-1 stimulation of bone marrow-derived macrophages resulted in a change in the electrophoretic mobility of syntaxin 7, suggesting a possible post-translational modification of the protein (discussed in detail in Chapter 4). VAMP7 also forms a complex with syntaxin 7, Vti1b and syntaxin 8 [296]. However, its expression in bone marrow-derived macrophages was not investigated in this study.

3.2.4 Promoter analysis of late endosomal SNARE genes

Control of transcriptional initiation is a pivotal mechanism for determining whether or not a gene is expressed. Gene transcription is largely controlled by the binding of transcription factors to specific elements in the promoter/enhancer regions (e.g. 5' flanking regions) of genes. Macrophages contain numerous transcription factors which are activated in response to different extracellular signals (e.g. CSF-1 and TLR ligands) [392-396]. Examples of transcription factors that are regulated by CSF-1 include Ets-2 and PU.1 [397]. As expression of the genes encoding syntaxin 7, Vti1b, syntaxin 8 and VAMP8 were up-regulated by CSF-1 in mouse bone marrow-derived macrophages, it was possible the genes possessed common transcription factor binding sites in their promoter regions. One way of predicting potentially functional transcription factor binding sites in genes is via bioinformatics-based promoter analysis.

The evolutionarily conserved region programme (http://ecrbrowser.dcode.org/) was created to: (i) identify candidate functional coding and non-coding elements in genes and (ii) visualise their relative genomic positions [398, 399]. The ECR programme permits the thorough annotation of functional elements in the genome through the portal to the University of California, Santa Cruz (UCSC) Genome Browser. The programme compares genomic sequences from various species (e.g. human, rat and dog) and highlights sequences that are conserved through evolution as well as identifies genomic regions that are rapidly diverging. Because functionally important transcription factor binding sites are likely to be conserved throughout evolution [400], the ECR programme was used first to identify evolutionarily conserved sequences in the 5' flanking regions of the syntaxin 7,

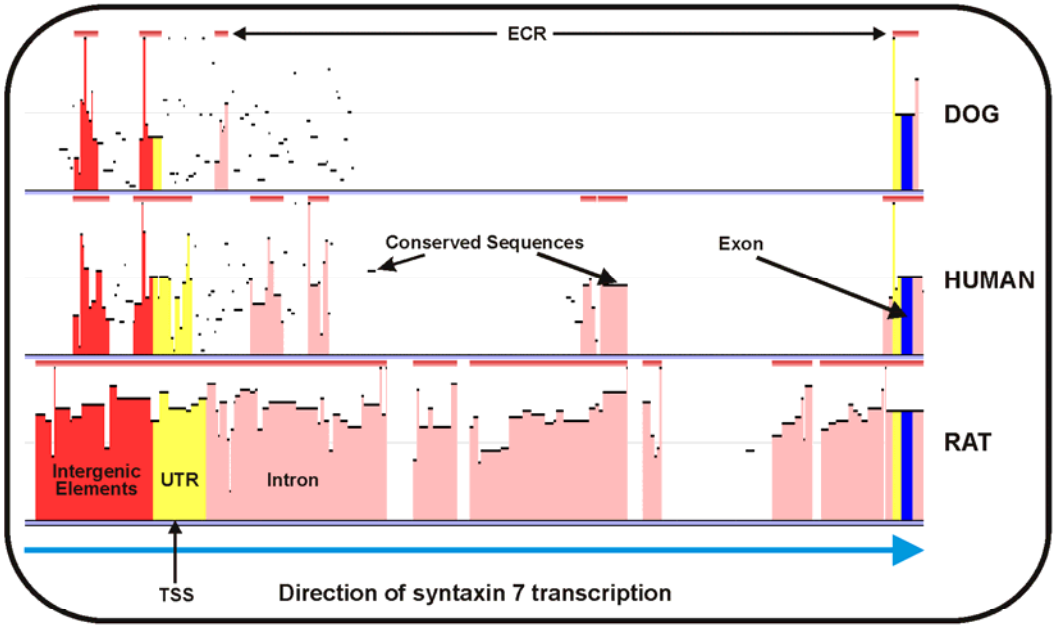
Vti1b, syntaxin 8 and VAMP8 genes. The default setting for the ECR was defined as a minimum 100 bp sequence length with 70% identity between mouse and the organisms examined (i.e. rat, human and dog). Analysis of the ECR of late endosomal SNARE genes revealed a high level of conservation between the mouse and rat genes, while the more evolutionarily distant species human and dog shared relatively low levels of conservation with the mouse genes (Figures 3.3A, 3.4A, 3.5A and 3.6A). Most of the variation between the syntaxin 7, Vti1b, syntaxin 8 and VAMP8 genes of mouse, rat, human and dog are found in introns, while coding exons and untranslated regions had a minimum of 70% identity.

The annotation of the transcription factor binding sites was then performed through the portal to the rVista programme (http://rvista.dcode.org/), which predicts potential transcription factor binding sites, from its database of 467 transcription factors [398, 399]. Bioinformatic analysis of the evolutionarily conserved 5' flanking regions of the four late endosomal SNARE genes across mouse, rat, dog and human revealed a number of binding sites for transcription factors that are known to be expressed in macrophages based on the published literature (Table 3.2). The percent identity between the mouse transcription factor binding sites of the late endosomal SNARE genes and the corresponding consensus sequences for each transcription factor binding site is also given in Table 3.2. The higher the percentage identity between the consensus sequence and the corresponding sequence of the late endosomal SNARE gene, the more likely it is that the site acts as a transcription factor binding site (TFBS). CAGE (Cap-analysis gene expression) analysis [401] was then performed in order to identify transcription start sites (TSS) and thus aid in the identification of TFBS that were most likely to be responsible for controlling the expression of the endosomal SNARE genes. In general, TFBS that are located upstream of TSS are more likely to be functional [402]. CAGE analysis identifies transcription start sites based on information obtained from the sequencing of the 5' ends of a large number of mRNA transcripts; the resulting sequences are referred to as CAGE tags. There are currently >7 million CAGE tags in the mouse FANTOM3 database (http://fantom31p.gsc.riken.jp/cage_analysis/mm5/); these tags correspond to various mRNA transcripts and are also known as transcription clusters (TCs). The FANTOM3 database contains 736,403 TCs. Syntaxin 7, Vti1b, syntaxin 8 and VAMP8 have 39, 17, 38 and 25 TCs, respectively. The top three TCs for syntaxin 7, Vti1b, syntaxin 8 and VAMP8 are given in Table 3.3. The 5'

flanking region of a gene (e.g. syntaxin 7 gene) that contains the largest number of CAGE tags is defined as the transcription start site [401]. For example, syntaxin 7 contains 2126 transcripts in the CAGE database. One cluster (T10F016CD30D) contains 2022 of the 2126 tags, while the second largest cluster (T10F016CD3A0) contains only 20 of the 2126 (Table 3.3). Hence, syntaxin 7 transcription is likely to start at position 23909133 on chromosome 10.

Analysis of the 5' flanking region of the syntaxin 7 gene identified seven potential TFBS, including two CCAAT boxes (Figure 3.3 and Table 3.2). Apart from an Ets-1 binding site, which was located downstream of the predicted TSS, the other six TFBS were found upstream of the TSS (Figure 3.3). The two CAAT box binding sites upstream of the TSS in the syntaxin 7 gene are 100% identical to its consensus sequence; however, the downstream Ets-1 binding site also shared a high identity (~90%) with its respective consensus sequence. The promoter region of the Vti1b gene contains six putative TFBS (Figure 3.4). The SP-1 and Ets-1 binding sites are located upstream of the putative TSS, whereas potential binding sites for DEAF1, ELK1, ZF5 and E2F1 are situated downstream of the predicted TSS. Analysis of the 5' flanking region of the syntaxin 8 gene predicted binding sites for eight transcription factors; binding sites for E2F1, cMyc/Max, AP1 and AP4 reside upstream of the TSS, while another AP4 binding site, along with binding sites for AP2 and ZF5 TFBS, are present downstream of the TSS (Figure 3.5). Although the syntaxin 8 gene has a binding site GABP/PEA3, this site overlaps the TSS and thus it is unlikely to serve as a TFBS. Finally, analysis of the VAMP8 gene identified five TFBS, all of which were located upstream of the predicted TSS (Figure 3.6). Notably, all five predicted TFBS in the VAMP8 gene shared less than 80% identity with their respective consensus sequences.

A



B

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                    Meis1
Mouse     -169   CACTTTGTCACCCATCAGAGCTTGGCTCCCCAGCAATTTTGCCAGTCCCTCCAA------
Human            CTCTACGTCAGGCTTCAAAGAACAGCTCCCTTGCAATTTTGCTGATACCCCCAAAGGAAA
                 * **  ****  * *** **    ******  **********   * ** ****

                                                                 CCAAT Box
Mouse     -115   GCTTCA----------------------GCTGTCACTG----------GCCAATGGGAGA
Human            GCTTCCTTGGCCTCCCCGCCACTCCCCCGCTGTCACTGTCGGGGCGCGGCCAATAGAAAA
                 *****                       **********          ****** * * *

                                                          NRF1
Mouse      -87   GACGCAAGCCCCGCCTCCTCCTTCCTGGGGCTGTGCCCCGAGCATGCGCAGTGGGCGTGC
Human            GGGGTGAACCCCGCCTTCTTCCT----GAGTTGTGCTGCGGGCATGCGCACTGGGCGTCC
                 *  *  * ******** ** * *    * * *****  ** ********* ******* *

                          AP4
                 CCAAT Box SREBP1      +1
Mouse      -27   TTGTGCCGCCGCCCCATCAGCTGAGACCGGCTGCGCCAGGCAGTGGGCCCCC--------G
Human            CCACGCCACCGCCCCATCAGCTGAGA---ATTGCA----GCTGAGGGCTCCCCCCATCCTG
                     *** ****************     ***     ** * **** ***        *

                                 Ets-1
Mouse       26   GGTGACAGCGGCTGGAGGTGGCCGAGGAAGCCGGCGAGGTCAGGGTAAGAGGAGACTGCA
Human            GGTGACGGCGGTCGGAGGTGTAGGAGGGAGCCGTGGAGGTCCAGGTAAGAGGACCCTGGG
                 ****** ****  *******   **** *****  ******  **********  ***

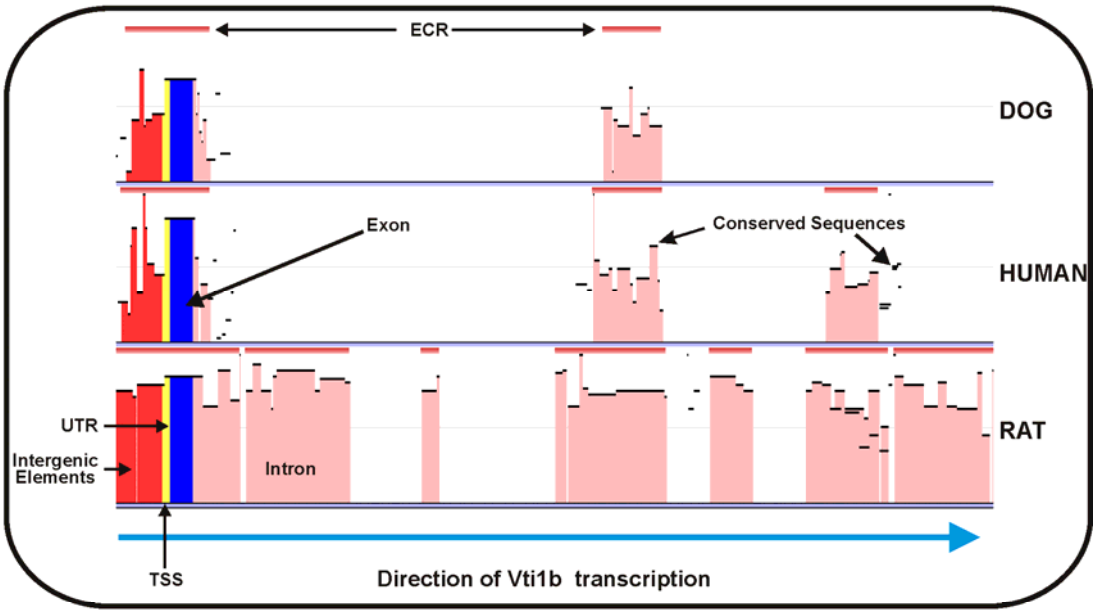
Mouse       86   GAGGC-----------GGGGGAGAGGGGGGCG-GGA--GGGACACC------------
Human            GAGGCCCGAGGGACCGCGGGCGACAGGGAGGCGCGGACGGGGACGCCGACGACGCGGAAG
                 *****            *** ** **** **** ***  ***** **
```

Figure 3.3 Evolutionarily conserved regions and potential transcription factor binding sites in the syntaxin 7 gene

A, The evolutionarily conserved 5' flanking regions of syntaxin 7 gene were identified using the ECR Browser. The display shows a pip-type conservation plot in which mouse DNA is represented in the horizontal axis as a grey shade, while multiple blastz alignments are displayed as short horizontal black lines. The length of vertical peaks indicates the level of nucleotide identity between base genome (mouse) and aligned genome (rat, dog or human). The horizontal blue line under the graph indicates the direction of gene transcription. Blue boxes correspond to positions of coding exons, while yellow boxes correspond to untranslated regions. Peaks within the conserved profile that do not correspond to transcribed regions are highlighted in red if they are intergenic (located between two different genes in a chromosome) or pale pink if they lie within an intron [399].

B, Potential transcription factor binding sites located in the 5' flanking region of the syntaxin 7 gene (using rVista) are labelled. Transcription factor binding sites that are evolutionarily conserved between mouse and human are boxed. The position of the predicted major transcription start site (TSS) is indicated by a black arrow.

A



B

```
                                                  SP1
Mouse     -260   TCCTCCCGGCCTGGTCCCGCCCCCGCCTCACCTGTCCCCGCGGTCCCCAGCAGCCGCTCC
Human            TCCCCACGGCGTGG-CCCACCCCCGCCTCACCGGTCCCCGCCGTCCCCAGCAGCCGCTCG
                 *** * **** *** *** ************ ******** ****************

                                                 Ets-1
Mouse     -200   GGCACCCCTTGTAAGTCTTCAAGGAGGCCGCGGAAGATCTCGTGCAGCTTCTCGAAATGC
Human            GGCACCCCTTGTAGGTCTTCATGGAGGCCGCGGAAGATCTCGTGCAGCTTCTCGAAATGC
                 ************ ******* ************************************

Mouse     -140   TCGGAGGAGGCGGCGGAGGCGGCCATGGCGCAGGTTGCGCGTGAGCTGCGGCCACCG---
Human            TCCGAGGAGGCGGCGGAGGAGGCCATGGCGCAGGCCGCGCTGGAGCAGCGGCCACCGAGA
                 ** ***************** **************  ****  **** **********

Mouse      -83   -----GGCGCTGGGCCCACTCCCACCTCAGCAATCAGCTGCCCAGCCCCGAAGCCATCAC
Human            TTCGGGGCCCTGGGCCCTTTCCTAGCCCGGCGGTCAGCCGCCCAGCCCAGTGGCCATAAC
                      *** ********  *** * * * **  ***** ********* *  ***** **

                                                 ELK1
                                            +1   DEAF1
Mouse      -28   TGCA---GCCGCACCTCGGCCTCCCGGAATCAGGCGCTTCCGG----------GGTCCT
Human            GGCGACCGCCGCACCACCGCCGCCCAGGACGAGGCTCTTCCGGAGCCGCGGCAGGGTCCT
                  **    ******** * *** *** * *  **** *******           ******

Mouse      +19   CG-GAGCCGGA-CGCCCTCCCCCT---GCTTGTCACTGCACGGGCGCCGGGCGCCAAAGC
Human            CTCGAGCCGGAACGCCCACCTTCTCCAGCCAGTGATTGCTGGAGCGCCGG-CGCGAAAGC
                 *  ******** ***** **  **   **  ** * ***  * ******* *** *****

                                                 ZF5        E2F1
Mouse      +74   CTGACAAGAAGCCGGGGCGGGG-ACGACACTCAGCTACTACAG-GGGCGCGGTCTCGCGC
Human            CTGACGAGAAGTCGGGAGACTGCGCGGC-CTAGGCGACTCCTGAGGGAGCGGTGCCGCGC
                 ***** ***** ****     *  ** * **  ** *** * * *** *****  *****
```

Figure 3.4 Evolutionarily conserved regions and potential transcription factor binding sites in the Vti1b gene

A, The evolutionarily conserved 5' flanking regions of Vti1b gene were identified using the ECR Browser. The display shows a pip-type conservation plot in which mouse DNA is represented in the horizontal axis as a grey shade, while multiple blastz alignments are displayed as short horizontal black lines. The length of vertical peaks indicates the level of nucleotide identity between base genome (mouse) and aligned genome (rat, dog or human). The horizontal blue line under the graph indicates the direction of gene transcription. Blue boxes correspond to positions of coding exons, while yellow boxes correspond to untranslated regions. Peaks within the conserved profile that do not correspond to transcribed regions are highlighted in red if they are intergenic (located between two different genes in a chromosome) or pale pink if they lie within an intron [399].

B, Potential transcription factor binding sites located in the 5' flanking region of the Vti1b gene (using rVista) are labelled. Transcription factor binding sites that are evolutionarily conserved between mouse and human are boxed. The position of the predicted major transcription start site (TSS) is indicated by a black arrow.

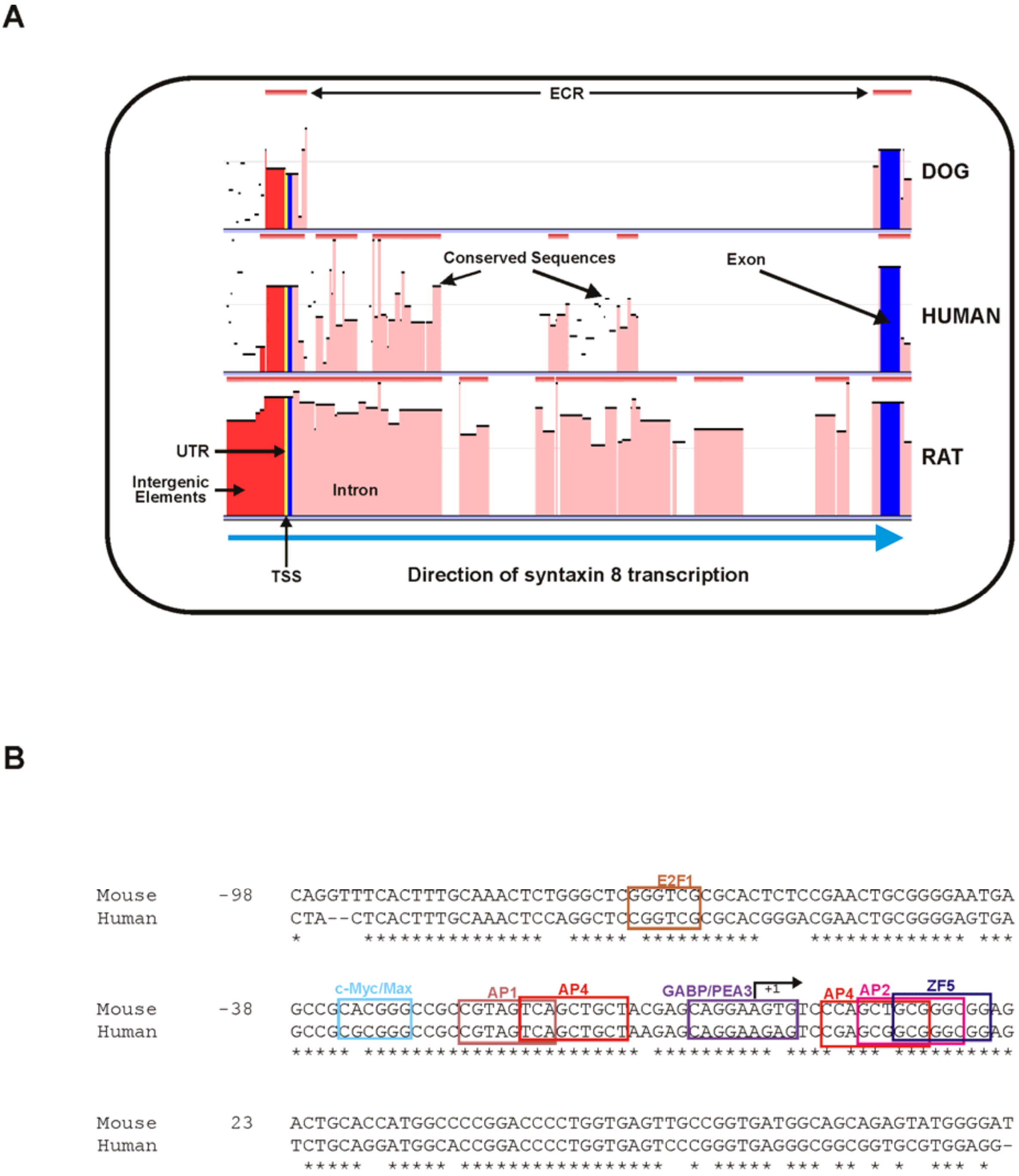
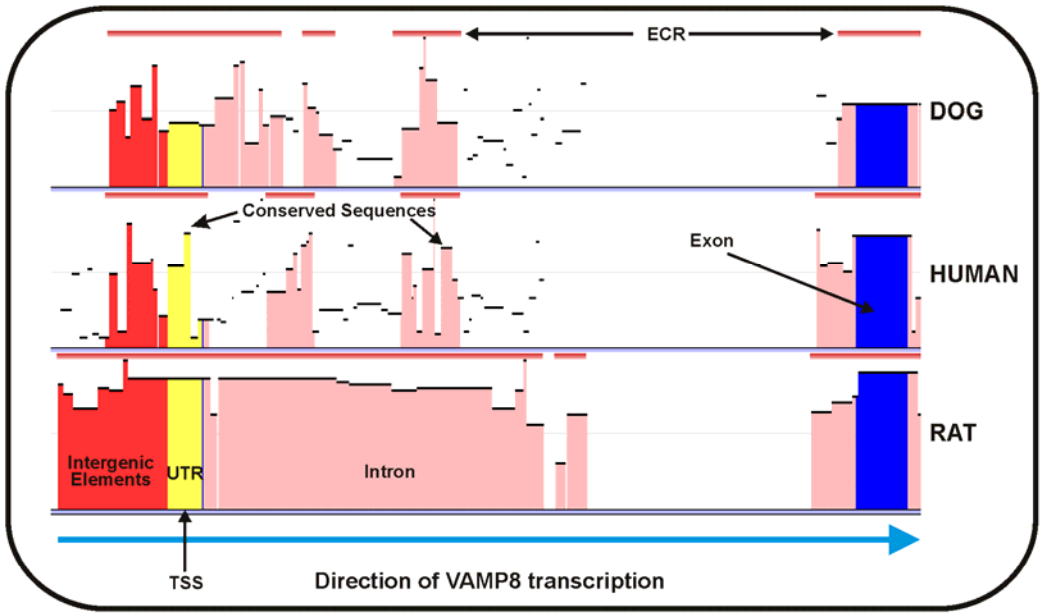

A
ECR
DOG
Conserved Sequences
Exon
HUMAN
UTR
Intergenic
Elements
Intron
RAT
TSS
Direction of syntaxin 8 transcription
B
E2F1
Mouse -98 CAGGTTTCACTTTGCAAACTCTGGGCTCGGGTCGCGCACTCTCCGAACTGCGGGGAATGA
Human CTA--CTCACTTTGCAAACTCCAGGCTCCGGTCGCGCACGGGACGAACTGCGGGGAGTGA
* *************** ***** ********** ************* ***
c-Myc/Max
AP1
AP4
GABP/PEA3
+1
AP4
AP2
ZF5
Mouse -38 GCCGCACGGGCCGCCGTAGTCAGCTGCTACGAGCAGGAAGTGTCCCAGCTGCGGGCGGAG
Human GCCGCGCGGGCCGCCGTAGTCAGCTGCTAAGAGCAGGAAGAGTCCGAGCGGCGGGCGGAG
***** *********************** ********** **** *** **********
Mouse 23 ACTGCACCATGGCCCCGGACCCCTGGTGAGTTGCCGGTGATGGCAGCAGAGTATGGGGAT
Human TCTGCAGGATGGCACCGGACCCCTGGTGAGTCCCGGGTGAGGGCGGCGGTGCGTGGAGG-
***** ***** ***************** * ***** *** ** * * *** *

Figure 3.5 Evolutionarily conserved regions and potential transcription factor binding sites in the syntaxin 8 gene

A, The evolutionarily conserved 5' flanking regions of syntaxin 8 gene were identified using the ECR Browser. The display shows a pip-type conservation plot in which mouse DNA is represented in the horizontal axis as a grey shade, while multiple blastz alignments are displayed as short horizontal black lines. The length of vertical peaks indicates the level of nucleotide identity between base genome (mouse) and aligned genome (rat, dog or human). The horizontal blue line under the graph indicates the direction of gene transcription. Blue boxes correspond to positions of coding exons, while yellow boxes correspond to untranslated regions. Peaks within the conserved profile that do not correspond to transcribed regions are highlighted in red if they are intergenic (located between two different genes in a chromosome) or pale pink if they lie within an intron [399].

B, Potential transcription factor binding sites located in the 5' flanking region of the syntaxin 8 gene (using rVista) are labelled. Transcription factor binding sites that are evolutionarily conserved between mouse and human are boxed. The position of the predicted major transcription start site (TSS) is indicated by a black arrow.

A



B

```
                                  NRSF          ATF
Mouse     -394   GGATCCAGCACCGAGGTCACGGGAGGAAGGAGGTGGAGAAGTGTCAGGAACCCCTGGCCC
Human            CCCACCAGCACAGAGGTCAGGGGAGGGAGG-----GAGAAGGAGTAGAAACACCTG-CCA
                  ******* ******* ****** ***     ******    ** *** **** **

Mouse     -334   GGAGTGC--GGAGCTATTTGTGGAGGAGTTGTCAAAAGTAAGGAGACAGAGGCAGCGCTG
Human            GGAGTGCTCAGAGCTATTTGCAGCCAAGTTGTCAAA-GAAGAGAGGCCATAGCAGCACTG
                 *******   **********  *   ********** * *  *** *    ***** ***

Mouse     -276   GCCCAACCCCGCCAGCCCTCTGACT--GCCGCCAACTTACCTTTCTCGACACAGGCAACC
Human            GC---------CCAGCCCTCTAACTAAGCCACCAACTCTCCTCCCCCGACACAGCCAACC
                 **         ********** ***  *** ******  ***  * ******** *****

Mouse     -218   CCCAGGCCCGATCCCCCTCTGGCAGCTGCTCTAACTCGCTCTTTTCTTGGTCCCCACGTC
Human            CCCAGTCCCAAGCCCCCTCTGGCTCCT-CTTTAAACCAGCCCTTTCCTAGTTCCCAGTTG
                 ***** *** * **********  ** ** ***  *   * **** * ** ****  *

Mouse     -158   ACTCACCATGTCTCGGAGCACTT-GGGCCTCTCA--AAGCAGAACTTCGCCTAGTCTGTT
Human            GCTCACCATGTCTCAGAGCACCCCAGGCCGGTCAGTAAGTGAA--TTCGCCTAGTCGGCT
                  ************ ******    ****  ***  ***   *  ********** * *

                                  ELK1                          ZF5  SP1
Mouse     -101   TCCACCCAAGGCAGCCACAGCTTCACTTCCTGCTTACTCCAACTTTCAGCCCGCCTCCTG
Human            TCCTCCCAGGGTGGCCCTCAGTTCACTTCCTGCTTGCTCCAACTTTCAGCCTGCC-CCCG
                 *** **** **  ***     ************** ************** *** ** *

                                                          +1
Mouse      -41   ACCATCCCGCGACTCCAGCCACTCCGGCTCCTGCCACACACGTAACTGCAGGTTTCTCCA
Human            CCCACCTCGCATCTCGAGTCGCTCCAGC--------CACACCTAACT-CAGGTTTCCCCA
```

Figure 3.6 Evolutionarily conserved regions and potential transcription factor binding sites in the VAMP8 gene

A, The evolutionarily conserved 5' flanking regions of VAMP8 gene were identified using the ECR Browser. The display shows a pip-type conservation plot in which mouse DNA is represented in the horizontal axis as a grey shade, while multiple blastz alignments are displayed as short horizontal black lines. The length of vertical peaks indicates the level of nucleotide identity between base genome (mouse) and aligned genome (rat, dog or human). The horizontal blue line under the graph indicates the direction of gene transcription. Blue boxes correspond to positions of coding exons, while yellow boxes correspond to untranslated regions. Peaks within the conserved profile that do not correspond to transcribed regions are highlighted in red if they are intergenic (located between two different genes in a chromosome) or pale pink if they lie within an intron [399].

B, Potential transcription factor binding sites located in the 5' flanking region of the VAMP8 gene (using rVista) are labelled. Transcription factor binding sites that are evolutionarily conserved between mouse and human are boxed. The position of the predicted major transcription start site (TSS) is indicated by a black arrow.

Table 3.2: Evolutionarily conserved TFBS in the 5' flanking regions of late endosomal SNARE genes

SNARE	Transcription Factor	Consensus Sequence	TFBS (relative to TSS)	% identity	Ref
Syntaxin 7	Meis1	TGACAG	-164 to -159	67	[403]
	CCAAT box	CCAAT	-98 to -94	100	[404]
	NRF1	GCGC(A/T)TGCGC	-48 to -39	90	[405]
	CCAAT box	CCAAT	-16 to -12	100	[406]
	SREBP1	ATCACGTGA	-13 to -5	78	[407]
	AP4	TCAGCTGTG	-12 to -4	89	[408]
	Ets-1	CCGCGGAAG	47 to 55	89	[409]
Vti1b	SP1	CCCCGCCCC	-246 to 237	90	[410]
	Ets-1	CCGCGGAAG	-173 to -165	100	[409]
	DEAF1	TTTCCG	6 to 11	83	[411]
	ELK1	ATCC(C/G)	7 to 11	80	[412]
	ZF5	GCGCGCGA	116 to 123	75	[413]
	E2F1	TTTCCCGC	124 to 131	75	[414]
Syntaxin 8	E2F1	TTTCCCGC	-69 to - 62	62	[414]
	c-Myc/Max	CACGTG	-34 to -29	83	[415]
	AP1	CTGA(C/G)T(A/C)A	-24 to -17	75	[416]
	AP4	TCAGCTGTG	-19 to -11	78	[408]
	GABP/PEA3	CAGGA(A/T)GTG	-5 to 4	100	[417]
	AP4	TCAGCTGTG	7 to 15	78	[408]
	AP2	(G/C)(G/C)(G/C)GCGGGC	10 to 18	89	[418]
	ZF5	GCGCGCGA	13 to 20	75	[413]
VAMP8	NRSF	TTCAGCACCACGGACAGCG	-391 to -373	68	[419]
	ATF	TGA(G/C)(G/C)TGC	-383 to -376	62	[420]
	ELK1	ATCC(C/G)	-80 to -76	60	[412]
	ZF5	GCGCGCGA	-53 to -46	62	[413]
	SP1	CCCCGCCCC	-53 to -45	78	[410]

Table 3.3: CAGE tags corresponding to mouse late endosomal SNAREs.

Gene	TC ID	Chromosome	TSS	CAGE Tags
syntaxin 7	T10F016CD30D	10	23909133	2022 of 2126
	T10F016CD3A0	10	23909280	20 of 2126
	T10F016D6D29	10	23948585	14 of 2126
Vti1b	T12R04704942	12	74467650	746 of 816
	T12R04704A38	12	74467896	34 of 816
	T12R04700A61	12	74451553	12 of 816
syntaxin 8	T11F04072C89	11	67579017	535 of 583
	T11F040C3517	11	67908887	5 of 583
	T11F040ACB95	11	67816341	4 of 583
VAMP8	T06R04540D95	6	72617365	631 of 694
	T06R0453F896	6	72611990	19 of 694
	T06R045404E0	6	72615136	5 of 694

3.2.5 Gene expression profiles of late endosomal SNAREs

By investigating the gene expression profiles of late endosomal SNARE proteins across different tissues and cell types it is likely that a more comprehensive understanding of their functions will be achieved. The RefExa database, which was established by the Laboratory of Systems Biology and Medicine at The University of Tokyo (http://www.lsbm.org/site_e/database/index.html#), is a comprehensive database of human gene expression profiles [421]. All expression profiles in this database were generated using Affymetrix HG-U133A gene chips. This database was used to determine the relative expression of the late endosomal SNAREs syntaxin 7, Vti1b, syntaxin 8 and VAMP8 in different human tissues and organs.

In addition to being expressed at relatively high levels in macrophages, syntaxin 7 was also highly expressed in heart, kidney and bone marrow; whereas its expression levels in lung and brain were lower (Figure 3.7A). High levels of Vti1b gene expression were present in macrophages as well as in brain (Figure 3.7B). In the case of syntaxin 8, its expression was relatively uniform across the tissues profiled (Figure 3.7C). VAMP8 gene expression was highest in macrophages, but absent or only expressed at very low levels in brain and heart (Figure 3.7D). Thus, analysis of the RefExa human gene expression database revealed that the late endosomal SNARE genes are expressed at relatively high levels in macrophages when compared to their expression in other cell types.

Symatlas is another expression database (http://symatlas.gnf.org/) that contains the mRNA expression profiles of human and mouse genes across a panel of 79 human and 61 mouse tissues [422]. The data deposited in the database were generated from analyses performed with custom-made Affymetrix gene chip arrays for human (GNF1H) and mouse (GNF1M) cells and tissues. Some of the genes in the Symatlas database contain more than one expression profile because multiple probe sets were used to evaluate their expression. For example, there are three gene expression profiles, from three different probe sets, for human syntaxin 7. In that specific case, the probe set that displayed the highest median fluorescence intensity, which is proportional to the level of gene expression, was chosen because it exhibited the greatest sensitivity in detecting syntaxin 7 mRNA. Searches of the

human dataset revealed that syntaxin 7 mRNA levels were highest in $CD14^+$ monocytes, which are precursor cells for tissue macrophages (Figure 3.8A). Although Vti1b was also highly expressed in $CD14^+$ monocytes, its highest level of expression was detected in brain (Figure 3.8B). The expression level of syntaxin 8 in $CD14^+$ monocytes was at least 7-fold greater than in any other tissue or cell type (Figure 3.8C). VAMP8 was expressed at high levels in $CD14^+$ monocytes, coinciding with its high expression in macrophages from the RefExa database (Figure 3.8D).

Analysis of the expression profiles in the mouse Symatlas database revealed that syntaxin 7 mRNA levels were highest in kidney, while lung and cerebral cortex also exhibited high levels of syntaxin 7 gene expression (Figure 3.9A). Mouse cerebral cortex had the highest expression levels of Vti1b; while on the other hand, Vti1b expression levels were lowest in bone marrow (Figure 3.9B). The expression levels of syntaxin 8 mRNA were relatively uniform across all the tissues profiled (Figure 3.9C), while VAMP8 expression was highest in lung (Figure 3.9D).

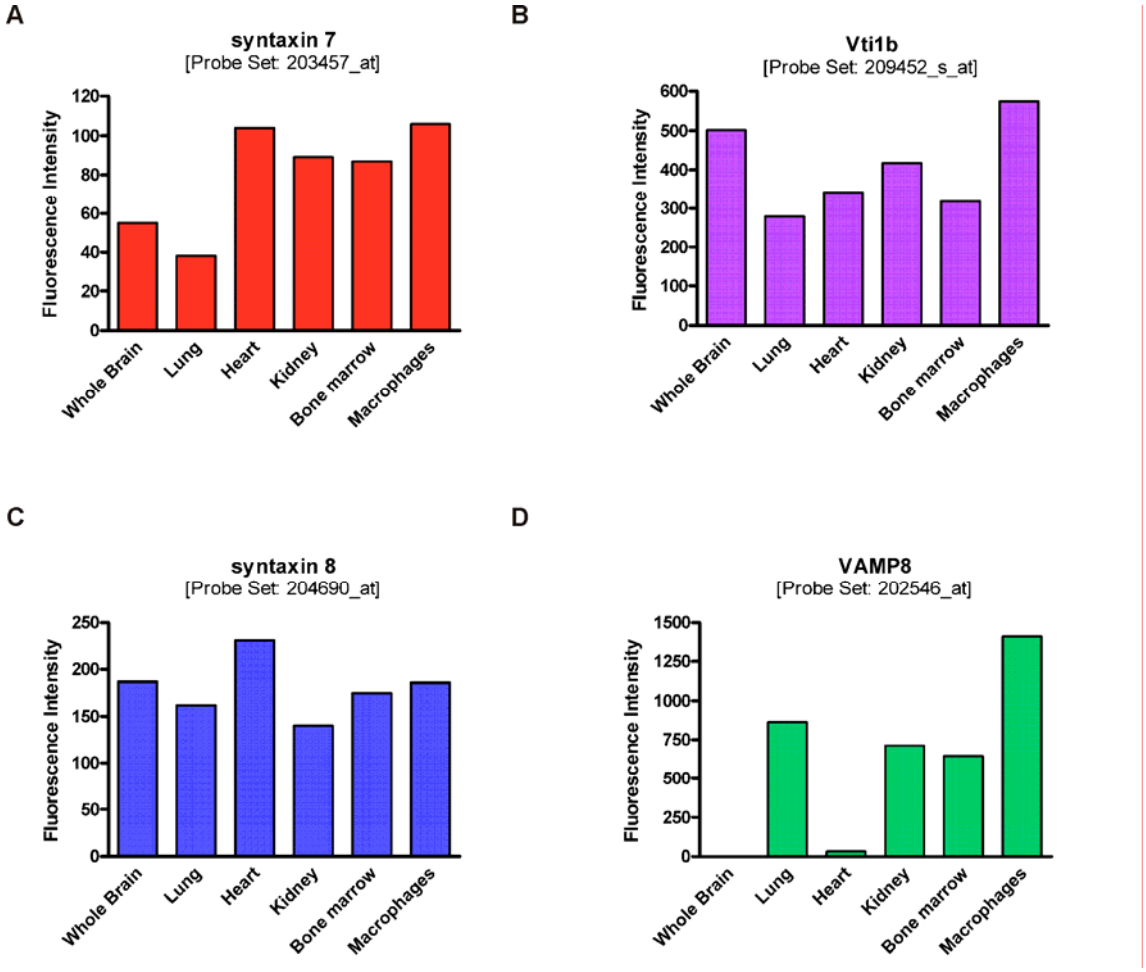


Figure 3.7: Gene expression profiles of *human* late endosomal SNAREs

The gene expression profiles of human late endosomal SNAREs were extracted from the RefEXA database. The fluorescence intensity values are proportional to the level of gene expression. When more than one gene expression profile was available from the database, the probe set that displayed the highest median fluorescence intensity was chosen. The name of the chosen probe set (in square brackets) is given under the gene name.

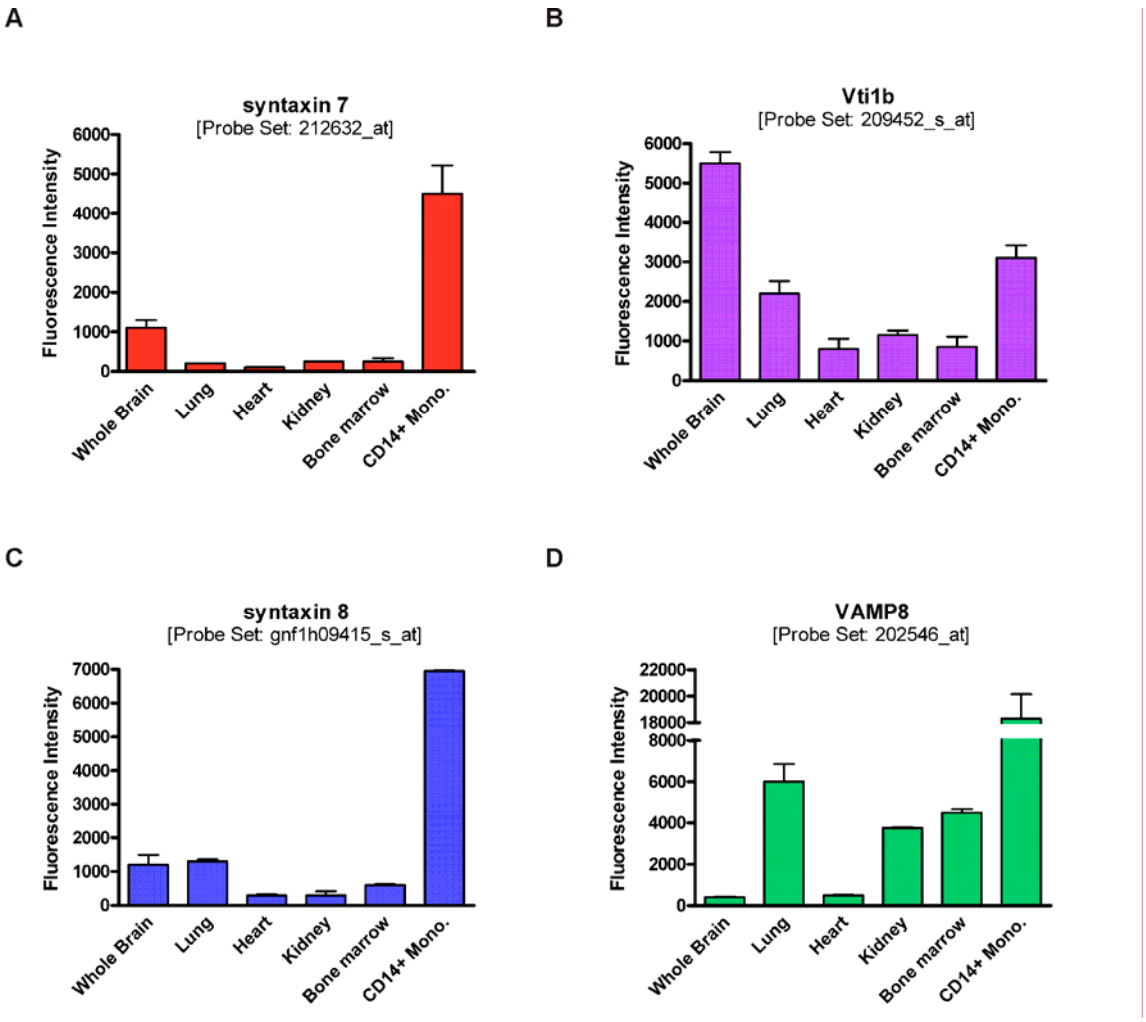


Figure 3.8: Gene expression profiles of *human* late endosomal SNAREs

The gene expression profiles of human late endosomal SNAREs were extracted from the Symatlas GNF1H, gcRMA-normalised dataset. The fluorescence intensity values are proportional to the level of gene expression. When more than one gene expression profile was available from the database, the probe set that displayed the highest median fluorescence intensity was chosen. The name of the chosen probe set (in square brackets) is given under the gene name.

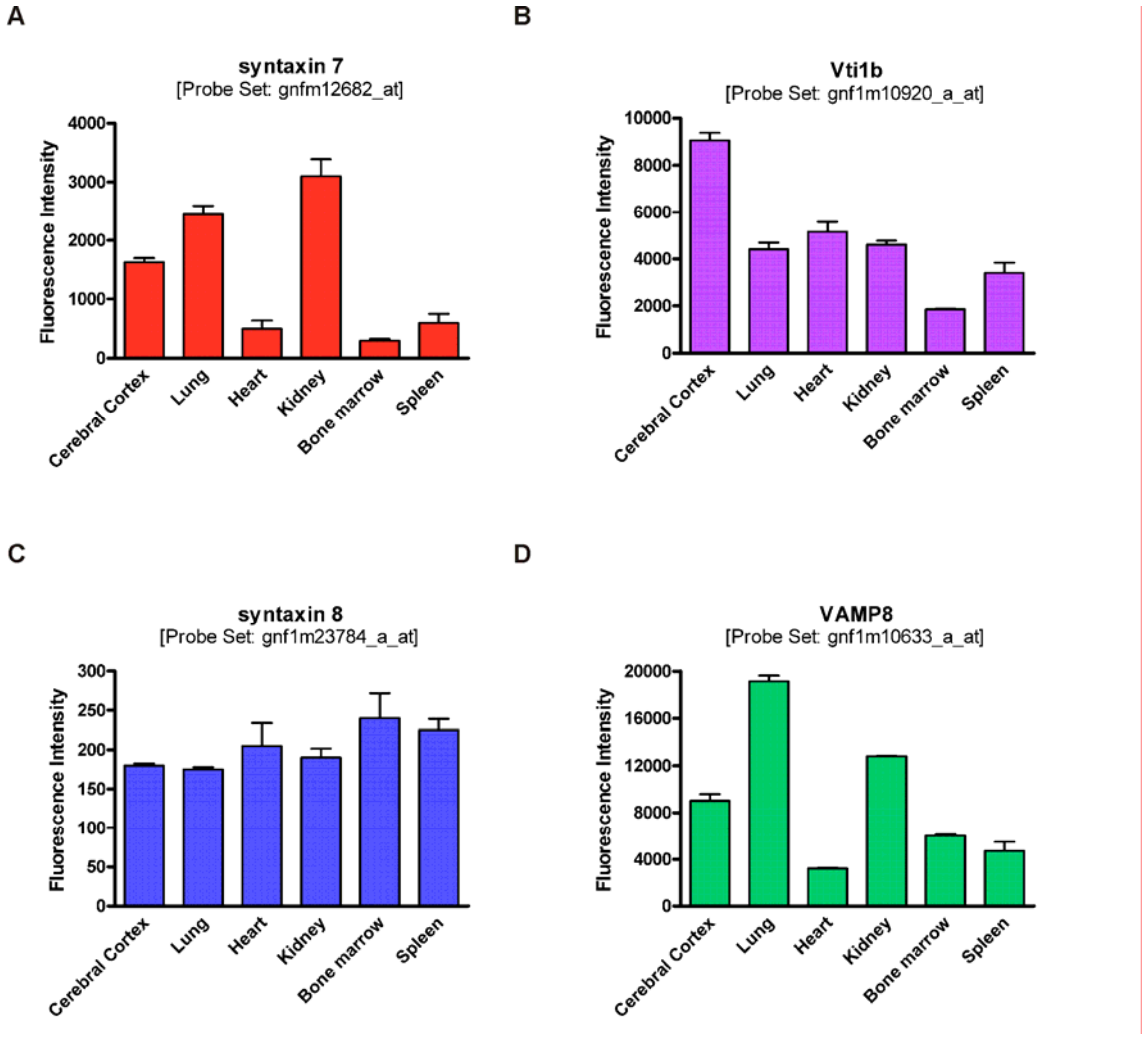


Figure 3.9: Gene expression profiles of *mouse* late endosomal SNAREs

The gene expression profiles of mouse late endosomal SNAREs were extracted from the Symatlas GNF1M, gcRMA-normalised dataset. The fluorescence intensity values are proportional to the level of gene expression. When more than one gene expression profile was available from the database, the probe set that displayed the highest median fluorescence intensity was chosen. The name of the chosen probe set (in square brackets) is given under the gene name.

3.2.6 Structural organisation and phylogenetic analysis of late endosomal SNARE proteins

ClustalW-based amino acid sequence alignment of mouse and human late endosomal SNARE proteins revealed a high degree of conservation through evolution. The mouse late endosomal Q-SNARE proteins syntaxin 7, Vti1b and syntaxin 8 exhibit more than 90% sequence identity with their human orthologues (Table 3.4). Similarly, the mouse and human orthologues of the R-SNARE protein VAMP8 share 86% sequence identity (Table 3.4). This high degree of sequence identity between human and mouse late endosomal SNARE orthologues suggest that their functional roles are conserved through evolution. Thus, knowledge from studying mouse SNARE proteins will likely contribute to our understanding of the roles of SNARE proteins in human biology and pathology.

Comparative protein sequence analysis of multi-protein families allows one to estimate the extent of molecular evolution and thereby infer evolutionary patterns of a genome. As mentioned in Chapter 1, the late endosomal SNARE proteins syntaxin 7, Vti1b and syntaxin 8 belong to the Q-SNARE family, whereas VAMP8 is a member of the R-SNARE family. Determining how closely these proteins are related to their sub-family members will be potentially helpful in developing a better understanding of their biological and hence physiological functions. The Molecular Evolutionary Genetics Analysis 3 (MEGA3) software provides a means by which DNA and protein sequence variation can be analysed from an evolutionary perspective [423]. The MEGA3 software utilises the ClustalW algorithm to align sequences and then infers phylogenetic relationships and evolutionary distances between proteins.

As Q-SNAREs and R-SNAREs vary significantly in their amino acid sequences, lengths and structures (Chapter 1 and Table 3.4), phylogenetic analysis of these two SNARE sub-families were performed individually. Phylogenetic analysis of the Q-SNAREs revealed that syntaxin 7 is most closely related to syntaxin 13 (Figure 3.10), which is a Qa-SNARE that plays a role in early endosome fusion and

Table 3.4: Structural organisation of mouse late endosomal SNAREs.

Late endosomal SNAREs (GenBank)	Classification		Length (amino acids)	Helical Domain			SNARE motif	TM Domain	Identity with human orthologue
	Functional	Structural		Ha	Hb	Hc			
Syntaxin 7 (NP_058077)	t	Qa	261	12-38	45-74	81-127	175-228	238-259	94%
Vti1b (NP_058080)	t	Qb	232	9-22	37-65	72-99	146-198	207-226	93%
Syntaxin 8 (NP_061238)	t	Qc	236	4-28	34-55	87-104	155-207	216-233	93%
VAMP8 (NP_058074)	v	R	101	-	-	-	13-65	76-99	86%

phagosome maturation [127, 424]. Syntaxin 8, on the other hand, is most closely related to syntaxin 6 and syntaxin 10 (Figure 3.10). Syntaxin 10 is localised to the *trans*-Golgi network (TGN), while syntaxin 6 is found on the TGN and early endosomes [282-284]. Vti1b arises from the same ancestral gene as Vti1a (Figure 3.10) and is present on tubules and vesicles in the TGN area and on endosomes, whereas Vti1a is found on the Golgi and TGN [425]. The phylogenetic analysis revealed that the plasma membrane associated syntaxins 1-4 all arose from a common ancestor (Figure 3.10). Furthermore, Q-SNARE sub-family members appear to have arisen from common ancestors. For example, the Qa-SNAREs syntaxins 1-5, 7, 11, 13, 16, 18 and 19 have a common ancestor (Figure 3.10). Likewise, Vti1a, Vti1b, GS27 and GS28, which belong to the Qb-SNARE sub-family, are grouped together (Figure 3.10). SNAP family proteins contribute two SNARE motifs to ternary SNARE complexes and are thus classified as Qbc-SNAREs. All members of the Qbc-SNARE family were grouped together in the phylogenetic tree (Figure 3.10). The remaining SNAREs in between the Qb- and Qbc-SNARE sub-families belong to the Qc-SNARE sub-family of SNARE proteins (Figure 3.10).

D12 is a recently characterised novel Q-SNARE protein from murine haematopoietic stem cells [253]; it is localised to the ER and can form a complex with the Qa-SNARE syntaxin 18 and Sec22b, an R-SNARE. D12 can also bind to VAMP7 and is implicated in the maintenance of proper lysosome function [253]. Although there is some controversy regarding the classification of D12 as a Q-SNARE, it shows significant homology to Use1p/Slt1p, which belongs to the Qc-SNARE family [253]. Furthermore, phylogenetic analysis indicated that D12 is more closely related to Qc-SNAREs than to Qa- or Qb-SNAREs (Figure 3.10).

Phylogenetic analysis of R-SNARE proteins revealed that VAMP8 is most closely related to VAMP7 (Figure 3.11), which mediates membrane fusion between late endosomes and lysosomes [296, 426]. Moreover, it appears that R-SNAREs which are closely related to each other share similar biological roles. For example, VAMP8 and VAMP7 are involved in endocytic pathways, while VAMP1 and VAMP2 play crucial roles in exocytic pathways in neuronal cells and adipocytes [291, 292, 427]. Both Sec22b and Ykt6, which are closely related in the phylogenetic analysis, function in trafficking events that involve the *trans*-Golgi network [281, 428].

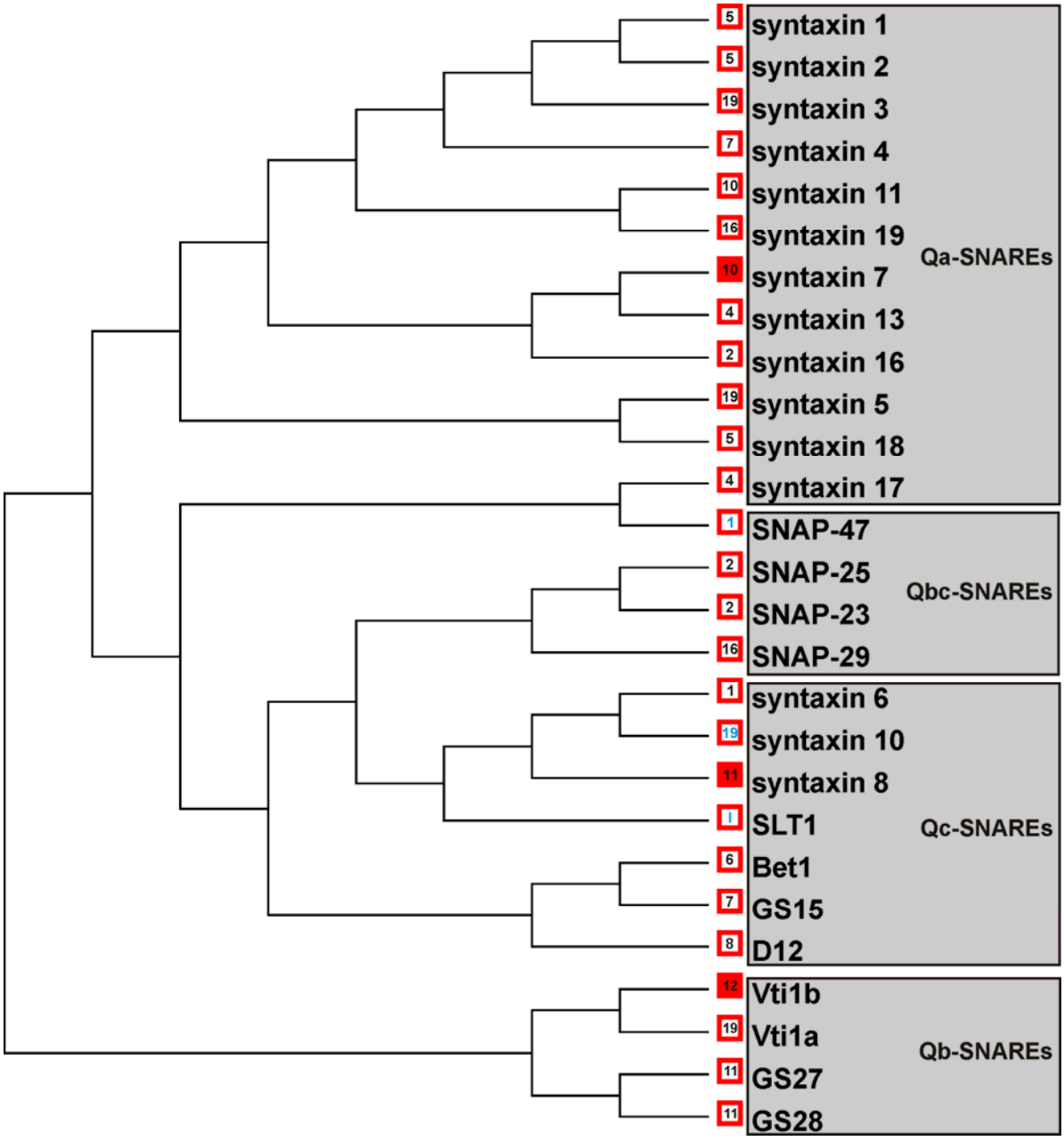


Figure 3.10: Phylogenetic tree of Q-SNARE proteins

The amino acid sequences of twenty four mouse Q-SNARE proteins, as well as human SNAP-47, syntaxin 10 and SLT1 for which no mouse homologues have been identified, were aligned by ClustalW analysis. The results of these multiple sequence alignments were used to draw a nearest neighbour-joining tree using the MEGA3 program [423]. The chromosome on which each gene is located is indicated inside the boxes. Late endosomal SNARE proteins are indicated with filled boxes. The human chromosomes on which SNAP-47, syntaxin 10 and SLT1 are located are indicated in blue font.

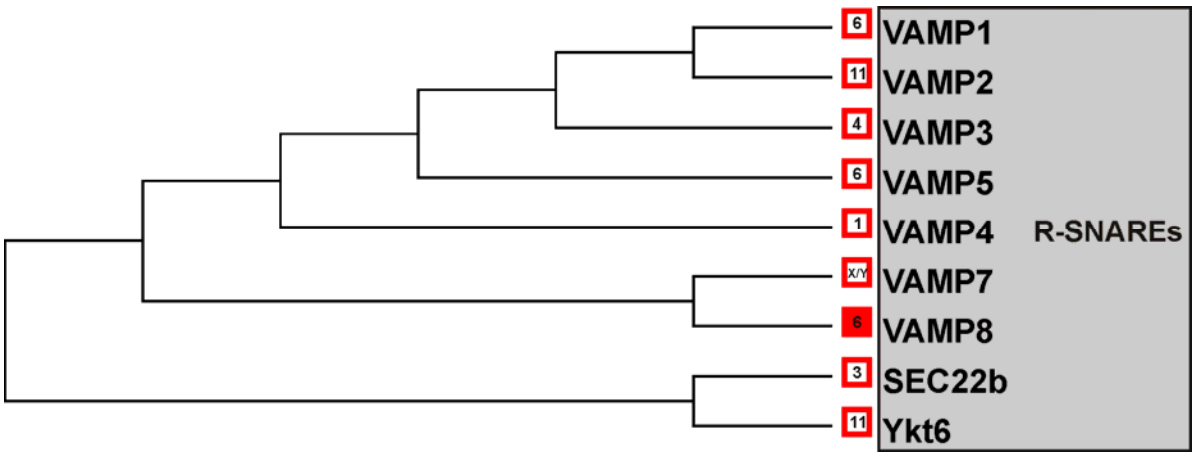


Figure 3.11: Phylogenetic tree of R-SNARE proteins.

The amino acid sequences of nine mouse R-SNARE proteins were aligned by ClustalW analysis. The results of the multiple sequence alignments were used to draw a nearest neighbour-joining tree using the MEGA3 program [423]. Late endosomal SNARE proteins are indicated with filled boxes. The chromosome on which each gene is located is indicated inside the boxes.

3.3 Discussion

Several earlier studies have revealed that CSF-1 modulates some of the effector functions of macrophages, such as cytokine secretion and phagocytosis [177, 184]. However, the molecular mechanisms by which CSF-1 modulates these functions remain largely unknown. The immune functions of macrophages involve a number of vesicle trafficking and membrane fusion events. Over the past few years SNARE proteins have emerged as key mediators of membrane tethering and fusion. Hence, I hypothesised that CSF-1 may modulate the effector functions of macrophages by regulating the expression of SNARE genes.

Our microarray study identified the syntaxin 7 gene as a CSF-1-regulated gene in mouse bone marrow-derived macrophages. Syntaxin 7 regulates membrane fusion between late endosomes and lysosomes [272, 391, 429-431] and is present on mature phagosomes in macrophages [424]. Furthermore, syntaxin 7 has recently been shown to be a component of a novel SNARE complex that forms in the ER-Golgi region of macrophages to traffic TNF to the plasma membrane in response to macrophage activation by the TLR4 ligand LPS [126, 240]. However, the precise role of syntaxin 7, as well as its regulation, in macrophages is poorly understood.

The extents to which syntaxin 7 mRNA levels were up-regulated by CSF-1 in bone marrow-derived macrophages were comparable by both microarray (~2.5-fold) and Real-Time PCR (~4-fold) analysis. Notably though, syntaxin 7 protein expression was only up-regulated ~1.5-fold following CSF-1 stimulation of mouse bone marrow-derived macrophages. This suggests that syntaxin 7 mRNA levels alone do not dictate syntaxin 7 protein levels; moreover, they suggest that the translation of syntaxin 7 transcripts may also be a rate-limiting factor in the synthesis of syntaxin 7. In contrast to syntaxin 7, the extents to which Vti1b mRNA and protein levels were up-regulated by CSF-1 largely correlated. The differences in the effects of CSF-1 on syntaxin 7 and Vti1b protein levels may well be due to differences in the efficiencies with which their respective mRNA transcripts are translated; alternatively the Vti1b protein may be more stable than syntaxin 7. Although the expression levels of late endosomal SNARE proteins were studied in bone marrow-derived macrophages future experiments should aim to confirm these results with macrophages from different origins (e.g. peritoneal macrophages) and from mice of

different genetic background (e.g. BALB/c, C3H/HeJ, etc.); examining their expression in human monocytes/macrophages would also be informative.

The mRNA levels of syntaxin 8 and VAMP8 were only modestly (~1.5-fold) up-regulated by CSF-1 in bone marrow-derived macrophages. Furthermore, no increase in syntaxin 8 protein levels was observed in response to CSF-1 stimulation. However, whilst CSF-1 had little influence on VAMP8 mRNA levels, VAMP8 protein levels were up-regulated ~4-fold by CSF-1. This observation suggests that the increased VAMP8 protein levels are likely to have been due to the more efficient translation of VAMP8 transcripts and/or an increase in VAMP8 protein stability. This issue, as well as the effects of CSF-1 on syntaxin 7 protein levels, could be explored further by performing pulse-chase labelling experiments in which bone marrow-derived macrophages are metabolically labelled briefly (i.e. "pulsed") with ^{35}S-methionine and/or ^{35}S-cysteine, in the presence and absence of CSF-1, and then incubated with unlabelled methionine and/or cysteine (i.e. "chased") for different periods of time. By assessing the levels of ^{35}S-methionine- and/or ^{35}S-cysteine-labelled VAMP8 (or any of the other late endosomal SNARE proteins) at different time points post-pulsing, it would be possible to establish the effects of CSF-1 on VAMP8 translation and protein stability. Micro RNAs (miRNAs) are rapidly emerging as key regulators of protein translation [432]. Thus, the possibility exists that CSF-1 may exert its effects on the expression of late endosomal SNARE proteins (e.g. VAMP8) by not only regulating their transcription but also by controlling the expression of miRNAs that regulate the translation of SNARE transcripts. Indeed, it has recently been suggested that miRNAs may control the translation of a number of SNARE proteins (e.g. syntaxin 1, SNAP-25 and VAMP2) which are involved in exocytosis [433]. Interestingly, miRNAs have recently been reported to control macrophage differentiation by regulating the translation of AML1, a transcription factor that governs the expression of the CSF-1 receptor [434].

Bioinformatics analysis of the gene expression profiles in the RefExa and Symatlas databases revealed that syntaxin 7, Vti1b, syntaxin 8 and VAMP8 are highly expressed, at least at the mRNA level, in human macrophages and CD14$^+$ monocytes. However, the gene expression profiles of the mouse arrays varied significantly from those of the human arrays. In the context of macrophages, both human arrays utilised relatively differentiated macrophages, whereas the mouse

arrays made use of bone marrow cells that are immature haematopoietic progenitor cells, containing macrophages lower in number as well as less advanced in maturation. Macrophage precursors have a limited ability to perform a range of immune functions (e.g. cytokine secretion and phagocytosis) compared to mature tissue macrophages due to the lack of specialised receptors, such as TLRs and FcγRs. Thus, high level gene expression of late endosomal SNAREs may not be required by bone marrow cells, whereas macrophages may require the expression of the genes at high levels in order to perform macrophage immune functions immediately and effectively upon pathogen invasion. Interestingly, syntaxin 8 was expressed at high levels in heart from the RefExa database, but the Symatlas database, in contrast, showed the lowest level of syntaxin 8 expression in heart (Figures 3.4C and 3.5C). This highlights the need for caution when interpreting microarray data. However, despite some inconsistencies between the gene expression data sets, the gene expression profiles from both the RefExa and Symatlas databases for human tissues and cells indicated that $CD14^+$ monocytes and macrophages express high levels of late endosomal SNARE genes.

The transcriptional regulation of SNARE proteins remains largely unknown. A limited number of studies have recently reported that the expression of some endosomal SNARE proteins (e.g. Vti1b, syntaxin 6, VAMP3) was up-regulated in response to the activation of macrophages by LPS [126]. Although the transcriptional mechanisms that regulated the expression of these SNARE proteins is not known, it was notable that their up-regulation mirrored the increase in TNF expression [126, 240]. Such findings suggest that the expression of inflammatory cytokines and SNARE proteins in macrophages may be co-regulated by the same transcription factor(s). Moreover, some of the pro-inflammatory effects of cytokines could potentially be partially explained by their ability to modulate the expression of SNARE proteins [240]. For example, IFNγ has been shown to up-regulate the expression of syntaxin 6, Vti1b and VAMP3 in macrophages [126, 240]. Whether anti-inflammatory cytokines (e.g. IL-10) exert, at least in part, their effects via the modulation of SNARE protein expression is yet to be established.

Although transcription of the syntaxin 7, Vti1b, syntaxin 8 and VAMP8 genes was regulated by CSF-1, albeit to different extents, no single transcription factor binding site was found to be common to all four genes. This suggests either that CSF-1 may regulate their expression by acting on different transcription factors or that

there might be other TFBS present in the 5'-flanking regions of the genes that were not identified by the ECR programme. Nonetheless, the finding that syntaxin 7 and Vti1b mRNA levels were up-regulated as early as 2 h post-CSF-1 stimulation, and they were maximally induced to similar levels by 8 h, suggests that these two genes may be regulated by a common transcription factor(s). Promoter analysis identified an Ets-1 TFBS in the 5'-flanking regions of the syntaxin 7 and Vti1b genes that could potentially control their expression. Ets family transcription factors (e.g. Ets-1, Ets-2, PU.1, ELK-1, GABP and PEA3), which are defined by the core Ets binding motif GGA(A/T), are known to regulate the expression of a number of CSF-1-induced genes [435, 436]. Amongst these CSF-1-regulated genes, Ets-1 regulates the expression of monocyte chemoattractant-1 (MCP-1), vascular cellular adhesion moleucle-1 (VCAM-1) and platelet-derived growth factor (PDGF) [437]. Thus, the presence of a putative Ets-1 binding site in the 5'-flanking regions of the syntaxin 7 and Vti1b genes would be consistent with their transcriptional regulation by CSF-1. ELK1 is another member of the Ets family of transcription factors and an ELK1 site is located downstream of the TSS of the Vti1b gene and upstream of the TSS of the VAMP8 gene. CSF-1 has been shown to enhance fos gene transcription by activating ELK1 in mouse osteoclasts, which arise from cells of the monocyte/macrophage lineage [438]. ELK1 is part of a transcription complex that is recruited to the TNF promoter, resulting in rapid TNF production following LPS stimulation of RAW 264.7 macrophages and THP-1 monocyte-derived macrophages [439]. Given that protein expression of both TNF and Vti1b were up-regulated in LPS activated macrophages, it is possible that common transcription factor binding sites (e.g. ELK1 binding site) present in the promoter regions of these genes may be regulated by LPS. However, until functional promoter analysis studies are undertaken we cannot exclude the possibility that the expression of the late endosomal SNARE genes is regulated by other transcription factors.

Syntaxin 8 and VAMP8 were similarly regulated at the mRNA level by CSF-1, suggesting they may have common transcription factor binding sites in their promoter regions. However, the ECR programme did not identify a transcription factor binding site(s) present in both of these genes but that was absent in syntaxin 7 and Vti1b. Given that the rVista tool examines only 467 out of approximately 1,400 transcription factors expressed in the human genome [398, 399], it is possible there may be other CSF-1-responsive target elements that control the expression of syntaxin 7, Vti1b, syntaxin 8 and VAMP8. It is also important to note that TFBS

prediction software, such as the ECR programme, only infer the potential for transcription factor binding, but not the functionality of a site. However, the initial use of bioinformatics tools to analyse promoters can significantly reduce the number of candidates that need to be tested experimentally. Putative promoter regions of late endosomal SNARE genes (i.e. 5' flanking regions of syntaxin 7, Vti1b, syntaxin 8 and VAMP8 genes) can be PCR-amplified from genomic DNA and cloned into a vector containing a reporter gene (e.g. luciferase) [440]. CSF-1-dependent RAW 264.7 macrophages can then be transfected with these plasmids and the effects of CSF-1 in driving the expression of luciferase reporter gene can be measured. Putative transcription factor binding sites (e.g. Ets-1 and Elk1 binding sites) can then be mutated in order to evaluate their role in driving gene transcription.

Phylogenetic analysis revealed evolutionary linkage among SNARE proteins. This analysis can also help in predicting the functions of poorly characterised SNARE proteins, since if they are closely linked to a relatively well characterised SNARE protein, then the SNARE proteins may have similar functions. Syntaxin 7 is most closely linked to the early endosome-localised SNARE protein syntaxin 13 (Figure 3.10). Both of these syntaxins have been shown to play roles in phagocytosis. Syntaxin 7 is involved in the interaction of late endosomes with mature phagosomes, whereas syntaxin 13 is recruited to nascent phagosomes and rapidly recycled off the phagosomes in RAW 264.7 macrophage cells [127, 441]. However, although Vti1a and Vti1b diverged from a common branch, they have distinct subcellular localisations and form different SNARE complexes. Vti1b is found in vesicles in the TGN region and endosomes and forms a complex with syntaxin 7, syntaxin 8 and VAMP8 [425]. Vti1a, on the other hand, is predominantly localised to the Golgi and the TGN and forms a complex with syntaxin 16, syntaxin 6 and VAMP4 [425]. Syntaxin 8 is closely linked to the TGN-localised SNAREs, syntaxin 6 and syntaxin 10. Interestingly, syntaxin 8 and syntaxin 6 were found to be separated from a common branch. In the ER-Golgi SNARE complex in which syntaxin 7 and Vti1b were conserved, while syntaxin 8 was replaced by its close paralogue syntaxin 6 [240]. Phylogenetic analysis of R-SNAREs revealed VAMP7 as a closely linked SNARE to VAMP8. VAMP7 is localised in lysosomes and forms a complex with syntaxin 7, Vti1b and syntaxin 8 during late endosome-lysosome fusion [295, 296]. These observations suggest that evolutionarily closely related

SNARE proteins can replace one another in a SNARE complex, although they may be localised to distinct organelles.

In summary, CSF-1 has been shown to enhance the expression of late endosomal SNAREs in bone marrow-derived macrophages. The putative promoter regions of the genes encoding these SNARE proteins contain predicted binding sites for several Ets family transcription factors, such as Ets-1 and ELK1, which may play a role in the CSF-1-induced up-regulation of late endosomal SNARE protein expression. Web-available microarray data suggests that late endosomal SNARE genes are relatively highly expressed in macrophages, which is consistent with one of the major functions of macrophages being the endocytosis and degradation of pathogens and apoptotic cells.

BIOCHEMICAL CHARACTERISATION OF THE CSF-1-INDUCED PHOSPHORYLATION OF SYNTAXIN 7

4.1 Introduction

Phosphorylation of SNARE proteins is emerging as an important mechanism to regulate the formation of specific SNARE complexes. The assembly of a neuronal SNARE complex consisting of syntaxin 1A, SNAP-25 and VAMP2 is controlled by the phosphorylation of the individual SNARE proteins by various protein kinases including casein kinase II (CKII), calcium/calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC) [307, 311, 442]. Phosphorylation of syntaxin 1A on Ser-14 (which is located N-terminal to the Ha domain) by CKII enhances its association with SNAP-25, whereas phosphorylation of SNAP-25 on Ser-187 (which is located in the C-terminal SNARE motif SN2; see Figure 1.11) by PKC hinders neuronal SNARE complex assembly [311, 443]. These phosphorylation events potentially explain, at least in part, the regulation of exocytosis, and hence neurotransmission, by CKII and PKC [307, 311, 318, 444].

In addition to the phosphorylation of SNARE proteins, the Habc domain of Qa-SNARE proteins can also regulate SNARE complex assembly. The N-terminal Habc domains of the Qa-SNARE proteins, syntaxin 1A and syntaxin 7, have been shown to fold back and interact with the SNARE motif to form a 'closed' conformation [298, 312]. Munc18-1 is able to bind to syntaxin 1A in this 'closed' conformation and essentially lock it in this conformation, thereby preventing syntaxin 1A from interacting with other SNARE proteins and thus inhibiting SNARE complex formation and subsequent membrane fusion [445]. Phosphorylation of Munc18-1 by cyclin-dependent kinase 5 has been shown to decrease its affinity for syntaxin 1A in rat brain and to 'free' syntaxin 1A to form a *trans*-SNARE complex with SNAP-25 and VAMP2 [446]. Similarly, phosphorylation of syntaxin 1A at Ser-188 by death-associated protein kinase (DAPK), a calcium/calmodulin-dependent protein kinase, decreases its binding with Munc18-1 in rat brain [309]. Given the structural similarities between syntaxin 1A and syntaxin 7 [264], it is likely that the interaction of syntaxin 7 with its partner SNAREs (e.g. syntaxin 8, Vti1b and VAMP8) may be regulated by a comparable mechanism.

As shown in Chapter 3, the stimulation of bone marrow-derived macrophages with CSF-1 resulted in an increase in the mRNA and protein levels of syntaxin 7 as well as a change in the electrophoretic mobility of syntaxin 7 when it was analysed by SDS-PAGE electrophoresis. This implies that CSF-1 not only regulates syntaxin 7

gene expression but that it also induces the post-translational modification of syntaxin 7, which may be important for controlling interactions between syntaxin 7 and other SNARE proteins and/or SNARE-binding proteins. This chapter examines the biochemical mechanisms underlying the CSF-1-induced post-translational modification of syntaxin 7.

4.2 Results

4.2.1 CSF-1 induces a rapid change in the electrophoretic mobility of syntaxin 7

Stimulation of bone marrow-derived macrophages with CSF-1 resulted in a change in the electrophoretic mobility of syntaxin 7 upon SDS-PAGE analysis (Figure 4.1). When the SDS-PAGE resolution was optimal, the presence of at least three isoforms of syntaxin 7, which will subsequently be referred to as isoforms 1, 2 and 3, could be detected. Isoform 1 refers to the lowest apparent molecular mass syntaxin 7 isoform, while the highest apparent molecular mass isoform is designated as isoform 3. Two syntaxin 7 isoforms, namely isoforms 1 and 2, were detected in bone marrow-derived macrophages that had been deprived of CSF-1, with isoform 1 being the most abundant (Figure 4.1). However, the levels of these isoforms were acutely regulated by CSF-1. The levels of isoform 2 increased within 5 min of CSF-1 stimulation, which was coupled to a corresponding decrease in the levels of the syntaxin 7 isoform 1 (Figure 4.1). Stimulation with CSF-1 beyond 10 min resulted in the almost total disappearance of isoform 1. Moreover, stimulation beyond 15 min gave rise to isoform 3, the isoform with the greatest apparent molecular mass (Figure 4.1). These findings are consistent with CSF-1 inducing the post-translational modification of syntaxin 7. It is also worth noting that the separation of all three isoforms of syntaxin 7 was dependent on the gel electrophoretic conditions (e.g. percentage of SDS-PAGE gel, electrophoresis voltage and duration). That is, the detected number of isoforms varied depending upon the nature of the experimental conditions.

The kinetics of the CSF-1-induced post-translational modifications of syntaxin 7 were compared with those for the activation of two major kinases downstream of

the CSF-1 receptor, specifically Akt and ERK. The kinetics of Akt and ERK phosphorylation coincided with the increased levels of the higher apparent molecular mass isoforms (i.e. isoforms 2 and 3) of syntaxin 7 (Figure 4.1). The levels of isoform 2 remained elevated at time points at which Akt and ERK phosphorylation had declined (Figure 4.1). Thus, the post-translational modification of syntaxin 7 in response to CSF-1 was sustained, whilst other signalling events were more transient.

4.2.2 CSF-1 induces the phosphorylation of syntaxin 7

The rapid appearance and/or increase of higher apparent molecular mass isoforms of syntaxin 7 (i.e. isoforms 2 and 3) in CSF-1-stimulated bone marrow-derived macrophages suggested that syntaxin 7 had been subjected to post-translational modification (e.g. phosphorylation, ubiquitination, sumoylation or palmitoylation) rather than proteolytic 'clipping'. Further, the translation of alternative syntaxin 7 mRNA transcripts would be unlikely to explain the rapid appearance of these additional syntaxin 7 isoforms. Given that activation of the CSF-1 receptor triggers the activation of various downstream protein kinases (e.g. Akt, ERK1/2 and Src family kinases), phosphorylation of syntaxin 7 was considered a likely explanation for the CSF-1-induced appearance of the different syntaxin 7 isoforms. In order to directly establish if syntaxin 7 was phosphorylated in response to CSF-1 stimulation, bone marrow-derived macrophages were incubated with [^{32}P]-orthophosphate for 6 h in order to metabolically label the intracellular pool of ATP. The macrophages were then stimulated with CSF-1 for 15 min, to trigger potential phosphorylation of syntaxin 7, or left unstimulated. Syntaxin 7 was subsequently immunoprecipitated from cell lysates and the immunoprecipitates subjected to SDS-PAGE analysis, followed by transfer to a PVDF membrane. Autoradiography of the membrane revealed that syntaxin 7 was phosphorylated in the absence of CSF-1 (Figure 4.2A). Notably though, CSF-1 stimulation resulted in a ~5-fold increase in the level of [^{32}P]-phosphate incorporated into syntaxin 7 (Figure 4.2A). Due to the difficulty in resolving syntaxin 7 into distinct isoforms (i.e. isoforms 1, 2 and 3), it was not possible to establish to what extent phosphorylation was associated with each individual isoform.

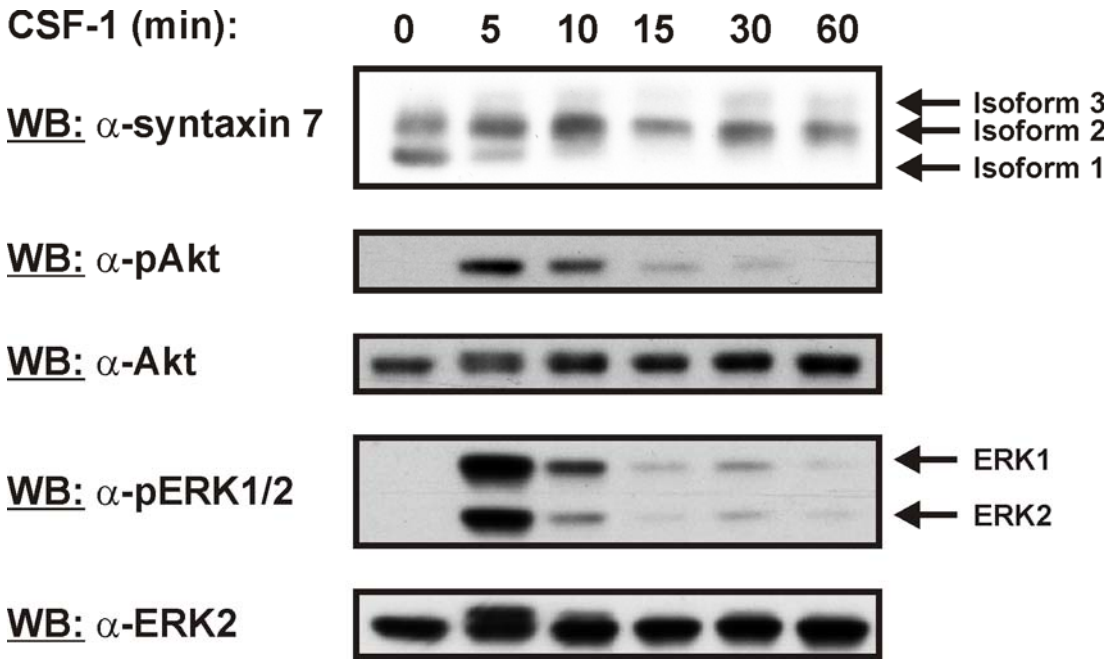
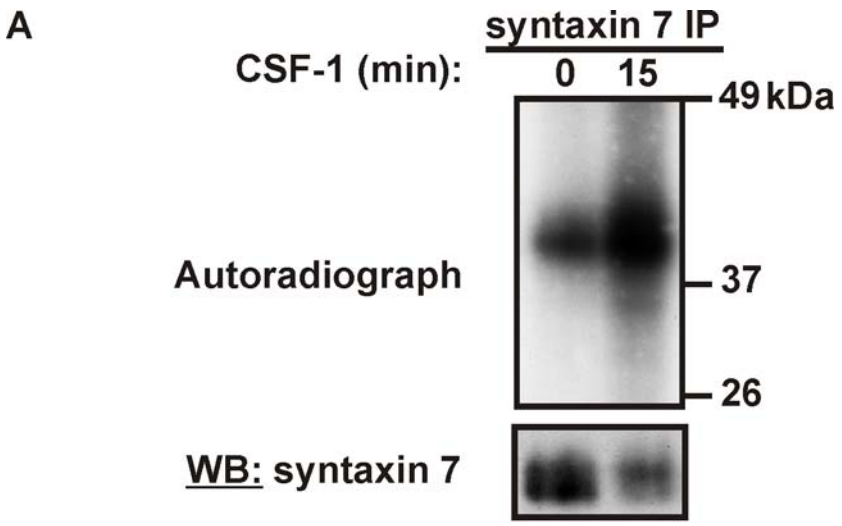


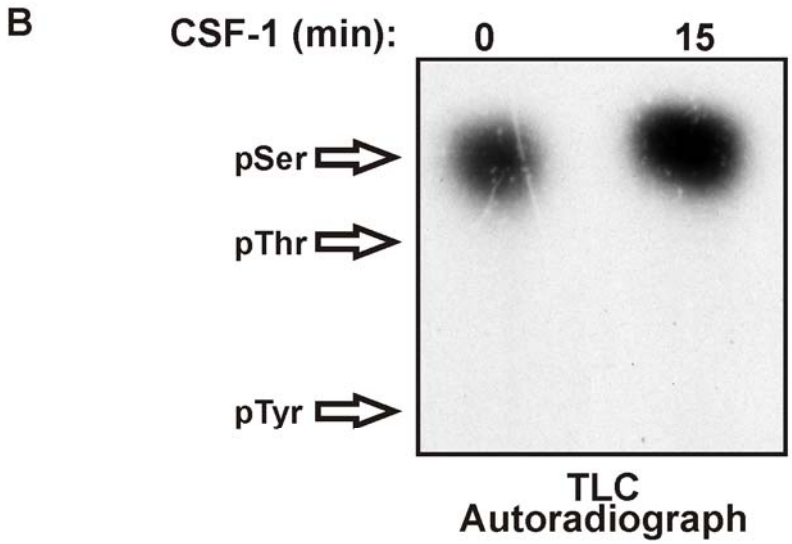
Figure 4.1: CSF-1 induced post-translational modification of syntaxin 7.

Bone marrow-derived macrophages were deprived of CSF-1 for 16 h and then stimulated with CSF-1 (5,000 U/ml) for the indicated times. The cells were lysed and aliquots of the cell lysates were subjected to Western blotting with anti-syntaxin 7, anti-phospho Akt, anti-Akt, anti-phospho ERK1/2 and anti-ERK2 antibodies (n = 3). Molecular masses: Akt ~60 kDa; ERK ~44 kDa; ERK2 ~42 kDa.

Although the CSF-1 receptor possesses an intrinsic tyrosine kinase activity that is activated upon ligand binding [174, 447], anti-phosphotyrosine Western blotting of anti-syntaxin 7 immunoprecipitates failed to reveal detectable tyrosine phosphorylation of syntaxin 7 in response to CSF-1 stimulation of bone marrow-derived macrophages (data not shown). One possibility was that CSF-1 had induced serine and/or threonine, rather than tyrosine, phosphorylation of syntaxin 7. In order to test this proposal, syntaxin 7 that had been purified from [^{32}P]-orthophosphate-labelled bone marrow-derived macrophages, via immunoprecipitation with anti-syntaxin 7 antibodies, was subjected to one-dimensional phosphoamino acid analysis. Consistent with the data arising from the Western blotting of syntaxin 7 immunoprecipitates with an anti-phosphotyrosine antibody, phosphoamino acid analysis failed to reveal the presence of [^{32}P]-phosphate-labelled tyrosine residues in syntaxin 7 (Figure 4.2B). Instead, syntaxin 7 was phosphorylated exclusively on serine residues in both CSF-1-deprived bone marrow-derived macrophages as well as in macrophages that had been stimulated with CSF-1 for 15 min (Figure 4.2B).

To ascertain whether the changes in the electrophoretic mobility of syntaxin 7 following CSF-1 stimulation was indeed due to its phosphorylation, syntaxin 7 that had been immunoprecipitated from CSF-1-stimulated and unstimulated bone marrow-derived macrophages was incubated *in vitro* with calf intestinal alkaline phosphatase. As shown in Figure 4.2C, syntaxin 7 from CSF-1-stimulated macrophages exhibited increased 'smearing' (i.e. higher molecular weight syntaxin 7 isoforms) when compared to syntaxin 7 from CSF-1-deprived macrophages. However, this 'smearing' was greatly diminished following the incubation of syntaxin 7 with calf intestinal alkaline phosphatase, suggesting that the appearance of the apparent higher molecular mass isoforms of syntaxin 7 (i.e. isoforms 2 and 3) following CSF-1 stimulation was indeed due to syntaxin 7 phosphorylation. It is important to note that the resolution of immunoprecipitated syntaxin 7 into two or three distinct isoforms was not as great as that achieved when cell lysates were directly subjected to SDS-PAGE.


A
syntaxin 7 IP
CSF-1 (min):
0
15
49 kDa
Autoradiograph
37
26
WB: syntaxin 7


B
CSF-1 (min):
0
15
pSer
pThr
pTyr
TLC
Autoradiograph

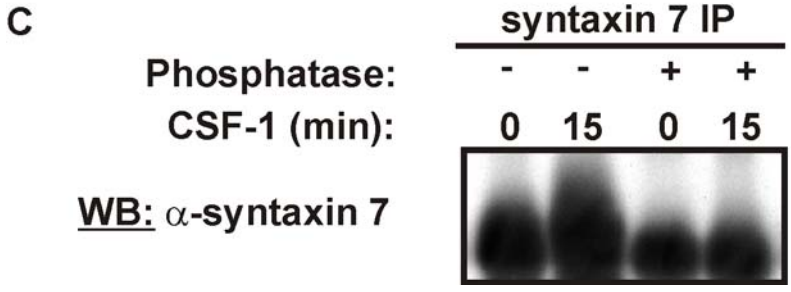

C
syntaxin 7 IP
Phosphatase:
-
-
+
+
CSF-1 (min):
0
15
0
15
WB: α-syntaxin 7

Figure 4.2: CSF-1 induced serine phosphorylation of syntaxin 7.

A, Bone marrow-derived macrophages were deprived of CSF-1 for 10 h and then incubated in phosphate-free medium supplemented with 3 mCi [^{32}P]-orthophosphate for a further 6 h. The macrophages were stimulated with CSF-1 for 15 min, or left unstimulated, and then syntaxin 7 was immunoprecipitated from lysates of the cells using anti-syntaxin 7 antibodies. Each immunoprecipitate was divided into two (representing 90% and 10% of the immunoprecipitate, respectively) and the samples subjected to SDS-PAGE, followed by transfer to a PVDF membrane. The section of the PVDF membrane onto which the '90% fractions' had been transferred was subjected to autoradiography (upper panel), while the other section of the membrane was subjected to Western blotting with anti-syntaxin 7 antibodies to determine the levels of syntaxin 7 that had been immunoprecipitated (lower panel) (n = 2).

B, Syntaxin 7 that had been immunoprecipitated from [^{32}P]-orthophosphate-labelled bone marrow-derived macrophages (Figure 4.2A) was excised from the PVDF membrane and hydrolysed in hydrochloric acid. The acid hydrolysates were then subjected to one-dimensional phosphoamino acid analysis via high voltage electrophoresis on a cellulose-coated thin layer chromatography plate. Following electrophoresis, the positions of the phosphoamino acid standards (phosphoserine, pSer; phosphothreonine, pThr; and phosphotyrosine, pTyr) were visualised by ninhydrin staining. The thin layer chromatography plate was then exposed to X-ray film to detect phosphorylated amino acids (n = 1).

C, Bone marrow-derived macrophages were deprived of CSF-1 for 16 h and then either stimulated with CSF-1 for 15 min or left unstimulated. Syntaxin 7 was immunoprecipitated from lysates of the cells with anti-syntaxin 7 antibodies. The immunoprecipitates were divided into two: one half of each immunoprecipitate was incubated with calf intestinal alkaline phosphatase (CIP) for 30 min at 37°C, while the other half of the immunoprecipitate was incubated in phosphatase reaction buffer alone. The immunoprecipitates were then subjected to Western blotting with anti-syntaxin 7 antibodies (n = 2).

4.2.3 CSF-1 induces phosphorylation of syntaxin 7 at multiple sites

The finding that CSF-1 induced the appearance, and/or increased the levels, of at least two additional isoforms of syntaxin 7 (Figure 4.1) suggested that syntaxin 7 may be phosphorylated at multiple sites upon activation of the CSF-1 receptor. In order to determine if this was the case, cell lysates of both CSF-1-stimulated and unstimulated bone marrow-derived macrophages were subjected to one- and two-dimensional SDS-PAGE followed by Western blotting with anti-syntaxin 7 antibodies.

Two-dimensional SDS-PAGE analysis revealed that CSF-1-deprived bone marrow-derived macrophages contained two differentially charged populations of syntaxin 7 (Figure 4.3A). These two populations potentially correspond to the two isoforms of syntaxin 7 (i.e. isoforms 1 and 2) that were detected by one-dimensional SDS-PAGE (Figure 4.3B), although the two populations did not exhibit noticeable differences in their electrophoretic mobility in the second dimension (i.e. in the SDS-PAGE dimension) (Figure 4.3A). Following CSF-1 stimulation, only one major population of syntaxin 7 was detected; furthermore, the pI of this syntaxin 7 was higher than that of syntaxin 7 from CSF-1-deprived bone marrow-derived macrophages (Figure 4.3A). It is unclear if the syntaxin 7 population with the highest pI corresponds to syntaxin 7 isoform 3 that was identified by one-dimensional SDS-PAGE (Figure 4.3B). Furthermore, all three populations appeared to contain sub-populations of syntaxin 7 (i.e. distinct syntaxin 7 ‘spots’ among each main syntaxin 7 population) (Figure 4.3A). This suggests that syntaxin 7 is phosphorylated at multiple sites; moreover, it implies that each syntaxin 7 ‘isoform’ (i.e. isoforms 1, 2 and 3) may be phosphorylated at more than one site.

4.2.4 Bioinformatics analysis of the conserved serine residues in syntaxin 7

It is generally thought that phosphorylation sites that are important for the function(s) of a protein are likely to be conserved throughout evolution. Thus, identification of serine residues in syntaxin 7 that are conserved across different species could help guide the identification of the CSF-1-induced phosphorylation

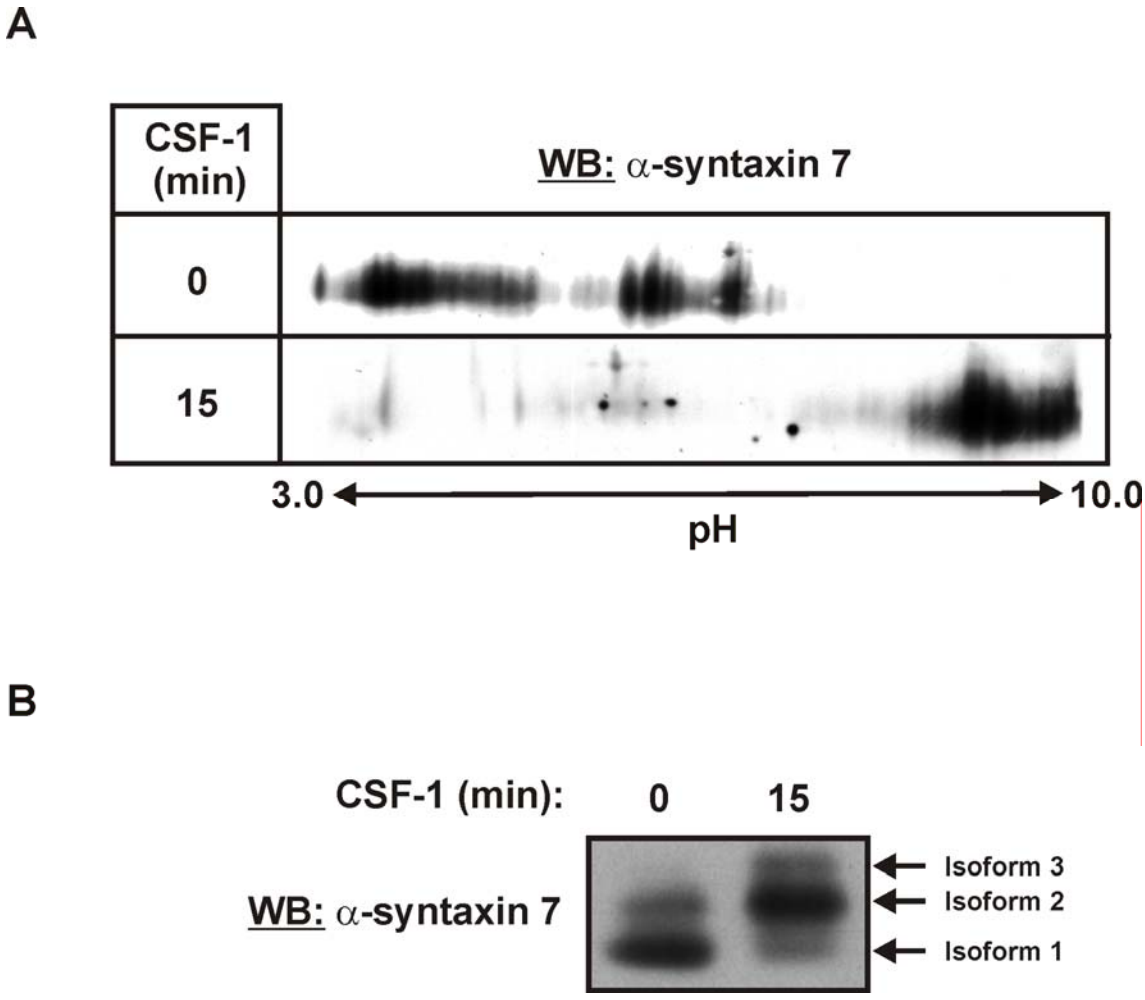


Figure 4.3: One- and two-dimensional SDS-PAGE analyses of syntaxin 7.

A, Bone marrow-derived macrophages were deprived of CSF-1 for 16 h and then stimulated with CSF-1 for 15 min or left unstimulated. Lysates of the cells were subjected to two-dimensional SDS-PAGE analysis followed by Western blotting with anti-syntaxin 7 antibodies (n = 2).

B, The same lysates from (***A***) were subjected to one-dimensional SDS-PAGE analysis followed by Western blotting with anti-syntaxin 7 antibodies (n = 2).

sites in syntaxin 7. ClustalW-based sequence alignment (www.ebi.ac.uk/clustalw/) [448] of mouse syntaxin 7 with that of human, rat and zebra fish syntaxin 7 identified ten serine residues that were evolutionarily conserved across all four species (Figure 4.4). Among the conserved serine residues, Ser-2 resides N-terminal to the Ha domain, whereas Ser-19 and Ser-20 are located in this domain. Ser-125 and Ser-126 are situated towards the C-terminal end of the Hc domain, while Ser-129 is located just outside the Hc domain. Ser-147 is in the linker region connecting the Hc domain to the SNARE motif. The three remaining serine residues (Ser-173, Ser-174 and Ser-205) reside in the SNARE motif of syntaxin 7 (Figure 4.4). In addition to these ten serine residues, which are conserved from human to zebra fish, ten other serine residues are conserved at least between mouse and human syntaxin 7 (Figure 4.4).

Crystallographic studies of neuronal and late endosomal SNARE complexes have revealed a marked similarity between the two complexes [264, 265]. Syntaxin 1A, the Qa-SNARE in the neuronal SNARE complex, is structurally similar to syntaxin 7 [264, 265]. Significantly, Ser-14 and Ser-188 of syntaxin 1A, both of which reside outside the SNARE motif, have previously been reported to be phosphorylated by CKII and DAPK, respectively [309, 310]. Given the close structural relationship between syntaxin 1A and syntaxin 7 as Qa-SNARE proteins, alignment of the amino acid sequence of syntaxin 1A with that of syntaxin 7 could also potentially identify candidate CSF-1-induced phosphorylation sites in syntaxin 7. However, the alignment revealed that Ser-14 and Ser-188 of mouse syntaxin 1A are not conserved in mouse syntaxin 7, although syntaxin 7 contained a threonine residue (Thr-161) at a position equivalent to Ser-188 in syntaxin 1A (Figure 4.5). Interestingly, two serine residues that are conserved in mouse, rat and human syntaxin 7, namely Ser-144 and Ser-173, are also conserved in mouse, rat and human syntaxin 1A (Figure 4.5). Notably, Ser-173 of syntaxin 7 lies just inside the N-terminal end of the SNARE motif and is conserved throughout evolution, whereas Ser-144 is located in the linker region between the Hc domain and SNARE motif (Figure 4.5).

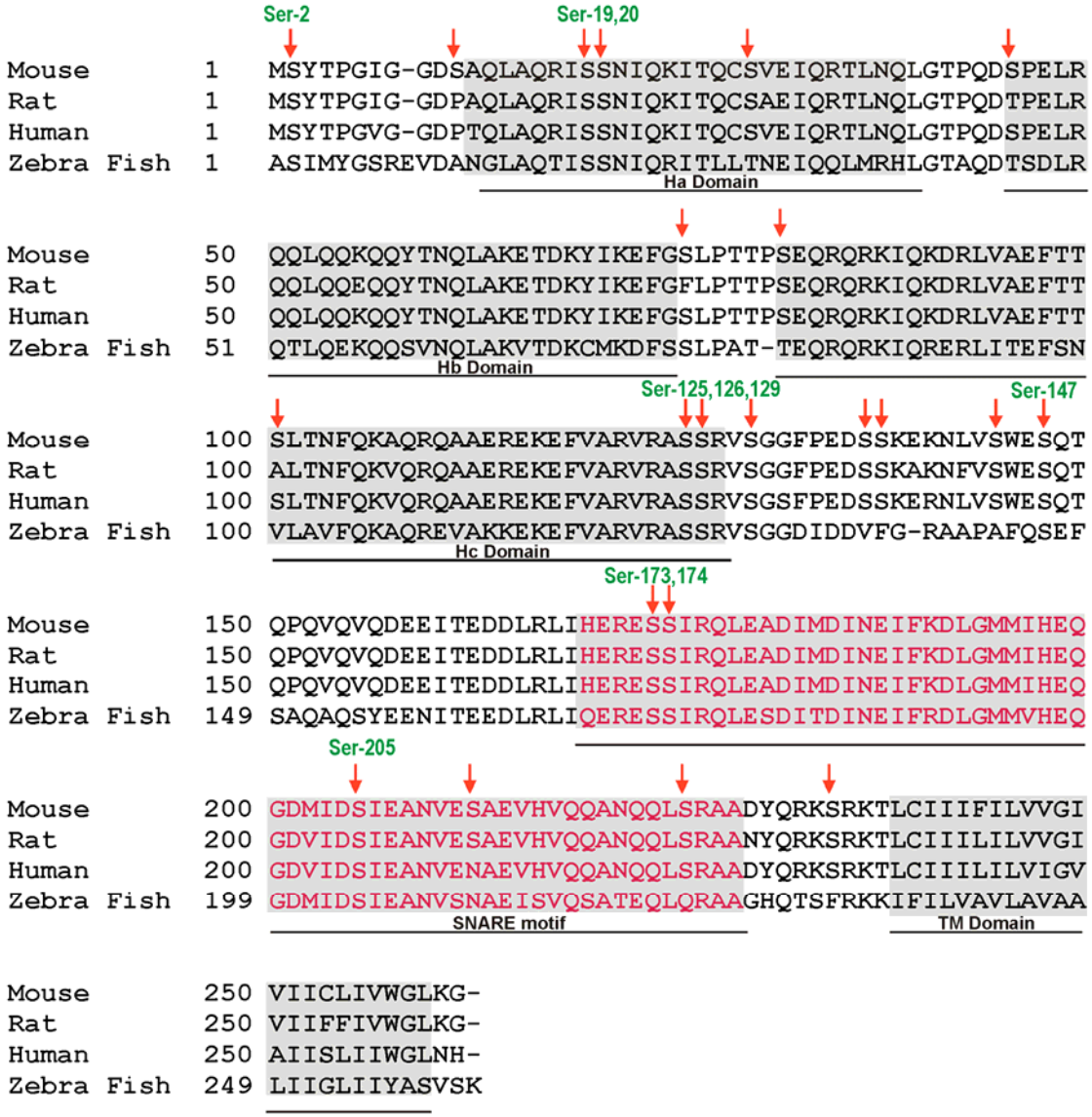


Figure 4.4: Sequence alignment of syntaxin 7 across species.

The amino acid sequences of mouse, rat, human, and zebra fish syntaxin 7 were aligned using the ClustalW algorithm. Serine residues in mouse syntaxin 7 are indicated with inverted arrows, while serine residues that are conserved across all four species are numbered. The Ha, Hb, Hc and transmembrane (TM) domains are shaded in grey, while the SNARE motif is highlighted in red.

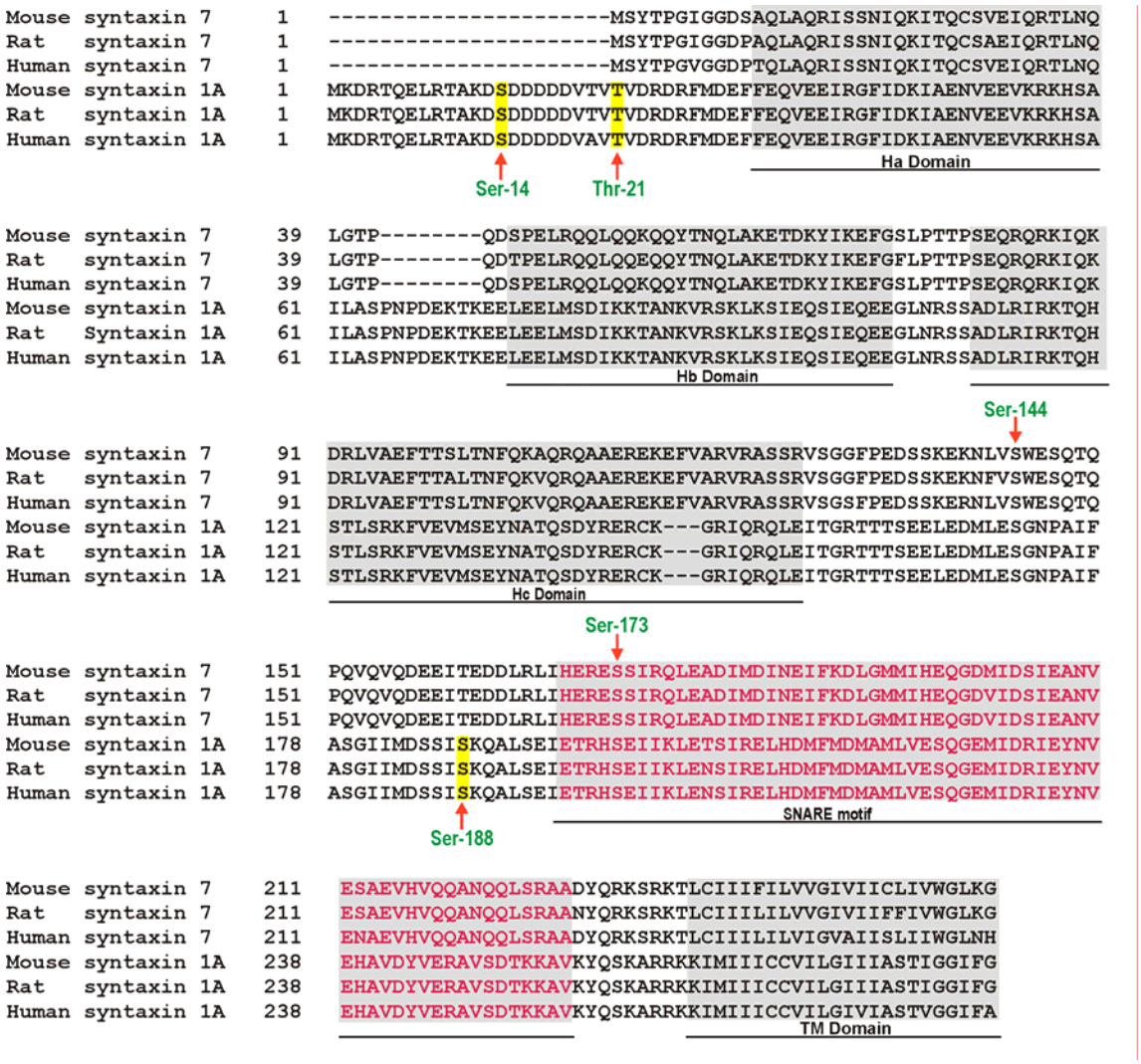


Figure 4.5: Sequence alignment of syntaxin 7 with syntaxin 1A.

The amino acid sequences of mouse, rat and human syntaxin 7 were aligned with those of mouse, rat and human syntaxin 1A using the ClustalW algorithm. Known serine phosphorylation sites of syntaxin 1A are indicated by upright arrows. Serine residues that are conserved between syntaxin 7 and syntaxin 1A are indicated with inverted arrows. The Ha, Hb, Hc and transmembrane (TM) domains are shaded in grey, while the SNARE motif is highlighted in red.

4.2.5 Bioinformatics analysis of potential phosphorylation sites in syntaxin 7

Submission of the mouse syntaxin 7 amino acid sequence to the NetPhos server (www.cbs.dtu.dk/services/NetPhos/) [449] identified a number of putative serine phosphorylation sites. The NetPhos 2.0 programme predicts phosphorylation sites in proteins by using algorithms that utilise experimentally verified phosphorylation sites in proteins from the literature as well as various databases, such as the Swiss-Prot database [449]. The output for the phosphorylation sites 'identified' is a probability score of between 0 and 1. Among the sites identified, Ser-125, Ser-126, Ser-129, Ser-173 and Ser-234 received the highest scores, and thus have the highest predicted probabilities of being phosphorylated *in vivo* (Figure 4.6A).

Another programme that can be used to predict phosphorylation sites in proteins is the NetPhosK 1.0 programme (www.cbs.dtu.dk/services/NetPhosK/). This programme predicts phosphorylation sites for specific protein kinases in proteins [450]. Although NetPhosK 1.0 searches only for phosphorylation sites in proteins for a relatively small proportion of the total number of protein kinases in the mouse and human genomes (e.g. protein kinases A, C and G, casein kinases I and II, Cdc2, Cdk5, p38 MAP kinase, Akt, and Rsk), Ser-20 and Ser-125 of syntaxin 7 were identified as likely sites of phosphorylation by PKC (Figure 4.6B). Similarly, Ser-126 was identified as a potential phosphorylation site for Akt (Figure 4.6B).

4.2.6 Activation of PKC triggers the phosphorylation of syntaxin 7

Given that PKC had the highest predicted probability to phosphorylate syntaxin 7, and that CSF-1 has been reported to trigger PKC activation in monocytes [451], bone marrow-derived macrophages were treated with the PKC activating agent, phorbol myristate acetate (PMA), to determine whether this agent also caused phosphorylation of syntaxin 7. Thus, bone marrow-derived macrophages were deprived of CSF-1 for 16 h and then stimulated with PMA for up to 60 min.

A

Residue	Score
Ser-2	0.007
Ser-11	0.011
Ser-19	0.079
Ser-20	0.416
Ser-29	0.760
Ser-45	0.956
Ser-75	0.148
Ser-81	0.196
Ser-100	0.063
Ser-125	0.990
Ser-126	0.994
Ser-129	0.996
Ser-136	0.959
Ser-137	0.978
Ser-144	0.980
Ser-147	0.030
Ser-173	0.997
Ser-174	0.981
Ser-205	0.760
Ser-212	0.451
Ser-225	0.191
Ser-234	0.997

B

Residue	Kinase	Score
Ser-11	cdc2	0.51
Ser-20	RSK	0.53
Ser-20	PKC	0.73
Ser-45	GSK3	0.50
Ser-75	DNAPK	0.50
Ser-75	PKA	0.58
Ser-125	PKC	0.90
Ser-126	RSK	0.51
Ser-126	Akt	0.72
Ser-129	cdc2	0.52
Ser-136	CKII	0.57
Ser-147	DNAPK	0.65
Ser-173	PKC	0.57
Ser-174	RSK	0.51
Ser-174	PKA	0.64
Ser-205	CKII	0.53
Ser-234	PKC	0.51

Figure 4.6: Predicted serine phosphorylation sites in syntaxin 7.

A, The amino acid sequence of mouse syntaxin 7 was analysed using the NetPhos 2.0 programme. Predicted serine phosphorylation sites in syntaxin 7 and their corresponding probability scores are shown. Serine residues with probability scores ≥ 0.990 are shown in red.

B, The amino acid sequence of mouse syntaxin 7 was analysed using the NetPhosK 1.0 programme. Predicted serine phosphorylation sites in syntaxin 7, the protein kinase predicted to phosphorylate the site, and their corresponding probability scores are shown. Serine residues with probability scores ≥ 0.700 are shown in red.

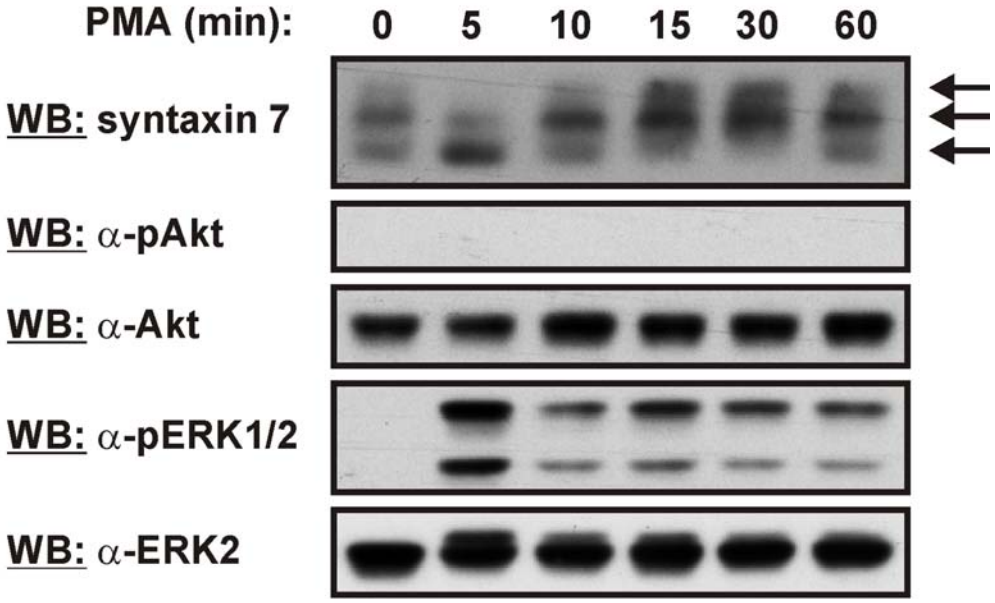
As shown in Figure 4.7A, this treatment triggered the rapid phosphorylation of syntaxin 7 as evidenced by the increased levels of isoforms 2 and 3 of syntaxin 7 and a corresponding decrease in isoform 1. The apparent decrease in syntaxin 7 phosphorylation at the 5 minute time-point varied between experiments. However, PMA reproducibly induced syntaxin 7 phosphorylation at the later time points. As expected [452], stimulation of the macrophages with PMA also triggered the activation of ERK1/2 but not that of Akt (Figure 4.7A).

Mouse bone marrow-derived macrophages express several different isoforms of PKC, including conventional isoforms (PKCα and PKCβ1), novel isoforms (PKCδ and PKCε) and an atypical isoform (PKCζ) [453]. PKCα, PKC β1, PKCδ and PKCε are sensitive to inhibition by GF109203X, whereas PKCζ activity is largely unaffected by GF109203X [453-455]. Pre-treatment of the macrophages with GF109203X appeared to partially block the PMA-induced phosphorylation of syntaxin 7 (Figure 4.7B). GF109203X also blocked the activation of ERK1/2 in response to PMA stimulation (Figure 4.7B).

4.2.7 CSF-1-induced phosphorylation of syntaxin 7 is primarily dependent on PI3-K activity

Partial inhibition of syntaxin 7 phosphorylation by the PKC inhibitor in response to PMA indicated that syntaxin 7 may be phosphorylated by other kinases that are activated upon CSF-1 stimulation. Therefore, a wide range of pharmacological inhibitors of various kinases that are activated upon CSF-1R signalling were tested. The bioinformatics analysis of syntaxin 7 identified Akt as a candidate kinase that could mediate its phosphorylation (e.g. Ser-126) in response to CSF-1 (Figure 4.6B). In fact, Ser-126 is found within a perfect consensus sequence (R-X-X-S/T-R) for phosphorylation by Akt [456]. There are three different isoforms of Akt: Akt1, Akt2 and Akt3 [457]. Although Akt1 and Akt2 are broadly expressed in most tissues and organs, Akt3 expression was highest in neuronal tissues [458-460]. Mouse bone marrow-derived macrophages expressed all three isoforms of Akt as assessed by Western blotting [458]. Inhibition of Akt activity with the Akt1 and Akt2

A


PMA (min):
0
5
10
15
30
60
WB: syntaxin 7
WB: α-pAkt
WB: α-Akt
WB: α-pERK1/2
WB: α-ERK2

B

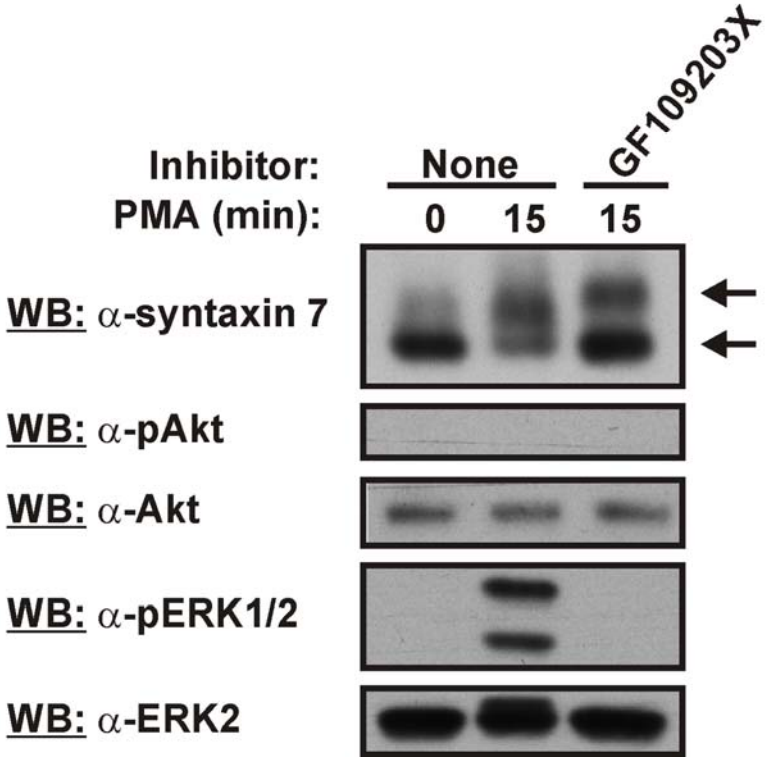

Inhibitor:
None
GF109203X
PMA (min):
0
15
15
WB: α-syntaxin 7
WB: α-pAkt
WB: α-Akt
WB: α-pERK1/2
WB: α-ERK2

Figure 4.7: PMA induces the phosphorylation of syntaxin 7.

A, Bone marrow-derived macrophages were deprived of CSF-1 for 16 h before being stimulated with 1 μM PMA for the indicated times. The cells were then lysed and aliquots of the lysates subjected to Western blotting with anti-syntaxin 7, anti-phospho Akt, anti-Akt, anti-phospho ERK1/2 and anti-ERK2 antibodies (n = 3).

B, Bone marrow-derived macrophages were deprived of CSF-1 for 16 h before being treated with either 0.1% DMSO or 5 μM GF109203X for 30 min. The macrophages were then stimulated with 1 μM PMA for 15 min or left unstimulated. The cells were lysed and aliquots of the lysates subjected to Western blotting with anti-syntaxin 7, anti-phospho Akt, anti-Akt, anti-phospho ERK1/2 and anti-ERK2 antibodies (n = 3).

specific inhibitor Akt VIII [461] impaired CSF-1-induced phosphorylation of syntaxin 7; in particular, it led to a reduction in the levels of syntaxin 7 isoforms 2 and 3 (Figure 4.8). In order to confirm the efficacy of the Akt inhibitor its ability to block CSF-1-induced phosphorylation of Akt was tested. As shown in Figure 4.8, the Akt inhibitor Akt VIII blocked the ability of CSF-1 to activate Akt.

Because Akt activation occurs in response to the activation of PI3-K [462], the ability of a PI3-K inhibitor, namely LY294002, to block CSF-1-induced phosphorylation of syntaxin 7 was also tested. LY294002 blocked the ability of CSF-1 to increase the levels of syntaxin 7 isoforms 2 and 3 (Figure 4.8). The levels of isoform 2 in macrophages that had been treated with LY294002 were lower than those in macrophages that had been deprived of CSF-1 but not treated with the PI3-K inhibitor (Figure 4.8). As expected [463], LY294002 completely blocked CSF-1-induced activation of Akt without impairing ERK1/2 activation (Figure 4.8).

PI3-K also activates the serine/threonine kinase p70 S6 kinase via the activation of PDK1 [464]. In contrast to Akt VIII, which impaired CSF-1-induced phosphorylation of syntaxin 7, rapamycin, which inhibits p70 S6 kinase, had no effect (Figure 4.8). Inhibitors of other serine/threonine kinases, such as MEK and p38 MAP kinase, also had no effect on the CSF-1-induced phosphorylation of syntaxin 7 (Figure 4.8). Blocking JNK activity increased the phosphorylation of syntaxin 7 but abrogated CSF-1-induced Akt phosphorylation (Figure 4.8). This suggested that JNK may negatively regulate the phosphorylation of syntaxin 7. Src family tyrosine kinases activate a number of serine/threonine kinases downstream of the CSF-1 receptor including ERK1/2 [465]. However, the Src family kinase inhibitor PP2 had no effect on syntaxin 7 phosphorylation (Figure 4.8).

4.2.8 Regulation of syntaxin 7 phosphorylation by PI3-K

Figure 4.8 shows that the PI3-K inhibitor (LY294002) blocked CSF-1-induced syntaxin 7 phosphorylation, whereas the PKC inhibitor (GF109203X) partially blocked it (Figure 4.7B), suggesting that there may be multiple sites of phosphorylation in syntaxin 7 that are differentially regulated by PI3-K and PKC.

This led to the investigation of the combined effects of both PI3-K and PKC inhibitors in blocking syntaxin 7 phosphorylation in response to CSF-1 stimulation. CSF-1 deprived bone marrow-derived macrophages were pre-treated with GF109203X or LY294002, or both, before being stimulated with CSF-1. As shown in Figure 4.9A, the PI3-K inhibitor blocked the CSF-1-induced phosphorylation of syntaxin 7. The PKC inhibitor, on the other hand, abolished the third and highest apparent molecular weight isoform of syntaxin 7. However, the ratio between the remaining two isoforms (i.e. isoform 1 and 2) remained constant in contrast to the syntaxin 7 isoforms from LY294002 pre-treated macrophages, where the protein levels of isoform 1 was greater than that of isoform 2 (Figure 4.9A). The combined effect of these two inhibitors on CSF-1-induced syntaxin 7 phosphorylation was comparable to that of the PI3-K inhibitor alone (Figure 4.9A), suggesting that PI3-K is the main kinase that regulates the phosphorylation of syntaxin 7 in response to CSF-1.

In agreement with the preceding inhibitor experiments, pre-treatment of bone marrow-derived macrophages with LY294002 resulted in a loss of the higher pI isoforms of syntaxin 7 when lysates of the macrophages were subjected to two-dimensional SDS-PAGE analysis (Figure 4.9B). Interestingly, pre-treatment of bone marrow-derived macrophages with the JNK inhibitor SP600125 resulted in a shift in syntaxin 7 towards higher pH (Figure 4.9B), implying a possibly enhanced phosphorylation of syntaxin 7. Moreover, this result was consistent with the anti-syntaxin 7 Western blot from SP600125 pre-treated macrophages that demonstrated the appearance of the highest molecular weight isoform of syntaxin 7 (Figure 4.8).

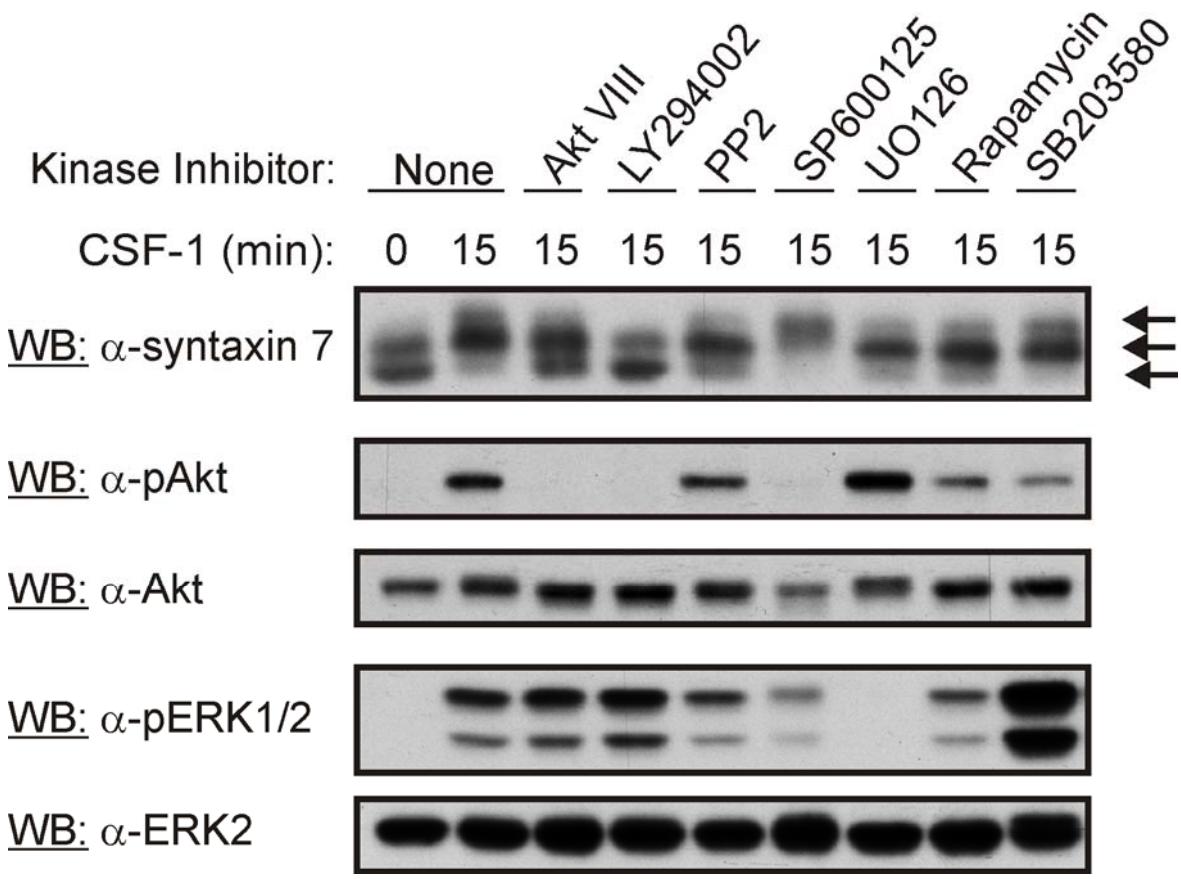
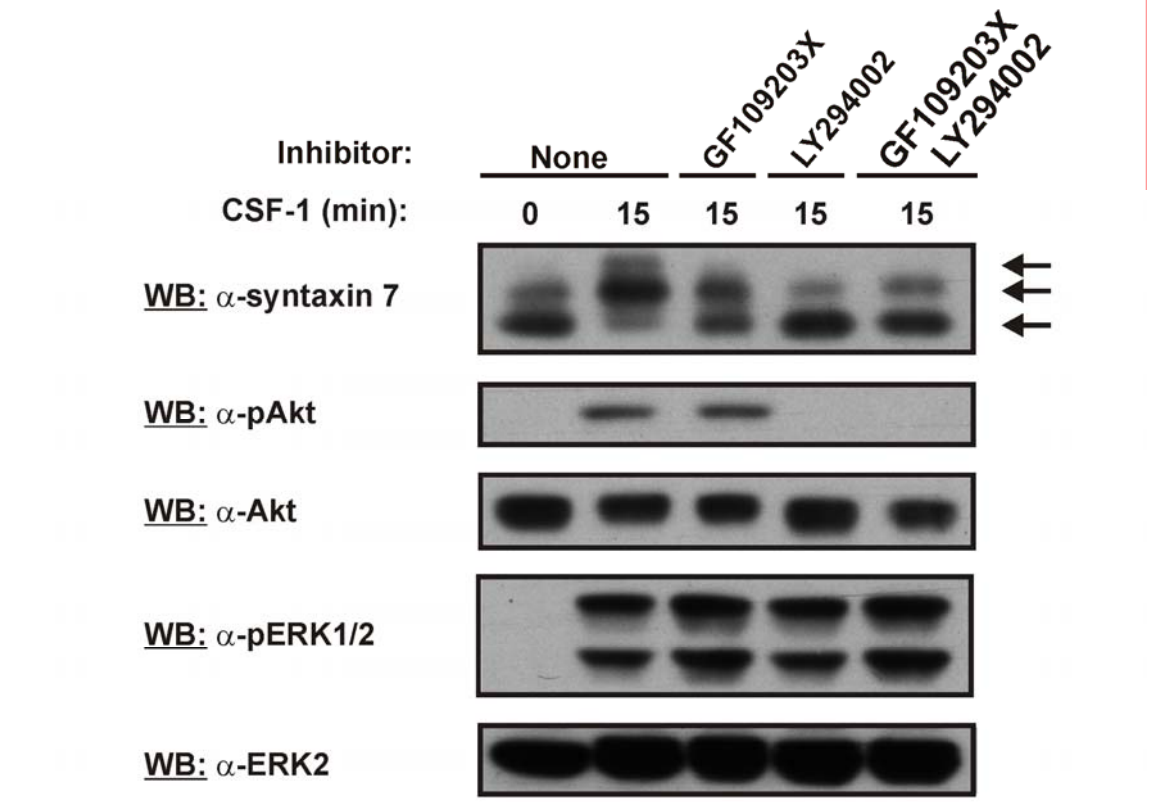


Figure 4.8: Effects of kinase inhibitors on the CSF-1-induced phosphorylation of syntaxin 7.

Bone marrow-derived macrophages were deprived of CSF-1 for 16 h before being treated with 0.1% DMSO, 5 μM Akt VIII (Akt 1/2 inhibitor), 10 μM LY294002 (PI3-K inhibitor), 20 μM PP2 (Src family kinase inhibitor), 50 μM SP600125 (JNK inhibitor), 10 μM UO126 (MEK inhibitor), 30 μM rapamycin (p70 S6K inhibitor) and 10 μM SB203580 (p38 MAPK inhibitor). The macrophages were then stimulated with CSF-1 (5,000 U/ml) for 15 min or left unstimulated. The cells were lysed and aliquots of the lysates subjected to Western blotting with anti-syntaxin 7, anti-phospho Akt, anti-Akt, anti-phospho ERK1/2 and anti-ERK2 antibodies (n = 3).

A


Inhibitor:
None
GF109203X
LY294002
GF109203X LY294002
CSF-1 (min):
0
15
15
15
15
WB: α-syntaxin 7
WB: α-pAkt
WB: α-Akt
WB: α-pERK1/2
WB: α-ERK2

B

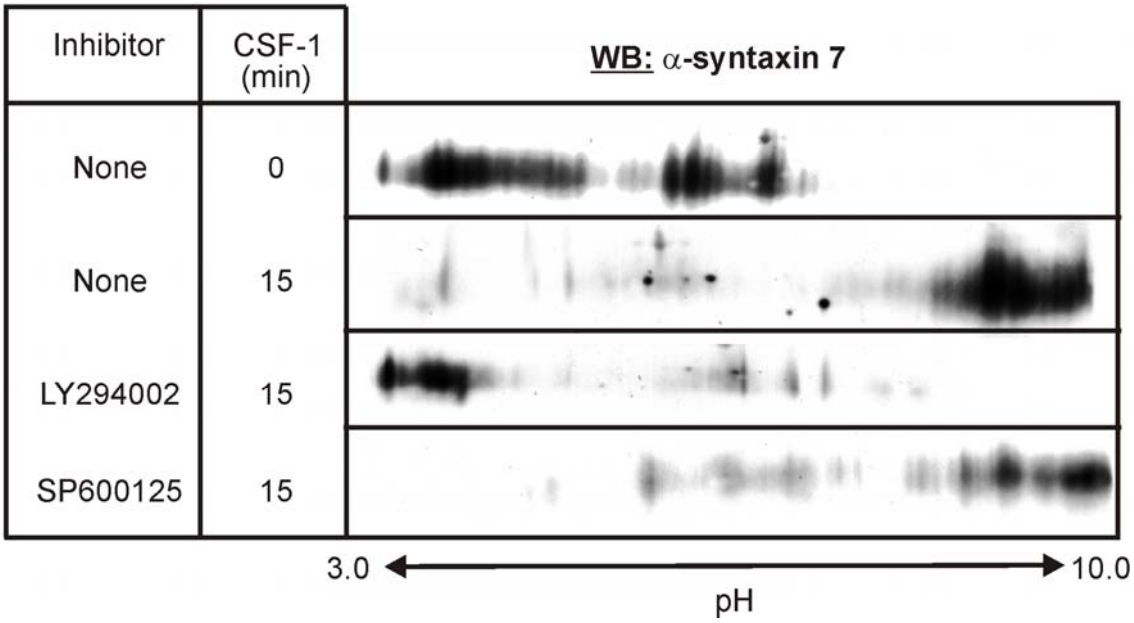

Inhibitor
CSF-1 (min)
WB: α-syntaxin 7
None
0
None
15
LY294002
15
SP600125
15
3.0
10.0
pH

Figure 4.9: Effects of PKC and PI3-K inhibitors on the CSF-1-induced phosphorylation of syntaxin 7.

A, Bone marrow-derived macrophages were deprived of CSF-1 for 16 h before being treated with 0.1% DMSO, 5 μM GF109203X, 10 μM LY294002 or both GF109203X and LY294002 together for 30 min. The macrophages were then stimulated with CSF-1 (5,000 U/ml) for 15 min or left unstimulated. The cells were lysed and aliquots of the lysates subjected to Western blotting with anti-syntaxin 7, anti-phospho Akt, anti-Akt, anti-phospho ERK1/2 and anti-ERK2 antibodies (n = 3).

B, Bone marrow-derived macrophages were deprived of CSF-1 for 16 h, treated with 0.1% DMSO, 10 μM LY294002 (PI3-K inhibitor) or 50 μM SP600125 (JNK inhibitor) for 30 min and then stimulated with CSF-1 for 15 min or left unstimulated. The cells were lysed and aliquots of the lysates subjected to two-dimensional SDS-PAGE analysis, followed by Western blotted with anti-syntaxin 7 antibodies (n = 2).

4.3 Discussion

Protein phosphorylation is a key post-translational modification that governs a multitude of biological processes. In this study, syntaxin 7 was found to undergo rapid serine phosphorylation following the stimulation of bone marrow-derived macrophages with CSF-1. This is the first member of the endosomal SNARE complex that has been demonstrated to be phosphorylated. By using pharmacological inhibitors of different kinases, we have provided evidence that the phosphorylation of syntaxin 7 is mediated by protein kinases that are activated in a PI3-K-dependent manner by CSF-1.

In addition to being phosphorylated in response to CSF-1, syntaxin 7 was also phosphorylated, albeit to a far lesser extent, in the absence of CSF-1 (Figure 4.2A). This CSF-1-independent phosphorylation of syntaxin 7 may have been induced by growth factors or other substances present in the foetal calf serum that was used to supplement the medium in which the bone marrow-derived macrophages were cultured. Alternatively, as bone marrow-derived macrophages grow as adherent cells, adhesion itself could have triggered the activation of protein kinases capable of phosphorylating syntaxin 7. Treatment of bone marrow-derived macrophages with the PI3-K inhibitor LY294002 appeared to partially inhibit the CSF-1-independent phosphorylation of syntaxin 7. Thus, suggesting the 'basal' phosphorylation of syntaxin 7, like its CSF-1-induced phosphorylation, is mediated by protein kinases downstream of PI3-K. Several prior studies have demonstrated that PI3-K is activated upon macrophage cell adhesion [466, 467].

The analysis of syntaxin 7 by one- and two-dimensional SDS-PAGE revealed that bone marrow-derived macrophages contain multiple isoforms of syntaxin, particularly following the stimulation of the macrophages with CSF-1 (Figure 4.3). The various isoforms appear to arise from the differential phosphorylation of syntaxin 7 rather than from other forms of post-translational modification (e.g. ubiquitination, sumoylation or palmitoylation) as the treatment of immunoprecipitated syntaxin 7 with calf intestinal alkaline phosphatase resulted in the disappearance of the apparent higher molecular mass isoforms of syntaxin 7 and a corresponding increase in the levels of the lower molecular mass syntaxin 7 isoform. Analysis of the phosphoamino acid contents of syntaxin 7 from CSF-1-

stimulated bone marrow-derived macrophages, as well as from macrophages that had been deprived of CSF-1, revealed that syntaxin 7 was phosphorylated exclusively on serine (Figure 4.2B).

Syntaxin 7 contains more than twenty serine residues and bioinformatics identified ten that were evolutionarily conserved between murine, human, rat and zebra fish syntaxin 7 (Figure 4.4). Further bioinformatics analysis predicted that Ser-125, Ser-126, Ser-129, Ser-173 and Ser-234 as likely sites for phosphorylation (Figure 4.6). Moreover, the analysis predicted that the phosphorylation of these sites was most likely to be mediated by Akt and PKC. Whilst pharmacological inhibitors of Akt and PKC suppressed the CSF-1-induced phosphorylation of syntaxin 7, the extent of suppression was not as great as that achieved with the PI3-K inhibitor LY294002 (Figures 4.8 and 4.9). Prior studies have demonstrated that Akt and PKC can become activated in response to the activation of PI3-K, although PKC can also be activated independently of PI3-K [468, 469]. Based on these findings, it is proposed that Akt and PKC are largely responsible for mediating the CSF-1-induced phosphorylation of syntaxin 7 in response to PI3-K activation, potentially via the PI3-K target, phosphoinositide-dependent kinase 1 [470, 471].

It is acknowledged that this conclusion is largely based on the effects of pharmacological inhibitors of PI3-K, Akt and PKC. Whilst these pharmacological agents effectively inhibit the targeted kinases, they may also affect other signalling pathways [472]. For example, the p38 MAP kinase inhibitor SB203580 potentiated the CSF-1-induced activation of ERK1/2 activation, while the JNK inhibitor SP600125 diminished its activation (Figure 4.8). Hence, additional approaches are required to more precisely define the roles of PI3-K, Akt and PKC as well as the specific isoforms involved in the phosphorylation of syntaxin 7. This could include testing the ability of recombinant forms of different Akt and PKC isoforms to phosphorylate syntaxin 7 *in vitro*. Ascertaining the effects of the siRNA-mediated silencing of PI3-K, Akt and PKC in bone marrow-derived macrophages on the ability of CSF-1 to induce the phosphorylation of syntaxin 7 would also be informative. Similarly, testing the ability of CSF-1 to induce the phosphorylation of syntaxin 7 in bone marrow-derived macrophages from knockout mice that are deficient in specific protein kinases (e.g. isoform-specific Akt and PKC knockout mice) could also help define the protein kinases that regulate and/or mediate the phosphorylation of syntaxin 7.

Despite the data above supporting roles for PI3-K, Akt and PKC in the phosphorylation of syntaxin 7, we cannot exclude the possibility that other protein kinases (e.g. CKII and DNA-dependent protein kinase), as predicted by the NetPhosK 1.0 programme, may also contribute to the phosphorylation of syntaxin 7. CKII, which phosphorylates syntaxin 1A at Ser-14 *in vivo* [310], was predicted to phosphorylate syntaxin 7 at Ser-136 and Ser-205, the former aligning with Ser-171 of syntaxin 1A. Furthermore, CKII activity has been shown to be important for Fcγ2A-receptor-mediated phagocytosis in murine peritoneal macrophages and for the secretion of pro-inflammatory cytokines, such as IL-1 and IL-6 [473, 474]. Interestingly, bone marrow-derived macrophages from mice lacking the catalytic subunit of DNA-dependent protein kinase exhibited defective production of IL-6 and IL-12 when stimulated with bacterial DNA [475]. Syntaxin 7 contains optimal consensus sites for CaMKII (R-X-X-S/T): one at the C-terminus of the Hc domain (amino acids 123 to 126; R-A-S-S), the other at the N-terminus of the SNARE motif (amino acids 171 to 174; R-E-S-S). These two sites are also conserved throughout evolution. Phosphorylation of syntaxin 7 by CaMKII would be particularly interesting as it has recently been reported to regulate the fusion between early endosomes in macrophages [476] and to be important for phagosome maturation [477]; it has also been shown to phosphorylate syntaxin 1A [311].

Our data on the PI3-K-dependent phosphorylation of syntaxin 7 may help to explain the importance of PI3-K for many vesicle trafficking and membrane fusion events. PI3-K has been shown to be a key regulator of vesicular trafficking in cells [478]. The first evidence of this came from the study of the Vps34 yeast protein during autophagy where cytoplasmic cargo is trafficked to the yeast vacuole (which is equivalent to the mammalian lysosome) for degradation. Vps34 shares sequence homology with the p110 catalytic subunit of mammalian PI3-K and exhibits similar kinase activity [479]. Deletion of the Vps34 gene in yeast results in the defective targeting of soluble proteins to the vacuole [479, 480], a process resembling vesicular transport from the *trans*-Golgi network to lysosomes in mammalian cells [481, 482]. PI3-K inhibitor studies have also revealed an important role for the kinase in the regulation of early endosome fusion by activating the early endosome GTPase Rab5 [483].

Both PI3-K and PKC play crucial roles in phagocytosis by macrophages. Activation of PI3-K has been shown to be important for the initiation of the engulfment of foreign particles by macrophages as it regulates the recruitment and fusion of recycling endosomes with the nascent phagocytic cup [132]. Mouse bone marrow-derived macrophages express the PKC isoforms α, β_1, δ, ε and ζ [453]. PKC α, β, γ, δ and ε have all been shown to localise to phagosomes during FcγR-mediated phagocytosis [484]. Notably, phagosome maturation was inhibited in RAW 264.7 macrophages over-expressing a dominant negative PKC α mutant, as defined by the impaired recruitment of Rab7 and LAMP1 to phagosomes [485]. Furthermore, latex bead-containing phagosomes that were isolated from the macrophages displayed impaired acquisition of cathepsins D and S, suggesting a role for PKC α at a later stage in phagosome maturation [485]. Activation of PKC β_2 is required for the phagocytosis of apoptotic thymocytes by alveolar and peritoneal mouse macrophages [484]. PKC ζ has been shown to be important for LPS-induced macrophage activation [486, 487]. Indeed, activation of PKC ζ was crucial for the secretion of TNF [487].

PI3-K and PKC have also been shown to be important for macropinocytosis in macrophages. CSF-1 stimulation of the BAC1.2F5 mouse macrophage cell line resulted in rapid macropinocytosis. However, pre-treatment of the cells with the PI3-K inhibitors LY294002 and wortmannin abrogated the maintenance and maturation of macropinosomes but did not inhibit the initiation of macropinosome formation [488]. Likewise, macropinocytosis in bone marrow-derived macrophages was inhibited by both LY294002 and wortmannin [489]. Pharmacological inhibitor studies in PMA-activated human monocyte-derived macrophages have also revealed important roles of PKC β and PKC δ in macropinocytosis [490].

Emerging literature indicates that the phosphorylation of SNARE proteins represents a key mechanism for controlling the assembly of SNARE complexes and hence for regulating membrane fusion events. For example, PKC ζ activity, via a PI3-K-dependent pathway, is required for the insulin-induced uptake of glucose by Glut4 transporters in rat adipocytes [491]. Furthermore, insulin stimulation resulted in the PKC ζ-induced phosphorylation of VAMP2 in Glut4 containing vesicles [442]. In neuronal cells the cytosolic Ca^{2+} concentration plays a major role in neurotransmission by activating Ca^{2+}-dependent kinases, which phosphorylate

neuronal SNARE proteins [492]. The impact of phosphorylation on SNARE protein activity is dictated by the site of phosphorylation. SNAP-25, a Q-SNARE family protein, has been shown to be phosphorylated by PKC on Thr-138 and Ser-187 [493, 494]. Thr-138 resides in the linker region that connects the two SNARE motifs of SNAP-25, while Ser-187 is located in the C-terminal SNARE motif. Although phosphorylation of Thr-138 had no impact on neuronal SNARE complex formation, phosphorylation of Ser-187 hindered the interaction of SNAP-25 with syntaxin 1A and VAMP2 [308]. CaMKII phosphorylates Ser-61 in the SNARE motif of VAMP2 but this did not affect the binding of VAMP2 to syntaxin1A in the presence of SNAP-25 [444]. The phosphorylation of SNARE proteins outside their SNARE motifs can also affect the assembly of SNARE complexes. In the steady state, the N-terminal Habc domain of syntaxin 1A, a Qa-SNARE protein like syntaxin 7, folds back and interacts with the SNARE motif to prevent syntaxin 1A from interacting with VAMP2 and SNAP-25 [304, 305]. However, phosphorylation of Ser-14 and Thr-21 results in the displacement of the Habc domain from the SNARE motif, thereby freeing the SNARE motif to facilitate neuronal SNARE complex formation [310, 311]. Phosphorylated Ser-188 also serves as a binding site for DAPK, which acts as a 'clamp' to prevent the interaction of the Habc domain of syntaxin 1A with its SNARE motif [309]. Thus, based on the work presented in this chapter, it is tempting to speculate that PI3-K, Akt and PKC may regulate the vesicle trafficking and membrane fusion events necessary for phagocytosis, macropinocytosis and cytokine secretion, at least in part, by controlling and/or mediating the phosphorylation of syntaxin 7.

In order to address the importance of phosphorylation in regulating the activity of syntaxin 7 (i.e. formation of late endosomal SNARE complexes), the sites at which it is phosphorylated must be identified first. As mentioned above, bioinformatics analysis of syntaxin 7 indicated that Ser-125, Ser-126, Ser-129, Ser-173 and Ser-234 were likely sites of phosphorylation by PKC and/or Akt. A recent large scale, mass spectrometric-based phosphorylation analysis of proteins in mouse liver identified Ser-125, Ser-126 and Ser-129 as sites of phosphorylation in syntaxin 7 [495]. This finding strengthens our contention that syntaxin 7 is likely to be phosphorylated at Ser-125, Ser-126 and/or Ser-129 in response to the stimulation of bone marrow-derived macrophages with CSF-1. Although phosphorylation of syntaxin 7 was found to be exclusive to serine residues in mouse bone marrow-derived macrophages, *Ballif et al* identified, via mass spectrometry, Thr-79 as a site

of phosphorylation site in syntaxin 7 [496]. Thus, depending on the stimulus, syntaxin 7 may be phosphorylated at different sites, which would potentially provide a mechanism for regulating its binding to different SNARE proteins and hence regulating the different immune functions of macrophages. How the phosphorylation of syntaxin 7 may regulate late endosomal SNARE complex formation is the focus of the next chapter.

EFFECTS OF CSF-1 ON THE ASSEMBLY AND SUBCELLULAR LOCALISATION OF LATE ENDOSOMAL SNARE COMPLEXES

5.1 Introduction

Studies on neuronal SNAREs have given important insights into the regulation of SNARE complexes and thus serve as a paradigm for the understanding of the assembly, function and trafficking of other SNARE complexes (e.g. the late endosomal SNARE complex) in other systems. For example, the assembly of neuronal SNARE protein complexes is regulated in part by the phosphorylation of the individual members of the complex [311, 338, 444]. In addition to interacting with other SNARE proteins, neuronal SNARE proteins have been shown to bind to a number of non-SNARE proteins, including SM (Sec1/Munc18-like) family proteins, synaptotagmins and 14-3-3 proteins [321, 369, 497]. Munc-18-1 binds to syntaxin 1A when syntaxin 1A is in its 'closed' conformation (see Figure 1.13), thereby preventing syntaxin 1A from interacting with other SNARE proteins (e.g. SNAP-25 and VAMP2) [304, 305]. Phosphorylation of syntaxin 1A is thought to displace Munc-18-1, resulting in syntaxin 1A adopting an 'open' conformation [304, 305]. In this conformation, syntaxin 1A can then interact with SNAP-25 and VAMP2 to form a functional *trans*-SNARE complex and mediate membrane fusion. Because syntaxin 1A is structurally similar to syntaxin 7, phosphorylation of syntaxin 7 may similarly regulate its interaction with other late endosomal SNARE proteins and/or with proteins that regulate the assembly of SNARE complexes.

In order to mediate their biological functions, SNARE proteins must reside at the correct subcellular localisation. Thus, establishing the localisation of SNARE proteins should provide insight into their functional roles. Prior studies have shown that syntaxin 7, syntaxin 8, Vti1b and VAMP8 are localised to late endosomes when ectopically expressed in rat kidney cells [270]. Notably though, syntaxin 7 was localised to early endosomes in alveolar macrophages [430]. Although SNARE proteins were originally thought to be organelle-specific, it has now become apparent that they are, in general, widely distributed throughout cells, but form unique SNARE complexes to mediate membrane fusion between organelles. For example, syntaxin 7 and Vti1b are part of the late endosomal SNARE complex, but are also localised to the ER-Golgi network and mediate membrane fusion with recycling endosomes by forming a unique SNARE complex with syntaxin 6 and VAMP3 to mediate cytokine secretion by activated macrophages [126, 240].

This chapter examines the role of CSF-1 in regulating the formation of late endosomal SNARE complexes. In addition, the effects of CSF-1 on the subcellular localisation of syntaxin 7, as well as that of its partner endosomal SNARE proteins, in bone marrow-derived macrophages are also investigated.

5.2 Results

5.2.1 Acute CSF-1 stimulation enhances the formation of endosomal SNARE complexes

In the preceding chapter it was demonstrated that CSF-1 induced the phosphorylation of syntaxin 7 at multiple sites. The use of pharmacological agents suggested that phosphorylation of syntaxin 7 was largely dependent on PI3-K activity and, to a lesser extent, on that of PKC. In order to determine if the CSF-1-induced phosphorylation of syntaxin 7 influenced the binding of syntaxin 7 to other late endosomal SNARE proteins, co-immunoprecipitation assays were performed. Briefly, syntaxin 7, syntaxin 8, Vti1b and VAMP8 were immunoprecipitated from cell lysates of both CSF-1-stimulated bone marrow-derived macrophages and unstimulated macrophages. The immunoprecipitates were then subjected to Western blotting with antibodies against the different endosomal SNARE proteins (Figure 5.1A). It is worth noting that the number of syntaxin 7 isoforms that were detected by Western blotting varied depending on the electrophoresis conditions (e.g. percentage of SDS-PAGE gel, electrophoresis time and voltage). In the absence of CSF-1, Vti1b, syntaxin 8 and VAMP8 were found to interact with syntaxin 7 (Figure 5.1B). However, the binding of these late endosomal SNARE proteins to syntaxin 7 was increased following CSF-1 stimulation. The binding of syntaxin 8 and VAMP8 to syntaxin 7 was increased ~5-fold by CSF-1, whereas the binding of Vti1b to syntaxin 7 was increased ~3-fold (Figure 5.1B) as determined by densitometry. Western blotting of control antibody immunoprecipitates suggested that the interactions between the late endosomal SNARE proteins were specific (Figure 5.1B).

Reciprocal immunoprecipitation assays also supported the above findings that CSF-1 enhanced the formation of endosomal SNARE complexes in bone marrow-

derived macrophages. The co-immunoprecipitation of syntaxin 7 with Vti1b was increased ~3.5-fold following CSF-1 stimulation, while the co-immunoprecipitation of syntaxin 8 and VAMP8 with Vti1b was increased ~2.5- and ~3.5-fold, respectively (Figure 5.1C). Immunoprecipitation studies with anti-syntaxin 8 antibodies also showed enhanced endosomal SNARE complex assembly in macrophages that had been stimulated with CSF-1. The binding of syntaxin 7 and Vti1b to syntaxin 8 was increased by ~2.5 and ~1.5-fold, respectively (Figure 5.1D). By contrast, co-immunoprecipitation of VAMP8 with syntaxin 8 was increased ~3.5-fold following CSF-1 stimulation of bone marrow-derived macrophages (Figure 5.1D).

Whilst the anti-VAMP8 antibody used in this study can detect VAMP8 in cell lysates by Western blotting, it does not immunoprecipitate SNARE complexes [429]. Thus, it was not possible to directly investigate the effects of CSF-1 on the binding of syntaxin 7, syntaxin 8 and Vti1b to VAMP8 in reciprocal immunoprecipitation assays using anti-VAMP8 antibodies.

5.2.2 Investigation of the effects of CSF-1 on endosomal SNARE complex formation by gel filtration chromatography

The data presented in Figure 5.1 suggested that CSF-1 enhanced the formation of endosomal SNARE complexes consisting of syntaxin 7, Vti1b, syntaxin 8 and VAMP8 in bone marrow-derived macrophages. However, the co-immunoprecipitation assays did not allow us to distinguish between the enhanced assembly of binary SNARE complexes (e.g. a syntaxin 7-Vti1b complex) and complexes that contained all four SNARE proteins. In order to investigate the potential formation of distinct SNARE complexes, cell lysates from bone marrow-derived macrophages that had been deprived of CSF-1, as well as macrophages that had been stimulated with CSF-1, were subjected to gel filtration chromatography.

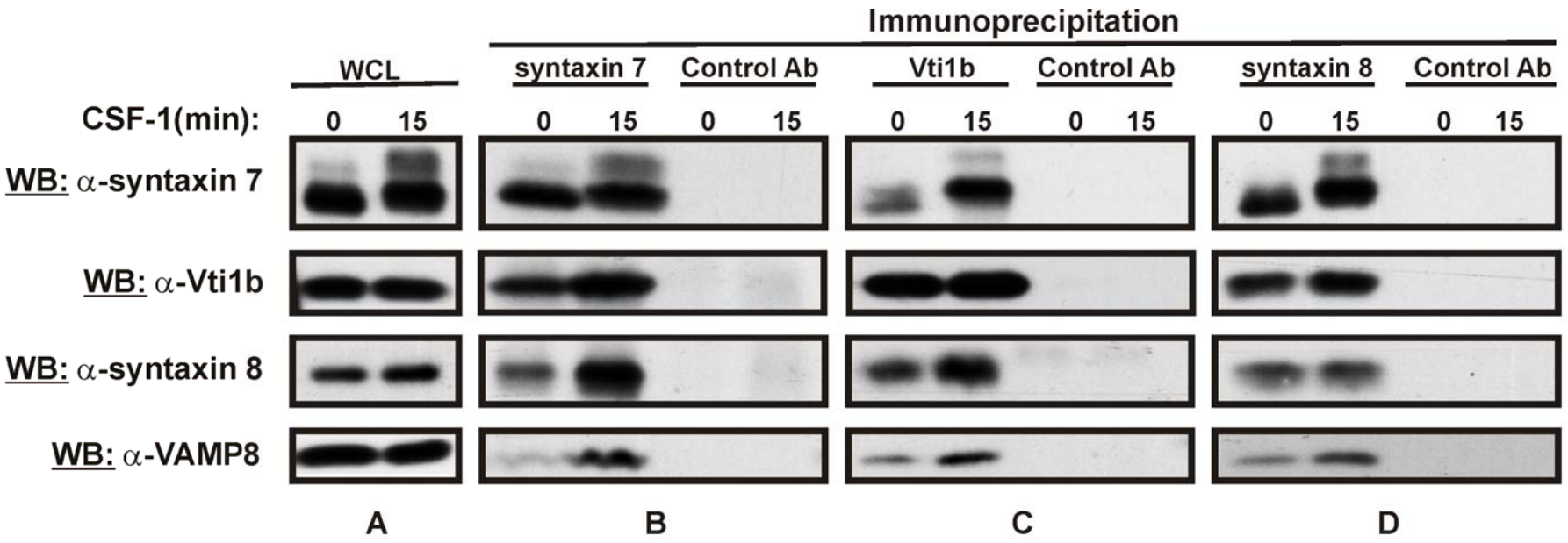


Figure 5.1: Effects of CSF-1 on late endosomal SNARE complex formation.

Bone marrow-derived macrophages were deprived of CSF-1 for 16 h before being stimulated with CSF-1 for 15 min or left unstimulated. The macrophages were then lysed and (***A***) the expression levels of syntaxin 7, Vti1b , syntaxin 8 and VAMP8 in the cell lysates measured by Western blotting. (***B***) Syntaxin 7 was immunoprecipitated from the cell lysates using anti-syntaxin 7 antibodies and the immunoprecipitates subsequently subjected to Western blotting with anti-syntaxin 7, anti-Vti1b, anti-syntaxin 8 and anti-VAMP8 antibodies. (***C***) Anti-Vti1b and (***D***) anti-syntaxin 8 immunoprecipitates were likewise subjected to Western blotting with anti-syntaxin 7, anti-Vti1b, anti-syntaxin 8 and anti-VAMP8 antibodies. The corresponding IgG control antibodies for syntaxin 7 (anti-IRAK-4 rabbit IgG polyclonal antibody), Vti1b (anti-paxillin mouse IgG_1 monoclonal antibody) and syntaxin 8 (anti-CSF-1R mouse IgG_2 monoclonal antibody) were also immunoprecipitated in (***B***), (***C***) and (***D***), respectively (n = 3).

Gel filtration chromatography fractionates proteins and complexes based on differences in size, thus potentially allowing the identification of endosomal SNARE complexes of different compositions in bone marrow-derived macrophages. Western blot analysis of fractions from the gel filtration column revealed that syntaxin 7, Vti1b, syntaxin 8 and VAMP8 from unstimulated bone marrow-derived macrophages co-eluted in a subset of fractions, namely fractions 26 to 28 (Figure 5.2A). When lysates from CSF-1-stimulated macrophages were subjected to the same analysis, a sub-fraction of all four SNARE proteins again co-eluted in fractions 26 to 28 (Figure 5.2B). The elution profile of syntaxin 7 from unstimulated bone marrow-derived macrophages exhibited a relatively uniform distribution that was centred on fraction 28 (Figure 5.2A). By contrast, CSF-1 stimulation appeared to shift slightly the elution profile of syntaxin 7 towards that of a protein/complex of a higher molecular mass (Figure 5.2B). Similarly, the elution profile of VAMP8 was shifted slightly towards that of a protein/complex of a higher molecular mass (Figure 5.2B). These apparent changes in the elution profiles of syntaxin 7 and VAMP8 are consistent with the immunoprecipitation data presented in Figure 5.1, which showed the increased immunoprecipitation of VAMP8, Vti1b and syntaxin 8 with syntaxin 7 in cell lysates of CSF-1-stimulated bone marrow-derived macrophages. Notably, fractions 22 to 24 from CSF-1-stimulated bone marrow derived macrophages, as well as from unstimulated macrophages, contained all three Q-SNARE proteins (i.e. syntaxin 7, Vti1b and syntaxin 8) but did not contain the R-SNARE VAMP8 (Figure 5.2), suggesting that all three Q-SNAREs may form a ternary complex in the absence of VAMP8. In addition, irrespective of whether the macrophages had been stimulated with CSF-1 or not, a sub-fraction of Vti1b and syntaxin 8 co-eluted in fraction 22 in the absence of syntaxin 7 (Figure 5.2). Such a finding suggests that Vti1b and syntaxin 8 may be capable of forming a constitutive binary complex in bone marrow-derived macrophages. However, it needs to be noted that as the ability of SNARE proteins in individual column fractions to co-immunoprecipitate with the other SNAREs in the same fractions was not tested, we cannot conclude that SNARE proteins which co-eluted were present in the same protein complex. Nonetheless, when combined with the earlier co-immunoprecipitation results, this analysis still provided valuable insights into the composition of endosomal SNARE complexes in bone marrow-derived macrophages.

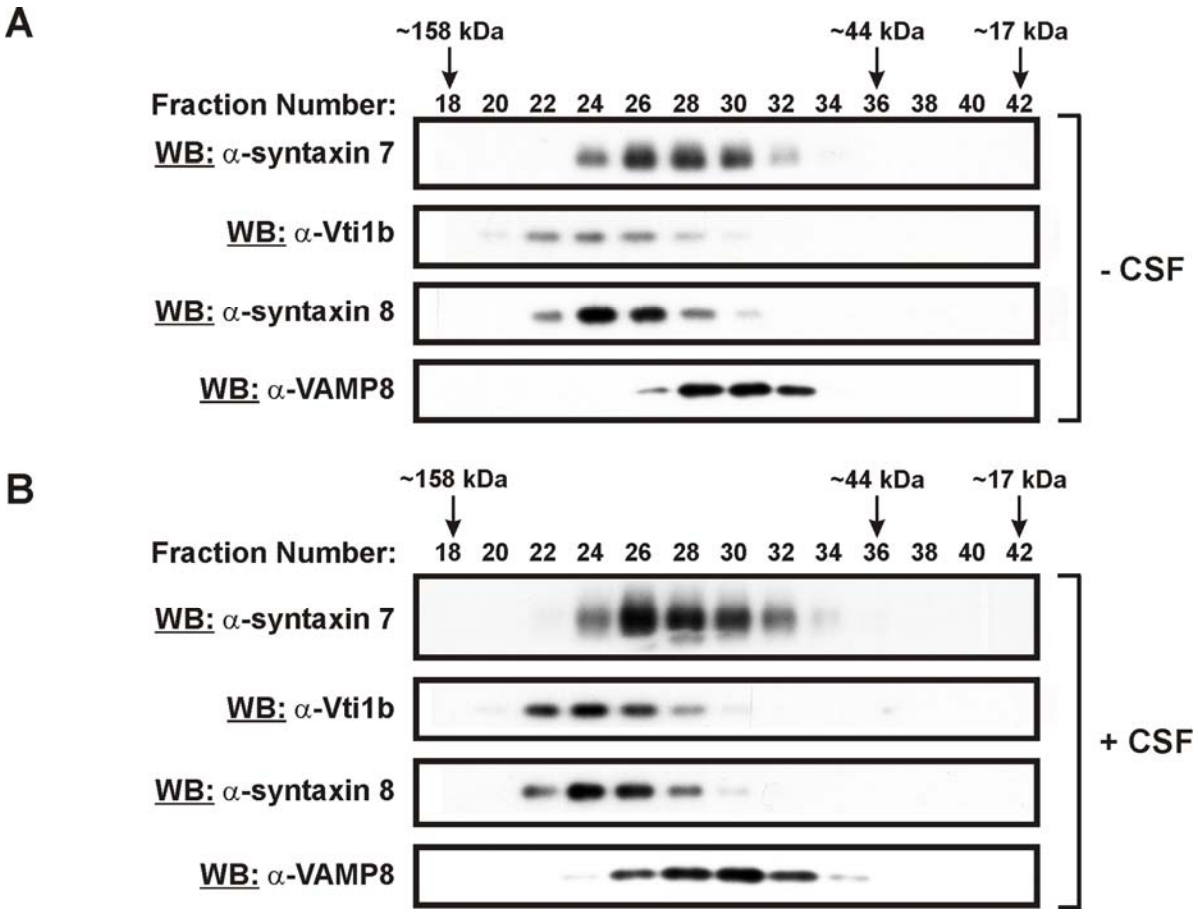


Figure 5.2: Analysis of late endosomal SNARE complexes by gel filtration chromatography.

Bone marrow-derived macrophages were deprived of CSF-1 for 16 h before being stimulated with CSF-1 for 15 min (***B***) or left unstimulated (***A***). The cells were then lysed and the cell lysates subjected to gel filtration chromatography on a Superose-6 column. Aliquots of alternate column fractions were subjected to Western blotting with anti-syntaxin 7, anti-Vti1b, anti-syntaxin 8 and anti-VAMP8 antibodies. The elution positions of column calibration standards are shown at the top.

5.2.3 Effect of a PI3-K inhibitor on late endosomal SNARE complex formation

In the preceding experiments it was demonstrated that CSF-1 enhanced the formation of late endosomal SNARE complexes in bone marrow-derived macrophages (Figures 5.1 and 5.2). Since CSF-1 induced the PI3-K-dependent phosphorylation of syntaxin 7 (Figure 4.7), it was possible that this phosphorylation of syntaxin 7 provided the biochemical basis for the enhanced assembly of endosomal SNARE complexes by CSF-1. To address this possibility, bone marrow-derived macrophages were treated with the PI3-K inhibitor LY294002 prior to CSF-1 stimulation and interactions between the late endosomal SNARE proteins were then monitored via immunoprecipitation assays. Consistent with the data presented in Figure 4.9, the CSF-1-induced hyperphosphorylation of syntaxin 7 was markedly decreased when bone marrow-derived macrophages were pre-treated with LY294002, as exhibited by the characteristic changes in electrophoretic mobility (Figure 5.3). The binding of syntaxin 7 to Vti1b was increased ~2.5-fold following CSF-1 stimulation (Figure 5.3), which was consistent with earlier data (Figure 5.1). However, when the macrophages were pre-treated with LY294002 the binding of syntaxin 7 to Vti1b was increased only ~1.2-fold in response to CSF-1 stimulation (Figure 5.3), suggesting that the PI3-K-dependent CSF-1-induced phosphorylation of syntaxin 7 enhanced its binding to Vti1b.

Similar to the case with Vti1b, CSF-1 stimulation of bone marrow-derived macrophages resulted in a ~1.5-fold increase in the binding of syntaxin 7 to syntaxin 8 (Figure 5.3); again this finding was consistent with the results presented in Figure 5.1. Notably though, not only was the ability of CSF-1 to enhance the binding of syntaxin 7 to syntaxin 8 completely inhibited by LY294002, but the levels of syntaxin 7 that co-immunoprecipitated with syntaxin 8 were lower than those that co-immunoprecipitated with syntaxin 8 from CSF-1-deprived macrophages (Figure 5.3). This data suggests that the PI3-K-dependent phosphorylation of syntaxin 7 is also important for the interaction of syntaxin 7 with syntaxin 8.

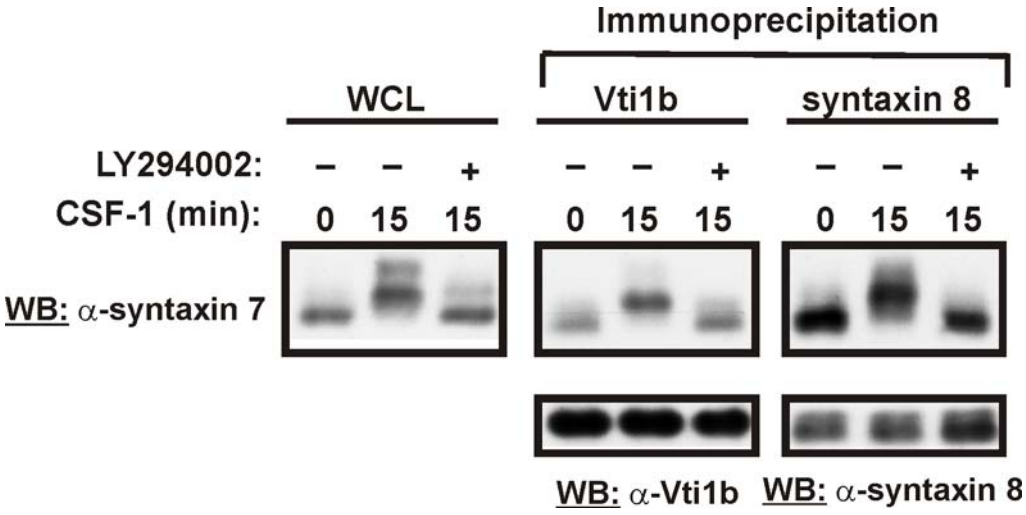


Figure 5.3: The effect of PI3-K-dependent phosphorylation of syntaxin 7 on its binding to other endosomal SNARE proteins.

Bone marrow-derived macrophages were deprived of CSF-1 for 16 h before being treated with 10 □M LY294002 (PI3-K inhibitor) or 0.1% DMSO for 30 min. The macrophages were stimulated with CSF-1 for 15 min or left unstimulated and then lysed. Vti1b and syntaxin 8 were immunoprecipitated from the cell lysates and the immunoprecipitates subjected to Western blotting with anti-syntaxin 7, anti-Vti1b and/or anti-syntaxin 8 antibodies.

5.2.4 Subcellular localisation of syntaxin 7 in macrophages

The targeting of SNARE proteins to the correct subcellular sites is required for them to carry out their specific functions. In turn, knowledge about the subcellular localisation of a particular SNARE protein may provide insight into its function. Consequently, the subcellular localisation of endogenous syntaxin 7 in bone marrow-derived macrophages was investigated by indirect immunofluorescent staining, followed by confocal microscopy. Briefly, bone marrow-derived macrophages were double stained with anti-syntaxin 7 antibodies and with either anti-early endosome antigen 1 (EEA1) or anti-lysosome associated membrane protein 1 (LAMP1) antibodies. EEA1 is typically used as a marker of early endosomes but can also be used as a marker of nascent phagosomes [498], whereas LAMP1 is used as a marker of late endosomes/lysosomes and mature phagosomes [499]. Images of the stained bone marrow-derived macrophages were captured using a Leica™ TCS SP2 confocal microscope.

The anti-syntaxin 7 antibodies stained vesicular structures that appeared to be distributed in the cytoplasmic region as well as in endosome-like elongated structures of the macrophages. Merged images of syntaxin 7 staining with the staining of EEA1 and LAMP1 revealed that syntaxin 7 largely co-localised with EEA1 in bone marrow-derived macrophages (Figure 5.4) and majority of LAMP1-stained organelles (Figure 5.5). These findings suggest that syntaxin 7 is present in all early endosomes and in a large portion of late endosomes in bone marrow-derived macrophages. The co-localisation of syntaxin 7 with EEA1 and LAMP1 in bone marrow-derived macrophages is in agreement with previous reports in which syntaxin 7 co-localised with LAMP1 in alveolar macrophages and with EEA1 and LAMP1 in rat kidney cells [270, 272, 500]. Notably though, CSF-1 did not appear to influence the subcellular localisation of syntaxin 7 in bone marrow-derived macrophages (Figure 5.4 and 5.5).

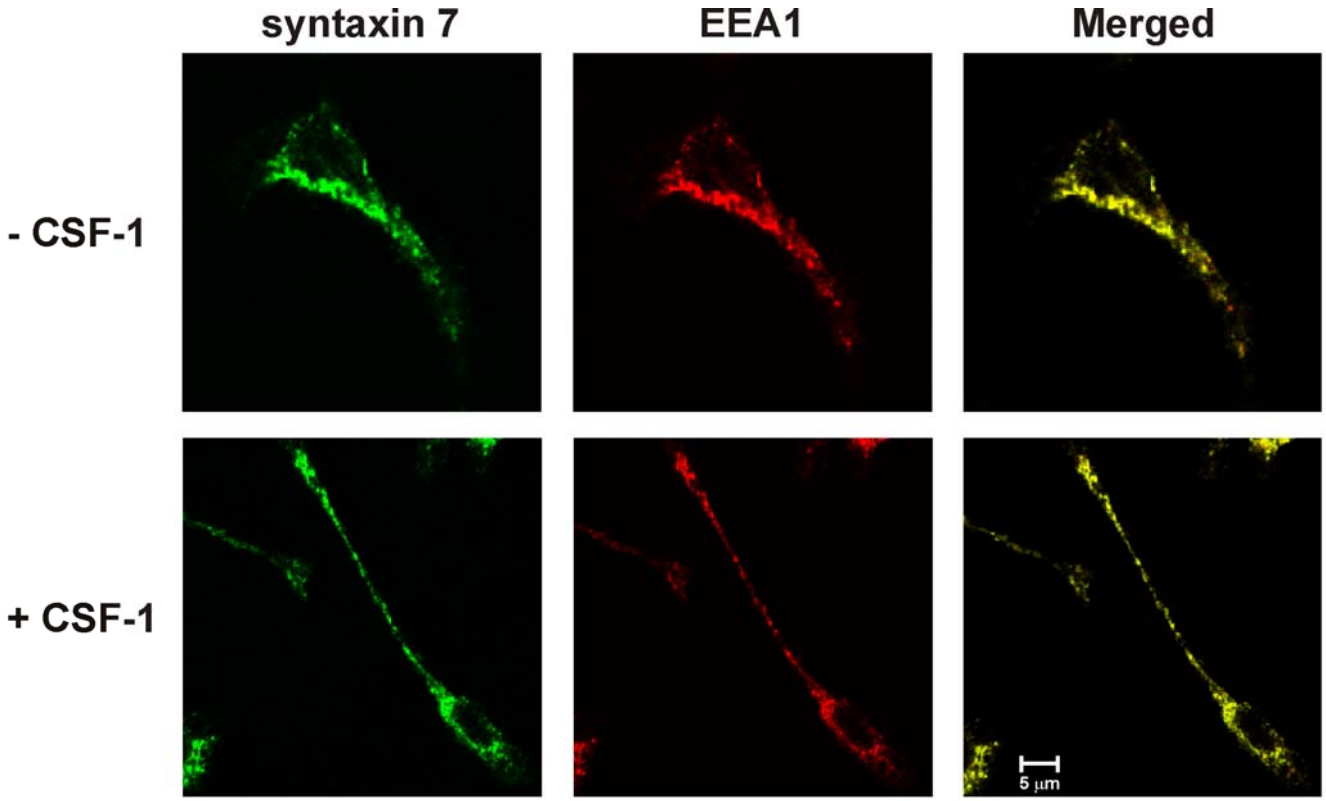


Figure 5.4: Co-localisation of syntaxin 7 with the early endosome marker early endosome antigen 1 (EEA1).

Bone marrow-derived macrophages were grown on glass coverslips. They were deprived of CSF-1 for 16 h and then either stimulated with CSF-1 for 15 min or left unstimulated. The cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton-X 100 and then stained with anti-syntaxin 7 (*green*) and anti-EEA1 (*red*) antibodies. The co-localisation of syntaxin 7 with EEA1 is shown as a *yellow* colour in the merged images. The images were acquired using a Leica™ confocal microscope in the *xy* plane.

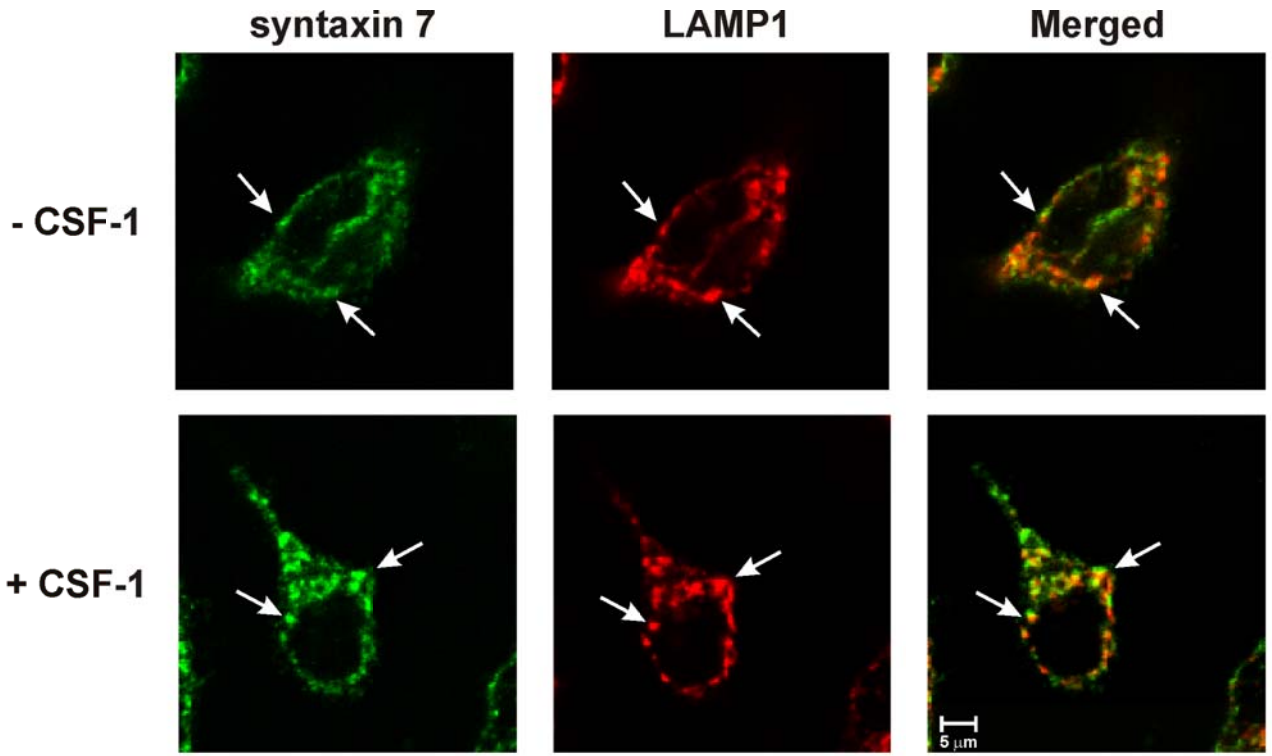


Figure 5.5: Co-localisation of syntaxin 7 with the late endosome/lysosome marker Lysosome Associated Membrane Protein 1 (LAMP1).

Bone marrow-derived macrophages were grown on glass coverslips. They were deprived of CSF-1 for 16 h and then either stimulated with CSF-1 for 15 min or left unstimulated. The cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton-X 100 and then stained with anti-syntaxin 7 (*green*) and anti-LAMP1 (*red*) antibodies. The co-localisation of syntaxin 7 with LAMP1 is shown as a *yellow* colour in the merged images. The images were acquired using a Leica™ confocal microscope in the *xy* plane. Arrows indicate co-localisation between syntaxin 7 and LAMP1.

5.2.5 The effects of CSF-1 on the co-localisation of syntaxin 7 with partner SNARE proteins.

Since CSF-1 stimulation of bone marrow-derived macrophages did not appear to regulate the subcellular localisation of syntaxin 7, it was of interest to determine if CSF-1 instead regulated the localisation of its partner late endosomal Q-SNAREs, namely Vti1b and syntaxin 8. Both Vti1b and syntaxin 8 are localised to endosome-like organelles in the cytoplasm as well as in the elongated tail structures of bone marrow-derived macrophages (Figures 5.6 and 5.7). Furthermore, both Vti1b and syntaxin 8 co-localised with syntaxin 7, although there was no obvious increase in their co-localisation following CSF-1 stimulation. However, the co-localisation of syntaxin 7 with syntaxin 8 appeared to be greater than that with Vti1b in bone-marrow derived macrophages. The Q-SNARE proteins that form a trans-SNARE complex are expected to reside in the same organelle membrane [501, 502]. Hence, it is perhaps not surprising that all three late endosomal SNARE proteins were co-localised, independently of CSF-1 stimulation.

Despite the co-localisation of Vti1b and syntaxin 8 with syntaxin 7, merged images revealed that some vesicular structures were free from staining with the anti-syntaxin 7 antibodies (Figures 5.6 and 5.7). This finding was in agreement with the gel filtration column chromatography experiments in which Vti1b and syntaxin 8 co-eluted in the absence of syntaxin 7 in some fractions (Figure 5.2), suggesting that both Vti1b and syntaxin 8 may form complexes independently of syntaxin 7 in different organelles. Indeed, Vti1b and syntaxin 8 were localised to vesicles other than endosomes, such as the *trans*-Golgi network (TGN), in rat kidney cells [425].

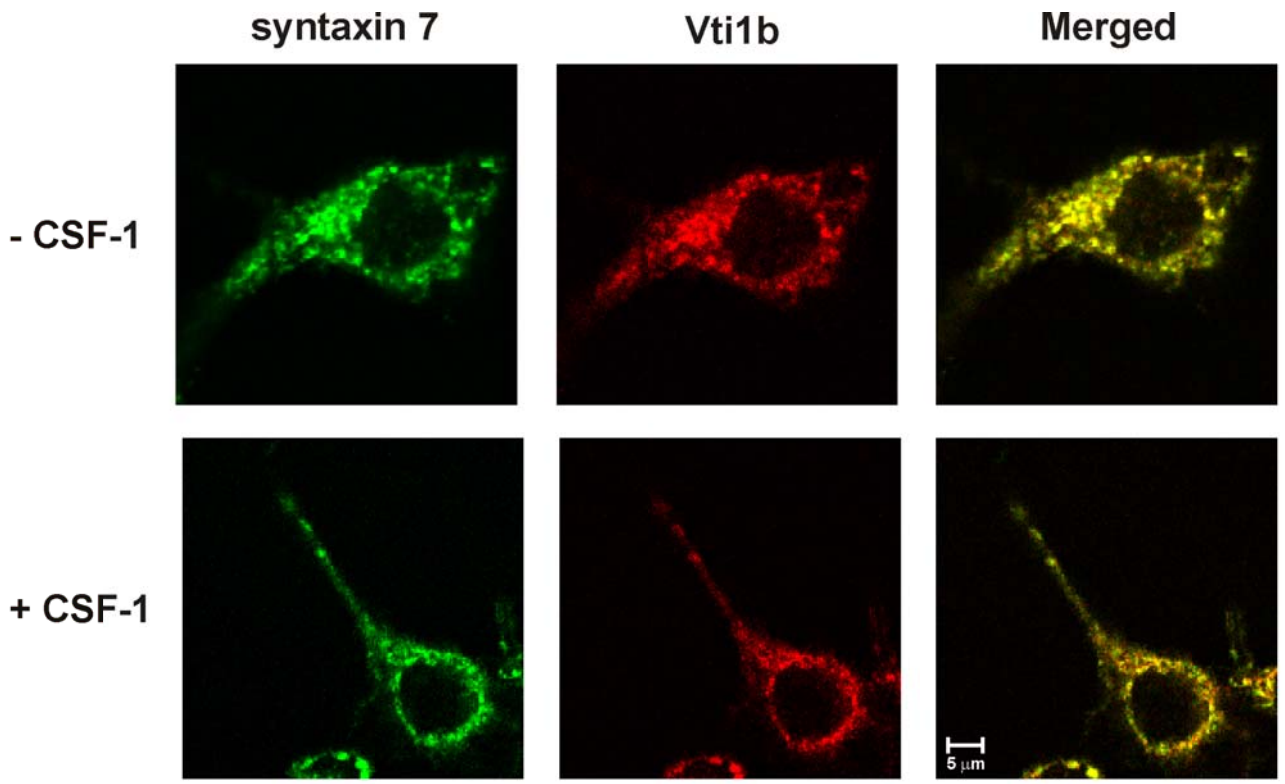


Figure 5.6: Co-localisation of syntaxin 7 with Vti1b.

Bone marrow-derived macrophages were grown on glass coverslips. They were deprived of CSF-1 for 16 h and then either stimulated with CSF-1 for 15 min or left unstimulated. The cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton-X 100 and then stained with anti-syntaxin 7 (*green*) and anti-Vti1b (*red*) antibodies. The co-localisation of syntaxin 7 with Vti1b is shown as a *yellow* colour in the merged images. The images were acquired using a Leica™ confocal microscope in the *xy* plane.

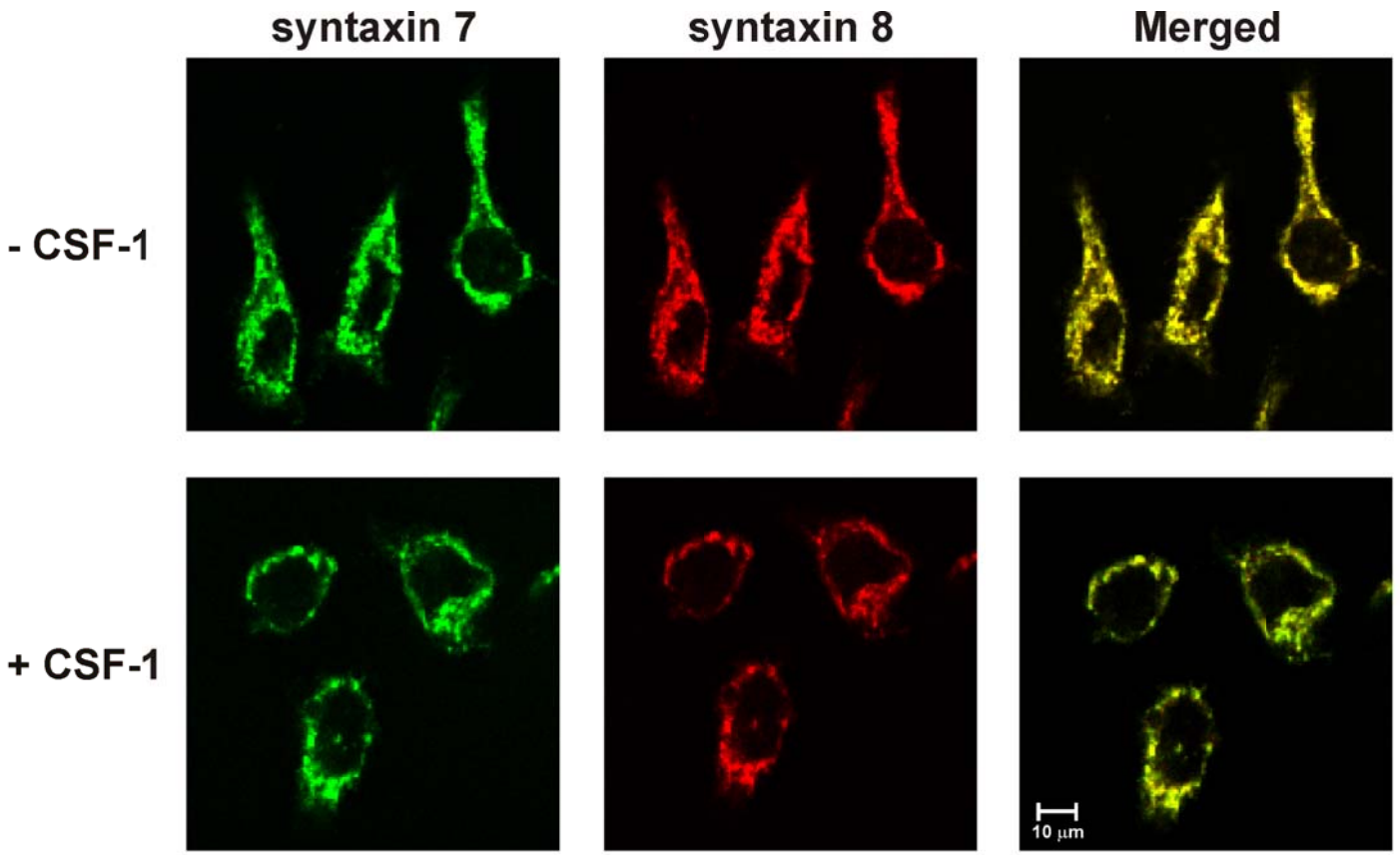


Figure 5.7: Co-localisation of syntaxin 7 with syntaxin 8.

Bone marrow-derived macrophages were grown on glass coverslips. They were deprived of CSF-1 for 16 h and then either stimulated with CSF-1 for 15 min or left unstimulated. The cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton-X 100 and then stained with anti-syntaxin 7 (*green*) and anti-syntaxin 8 (*red*) antibodies. The co-localisation of syntaxin 7 with syntaxin 8 is shown as a *yellow* colour in the merged images. The images were acquired using a Leica™ confocal microscope in the *xy* plane.

5.3 Discussion

The data presented in this chapter suggest that CSF-1 increased the formation of late endosomal SNARE complexes composed of syntaxin 7, Vti1b, syntaxin 8 and VAMP8 in bone marrow-derived macrophages. Pre-treating bone marrow-derived macrophages with the PI3-K inhibitor, LY294002, decreased the co-immunoprecipitation of syntaxin 7 with Vti1b and syntaxin 8. Given that this inhibitor also blocked the CSF-1-induced phosphorylation of syntaxin 7 (Figure 4.8), this post-translational modification to syntaxin 7 may represent an important regulatory mechanism for governing the formation of late endosomal SNARE proteins in macrophages.

Although immunoprecipitation assays are useful for detecting complex formation between proteins, they do not necessarily indicate whether all detected proteins are present in the same complex (e.g. a syntaxin 7-Vti1b-syntaxin 8-VAMP8 complex) or whether there are multiple complexes containing only some of the proteins (e.g. syntaxin 7-Vti1b and syntaxin 7-syntaxin 8 complexes, etc.). On the other hand, fractionation of cell lysates by gel filtration chromatography enables investigation of the composition of potentially distinct SNARE complexes. For example, if all four endosomal SNARE proteins were present in a single complex they would be expected to co-elute, at least, in some fractions. Alternatively, if they form distinct dimeric or trimeric complexes, then the elution profiles of the different late endosomal SNARE proteins may not overlap or only partially overlap. The elution profiles of all four endosomal SNARE proteins did not completely overlap, either in the presence or absence of CSF-1. However, there was a greater extent of overlap in cell lysates from CSF-1-stimulated bone marrow-derived macrophages (Figure 5.2), which was consistent with the increased co-immunoprecipitation of Vti1b, syntaxin 8 and VAMP8 with syntaxin 7 following CSF-1 stimulation (Figure 5.1). Gel filtration chromatography further revealed that Vti1b and syntaxin 8 largely co-eluted irrespective of whether the macrophages had been stimulated with CSF-1 or not. Co-immunoprecipitation studies also identified an interaction between Vti1b and syntaxin 8 in the absence of CSF-1 (Figure 5.1C and D), although the level of this interaction was augmented by CSF-1 stimulation. Interestingly, syntaxin 8 levels in cells from Vti1b-deficient mice are markedly reduced [503]. Thus, the binding of Vti1b to syntaxin 8 may increase the stability of syntaxin 8.

Accumulating evidence suggests that phosphorylation of SNARE proteins plays an important role in modulating interactions between SNARE proteins, as well as with proteins outside the SNARE family [311, 338, 444]. For example, phosphorylation of syntaxin 1A and SNAP-25 regulates the assembly of neuronal SNARE complexes (discussed in Chapter 1). The Habc domain of syntaxin 1A interacts with the SNARE motif, thereby hindering the interaction of syntaxin 1A with SNAP-25 and VAMP2 [312]. However, phosphorylation of syntaxin 1A leads to the dissociation of the Habc domain from the SNARE domain, thereby resulting in an increase in the affinity of syntaxin 1A for SNAP-25 and VAMP2 [309]. In agreement with this model for syntaxin 1A regulation, removal of the Habc domain enhanced the assembly of complexes between syntaxin 1A, SNAP-25 and VAMP2 *in vitro* [312]. These studies suggest that the interaction between the SNARE motif and the Habc domain negatively regulates SNARE complex assembly and, at least in neuronal cells, phosphorylation of syntaxin 1A modulates this interaction.

Like syntaxin 1A, the Habc domain in the N-terminal region of syntaxin 7 interacts with the SNARE domain to inhibit SNARE complex assembly; deletion of the Habc domain releases syntaxin 7 from this inhibitory constraint [298]. Interestingly, Vti1b and syntaxin 8 also contain Habc domains but these do not interact with their SNARE domains [299]. Hence, the regulation of SNARE complex formation by interactions between the Habc domain and the SNARE domain may be a unique attribute of Qa-SNAREs (e.g. syntaxin 1A and syntaxin 7). As discussed in the preceding chapter, the stimulation of bone marrow-derived macrophages with CSF-1 triggered the rapid phosphorylation of syntaxin 7. Thus, the enhanced binding of syntaxin 7 to Vti1b, syntaxin 8 and VAMP8 following the stimulation of bone marrow-derived macrophages with CSF-1 could potentially be explained by the PI3-K-dependent phosphorylation of syntaxin 7 inducing a conformational change in syntaxin 7 that enhances its binding to Vti1b, syntaxin 8 and VAMP8. More precisely, in its non-phosphorylated state the Habc domain of syntaxin 7 may interact with the SNARE domain such that in this ‘closed’ conformation syntaxin 7 would have a reduced capacity to bind to other SNARE proteins (Figure 5.8). However, the CSF-1-induced phosphorylation of syntaxin 7 could lead to syntaxin 7 adopting an ‘open’ conformation that allows it to more efficiently interact with Vti1b, syntaxin 8 and VAMP8 to form functional endosomal SNARE complexes (Figure 5.8). If phosphorylation does indeed regulate the conformation of syntaxin, and hence its ability to bind to other SNARE proteins, it follows that the basal

phosphorylation of syntaxin 7 in CSF-1-deprived macrophages may be sufficient to allow the assembly of low levels of endosomal SNARE complexes consisting syntaxin 7, Vti1b, syntaxin 8 and VAMP8. Thus, it is hypothesised that bone marrow-deprived macrophages may contain a mixed population of both non-phosphorylated ('closed') syntaxin 7 and phosphorylated ('open') syntaxin 7 that have different abilities to form late endosomal SNARE complexes (Figure 5.8). Further, the relative levels of the 'closed' and 'open' conformations of syntaxin 7 would be controlled by the CSF-1 receptor, via PI3-K (Figure 5.8)

The site of phosphorylation in SNARE proteins influences the regulation of SNARE complex formation: an example of this occurs in the case of SNAP-25. The phosphorylation of Ser-187 within the SNARE domain of SNAP-25 by PKC in PC12 cells reduced its binding to syntaxin 1A [308, 504]. Furthermore, mutational analysis revealed that phosphorylation of Ser-187 resulted in the inhibition of neuronal SNARE complex formation [307]. However, such a reduction in complex formation was not observed following the PKA-mediated phosphorylation of Thr-138, which is located outside the SNARE motif [494]. Since phosphorylation of syntaxin 7 correlated with its enhanced binding to Vti1b, syntaxin 8 and VAMP8, the CSF-1-induced phosphorylation sites in syntaxin 7 are likely to be located outside the SNARE motif.

Alignment of the amino acid sequences of syntaxin 7 from different species identified ten conserved serine residues. Further bioinformatics analysis predicted that Ser-173 of syntaxin 7 was a likely site of phosphorylation by PKC. Significantly, Ser-173 is located at the N-terminal end of the SNARE motif, which is connected to the Habc domain via a linker region that has been shown to be highly flexible [312]. Thus, phosphorylation of residues in this region could potentially induce conformational changes in syntaxin 7 that modulate the interaction of the Habc domain with the SNARE domain. However, syntaxin 7 was found to be phosphorylated at multiple sites in response to CSF-1 stimulation. Hence, phosphorylation of sites other than Ser-173 may also regulate the interaction of syntaxin 7 with other late endosomal SNARE proteins. Indeed, the recent discovery that syntaxin 7 is phosphorylated at Ser-125, Ser-126 and Ser-129 in mouse brain [495] raises the possibility that the phosphorylation of these sites may also contribute to the regulation of syntaxin 7 binding. Ser-125 and Ser-126 are located at the very C-terminal end of the Habc, while Ser-129 is situated just C-

terminal of the domain. Consequently, it could be hypothesised that the phosphorylation of these serine residues modulates the ability of the Habc domain to interact with the SNARE domain. Identification of the sites in syntaxin 7 that are modified, together with mutational studies, will be necessary to address this hypothesis.

The phosphorylation of SNARE proteins not only influences their interaction with other SNAREs and binding partners (e.g. Munc18-1) but can also modulate their subcellular localisation. For example, phosphorylated syntaxin 1A localised to discrete domains along a subset of axons following its phosphorylation on Ser-14 by CKII [311]. In bone marrow-derived macrophages, syntaxin 7 localised to both early and late endosomes. Previous subcellular localisation studies in epithelial cells (e.g. MDCK and NRK cells) revealed that syntaxin 7 was distributed throughout the endocytic pathway but concentrated in late endosomes and lysosomes [430]. In addition, syntaxin 8 and Vti1b were found to be largely co-localised with syntaxin 7 (Figures 5.6 and 5.7). However, vesicular structures that were free of syntaxin 7 can be explained as both Vti1b and syntaxin 8 were previously shown to localise to organelles other than late endosomes. Syntaxin 8 co-localised with the transferrin receptor (TfR) and Rab 11, which are markers for recycling endosomes, in COS-7 cells, suggesting it can function in recycling endosomes [284]. In contrast, Vti1b co-localised with β-COP, a non-clathrin coated vesicle-associated protein mediating transport between the ER and the Golgi complex, and which is also involved in intra Golgi transport in RAW 264.7 cells, where it functions in TNF trafficking [240]. Supporting previous findings, the partial co-localisation of syntaxin 7 with early and late endosomes suggests it has a functional role in membrane fusion events during early and late stages of endocytic pathways [430]. Furthermore, syntaxin 7 was reported to be recruited to mature phagosomes in RAW 264.7 macrophages [127]. Other data and the localisation data presented above suggest a possible functional role for syntaxin 7 in the early and late endocytic and/or phagocytic pathways of macrophages.

Vti1b is known to interact with a number of SNARE proteins forming various SNARE complexes in different organelles. For example, Vti1b interacts with syntaxin 7, syntaxin 8 and VAMP8 in late endosomes in bone marrow-derived macrophages and B16 melanoma cells [429], while in RAW 264.7 cells Vti1b forms

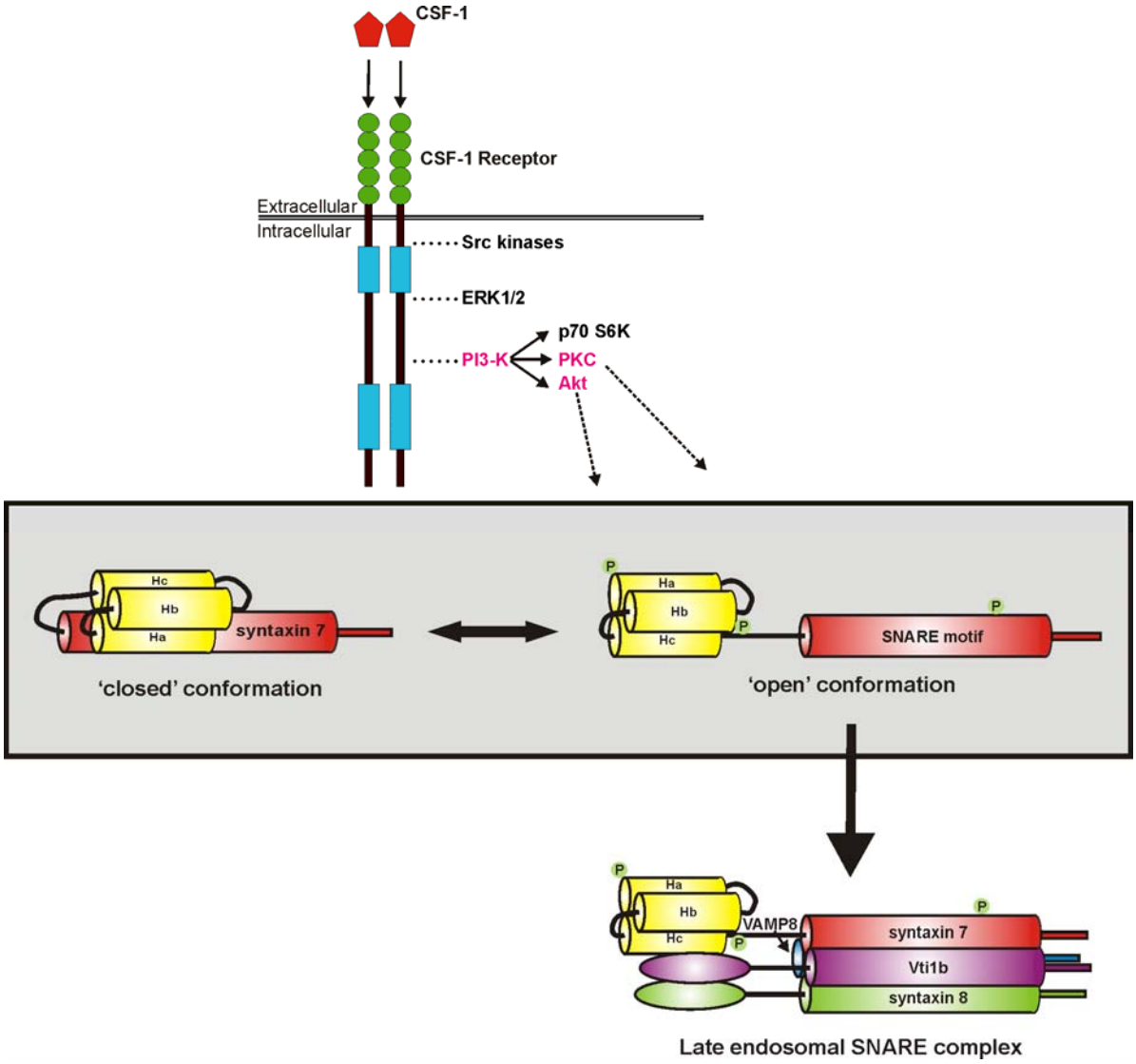


Figure 5.8: Proposed model for the regulation of late endosomal SNARE complex assembly by CSF-1.

In the absence of CSF-1, bone marrow-derived macrophages contain predominantly syntaxin 7 that is in a 'closed' conformation by virtue of the Habc domain interacting with the SNARE domain. A small sub-population of syntaxin 7 exists in an 'open' conformation due to its basal phosphorylation. Thus even in the absence of CSF-1, bone marrow-derived macrophages contain low levels of late endosomal SNARE complexes. In response to CSF-1, kinases including PI3-K, Akt and PKC are activated and they induce further phosphorylation of syntaxin 7, resulting in a greater proportion of syntaxin 7 adopting an 'open' conformation. This in turns leads to the enhanced interaction of syntaxin 7 with its SNARE binding partners Vti1b, syntaxin 8 and VAMP8, and hence to increased levels of late endosomal SNARE complexes.

a complex with syntaxin 7, syntaxin 6 and VAMP3 in the *trans*-Golgi network [126, 240, 425, 429]. Further, syntaxin 8 is not confined exclusively to endosomes; it has been found in TGN membranes, endosomes and numerous tubular vesicles dispersed thorough out the cytoplasm in COS-7 cells [272]. The promiscuity of Vti1b and the differential localisation of syntaxin 8 may therefore explain their partial co-localisation with syntaxin 7. In addition, the partial localisation of syntaxin 7 with syntaxin 8 and Vti1b suggests that syntaxin 7 and other endosomal SNARE proteins are not exclusive to endocytic/phagocytic pathways but may also be involved in other trafficking pathways.

Phosphorylation of syntaxin 7 did not appear to result in a change in its subcellular localisation nor in that of its interacting SNARE partners, Vti1b and syntaxin 8, in bone marrow-derived macrophages. However, co-immunoprecipitation studies revealed the increased binding of syntaxin 7 to Vti1b and syntaxin 8 in response to CSF-1. The CSF-1-induced phosphorylation of syntaxin 7 peaks approximately 15 min post stimulation; thus, stimulation time periods beyond 15 min were not investigated. It is therefore possible that a change in syntaxin 7 localisation may occur at a later time point following CSF-1 stimulation. However, instead of regulating the 'gross' subcellular localisation of syntaxin 7, CSF-1 may instead govern complex formation between syntaxin 7 and co-localised Vti1b, syntaxin 8 and VAMP8. Other techniques, such as intramolecular fluorescence resonance energy transfer (FRET), have been used in a number of studies to quantify molecular dynamics between SNARE proteins. For example, FRET was utilised to examine the formation of neuronal SNARE complexes, where syntaxin 1A and VAMP2 were labelled separately with fluorescent 'donor' and 'acceptor' probes and changes in their interactions in response to Ca^{2+} measured [505, 506]. A similar approach could be used to assess the effect of CSF-1 on the interaction of syntaxin 7 with its partner SNAREs in intact bone marrow-derived macrophages.

In summary, the CSF-1-induced phosphorylation of syntaxin 7 appeared to enhance its interaction with Vti1b and syntaxin 8 in bone marrow-derived macrophages. However, acute CSF-1 stimulation did not lead to any overt changes in the subcellular localisation of syntaxin 7 nor on its co-localisation with Vti1b or syntaxin 8. How future studies which address further the biological significance of the CSF-1-induced phosphorylation of syntaxin 7 will be discussed in the next chapter.

GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 Discussion

6.1.1 Regulation of SNARE protein expression

Whilst CSF-1 is known to play a crucial role in the development of macrophages from haematopoietic progenitor cells, its role in modulating the immune functions of mature macrophages (e.g. cytokine secretion, phagocytosis and antigen-presentation) has been largely underappreciated [177, 182, 184, 507]. The immune functions of macrophages involve a number of vesicle trafficking and membrane fusion events, which are common but integral requirements for macrophages to perform their immune functions. The hypothesis tested in this thesis was that CSF-1 modulates the immune functions of macrophages by regulating the expression and/or activity of proteins (e.g. SNARE proteins) involved in exocytosis and endocytosis via its ability to control the activity of various kinases, including ERK1/2 and PI3-K, and transcription factors (e.g. Ets family transcription factors).

The expression of the endosomal SNARE proteins syntaxin 7, Vti1b and VAMP8 in bone marrow-derived macrophages was found to be up-regulated by CSF-1. It has previously been shown that Vti1b, syntaxin 6 and VAMP3 expression is increased in LPS-activated RAW 264.7 macrophages, and that the increase in their expression resulted in enhanced TNF secretion [126, 240]. Moreover, when RAW 264.7 macrophages were pre-treated with IFNγ, which enhances TNF release upon macrophage activation, the expression levels of specific SNARE proteins (e.g. Vti1b, syntaxin 6 and VAMP3) were not only increased, but the kinetics of their LPS-induced expression were also accelerated. IFNγ also enhances the secretion of other proinflammatory cytokines (e.g. IL-6) by LPS-activated macrophages [126, 240, 341]. The release of TNF, IL-1α and IL-12 by activated macrophages stimulates NK cells to secrete IFNγ in a positive feedback loop to propagate Th1 responses [508]. In contrast, the secretion of IL-10 by macrophages counter-regulates these responses via its immunosuppressive effects on macrophages and Th1 cell differentiation [508]. The effects, if any, of IL-10 on the expression of the SNARE proteins that mediate the trafficking and release of proinflammatory cytokines by macrophages (e.g. Vti1b, syntaxin 4 and VAMP3) have not been reported. Nonetheless, the data presented in this thesis, together with the recent work of others, suggests that the immunomodulatory effects of growth factors (e.g. CSF-1) and cytokines (e.g. IFNγ) on macrophages are likely to be mediated in part

via their ability to influence the expression of specific SNARE proteins and/or the assembly of distinct SNARE complexes.

6.1.2 Regulation of syntaxin 7 function via phosphorylation

It is generally assumed that the assembly of SNARE complexes must be tightly regulated in order for endocytic (e.g. phagocytosis) and exocytic (e.g. cytokine secretion) processes to occur in a coordinated manner. The assembly of the neuronal SNARE complex is governed by both intrinsic factors (e.g. the conformations of the SNARE proteins) and extrinsic factors (e.g. regulatory proteins; e.g. Munc 18-1). Protein phosphorylation is central to these regulatory factors [305, 309, 509]. Metabolic labelling experiments together with phosphoamino acid analysis and one- and two-dimensional SDS-PAGE analysis revealed that multiple serine residues in syntaxin 7 are phosphorylated in response to CSF-1 (Figure 4. 3). Identification of these phosphorylation sites will be crucial in determining the biological significance of phosphorylation with regards to syntaxin 7 function. Syntaxin 7 contains twenty two serine residues, ten of which are conserved in mouse, rat, human and zebra fish syntaxin 7 (Figure 4.4). The NetPhos programme predicted that Ser-125, Ser-126, Ser-129 and Ser-234 had the highest probabilities of being phosphorylated (Figure 4.6). Interestingly, a recent study identified Ser-125, Ser-126 and Ser-129 as phosphorylation sites in syntaxin 7 [495]. Although phosphorylation of syntaxin 7 in mouse bone marrow-derived macrophages was found to be exclusive to serine residues (Figure 4.2B), *Ballif et al* identified Thr-79 as a phosphorylation site in syntaxin 7 from mouse brain [496]. These studies suggest that different stimuli, in different cell types, may result in the phosphorylation of syntaxin 7 at different sites. Furthermore, the phosphorylation of syntaxin 7 at different sites may result in the formation of different SNARE complexes. For example, phosphorylation at one site may result in syntaxin 7 forming a complex with Vti1b, syntaxin 8 and VAMP8/VAMP7 to mediate the trafficking of endocytosed/phagocytosed particles to lysosomes, while phosphorylation at other site/s may favour syntaxin 7 forming a complex with Vti1b, syntaxin 6 and VAMP3 to mediate the exocytosis of cytokines (e.g. TNF) by macrophages.

6.1.3 The roles of PI3-K, Akt and PKC in macrophage functions

The CSF-1-induced phosphorylation of syntaxin 7 in bone marrow-derived macrophages was found to be dependent on PI3-K activity (Figure 4.). Furthermore, syntaxin 7 phosphorylation was largely mediated and/or regulated by Akt and PKC, protein kinases which function downstream of PI3-K [470]. PI3-K has been shown to regulate membrane trafficking during phagocytosis and macropinocytosis in macrophages, at least in part by regulating actin polymerisation [162]. Whilst inhibition of PI3-K activity with the pharmacological agents wortmannin and LY294002 did not block the initiation of phagocytosis, it inhibited the closure of the phagocytic cup [510]. PI3-K activity is also important for the fusion of intracellular exocytic vesicles with the membranes of newly forming phagosomes [478]. Furthermore, blocking PI3-K activity with wortmannin hindered the fusion of late endosomes with lysosomes [234], a process in which syntaxin 7 mediates membrane fusion by forming a complex with Vti1b, syntaxin 8 and VAMP7 [296]. In addition to PI3-K, its downstream kinases Akt and PKC are also known to regulate phagocytosis in macrophages. For example, the clustering of FcγRs resulted in phosphorylation of Akt in bone marrow-derived macrophages [204], while bone marrow-derived macrophages from transgenic mice expressing a constitutively active Akt mutant (i.e. myristoylated Akt) showed enhanced FcγR-mediated phagocytosis when compared to macrophages from wild-type control mice [511]. RAW 264.7 macrophages over-expressing a dominant negative PKCα mutant displayed impaired acquisition of the lysosomal enzymes cathepsins D and S, suggesting that PKCα plays a role during the later stages of phagosome maturation [485]. PKCβ_2 has been shown to be necessary for the trafficking of influenza virus to lysosomes in HeLa cells [512]. In cells lacking PKCβ_2 activity, the virus accumulated in late endosomes, whilst recycling of the transferrin receptor to the plasma membrane was not affected [512], consequently PKCβ_2 may play a specific role in the late endosome/lysosome trafficking step.

In addition to their roles in phagocytosis, PI3-K, Akt and PKC have been implicated in regulating cytokine secretion by macrophages. Blocking PI3-K activity with LY294002 reduced TNF and IL-6 release by bone marrow-derived macrophages infected with *Mycobacterium smegmatis* [513]. Furthermore, inhibition of PI3-K activity by LY294002 inhibited the release of the chemokine CCL2 by IL-18-

activated mouse peritoneal macrophages [514]. Blockade of PI3-K activity in IL-18-activated mouse peritoneal macrophages inhibited phosphorylation of p65 subunit of NF-κB that is required for maximal transcriptional activity of NF-κB [514]. Akt has been reported to enhance the nuclear translocation of NF-κB through phosphorylation and activation of IκB kinase in RAW 264.7 macrophages [515]. Furthermore, NF-κB activation, via PI3-K and PKC signalling pathways, up-regulated TNF gene expression in gp350-stimulated human peripheral blood monocyte-derived macrophages [516]. In addition, anti-sense oligonucleotides against PKCδ increased IL-12 secretion, depletion of PKCε decreased IL-12 secretion, while PKCα had no effect in IL-12 secretion in LPS-activated mouse peritoneal macrophages [517]. Given the membrane trafficking roles of PI3-K, Akt and PKC, these kinases may not control cytokine secretion by solely controlling cytokine expression but the proteins (e.g. SNARE proteins) that mediate cytokine secretion. Based on the results presented in this thesis, we hypothesise that the phosphorylation of syntaxin 7 by Akt and PKC governs the interaction of syntaxin 7 with its partner late endosomal SNARE proteins. Thus, the regulation of membrane fusion during phagocytosis and cytokine secretion by PI3-K in macrophages may relate to its regulation of syntaxin 7 phosphorylation by Akt and PKC.

6.1.4 Binding partners of syntaxin 7

SNARE-binding proteins, such as Munc18-1, complexin and synaptotagmin, are important regulators of the assembly of neuronal SNARE complexes and hence of membrane fusion in neurons [319, 509]. Munc18-1 binds to syntaxin 1A when it is in a 'closed' conformation and thereby prevents SNARE complex assembly, while complexin prevents the final membrane fusion step (Figure 1.12). Synaptotagmin competes with complexin and displaces it from the SNARE complex upon an increase in intracellular Ca^{2+} in order to promote the final membrane fusion step [257]. Thus, in addition to regulating the expression of syntaxin 7, CSF-1 may also govern the interaction of syntaxin 7 with SNARE-binding proteins via the phosphorylation of syntaxin 7 and/or by modulating the activities of SNARE-binding proteins. Few studies have investigated syntaxin 7 binding regulatory proteins; however, a yeast-two-hybrid study by Kim and colleagues showed an association between syntaxin 7 and human vacuolar protein sorting 11 (hVPS11) and hVPS18 proteins [499]. Although little is known about the human homologues of VPS

proteins, yeast VPS11 and VPS18 have been shown to mediate transport from endosomes to vacuoles (equivalent to mammalian lysosomes) [518]. It would therefore be worthwhile investigating if CSF-1 may regulate interactions between syntaxin 7 and hVPS proteins in macrophages.

The enhanced assembly of late endosomal SNARE complexes by CSF-1 may also be a consequence of changes in the trafficking of newly synthesised SNARE proteins to the appropriate locations. For example, adaptor protein complex proteins (e.g. APC-1, APC-2 and APC-3) play important roles in the sorting of proteins to various organelles (e.g. late endosome and the *trans*-Golgi network) by binding to 'sorting motifs' in cargo proteins [519]. Di-leucine-based sorting motifs in endosomal/lysosomal-targeted proteins are typically composed of an acidic residue (e.g. aspartic acid or glutamic acid) followed by a pair of leucine residues. APC-1 is involved in protein transport from the TGN; by contrast, APC-2 mediates the formation of clathrin-coated vesicles at the plasma membrane and is involved in endocytosis, whereas APC-3 sorts proteins to late endosomes [520]. APC-1 has been shown to interact with VAMP4; the cytoplasmic domain of VAMP4 contains a di-leucine motif that may serve as a sorting signal for APC-1 [294]. The interaction of VAMP4 with APC-1 was increased following the phosphorylation of VAMP4 (at Ser-30) by CKII and was found to be important for the intracellular trafficking of VAMP4 to the TGN [335]. Many SNARE proteins, including syntaxin 5, 6, 7 and 8, that localise to the TGN or to endosomes have putative di-leucine-based motifs in their cytoplasmic domains [336]. Mouse syntaxin 7 contains a putative di-leucine-based motif in its cytoplasmic domain (amino acids 162-168; Glu-Asp-Asp-Leu-Arg-Leu-Iso) [333] and this motif is conserved across human, rat and zebra fish syntaxin 7 (Figure 4.5). Notably, APC-3, which sorts proteins to late endosomes, was identified as a CSF-1-induced gene in the microarray experiment described in this thesis (Table 3.1). Thus, CSF-1 may up-regulate the expression of proteins, such as APC-3, that are required for the targeting of SNARE proteins (e.g. syntaxin 7) to late endosomes/lysosomes in order to promote SNARE complex assembly. Future studies investigating potential interactions between syntaxin 7 and APC-3 may shed light on how syntaxin 7 is targeted to the correct organelle(s) in order to mediate the immune functions of macrophages.

6.1.5 Inhibition of SNARE functions by bacterial pathogens

A number of bacterial pathogens have evolved strategies to survive in macrophages, one of which is the secretion of toxins and proteins that interfere with phagocytic pathways. *M.tuberculosis* produces a variety of lipids including mannose-capped lipoarabinomannan (ManLAM) that target the phagosome maturation process [362]. EEA1 is required for phagosome maturation and is recruited to the phagosome in a PI3-K-dependent manner [521]. Notably though, the recruitment of EEA1 to phagocytosed ManLAM-coated latex beads was reduced in comparison to control beads. In addition, ManLAM reduced the acidification of phagosomes, which is necessary for the efficient lysosomal degradation of pathogens [521].

Some intracellular pathogens also secrete phosphatases in order to evade the microbicidal mechanisms of macrophages. Salmonella, Shigella and Yersinia have evolved type III secretion systems to deliver proteins into the interior of cells [91]. These secreted proteins, such as Yersinia outer proteins (Yop), interfere with the phagocytic pathway [522]. For example, YopH, which is a protein tyrosine phosphatase, is a potent inhibitor of FcγR- and β_1 integrin-mediated phagocytosis [523]. In another example, *M.tuberculosis* secretes the phosphatase SapM which dephosphorylates PtdIns(3)*P* and thus halts phagosome maturation [524]. This thesis showed that CSF-1 induced the PI3-K-dependent phosphorylation of syntaxin 7 and the assembly of late endosomal SNARE complexes (Figure 5.1), consistent with PI-3K regulating endocytic trafficking in macrophages. It is therefore feasible that pathogens could potentially perturb their trafficking to lysosomes by inhibiting the phosphorylation of syntaxin 7.

Another way in which pathogens interfere with host function is by cleaving SNARE proteins and thereby preventing membrane fusion events. The clostridial toxins botulinum neurotoxin (BoNT) and tetanus toxin (TeNT), which cause botulism and tetanus in humans [525], are proteases that cleave SNARE proteins and prevent neurotransmitter release [526]. For example, BoNT-A, -C and -E cleave SNAP-25, BoNT-C cleaves syntaxin 1A and BoNT-B, -D, -F and -G and TeNT cleave VAMP2 [527]. Although the effects of clostridial toxins have been primarily studied with respect to neuronal SNARE proteins, they have recently been investigated in the context of non-neuronal SNAREs. Syntaxin 4, SNAP-23 and VAMP7 form a

SNARE complex to mediate the Ca^{2+}-triggered exocytosis of lysosomes by mouse rat kidney cells [528]; proteolytic cleavage of SNAP-23 by BoNT-E was found to block this exocytic process. Given the likely importance of syntaxin 7 in phagocytosis and cytokine secretion that it would be potentially interesting to see if pathogens have any effects on syntaxin 7 localisation and/or its expression levels.

6.1.6 Therapeutic potential of SNARE proteins

The fundamental involvement of SNARE proteins in the immune functions of macrophages raises the possibility that they may represent targets for drug development to treat various inflammatory and infectious diseases. SNARE proteins, such as Vti1b, syntaxin 6 and VAMP3 have been shown to mediate membrane fusion of vesicles containing the pro-inflammatory cytokines TNF and IL-6 in macrophages [341]. As the excessive production of these pro-inflammatory cytokines is linked to a number of pathological conditions, including sepsis, chronic obstructive pulmonary disease and rheumatoid disease, the targeted inhibition of specific SNARE proteins, and the subsequent inhibition of SNARE complex assembly, may have potential in the treatment of such diseases.

Defects in phagocytosis have been linked to a number of diseases. For example, the phagocytosis of protein aggregates, such as amyloid-β by macrophages, is normal in healthy individuals but impaired in patients with Alzheimer's disease [529]. Notably, CSF-1 has been shown to increase the phagocytosis of amyloid-β by mouse microglia [184], which based on this thesis might be explained by the ability of CSF-1 to modulate the expression of late endosomal SNARE proteins. The defective phagocytosis of apoptotic cells by peripheral blood-derived macrophages plays a key role in the pathogenesis of systemic lupus erythematosus [530]. Given the importance of SNARE proteins for phagocytosis, gene therapy-based approaches to restore the functions of SNARE proteins may be of value in treating patients with diseases that are a result of impaired phagocytosis.

As discussed earlier, intracellular pathogens, such as *M.tuberculosis* and *L.monocytogene*s, are able to subvert the microbicidal properties of macrophages and use them as an intracellular niche in which to hide from the immune system. These pathogens secrete toxins that interfere with phagosome maturation.

Importantly, clostridial toxins have been shown to cleave SNARE proteins and prevent membrane fusion events. Hence, 'biological' drugs based on bacterial toxins may also be of value in treating inflammatory diseases by preventing the membrane fusion events necessary for the secretion of proinflammatory cytokines by macrophages.

6.2 Future Directions

6.2.1 Identification of syntaxin 7 phosphorylation sites

Phosphorylation of syntaxin 7 is likely to represent a key regulatory mechanism for controlling the assembly of late endosomal SNARE complexes. Bioinformatics analysis of syntaxin 7 identified Ser-125, Ser-126, Ser-129 and Ser-234 as likely sites of phosphorylation. Several approaches could be taken to directly establish if these sites are indeed phosphorylated, including mutational studies and mass spectrometry. For example, Ser-125, Ser-126, Ser-129 and/or Ser-234 could be substituted for alanine or phosphomimetic aspartate residues; these syntaxin 7 mutants would then be expressed in bone marrow-derived macrophage using a retroviral-based expression system that has been established in our laboratory [215]. SDS-PAGE analysis, as well as phosphopeptide mapping [531], of the syntaxin 7 mutants from macrophages that had been metabolically labelled with [^{32}P]-orthophosphate (as described in Chapter 4) would potentially establish if Ser-125, Ser-126, Ser-129 and/or Ser-234 are phosphorylated. Mass spectrometry has been used recently to identify phosphorylation sites in syntaxin 7 from mouse liver cells [495]. A similar approach could be taken to identify phosphorylation sites in syntaxin 7 from mouse bone marrow-derived macrophages. The importance of serine phosphorylation of syntaxin 7 for the assembly of late endosomal SNARE complexes could be examined by testing the ability of Vti1b, syntaxin 8 and VAMP8 to co-immunoprecipitate with the various syntaxin 7 phosphorylation site mutants when expressed in bone marrow-derived macrophages.

6.2.2 The importance of syntaxin 7 for the effector functions of macrophages

One way in which the functional role of syntaxin 7 in macrophages can be investigated is by selectively reducing its expression via RNA interference. Several recent studies have utilised siRNAs to 'knock-down' the expression levels of SNARE proteins to investigate their roles in macrophages. For example, the role of VAMP3 in TNF secretion and phagosome formation in RAW 264.7 macrophages was investigated using siRNAs that target VAMP3 mRNA transcripts [126]. Recently, other members of the laboratory have achieved highly effective siRNA-mediated silencing of syntaxin 7 expression in RAW 264.7 cells. Another approach that could be used to investigate the biological role of syntaxin 7 is to over-express wild-type and a dominant-negative mutant of syntaxin 7 in macrophages (Figure 6.1A). As the transmembrane domain (TMD) of SNARE proteins is important for their anchoring to membranes, truncated forms of SNAREs lacking the TMD can act in a dominant-negative fashion and out compete the endogenous form of the SNARE protein [126, 240]. The wild-type and TMD truncation mutants are typically expressed as green fluorescent protein (GFP)-fusion proteins as this allows the subcellular localisations of the proteins to be easily monitored as well as providing a means for purifying the transfected macrophages by FACS-based cell sorting if the need arises. Macrophages over-expressing GFP-syntaxin 7 would be expected to have enhanced immune functions (e.g. cytokine secretion, phagocytosis and/or macropinocytosis), whereas GFP-syntaxin 7 ΔTMD may act as a dominant negative mutant and therefore diminish the effector functions of macrophages. As shown in Figure 6.2B, RAW 264.7 macrophages were successfully transfected with vectors expressing GFP-syntaxin 7 and GFP-syntaxin 7 ΔTMD. In both cases, the transfection efficiencies were in the order of ~20% (Figure 6.1B). This level of transfection would be sufficient to enable the GFP-positive RAW 264.7 macrophages to be purified by FACS-based cell sorting and used in functional assays (e.g. cytokine secretion, phagocytosis and/or macropinocytosis).

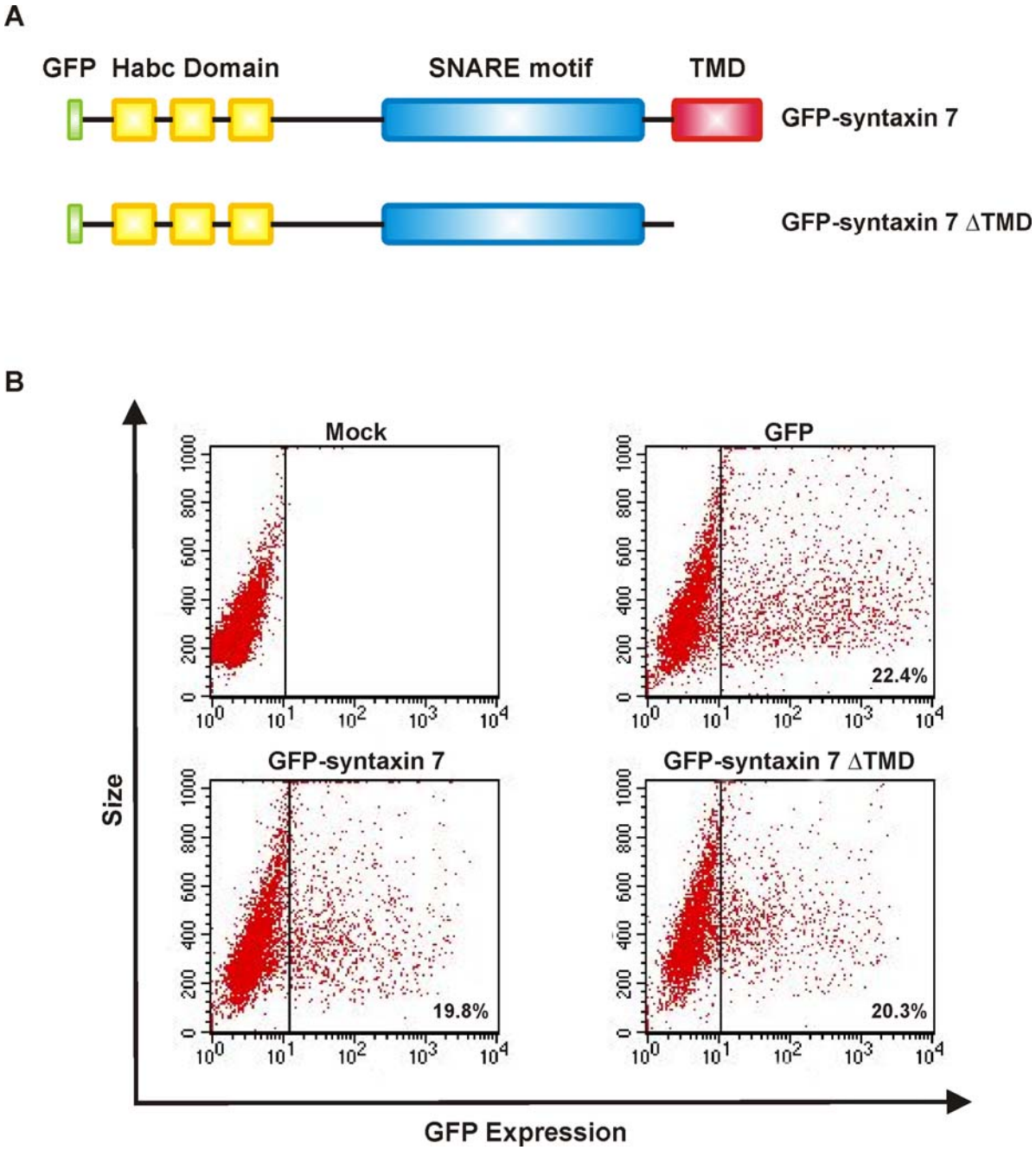


Figure 6.1: Over-expression of wild-type and a dominant-negative mutant of syntaxin 7 in RAW 264.7 macrophages.

A, Schematic representation of GFP-tagged wild-type syntaxin 7 (GFP-syntaxin 7) and a GFP-tagged ΔTMD truncation mutant of syntaxin 7 (GFP-syntaxin 7 ΔTMD). ***B***, RAW 264.7 macrophages were electroporated with vectors expressing GFP alone, GFP-syntaxin 7 or GFP-syntaxin 7 ΔTMD, or electroporated in the absence of vector (mock). Transfection efficiencies were determined by flow cytometry and the values indicated (n = 1).

As described in Chapter 1, when activated with LPS macrophages secrete various cytokines, such as TNF, IL-6 and IL-10. It was reported that syntaxin 7 may form a complex with Vti1b, syntaxin 6 and VAMP3 to facilitate TNF secretion by LPS-activated macrophages [126, 240]. Therefore, it would be worthwhile directly testing the potential involvement of syntaxin 7 in cytokine secretion from macrophages that express wild-type and mutant forms of syntaxin 7. However, before testing the impact of siRNA-mediated silencing of syntaxin 7 and/or over-expression of a GFP-syntaxin 7 ΔTMD on cytokine secretion, a preliminary experiment was performed to assess the kinetics of LPS-induced cytokine secretion by RAW 264.7 macrophages. TNF, IL-6 and IL-10 was readily detected in tissue culture supernatants within 4 h of LPS stimulation (Figure 6.2). However, maximum levels cytokines were not reached at least for another 4 h. The necessary systems are now in place to test the importance of syntaxin 7 for cytokine secretion by macrophages.

The identification of syntaxin 7 in phagosomal membranes suggests that it may play a role in phagocytosis [127]. The successful over-expression of GFP-syntaxin 7 and GFP-syntaxin 7 ΔTMD in RAW 264.7 cells, as well as siRNA-mediated gene silencing, will allow the role of syntaxin 7 in phagocytosis to be studied further; for example, by measuring the ability of the macrophages to phagocytose a range of particles and cells (e.g. latex beads, zymosan, bacteria and apoptotic cells). Similar to phagocytosis, the role of syntaxin 7 in macropinocytosis could be ascertained by determining the impact on the uptake of lucifer yellow [380, 507]. Fluorescently-tagged proteins (e.g. FITC-labelled BSA) could also be used to measure the macropinocytic activity of macrophages, as well as their ability to traffic the endocytosed proteins to lysosomes for degradation [532].

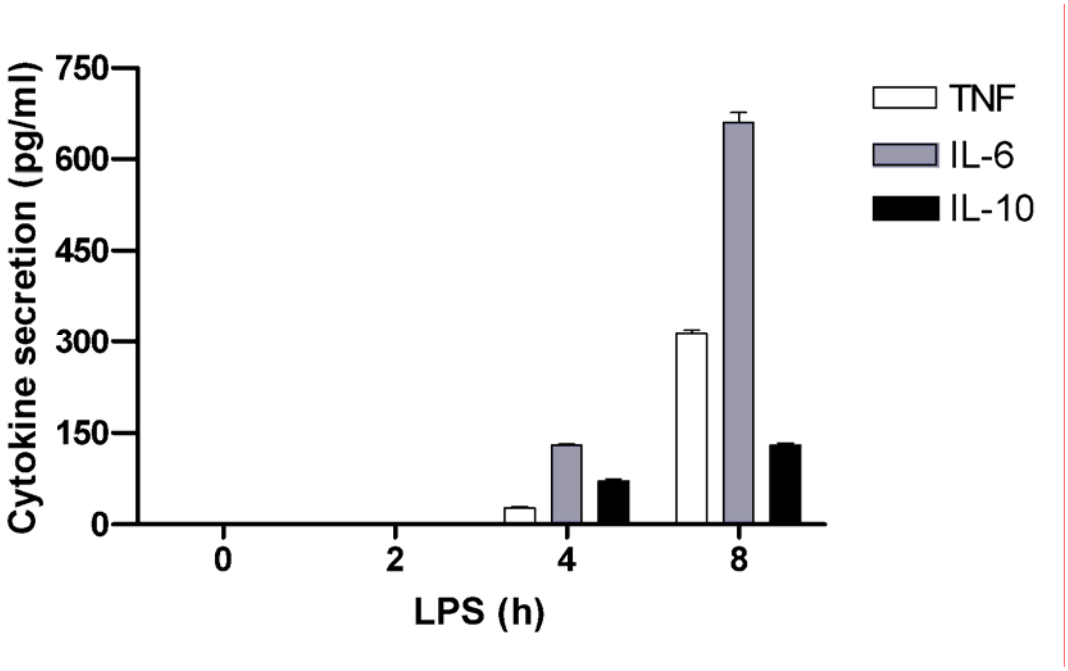


Figure 6.2: Cytokine secretion by LPS-activated RAW 264.7 macrophages.

RAW 264.7 macrophages were stimulated with 100 ng/ml LPS for the indicated periods of time. The medium was collected and the levels of TNF, IL-6 and IL-10 measured by ELISA (n = 1).

6.2.3 The importance of syntaxin 7 phosphorylation for the effector functions of macrophages

If phosphorylation of syntaxin 7 at specific sites is required for late endosomal SNARE complex formation, the functional significance of syntaxin 7 phosphorylation could be investigated by expressing wild-type and serine-to-aspartic acid mutants of syntaxin 7 in RAW 264.7 macrophages and/or bone marrow-derived macrophages. The serine-to-aspartatic acid syntaxin 7 mutants would potentially act as gain-of-function mutants and enhance cytokine secretion, phagocytosis and/or macropinocytosis. The serine-to-alanine syntaxin 7 mutants, however, are unlikely to exert a biological effect as they would not be expected to be capable of binding to Vti1b, syntaxin 8 and/or VAMP8, which would be necessary for them to act in a dominant-negative manner. Although the creation of syntaxin 7 knockout mice has not yet been reported, the reconstitution of bone marrow-derived macrophages from such mice with wild-type and serine-to-aspartatic acid syntaxin 7 mutants would be anticipated to restore syntaxin 7 function. Conversely, reconstitution of the macrophages with serine-to-alanine mutants would not be expected to restore syntaxin 7 function and thereby provide further evidence as to the importance of syntaxin 7 phosphorylation for the effector functions of macrophages.

6.2.4 Identification of syntaxin 7 binding partners

14-3-3 proteins are phosphoserine/phosphothreonine-binding proteins that have been found to interact with more than 200 proteins, including various proto-oncogenes and signalling proteins [533]. The binding of 14-3-3 proteins can alter the localisation, stability, phosphorylation state, activity and/or the interaction of target proteins with other proteins [533]. Recently, 14-3-3 proteins have been shown to interact with AS160, a syntaxin 4 binding protein, and serve as a scaffolding protein for membrane fusion [534, 535]. 14-3-3 proteins are highly conserved and ubiquitously expressed in eukaryotes, with seven isoforms (β, γ, ε, σ, ζ, τ and η; the phosphorylated forms of β and γ were initially described as α and δ, respectively) in mammals [533]. 14-3-3 proteins recognise two defined phosphorylation-dependent binding motifs in target proteins: RSXpSXP and RXXXpSXP [536].

A number of protein kinases, including PKC and Akt, have been shown to phosphorylate sites in several proteins (e.g. insulin receptor substrate-1 and the pro-apoptotic protein BAD) that bind to 14-3-3 proteins [533]. Because the phosphorylation of syntaxin 7 in bone marrow-derived macrophages is dependent on PKC and Akt activity, 14-3-3 proteins could potentially be involved in the regulation of syntaxin 7 (e.g. its binding to other SNARE proteins and/or its subcellular localisation). Syntaxin 7 does not contain a consensus 14-3-3 binding motifs; however, phosphorylation-dependent sites that diverge significantly from RSXpSXP and RXXXpSXP have been identified in bone fide 14-3-3-binding proteins (e.g. IGF-1 receptor and IRS-1) [537-539]. In a preliminary experiment, 14-3-3 was found to co-immunoprecipitate with syntaxin 7 from lysates of bone marrow-derived macrophages (Figure 6.3A). Notably, the co-immunoprecipitation of 14-3-3 with syntaxin 7 was not dependent on CSF-1 stimulation of the macrophages. This suggests that the basal phosphorylation of syntaxin 7 was sufficient for its interaction with 14-3-3. In order to examine further the binding of 14-3-3 to syntaxin 7, bone marrow-derived macrophages were treated with the PI3-K inhibitor LY294002. As shown in Figure 6.3B, the interaction of 14-3-3 with syntaxin 7 was not impaired by this treatment, implying that 14-3-3 constitutively interacts with syntaxin 7 in bone marrow-derived macrophages independently of the PI3-K-regulated phosphorylation of syntaxin 7. This interaction potentially provides an explanation for the constitutive phosphorylation of syntaxin 7 in bone marrow-derived macrophages, as the binding of 14-3-3 may protect serine residues in syntaxin 7 from dephosphorylation by phosphatases.

The biological significance of the interaction of 14-3-3 with syntaxin 7 remains to be established. One important mechanism through which 14-3-3 regulates cellular functions is by modulating the localisation of its target proteins [537, 538]. Indeed, it has been shown that 14-3-3 binding can sequester the target protein to a particular subcellular compartment and that the release of 14-3-3 then allows the target protein to relocate. This relocation is often due to the exposure of an intrinsic subcellular targeting sequence that is masked by the binding of 14-3-3 [533]. Thus, the binding of syntaxin 7 to 14-3-3 may influence its subcellular localisation.

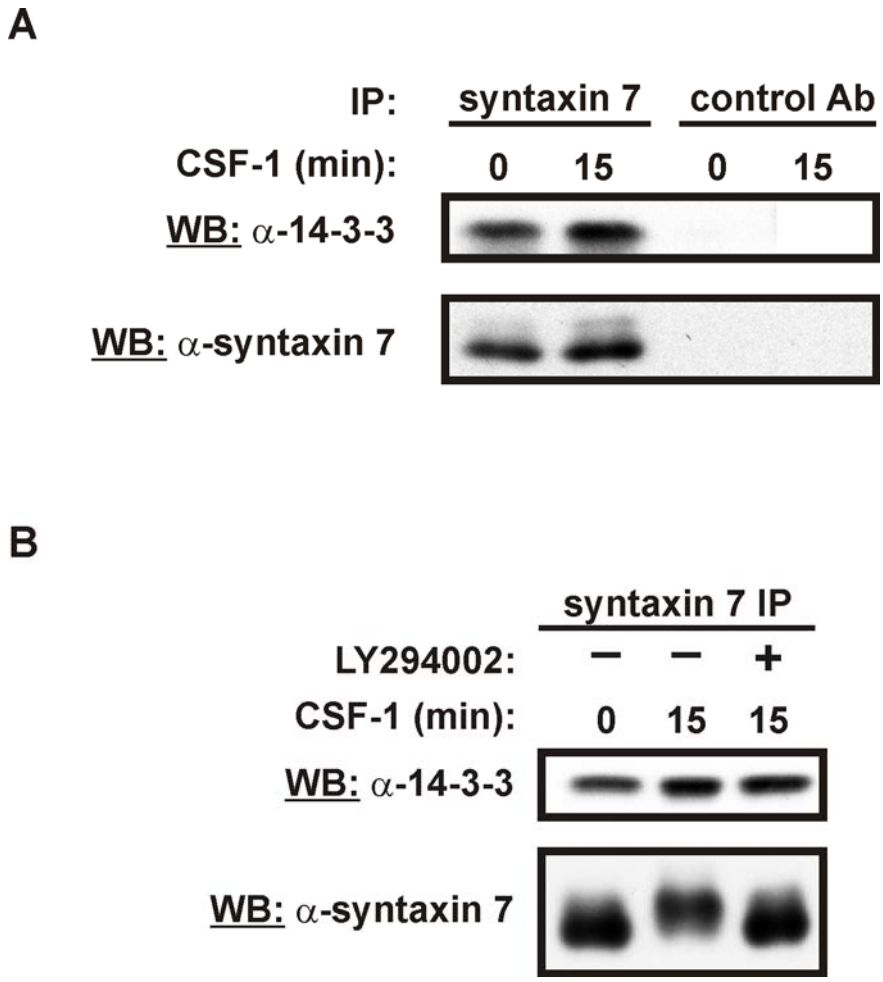


Figure 6.3: Syntaxin 7 interacts with 14-3-3 proteins.

A, Bone marrow-derived macrophages were deprived of CSF-1 for 16 h and then stimulated with CSF-1 for 15 min or left unstimulated. The macrophages were lysed and syntaxin 7 immunoprecipitated from the cell lysates using anti-syntaxin 7 antibodies. The corresponding IgG control for syntaxin 7 was also immunoprecipitated. The immunoprecipitates were then subjected to Western blotting with anti-14-3-3 and anti-syntaxin 7 antibodies. ***B***, Bone marrow-derived macrophages were deprived of CSF-1 for 16 h before being treated with either 10 □M LY294002 or 0.1% DMSO for 30 min. The macrophages were stimulated with CSF-1 for 15 min or left unstimulated and then lysed. Syntaxin 7 was immunoprecipitated from the cell lysates and the immunoprecipitates subjected to Western blotting with anti-14-3-3 and anti-syntaxin 7 antibodies (n = 1). The anti-14-3-3 antibody used recognises all seven isoforms of 14-3-3.

6.3 Summary

The endosomal SNARE protein syntaxin 7 forms a complex with Vti1b, syntaxin 8 and VAMP8 to mediate membrane fusion among late endosomes [264]. VAMP8 is replaced by VAMP7 during fusion between late endosomes and lysosomes [296]. Data was presented in this thesis which indicated that the expression of syntaxin 7, at both the mRNA and protein level, was up-regulated by CSF-1 in bone marrow-derived macrophages. Furthermore, CSF-1 promoted the assembly of late endosomal SNARE complexes. Syntaxin 7 has been reported to be localised to early and late endosomes as well as to phagosomes [270, 430]. Consistent with these earlier studies, syntaxin 7 was also found to be localised to early and late endosome in bone marrow-derived macrophages. Although the localisation of syntaxin 7 implies a role in endocytic/phagocytic pathways, its exact biological role(s) in macrophages still needs to be defined.

Recently, mass spectrometric-based studies have revealed that mouse syntaxin 7 is phosphorylated at Thr-79, Ser-125, Ser-126 and/or Ser-129 *in vivo* [495]. In this study, syntaxin 7 was found to be phosphorylated in bone marrow-derived macrophages, with the extent of phosphorylation increased following CSF-1 stimulation. The phosphorylation of syntaxin 7 was exclusive to serine residues; furthermore, phosphorylation occurred at multiple sites and was dependent on the activity of PI3-K, Akt and PKC. Although the functional importance of syntaxin 7 phosphorylation remains unknown, inhibition of PI3-K activity reduced the levels of late endosomal SNARE complexes in bone marrow-derived macrophages. As phosphorylation of neuronal SNARE proteins have been shown to modulate SNARE complex assembly through non-SNARE binding partners, phosphorylation of syntaxin 7 may likewise represent a key regulatory mechanism to control the assembly of late endosomal SNARE complexes. It is anticipated that the experiments outlined above will help to elucidate the function and regulation of syntaxin 7.

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