MAMMALIAN CHITOTRIOSIDASE AND ITS ROLE IN INNATE IMMUNITY

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ABSTRACT

The main aim of this thesis was to characterize mammalian chitotriosidase in terms of its function in innate immunity. This was achieved using several different approaches. In terms of gene expression, it was demonstrated that chitotriosidase is highly expressed in human peripheral blood monocyte-derived macrophages and can be induced further by β-glucan-containing PAMPs. This suggests that the enzyme may be involved specifically in anti-fungal immunity. Furthermore, chitotriosidase activity was detected in neutrophil pellets and the supernatants of cells treated with prolactin (consistent with its putative role in immune modulation) and the degranulating compound fMLP.

The presence of chitotriosidase activity was also demonstrated in human tears; previously it has been shown that there are high levels of expression in the lachrymal glands of the eye, that also express lysozyme. As lysozyme has been shown to have antimicrobial activity in combination with other compounds and has additional chitinase activity, it was postulated that these enzymes may produce synergistic effects. The addition of recombinant human chitotriosidase, however, had no additional effect against Gram-positive or Gram-negative bacteria, when added with sub-optimal doses of lysozyme.

It has been suggested that the loss of the mutant chitotriosidase genotype in two *meso*-endemic West African countries demonstrates that the enzyme plays a critical role in malaria prognosis. Furthermore, the homozygous mutant genotype has been linked with susceptibility to filarial infection. However in the current study of Papua New Guinean populations, it was shown that there is no correlation between genotype and the extent of hookworm infection. Furthermore, the frequency of the mutant allele was maintained at a level similar to those in non *meso*-endemic countries, suggesting that the enzyme does not play a critical role in the outcome of malaria.

Phylogenetic analyses of the mammalian chitinase/chi-lectin gene cluster demonstrated that these genes have a highly conserved gene signature and may have evolved from a common AMCase ancestor gene. This would be consistent with the requirement for a chitin-digesting enzyme in primitive mammals. Through duplication and mutations events, this gene most likely produced subsets of other chitinases and chi-lectins, the latter evolving non-enzymatic functions, courtesy of the conserved α/β TIM-barrel domain.

Publications

Hall, A.J., et al., *Chitotriosidase deficiency is not associated with human hookworm infection in a Papua New Guinean population.* Infect Genet Evol, 2007. **7**(6): p. 743-7.

Hall, A.J., et al., *Human chitotriosidase is expressed in the eye and lacrimal gland and has an antimicrobial spectrum different from lysozyme*. Microbes Infect, 2008. **10**(1): p. 69-78.

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LIST OF ABBREVIATIONS

4-MU	4-methylumbelliferone
AMCase	acidic mammalian chitinase
ANOVA	analysis of variance
APC	antigen presenting cell
APRT	adenine phosphoribosyltransferase
cDNA	complementary DNA
CpG	cytosine-phosphate-guanine
CHIA	acidic mammalian chitinase
CHIT	chitotriosidase
C_t	cycle threshold
DMF	dimethylformamide
EDTA	ethylenediaminetetraacetic acid
epg	eggs per gram
fMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
*F*st	fixation index
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony stimulating factor
HRP	horse radish peroxidase
IFN	interferon
IMDM	Iscove's modified dulbecco's medium
Indel	insertion-deletion
kDa	kilodalton
LPS	lipopolysaccharide
LSD	least significant difference
MACS	magnetic cell sorting
MDM	monocyte-derived macrophage
MR	mannose receptor
NCBI	national center for biotechnology information
PAGE	polyacylamide-gel electrophoresis
Pam_3Cys	tripalmytoyl-cysteinyl-seryl-(lysyl)3-lysine
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
pI	isoelectric point
PM	peritrophic membrane
PMN	polymorphonuclear leukocyte
PRL	prolactin
PRP	platelet-rich plasma
PRR	pattern recognition receptor

qPCR	quantitative polymerase chain reaction
QTDT	quantitative transmission disequilibrium testing
RES	reticuloendothelial system
REST-MCS	relative expression software-multiple condition solver
RIN	RNA integrity number
RPMI	Roswell Park memorial institute
RT-PCR	reverse-transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
Sf9	Spodoptera frugiperda
Syk	spleen tyrosine kinase
TBS	tris buffered saline
TIM	triosephosphateisomerase
TLR	toll-like receptor
T_H	T helper cell
TNF	tumour necrosis factor

1. INTRODUCTION

1.1 OVERVIEW OF THE INNATE IMMUNE SYSTEM

The immune system consists of a diverse but interconnected group of defence mechanisms that prevent an individual from infection and disease. Many of these constituents are present from birth or act without the need for prior exposure to foreign material. These include physical barriers like the skin and mucous membranes, production of antimicrobial compounds and cellular killing processes. Collectively these mechanisms are defined as innate immunity and share certain characteristics that differentiate them from acquired immunity, the other main branch of the immune system.

Innate immunity is evolutionary ancient; the same molecular processes are present in both animals and plants showing that it developed before these kingdoms diverged. The importance of innate immunity is shown by the fact adaptive immunity only appeared 450 million years ago and exclusively in jawed vertebrates (Agrawal et al., 1998). Mutations in innate immunity genes are also usually lethal. In comparison, acquired immunity is slower to develop but is required for the removal of pathogens that have evolved to evade the innate response. Through somatic rearrangement of antigen receptor genes, the adaptive immune system is capable of recognizing an unlimited variety of proteins and

carbohydrates (Schatz et al., 1992); however without underlying innate immunity, these processes are ineffective.

The critical first line of protection is the skin; the importance of which is illustrated by the susceptibility of burns patients to infection. In addition to being a mechanical barrier, the skin provides resistance to invading pathogens through the presence of keratin that is indigestible and therefore protects the underlying epidermis. Bactericidal and fungicidal secretions in sweat containing antimicrobial peptides and proteins (AMPs) (Schroder and Harder, 2006) and the high concentration of salt are lethal to many microorganisms.

The spread of infection into the systemic circulation results in the initiation of an acute phase response which includes expression of the C-reactive protein (CRP) in response to the production of cytokines including interleukin 6 (IL-6) (Pepys and Hirschfield, 2003). CRP acts as an opsonin by marking foreign material for removal by immune cells expressing the CRP-receptor (Mold et al., 1982). The process of opsonization by CRP, and numerous other opsonins including immunoglobulin G (IgG) and immunoglobulin A (IgA), leads the activation of the complement system (Ravetch and Bolland, 2001).

Complement consists of plasma enzymes that become sequentially activated, resulting in various antimicrobial activities within the acute

phase response. These include degranulation of histamine-containing mast cells, leading to increased vascular permeability and induction of chemotaxis which result in recruitment of immune cells to these infected tissues. The end-point of the complement cascade is the production of membrane attack complex (MAC) that forms pores in the microbial membrane resulting in cell lysis (Song et al., 2000).

At a cellular level, the innate immune system consists of polymorphonuclear leukocytes (PMNs), macrophages, dendritic cells and mast cells. PMNs are subdivided into neutrophils, eosinophils and basophils and are identifiable by their distinctive multi-lobed nucleuses. The neutrophil, the most common blood leukocyte, contains granules that are broadly divided into three subsets: azurophil, specific and gelatinse (as shown in Table 1.1) based on their morphology and content (Borregaard et al., 1993). The compounds of neutrophil granules can also be divided into three groups: serine endopeptidases, acid hydrolases and metalloproteinases; based on their antimicrobial mechanism of action (Borregaard and Cowland, 1997).

Table 1.1 The main granule contents of human neutrophils. Azurophil granules can be further subdivided into defensin-rich and defensin-poor granules (Borregaard and Cowland, 1997). Serine endopeptidases are a group of enzymes characterized by a conserved serine residue in their active site; acid hydolases include phosphatases, glycosidases, nucleases and lipases and have low pH optimum; metalloproteinases are endopeptidases that contain zinc ions in their active site.

	Azurophil Granules	**Specific Granules**	**Gelatinase Granules**
Serine Endopeptidases			
	elastase cathepsin G Proteinase 3		
Acid hydrolases			
	β-glucuronidase *β*-glycerophosphatase N-acetyl-β-glucosaminidase α-mannosidase cathepsin B cathepsin D		
Metalloproteinases			
		collagenase gelatinase	gelatinase
Other proteins			
	myeloperoxidase lysozyme azurocidin bacterial-permeability increasing protein (BPI) defensins	lysozyme histaminase lactoferrin Vitamin B_{12} - binding protein	lysozyme β_2-micoglobulin acetyltransferase

Neutrophils are also capable of phagocytosis, a process that begins with the recognition of antibody- or complement-opsonized particles by Fc receptors (FcR) or complement receptors (CR) respectively, followed by ingestion of foreign material through the formation of a phagosome. Intracellularly, ligand binding results in receptor phosphorylation and the associated immunoreceptor tyrosine-based activating motif (ITAM)-containing subunits by Src family kinases (Gresham et al., 2000). Phosphorylated ITAMs then become docking sites for spleen tyrosine kinase (Syk), which recruits a number of adaptor proteins to the receptor complex (Crowley et al., 1997).

The activation of other kinases including phosphatidylinositol 3-kinase (PI3K) is required for lipid remodelling and formation of the phagosomal cup (Marshall et al., 2001). Ultimately, localized polymerization of F-actin filaments leads to the internalization of particles, a process regulated by the Rho family of GTPases (Caron and Hall, 1998). The processing of phagocytosed material requires subsequent fusion between the phagosome and lysosome, to form a phagolysosome.

The production of a number of reactive oxygen-based compounds during the process of respiratory burst results in microbial killing in phagocytic cells. NADPH is redox-coupled to molecular oxygen by phagocyte NADPH oxidase (Phox), to generate superoxide anions (O_2^-) and subsequently hydrogen peroxide (H_2O_2), by superoxide

dismutase (Babior et al., 2002). In the presence of myeloperoxidase, hypochlorous acid (HOCl) is formed from H_2O_2, which can spontaneously break down to produce the toxic hypochlorite (OCl-) and singlet oxygen ($^1O_2$) (Panasenko, 1997). The antimicrobial activity of radicals occurs by oxidation of the electron carriers within mitochondrial respiratory chains and specific amino acid residues within the active site of (for example) bacterial protein tyrosine phosphatases, rendering them inactive (Lee et al., 1998, Fu et al., 2003) .

In addition to ROS, there are reactive nitrogen species (RNS) with antimicrobial activity that is derived from nitric oxide radicals (NO·). Following the stimulation of macrophages with bacteria, tumour necrosis factor-α (TNF-α) induces expression of inducible nitric oxide synthase (i-nos) via an autocrine mechanism (Weisz et al., 1994). NO· is a relatively weak anti-bacterial compound, but has been shown to have several immune-regulatory functions including effects on neutrophil recruitment through changes to P-selectin recruitment and reduced superoxide production through a direct inhibition on NADPH oxidase, suggesting that it has a role in protection against tissue damage by ROS (Armstead et al., 1997, Clancy et al., 1992). Furthermore, NO· and O_2^- form peroxynitrite ($ONOO^-$) that is highly bactericidal in macrophages; in particular the protonated form (ONOOH) that decomposes to form several other toxic radicals. In the presence of transition metals, $ONOO^-$ can form the hydroxyl anion

(OH^-) and nitronium ion (NO^+), which nitrates protein tyrosine residues (Beckman et al., 1992).

To prevent further tissue damage at sites of inflammation by the release of free radicals and hydrolytic enzymes, neutrophils undergo apoptosis, resulting in the formation of apoptotic bodies that are phagocytosed by other immune cells including macrophages (Savill et al., 1989). Following degradation of phagocytosed material, processed peptides are presented to T_H lymphocytes by antigen-presenting cells, leading to T-cell activation and the acquired immune response. The process of antigen presentation is dependent on the expression of major histocompatibility complex (MHC) molecules on the cell surface (Bach et al., 1976). There are two types of MHC molecule: MHC I are expressed on most virally infected cells allowing recognition by cytotoxic T (T_c) cells, whereas MHC II molecules are exclusively presented on professional APCs. The highest levels of MHC II expression are found, however, on dendritic cells that are located in the tissues exposed to the external environment (Banchereau and Steinman, 1998). These include the epidermis (where they are called Langerhans cells), the lung, nose, stomach and intestines and their dendrites. This allows close interaction with antigen and other immune cells, providing an ideal environment for antigen presentation.

In contrast to neutrophils and dendritic cells, macrophages are primarily located in secondary lymphoid organs such as the lymph

nodes and are derived from haematopoietic stem cells that differentiate through precursor cells prior to entering the tissues (van Furth et al., 1972). The first stages of differentiation occur in the bone marrow and lead to the production of monoblasts and promonocytes. Monoblasts express antibody receptors but rarely ingest opsonized bacteria whereas the more developed promonocytes are capable of phagocytosis and contain antimicrobial granules. Each promonocyte divides to form two monocytes that remain in the bone marrow for less than 24 hours before entering the peripheral blood where they become distributed between circulating and marginating pools. The monocyte half-life is approximately 70 hours prior to migration into extravascular tissues where further differentiation results in macrophages (Sutton and Weiss, 1966, Metcalf, 1997).

The heterogeneity of macrophage phenotype and function *in vivo* is dependent on the distribution of monocytes to different tissues resulting in subsets of cells that are suited to their local environment and physiological role. Alveolar macrophages, for example, are present in the lung and are required for preventing infection from particulate matter in the airway. The production of different macrophage subsets is also dependent on the presence of polypeptides and cytokines including macrophage colony stimulating factor (M-CSF) or a combination of other polypeptide growth factors: granulocyte-macrophage colony stimulating factor (GM-CSF), Interleukin –1 (IL-1), Interleukin- 3 (IL-3), Interleukin- 6 (IL-6), stem cell factor (SCF),

leukaemia inhibitory factor (LIF) and Interferon- γ (IFN-γ) *in vivo* (Lee, 1992, Metcalf, 1989, Wiktor-Jedrzejczak and Gordon, 1996).

1.2 PAMPs AND PRRs

Innate immune cells have the means to recognize microorganisms containing specific structures through the expression of germ-line encoded pattern recognition receptors (PRRs) (Teixeira et al., 2002). PRRs recognize small non-self motifs from lipids, complex sugars, proteins or nucleic acids, termed pathogen-associated molecular patterns (PAMPs). The limited variation between PAMPs that are derived from the same microbial class allows recognition by invariant PRRs. Furthermore, PAMP structures are typically required for microbial survival and this precludes the production of mutants that could evade immune detection through this process (Teixeira et al., 2002) (Roeder et al., 2004a).

1.2.1 Toll-Like Receptors

Toll-like receptors are a large class of single membrane-spanning PRRs that recognize a wide variety of molecular patterns. This occurs partly as a result of leucine-rich repeat (LRR) motifs that allow the formation of numerous binding sites. LRRs typically contain 20-29 amino acids with irregularly spaced leucine residues forming β-sheets that make a solenoid horse-shoe structure, flanked by α-helices and

stabilized by disulphide bonds (Kedzierski et al., 2004). TLRs also contain an intracellular carboxy-terminal sequence consisting of approximately 200 amino acids known as the Toll/interleukin-1 receptor (TIR) homology domain. The IL-1/TLR super-family contains a diverse group of receptors, however similar TIR sequence homology suggests that they are part of an ancient host response system (O'Neill and Dinarello, 2000).

Figure 1.1 illustrates TLR signal transduction pathways and intracellular proteins required for activation. The different TLRs identified to date are shown in Figure 2.2. TLRs recognise and are activated by PAMPs from a range of different bacteria and fungi, allowing variable gene expression patterns in response to these stimuli.

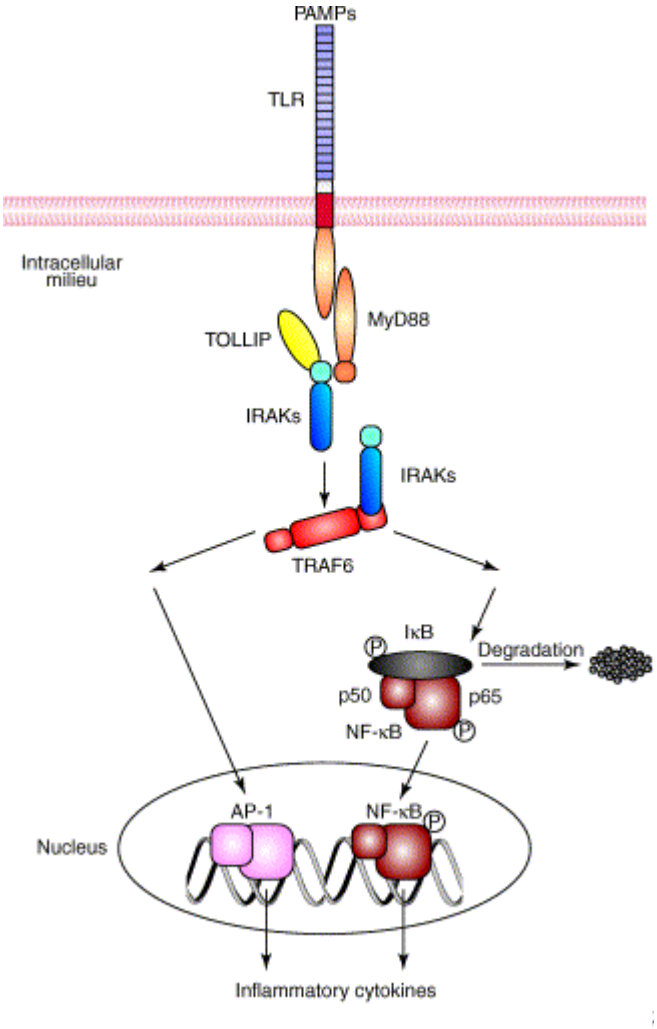

Figure 1.1 The TLR signal transduction pathway. Following ligand binding the TLR recruits the adaptor protein myeloid differentiation marker 88 (MyD88) that interacts with the TIR domain via its C-terminal module. At the N terminal end of MyD88, a death domain (dd) module recruits the IL-1 receptor associated kinase (IRAK) to the TLR. Subsequently IRAK interacts with tumour necrosis factor 6 (TRAF6) that induces activating protein-1 (AP-1) and nuclear factor kappa B (NF-κB) transcription. This results in the production of pro-inflammatory cytokines and T-helper (Th) cell development. From (Roeder et al., 2004a).

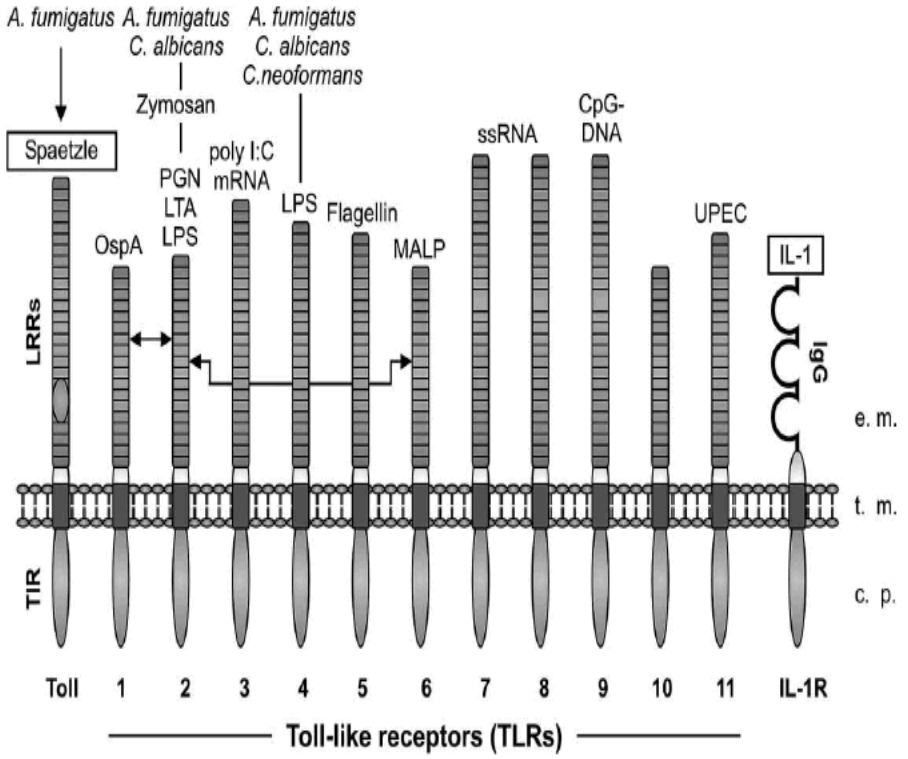

Figure 1.2 The role of TLRs in the recognition of fungi and bacteria. Schematic representation showing the *Drosophila*, TLR1 to TLR11 and the IL-1 (IL-1R) receptors. LRRs and transmembrane domains are represented as boxes, TIRs are represented as ovals. Arrows depict cooperation between receptors including zymosan activation of TLR-2 and TLR-6 heterodimers. Abbreviations: c.p.- cytoplasm, CpG DNA- unmethylated CG motifs, e.m.- extracellular milieu, LPS- lipopolysaccharide, LTA- lipoteichoic acid, MALP- macrophage-activating lipopeptide, OspA- triacyl lipopeptide outer surface protein A, PGN- peptidoglycan, poly IC- double stranded RNA, t.m.- transmembrane domain, UPEC- uropathogenic *E.coli.* From (Roeder et al., 2004b).

TLR-1

TLR-1 is highly expressed in the spleen and on peripheral blood cells (Zarember and Godowski, 2002) and acts as a co-receptor for TLR-2 in response to triacylated lipopeptides (Yamamoto et al., 2002). TLR-1 may also have a role in the regulation of the overall TLR response by inhibiting TLR-2 mediated signalling following binding with phenol soluble modulin from *Staphylococcus epidermidis* (*S. epidermidis*) (Hajjar et al., 2001). TLR-6 is similar to TLR-1 in that it acts as a co-receptor for example with TLR-2 to recognise zymosan (Ozinsky et al., 2000).

TLR-2

TLR-2 is also involved in the recognition of Gram-positive bacteria, mycobacteria and yeast and mediates the LPS response in the species *Leptospira interrogans* (Werts et al., 2001) and *Porphyromonas gingivalis* (Hirschfeld et al., 2001); however recognition of the most common LPS species is dependent on TLR-4 (Takeuchi et al., 1999). TLR-2 is highly expressed on the surface of neutrophils and can be up-regulated by treatment with GM-CSF and G-CSF. Moreover GM-CSF enhances IL-8 production and the superoxide priming of neutrophils to TLR-2 agonists including zymosan and peptidoglycan (Kurt-Jones et al., 2002). Mice deficient in TLR-2 are more resistant to disseminated candidiasis and this may result from TLR-2 induced immune suppression via the release of IL-10 and subsequent activation of regulatory T cells (T_{regs}) (Netea et al., 2004).

The recognition of peptidoglycan was initially believed to be dependent on TLR-2 in combination with TLR-1 or TLR-6 (Takeuchi et al., 1999) (Schwandner et al., 1999). However, a study of eight different bacteria including *Escherichia coli* and *Pseudomonas aeruginosa* using highly purified peptidoglycan, showed that recognition is not TLR dependent and that activation of TLR-2 likely resulted from the presence of lipoteichoic acids (Travassos et al., 2004). Furthermore, it has been suggested that the recognition of peptidoglycan is dependent on the intracellular NOD receptors; that recognize the GlcNAc-MurNAc motif of peptidoglycan (Travassos et al., 2004, Girardin et al., 2003).

TLR-3

Double-stranded RNA is recognized by TLR-3 and results in transcription of IFN-β. This process is independent of MyD88 and instead relies on the TIR- containing adaptor molecule (TICAM)-1 (Oshiumi et al., 2003). It has been suggested that abnormally high levels of RNA resulting from viral infection induce this response (Matsumoto et al., 2002).

TLR-4

TLR-4 is required for the recognition of LPS, the primary constituent on Gram-negative bacterial membranes, as previously it has been shown that TLR-4 knockout mice and humans with the mutant

genotype are hypo-responsive to LPS (Hoshino et al., 1999) (Arbour et al., 2000).

The receptor proximal intracellular signalling pathways for LPS are shown in figure 1.1. The adaptor molecule MyD88 associates with TLR-4, followed by the recruitment and activation of IL-1R-associated kinase 1 (IRAK-1). Initiation of gene transcription by NF-κB, AP-1 and early growth response protein 1 (Egr-1) is dependent on the phosphorylation events following the activation of mitogen-activated protein kinases (MAPK) (LoGrasso et al., 1997). The kinases required in LPS signalling and for other cellular responses are extracellular signal-regulated kinases 1 and 2 (ERK1/2), MAPK p38 (the p38α and δ isoforms), and c-Jun N-terminal kinases (JNKs) (Ashwell, 2006).

Different MAPK isoforms share the amino acid sequence Thr-Xxx-Tyr in which the threonine and tyrosine residues are phosphorylated by MAPK-kinase (MKK), itself phosphorylated upstream by MKK-kinase (MKKK) (Gum et al., 1998). The 'X' amino acid residue defines the different MAPK isoform: in p38 MAPK it is glycine, in JNK it is proline and in ERK it is glutamic acid (Ashwell, 2006). This Thr-Xxx-Tyr motif is located near to both ATP and substrate binding sites within the activation loop. Once activated by the sequential addition of a phosphate to the tyrosine and threonine by MKK, MAPK are then able to phosphorylate other kinases and transcription factors (Gum et al., 1998, LoGrasso et al., 1997). This MAPK signalling cascade is

regulated by PI3K and the inositol phosphatase SHIP. PI3K and SHIP activate and inhibit the serine/threonine kinase Akt respectively. This allows a regulated response to LPS induced cell activation (Fang et al., 2004).

TLR-5 and TLR-9

TLR-5 recognises flagellin: the monomeric unit of bacterial flagella that is required for motility and adhesion, from both gram-positive and gram-negative bacteria (Gewirtz et al., 2001) (Liaudet et al., 2002). Activation of TLR-5 signalling results in human dendritic cell maturation and the expression of chemokines including IL-8 by neutrophils (Means et al., 2003). TLR-9 is an intracellular PRR and detects the presence of viral and bacterial infection in endosomes through recognition of unmethylated CpG motifs that are not present in vertebrates. Ligand binding induces an appropriate cell-mediated T_H-1 response (Shirota et al., 2001).

TLR-7, TLR-8 and TLR-10

The natural ligands for TLR-7, TLR-8 and TLR-10 have yet to be identified. TLR-7 is highly expressed on lung, placenta, spleen and peripheral blood leukocytes (Zarember and Godowski, 2002) and recognizes anti-viral imidazoquinoline compounds (Hemmi et al., 2002). TLR-8 is highly expressed in peripheral blood leukocytes and the lung. TLR-10 is found in lymphoid tissue cells such as the spleen,

lymph nodes and thymus. TLR-10 has a similar sequence homology to TLR-1 and TLR-6 suggesting it may act as a co-receptor.

1.2.2 Dectin-1

The recognition of β-1,3- and β-1,6- glucans is dependent on a non-TLR PRR called dectin-1(Brown et al., 2002). Previously the non-opsonic recognition of β-glucans was believed to require either the mannose receptor (MR) or complement receptor 3 (CR3); however experiments with CR3 knockout and MR antagonized murine macrophages showed that they are still able to bind zymosan in the absence of these receptors (Ariizumi et al., 2000) (Herre et al., 2004). Furthermore, macrophages from dectin-1 knock-out mice also have a severely impaired ability to recognize zymosan particles. Prior treatment with mouse serum opsonins that restore zymosan binding through complement fail to induce an inflammatory response as assessed by TNF production. In addition, dectin-1 knock-out mice treated with *Candida albicans* (*C. albicans*) have a significantly reduced survival rate at sub-lethal doses compared to wild type mice and have higher fungal burdens prior to death (Taylor et al., 2007) In humans, the potential importance of dectin-1 in neutrophil responses to β-glucans has recently been shown. Soluble β-glucan and anti-dectin-1 monoclonal antibodies have been shown to inhibit binding and phagocytosis of zymosan particles, block reactive oxygen intermediate (ROI) production and attenuate *C. albicans* killing (Kennedy et al., 2007).

Dectin-1 was the first non-TLR PRR shown to mediate its own intracellular signalling. The process by which this occurs is dependent on the Y*XX*L ITAM motif sequence in humans (Brown, 2005). Phosphorylation of a single ITAM sequence containing one phosphotyrosine is insufficient to induce spleen tyrosine kinase (Syk) interaction suggesting that two dectin-1 molecules may be required for this activity (Rogers et al., 2005). However the requirement for Syk recruitment appears to be cell specific and vary between processes. For example in some macrophage subsets, dectin-1 is required only for the induction of respiratory burst whereas $Syk^{-/-}$ macrophages can phagocytose zymosan normally (Underhill et al., 2005). Syk-independent dectin-1 processes require dual signalling with for example TLR-2 for IL-12 and TNF production (Herre et al., 2004). Additionally, recognition of the pathogenic fungus *Coccidioides posadasii* (*C. posadasii*) spherules is dependent on TLR-2 and dectin-1 collaboration in macrophages (Viriyakosol et al., 2005). Dectin-1 mediated signal transduction is shown in figure 1.3.

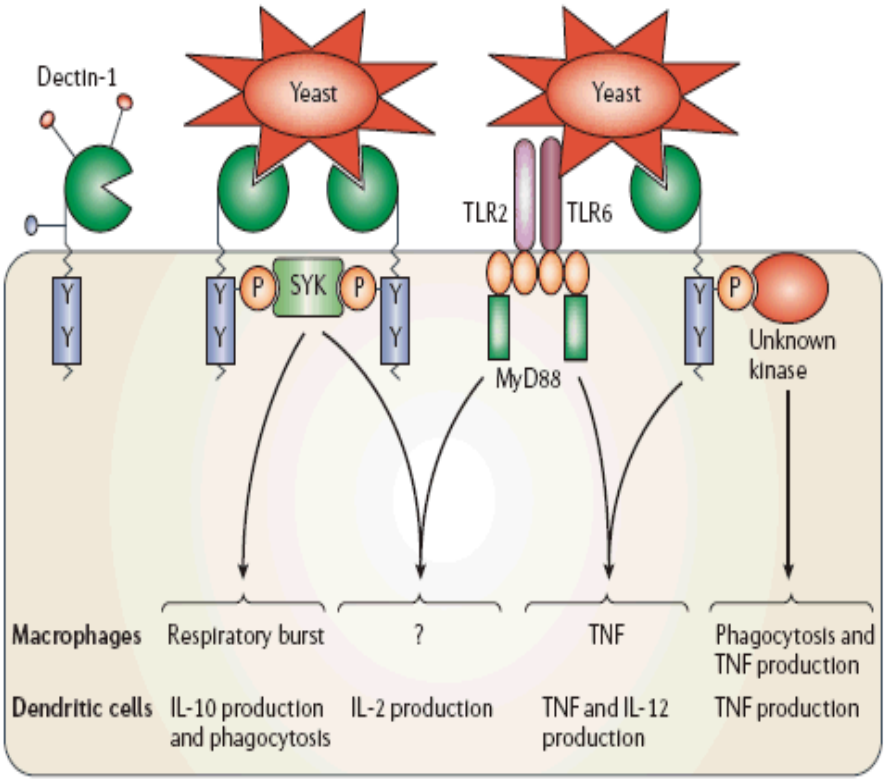

Figure 1.3 Dectin-1 mediated signal transduction. Following ligand binding, dectin-1 becomes tyrosine phosphorylated by SRC kinases leading to an intracellular signalling cascade. The interaction of SYK kinase with a single tyrosine on dectin-1 suggests that SYK bridges two dectin-1 molecules. Cellular responses resulting from dectin-1 ligand binding can be independent of SYK in macrophages but dependent on TLR signalling for the production of TNF or via an unidentified mechanism for phagocytosis. These signalling pathways vary between subsets of macrophages (Brown, 2005).

1.2.3 *Candida albicans* and immune evasion

The importance of the dectin-1 receptor in recognition of β-glucans has been demonstrated; however, *in vivo*, certain species have the capacity to mask their structural carbohydrates and thereby evade immune detection. Ultimately, anti-fungal immunity is dependent on phagocytosis, the secretion of antimicrobial chemicals and the

activation of a T helper 1 (T_H1) response that up-regulates these processes (Romani, 2004). This leads to respiratory burst and the production of reactive oxygen intermediates that induce nucleic acid breaks and lipid peroxidation in fungi (Mansour and Levitz, 2002).

Candida albicans (*C. albicans*) is a commensal fungus and saprophyte that becomes an aggressive pathogen in immuno-compromised or immuno-suppressed individuals. The pathogenicity of *C. albicans* is a result of complex processes, but is dependent on the cell wall constituents providing the main interface for host-pathogen interaction. The resulting cross-talk between *C. albicans* and the immune system is therefore dependent on the chemical composition of the cell wall polysaccharides known as the 'glycan code' (Masuoka, 2004).

The *C. albicans* cell wall consists of between four and eight layers that are distinguishable by their electron density. The outer layer consists largely of mannoproteins that may be linked to β-1,6-glucan chains in regions that are highly N- or O-glycosylated (Klis, 1994). In the yeast form of *C. albicans*, β-glucan exposed on the surface following budding forms a scar; however following switching to the hyphal form this becomes masked and therefore prevents immune detection by dectin-1 (Gantner et al., 2005). *Cryptococcus neoformans* (*C. neoformans*), avoids immune detection by masking β-glucans through production of an extracellular capsule (Kozel, 1995).

In addition to the β-glucans, the inner layers of fungal cell walls, including *C. albicans*, contain chitin: a major structure component that is required to maintain the integrity of the cell wall, forming a matrix with β-1,3 and β-1,6 glucans and mannoproteins. The process of fungal recognition and the interactions between cell wall components and PRRs will be discussed further in Chapter 3.

1.3 CHITINASES

1.3.1 Structure of chitin

Chitin is a long-chain biopolymer of *N*-acetylglucosamine (NAG) units joined by β-1,4 linkages (figure 1.4) to form a hard and semi-transparent material that is structurally similar to cellulose.

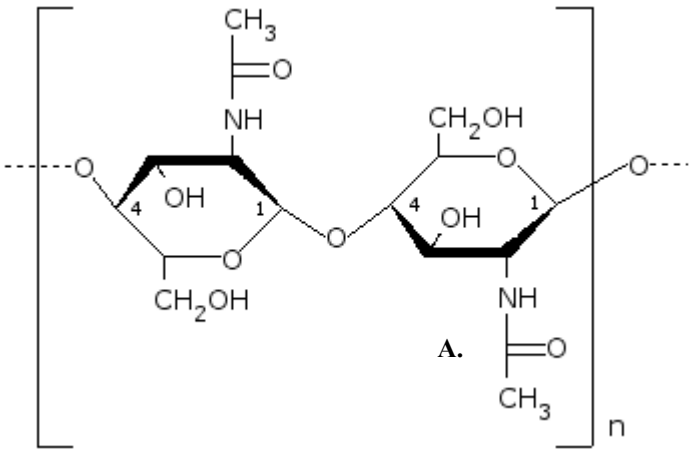

Figure 1.4 The structure of chitin. Multiple acetylglucosamine molecules form long chains via β-1,4 linkages. (^{A} -Acetylamine group) Chitin is stronger than the structurally similar cellulose because of the additional acetylamine groups that allow increased hydrogen bonding (http://en.wikipedia.org/wiki/Image:Chitin_fixed.png).

In nature, chitin exists in several different forms. *N*- acetylglucosamine units can arrange in anti-parallel strands (α- form), parallel strands (β- form) or a combination of both (γ- form). The tight packing of the α- form produces microfibrils that consist of up to twenty single chitin

chains, stabilized by a high degree of hydrogen bonding. In the β- and γ- forms the microfibrils are less compact, resulting in more hydrogen bonds with water and ultimately a weaker structure (Merzendorfer and Zimoch, 2003).

Chitin is a component of a diverse range of organisms. In insects, it is present in large quantities in the exoskeleton as a scaffold for cuticle proteins. Chitin is also present in peritrophic matrix of the midgut, forming a protective barrier between the epithelium and the bolus (Merzendorfer and Zimoch, 2003).. In addition, chitin turnover allows the formation and degeneration of the septum, prior to fungal cell division (Debono and Gordee, 1994) (Adams, 2004) (Cabib et al., 2001). Chitin is also present in the oocytes, zygotes and microfilariae of *Brugia malayi* and its turnover is also an important process that allows growth of this filarial nematode (Fuhrman and Piessens, 1985, Schraermeyer et al., 1987). The presence of chitin in nematodes will be discussed further in chapter 5.

1.3.2 Classification and role of chitinases

Chitinases can be divided into two main groups based on their mechanism of action. Endochitinases produce low molecular weight multimers of GlcNAc units. Exochitinases can be further subdivided into those that catalyze the release of diacetylchitobiose from chitin polymers and those that further degrade the products of endochitinases to produce chitin monomers.

In terms of sequence homology and tertiary structure, chitinases belong to seven classes divided between family 18 and family 19 of the glycosyl hydrolases. Classes I, II, IV, VI and VII are present in plants and belong to family 19; Classes III and V that include those from plants, bacteria, fungi and mammals, form part of family 18 (Henrissat and Bairoch, 1993) (Kasprzewska, 2003). There is also a wide variation in the role that chitinases have across different species.

Fungi

A number of fungal chitinase genes have been identified that are required for autolysis, nutrition and morphogenesis (Duo-Chuan, 2006). In *Saccharomyces cerevisiae* (*S. cerevisiae*), yeast division is dependent on the production of a chitin disk in the bud site septum between mother and daughter cell by chitin synthase II (*CHS-II*), followed by endochitinase activity to produce cell separation. *CTS-1* gene mutants are unable to induce cell separation resulting in pseudohyphae formation (Kuranda and Robbins, 1991) (King and Butler, 1998). *S. cerevisiae* also produces a chitinase synthase from a second gene (*CTS2*) that is required for spore wall biosynthesis (Giaever et al., 2002).

Chitinase-encoding genes have also been identified in filamentous fungi. For example, the *Aspergillus fumigatus* (*A. fumigatus*) genome contains at least 11 conserved chitinase active site domains of the

family 18 glycosyl hydrolase type (Jaques et al., 2003). Instead of morphogenesis, these chitinases may be required for exogenous chitin digestion as a source of energy and carbon for growth (Adams, 2004).

In *C. albicans* there are at least four chitinase encoding genes (*CHT1-4)* that are distinct from those present in *S. cerevisiae*, although *CHT3* appears to be a homologue of *CTS1* (Dunkler et al., 2005). The expression of *CHT2 and CHT3* is down-regulated in the hyphal form and deletion of these genes results in increased hyphal growth on solid media suggesting that regulation of these genes is important in switching between forms (Dunkler et al., 2005, McCreath et al., 1996).

Bacteria

Chitin is particularly abundant in the marine environment as it is produced by zooplankton and phytoplankton species. The diversity of chitinase specificities produced by *Bacillus circulans* (*B. circulans*), *Serratia marcescens* (*S. marcescens*), *Vibrio harveyi* (*V. harveyi*) *and Alteromonas sp*. reflects the alternative forms of chitin available to these species and suggests that different chitinases are required for effective utilization of these substrates and their by-products (Fuchs et al., 1986, Harpster and Dunsmuir, 1989, Jones et al., 1986) (Svitil et al., 1997) (Orikoshi et al., 2005).

The process by which degradation occurs by bacteria is complex and involves the combined activity of different chitinases. For example *S.*

marcescens produces both an endochitinase (Chitinase A) and an exochitinase (Chitinase B) that act synergistically to degrade chitin (van Aalten et al., 2000).

In addition to providing a carbon source, chitinases from soil-based bacteria are involved in controlling the spread of fungal diseases of plants. This is achieved through the cleaving of chitin in fungal cell walls (Hoster et al., 2005). Furthermore, two chitinases from *Pseudomonas aeruginosa* (*P. aeruginosa*) have been shown to contain additional lysozyme activity against other bacteria (Wang and Chang, 1997).

Insects

In insects, chitin regeneration is required for development of the exoskeleton and therefore growth. During cuticle replacement, chitinases synthesized by epidermal cells accumulate in the fluid between the epidermis and the old cuticle. Chitin production in these structures is also dependent on chitin synthases that are located in the apical tips of microvilli found in the larval midgut. Mutations in the insect chitin synthase gene (*CHS-1*), which encodes *LsCHS-1* in *Drosophila*, leads to severe disruption at the head of the cuticle (Merzendorfer and Zimoch, 2003).

Plants

Plant chitinases are involved in pathogen defence and in particular the protection of germinating seeds and the stress response (Cohen-Kupiec and Chet, 1998) (Patil et al., 2000). Higher plants synthesize seven classes of chitinases that differ in structure, substrate specificity and catalysis mechanisms. Many have additional lysozyme-like activity and are capable of cleaving the β1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues of peptidoglycan, suggesting a role in protection against Gram-positive bacteria. Chitinases present in the plant apoplasm are released following cell death as a result of fungal hyphae penetration in to the intracellular space. Fungi have evolved to counteract this activity by chemical inhibition, leading to the development of active site modifications in class III chitinases (Kasprzewska, 2003).

Many plant chitinases do not have anti-fungal activity as shown *in vitro* suggesting alternative roles to immunity. These include growth and development as plant chitinases hydrolyze arabinogalactan proteins (AGPs) and it is therefore assumed that they are capable of degrading other *N*-acetylglucosamine containing glycoproteins in the plant cell wall. However the exact endogenous substrates have yet to be identified (Kasprzewska, 2003).

1.3.3 Mammalian chitinases

Mammalian genomes contain family 18 chitinase genes; however the absence of chitin and chitin sythases suggests that they have an alternative function to regeneration and growth, as seen in insects and fungi.

The active site of family 18 chitinases contains the consensus sequence: D*xx***D***x*D*x***E**. The last aspartic acid and central glutamic acid residues appear to be essential for catalysis as shown in site directed mutagenesis studies (Watanabe et al., 1993). Additionally, naturally occurring mutations in the active site have resulted in proteins that can bind but are unable to cleave chitin, called chi-lectins.

Family 18 chitinases share structural similarities including the common eight stranded α/β_8 (TIM) barrel. This protein motif, first characterized in the glycosidic enzyme triosephosphate isomerase, consists of 8 anti-parallel β-strands (β1-8) laid out in staves that are tilted at 30º to the central axis and surrounded by 8 α helices (Terwisscha van Scheltinga et al., 1996, Fusetti et al., 2002). At the C- terminus of family 18 chitinases there is an additional α/β insertion domain consisting of 5 anti-parallel β-sheets intercalated with short α/β strands.

1.3.4 Chitotriosidase

Chitotriosidase was initially identified in patients with Gaucher disease: a lysosomal storage disorder resulting from a mutation in the glucocerebrosidase gene, leading to accumulation of lipid- laden macrophages (Hollak et al., 1994). Subsequently chitotriosidase has been purified and cloned from a Gaucher diseased spleen and human macrophage cDNA library respectively (Renkema et al., 1995, Boot et al., 1995).

Chitotriosidase exists in isoforms that can be differentiated in terms of molecular mass and isoelectric point. The major form is a 50 kDa protein with a pI range of 5.0-7.2 that varies on the extent of *O*- linked glycosylation at the carboxyl end of the protein. The minor isoform is a 39 kDa enzyme with a discrete pI of 8.1 (Boot et al., 1998) (Renkema et al., 1997). Metabolic labeling experiments with cultured macrophages show that the 50 kDa isoform is synthesized and secreted with a small fraction undergoing post-translational modification or RNA processing to form the 39 kDa isoform that accumulates in lysosomes. The re-routing process of 39 kDa chitotriosidase is not fully understood (Renkema et al., 1997).

The 39 kDa form of chitotriosidase consists of a core domain with a $(\beta/\alpha)_8$ barrel that is similar to other family 18 chitinases, but without the α1 helix. The presence of an additional α/β domain consisting of

six anti-parallel β- strands and one α- helix inserted between β7 and α7 give chitotriosidase a groove structure (Fusetti et al., 2002).

The 50 kDa isoform binds strongly to chitin particles at the chitin binding domain, prior to hydrolysis by the catalytic domain. The absence of the binding domain in the 39 kDa form may reflect the close proximity that the enzyme has to endocytosed material in lysosomes. Moreover, it has been suggested that the presence of the binding domain may interfere with this process (Renkema et al., 1997).

The chitotriosidase active site is lined with solvent exposed aromatic side chains that stack against the hydrophobic face of the sugars. The elongated binding groove is suitable for binding long chitin polymers and contains the active site motif D*xx*D*x*D*x*E in common with other Family 18 chitinases; this is present at the end of the β4 strand (Fusetti et al., 2002). The exact substrate specificity of chitotriosidase is unknown, however it hydrolyzes 4-methylumbelliferyl β-D-Chitooligosaccharides and degrades polymeric chitin into dimers (Renkema et al., 1995).

Chitotriosidase cleaves both colloidal chitin and the *C. albicans* cell wall chitin and inhibits *Cryptococcus neoformans* proliferation suggesting a role against chitinous human pathogens (Boot et al., 2001) (van Eijk et al., 2005). Furthermore, increased plasma chitotriosidase levels have been found in neonates with fungal infections.

Additionally, clinical prognosis reflects chitotriosidase levels in plasma and urine with improving condition leading to decreased chitotriosidase activity (Labadaridis et al., 2005). In guinea pigs, there is an increase in chitinase activity following systemic *Aspergillus fumigatus* infection (Overdijk et al., 1996) and plasma chitotriosidase levels are also elevated in children with acute *Plasmodium falciparum* malaria (Barone et al., 2003).

Chitotriosidase is highly expressed in phagocytes and in particular in macrophages in the later stages of differentiation from monocytes (Boot et al., 1995). In addition to constitutive expression, chitotriosidase has shown to be inducible by Granulocyte- monocyte colony stimulating factor (GM-CSF), IFN-γ, TNF-α, LPS and the hormone prolactin (Hashimoto et al., 1999, van Eijk et al., 2005) (Malaguarnera et al., 2005, Malaguarnera et al., 2004). The induction of chitotriosidase expression in macrophages will be discussed further in Chapter 3.

The chitotriosidase gene (*CHIT1*) extends for twelve exons on chromosome 1 in humans at position 1p31-q32. In approximately 6% of individuals from European Caucasian populations there is a mutation in exon 10 of the *CHIT1* gene that leads to an 87 nucleotide deletion through activation of a cryptic 3' splice site. The resulting protein lacks the residues Val-344 to Gln-372 causing a misfold in the formation of the TIM-barrel catalytic core. In addition, the mutation

results in the deletion of Trp-358: a Family 18 conserved residue at the end of the β8 strand. Macrophages from *CHIT1* mutants express only small amounts of mRNA and virtually no protein (Boot et al., 1998, Fusetti et al., 2002).

The ratios of homozygous mutants and heterozygous carriers of the *CHIT1* mutation to wildtype individuals vary across different populations. For example, in the African countries of Benin and Burkina Faso there is a total absence of homozygous mutants and virtually no heterozygous carriers (Malaguarnera et al., 2003). These countries are endemic for *P. falciparum* malaria and gastrointestinal helminthes and therefore it has been suggested that the *CHIT1* genotype confers protection against these infections. The mutant *CHIT1* genotype has also been linked with susceptibility to lymphatic filariasis in India, however this was not confirmed by a later study in Papua New Guinea (Choi et al., 2001) (Hise et al., 2003).

1.3.5 Acidic Mammalian Chitinase

Acidic mammalian chitinase (AMCase) was the second human chitinase identified in man and is capable of degrading chitinous substrates and fungal cell wall chitin (Boot et al., 2001) (Boot et al., 2005b). The location of the AMCase gene (CHIA) on human chromosome 1 and the sequence homology and conservation of intron-exon boundaries with *CHIT1*, suggests that these genes arose from a duplication event in an ancestor gene (Boot et al., 2001) (discussed in

detail in chapter 2). In common with other mammalian chitinases, AMCase is also a family 18 glycosyl hydrolase and contains the 8-stranded α/β (TIM)-barrel catalytic core and C-terminal chitin binding domain. In contrast to chitotriosidase (that has a pH optimum of between 5-7, shown in chapter 4) AMCase is most active in highly acidic environments, consistent with its expression in the stomach (Boot et al., 2001) (Boot et al., 2005b). The expression of AMCase in the lung suggests that the enzyme may have a dual function in digestion of chitinous substrates and host defence. Increased expression in AMCase has been demonstrated in asthma, suggesting that it may play a role in the development of this disease (Reese et al., 2007) (Zhu et al., 2004).

1.4 AIMS AND OBJECTIVES

The aim of this research is to characterize mammalian chitinases. In particular, this work will focus on chitotriosidase in terms of its expression and release by cells of the innate immune system and its potential protective function against bacterial, fungal and parasitic infections.

This will be achieved by several different approaches. These are the objectives:

- Identifying chitinase and related genes from different mammalian species and using a phylogenetic approach to evaluate evolutionary relationships, with particular regard to exon structure.

- Determining whether *CHIT1* mRNA expression is constitutive in monocytes or macrophages and whether it can be up-regulated by microbial (fungal or bacterial) PAMPs. This will be achieved by measuring relative changes in *CHIT1* expression in monocyte-derived macrophages exposed to live *C. albicans*, fungal and bacterial PAMPs and during the process of *in vitro*-differentiation from peripheral blood monocytes, by quantitative real time reverse transcription PCR (qRT^2-PCR).

- Showing the effects of prolactin and fMLP, a hormone with immune modulatory properties and a synthetic peptide respectively, on the release of active chitotriosidase enzyme, determined by a 4-MU chitotrioside substrate cleaving assay.

- Assessing the antimicrobial activity of recombinant chitotriosidase and an active-site mutant with no chitinolytic activity. This will be achieved by treating a panel of Gram-positive and Gram-negative bacteria with both forms of chitotriosidase and determining viability with an ATP-luciferase assay.

- Assessing whether recombinant chitotriosidase and lysozyme in combination have synergistic or additive effects against bacterial viability.

- Determining whether the *CHIT1* genotype is associated with protection against infection with *N. americanus* and malaria in a human population. This will be achieved by analyzing the association between *CHIT1* genotype and N. americanus faecal egg loads (by transmission disequilibrium testing) to show whether the enzyme has a significant role in the outcome of this infection and correlating *CHIT1* genotype with malarial parasitaemia, using a logistic regression model.

2. PHYLOGENETIC ANALYSES OF MAMMALIAN CHITINASES

2.1 INTRODUCTION

Since the discovery and cloning of the first human chitinase (chitotriosidase) by Boot *et al*. (1995), and the finding of related genes in its proximity, chitinases and chi-lectins have received increasing attention. AMCase is currently discussed as an important factor involved in the pathology of asthma, but its precise role remains to be ascertained. Two conflicting recent publications have suggested that AMCase has an exacerbating (Zhu et al., 2004) or a protective role in asthma (Reese et al., 2007). The direct neighbour of chitotriosidase, Chi3l1, appears to be implicated in several particularly malignant cancers (Bergmann et al., 2005) and in rheumatoid arthritis (Vos et al., 2000). Ym1 and Ym2 have also been described in murine infection and asthma models (Zhao et al., 2005) (Falcone et al., 2001). However, despite the strong and steadily increasing interest in these genes, there has been much confusion regarding the relationship between those of humans and, for example, rodents. This has led to the misidentification of genes based only on partial sequence homology. For example, the human AMCase gene has recently been shown not to be the ortholog of mouse YM1 (Boot et al., 2005a), as had previously been believed (Raes et al., 2005).

It has now become clear that mice and rats, and perhaps also other rodents, have additional members in the chitinase chilectin gene cluster (CCGC) that are not found in other mammals. This is an important caveat when extrapolating data from murine or rat models to the human situation. The issue is further complicated by the usage of alternative names for the same gene, such as HC-GP39 and YKL-40 for Chi3l1, or TSA1902 and AMCase for CHIA. The identities of and relationships between members of the mammalian CCGC are therefore in need of further clarification.

The evolutionary relationships between members of the mammalian CCGC have recently been studied. Bussink *et al*. (2007) demonstrated that all chitinase and chi-lectin orthologs are derived from a common active ancestral gene that underwent duplication events to produce AMCase and chitotriosidase genes. Further duplication and mutation events, the latter resulting in loss of enzymatic function, produced four subsets (clades) of chitinase/chilectin orthologs: the enzymatically inactive oviductins and chi-lectins branching from AMCase and chitotriosidase respectively.

Funkhouser *et al*. (2007) categorized mammalian chitinase and chi-lectin genes into four subsets. In common with Bussink *et al*. (2007), they demonstrated that these genes have a common ancestor, however in their model an early mutation led to the formation of the oviductins as a separate subset. The remaining subsets described by Funkhouser *et*

al. (2007) are the AMCases and related chi-lectins, the chitotriosidases and the remaining chi-lectins. This model also shows the recently identified CHID1 genes are present in a separate branch. However, CHID1 may be unrelated to the other genes in the cluster, as it is located on *Homo sapien* chromosome 11 (p15.5), whereas other members are present on chromosome 1 (Funkhouser and Aronson, 2007) (Jin et al., 1998). No attempts have been performed so far to clarify the nature of the relationship of CHID1 with the members of the CCGC. The identification of CHID1 orthologs has led to the possibility that other genes within the cluster have yet to be described. Furthermore, it is possible that other chitinases or chi-lectins can be identified in the human genome, perhaps located outside the CCGC.

2.2 OBJECTIVES

The main aim of the work was to characterize the phylogenetic relationships between members of the mammalian chitinase/chi-lectin gene cluster. This was achieved using the following methods:

The hidden Markov models (HMM) of the superfamily database (Wilson et al., 2007) were used to search for unknown chitinase and chi-lectin genes in the mammalian genome. This approach allows probability of hidden features to be predicted within the protein structure and is therefore more effective than using PSI-BLAST, which primarily compares conserved residues within particular motifs.

Phylogenetic trees of members of the mammalian chitinase and chi-lectin gene cluster were generated using the conserved GH18 domains $(\alpha/\beta)_8$ (TIM) barrel) with Minimum Evolution and Neighbor-Joining methods. During the preparation of this chapter, Funkhouser *et al*. (2007) and Bussink *et al*. (2007) demonstrated phylogenetic relationships of these genes by comparing GH18 $(\alpha/\beta)_8$ (TIM) barrel) domains and therefore the outcomes of these analyses were compared.

The exon sizes and structures of different members of this cluster were compared to demonstrate that chi-lectins have evolved through the loss of exon 11, which encodes the chitin-binding domain in active chitinases.

2.3 METHODS

2.3.1 Mapping of chitinase and chi-lectin genes on human and mouse chromosomes

Chitinase and chi-lectin genes were mapped by identifying human and mouse chitinase and chi-lectin genes using Basic Local Alignment and Search Tool (BLAST) searches and creating a cytogenetic location map (NCBI Build 36.1) (by Dr. Franco Falcone).

2.3.2 Determination of chitinase and chi-lectin homologues using Hidden Markov models (HMMs) of the SUPERFAMILY database (v.1.69)

The SUPERFAMILY database (Wilson et al., 2007) was used to identify mammalian chitinase and chi-lectin homologues in five species: *Bos taurus*, *Gallus gallus*, *Homo sapiens*, *Mus musculus* and *Rattus norvegicus*. This was achieved by comparing the structural and functional protein motifs of the three domains of *Homo sapiens* AMCase (GH18 TIM α/β barrel domain, chitin binding domain and αβ insertion domain (as described in section 1.3.3), identified using NCBI protein BLAST, with those of other proteins using Hidden Markov models (HMMs).

2.3.3 Generation of phylogenetic trees using Molecular Evolutionary Genetics Analysis software v.4.0 (MEGA4)

Protein sequences for mammalian chitinases and chi-lectins were identified using NCBI BLAST (http://www.ncbi.nlm.nih.gov) and ENSEMBL (www.ensembl.org) databases. In a few cases where the predictions appeared to be ambiguous, the sequences were obtained from published work rather than the predicted gene sequences, where such publications were available. Transcripts were aligned in MEGA4 (Tamura et al., 2007) using the ClustalW function and phylogenetic trees were generated using the Minimum Evolution and Neighbor-Joining methods, with 1000 replications to produce bootstrap values. For each method, trees were rooted with *Gallus gallus* CBPch04 as the out-group, or left unrooted.

2.3.4 Exon-Intron analysis using PP Hwang's Exon-Intron Drawer v.2.0b

The exon-intron composition of mammalian chitinases and chi-lectins was determined by using peptide and exon transcripts from ENSEMBL. Sequence regions corresponding to the chitin-binding domain, GH18 (TIM α/β barrel) domain and active site were identified using PP Hwang's Lab Exon-Intron Drawer (www.ecophysiology.org.tw/EI2/). and was used to depict transcripts to allow comparison between genes.

2.3.5 Alignment of c-terminal domains using ClustalW

Mammalian chitinase and chilectin peptide sequences were aligned using ClustalW 2.0 (EMBL-EBI) (by Dr. Franco Falcone) to allow the comparison of chitinase and chi-lectin orthologs.

2.4 RESULTS

2.4.1 The chitinase/chi-lectin gene cluster is located on chromosome 1 in humans and chromosomes 1 and 3 in mice

Using the NCBI Build 36.1 cytogenetic location map and ENSEMBL searches, chitinase and chi-lectin genes from humans and mice were mapped to chromosome 1 and chromosomes 1 and 3 respectively. In humans, chitinase and chi-lectin genes are present in two separate regions of the chromosome 1 (p13 and q32) (Fig. 2.1). In mice, the genes are segregated into regions 3F2.2-3F3 on chromosome 3 and 1E4 on chromosome 1.

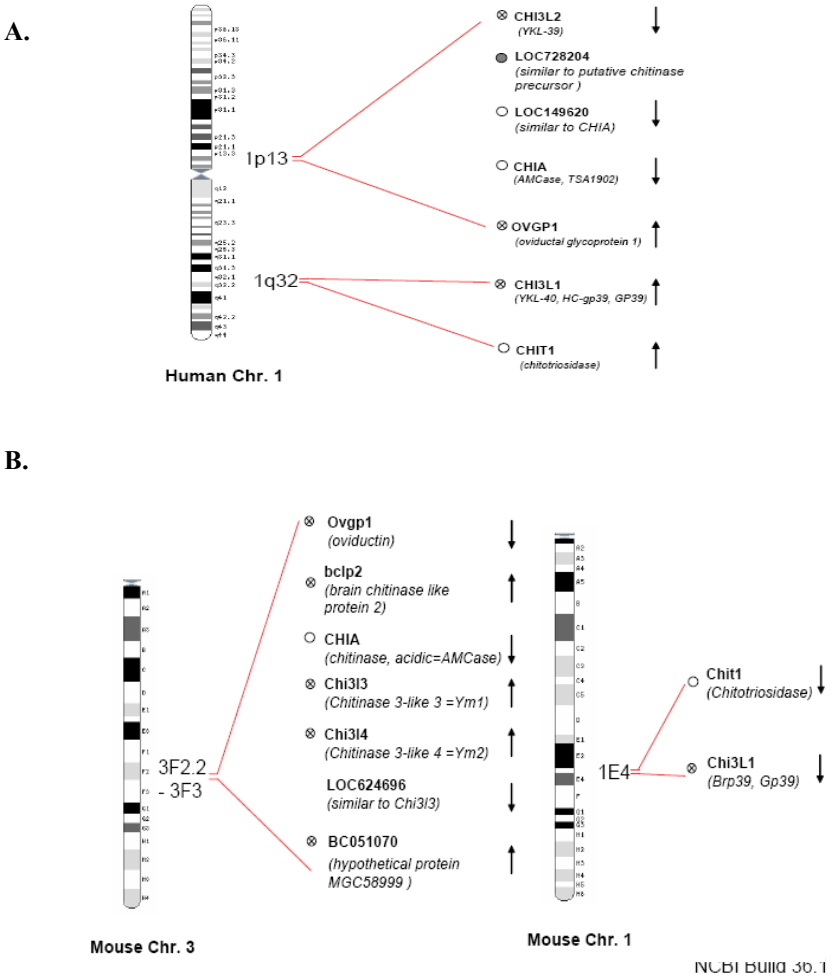

Figure 2.1 Chitinase and chi-lectin gene clusters on human chromosome 1 (A) and mouse chromosomes 1 and 3 (B). Arrows indicate gene orientation, white circles represent active or potentially active chitinases and crossed circles represent chi-lectins. (from NCBI Build 36.2; Dr. Franco Falcone).

2.4.2 Search for new members of the mammalian chitinase/chi-lectin gene cluster using SUPERFAMILY HMM database

To determine whether any members of the mammalian CCGC have yet to be identified, or related chitinases can be found outside the cluster, the SUPERFAMILY HMM library was used to search for domains (GH18 TIM α/β barrel domain, chitin binding domain and αβ domain) shared with *Homo sapiens* AMCase. Gene sequences that have been identified but not fully characterized were submitted to protein BLAST and closest matches were determined by the BLAST score and E value (to determine the likely-hood of this occurring by chance).

As shown in table 2.1, no new chitinases or chi-lectins of similar homology were found using this approach. Furthermore, the recently described CHID1 was not identified. This was an expected outcome as the sequence homology of this with other GH18 genes is low (Funkhouser and Aronson, 2007). This result suggests that there may be no other chitinases or chi-lectins in the human genome other than those already described.

Table 2.1 Chitinase and chi-lectin homologues identified using hidden Markov models (HMMs) of the SUPERFAMILY database for *Bos taurus*,*Gallus gallus* and *Homo sapiens* (A); *Mus musculus* and *Rattus norvegicus* (B).

A.

Species	Gene Name/ENS	Protein	Blast score/E value
Bos taurus	CHIA	AMCase	
	CHI3L1	Hcgp39	
	CHI3L2	YKL39	
	OVGP1	Oviductin	
	ENSBTAP00000032067	CHIT1	608/ 2e-172
	NM_174699.2	AMCase precursor	538/2e-151
	LOC513513	YKL39	723/0
Gallus gallus	CHIA	AMCase	
	ENSGALP00000005559	CBPch04	904/0
	ENSGALP00000005557	Eosinophil chemotactic cytokine	931/0
	ENSGALP00000005556	CBPch04	942/0
	XR_026654.1	Eosinophil chemotactic cytokine	721/0
	NP_989760.1	Eosinophil chemotactic cytokine	947/0
	ENSGALP00000005553	CBPch04	956/0
Homo sapiens	CHIA	AMCase	
	CHIT1	Chitotriosidase	
	CHI3L1	Hcgp39	
	CHI3L2	YKL39	
	OVGP1	Oviductin	
	Q9BZP6-2	chitinase family protein 3	855/0
	Q59HH5_HUMAN	Oviductin precursor	1508/0
	Q9BZP6-2	AMCase	753/0
	ENSP00000358758	Eosinophil chemotactic cytokine	629/7e-179
	ENSP00000356197	CHIT1	530/3e-149

B.

Species	Gene Name/ENS	Protein	Blast score/E value
Mus musculus	CHIT1	Chitotriosidase	
	CHIA	AMCase	
	CHI3L1	Hcgp39	
	CHI3L2	YKL39	
	CHI3L3	Ym1	
	CHI3L4	Ym2	
	OVGP1	Oviductin	
	BC051070	LOC229688 (BYm?)	928/0
	Q9D7Q1-2	Chitotriosidase	803/0
	ENSMUSP00000059167	Chitobiase	669/0
	Bclp2	Brain Chitinase-like protein 2	449/5e-125
	NP_001074606.1	LOC69983	3824/0
Rattus norvegicus	CHIT1	Chitotriosidase	
	CHIA	AMCase	
	CHI3L1	Hcgp39	
	CHI3L2	YKL39	
	CHI3L3 pred	Ym1	
	CHI3L4	Ym2	
	ENSRNOP00000044947	AMCase precursor	907/0
	ENSRNOP00000052822	AMCase precursor	873/0
	NP_446012.1	Chitinase 3-like 1 isoform	824/0
	ENSRNOP00000052813	AMCase	783/0
	RGD1309110_predicted	AMCase	909/0
	ENSRNOP00000052820	Chitinase 3-like protein 3 precursor	796/0
	ENSRNOP00000052812	AMCase	775/0
	ENSRNOP00000045596	Chitinase 3-like 3 precursor	388/2e-106
	RGD1309110_predicted	AMCase	909/0

2.4.3 Phylogenetic trees of mammalian chitinases and chi-lectins

The phylogenetic trees generated are shown in figures 2.2-2.5. Using the Minimum-Evolution method (figures 2.2 and 2.3), with and without the *Gallus gallus* CBPch04 out-group, the subsets shown are similar to those described by Funkhouser *et al*. (2007). The gene cluster is divided into orthologs consisting of the oviductins, the AMCases, the chitotriosidases, the Yms and the chi-lectins. The AMCases and Yms are closely related, with high bootstrap support.

The oviductins form an exclusive subset or clade that appears to have evolved from an ancient chitinase ancestor gene. Furthermore, the chitinases and chi-lectins form separate, but closely related subsets, suggesting a more recent common ancester. The use of *Gallus gallus* CBPch04 to root the phylogenetic tree (figure 2.3) appears to be a suitable choice of outgroup as the tree reflects that of figure 2.2.

The phylogenetic trees generated using the Neighbor-Joining method (figures 2.4 and 2.5), demonstrate similar subsets to those shown using the Minimum-Evolution method and that described by Funkhouser *et al*. (2007). In contrast to the phylogenetic analysis of Bussink *et al*. (2007), our analysis suggests that the AMCase orthologs are most closely related to the ancestral chitinase and this is particularly evident in the rooted trees (figures 2.3 and 2.5). In these models, the ancestral

AMCase duplicated to form another chitinase gene that formed chitotriosidase orthologs and, via mutation events, the oviductins. A Subsequent mutation in the chitotriosidase gene led to the formation of the chi-lectins, whereas further gene duplication events followed by mutations in the original AMCase, which occurred after speciation, formed the Ym orthologs in rodents. This is in contrast with the analyses of Bussink *et al*. (2007), who suggested that the primitive chitinase underwent duplication events to form AMCase and chitotriosidase subsets, prior to loss of enzymatic function mutations that resulted in chi-lectins, Yms and oviductins.

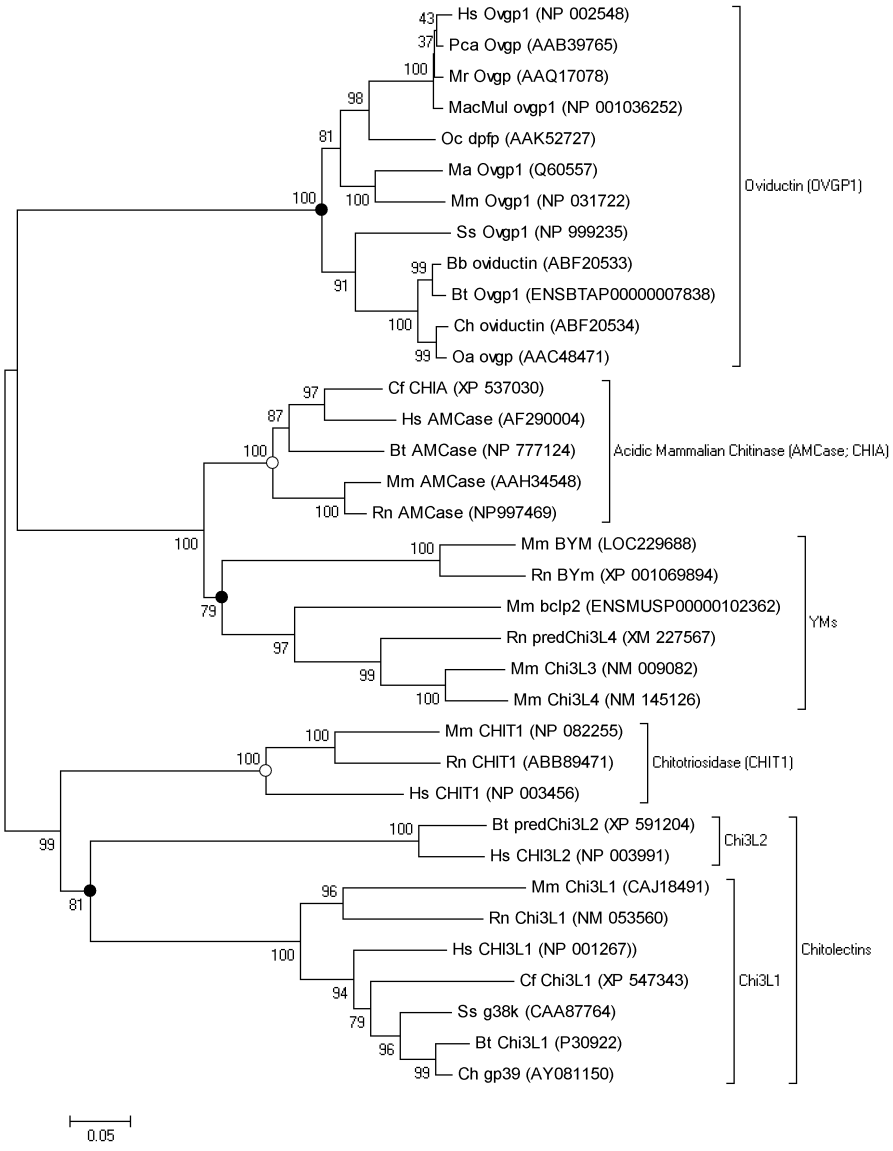

Figure 2.2 The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky and Nei, 1992). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl, 1965) and are in the units of the number of amino acid substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Species abbreviations: Gg- *gallus gallus*, Rn- *Rattus norvegicus*, Mm- *Mus musculus*, Hs- *Homo sapiens*, Bt- *Bos taurus*, Bb- *Bubalus bubalus*, Ch-*Capra hircus*, Ss- *Sus scrofa*, Cf- *Canis familiaris*, PCa- *Papio anubis*, MacMul- *Macaca mulatta*, Oc- *Oryctolagus cuniculus*, Ma- *Mesocricetus auratus*. Hypothetical ancestors with enzymatic activity are denoted with a white circle; ancestors without enzyme activity with a black circle.

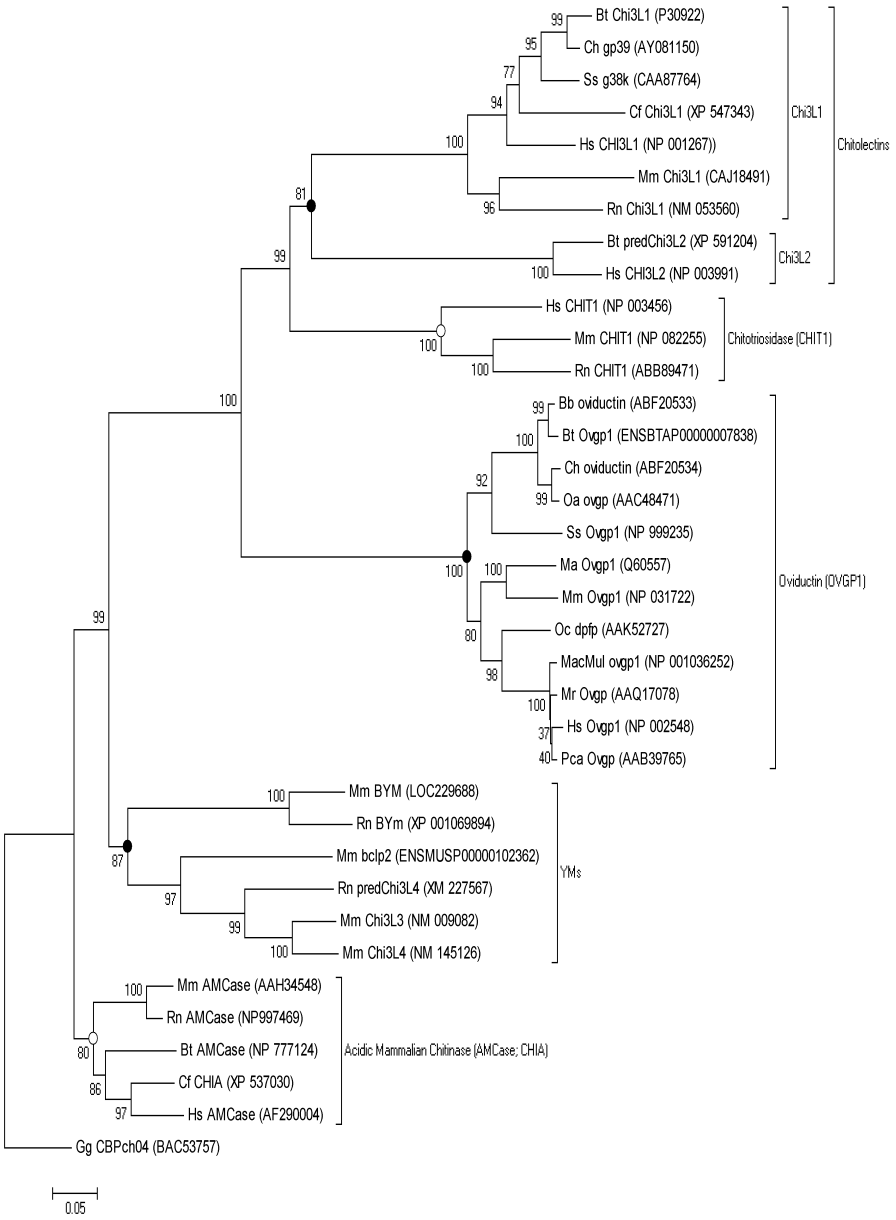

Figure 2.3 The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky and Nei, 1992). The tree is rooted with with *Gallus gallus* CBPch04 as the out-group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl, 1965) and are in the units of the number of amino acid substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Species abbreviations as in figure 2.2 Hypothetical ancestors with enzymatic activity are denoted with a white circle; ancestors without enzyme activity with a black circle.

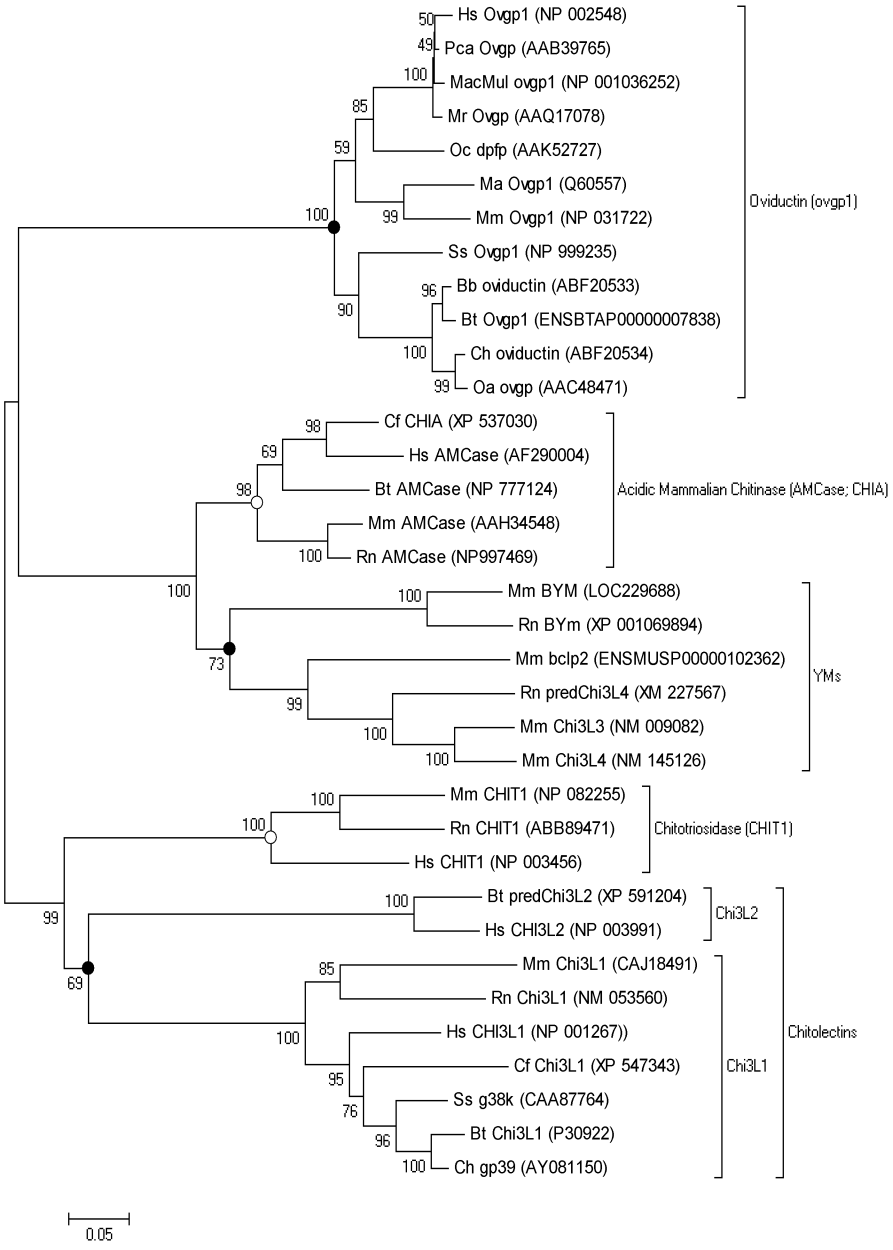

Figure 2.4 The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl, 1965) and are in the units of the number of amino acid substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

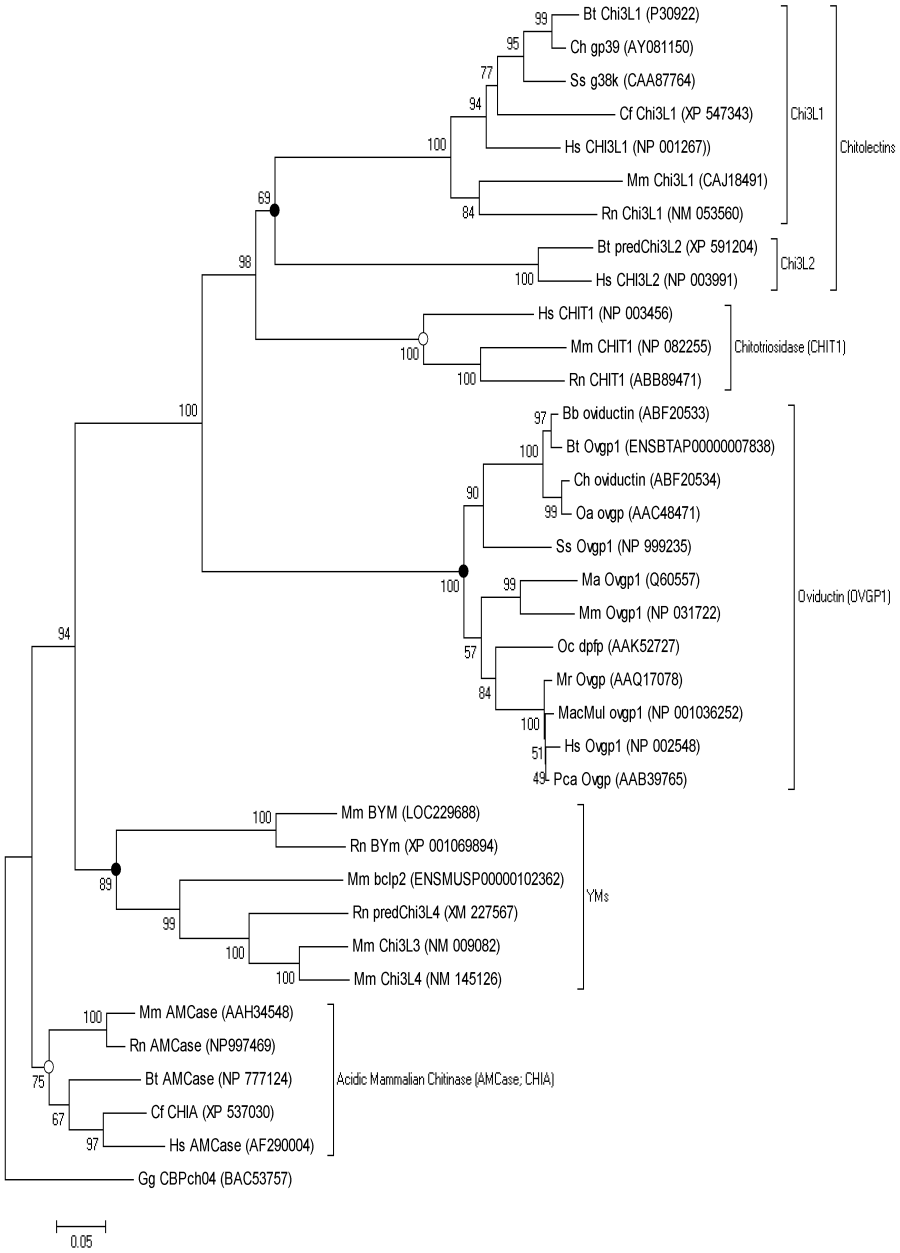

Figure 2.5 The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The tree is rooted with with *Gallus gallus* CBPch04 as the out-group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl, 1965) and are in the units of the number of amino acid substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

2.4.4 Exon-intron analyses and sequence alignment of mammalian chitinase and chi-lectin genes

The exon-intron structures of mammalian chitinases and chi-lectins were derived using ENSEMBL peptide and exon transcripts and PP Hwang's exon-intron drawer (Figures 2.6 and 2.7). The key feature of this analysis is the presence of the conserved secretory signal that covers the first three exons. These figures also illustrate the absence of exon 11 in chi-lectins that encode the chitin-binding domain.

The sizes of mammalian chitinase and chi-lectin gene exons are shown in table 2.2. Exons 2 and 4 of all genes studied, with the exception of *Rattus norvegicus* AMCase and *Homo sapiens* CHID1, are perfectly conserved in terms of their size. The likely unrelated CHID1 consists of 13 exons that show no similarity, in terms of size, with other members of the mammalian chitinase/chi-lectin cluster. This clearly discounts a phylogenetic relationship with the members of the CCGC, a fact that cannot be stated when basing the phylogenetic analysis on sequence homology alone.

The multiple sequence alignment (figure 2.8A) shows that exons 9 and 10 of the genes from the mammalian chitinase/chi-lectin cluster align, with numerous conserved residues. Figures 2.8B and 2.8C show that the chi-lectins have apparently lost exon 11, the region encoding the chitin-binding domain. The presence of residual amino acids at the

start of exon 11 in *Mus musculus* Chi3l3 and Chi3l4, which are not present in Chi3l1 orthologues, suggests that these mutations occurred separately and this is supported by the phylogenetic tree analyses that show these genes in separate subsets.

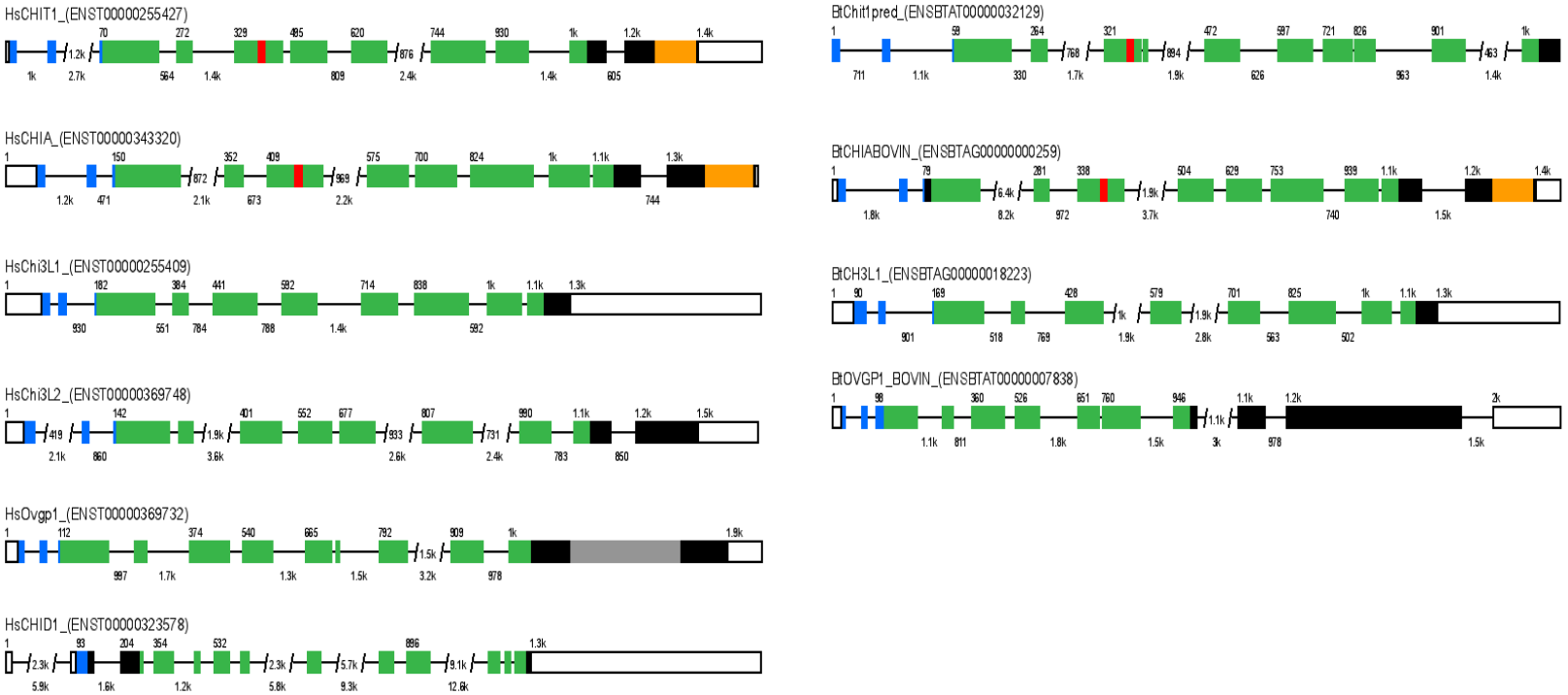

Figure 2.6 Exon-Intron analysis of mammalian (Hs-*Homo sapiens*, Bt- *Bos taurus*) chitinases and chi-lectins using PP Hwang's Exon-Intron Drawer. Blue: classical secretory leader peptide; green: GH18 chitinase domain; red: chitinase active site; orange: chitin binding domain (CBD); grey: Ser-Thr rich mucin domain.

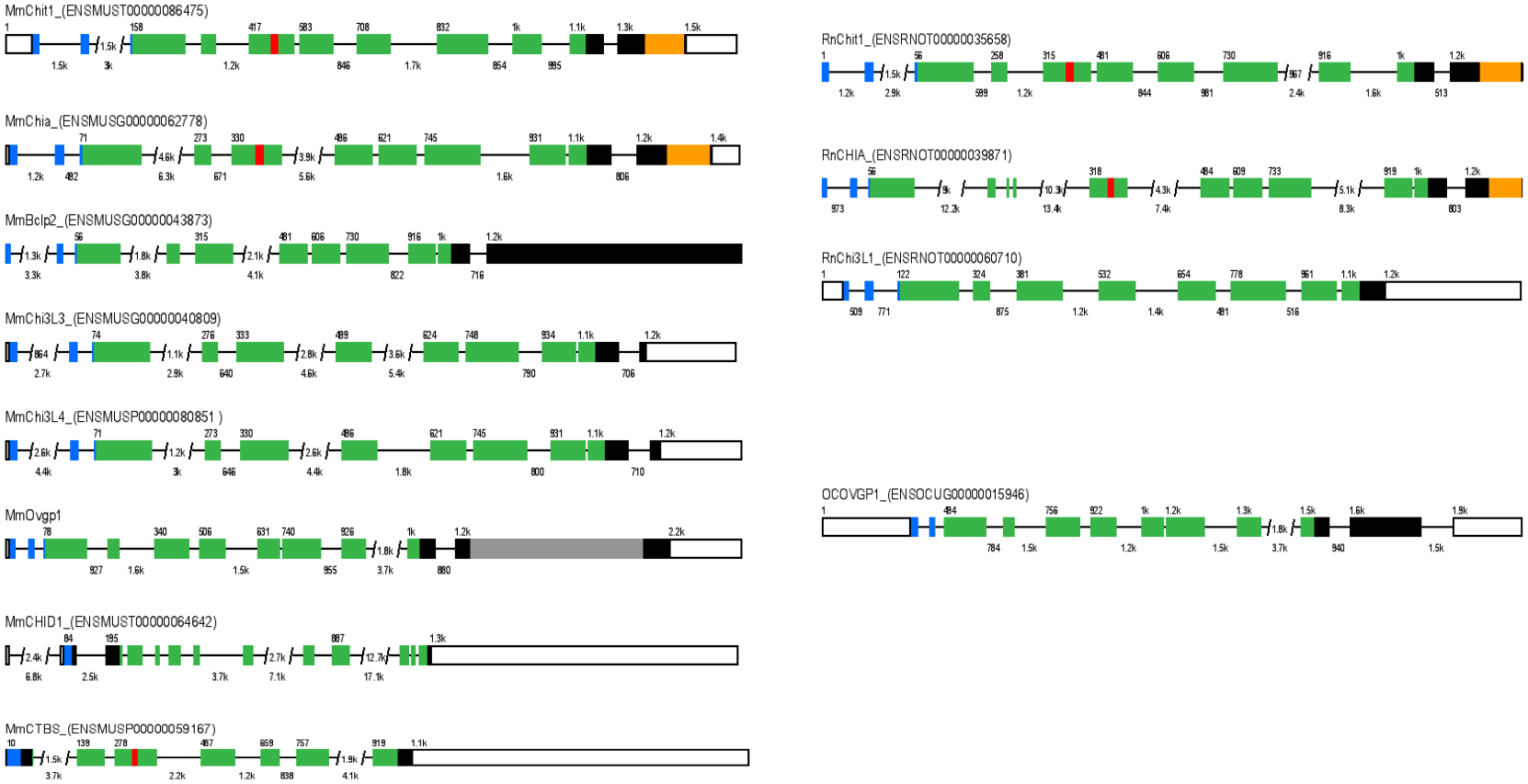

Figure 2.7 Exon-Intron analysis of mammalian (Mm-*Mus musculus*, Rn- *Rattus norvegicus*, Oc- *Oryctolagus cuniculus*) chitinases and chi-lectins using PP Hwang's Exon-Intron Drawer. Blue: classical secretory leader peptide; green: GH18 chitinase domain; red: chitinase active site; orange: chitin binding domain (CBD); grey: Ser-Thr rich mucin domain.

Table 2.2 Exon sizes of mammalian chitinases and chi-lectins, determined using the Ensembl database. With the exceptions of RnCHIA, HsCHID1 and the CTBS orthologs, all genes have perfectly conserved exon sizes of 30bp and 57bp in exons 2 and 4 respectively.

Gene name/ Exon number	1	2	3	4	5	6	7	8	9	10	11	12	13
HsCHIT1	37	30	202	57	166	125	124	186	114	127	467	X	X
MmCHIT1	127	30	202	57	166	125	124	186	108	127	436	X	X
RnCHIT1	25	30	202	57	166	125	124	186	108	127	513	X	X
BtCHIT1		30		57									
HsCHIA	119	30	202	57	166	125	124	186	120	142	263	X	X
MmCHIA	40	30	202	57	166	125	124	186	120	142	342	X	X
RnCHIA	25	30	202	34+11 +15	166	125	124	186	120	142	245	X	X
BtCHIA		30		57									
HsChi3L1	151	30	202	57	151	122	124	183	117	784	X	X	X
MmChi3L1	89	30	205	57	151	122	124	183	117	548	X	X	X
RnChi3L1	81	30	202	57	151	122	124	183	117	591	X	X	X
BtChi3L1	138	30	202	57	151	122	124	183	117	626	X	X	X
CfChi3L1	55	30	202	57	151	125	124	183	117	126	X	X	X
HsChi3L2	111	30	202	57	151	125	130	183	117	140	(222)		
HsOvgp1	81	30	205	57	166	125	109	186	117	136	1029	X	X
MmOvgp1	39	30	205	57	166	125	109	186	117	136	1355	X	X
RnOvgp1		30		57									
BtOvgp1	17	30	205	57	166	125	109	186	117	136	846	X	X
OcOvgp1	36	30	205	57	166	125	109	186	117	136	>344	X	X
MmChi3L3	43	30	202	57	166	125	124	186	120	142	318	X	X
MmChi3L4	40	30	202	57	166	125	124	186	120	142	318	X	X
HsCHID1	49	154	150	133	45	107	62	93	102	156	81	43	1561
HsCTBS	177	139	209	172	98	162	201	X	X	X	X	X	X
MmCTBS	129	139	209	172	98	162	192	X	X	X	X	X	X
RnCTBS	132	139	209	172	98	192	X	X	X	X	X	X	X

A.

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BtChi3L1    ICDFLH-GATTHRFRDQQVPYATKGNQWVAYDDQESVKNKARYLKNRQLAGAMVWALDLD 59
HsChi3L1    ICDFLR-GATVHRILGQQVPYATKGNQWVGYDDQESVKSKVQYLKDRQLAGAMVWALDLD 59
MmChi3L1    ICDFLK-GAEVHRLSNEKVPFATKGNQWVGYEDKESVKNKVGFLKEKKLAGAMVWALDLD 59
MmCHIA      ICTFLRSGATEVWDASQEVPYAYKANEWLGYDNIKSFSVKAQWLKQNNFGGAMIWAIDLD 60
RnCHIA      ICTFLRNGATQDWDAPQEVPYAYKGNEWVGYDNIKSFSVKAQWLKQNNFGGAMIWAIDLD 60
HsCHIA      ICTFLKNGATQGWDAPQEVPYAYQGNVWVGYDNIKSFDIKAQWLKHNKFGGAMVWAIDLD 60
BtCHIA      ICAFLKDGATEAWDDSQNVPYAYKGTEWVGYDNVNSFRIKAQWLKENNFGGAMVWAIDLD 60
MmChi3L4    ICTFLNEGATEIFDATQEVPYAYLGNEWVGYDNVRSFKLKAQWLKDNNLGGAVVWPLDMD 60
MmChi3L3    VCTFLNEGATEVWDAPQEVPYAYQGNEWVGYDNVRSFKLKAQWLKDNNLGGAVVWPLDMD 60
MmChit1     ACSWK----ERHRIEDQKVPYAFQDNQWVSFDDVESFKAKAAYLKQKGLGGAMVWVLDLD 56
HsChit1     VCSWKG--ATKQRIQDQKVPYIFRDNQWVGFDDVESFKTKVSYLKQKGLGGAMVWALDLD 58
HsChi3L2    ICQFLKG-AKITRLQDQQVPYAVKGNQWVGYDDVKSMETKVQFLKNLNLGGAMIWSIDMD 59
             * :            ::**:    . *:.::: .*.  *. ::*.. :.**::* :* *
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B.

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BtChi3L1    DFRGTFCGQNLAFPLTSAIKDVLAEV---------------------------------- 85
HsChi3L1    DFQGSFCGQDLRFPLTNAIKDALAAT---------------------------------- 85
MmChi3L1    DFQGT-CQPKEFFPLTNAIKDALA------------------------------------ 82
MmCHIA      DFTGSFCDQG-KFPLTSTLNKALGISTEGCTAPDVPSEPVTTPP---GSGSGGGSSGGSS 116
RnCHIA      DFTGSFCDQG-KFPLTSTLNKALDIPTAGCTAPDLPSEPVTTPP---GSGSGGGSSGGGS 116
HsCHIA      DFTGTFCNQG-KFPLISTLKKALGLQSASCTAPAQPIEPITAAPSGSGNGSGSSSSGGSS 119
BtCHIA      DFTGTFCNQG-KFPLINTLKDALGLKSATCNASTQSSEP-NSSP---GNESGSGNKSSSS 115
MmChi3L4    DFSGSFCHQG-RFPLTTTLKRDLNVHSASCKASYRGEL---------------------- 97
MmChi3L3    DFSGSFCHQR-HFPLTSTLKGDLNIHSASCKGPY-------------------------- 93
MmChit1     DFKGSFCNQG-PYPLIRTLRQELNLPSETPRSPEQIIPEPRPSS--------MPEQGPSP 107
HsChit1     DFAGFSCNQG-RYPLIQTLRQELSLPYLPSGTPELEVPKPGQPS--------EPEHGPSP 109
HsChi3L2    DFTGKSCNQG-PYPLVQAVKRSLGSL---------------------------------- 84
            ** *  *     :**  ::.  *
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C.

```
BtChi3L1    ------------------------------------------------------
HsChi3L1    ------------------------------------------------------
MmChi3L1    ------------------------------------------------------
MmCHIA      GGSGFCADKADGLYPVADDRNAFWQCINGITYQQHCQAGLVFDTSCNCCNWP 168
RnCHIA      EGSGFCAGKADGLYPVADDRNAFWHCINGITYQQHCQAGLVFDTSCNCCNWP 168
HsCHIA      GGSGFCAVRANGLYPVANNRNAFWHCVNGVTYQQNCQAGLVFDTSCDCCNWA 171
BtCHIA      EGRGYCAGKADGLYPVADNRNAFWNCVNGITYKQNCLTGLVFDTSCHCCNWA 167
MmChi3L4    ------------------------------------------------------
MmChi3L3    ------------------------------------------------------
MmChit1     GLDNFCQGKADGVYPNPGDESTYYNCGGGRLFQQSCPPGLVFRASCKCCTWS 159
HsChit1     GQDTFCQGKADGLYPNPRERSSFYSCAAGRLFQQSCPTGLVFSNSCKCCTWN 161
```

Figure 2.8 Multiple sequence alignment of mammalian chitinases and chi-lectins using ClustalW 2.0 software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). A) exons 9 and 10; B) exons 10 and 11; C) exon 11. Blue- exon 9, Black- exon 10, green exon 11. Consensus symbols: "*"- residues from sequences all identical in that column, ":"- conserved substitutions observed, "."- semi-conserved substitutions observed. The alignment is missing *Rattus norvegicus* chitotriosidase as the transcript is not available.

2.5 DISCUSSION

Using the hidden Markov models of the SUPERFAMILY database, no new chitinases or chi-lectin genes were identified in the mammalian genomes investigated (Table 2.1). The recently described CHID1 gene was not found using this approach, providing further evidence that it is not homologous to others in the cluster. This is consistent with previous phylogenetic analyses that showed CHID1 orthologs to have a low sequence homology to other genes in the cluster (Funkhouser and Aronson, 2007).

The phylogenetic trees shown in figures 2.2 to 2.5, demonstrate that the mammalian chitinase/chi-lectin gene cluster may have originated from a single AMCase-like ancestor gene that underwent subsequent duplication and mutation events to form subsets of chitinase and chi-lectin genes. This analysis is similar to that of Funkhouser *et al*. (2007) who demonstrated that the ancestor gene duplicated to form AMCases and chitotriosidases in one branch and enzymatically inactive oviductins in the other. In the current analysis, however, the original AMCase gene underwent duplication to form the chitotriosidases and further duplication and mutation events to form the Ym orthologs in mice and rats. The chitotriosidase gene underwent further gene duplications and mutations to form the chi-lectins and oviductins.

The multiple sequence alignment (figure 2.8) provides further evidence that Ym1/Ym2 have undergone mutation and chitin-binding domain

loss in a separate event from the chi-lectins. Ym1 and Ym2 still retain the cysteine and a few amino acids of the exon 11 of AMCase (or their common ancestor), so still have 11 exons, but functionally are the equivalent of the chi-lectins that have lost the totality of the terminal exon. This could have been caused by an imperfect gene duplication in the chi-lectins as opposed to a mutation leading to a premature stop codon in Ym1 and Ym2.

Figure 2.9 shows a schematic representation of the evolution of mammalian chitinase and chi-lectin genes, based on our phylogenetic analysis

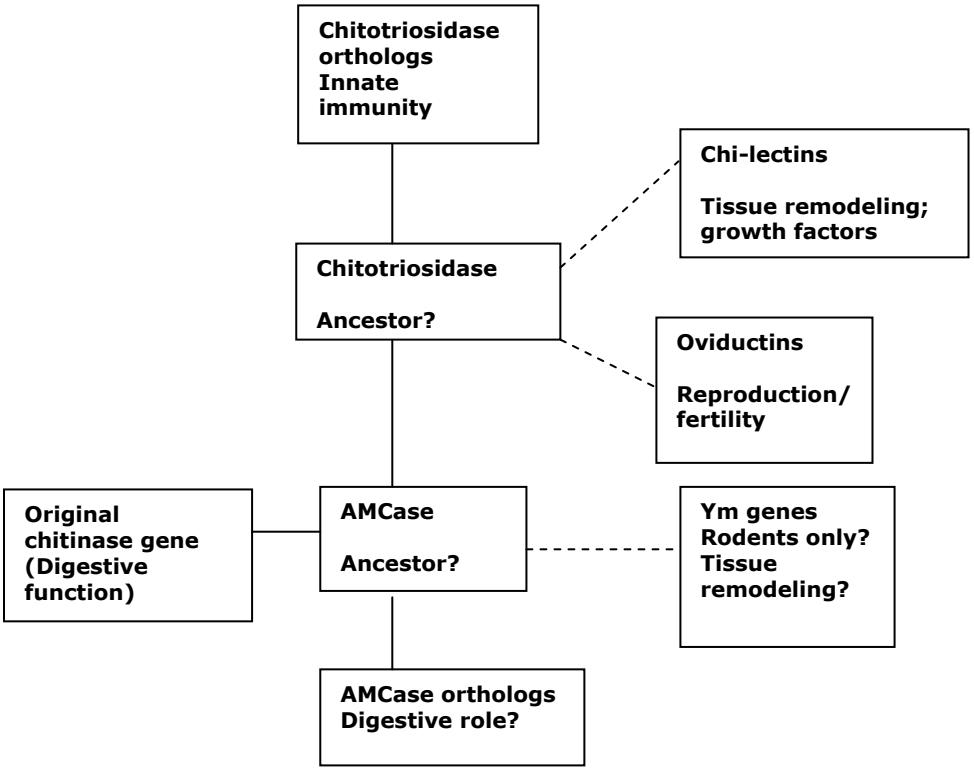

Figure 2.9 Schematic representation of the evolution of mammalian chitinases and chi-lectins based on the phylogenetic analyses presented in figures 2.2-2.5. Dotted lines represent mutations leading to loss of enzymatic function.

The evolution of the mammalian chitinase/chi-lectin cluster from an AMCase ancestor gene suggests that the original chitinase might have had a digestive function, as has been suggested for the current mammalian orthologs (Boot et al., 2001). The requirement for this would be consistent with the digestion of chitinous material by ancient mammalian species. This is also compatible with the distribution of AMCase expression in the body (Boot et al., 2005b), e.g. in parts of the stomach, and its adaptation to low pH (Boot et al., 2001). This role however cannot account for the role that AMCase might play e.g. in the lung. After several gene duplication events, followed by active site mutations and terminal exon loss in a subset of CCGC genes, the genes derived from the AMCase ancestor have evolved various functions. As will be shown in the next two chapters, it can be suggested that the current role of chitotriosidase is to be found in innate immunity. Thus, in this evolutionary model, a digestive enzyme has duplicated and evolved to fulfil new roles in mammals. However the opposite process, the recruitment of an ancient innate immunity gene for digestive purposes, has also been described for lysozyme in cows (Jolles et al., 1984, Dobson et al., 1984).

The exon-intron analyses (figures 2.6-2.8; table 2.2) indicate that members of the chitinase/chi-lectin gene cluster share a highly conserved, characteristic gene signature. This is exemplified by the conserved exon sizes (30bp and 57bp) of exons 2 and 4 respectively and the secretory signal that spans exons 1-3. As shown in Table 2.2,

these features are common to all member of the cluster in mammalian species except *Rattus norvegicus* AMCase, *Homo sapiens* CHID1, that has a distinct 13 exon gene structure, and *Homo sapiens* CTBS.

The preservation and divergence of mammalian chitinase and chi-lectin genes may have resulted from the presence of the $(\alpha/\beta)_8$ TIM barrel (described in section 1.3.3 and figure 5.2) that is a conserved motif with a high level of structural stability. The significance of this is that the structural fold, which is one of the most commonly encountered protein folds in nature, is particularly resistant to mutations that allow small changes in amino acid sequence which otherwise may affect its conformation, and therefore allow proteins containing this motif to acquire new functions following duplication and mutation events (Wise and Rayment, 2004). This is shown in the evolution of chi-lectins that despite losing a whole exon as well as a functional active site, have evolved entirely new functions, including the proliferative effect of Chi3L1 on connective tissues (Recklies et al., 2002).

3. INDUCTION OF *CHIT1* EXPRESSION BY PATHOGEN ASSOCIATED MOLECULAR PATTERNS (PAMPS) AND *C. ALBICANS*

3.1 INTRODUCTION

The human chitotriosidase gene (*CHIT1*) is highly expressed in the lymph nodes, bone marrow and lung (Boot et al., 2005b). There is also a high level of expression in the eye and in particular in the lachrymal glands. Additionally, there are measurable amounts of chitotriosidase activity in human reflex tears (Hall et al., 2007a) and (chapter 4). At a cellular level, constitutive expression occurs in macrophages but not in monocytes, as confirmed by northern blot analysis (Boot et al., 1995). The enzyme is also present in mature granulocytes where it is stored in the specific granules (van Eijk et al., 2005) (Boussac and Garin, 2000).

It has been demonstrated that *CHIT1* expression can be induced by TNF-α, LPS, prolactin and IFN-γ; with maximum increases in expression observed within two to four hours of treatment in mature macrophages (Malaguarnera et al., 2005) (Malaguarnera et al., 2004). However prolonged treatment with IFN-γ over twenty-four to forty-eight hours prevents induction of *CHIT1* and the cytokine also inhibits expression in differentiating monocytes (van Eijk et al., 2005).

Granulocyte macrophage colony-stimulating factor (GM-CSF) has several effects on macrophages *in vitro*. In regard to chitinases, it induces *CHIT1* expression in cells already expressing the enzyme (van Eijk et al., 2005). It also facilitates the survival of differentiating monocytes in long term culture (Eischen et al., 1991), a process that may account for increases in chitinase activity observed in culture supernatants. GM-CSF also causes release of chitotriosidase through degranulation of the specific granules of neutrophils (van Eijk et al., 2005). The observation that GM-CSF administration improves the prognosis of individuals suffering from invasive fungal infections (Jones, 1999) may therefore in part be explained by increased chitotriosidase activity.

The observation that chitotriosidase levels in plasma and urine are elevated in neonates with *C. albicans* and *A. niger* infection and that increased chitinase activity is detected in blood and tissues of guinea-pigs with *A. fumigatus* infection (Labadaridis et al., 2005) (Overdijk et al., 1999, Overdijk et al., 1996) supports the hypothesis that chitotriosidase is involved in anti-fungal immunity. Elevated activity in tissues may result from increased macrophage differentiation and therefore increased constitutive expression or by subsequent priming and activation by T cells and cytokines including IL-3 following infection (North, 1974) (Frendl, 1992).

Further increases in *CHIT1* expression and chitotriosidase activity may also occur as a result of innate immune recognition, resulting from the interaction between pathogen associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs), as previously described. In fungi, the main PAMPs are structural components of the cell wall and their location and immunogenicity ultimately affect the outcome of the host response.

Fungal recognition is greatly dependent on the interaction between cell wall components including β-glucan and specific PRRs. In *C. albicans*, pathogenicity is enhanced by switching from yeast to hyphal growth. It has been shown that the main non-opsonic β-glucan receptor (dectin-1) is unable to recognize *C. albicans* hyphae (Gantner et al., 2005). However in the yeast form, β-glucan becomes exposed following the formation of permanent bud scars that allow interaction with dectin-1 (Brown et al., 2002).

Dectin-1 was the first non-TLR PRR discovered that can mediate its own signaling; however many responses including the induction of TNF-α and IL-12 are dependent on collaborative signals between dectin-1 and TLR-2 (Brown et al., 2003) (Rogers et al., 2005) (Steele et al., 2003). In terms of intracellular signaling, dectin-1 independent responses usually involve activation of SH2-domain-containing protein spleen tyrosine kinase (Syk). However the utilization of non-Syk

pathways by dectin-1 appears to be dependent on collaborative interaction with TLRs (Brown, 2006).

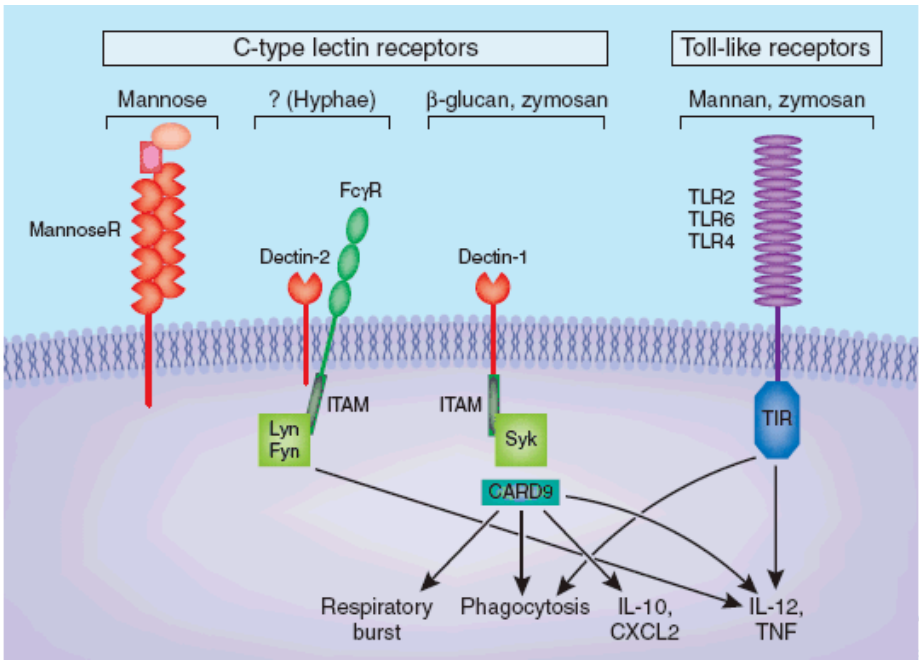

Figure 3.1 Membrane receptors and signaling pathways involved in fungal recognition. Dectin-1 signaling is dependent on Syk tyrosine kinase or collaborative signaling with TLRs. CARD9 is a linker molecule required in dendritic cell signaling for activation of NF-κB. Dectin-2 is a recently identified PRR and appears to be involved in hyphal recognition, however its specific PAMPs have yet to be identified. Dectin-2 lacks an intracellular signaling motif and therefore is likely to collaborate with FcγR (Dostert and Tschopp, 2007).

In addition to fungal recognition, it has recently been shown using knock-out mice that mouse dectin-1 and TLR-2 collaboration may be involved in certain anti-bacterial responses including TNF-α production in macrophages in response to *Mycobacterium smegmatis* (*M. smegmatis*) and *Bacillus Calmette – Guerin* (BCG) (Yadav and Schorey, 2006). This suggests that dectin-1 in humans and its signaling with TLR-2 may not be limited to anti-fungal immunity.

The recognition of fungal PAMPs by PRRs does not exclusively involve dectin-1 TLR interactions. In other cell types including

dendritic cells, recognition of β-glucan is achieved by receptors including complement receptor 3 (CR3) and natural killer (NK) cells that do not express dectin-1 (Taylor et al., 2002). The mannose receptor (MR) is required by dendritic cells for internalization and clearance of fungal particles, however this process appears to be dependent on prior opsonization (Romani, 2004).

In addition to the process of fungal recognition shown in figure 3.1, TLRs are also involved in the interactions with other microbial PAMPs. In Gram-positive bacteria, TLR-2 is required for the recognition of peptidoglycan and lipoteichoic acid (Schwandner et al., 1999). However experiments with microglia from TLR-2 knockout mice showed that this receptor had no effect on the production of pro-inflammatory cytokines in response to *Staphylococcus aureus* (*S. aureus*) (Kielian et al., 2005) suggesting a more complex system of detection *in vivo*. The recognition of LPS from *Escherichia coli* (*E. coli*) and that of most Gram- negative bacteria is dependent on TLR-4. Mutations in the TLR-4 gene in humans are associated with impaired immune response to LPS (Arbour et al., 2000).

3.2 OBJECTIVES

It has previously been shown by northern blot that constitutive chitotriosidase expression increases as macrophages differentiate from peripheral blood monocytes *in vitro* (Boot et al., 1995). The first objective of this work was to use RT-qPCR to measure relative changes in chitotriosidase expression over a period of seven days during monocyte differentiation, in the presence of GM-CSF.

In addition to constitutive expression in monocyte derived macrophages, it has been demonstrated that *CHIT1* expression can be induced by the cytokines IFN-γ and TNF-α, the peptide hormone prolactin and LPS from the membrane of Gram-negative bacteria (Malaguarnera et al., 2005) (Malaguarnera et al., 2004). The second objective was to measure relative changes in *CHIT1* and TNF-α (as a positive control) expression following treatment with fungal and bacterial PAMPs and live *C. albicans* by RT-qPCR.

Prolactin is a peptide hormone that is secreted by acidophilic cells of the adenohypophysis (Herlant and Pasteels, 1959) where its production is linked to physiological functions in growth and development. It is also found in the uterine lining and breast tissues (Ben-Jonathan et al., 1996), reflecting its role in lactogenesis and other reproductive processes. Immune cells are one of the main sources of prolactin in these tissues and this, coupled with the expression of the prolactin

receptor (PRL-R) on leukocytes and induction of macrophage activation and T lymphocyte function, suggests that prolactin acts as a cytokine as well as a peptide hormone (Malaguarnera et al., 2004) (Ben-Jonathan et al., 1996).

The intracellular mechanisms by which prolactin exerts its immunomodulatory effects primarily involve the p38 mitogen-activated protein kinase (MAPK) biochemical pathway (as described previously for LPS) (Dogusan et al., 2001) and the Janus kinase/ signal transducers and activators of transcription (JAK/Stat) pathway. JAK/Stat is activated by PRL-R dimerization and requires the translocation of Stat-1, 3 and 5 molecules into the nucleus (DaSilva et al., 1996), resulting in upregulated gene expression and pro-inflammatory effects including generation of ROS and degranulation (Clevenger et al., 1998).

N-formylated peptides are derived from mitochondrial and bacterial proteins and are potent neutrophil activators. The synthetic peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) binds to the G-protein-coupled receptor Formyl peptide receptor 1 (FPR1) (Boulay et al., 1990) and induces changes in gene transcription through signaling mechanisms dependent on tyrosine phosphorylation by the kinases MAPK p38 and PI3K (as described in section 1.2.1) (LoGrasso et al., 1997). However processes including neutrophil degranulation following stimulation by fMLP appear to require the Src family

kinases Fgr and Hck (Mocsai et al., 1999). Furthermore, the release of granular contents appears to be dependent on more receptor-distal signaling molecules including protein tyrosine phosphatase non-receptor type 9 (PTPN9) and soluble NSF attachment receptor (SNARE) kinase, which are involved in membrane fusion between the granule and the plasma membrane, prior to exocytosis (Lacy and Eitzen, 2008).

The final objective of this work was to test the effects of prolactin and fMLP on the release of chitotriosidase from neutrophil granules using a 4-MU chitotrioside assay.

3.3 METHODS

3.3.1 Isolation of human mononuclear cells and granulocytes with Percoll

Human peripheral blood was drawn from healthy donors, mixed with 3.2% sodium citrate and centrifuged at 350x*g* for 20 minutes. Platelet-rich plasma (PRP) was removed using a sterile pipette and the pellet was resuspended in 6mls of a 6% dextran (Dextran 500, Amersham Biosciences) solution and made up to 50ml with NaCl buffer. Following a 25 minute incubation period at room temperature, the leukocyte-rich upper layer was removed and centrifuged at 350xg for 6 minutes in NaCl buffer.

Percoll (Amersham Biosciences) gradients were prepared using a stock isotonic (90%) solution and diluting in DPBS (without Mg^{2+} / Ca^{2+}) (Cambrex) to produce 81%, 70% and 55% Percoll solutions. The leukocyte rich pellet was resuspended in 55% Percoll and gradients were prepared by overlaying this on the 70% and 81% Percoll layers and cell fractions produced by centrifuging at 720xg for 20 minutes without brake.

Mononuclear cells were isolated from the resulting 55/70% interface and granulocytes were isolated from the 70/81% interface. Cells were washed twice in DPBS and cell number was determined using a

Neubauer haemocytometer. Cell purity was ascertained by flow cytometry and shown to be in excess of 95%.

3.3.2 Preparation of autologous serum

Autologous serum was prepared for use in macrophage culture from human monocytes. Calcium chloride ($CaCl_2$) was added to PRP (220µl/ 10ml) in glass tubes, incubated at 37ºC for 1 hour and centrifuged at 4000x*g* for 15 minutes to remove fibrin clots. Autologous serum was stored in aliquots at -20ºC.

3.3.3 Isolation of monocytes with ficoll-paque plus

Human peripheral blood was drawn from healthy donors, mixed with 3.2% sodium citrate and diluted in an equal volume of DPBS. Diluted blood was overlayed on ficoll (StemCell Technologies) (1:1.5) and centrifuged at 400xg for 30 minutes without brake. The mononuclear cell layer was removed from the plasma/ficoll interface and washed twice in DPBS.

3.3.4 Purification of monocytes and neutrophils

Monocytes were isolated from mononuclear cells by $CD14^+$ selection and neutrophils from granulocytes by $CD16^+$ selection using magnetic cell separation (MACS) following the manufacturers instructions (Miltenyi Biotec). Purified monocytes were washed twice in DPBS and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma-Aldrich). IMDM was supplemented with 5% autologous

serum, 1% penicillin- streptomycin (Gibco), 1% L-glutamine (Sigma-Aldrich) and 5 ng/ml of GM-CSF (Peprotech). To prevent the potential activity of any contaminating endotoxin, 20μg/ml of the antibiotic Polymyxin B (Sigma-Aldrich) was added.

3.3.5 Treatment of macrophages with *C. albicans* and PAMPs

Following 8 days of culture to allow differentiation, macrophages were treated with either live or heat killed *C. albicans* (SC 5314 clinical isolate (Gillum et al., 1984), or fungal or bacterial PAMPs. Treated cells and untreated controls were incubated at 37°C with 5% CO_2 for 2-8 hours in the presence of 20μg/ml Polymyxin B, with the exception of those treated with LPS. Macrophages were cultured with PAMPs at the following concentrations: (*S*)-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-*N*-palmitoyl-(*R*)-Cys-(*S*)-*S*)-Lys_4-OH trihydrochlo-ride (Pam_3Cys, Alexis Corporation): 5 μg/ml. Recombinant flagellin (Alexis Corporation): 50 ng/ml; zymosan (Sigma-Aldrich), laminarin (Sigma-Aldrich) and mannan (Sigma-Aldrich): 100 μg/ml. LPS 055:B5 (Sigma-Aldrich): 50 ng/ml. Peptidoglycan (from *B. subtilis*, Fluka): 100 ng/ml. Prolactin (recombinant human, PeproTech): 25ng/ml.

3.3.6 RNA extraction and reverse transcription

Following incubation, RNA was isolated from macrophages using the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich) following the manufacturer's instructions. RNA quality was assessed

using a 2100 Bioanalyzer (Agilent Technologies) and only RNA integrity numbers (RINs) higher than 7 were deemed acceptable for RT-qPCR (Fleige and Pfaffl, 2006). Generation of cDNA from total RNA was achieved using the GeneAmp RNA PCR core kit (Applied Biosystems) as directed by the manufacturer.

3.3.7 Primer design and quantitative PCR optimization

Primers were designed using the Primer3 software (Rozen and Skaletsky, 2000) and following the guidelines for RT-qPCR with SYBR-green I fluorescence (Ponchel et al., 2003). For each primer pair one primer was designed across an exon boundary to prevent amplification of genomic DNA. To ensure the absence of near and exact matches, primer sequences were checked for regions of similarity using nucleotide blast ('search for short, nearly exact matches') on the NCBI database.

Primer validity was determined using the melt curve functionality with the Icycler IQ multicolor real-time PCR detection system (Optical system software 3.1, Bio-Rad) and annealing temperatures were raised to 62°C to prevent the formation of unspecific products. Gene efficiency values were determined by 5x5-fold serial dilutions of macrophage cDNA. Each RT-qPCR reaction contained the following: 1x iQ SYBR Green Supermix (Biorad), 200nM forward and 200nM reverse primers (Sigma-Genosys), 5ng cDNA and DEPC-treated water to a final volume of 25µl.

Table 3.1 Forward and reverse primer sequences used in RT-qPCR experiments.

Gene	Sequence	Product (bp)
Hs *CHIT1* for	**5'-CCCTGGTACAGGACTTGGC-3'**	
Hs *CHIT1* rev	**5'-ACCTCGTATCCAGCATCCAC-3'**	**121**
Hs *TNF-α* for	**5'-CCATGTTGTAGCAAACCCTCA-3'**	
Hs *TNF-α* rev	**5'-TGAGGTACAGGCCCTCTGAT-3'**	**130**
Hs *GAPDH* for	**5'-CGTGGAAGGACTCATGACC-3'**	
Hs *GAPDH* rev	**5'-GAGGCAGGGATGATGTTCTG-3'**	**120**
Hs *APRT* for	**5'-CTGGAGTACGGGAAGGCTG-3'**	
HS *APRT* rev	**5'-AGATCATCCACGACGACCAC-3'**	**80**

3.3.8 Data analyses

Relative expression ratios and statistical significance were determined using the Relative Expression Software Tool: Multiple Condition Solver (REST-MCS) (Pfaffl et al., 2002) with GAPDH and APRT as reference genes. The pair-wise fixed reallocation randomisation test used 2000 permutations to determine *P*- values. Data was presented using SigmaPlot 8.0.

3.3.9 Neutrophil degranulation studies

Neutrophils were washed twice in DPBS (Cambrex) and resuspended in Roswell Park Memorial Institute (RPMI) medium (Sigma) at a density of $1x10^6$/ml. Cells were incubated at 37°C with human recombinant prolactin (Peprotec) (5-100ng/ml), fMLP (Sigma) (2-1000nM) or left untreated. After 15 minutes, neutrophils were spun at 300*xg* for 10 minutes and supernatants were removed. Remaining pellets were sonicated and chitotriosidase activity was determined using a 4-MU chitotrioside assay: 4-Methylumbelliferyl β-D-N,N′,N′-triacetylchitotrioside hydrate (4-MU chitotrioside) (Sigma) was dissolved 1:1 in DMF and water to form a 2.23mM (100x) stock solution. 4-MU was diluted in citrate-phosphate buffer (pH 5.2) and mixed with 1µl of supernatant, lysate or enzyme solution in triplicate and incubated for 30 minutes at room temperature with 4-MU chitotrioside plus buffer and buffer alone as negative controls. After 30 minutes, the reaction was stopped with 2mls of glycine buffer (pH 10.6) and the tubes were placed on ice for 2 minutes. The fluorescence was measured on an MFX microtiter plate fluormeter with Revelation 4.21 software (Dynex Technologies) with excitation at 365nm and emission at 450nm.

3.4 RESULTS

3.4.1 Expression of chitotriosidase RNA during *in vitro* differentiation of peripheral blood monocytes to macrophages

Firstly the relative changes in expression of *CHIT1* RNA between freshly isolated peripheral blood monocytes and those cultured between 1-7 days were determined by RT-qPCR. *CHIT1* expression increased steadily over this period, reaching an approximately 1000-fold increase compared to day 0 at the end of the week (Fig. 3.2A). This is consistent with the elevated chitotriosidase mRNA expression shown by Northern blot analysis over 21 days of culture (Boot et al., 1995).

Differentiated macrophages therefore express high amounts of *CHIT1* constitutively, and any further induction will be from an already elevated baseline. Conversely, the level of TNF-α expression in unstimulated macrophages is low and significantly decreases over the same period (Fig. 3.2B). The roles TNF-α in inducing apoptosis, cell proliferation and inflammation require its expression to be highly controlled to prevent the development of persistent inflammatory response and subsequent autoimmune diseases including rheumatoid arthritis (Locksley et al., 2001). A low level of expression is therefore consistent with this notion. Reduced TNF-α expression may also result from the removal of contaminating endotoxin through the addition of Polymyxin B to the culture medium.

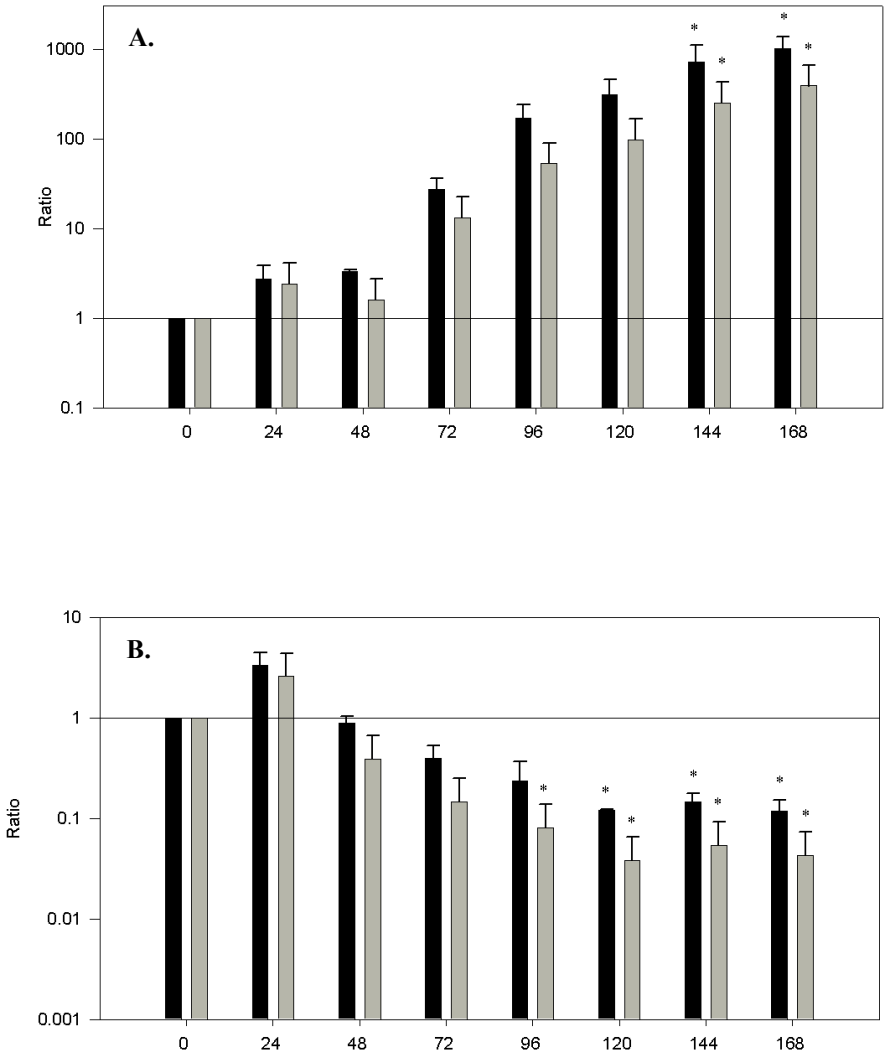

Figure 3.2 Detection of chitotriosidase (A) and *TNF-α* (B) expression in differentiating peripheral blood monocytes over 7 days by quantitative PCR. Ratios were calculated using the Pfaffl equation: ratio= $(E\text{target})^{\Delta CP_{target}(control\text{-}sample)} / (E\text{ref})^{\Delta CP_{ref}(control\text{-}sample)}$. Black bars: normalization with GAPDH; grey bars: normalization with APRT. Statistical analyses were performed using one-way ANOVA followed by LSD post-hoc test; **p* <0.05 time 0 vs. subsequent time point.

A comparison of mean RT-qPCR cycle threshold values (C_t) of each gene for unstimulated macrophages from twenty- six different experiments shows that in 90% of the cases the relative expression levels were: GAPDH> *CHIT1*> APRT>> TNF-α, and in 5% of the cases *CHIT1* levels exceeded GAPDH levels in the same sample. In stimulated samples where *CHIT1* was induced, the levels of expression were invariably raised above GAPDH levels, whereas TNF-α levels, despite the strong relative increase in comparison to resting macrophages, were not. This shows that *CHIT1* expression is both highly expressed constitutively and also inducible with appropriate stimuli.

3.4.2 Induction of chitotriosidase mRNA expression by PAMPs in macrophages and enzymatic activity in neutrophils

The effect of different fungal and bacterial PAMPs on monocyte-derived macrophages has been studied. Chitotriosidase RNA expression levels were measured by RT-qPCR, and normalized using GAPDH and APRT as reference genes (as described in Methods). Figures 4.2-4.4 show the expression levels of chitotriosidase and TNF-α mRNA every two hours after stimulation with fungal PAMPs.

Laminarin and zymosan (Fig. 3.3A and 3.4A), which consist of β-glucan and a mix of β-glucan and mannan respectively, induced an approximately sixteen-fold mean increase in *CHIT1* expression, whereas mannan (Fig. 3.5A) had no significant effect.

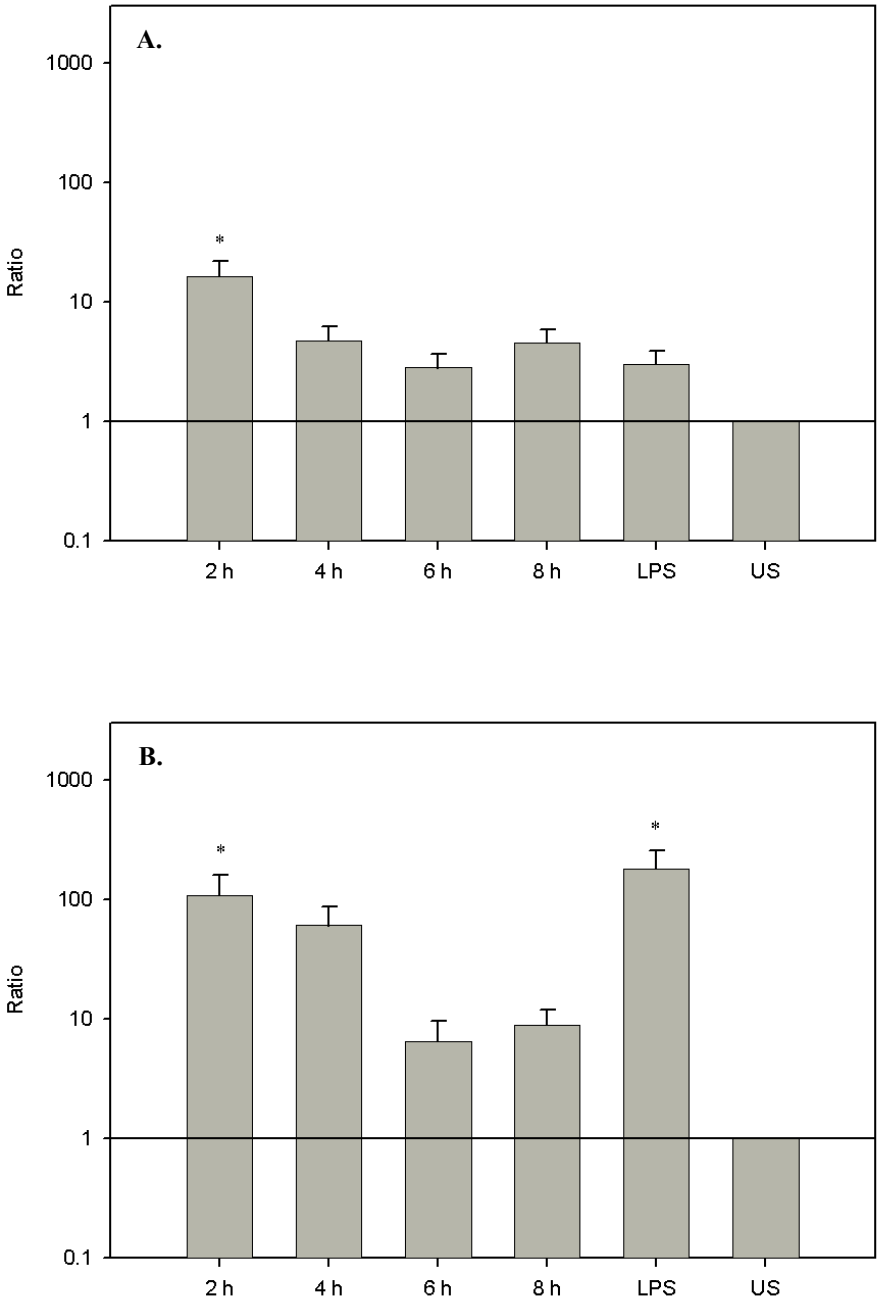

Figure 3.3 Time course of chitotriosidase (A) and TNF-α (B) mRNA expression after incubation of peripheral blood monocyte derived macrophages with 100 μg/ml of laminarin. Following quantitative PCR, duplicate *Ct* values for each treatment were normalized using the housekeeping genes GAPDH and APRT and expression ratios determined using the Pfaffl equation (Pfaffl, 2001). Each experiment was run in triplicate and repeated with three different donors with comparable results. Statistical significance between treated and untreated cells was determined by REST-MCS (Pfaffl et al., 2002); **P* <0.05.

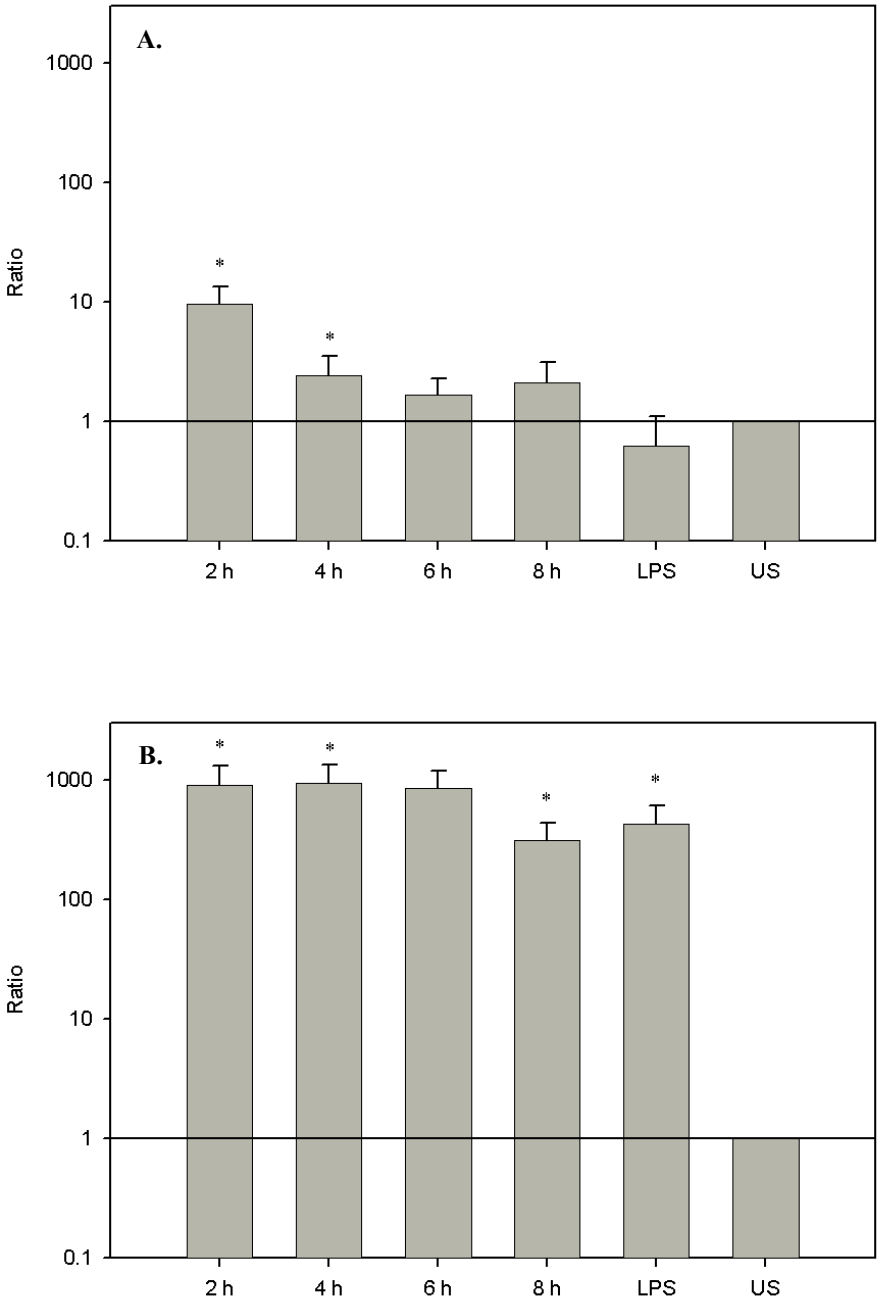

Figure 3.4 Time course of chitotriosidase (A) and TNF-α (B) mRNA expression after incubation of peripheral blood monocyte derived macrophages with 100 μg/ml of zymosan. Following quantitative PCR, duplicate *Ct* values for each treatment were normalised using the housekeeping genes GAPDH and APRT and expression ratios determined using the Pfaffl equation (Pfaffl, 2001). Each experiment was run in triplicate and repeated with three different donors with comparable results. Statistical significance between treated and untreated cells was determined by REST-MCS (Pfaffl et al., 2002); **P* <0.05.

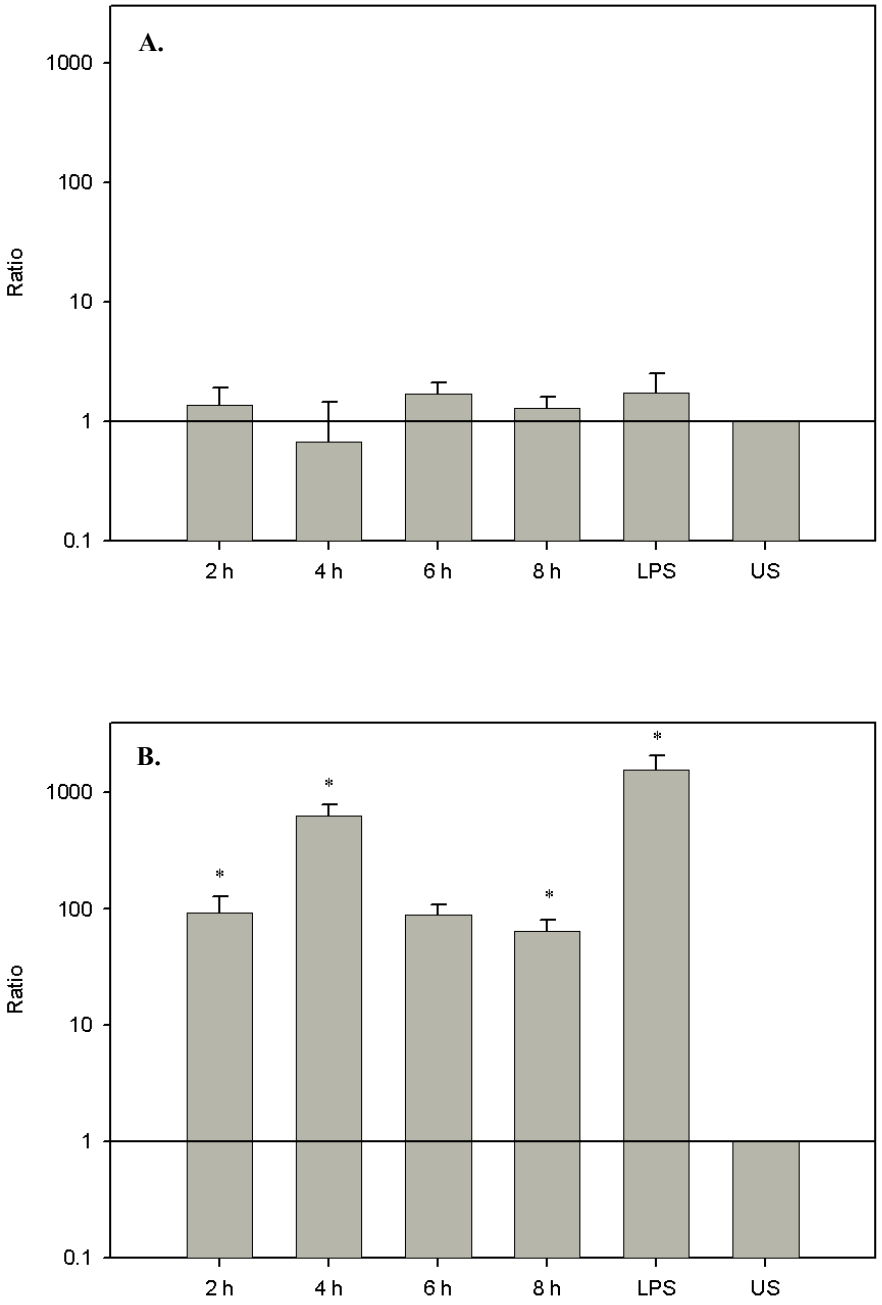

Figure 3.5 Time course of chitotriosidase (A) and TNF-α (B) mRNA expression after incubation of peripheral blood monocyte derived macrophages with 100 μg/ml of mannan. Following quantitative PCR, duplicate *Ct* values for each treatment were normalised using the housekeeping genes GAPDH and APRT and expression ratios determined using the Pfaffl equation (Pfaffl, 2001). Each experiment was run in triplicate and repeated with three different donors with comparable results. Statistical significance between treated and untreated cells was determined by REST-MCS (Pfaffl et al., 2002); **P* <0.05.

LPS has previously been shown to induce *CHIT1* expression and was therefore included as a positive control (50ng/ml) (Di Rosa 2005). As shown in figure 3.6, LPS induced TNF-α expression on average for all experiments, but had no significant effect on *CHIT1* expression. This however is consistent with our previous suggestion that *CHIT1* is induced primarily by fungal and not bacterial PAMPs.

The bacterial PAMPs tested for their effects on *CHIT1* expression were recombinant flagellin, peptidoglycan and Pam_3Cys. As shown in figures 3.7A-3.9A, the bacterial PAMPs induced no significant changes in *CHIT1* expression at any time point over the eight hours. There was a significant increase in TNF-α expression following bacterial PAMP treatment on average for at least one time point compared to the untreated control.

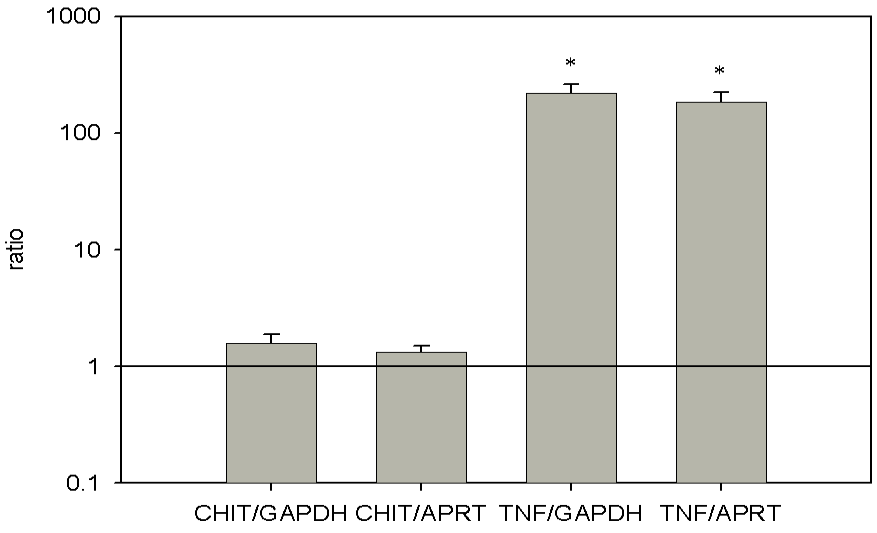

Figure 3.6 Changes of chitotriosidase and TNF-α mRNA expression levels in MDMs stimulated with 50 ng/ml LPS (n=26) compared to unstimulated cells (a ratio of 1 indicates no significant change in gene expression between treatments). GAPDH and APRT were used as reference genes for normalization, with similar results. While LPS up-regulated TNF-α more than 100-fold, it had no effect on *CHIT1* expression. **P*<0.05 using one-way ANOVA.

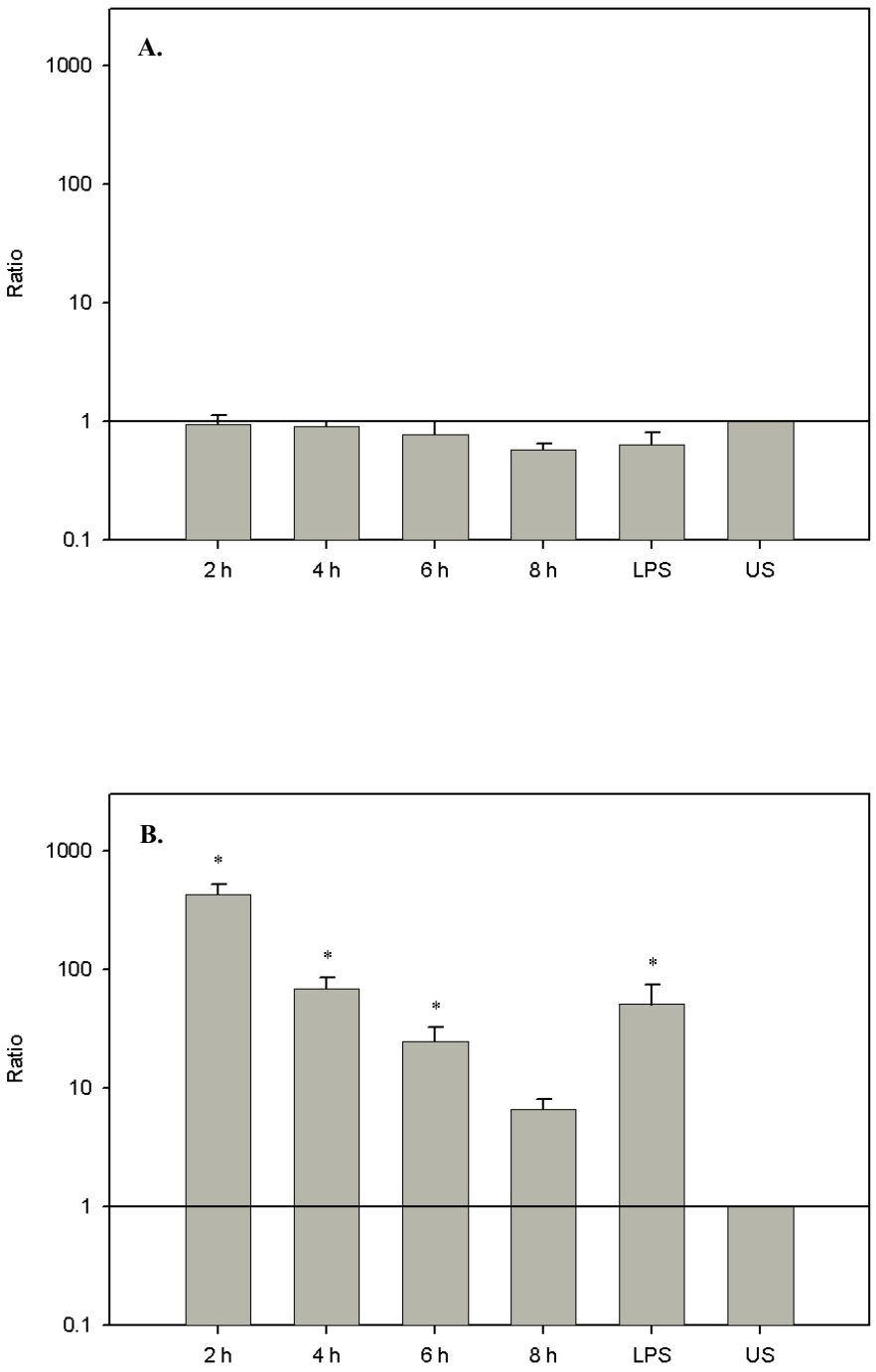

Figure 3.7 Time course of chitotriosidase (A) and TNF-α (B) mRNA expression after incubation of peripheral blood monocyte derived macrophages with 50ng/ml recombinant flagellin. Following quantitative PCR, duplicate *Ct* values for each treatment were normalised using the housekeeping genes GAPDH and APRT and expression ratios determined using the Pfaffl equation (Pfaffl, 2001). Each experiment was run in triplicate and repeated with three different donors with comparable results. Statistical significance between treated and untreated cells was determined by REST-MCS (Pfaffl et al., 2002); **P* <0.05.

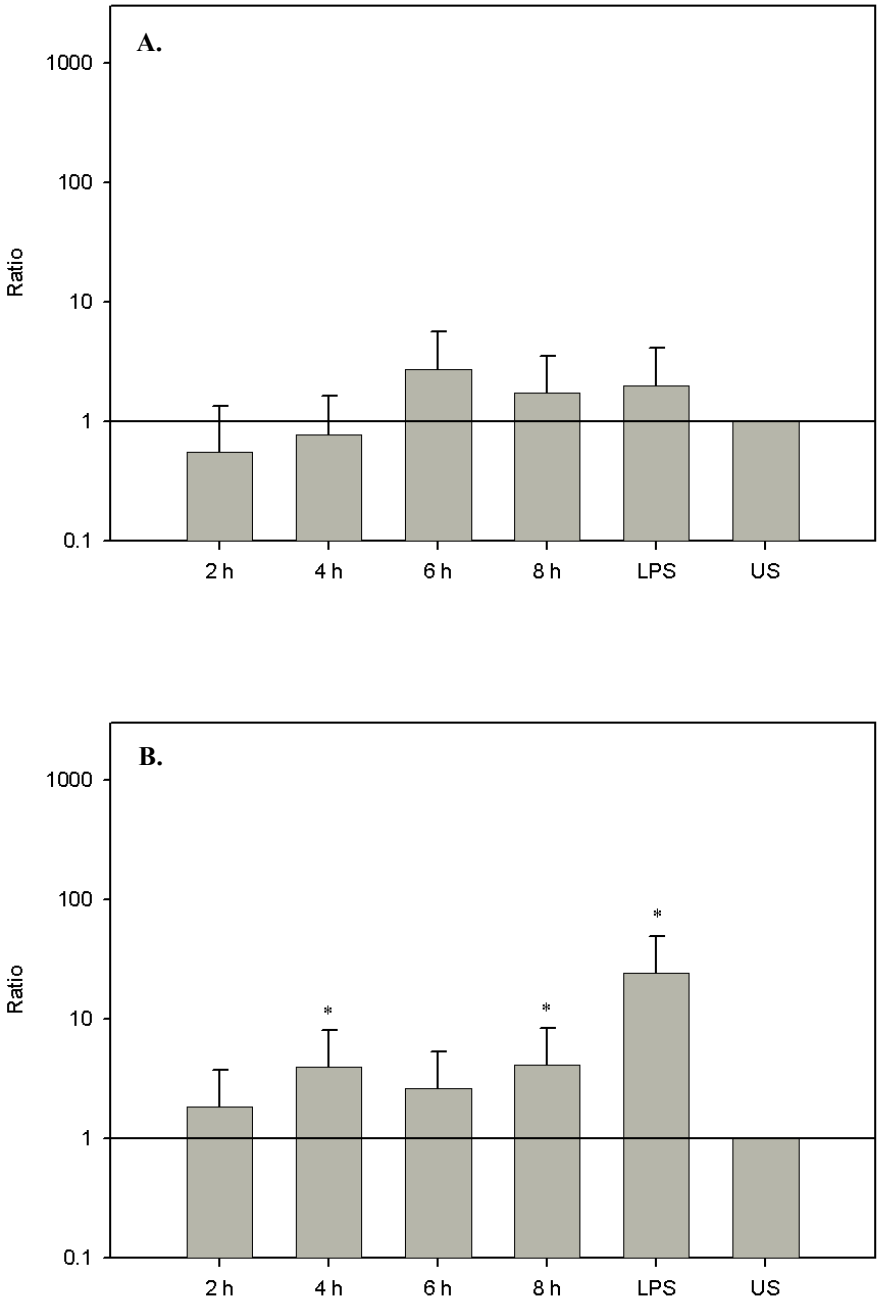

Figure 3.8 Time course of chitotriosidase (A) and TNF-α (B) mRNA expression after incubation of peripheral blood monocyte derived macrophages with 100ng/ml peptidoglycan. Following quantitative PCR, duplicate *Ct* values for each treatment were normalised using the housekeeping genes GAPDH and APRT and expression ratios determined using the Pfaffl equation (Pfaffl, 2001). Each experiment was run in triplicate and repeated with three different donors with comparable results. Statistical significance between treated and untreated cells was determined by REST-MCS (Pfaffl et al., 2002); **P* <0.05.

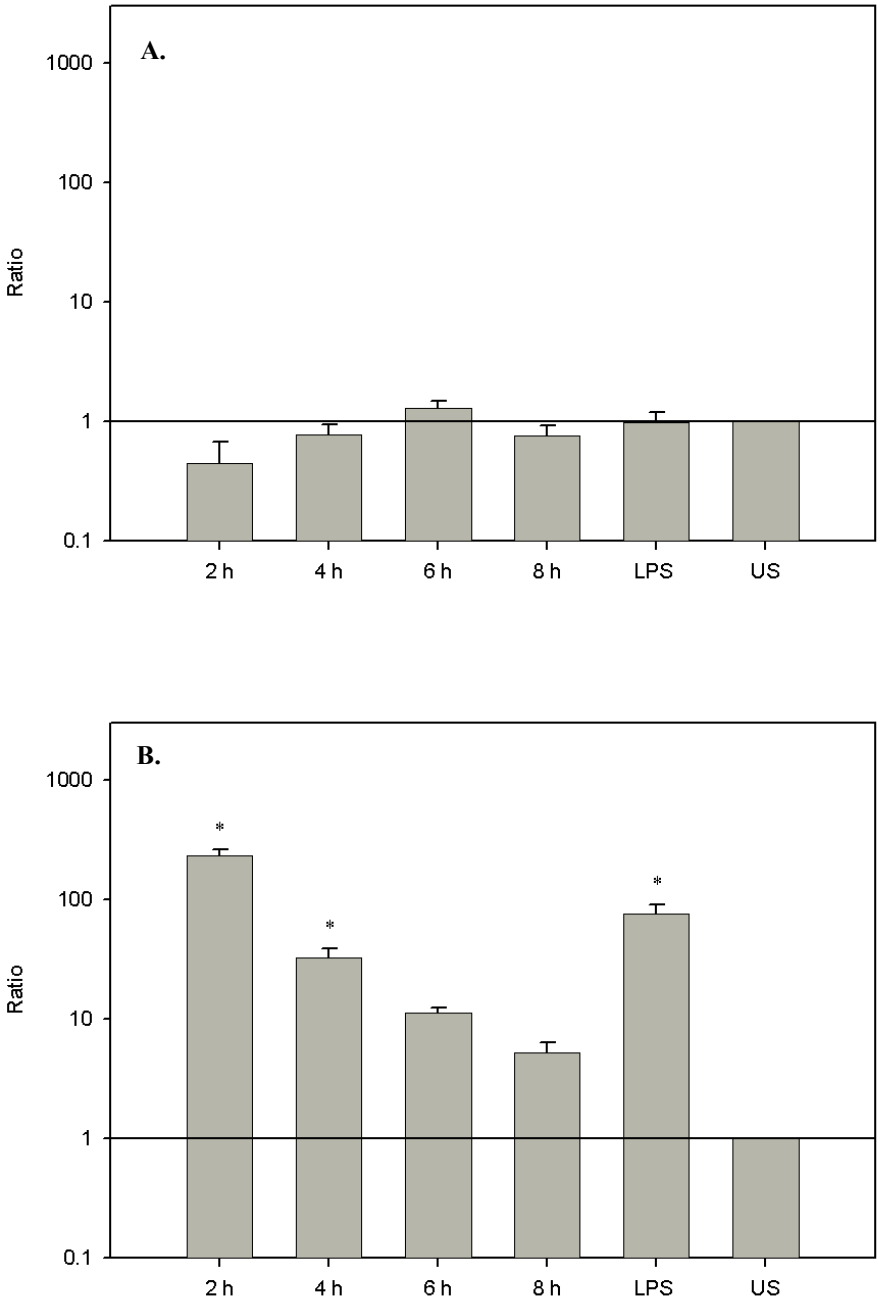

Figure 3.9 Time course of chitotriosidase (A) and TNF-α (B) mRNA expression after incubation of peripheral blood monocyte derived macrophages with 5μg/ml Pam_3Cys. Following quantitative PCR, duplicate *Ct* values for each treatment were normalised using the housekeeping genes GAPDH and APRT and expression ratios determined using the Pfaffl equation (Pfaffl, 2001). Each experiment was run in triplicate and repeated with three different donors with comparable results. Statistical significance between treated and untreated cells was determined by REST-MCS (Pfaffl et al., 2002); **P* <0.05.

In addition to fungal PAMPs, the effect of live *C.albicans* on *CHIT1* and TNF-α expression was determined in monocyte-derived macrophages. As shown in figure 3.10A and 3.10B, dilutions of the yeast (that form hyphae during incubation) had no significant effect on *CHIT1* and may have inhibited TNF-α expression. A similar result was observed using a fixed number of *C. albicans* yeast cells over a time course of 2-8 hours (Fig. 3.10C and 3.10D). However after two hours of culture, there appears to be a small but significant induction of *CHIT1*. To confirm this effect, further experiments would need to be conducted.

The presence of chitotriosidase activity in neutrophils pellets is shown in figures 3.11A, 3.11B and 3.11C. Figure 3.11A shows activity (in terms of 4-MU chitotrioside cleavage) in a sonicated neutrophil pellet. Figure 3.11B demonstrates that the fMLP treatment increases the chitotriosidase activity in neutrophil supernatants in a dose-dependent manner, suggesting that it induces activation and degranulation. A similar dose-dependent effect is observed following treatment with prolactin (Fig. 3.11C), demonstating a potential role for the hormone in immune modulation.

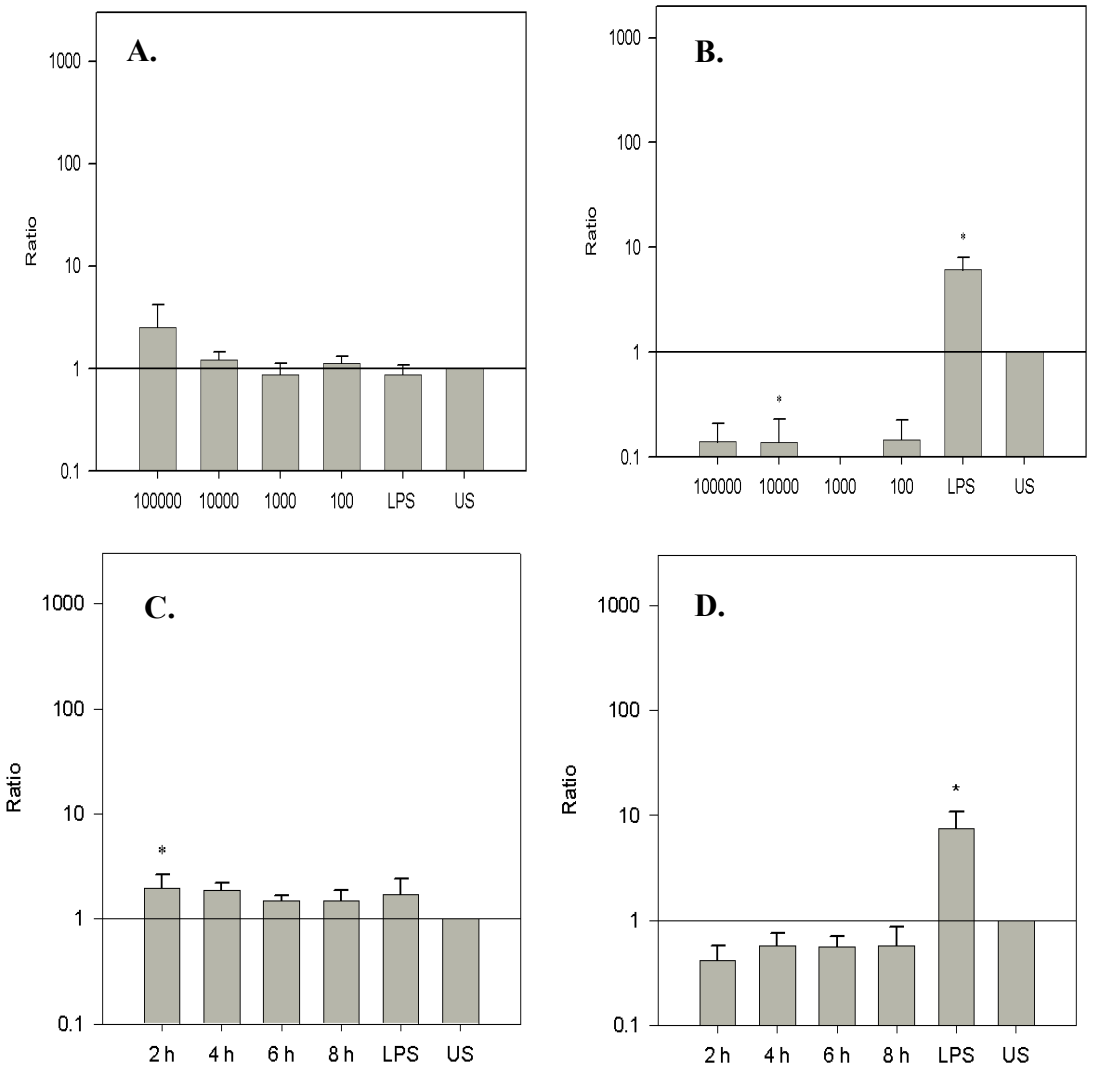

Figure 3.10 Live *C. albicans* does not up-regulate chitotriosidase (A) or TNF-α (B) mRNA transcription in MDMs. 10^6 MDMs were incubated with 100-100,000 live *C. albicans* yeast cells under conditions which induce hyphal growth (37°C and 10% foetal calf serum). 100,000 live *C. albicans* cells were incubated for up to 8h and samples taken for RNA extraction bi-hourly. A small increase of chitotriosidase expression was seen after 2h of co-incubation (C), but only with the highest dose used. *C. albicans* did not significantly change TNF-α gene expression (D). Statistical significance between treated and untreated cells was determined by REST-MCS; **P* <0.05.

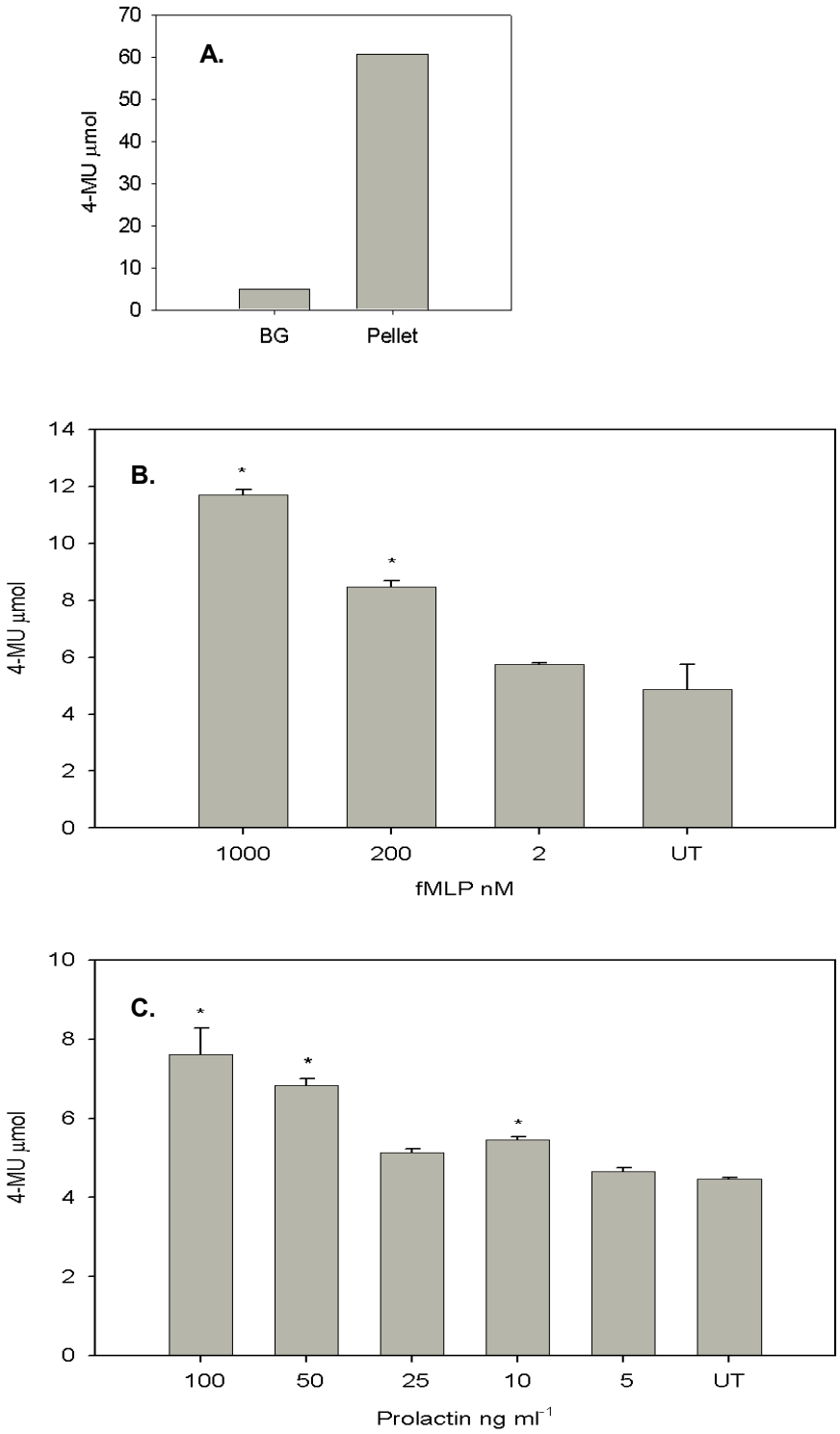

Figure 3.11 Chitotriosidase activity in human peripheral blood neutrophils determined using 4-MU chitotrioside assay. Untreated sonicated neutrophil pellets (A), supernatants from neutrophils treated for 15 minutes with human recombinant fMLP (B) or prolactin (C). Statistical analyses were carried out by one-way ANOVA and post hoc LSD test (*significance at $P<0.05$ compared to untreated) using SPSS v11.5.

3.5 DISCUSSION

It has previously been shown that *CHIT1* is constitutively expressed in monocyte-derived macrophages and that levels of expression rise during differentiation (Boot et al., 1995). The results from this work support this and show that after a week of culture there is close to a 1000-fold increase in *CHIT1* mRNA expression. Addition of GM-CSF to the culture medium has been shown to amplify mRNA and chitotriosidase protein levels and enzymatic activity (van Eijk et al., 2005). Therefore it is possible that the addition of 5ng/ml of GM-CSF and 5% autologous serum, containing growth factors and cytokines present *in vivo*, are responsible for this increase.

Recent research has pointed to a role for chitotriosidase in anti-fungal immunity. The enzyme can degrade the *C. albicans* cell wall and inhibit growth of *C. neoformans* (Boot et al., 2001) (van Eijk et al., 2005). Moreover, treatment of fungal infections in immune-compromised individuals with GM-CSF significantly improves prognosis. It can be speculated that this response may at least in part result from chitotriosidase activity (Jones, 1999). In terms of chitotriosidase induction, it has been shown that intravenous infection of guinea pigs with *Aspergillus fumigatus* leads to increased serum and tissue levels, in particular in the spleen (Overdijk et al., 1999).

Laminarin is a storage polysaccharide found in brown algae that consists of β1-3 and to a lesser extent β1-6 glucan and is similar to that found in the cell wall of *C. albicans*. Laminarin induced up to a 16.2-fold increase in *CHIT1* mRNA and this was observed after two hours of incubation with mature macrophages. A similar result was seen using zymosan that consists of β-glucan, mannan and mannoproteins derived from yeast cell walls. The maximum amount of *CHIT1* induction following zymosan treatment occurred after two hours (9.6-fold increase). Both laminarin and zymosan were capable of inducing between 100-1000-fold increases in TNF-α expression and this is consistent with the role of the cytokine in promoting an inflammatory response *in vivo*.

Mannan is a polysaccharide of mannose monomers that is a large constituent of the mannoproteins found in the outer layer of fungal cell walls (Horisberger and Clerc, 1988). Mannan did not significantly induce *CHIT1* expression but did lead to a 100-1000-fold increase in TNF-α expression. The induction of *CHIT1* by β-glucan but not mannan is consistent with the suggestion that pathogens may avoid recognition by masking their β-glucan (Brown and Gordon, 2003). Removing the outer mannan-rich layer using caspofungin and therefore exposing the underlying β-glucan has been shown to elicit a stronger immune response as shown by a 3-4-fold increase in TNF-α expression (Wheeler and Fink, 2006).

As previously described, it has been shown that TNF-α also induces chitotriosidase expression (Malaguarnera et al., 2005) that is strongly produced by PAMP-stimulated macrophages. It could therefore be suggested that *CHIT1* is indirectly induced as a result of an autocrine TNF-α mechanism, rather than via dectin-1 or other receptors. This autocrine mechanism theory can be ruled out for the following two reasons. Firstly, chitotriosidase mRNA induction peaks within two hours post stimulation. This time period is too short for an autocrine stimulation mechanism, unless TNF-α is preformed. TNF-α is produced by a variety of cell types but only mast cells, which were not present, are known to store it preformed (Galli et al., 1999). In addition, other tested PAMPs such as mannan, peptidoglycan, Pam_3Cys and flagellin, despite inducing a strong up-regulation of TNF-α expression, did not induce any significant changes in *CHIT1* expression. Therefore it is unlikely that these mechanisms are responsible for the induction of *CHIT1*.

Previously it has been shown that candidiasis, aspergillosis or cryptococcosis infection leads to high levels of circulating β-glucans (Obayashi, 1996). The observation that β-glucans induce chitotriosidase expression could account therefore for the increased chitinase levels in serum and tissues of systemically infected guinea pigs (Overdijk et al., 1999). The tolerance of high levels of chitotriosidase by individuals with Gaucher's disease and the

demonstration that recombinant chitotriosidase is protective against systemic fungal infections in *in vivo* animal models has led to the suggestion that it could be used in high doses against disseminated candidiasis in humans (Bussink et al., 2006, van Eijk et al., 2005).

The lack of *CHIT1* induction following culture with live *C. albicans* is consistent with the protection of β-glucan from the immune system by the mannan-rich layer. In the yeast form, the formation of budding scars can lead to β- glucan becoming exposed at the surface of the cell wall, however at 37°C and in the presence of serum, the hyphal form produces filaments and this no longer occurs (Brown and Gordon, 2003). Furthermore, the protection of the β-glucan layer in the hyphal form prevents dectin-1 interaction and activation and therefore precludes macrophage recognition and subsequent pro-inflammatory response (Brown et al., 2002) (Gantner et al., 2005).

The bacterial PAMPs tested had no significant effect on *CHIT1* expression. Previously it has been shown that plasma chitotriosidase levels are raised in children with both fungal and bacterial (Gram-positive and Gram-negative) infection (Labadaridis et al., 2005). However this may result from a globally increased level of phagocyte activation. In addition, the mutant *CHIT1* allele has been linked with susceptibility to Gram-negative bacterial infection in children with leukaemia (Lehrnbecher et al., 2005) and this may reflect a role for chitotriosidase either alone or in combination with antimicrobial

compounds against bacteria. As expected, TNF-α was strongly induced by all bacterial PAMPs.

The detection of chitotriosidase activity in supernatants from neutrophils treated with prolactin suggests that degranulation has occurred (Fig. 3.11b). The significance of this is unclear, however chitotriosidase activity is detectable in the colostrum of breast-feeding women and in particular those of African origin (Musumeci et al., 2005). The colostrum serves as a transport system for bioactive molecules between mother and infant and therefore it is possible that chitotriosidase expression or release is induced by localized prolactin and, in addition to many other bioactive compounds, provides an initial antimicrobial protection.

In summary, the human *CHIT1* gene is highly expressed in mature monocyte-derived macrophages and can be induced further by fungal but not bacterial PAMPs. This is consistent with the suggested role of chitotriosidase in anti-fungal immunity, however the mechanisms of action are not fully understood. Live *C. albicans* had no effect on *CHIT1* expression and this may have been caused by shielding of the β-glucan layer through the formation of filaments. Chitotriosidase activity is detectable in pellets and in the supernatants of neutrophils treated with fMLP and prolactin and is most likely derived from degranulation of specific granules where it is stored (van Eijk et al., 2005).

4. ANTIMICROBIAL ACTIVITY OF RECOMBINANT HUMAN CHITOTRIOSIDASE

4.1 INTRODUCTION

Constitutive chitotriosidase expression by tissues including the bone marrow, lung and lymph nodes (Boot et al., 2005b) and secretion by macrophages and neutrophils (Boot et al., 1995) points to a role in innate immunity and this hypothesis is supported by the role of chitinases in other organisms. Plant chitinases for example are pathogenesis related proteins (PRPs) and are induced following infection with bacteria, fungi and viruses (Collinge et al., 1993) (Kasprzewska, 2003). Chitinases from soil-based bacteria prevent the spread of fungi on plants by cell wall cleavage (Hoster et al., 2005).

In support of a role in innate immunity it has been shown that plasma and urine chitotriosidase levels are elevated in neonates with various fungal (*Candida albicans* and *Aspergillus niger*) and bacterial (*Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Serratia marcescens* and *Citrobacter sp*.) infection and in guinea pigs infected with systemic *Aspergillus fumigatus* (Labadaridis et al., 2005, Overdijk et al., 1999). However increased levels of chitotriosidase may result from induction of *CHIT1* expression by TNF-α, a process that has been demonstrated *in vitro* (Malaguarnera et al., 2005). Increased TNF-α expression and secretion is induced by a variety of products resulting from microbial infection including

bacterial LPS and zymosan from fungal cell walls (Beutler and Cerami, 1987) (Underhill, 2003).

In order to determine whether chitotriosidase has a critical role in antimicrobial immunity the discovery of a phenotype resulting from individuals that do not produce active enzyme would be useful. To date, no-one has produced *CHIT1*$^{-}$ knockout mice to serve this purpose, although there is a naturally occurring mutation in the human population that has been linked with susceptibility to various infections including lymphatic filariasis (Choi et al., 2001) and malaria (Malaguarnera et al., 2003). However, this has not been substantiated by other studies (Hise et al., 2003) (Chapter 5). Additionally the mutant *CHIT1* genotype does not increase susceptibility to candidiasis in adult leukaemia patients however children with the mutant *CHIT1* genotype and undergoing therapy for acute myeloid leukemia (AML) are more susceptible to Gram-negative bacterial infection (Choi et al., 2005) (Lehrnbecher et al., 2005).

In terms of activity, chitotriosidase has been shown to have several anti-fungal effects. Firstly it is capable of degrading both colloidal and fungal cell wall chitin as shown through its degradation of *C. albicans* spheroplasts (Boot et al., 2001). Furthermore, chitotriosidase is able to prevent hyphal switching in *C. albicans* (albeit at high concentrations of 1mg/ml) and inhibit growth of *Cryptococcus neoformans* and *Mucor rouxii* (van Eijk et al., 2005). *In vivo* it has been shown that treatment

of neutropenic mice with recombinant human chitotriosidase significantly increased rates of survival (van Eijk et al., 2005).

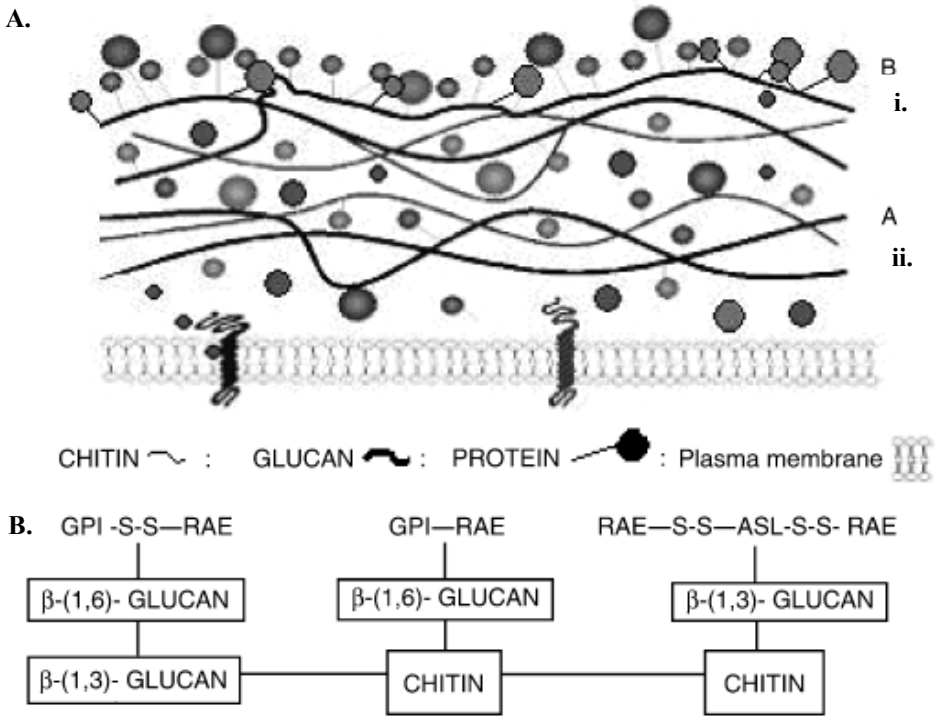

Figure 4.1 Schematic representation of the *C.albicans* cell wall. **A)** The outer layer is enriched with a variety of proteins (i), the inner layer is composed of covalently linked β1,3- and β1,6 glucan, chitin microfibrils and proteins (ii). **B)** Outer cell wall proteins include glycosyl phosphatidylinositol proteins (GPI), Alkali sensitive bond proteins (ASL) and reducing agent extractable (RAE) proteins (mannoproteins) and are linked to short β1,6 and β1,3 glucan chains and chitin. (Adapted from (Ruiz-Herrera et al., 2006).

As shown in figure 4.1, *C. albicans* has a cell wall that primarily consists of proteins, β- glucan and chitin that are involved in protecting the fungus from the immune response whilst simultaneously initiating direct interaction with host cells potentially leading to pathogenicity. *C. albicans* is a dimorphic yeast and therefore can switch between yeast and hyphal forms in the presence of particular stimuli (including temperature and serum) (Hudson et al., 2004). In the yeast form, *C. albicans* produces pseudo-hyphae (through apical division) leading to

the exposure of β-glucan through the formation of bud-scars. Therefore the yeast form is more susceptible to host defence processes than the hyphal form that contains a continuous layer of mannoprotein (Gantner et al., 2005).

Another well-defined fungal cell wall is that of *Saccharomyces cerevisiae* (*S. cerevisiae*). Like *C. albicans*, the *S. cerevisiae* cell wall consists of an inner layer of chitin, covered on the exterior side by β-glucans and mannoproteins (Theis and Stahl, 2004). The location of chitin in *C. albicans*, *S. cerevisiae* and cell walls from other fungi is an important consideration when considering the role of chitinases in antifungal immunity. The presence of networks of β1,6 and β1,3 glucans prevents the access of chitinases to the chitin layer and this would therefore preclude the action of human chitotriosidase against fungi. However, plant chitinases have been shown *in vitro* to have an antifungal effect when added with β-1,3 glucanases (Arlorio, 1992). In humans, no glucanase genes have been identified however lysozyme has been shown to be active against a range of fungi including *C. albicans* via a mechanism that involves hydrolysis of N-glycosidic bonds linking polysaccharides to proteins in the cell wall (Samaranayake et al., 1997).

Lysozyme is primarily active against Gram-positive bacteria through cleavage of β- 1,4 linkages between N-acetyl glucosamine and N-acetylmuramic acid in the exposed peptidoglycan layer. However,

acting alone it has a weak bactericidial activity. In Gram-negative bacteria, lysozyme activity is inhibited by the presence of an outer membrane containing LPS which protects the peptidoglycan, however this can be increased by the addition of membrane disruptive agents including EDTA and SDS (Iacono et al., 1983).

Lysozyme and chitotriosidase are both highly expressed in tissues of the human eye and in particular the lachrymal glands (Malik et al., 1970) (Hall et al., 2007a) that produce tear fluid in part to prevent infection. The co-expression of lysozyme and other antimicrobial compounds that together have synergistic or additive effects is well known. For example in airway surface liquid (ASL), which is similar in constitution to tear fluid, the interactions between different antimicrobial compounds are required for the greatest anti-bacterial effects and additionally provide levels of redundancy (Singh et al., 2000).

There may however be significant differences between the antimicrobial activities of ASL and tear fluid. It has been suggested that patients with cystic fibrosis are more susceptible to lung infections as a result of raised salt concentration and therefore increased ionic strength. In support of this hypothesis it has been shown that increasing salt concentration reduces synergistic and additive anti-bacterial effects against *Escherichia coli* (*E. coli*) that are independent of the effects on individual compounds (Singh et al., 2000). However the addition of

salt had no effect on activity of tears against *Pseudomonas aeruginosa* (*P. aeruginosa*) at comparable concentrations suggesting variability between tear and ASL constituents (Fleiszig et al., 2003).

4.2 OBJECTIVES

The activity of human recombinant chitotriosidase against fungal pathogens including *C. albicans* has been demonstrated. However, the anti-bacterial effects have yet to be investigated. Therefore the main objective of this work was to test the *in vitro* effects of recombinant chitotriosidase and active site mutant on the viability of several Gram-positive and Gram-negative bacteria. As lysozyme is sensitive to ionic strength, activity of the recombinant chitotriosidase was tested at a range of NaCl concentrations. In addition, recombinant chitotriosidase was tested in combination with lysozyme to determine whether there was a synergistic anti-bacterial effect. As the lachrymal glands have been identified as expressing high levels of chitotriosidase, human tears were tested for chitinase activity.

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4.3 METHODS

4.3.1 Generation of recombinant human chitotriosidase and active site mutant

Recombinant human chitotriosidase and active site mutant were generated (by Dr. Shaun Morroll and Dr. Franco Falcone) using the pMIB/V5-His vector kit (Invitrogen) with full length Hs*CHIT1* generated by reverse transcription PCR (RT-PCR) from neutrophil RNA. Site-directed mutagenesis was used to generate an active site mutant of chitotriosidase. Mutagenic primers were designed in order to change the active site consensus sequence from DGLDLDWE to DGLDLNWQ thus removing the aspartic and glutamic acid residues required for activity. Following sequence confirmation, the construct clone was transfected in to Sf9 insect cells and grown as a monolayer and suspension culture in Ex-cell 420 medium (JRH Biosciences).

4.3.2 Purification of recombinants

The Sf9 insect cell suspension was pelleted at 3350g for 20min. The supernatant was collected, dialysed against 20 vol. of water for 24-48hrs at 4°C using 12-14KDa cut-off dialysis tubing. The supernatant was re-centrifuged as before and then filtered through a 0.22μm filter, finally lyophilized to a fine powder. The powder was reconstituted in 1xNi-NTA binding buffer and purified using Novagen's His•Bind® Resin as directed by the manufacturer.

4.3.3 Western Blot and Silver Stain

The in-frame synthesis and purity of recombinant human chitotriosidase was confirmed by Western blot for the V5 epitope. Briefly, protein concentration was measured using a Nanodrop spectrophotometer (ND-1000 UV-Vis) and samples were diluted to 25μg/ml in DPBS (Cambrex). 10μl of recombinant chitotriosidase (250ng) was resuspended in an equal volume of reducing sample buffer and denatured at 100°C for 10 minutes. Chitotriosidase and dual color precision plus markers (Bio-Rad) were loaded onto a 10% SDS-PAGE gel and run at 80V for 30 minutes on a Mini-protean III transfer cell (Bio-Rad). Samples were blotted onto a nitrocellulose membrane (Sigma-Aldrich) for 13 hours at 26V. The blot was then placed in tris-buffered saline (TBS) with 5% w/v milk powder and incubated for 1 hour at 4°C. The primary anti-V5 antibody (Invitrogen) was added at a dilution of 1:5000 and the blot was incubated overnight. The blot was washed twice with TBS/0.05% Tween 20 and incubated for a further 2 hours in secondary anti-mouse IgG-HRP (1:1000) (Pharmingen). Finally the blot was washed three times in TBS/0.05% Tween 20, once in TBS without Tween, and developed in substrate buffer (1mg/ml 4-chloro-1-napthol, 30% ethanol and 0.06% hydrogen peroxide in TBS).

The purity of the recombinant human chitotriosidase was determined by silver staining using the same SDS-PAGE protocol as for Western blotting. Following electrophoresis the gel was washed three times for 20 minutes in 30% ethanol, 1 minute in 0.02% sodium thiosulphate

(Sigma) and three further washes in deionised water for 20 seconds. The gel was developed in 3% sodium carbonate (Sigma), 0.05% formaldehyde and 0.0004% sodium thiosulphate (Sigma) for 5 minutes followed by two washes in deionised water for 1 minute. The reaction was stopped using 2% EDTA (Sigma) for 20 minutes and the gel was washed with deionised water prior to storage in 2% glycerol (Sigma).

4.3.4 Measurement of chitinase activity

Enzymatic activity of recombinant human chitotriosidase was determined by incubating 10µl of enzyme with 100µl of 22µmol/L of substrate 4- methylumbelliferyl-β-d-N,N,N', N''triacetylchitotriose (Sigma) and taking readings of relative fluorescence units (RFU) at 450nm every 120 seconds on a MFX microtiter plate fluorometer with Revelation 4.21 software (Dynex Technologies). The rate of 4-MU production was determined by plotting a standard curve of 4-MU salt (Sigma) concentration versus relative fluorescence units (RFU) minus 4-MU and buffer alone.

The activity of the recombinant chitotriosidase was tested in different buffers: 0.1M glycine-HCL (pH 2.7), 100 mM sodium acetate (pH 4.0), 100 mM/200 mM citrate-phosphate buffer (pH 5.2), phosphate buffered saline (pH 7.2) and 100 mM glycine-NaOH (pH 9.0 and 10.5). All data analyses and regression plots were done using SigmaPlot 8.0. The effect of salt on recombinant chitotriosidase activity was measured using the same method. Sodium chloride (NaCl)

was added to citrate- phosphate buffer (pH 5.2) at concentrations between 0.08 M and 2 M.

4.3.5 Antimicrobial activity assessment

The effect of human recombinant chitotriosidase and lysozyme on bacterial viability was determined using the BacTiter-Glo ATP luciferase assay (Promega) and Lucy 1 multiplate reader (Anthos) with Stingray software (Dazdaq). Six gram-positive bacterial strains: *Bacillus cereus* (ATCC11778), *Bacillus subtilis* (ATCC6633), *Listeria innocua* (ATCC33090), *Staphylococcus aureus* (ATCC29213) and *Staphylococcus aureus* OatA$^{+/-}$ (ATCC35556) and two gram-negative bacterial strains: *Escherichia coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC27853) were incubated overnight in a shaking incubator at 37°C and then diluted 1:100 in low-salt LB (pH 6.0) and incubated for a further 2 hours. Cultures were then aliquoted into 96 well tissue culture plates and start point readings were taken in triplicate. Plates were then incubated for a further 4 hours in the presence of lysozyme, recombinant chitotriosidase or kanamycin (as a positive control).

The effect of chicken egg white lysozyme (Sigma) was tested at a range of concentrations (50μg to 0.39μg/ml and 3.12μg to 25ng/ml) on bacterial viability with the antibiotic kanamycin (50μg/ml) as positive control and untreated bacteria in triplicates. Sub-optimal doses of lysozyme were tested alone and in combination with recombinant

human chitotriosidase (25μg/ml) using the same protocol. Statistical analyses were carried out by One-way ANOVA and post hoc LSD test (significance at $P<0.05$) using SPSS v11.5.

4.3.6 Tear collection and chitotriosidase activity determination

Tears were obtained from four subjects by inducing lachrymation with a chemical stimulus and collecting the fluid with a pipette tip. Chitotriosidase activity in tear fluid was determined using the previously described 4-MU chitotrioside assay (4.3.4).

4.4 RESULTS

It has previously been shown that *CHIT1* is highly expressed in the eye and in particular the lachrymal gland Therefore the activity of chitotriosidase was measured in tear fluid. The results are shown in Fig. 4.2. However it needs to be stressed that tear fluid, like other body fluids, will also contain lysozyme, which also (albeit with lower affinity) has the ability to cleave the chitotriosidase substrate. Therefore, the presence of chitotriosidase activity in tear fluid, although consistent with gene expression in the lachrymal gland, cannot be considered as sufficient evidence of its presence in tears. Detection of the protein (eg. by Western blot) was not possible as there are no commercially available antibodies to chitotriosidase and mice treated with the recombinant failed to mount an antibody response.

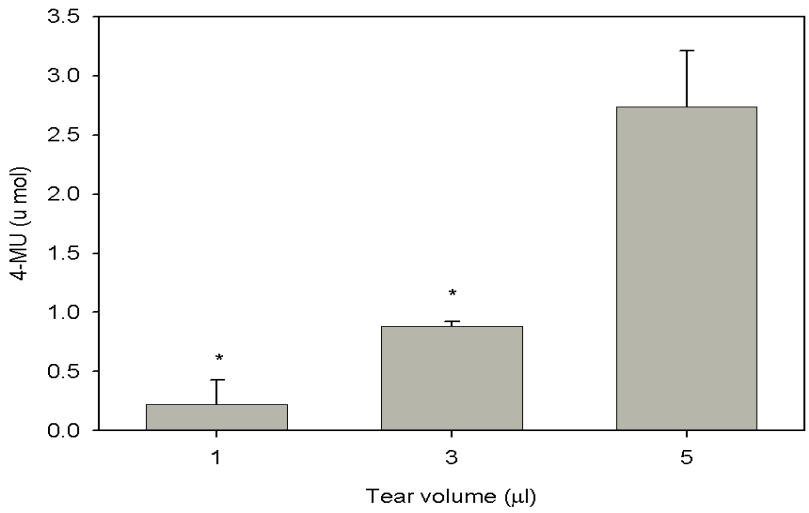


Figure 4.2 Chitinase activity in human tear fluid (n=4 experiments). Tears were collected and the chitinase activity was measured using the 4-MU chitotrioside method previously described. Data were analysed by a one-way ANOVA followed by posthoc LSD test using SPSS v11.5. * indicates a significant difference, compared with the highest volume. *P*=<0.05.

Recombinant histidine-tagged chitotriosidase protein (wildtype and mutant) was purified using Ni-NTA affinity chromatography and shown on a silver stained SDS-PAGE gel. There was no visible contaminant (Fig. 4.3A) and the recombinant protein reacted with an anti-V5 antibody, confirming in–frame expression (Fig. 4.3B) of wildtype and active site mutant chitotriosidase.

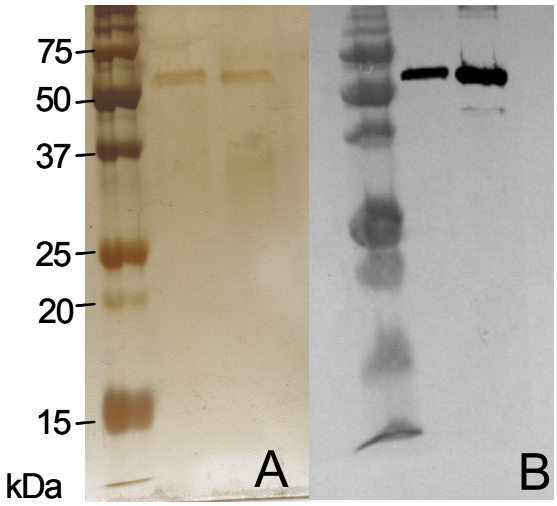
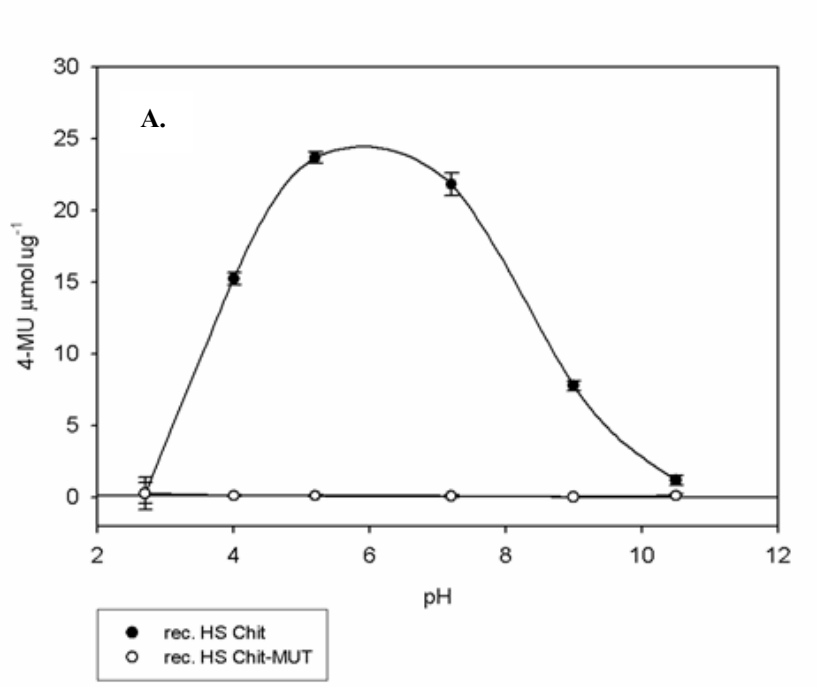


Figure 4.3 (A) Silver-stained 12% SDS-PAGE of recombinant wildtype (middle lane) and mutant (right lane) chitotriosidase. There is no apparent contamination other than the expected band. The molecular weight of the recombinant protein is slightly larger than the expected 50 kDa, as the expression vector adds a his tag and a V5-epitope to the C-terminus of the protein. (B): Western Blot with an anti-V5 detecting antibody, confirming in-frame synthesis of recombinant wildtype (middle lane) and mutant (right lane) chitotriosidase.

The recombinant chitotriosidase has a similar broad range of activity at intermediate pH as one previously produced using transient transfection of COS-1 cells (Boot et al., 2001). Recombinant chitotriosidase is completely inactive at low pH but has elevated activity between pH 4.0 and 8.0 with an optimum pH of between 5.0 and 6.0. At increasingly alkali pH, the rate of decline in activity is less significant than at acid pH (Figure 4.4A), (Boot et al., 2001). The maximum activity of chitotriosidase at an intermediate pH is consistent with its expression pattern and a role in innate immunity. In comparison, acidic mammalian chitinase (AMCase) is most active at very low pH which is consistent with its expression in the stomach (Boot et al., 2001).

Figure 4.4B is a time course at (pH 5.2) showing the kinetics of chitotrioside cleavage. The specific activity of recombinant chitotriosidase was calculated using the Lambert-Beer equation $A= \varepsilon cd$, where A = absorbance, ε = molar absorbtivity (M^{-1} cm^{-1}), d = path length (cm), c = concentration (M), yielding a value of 1260 μmol mg^{-1} min^{-1}.



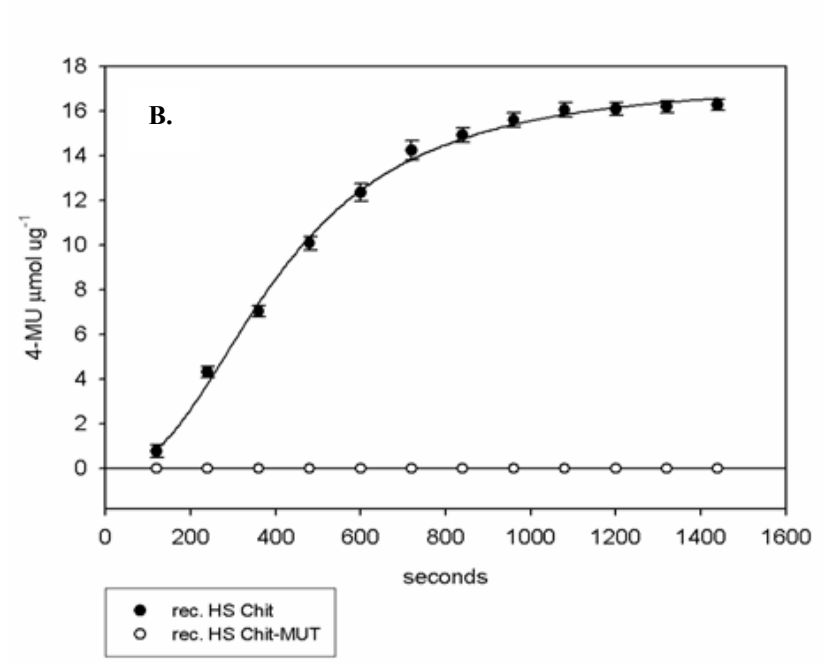


Figure 4.4 A) The pH curve indicates a broad activity with a pH optimum between 5.2 and 7.2. **B)** Enzymatic activity of recombinant human chitotriosidase and active site mutant obtained from stably transfected Sf9 insect cells. The results confirm that our recombinant enzyme is fully active and that the added detection/purification tag does not interfere with enzymatic activity (against chitotrioside substrates). As expected, the active site mutant (open circles) had no enzymatic activity.

These results also confirmed that the V5 epitope and his tag, added by the pMIB expression vector, do not interfere with enzymatic activity against chitotrioside. Previously it has been shown that the 50 kDa form is capable of cleaving both chitotriose and colloidal chitin, whereas the 39 kDa isoform (which lacks the chitin binding domain at the C- terminal end of the enzyme) is less capable of hydrolysing colloidal chitin (Renkema et al., 1997). This is consistent with the re-routing of the 39 kDa isoform of chitotriosidase to lysosomes where the close proximity of the enzyme with substrate removes the need for a chitin binding domain (Fusetti et al., 2002). With regard to the recombinant chitotriosidase, it is possible that the enzymatic activity against the chitotrioside substrate (shown in figure 4.4) is entirely derived from the active site. Moreover it is possible that His tag and V5 epitope at the C- terminal end could interfere with the chitin binding domain and therefore reduce activity against substrates where tight binding is required.

Previously it has been shown that lysozyme activity is dependent on salt concentration with maximal activity observed between 50mM and 120mM for sodium chloride (NaCl) with no bacterial lysis occurring at 500mM (Smolelis and Hartsell, 1952). As shown in Figure 4.5, chitotriosidase is significantly less sensitive to NaCl concentration than lysozyme as it is still highly active at 2M NaCl.

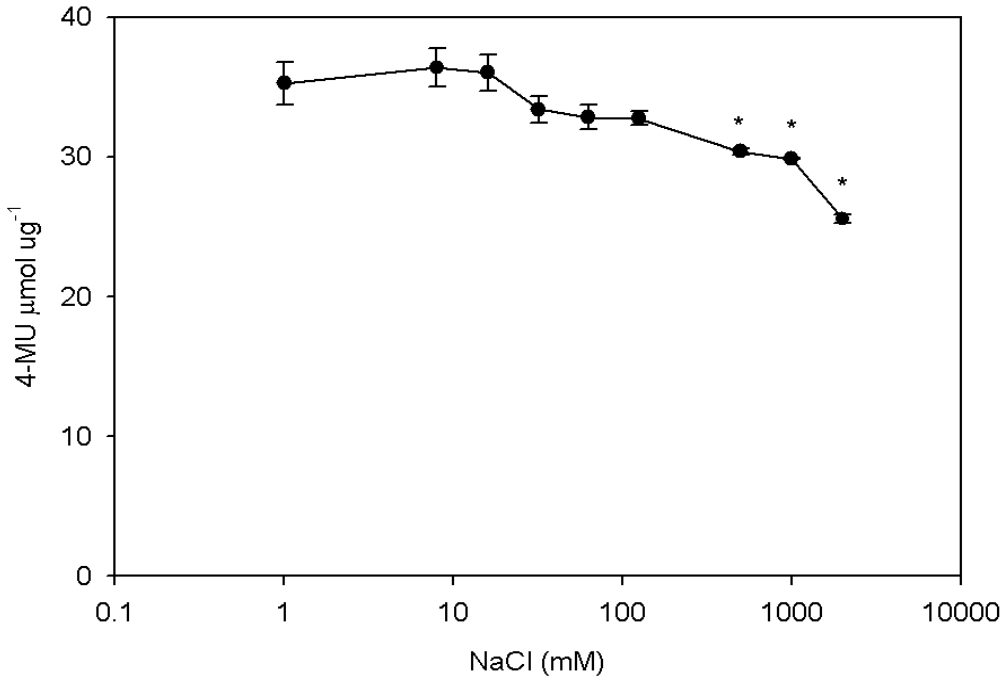


Figure 4.5 The effect of increasing concentration of NaCl on the activity of recombinant chitotriosidase. Data were analysed by a one-way ANOVA followed by posthoc LSD test using SPSS v11.5. * indicates a significant difference, compared with untreated chitotriosidase, at P=<0.05.

The anti-bacterial activity of the recombinant chitotriosidase and active site mutant was assessed both alone and in combination with lysozyme to determine whether there is a synergistic effect of these enzymes together. Gram- negative bacteria are more resistant to lysozyme than Gram- positives due to the presence of an outer layer of LPS protecting their peptidoglycan from hydrolysis. However regarding chitotriosidase, the mutant *CHIT1* genotype has been linked to susceptibility to Gram- negative bacterial infection in children undergoing therapy for acute myeloid leukaemia (AML) (Lehrnbecher

et al., 2005). Therefore both Gram-negative and Gram-positive bacterial genera were examined.

All bacteria were sensitive to 50 μg/ml Kanamycin, which was used as positive control in all experiments. First, a lysozyme sensitivity curve was made using serial dilutions of hen egg white lysozyme for the seven bacterial strains. As can be seen in figure 4.6A, *B. subtilis* was highly susceptible to the effects of lysozyme, with inhibition of growth at all tested dilutions. *L. innocua* (Fig. 4.9A) showed intermediate sensitivity to lysozyme, with partial, dose-dependent growth inhibition at all concentrations. Wildtype *S. aureus*, *B. cereus*, and the two tested Gram-negative strains *E. coli* and *P. aeruginosa* were completely insensitive to lysozyme (Fig. 4.8A, 4.10A, 4.11A, 4.12A).

The mutant *S. aureus oatA*-deficient strain, which lacks an O-acetyl transferase that acetylates the C6 hydroxy group of muramic acid in peptidoglycan, displayed increased sensitivity to lysozyme at the two highest concentrations (Fig. 4.7A), in line with previous findings (Bera et al., 2005) (Bera et al., 2006).

Based on the sensitivity to lysozyme identified in the essay, the growth inhibition experiments were repeated with a suboptimal concentration of lysozyme (0.05 μg/ml for *B. subtilis* and 50μg/ml for all others) in the presence of 25 μg/ml recombinant wildtype or mutant chiotriosidase. As shown in figures 4.6B, 4.7B, 4.8B, 4.9B, 4.10B,

4.11B and 4.12B) the addition of chitotriosidase to a suboptimal concentration of lysozyme did not increase growth inhibition in any of the tested bacterial strains, neither did the presence of chitotriosidase alone. In addition the chitotriosidase mutant also did not show any effect. These experiments clearly show that chitotriosidase, at least in the μg range, does not have any effect on bacterial growth, even when used in combination with lysozyme.

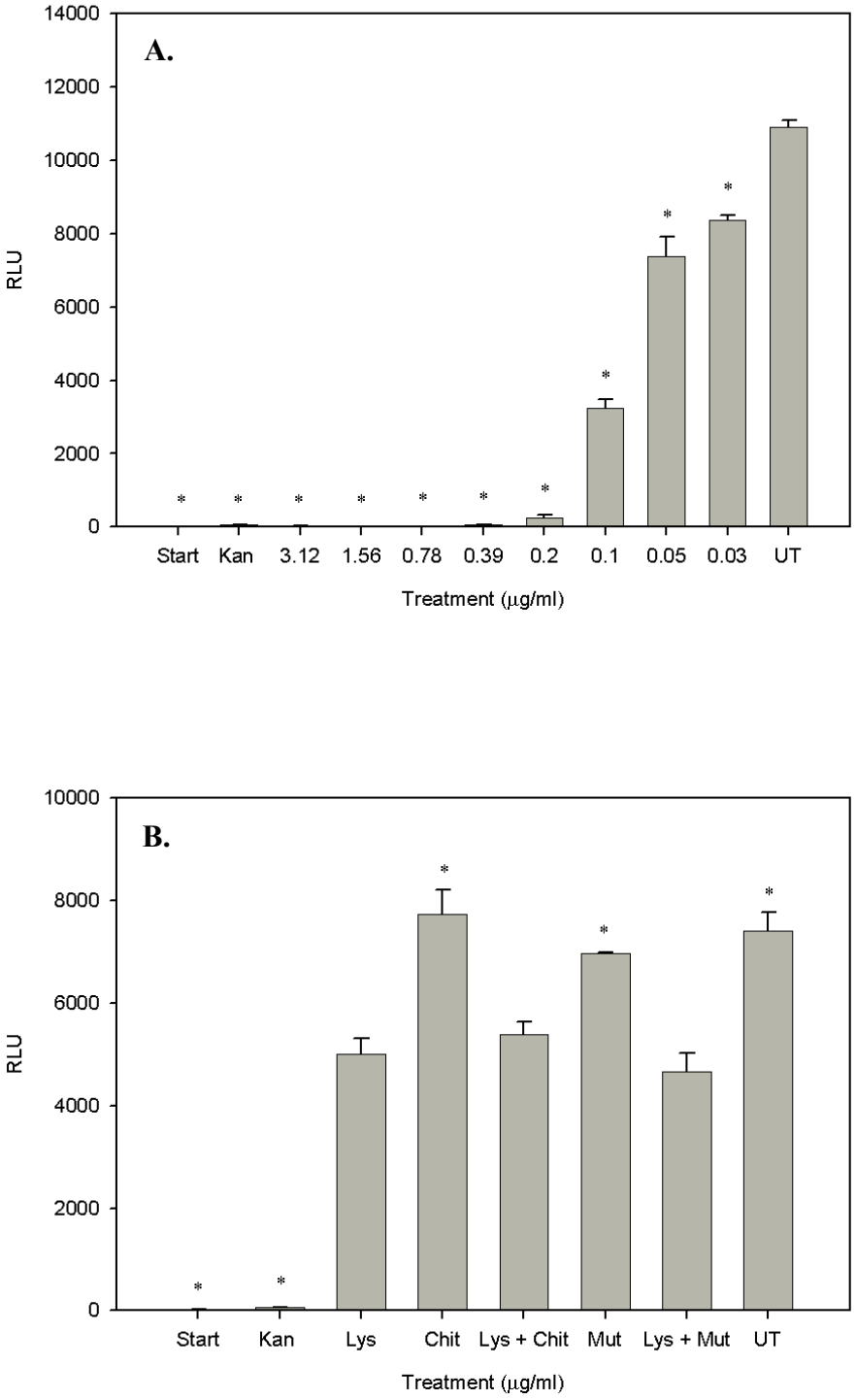


Figure 4.6 Antimicrobial growth inhibition of *B. subtilis* by egg-white lysozyme alone (A) or chiotriosidase, alone or in combination with a sub-optimal dose of lysozyme (B). The significance for the lysozyme charts is compared to the untreated control and the chitotriosidase assays compared to lysozyme alone. Experiments were repeated at least twice with triplicate samples with comparable results. (Kan= kanamycin 50µg/ml, Lys= lysozyme 0.05µg/ml, Chit & Mut= recombinant chitotriosidase and active site mutant 25µg/ml). Statistical analyses were carried out by one-way ANOVA and post hoc LSD test (* significance at $P<0.05$) using SPSS v11.5.

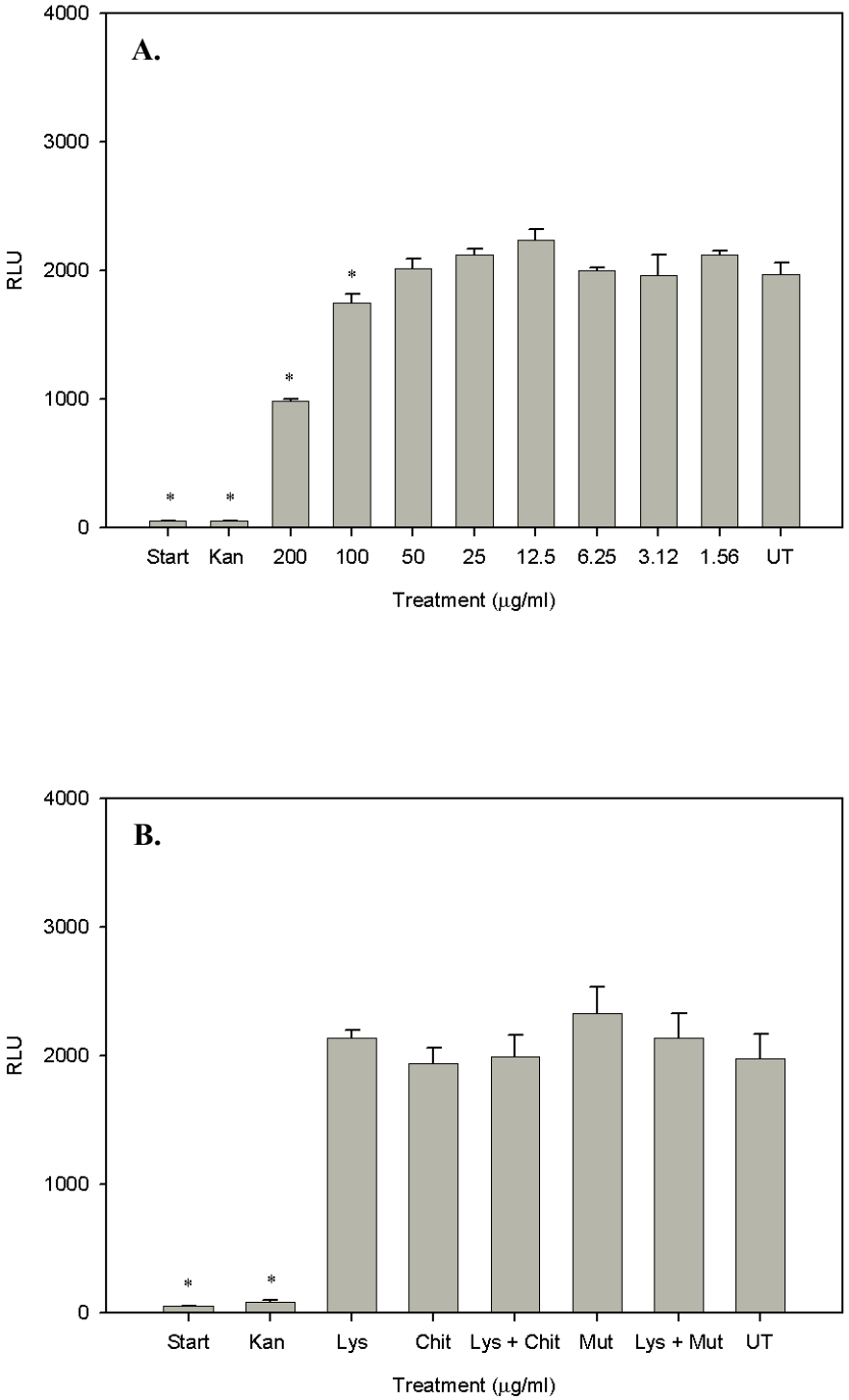


Figure 4.7 Antimicrobial growth inhibition of *S. aureus oatA*$^{-}$ by egg-white lysozyme alone (A) or chiotriosidase, alone or in combination with a sub-optimal dose of lysozyme (B). The significance for the lysozyme charts is compared to the untreated control and the chitotriosidase assays compared to lysozyme alone. Experiments were repeated at least twice with triplicate samples with comparable results. (Kan= kanamycin 50μg/ml, Lys= lysozyme 50μg/ml, Chit & Mut= recombinant chitotriosidase and active site mutant 25μg/ml). Statistical analyses were carried out by one-way ANOVA and post hoc LSD test (* significance at $P<0.05$) using SPSS v11.5.

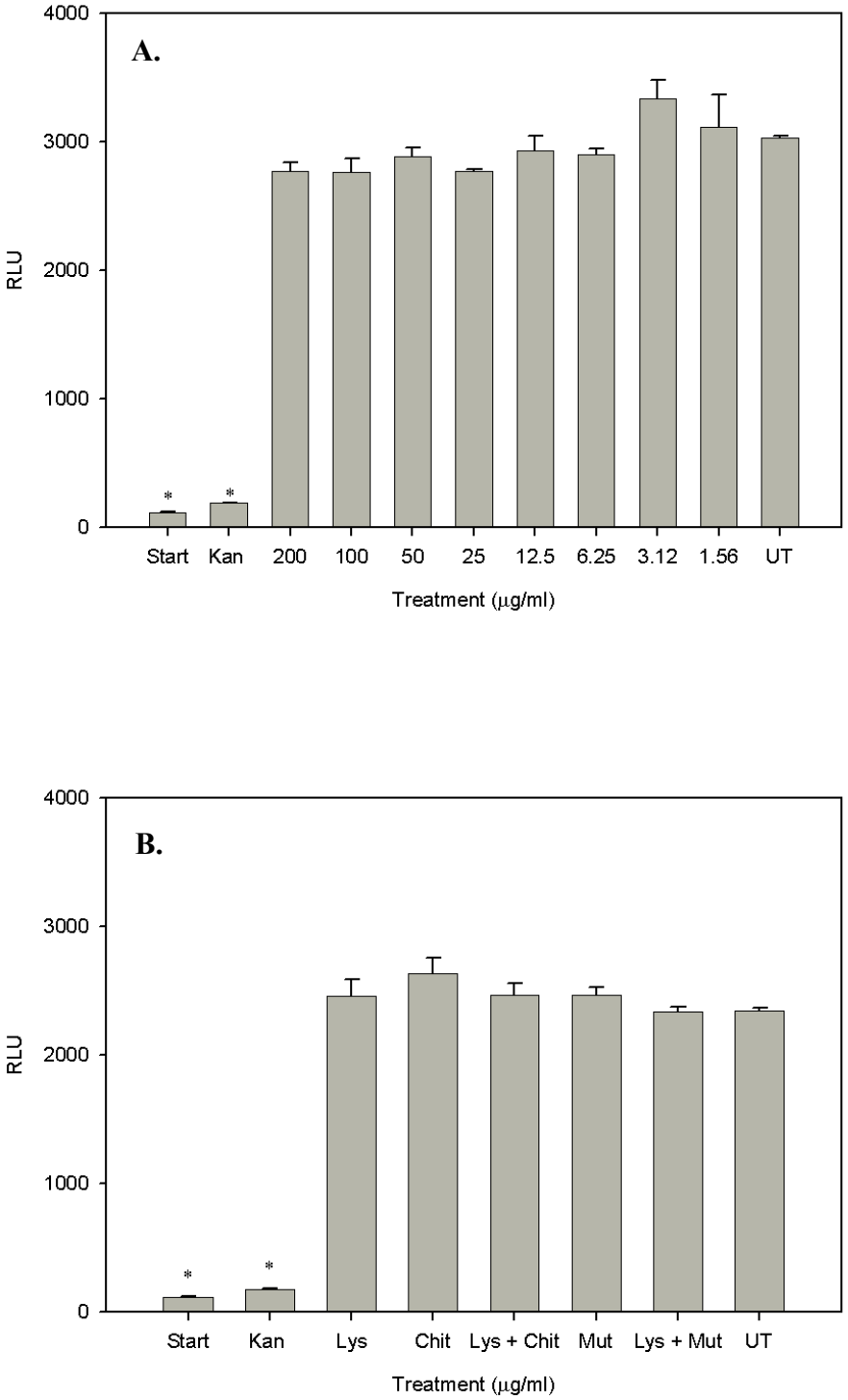


Figure 4.8 Antimicrobial growth inhibition of *S. aureus* wild-type by egg-white lysozyme alone (A) or chiotriosidase, alone or in combination with a sub-optimal dose of lysozyme (B). The significance for the lysozyme charts is compared to the untreated control and the chitotriosidase assays compared to lysozyme alone. Experiments were repeated at least twice with triplicate samples with comparable results. Experiments were repeated at least twice with triplicate samples with comparable results. (Kan= kanamycin 50μg/ml, Lys= lysozyme 50μg/ml, Chit & Mut= recombinant chitotriosidase and active site mutant 25μg/ml).Statistical analyses were carried out by one-way ANOVA and post hoc LSD test (* significance at $P<0.05$) using SPSS v11.5.

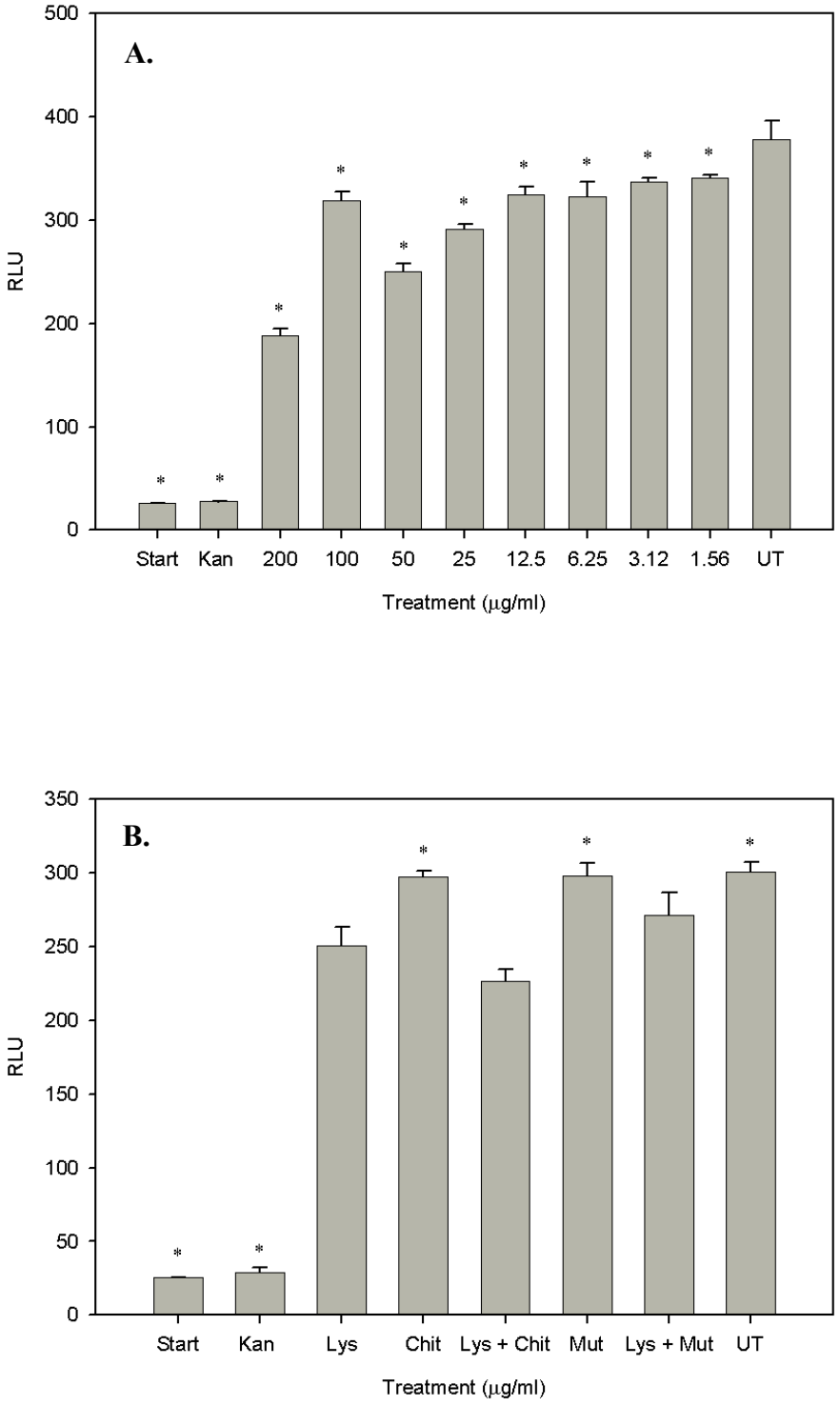


Figure 4.9 Antimicrobial growth inhibition of *L. innocua* by egg-white lysozyme alone (A) or chiotriosidase, alone or in combination with a sub-optimal dose of lysozyme (B). The significance for the lysozyme charts is compared to the untreated control and the chitotriosidase assays compared to lysozyme alone. Experiments were repeated at least twice with triplicate samples with comparable results. Experiments were repeated at least twice with triplicate samples with comparable results. (Kan= kanamycin 50μg/ml, Lys= lysozyme 50μg/ml, Chit & Mut= recombinant chitotriosidase and active site mutant 25μg/ml).Statistical analyses were carried out by one-way ANOVA and post hoc LSD test (* significance at $P<0.05$) using SPSS v11.5.

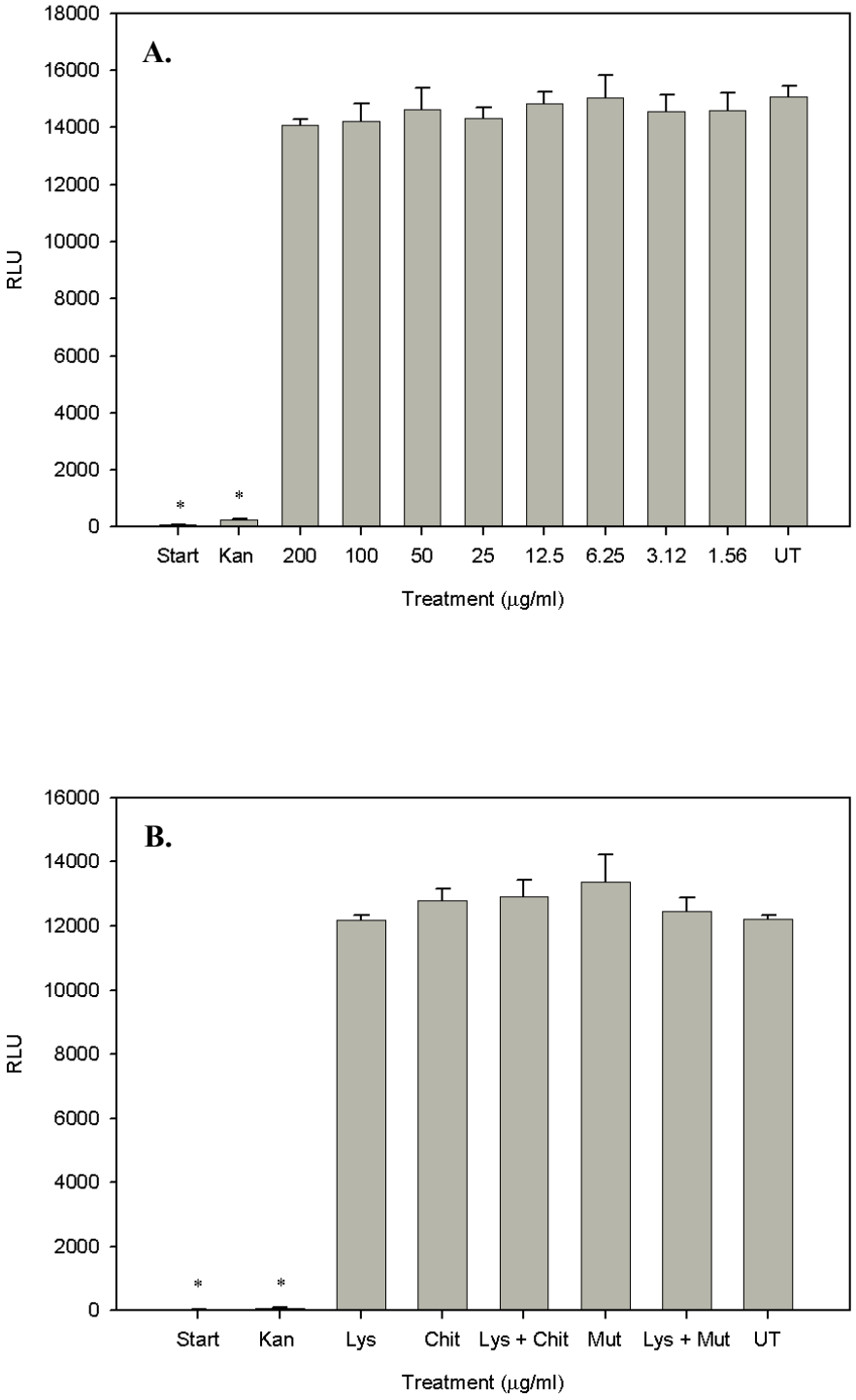


Figure 4.10 Antimicrobial growth inhibition of *B. cereus* by egg-white lysozyme alone (A) or chiotriosidase, alone or in combination with a sub-optimal dose of lysozyme (B). The significance for the lysozyme charts is compared to the untreated control and the chitotriosidase assays compared to lysozyme alone. Experiments were repeated at least twice with triplicate samples with comparable results. Experiments were repeated at least twice with triplicate samples with comparable results. (Kan= kanamycin 50µg/ml, Lys= lysozyme 50µg/ml, Chit & Mut= recombinant chitotriosidase and active site mutant 25µg/ml). Statistical analyses were carried out by one-way ANOVA and post hoc LSD test (* significance at $P<0.05$) using SPSS v11.5.

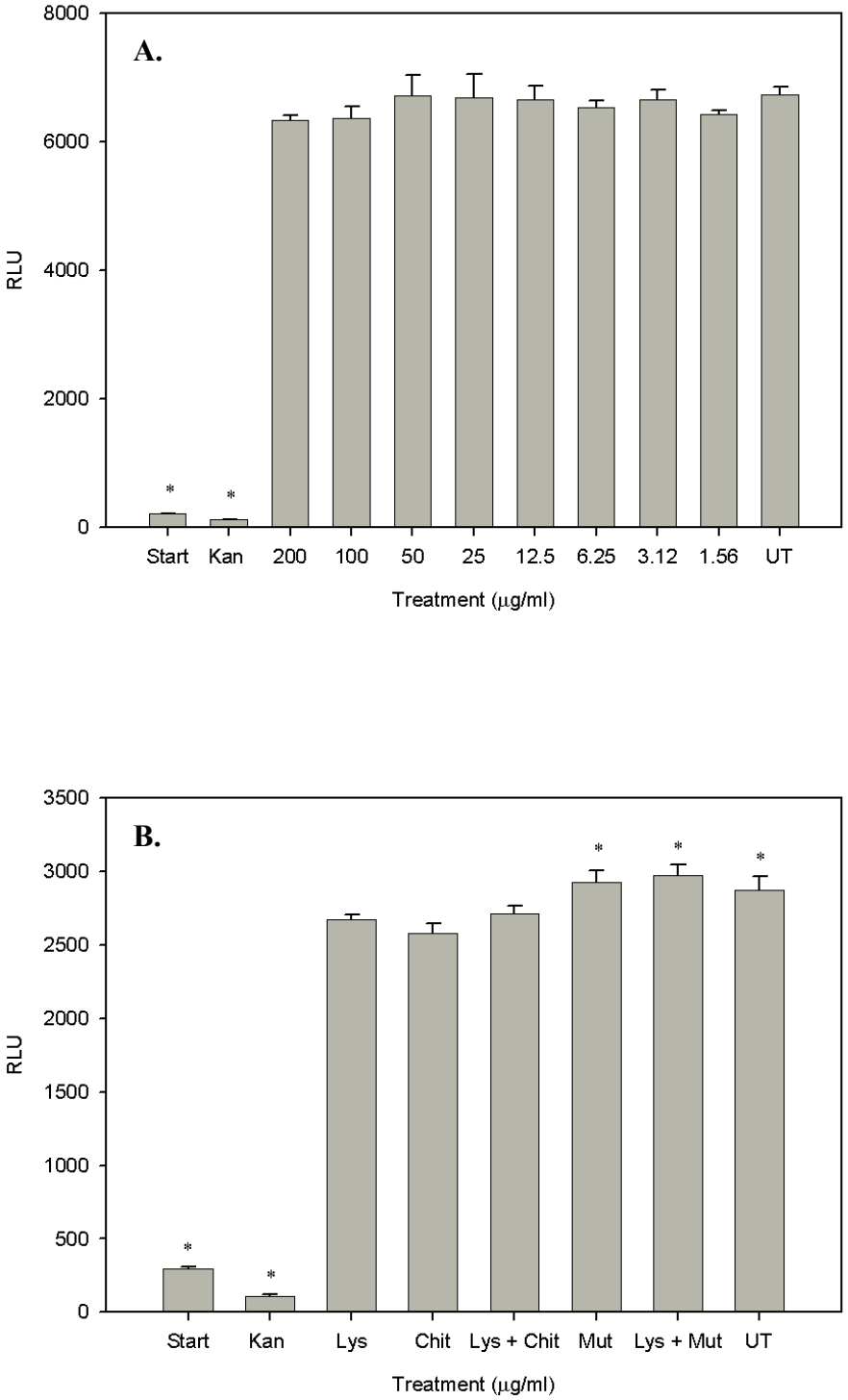


Figure 4.11 Antimicrobial growth inhibition of *E. coli* by egg-white lysozyme alone (A) or chiotriosidase, alone or in combination with a sub-optimal dose of lysozyme (B). The significance for the lysozyme charts is compared to the untreated control and the chitotriosidase assays compared to lysozyme alone. Experiments were repeated at least twice with triplicate samples with comparable results. Experiments were repeated at least twice with triplicate samples with comparable results. (Kan= kanamycin 50μg/ml, Lys= lysozyme 50μg/ml, Chit & Mut= recombinant chitotriosidase and active site mutant 25μg/ml). Statistical analyses were carried out by one-way ANOVA and post hoc LSD test (* significance at $P<0.05$) using SPSS v11.5.

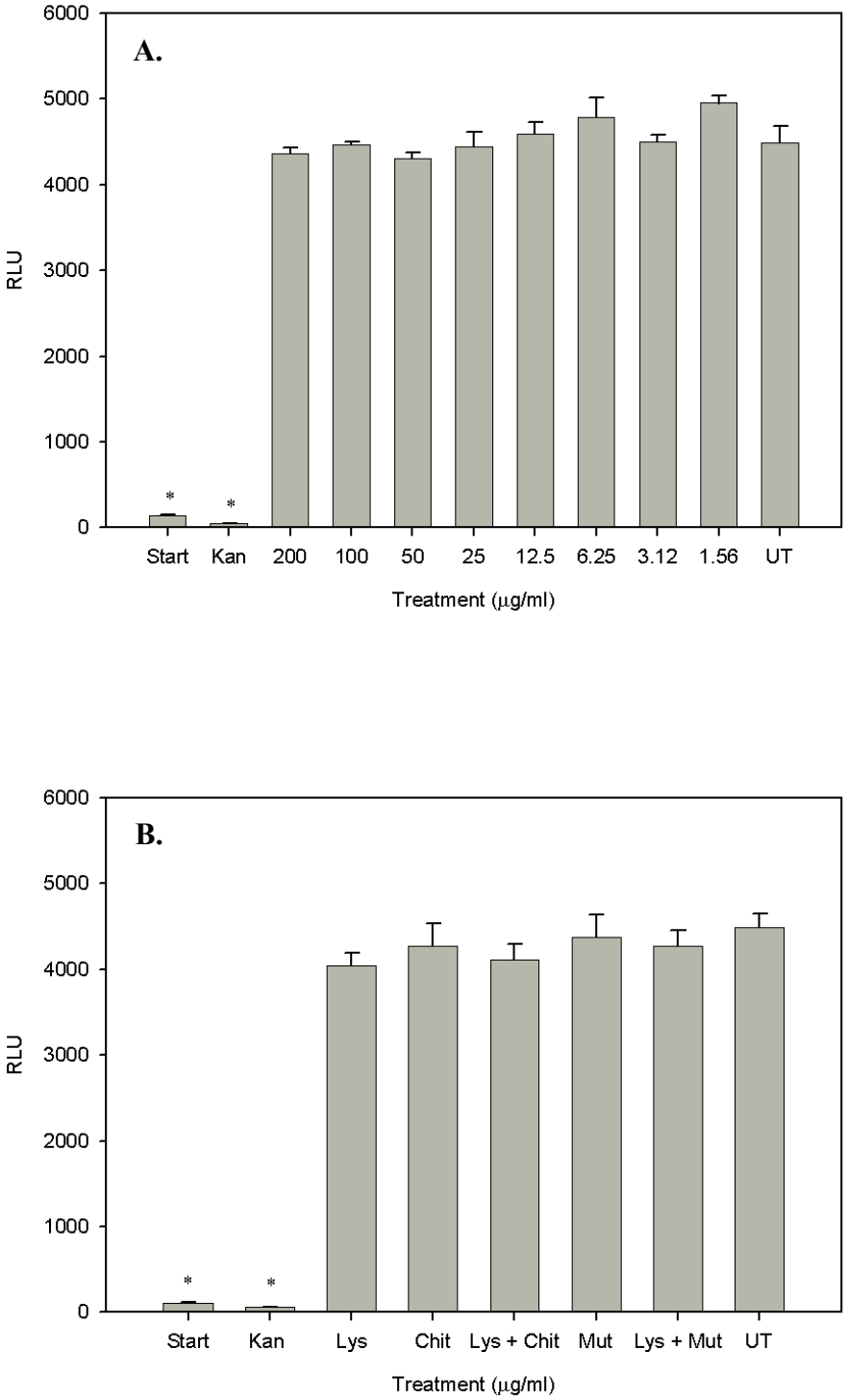


Figure 4.12 Antimicrobial growth inhibition of *P. aeruginosa* by egg-white lysozyme alone (A) or chiotriosidase, alone or in combination with a sub-optimal dose of lysozyme (B). The significance for the lysozyme charts is compared to the untreated control and the chitotriosidase assays compared to lysozyme alone. Experiments were repeated at least twice with triplicate samples with comparable results. Experiments were repeated at least twice with triplicate samples with comparable results. (Kan= kanamycin 50μg/ml, Lys= lysozyme 50μg/ml, Chit & Mut= recombinant chitotriosidase and active site mutant 25μg/ml). Statistical analyses were carried out by one-way ANOVA and post hoc LSD test (* significance at $P<0.05$) using SPSS v11.5.

4.5 DISCUSSION

Previously it has been shown that lysozyme and chitotriosidase are highly expressed in the lachrymal glands of the human eye (Malik et al., 1970), (Hall et al., 2007a). The presence of chitotriosidase activity is detectable in human tears (Figure 4.2), however lysozyme has a lower but significant chitotrioside cleaving capability and therefore this evidence alone cannot be used to confirm that chitotriosidase is secreted in tear fluid.

Lysozyme has documented antimicrobial effects against Gram-positive bacteria and, in sub-optimal doses in combination with other compounds, against Gram-negative bacteria and fungi (Ellison and Giehl, 1991) (Nishiyama et al., 2001). In addition to anti-fungal activity, it has been shown that chitotriosidase may have a role in anti-bacterial immunity as a previous study showed that children with the mutant *CHIT1* genotype and undergoing invasive AML treatment were more susceptible to infection with Gram- negative bacteria (Lehrnbecher et al., 2005). Therefore in the present study the activity of recombinant human chitotriosidase, alone and in combination with sub-optimal concentrations of lysozyme, was tested on the viability of six Gram- positive and two Gram-negative bacterial species.

Firstly the recombinant human chitotriosidase was characterized. The in-frame expression and purity of the enzyme were determined by

Western blot for the V5 epitope and silver staining respectively (Figure 4.3). The molecular weight of the recombinant is slightly greater than 50 kDa isoform courtesy of the his tag and V5 epitope present at the C terminal of the protein. The enzymatic activity of chitotriosidase was confirmed using a 4-MU chitotrioside substrate assay and specific activity was determined to be 1260 μmol/mg/min and a maximum activity range between pH 5 and 7. The active site mutant was enzymatically inactive.

The current study shows that although the recombinant chitotriosidase is enzymatically active, it does not have an anti-bacterial effect on any of the species tested (at 25 μg/ml). Moreover, chitotriosidase has no additive or synergistic effect when added in combination with sub-optimal doses of lysozyme regardless of the susceptibility to this enzyme. Therefore this data does not support the previously described study linking the absence of functional chitotriosidase with increased Gram- negative bacterial infections (Lehrnbecher et al., 2005).

The importance of these anti-bacterial compounds and their interactions in tears *in vivo* is complicated by the presence of several hundred other proteins present in the fluid (de Souza et al., 2006). These include the secretory phospholipases A_2 ($sPLA_2$); members of the PLA_2 family that hydrolyze sn-2 acyl bonds in cell membrane phospholipids to release arachidonic acid: a precursor of eicosanoids (prostaglandins, prostacyclins, leukotrienes and thromoboxanes), many

of which have pro-inflammatory activities (Murakami and Kudo, 2001). Recently it has been demonstrated that sPLA2, and in particular group IIA $sPLA_2$, are primarily responsible for the removal of certain Gram-positive species (for example *Listeria monocytogenes*), suggesting that lysozyme, present at much higher concentrations, has only a secondary effect (Qu and Lehrer, 1998). The activity of $sPLA_2$ is derived from its high positive surface charge that allows penetration of the Gram-positive bacterial cell wall, leading to bacterial lysis (Beers et al., 2002).

It is therefore likely that the antimicrobial activity of chitotriosidase is anti-fungal rather than anti-bacterial. This hypothesis is supported by the results from chapter 3 showing that *CHIT1* expression in human monocyte derived macrophages is induced by fungal but not bacterial PAMPs and that chitotriosidase has activity against multiple fungal species including *C*. albicans, *C*. *neoformans* and *M*. *rouxii* (Boot et al., 2001, van Eijk et al., 2005).

Complete activity of human chitotriosidase may be dependent on both the active site and chitin-binding domain. Figure 4.3 shows that our recombinant chitotriosidase is capable of cleaving chitotrioside substrates, however this activity may come completely from the active site. The addition of the his-tag and V5 epitope at the C-terminus of the enzyme may inhibit the chitin-binding domain. It has previously been shown that this domain that is present in the 50 kDa isoform but

truncated in the 39 kDa isoform of chitotriosidase is required for tight binding to chitin (Renkema et al., 1997). Furthermore the 39 kDa form that accumulates in lysosomes, may not require the chitin-binding domain to degrade short chitin oligosaccharides.

Proteins that primarily consist of chitin-binding domains exist and have antimicrobial activities. Two examples in plants and invertebrates are hevein and tachycitin respectively. The structure of tachycitin is shown in figure 4.13. The key features are the three and two stranded β-sheets. The presence of key aromatic residues in the β4, β5 and α1 strands suggests that this region forms the chitin binding site in the C-terminal end of the protein.

Hevein and tachycitin are structurally similar and have the chitin binding motif (Suetake et al., 2000). As these proteins do not contain an active site, their anti-fungal activity is derived from chitin binding in the cell wall that prevents remodelling required for growth. As bacteria lack chitin, it has been suggested that the anti-bacterial effect of the proteins comes from their oligomerisation resulting in bacterial agglutination (Suetake, 2002).

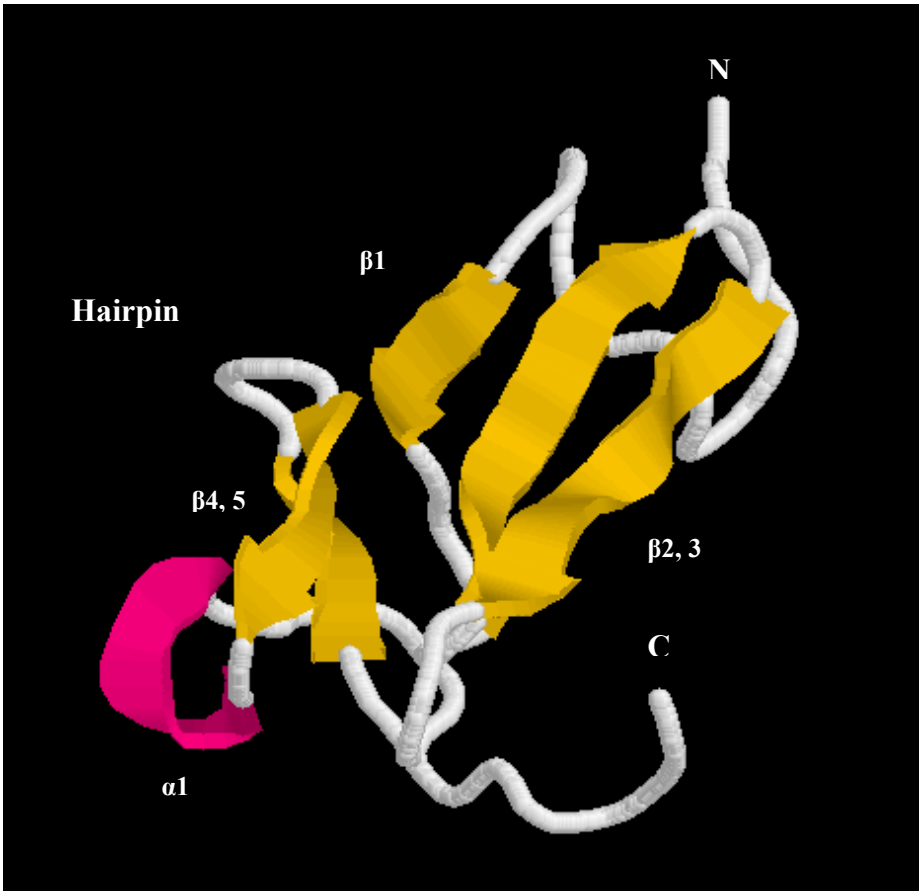


Figure 4.13 The structure of chitin binding protein tachycitin consisting of 3 stranded and 2 stranded anti-parallel β- sheets (yellow), α- helical turn (pink) and 5 disulphide bridges (grey). (Adapted from (Suetake et al., 2000) using Raswin v2.6)

It has been suggested that the chitin-binding domain of chitotriosidase is similar to that of chitinase B (ChiB) in *Serratia marcescens* (*S. marcescens*) (Fusetti et al., 2002). The ChiB chitin binding domain consists of three anti-parallel β strands with exposed aromatic residues (Trp479 and Tyr481) required for substrate binding (van Aalten et al., 2000). The structural and functional similarities between conserved chitin binding proteins like tachycitin and hevein and the C- terminal regions of ChiB and chitotriosidase suggests that these chitin binding domains are required for tight substrate binding and therefore full activity against complex polysaccharides.

Defining a role for a fully active chitotriosidase *in vivo* that includes both active site and chitin binding domain is complicated by the lack of an obvious phenotype in *CHIT1* mutants. The presence and absence of the *CHIT1* homozygous mutant genotype has been linked with susceptibility to lymphatic filariasis and malaria, respectively (Choi et al., 2001, Malaguarnera et al., 2003). However, variant *CHIT1* genotypes have not been associated with disease prognosis resulting from fungal infections. For example, the mutant *CHIT1* genotype does not increase the risk of candidiasis in leukaemia patients (Choi et al., 2005) and the presence of the mutant genotype has no effect on survival from *Candida* sepsis (Masoud et al., 2002).

The absence of a phenotype resulting from chitotriosidase deficiency in non-immune suppressed individuals may be explained several ways. Firstly it is possible that chitotriosidase has a non- immune function, for example in the digestion of chitinous material. A second human chitinase called acidic mammalian chitinase (AMCase) is highly expressed in the gastrointestinal tract and shows maximum activity at pH 2 suggesting a digestive function (Boot et al., 2001). A similar role of chitotriosidase seems unlikely considering the expression pattern of the gene in macrophages and neutrophils and maximum activity being observed at pH 6.

It is also possible that through evolution, several tiers of redundancy have developed that mask the development of a phenotype resulting

from the *CHIT1* mutation. Innate immunity gene mutations are often fatal as a result of the requirement for an immediate response following infection and the complexity of the interaction between the innate and adaptive immune systems. However, as stated earlier, chitinases in other species are known to act in synergy, for examples with glucanases in plants (Moravcikova, 2004) and therefore an obvious phenotype may only develop in the presence of concurring mutations.

A further possibility is that *in vivo* and at physiological concentrations, chitotriosidase has a non-critical supporting role in innate immunity. Previous studies showing that chitotriosidase inhibits fungal growth and hyphal switching were conducted at high concentrations (1 mg/ml) (van Eijk et al., 2005) and may reflect a general inhibitory effect caused by excessive membrane binding. One possibility is that chitotriosidase shares a function with lysozyme by opsonising Gram-negative bacteria and fungi for subsequent removal by phagocytosis (Zhukovskaia and Likina, 1966). This would explain the previously described link between the mutant *CHIT1* genotype and susceptibility to bacterial infection (Lehrnbecher et al., 2005) and the absence of a direct anti-bacterial effect in the current study.

In summary, chitotriosidase activity is detectable in human tears and this is consistent with the high expression of the *CHIT1* gene in human lachrymal glands. Human recombinant chitotriosidase activity is not

inhibited by high concentrations of salt but the enzyme had no effect on the viability of bacteria suggesting that its role in innate immunity is distinct from lysozyme. However, as full activity of chitotriosidase may be dependent on the chitin binding domain and active site, this can be clarified using untagged recombinant *CHIT1* and mutant and also a recombinant truncated protein without the chitin binding domain.

5. ALLELIC FREQUENCY OF THE 24BP MUTATION IN *CHIT1* IN A POPULATION FROM A HOOKWORM-ENDEMIC REGION OF PAPUA NEW GUINEA

5.1 INTRODUCTION

The genes of all identified human chitinase family members are located on chromosome 1. The chitotriosidase (*CHIT1*) gene consists of 12 exons and is present at 1q31-32, as shown by linkage analysis and fluorescence *in situ* hybridisation (Eiberg and Den Tandt, 1997) (Boot et al., 1998). Translation of *CHIT1* produces a 50-kDa protein; a proportion of which is routed to lysosomes, via a process that is not fully understood (Renkema et al., 1995). The lysosomal protein has a molecular mass of 39-kDa, resulting from the removal of the C-terminal domain by proteolytic processing (Boot et al., 1995). The presence of different amounts of sialic acid capped O-linked glycans in the 50-kDa isoform results in heterogeneous isoelectric points of 6.5 and 7.2, whereas the 39-kDa protein is not glycosylated and has a pI of 8.0 (Renkema et al., 1995, Renkema et al., 1997).

The production of the 50-kDa isoform of chitotriosidase is dependent on the splicing out of exon 11. In the macrophages of some individuals, transcription of exon 11 leads to activation of a stop codon resulting in small amounts of mRNA encoding a 40-kDa protein. This

form is almost identical to the 39-kDa isoform and is C-terminal truncated (Renkema et al., 1997).

There are several mutations that have been described in the *CHIT1* gene. These include the single nucleotide polymorphisms (SNPs) G102S, A442G; that are present in all continents and G354R and A442V that occur at low levels exclusively in African populations. Reduced chitotriosidase activity has been detected in the serum of individuals with the latter two mutations only (Lee et al., 2007) (Bierbaum et al., 2006). Lee *et al*. (2006) suggest that these polymorphisms may have development as a result of the preservation of the mutant genotype. This hypothesis is supported by the possibility that this version of the gene confers protection against malaria as active chitotriosidase in the serum could increase malarial tranmit through mosquito vectors by preventing the formation of the chitinous peritrophic membrane (Di Luca et al., 2006) .

The most significant mutation in the *CHIT1* gene occurs in exon 10, where alternative splicing occurs in a number of individuals, resulting from a 24 base-pair tandem repeat. This mutation leads to activation of a cryptic 3' splice site, resulting in the production of an abnormal mRNA with an inframe 87 nucleotide deletion. The mutant protein lacks amino acids Val-344 to Gln-372 that are required to form part of the catalytic core, causing a mis-fold in the triosephosphateisomerase (TIM)-barrel (Boot et al., 1998) (Fusetti et al., 2002). However, *in*

vivo, macrophages from chitotriosidase deficient individuals express only small amounts of mRNA and secrete virtually no chitotriosidase protein (Boot et al., 1998).

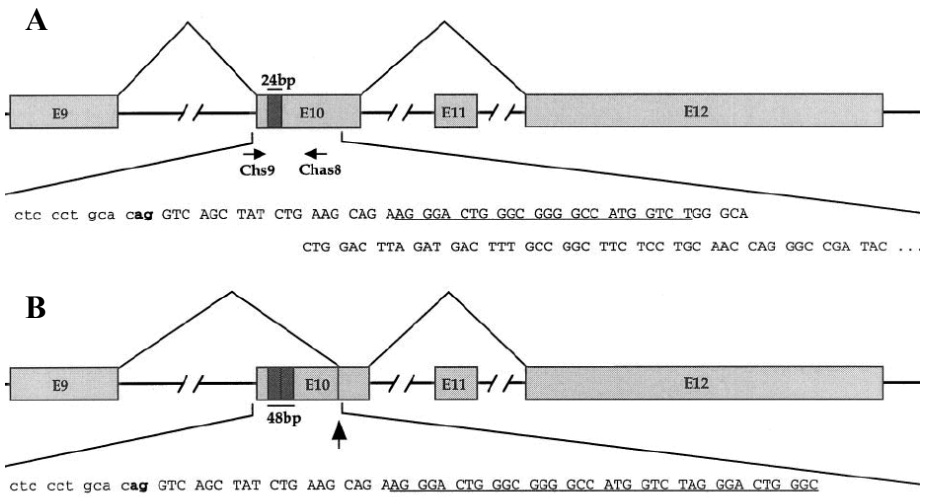


Figure 5.1 Defect in the *CHIT1* gene in chitotriosidase-deficient individuals. Overview of the relevant section of the *CHIT1* gene. (A) The normal *CHIT1* gene. Arrows represent the position of primers used to amplify genomic DNA. Arrows above the gene show normal splicing in which exon 11 is skipped. (B) In the mutant gene there is a 24bp duplication in exon 10 leading to activation of a cryptic 3' splice site (vertical arrow). The 24bp fragment in the normal gene and the 48bp fragment in the mutant gene are underlined (Boot et al., 1998).

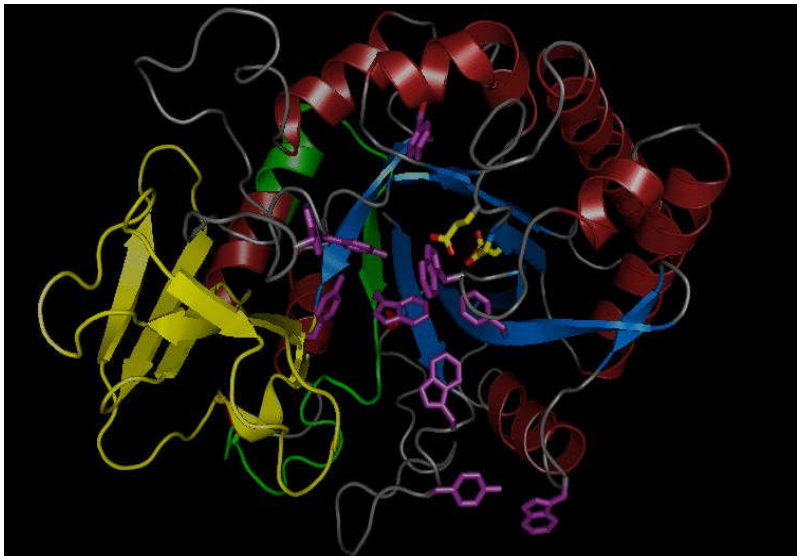


Figure 5.2 Secondary structure of the (hypothetical) mutant chitotriosidase. The 24bp mutation in exon 10 of the *CHIT1* gene results in an mRNA with an inframe 87 nucleotide deletion resulting from the activation of an cryptic 3' splice site. The mutant protein lacks amino acids 344-372 (shown in green), which are required for the formation of the TIM barrel core. Also shown are the the α/β domain (yellow), the $(\alpha\beta)_8$ barrel (blue/red) (from: http://davapc1.bioch.dundee.ac.uk/)

In Dutch and Ashkenazi Jewish populations, 6% of individuals are homozygous for the mutant allele (Table 5.1), whereas 35% and 34% respectively, are heterozygous carriers (Boot et al., 1998) with a mutant allele frequency of 0.23. A similar allelic frequency is found in Portugal (0.22), Sicily (0.27) and Sardinia (0.21) (Malaguarnera et al., 2003). However in two West African countries, Benin and Burkina Faso, that are *meso*-endemic for *Plasmodium falciparum* (*P. falciparum*) malaria and endemic for gastrointestinal helminths, a total absence of the homozygous mutation and significant reduction in heterozygous individuals has been shown, with a mutant allele frequency of 0.00 and 0.02 respectively (Malaguarnera et al., 2003). This observation led to the hypothesis that the wild-type *CHIT1* genotype may confer resistance to individuals with protozoan or helminth infections, common in tropical countries (Malaguarnera et al., 2003). The potential mechanism by which protection occurs is unclear. However chitin, the natural endogenous substrate of chitotriosidase, has been identified in the egg-shell of parasitic and free-living nematodes and in the microfilarial sheath surrounding the first stage larvae of filarial nematodes (Fuhrman and Piessens, 1985). More recently, the pharynx of the non-filarial nematode *Caenorhabditis elegans* (*C. elegans*) and *Oesophagosomum dentatum* (*O. dentatum*) have been shown to contain chitin (Zhang et al., 2005) (Neuhaus, 1997).

Chitin synthase (*chs*) genes have been identified in *Caenorhabditis elegans* (*C. elegans*) and the filarial species *Brugia malayi* (*B. malayi*) and *Dirofilaria immitis* (*D. immitis*) (Harris et al., 2000). *C. elegans* expresses *chs*-1 and *chs*-2, which encode eggshell and pharynx chitin respectively. Gene knockdown of *chs*-2 in *C. elegans* leads to loss of pharyngeal function and starvation (Zhang et al., 2005). This suggests that host chitotriosidase could potentially disrupt feeding by parasitic nematodes.

In support of the theory that chitotriosidase could interfere with filarial survival, a previous study has shown a significant association between susceptibility to lymphatic *Wuchereria bancrofti* infection and the homozygous mutant genotype in an Indian population (Choi et al., 2001). However a similar study of lymphatic filariasis from Papua New Guinea showed no link with genotype (Hise et al., 2003).

Table 5.1 Genotype and allele frequencies of the *CHIT1* mutation in different populations.

Population	Subjects	% wt/wt	% wt/mt	%mut/mt	Wt allele frequency	Mt allele frequency	Reference
Sicily	100	51	43	6	0.73	0.27	(Malaguarnera et al., 2003)
Sardinia	107	64	32	4	0.79	0.21	(Malaguarnera et al., 2003)
Portugal	295	60	37	3	0.78	0.22	(Rodrigues et al., 2004)
Holland	171	59	35	6	0.77	0.23	(Boot et al., 1998)
Ashkenazi Jews	68	60	34	6	0.77	0.23	(Boot et al., 1998)
South India	67	31	58	10	0.60	0.40	(Choi et al., 2001)
Papua New Guinea	906	77	22	1	0.88	0.12	(Hise et al., 2003)
Chinese Han	82	15	55	30	0.42	0.58	(Chien et al., 2005)
Benin	100	100	0	0	1.00	0.00	(Malaguarnera et al., 2003)
Burkina Faso	100	98	2	0	0.98	0.02	(Malaguarnera et al., 2003)

5.2 OBJECTIVES

Necator americanus (*N. americanus*) is a human hookworm species that is common in tropical and sub-tropical countries. Morbidity as a result of infection is primarily due to iron-deficiency anaemia (Stoltzfus et al., 1997). Previous studies investigating the susceptibility of individuals with the mutant *CHIT1* genotype to infection have focused on filarial nematodes. In the present study, 693 people from five villages in the Madang Province of Papua New Guinea were genotyped for the *CHIT1* mutation. Association between faecal egg counts and *CHIT1* genotype were investigated by transmission disequilibrium testing.

As it has previously been suggested that the wild-type *CHIT1* genotype may confer protection against malaria based on a low frequency of the mutant allele in *meso*-endemic Burkina Faso and Benin (Malaguarnera et al., 2003), allelic frequency of the mutant *CHIT1* allele was compared to that of previously reported populations. Furthermore, the correlation between *CHIT1* genotype and malarial parasitaemia was investigated using a logistic regression model.

5.3 METHODS

5.3.1 Geographical location and study population

The study population consisted of 5 villages (Bauri, Welwel, Mawan, Haven and Wasab) in lowland Madang Province, Papua New Guinea. Study samples and pedigree data were obtained in 1998 and the process of collection has been previously described (Quinnell et al., 2004). Briefly, villages were censused, pedigree information collected and sample containers for faecal collection offered to all individuals aged 4 years and above. Faecal egg counts were performed using a modified McMaster salt flotation method and results were expressed as eggs per gram (epg) of faeces (Quinnell et al., 2004). Venous blood was then collected, and all individuals were offered treatment with albendazole or pyrantel pamoate.

DNA was extracted from buffy coats using phenol/chloroform. In September 2001, a second faecal sample was taken from 3 of the 5 villages and re-infection hookworm burden determined; some previously untreated individuals provided samples in 2001. Hookworm epg has been shown to be a heritable phenotype in the study population (Breitling and Quinnell, unpublished results). The study was approved by the Medical Research Advisory Committee of Papua New Guinea and informed consent was received from all subjects or their parents.

5.3.2 Chitotriosidase genotyping by PCR

Thermal cycling reactions consisted of Reddy Mix PCR master mix (ABgene), 4.5pmol forward and 4.5pmol reverse chitotriosidase primers (Forward: AGCTATCTGAAGCAGAAG Reverse: GGAGAAGCCGGAAAGTC) (Sigma) (Boot et al., 1998) and 2µl genomic DNA in a final volume of 15µl. Samples were run in 96 well PCR plates (ABgene) on a Peltier PTC-200 Thermal Cycler (MJ Research). The thermal cycling protocol was: 94ºC for 5 minutes; 35 cycles of 94ºC for 30 seconds, 54ºC for 30 seconds and 72ºC for 30 seconds; and 72ºC for 5 minutes. Thermal cycling products with 10 base pair markers (Promega) were separated on 4% high resolution agarose (Sigma) gels in 1x TBE buffer and ethidium bromide. Gels were viewed using the Gene Genius Bio-imaging System and Gene Snap software (Syngene). The size of wild-type product is 75 base pairs whereas the mutant product is 99 base pairs due to the mutant allele containing a 24 base pair duplication in exon 10 (Boot et al., 1998).

5.3.3 Statistical analyses

Associations between faecal egg counts and *CHIT1* genotype were tested by transmission disequilibrium testing using the programme QTDT (Abecasis et al., 2000). Both total and within-family associations were examined, the latter controlling for any population stratification, including polygenic and locus-specific variance components. Faecal egg counts were log-transformed before analysis, and age, age^2, sex, village, year and faecal consistency included as

covariates. Empirical P values were calculated after 1000 permutations.

Binary logistic regression was used to correlate Chit genotype with the presence of malaria merozoites in blood smears. Data analysis was achieved using SPSS software. Malaria species (*P. falciparum* and *P. vivax*) were analysed individually and together. In each analysis, malaria species was designated as the dependent variable and age age^2, sex, village and genotype were covariates and village was categorical.

5.4 RESULTS

In total 693 individuals from Papua New Guinea were genotyped for the chitotriosidase 24 base-pair duplication. The integrity of the pedigree data was tested using Pedcheck (O' Connell, JR 1998), a program for detecting incompatibilities in pedigree data, and was found to be consistent. There were no level 0, 1 or 2 errors demonstrating the absence of duplicate entries and impossible pedigrees and the presence of the correct number and gender of parents.

5.4.1 Allelic frequency of the mutant *CHIT1* genotype in the study population

The allelic frequency of the mutation in 279 unrelated individuals, determined by PCR genotyping was 0.251. The percentage of individuals who were homozygous mutant was 5.7% (16), heterozygous 38.7% (108) and homozygous wild-type 55.6% (155).

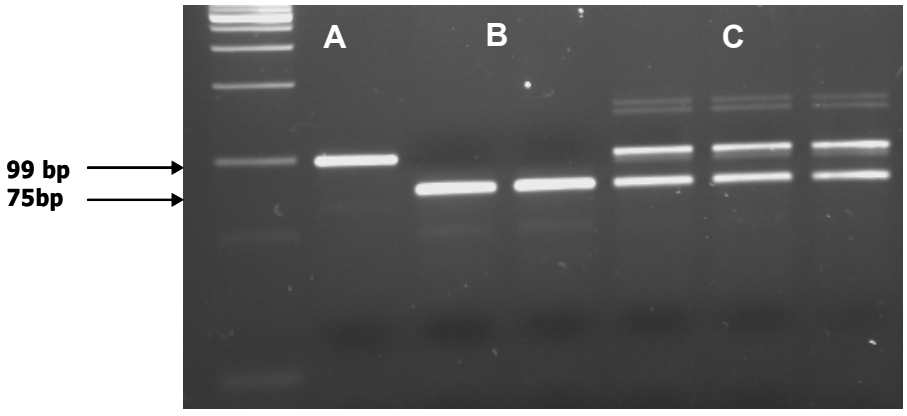


Figure 5.3 Image from high resolution agarose gel showing detection of different *CHIT1* genotypes by PCR of genomic DNA. Bands show different product sizes resulting from the 24bp duplication in the mutant allele. Samples from homozygous mutant (A), homozygous wild-type (B) and heterozygous (C) individuals are shown. Additional larger fragments are formed by the hybridisation of normal and mutant strands in genomic DNA from heterozygotes (Boot et al., 1998). Each lane represents a different individual.

Genotype frequencies did not differ from those expected under the Hardy-Weinberg equilibrium (χ^2= 0.25, *P*= 0.62). Both egg counts and *CHIT1* genotypes were available for a total of 602 individuals (574 pre-treatment and 210 individuals post-treatment). The prevalence and intensity of infection (mean egg count) in these individuals were 81% (2574 epg) pretreatment, and 92% (1727 epg) post-treatment.

5.4.2. Association between *CHIT1* genotype and *N. americanus* infection

There was no significant association between *CHIT1* genotype and egg count, using either the total (pre-treatment χ^2=0.66, p>0.1, post-treatment χ^2=0.18, p>0.1) or within-family test of association (pre-treatment χ^2=0.03, p>0.1, post-treatment χ^2=0.07, p>0.1).

5.4.3. Association between *CHIT1* genotype and malaria

The association between *CHIT1* genotype and number of *Plasmodium* merozoites was quantified using binary logistic regression. Genotype was treated as a categorical variable (5.2A) with genotypes treated discretely or as a continuous variable (5.2B) with heterozygotes treated as between two homozygotes. The results show that there was no significant association between genotype and merozoite number using either type of data analysis.

Table 5.2 Logistic regression showing correlation between malaria and CHIT1 genotype with genotype as a categorical variable. (A) and genotype as a continuous variable (B).

A

Plasmodium species	***P* values malaria infection *vs.* genotype**
P. falciparum (asexual stage/*gametocytes*)	0.447
P. vivax	0.257
P. falciparum + *P. vivax*	0.361

B

Plasmodium species	***P* values malaria infection *vs.* genotype**
P. falciparum (asexual stage/gametocytes)	0.444
P. vivax	0.119
P. falciparum + *P. vivax*	0.875

5.5 DISCUSSION

The results from the present study in the Madang Province of Papua New Guinea indicate that chitotriosidase does not have a critical role in protection against *N. americanus* and malaria. A comparison of the allelic frequency of the 24 base-pair *CHIT1* duplication in unrelated individuals from our data (0.25) with other populations shown in table 5.1 shows similarity with studies from Sicily and Sardinia (0.73 & 0.79) (Malaguarnera et al., 2003), Portugal and Holland (0.22 & 0.23) and the Ashkenazi Jews (0.23) (Rodrigues et al., 2004) (Boot et al., 1998). This, in combination with the mutant allele frequency matching that expected with the Hardy-Weinberg principle, suggests that there is no hereditary advantage for the wild-type allele in this population.

It has previously been shown that there is a low incidence of the heterozygous and mutant *CHIT1* genotypes in the West African countries of Benin and Burkina Faso. Furthermore it has been suggested this has resulted from selective pressure on the *CHIT1* gene, caused by the presence of widespread parasitic infection and *meso*-endemic status of this region (Malaguarnera et al., 2003). In terms of filarial infection, this theory is supported by a study of individuals in South India that linked the mutant *CHIT1* genotype to susceptibility to infection with *Wuchereria bancrofti* (*W. bancrofti*) (Choi et al., 2001).

However, the results from the current study are not consistent with this hypothesis, showing that in this Papua New Guinean population the mutant allele frequency is similar to the non *meso*-endemic populations

previously described. Furthermore, the fixation-index (*F*st) value, a measure of genetic variation, has been determined (by Dr. Rupert Quinnell), for the *CHIT1* locus. The value of 0.11 is similar to the median value of 210 insertion-deletions (indels) across the genome (Hall et al., 2007b). Similar *F*st values with other genes suggests selective neutrality as opposed to natural selection which can deviate this value (Akey et al., 2002). Ultimately this would indicate that there has not been directional selection at this locus across different geographical areas and that the worldwide variation in allele frequencies is consistent with genetic drift.

Assuming that the *CHIT1* gene is unaffected by selective pressure, there are several possible explanations for the absence of the mutant genotype in Benin and Burkina Faso. In neutral traits, the main factor causing fluctuation in allelic distribution over time is genetic drift. This occurs randomly, however it would be expected that over an undefined period of time that variant genotypes would be removed from the population resulting in homozygosity. In small populations, and those with a limited breeding pool, this process will occur at a faster rate as each random event has a higher impact on the overall allelic distribution. Therefore it is possible that in Benin, Burkina Faso and other undefined populations, the mutant *CHIT1* genotype has been lost by this process. Furthermore, genetic drift in West Africa could have been accentuated by the migration of people of East African descent to Europe and Asia (Piras et al., 2006).

In addition to demonstrating that the allelic frequency of the *CHIT1* mutation in the Madang Province of Papua New Guinea is similar to populations previously reported that are not endemic for malaria and gastrointestinal parasites, this study shows that there was no association between *CHIT1* genotype and *N. americanus* hookworm burden. Previous studies have focussed on the susceptibility of the mutant genotype to infection with filariasis as chitin, the potential endogenous substrate, is present in the cuticle of mature *W. bancrofti* (Araujo et al., 1993). In addition, filarial nematodes including *Brugia malayi* contain chitin in their microfilariae, oocytes and zygotes (Fuhrman and Piessens, 1985, Schraermeyer et al., 1987). Chitin turnover, required for sheath development, is an important process in the lifecycle of these species.

It is therefore possible that if chitotriosidase can access and cleave the chitin present in these structures, the growth and development of filarial nematodes would be interrupted. This could explain the observed susceptibility to *W. bancrofti* of individuals with the mutant genotype (Choi et al., 2001). However this issue is in need of further clarification as a subsequent study by Hise *et al*. (2003) in a region endemic for bancroftian filariasis in Papua New Guinea did not find any significant correlation with infection status or disease phenotype.

In contrast to filarial worms, gastrointestinal nematodes such as *N. americanus* do not have a microfilarial stage, however chitin has been identified in their eggshells, including those of *Ascaris lumbricoides*

(Sromova and Lysek, 1990). The importance of chitin in the lifecycle of these species is less well defined and it is also likely that eggshell chitin is not accessible to the host chitotriosidase. Chitin has, however, been identified in the pharynx of *O. dentatum*, a closely related species to *N. americanus* (Neuhaus, 1997). Macrophage and neutrophil derived chitotriosidase may have access to pharyngeal chitin during the larval stage and as blood-feeding adults.

As part of this study, the association between *CHIT1* genotype and malaria intensity was also investigated and showed no significant correlation between the gene and the extent of infection. This association was investigated by making single time point assessments of parasitaemia (presence of blood merozoites) in blood smears and correlating them with genotype, using logistic regression. The limitation of this analysis was that it was based on a single time point assessment rather than repeated measurements. As parasitaemia is very variable, it is difficult to make a reliable statement regarding the effect of the *CHIT1* genotype on malarial infection, and therefore further studies are needed.

Chitotriosidase levels are elevated in the serum of individuals with acute *P. falciparum* malaria, possibly reflecting the activation of the reticulo-endothelial system (Barone et al., 2003). However the allelic frequency found in the present study of Papua New Guinea was virtually identical to that seen in countries that are not *meso*-endemic

for malaria, precluding a strong protective effect of the wild-type *CHIT1* genotype against mortality.

In addition, it is possible that high levels of chitotriosidase in serum may increase malarial ookinete transit through *Anopheles*, the genus of mosquito vectors required to complete the malaria lifecycle, by interrupting the formation of the chitin-containing midgut peritrophic membrane (PM). The feeding of blood from patients with elevated chitotriosidase activity, including those with Gaucher disease, appears to degrade the PM between 16-24 hours post-treatment (Di Luca, 2007, 2006). The PM acts as a barrier between the undigested material and the epithelium; movement of malarial ookinetes is dependent on endogenous chitinases, present at the apical ends of these zygotes (Shahabuddin, 1994).

Human *CHIT1* and chitinase from *P. falciparum* (PfCHT1) have a sequence homology of only 14%, however the presence of shared sequence motifs and flanking sequences have led to the suggestion of functional homology between them (Vinetz et al., 1999). The idea of cooperation between malarial and human chitinases in degrading the PM of *Anopheles* is supported by the high transmissibility of malaria in countries like Benin and Burkina Faso where the wildtype *CHIT1* is most prevalent.

In summary, our data suggest that the chitotriosidase genotype does not play a major role in protection against hookworm infection, but this may be different in the case of lymphatic or cutaneous filariasis. The allelic frequency found precludes a strong protective effect against malaria and suggests that variability in allelic frequency may be caused solely by genetic drift. A similar genotype frequency is found in several European populations as well as the indigenous population in Papua New Guinea, suggesting that the *CHIT1* mutation arose before these ethnic groups diverged. The loss of the 24 base-pair mutant *CHIT1* genotype in Benin and Burkina Faso may have been caused by genetic drift, rather than selective pressure as had previously been suggested.

6. GENERAL DISCUSSION

The aim of this work was to characterize mammalian chitinases and in particular chitotriosidase. The originally envisaged approach was the following:

1. Determine the expression pattern of the chitotriosidase (*CHIT1*) gene at the tissue and cellular level; 2. Study *CHIT1* gene expression in macrophages, which were known to express *CHIT1* following treatment with fungal and bacterial PAMPs; 3. Study the putative anti-fungal and anti-bacterial activity of a recombinant chitotriosidase; 4. Investigate the potential protective effect of the enzyme against malarial and nematodal parasitic infections; 5. Clarify the phylogenetic relationships between members of the mammalian chitinase/chi-lectin gene cluster.

Is chitotriosidase inducible?

The high levels of chitotriosidase expression demonstrated in the lymph nodes, bone marrow, lung and lachrymal glands suggest a potential role for the enzyme in innate immunity (Boot et al., 2005b, Hall et al., 2007a). Furthermore, it has been shown that, at a cellular level, constitutive expression occurs in macrophages and increases during the process of *in vitro* differentiation from peripheral blood monocytes (Boot et al., 1995). In chapter 3 (figure 3.2), this process is shown over the course of seven days (by qRT^2-PCR), resulting in close to a 1000-fold increase in mRNA expression over this period.

In addition to high levels of constitutive expression, the *CHIT1* gene has also been shown to be inducible by cytokines (TNF-α and IFN-γ) and prolactin (Malaguarnera et al., 2004) (Malaguarnera et al., 2005). Furthermore, as chitotriosidase levels are raised in children with fungal and bacterial infections (Labadaridis et al., 2005) and the mutant *CHIT1* genotype has been linked with susceptibility to Gram-negative bacterial infection in children with leukaemia (Lehrnbecher et al., 2005), monocyte-derived macrophages were treated with a range of fungal and bacterial PAMPs and live *C. albicans* and changes in *CHIT1* expression were determined.

The fungal cell wall components that primarily consist of β-glucans (laminarin and zymosan) significantly induced *CHIT1* expression, whereas mannan and live *C. albicans* did not. This lack of *CHIT1* induction is consistent with the hypothesis that hyphal switching in particular fungal species allows evasion from the immune response by protecting the immunogenic β-glucan under the mannan-rich layer (Gantner et al., 2005).

The reason for the absence of *CHIT1* induction in macrophages following treatment with bacterial PAMPs is unclear. However, recently it has been shown that *CHIT1* expression is inducible by MDP (an active peptidoglycan motif) and not the TLR agonist PAM3Cys, in mature macrophages (van Eijk et al., 2007). As described in the introduction (section 1.2.1), the recognition of peptidoglycan, initially

thought to be a TLR-2 dependent response, appears to be dependent on intracellular NOD receptors (Girardin et al., 2003). Therefore it has been suggested that NOD2 is primarily required for macrophage activation in response to bacterial PAMPs (van Eijk et al., 2007). This may explain the observed lack of *CHIT1* induction in macrophages treated with bacterial TLR agonists (figures 3.7-3.9). The lack of activity observed following treatment with peptidoglycan may be explained by experimental conditions; in the method of van Eijk *et al*. (2007), 20μg of pure MDP was used to induce a response, whereas for this study only 100ng/ml of *B*. *subtilis* peptidogylcan was used (figure 3.8).

Previously it has been demonstrated that LPS induces *CHIT1* (Malaguarnera et al., 2005); however in the current work, there was no significant induction in macrophages from 26 donors treated with a fixed concentration of 50ng/ml (figure 3.6). The use of Polymyxin B in control samples to prevent endotoxin activity and the fact that LPS induced strong levels of TNF-α mRNA, confirms the validity of the analysis. However, in future experiments it would be useful to monitor LPS levels throughout the experiment in culture media and this could be achieved using the Limulus Amebocyte Lysate (LAL) test that produces clots which can be quantified using a turbidity assay, in the presence of endotoxin levels as low as 1ng.

Previously it has been shown that *CHIT1* expression is inducible by TNF-α (Malaguarnera et al., 2005). As discussed previously (Chapter

3), the induction of *CHIT1* expression in response to treatment with fungal PAMPs is unlikely to have resulted from an autocrine TNF-α mechanism, due to the time-scale required for this to occur. To confirm this, the production of TNF-α protein could be monitored by using an enzyme-linked immunosorbent assay (ELISA). This could be achieved as follows: 1) Coat polystyrene microtiter plates with anti-human TNF-α antibody. 2) Add tissue culture supernatants at different experimental time points, in triplicate. 3) Incubate to allow TNF-α antibody interaction and wash to remove non-specific binding. 4) Incubate with biotinylated secondary polyclonal antibody and wash off excess. 5) Add horseradish peroxidase-streptavidin (Avidin-HRP), incubate and wash. 6) Add substrate solution (tetramethylbenzidine) and measure optical density (OD). 7) Quantify TNF-α production using standard curve of recombinant enzyme versus OD.

Macrophages express two distinct types of TNF receptor: TNF-R1 and TNF-2 (Tartaglia et al., 1991). Following ligand binding, these receptors form trimers that induce a conformational change allowing dissociation of the inhibitory protein SODD and thereby allowing association with the adaptor protein TRADD (Hsu et al., 1995). The binding of TRADD allows further proteins to bind resulting in the activation of signalling pathways that lead to the activation of transcription factors. As shown previously (Figure 1.1), one of the most important transcription factors is NF-κB, which results in the expression of many immune effectors.

The lack of induction by bacterial PAMPs does not necessarily indicate a lack of anti-bacterial function as children with the mutant *CHIT1* genotype and undergoing therapy for acute myeloid leukaemia, have an increased susceptibility to Gram-negative bacterial infections (Lehrnbecher et al., 2005). This would suggest that in non-immune compromised individuals, other proteins compensate for the lack of chitotriosidase activity (as discussed in section 4.5).

Does chitotriosidase play a role in innate immunity?

Although it does not appear obvious to assume that chitotriosidase has a role in anti-bacterial immunity, as bacteria do not contain chitin, an important consideration is that lysozyme, which is primarily a muramidase, also has a weaker chitinase activity (Den Tandt et al., 1988). This most likely reflects the chemical similarity between chitinase and lysozyme substrates; chitin consisting of polymeric N-acetylglucosamine and peptidoglycan consisting of repeating N-acetylglucosamine and acetylmuramic acid disaccharide units. Furthermore hevamine, a chitinase produced by *Hevea brasiliensis*, has dual chitinase/muramidase activity (Rozeboom et al., 1990). Finally, chitotriosidase has been shown to have additional transglycosidase activity (Aguilera et al., 2003). This suggests that chitotriosidase may have antimicrobial properties that are not entirely dependent on chitinase activity.

In terms of discussing the function of chitotriosidase, it also has to be taken into account that several innate immunity effectors act in synergy with one another. In plants, chitinases act against fungal pathogens in synergy with beta-glucanases (Mauch et al., 1988), most likely to allow access to the chitin in the cell wall that is usually masked by mannoproteins and β-glucan. Currently no human genes encoding beta-glucanases have been identified and it has been demonstrated that recombinant chitotriosidase does not bind to the β-glucans curdlan and lichenan (Tjoelker et al., 2000). However, as lysozyme and chitotriosidase have similar cellular expression patterns and are highly expressed in the lachrymal glands (Malik et al., 1970) (Hall et al., 2007a) it was decided to test the enzymes for any synergistic antimicrobial activity.

Lysozyme was active against several bacterial strains: *B. subtilis*, *L. innocua* and *S. aureus* (oatA-deficient strain) (Bera et al., 2005) but not the Gram negative strains, *E. coli* and *P. aeruginosa*. Sub-optimal doses of lysozyme were tested in combination with recombinant chitotriosidase (25μg/ml) in sensitive strains and in high concentrations (50μg/ml) in non-sensitive strains. Previously it has been shown that lysozyme is active against Gram positive bacteria and, in combination with other antimicrobials, against Gram negative bacteria and fungi (Ellison and Giehl, 1991) (Nishiyama et al., 2001).

The conditions in which lysozyme is present in *in vitro* assays differs from that in tear fluid. Recently it has been demonstrated that the

antimicrobial peptide LL-37 requires the addition of carbonate to become active in the presence of physiological salt concentrations, and as tear fluid is carbonate-buffered, it may explain the lack of lysozyme antimicrobial activity in NaCl buffer alone (Dorschner et al., 2006).

The compounds of the aqueous part of tear film are poorly defined, but are likely to include at least 500 different proteins (de Souza et al., 2006), suggesting functional redundancy *in vivo*. Furthermore, the constituents of tear fluid vary according to the method of collection. It has been suggested that invasive methods can stimulate the conjunctiva and result in the release of plasma proteins from the blood via serum leakage (Ohashi et al., 2006). The method of tear fluid collection by de Souza *et al*. (2006) involved using a glass microcapillary tube without making contact with parts of the eye to prevent conjunctiva stimulation. Using this method of collection and mass spectrometry, de Souza et al (2006) did not identify chitotriosidase in basal tears. It is possible, however, that this was due to loss of the protein during the *in situ* digestion process. The presence of high levels of *CHIT1* in the lachrymal glands and the detection of the active enzyme in tear fluid (Hall et al., 2007a) (Figure 4.2) could be verified at the protein level through the generation of *CHIT*-specific antibodies. This could be achieved by repeatedly innoculating rabbits with recombinant human chitotriosidase and isolating the antibodies from the serum.

Of the total amount of protein in tears, it is believed that between 20-40% is lysozyme and that the typical concentration, between 0.6 and

2.6mg/L, is high enough to have an anti-bacterial effect (Ohashi et al., 2006). Many of the remaining proteins expressed by the lachrymal gland are found at much lower concentrations, however as discussed in chapter 4, antimicrobial compounds such as $sPLA_2$ are still capable of removing Gram-positive bacteria. As chitotriosidase and lysozyme (in addition to muramidase activity) hydrolyse N-acetylglucosamine chains, it may be expected that for significant antimicrobial activity, their concentration *in vivo* would be comparable. Therefore, it would be useful to determine the concentration of chitotriosidase in basal and reflex tears.

The anti-bacterial activity of chitotriosidase *in vivo* may be dependent on the presence of the active site and chitin-binding domain. Cleavage of chitotrioside substrates may be solely achieved through the active site, as observed in the lysosomal 39 kDa isoform (Renkema et al., 1997) However, for full activity against chitin-containing cell walls and other forms of insoluble chitin, the chitin-binding domain, consisting of the 49 amino acid C-terminal sequence, is required (discussed in section 4.5) (Tjoelker et al., 2000). It is possible that the production of recombinant chitotriosidase using the pMIB/V5-His vector kit, which adds a V5 epitope and a non-cleavable his-tag to the C-terminal, has resulted in interference with the chitin-binding domain (the gene transcript and protein sequence are shown in the appendix). This issue could be circumvented using a thrombin cleavable His-tag, which is commercially available.

Assuming that the added tags interfered with chitin binding, it would be interesting to study whether an untagged truncated recombinant chitotriosidase corresponding to the chitin-binding domain has antimicrobial activity. Tachycitin is an invertebrate protein that consists of only of an enzymatically-inactive chitin-binding domain (Kawabata et al., 1996). Proteins like this derive their anti-fungal activity by binding to cell wall chitin and thereby inhibiting the remodelling required for growth. In anti-bacterial immunity, these proteins may cause bacterial agglutination, a similar process to that of antibodies, resulting in more efficient phagocytosis.

In tachycitin, amidation of the C-terminus is required for full functionality. A comparison of native and recombinant tachycitin showed that whilst both bind chitin and are anti-fungal, only the native protein is anti-bacterial and this is due to addition of the post-translational C-terminal amide in the native form (Suetake, 2002). This suggests that subtle changes to the protein structure can cause alterations in the antimicrobial function and may therefore also explain the lack of activity in the recombinant chitotriosidase.

Chitotriosidase as marker of immunological disease?

Chitotriosidase has been suggested as a useful biomarker for several immune disorders. These include Gaucher disease, a lysosomal storage disorder caused by mutations in the glucocerebrosidase gene, resulting in the accumulation of glucocerebroside in macrophages. Chitotriosidase levels in patients with Gaucher disease can reach 600

times the plasma concentration observed in healthy individuals. Through the usage of a recently described 4MU-deoxychitobiose assay, it has been suggested that plasma chitotriosidase levels may be a useful marker of disease prognosis (Schoonhoven et al., 2007). The high levels of chitotriosidase tolerated by individuals with Gaucher disease also suggests that the enzyme may be a useful therapeutic agent, with low toxicity, for the treatment of fungal infections. It is however very important to consider the widespread mutations affecting chitotriosidase activity (discussed in chapter 5), when using chitotriosidase activity measurements e.g. in serum for diagnostic purposes.

The monitoring of plasma chitotriosidase levels has been suggested as a diagnostic marker for other diseases that involve macrophage activation. These include the severity of ischaemic stroke (Sotgiu et al., 2005) and β-thalassemia, a blood disease caused by mutations in the HBB gene that encodes β-globulin (Altarescu et al., 2002), for which chitotriosidase levels have been suggested as a marker for recovery (Maccarone et al., 2001). In patients with sarcoidosis, a systemic granulomatous disease, raised levels of chitotriosidase have been identified in both the bronchoalveolar lavage (BAL) and the serum and these appear to reflect the progression of the disease (Bargagli et al., 2007a, Bargagli et al., 2007b). Furthermore, it has been shown that levels correlate with the stages of radiotherapy and the degree of lung infiltration. Therefore it has been suggested that chitotriosidase may be

involved in the pathogenesis of the disease in addition to its potential use as a clinical marker (Bargagli et al., 2007b).

Future directions

To characterise further the potential role of chitotriosidase in innate immunity, there are several experiments that could be performed. Regarding the pattern of *CHIT1* expression, it would be useful to conduct further work using the yeast form of *C. albicans* where, due to the exposure of immunogenic β-glucans on the cell wall surface during the production of pseudo-hyphae, it is regarded to be more susceptible to immune recognition. Furthermore it would be interesting to measure changes in the expression of the β-glucan receptor dectin-1 in macrophages and use dectin-1 blocking antibodies to demonstrate the importance of this process in fungal recognition.

In terms of activity, the synergistic effect of chitotriosidase and other antimicrobial components could be investigated. As shown in chapter 4, the addition of recombinant chitotriosidase had no effect on the viability of Gram-positive or Gram-negative bacteria when incubated alone or in combination with lysozyme. Assuming that the addition of the his-tag and V5 epitope do not interfere with the chitin-binding domain, it can be hypothesized that if chitotriosidase has an anti-bacterial effect in the eye, it could involve other antimicrobial compounds including $sPLA_2$. Therefore a similar luminescence viability assay could be devised using different combinations of antimicrobial compounds in addition to recombinant chitotriosidase.

In the absence of any association between *CHIT1* genotype and malaria or hookworm infection being demonstrated (chapter 5), it would be interesting to determine whether any observable phenotype results from the loss of the active protein as a result of the 24 base-pair mutation in exon 10 of the gene. Previously it has been demonstrated that individuals with this mutation are more susceptible to infection with *W. bancrofti*, a filarial nematode that contains large amounts of chitin in several life stages (Choi et al., 2001). Therefore it would be useful to genotype a larger population that is endemic for this type of filarial nematode and correlate this with the severity of infection at different stages of the life-cycle.

In summary, the work presented in this thesis provides evidence to suggest that chitotriosidase has a role in innate immunity. It has been demonstrated that *CHIT1* expression increases dramatically during the monocyte to macrophage differentiation process. Furthermore, *CHIT1* can be induced following treatment with fungal (but not bacterial) PAMPs and, following the observation that the gene is expressed in lachrymal glands, chitotriosidase activity is detectable in tears (Hall et al., 2007a). With regard to the cluster of mammalian chitinases/chi-lectins, it was demonstrated that all genes may have evolved from an ancestor AMCase, which duplicated to form chitotriosidase and other members of the chitinase chilectin gene cluster.

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APPENDIX I

Buffers and solutions

Resolving gel (12%)

Acrylamide (30%: 0.8% w/v)	4.0ml
1.5m Tris- HCl pH 8.8	2.5ml
Distilled water	3.34ml
10% SDS	0.1ml
N'N'N'-tetramethylethylenediamine (TEMED)	5µl
Ammonium persulphate (APS)	50µl (10%)

Stacking gel

Acrylamide (30%:0.8% w/v)	0.65ml
0.5M Tris-HCl pH 6.8	1.25ml
Distilled water	2.98ml
10% SDS	0.05ml
TEMED	10µl
APS	25µl of
10%	

SDS-PAGE electrode buffer

Tris	30g
Glycine	144g
SDS	10g
Distilled water to 10L	

2x Reducing sample buffer

0.5M Tris-HCL pH 6.8	2ml
Glycerol	2ml
10% SDS	4ml
1% Bromophenol blue	0.2ml
Dithiothreitol (100mM)	0.154g
Distilled water	2ml

Horseradish Peroxidase substrate

Chlornapthol	50mg in10ml ethanol
TBS	50ml
Hydrogen peroxide	30µl

Tris- buffered saline

NaCl	90g
TRIS	6.21g
Distilled water	up to 1 Litre

Silver stain developer

Sodium Carbonate	15g
Sodium Thiosulphate	2mg
Formaldehyde	0.25ml
Distilled water	up to 1 Litre

Glycine- HCl (pH 2.7)

0.1M Glycine- HCL	11.1g
Distilled water	800ml

Na- acetate (pH 4.0)

0.1M Acetic acid	847ml
0.1M Sodium acetate	153ml

Citrate-Phosphate buffer (pH 5.2)

0.2M Na_2HPO_4	26.7ml
0.1M Citrate	23.3ml

Glycine- NaOH (pH 9.0 and 10.5)

0.1M Glycine
0.1M Sodium hydroxide

6% Dextran Solution

Dextran 500	1.5g
NaCl buffer (0.9%)	25ml

90% isotonic Percoll

Percoll	18mls
DPBS (10x w/o Ca/Mg^{++})	2mls

MACS buffer

BSA	0.5%
EDTA	2mM
DPBS	up to 50mls

4-methylumbelliferyl β- D-N, N', N'-triacetylchitotrioside (4-MU chitotrioside)
(2.23mM) (100x)

4-MU stock	1mg
DMF: water (1:1)	570µl
	Heat to 40ºC

APPENDIX II

Recombinant chitotriosidase with V5-epitope and His_6 tag

pMIB/V5-His/Hs Chit1

Homo sapien (Hs) Chitotriosidase (CHIT1) (Acc. No. NM_003465) cloned into pMIB/V5-His (invitrogen) using Hind111 and Xba1 restriction sites.

CGCGCCTATAAATACAGCCCCGCAACGATCTGGTAAACACAGTTGAACAGCATCTGT
TCgAATTTAAAGCTACC

ATCAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGTATACATTTCTTACATC
TATGCCGGCATGCTAAGCTTCGCAAAACTGGTCTGCTACTTCACCAACTGGGCCCAG
TACAGACAGGGGGAGGCTCGCTTCCTGCCCAAGGACTTGGACCCCAGCCTTTGCACC
CACCTCATCTACGCCTTCGCTGGCATGACCAACCACCAGCTGAGCACCACTGAGTGG
AATGACGAGACTCTCTACCAGGAGTTCAATGGCCTGAAGAAGATGAATCCCAAGCTG
AAGACCCTGTTAGCCATCGGAGGCTGGAATTTCGGCACTCAGAAGTTCACAGATATG
GTAGCCACGGCCAACAACCGTCAGACCTTTGTCAACTCGGCCATCAGGTTTCTGCGC
AAATACAGCTTTGACGGCCTTGACCTTGACTGGGAGTACCCAGGAAGCCAGGGGAGC
CCTGCCGTAGACAAGGAGCGCTTCACAACCCTGGTACAGGACTTGGCCAATGCCTTC
CAGCAGGAAGCCCAGACCTCAGGGAAGGAACGCCTTCTTCTGAGTGCAGCGGTTCCA
GCTGGGCAGACCTATGTGGATGCTGGATACGAGGTGGACAAAATCGCCCAGAACCTG
GATTTTGTCAACCTTATGGCCTACGACTTCCATGGCTCTTGGGAGAAGGTCACGGGA
CATAACAGCCCCCTCTACAAGAGGCAAGAAGAGAGTGGTGCAGCAGCCAGCCTCAAC
GTGGATGCTGCTGTGCAACAGTGGCTGCAGAAGGGGACCCCTGCCAGCAAGCTGATC
CTTGGCATGCCTACCTACGGACGCTCCTTCACACTGGCCTCCTCATCAGACACCAGA
GTGGGGGCCCCAGCCACAGGGTCTGGCACTCCAGGCCCCTTCACCAAGGAAGGAGGG
ATGCTGGCCTACTATGAAGTCTGCTCCTGGAAGGGGGCCACCAAACAGAGAATCCAG
GATCAGAAGGTGCCCTACATCTTCCGGGACAACCAGTGGGTGGGCTTTGATGATGTG
GAGAGCTTCAAAACCAAGGTCAGCTATCTGAAGCAGAAGGGACTGGGCGGGGCCATG
GTCTGGGCACTGGACTTAGATGACTTTGCCGGCTTCTCCTGCAACCAGGGCCGATAC
CCCCTCATCCAGACGCTACGGCAGGAACTGAGTCTTCCATACTTGCCTTCAGGCACC
CCAGAGCTTGAAGTTCCAAAACCAGGTCAGCCCTCTGAACCTGAGCATGGCCCCAGC
CCTGGACAAGACACGTTCTGCCAGGGCAAAGCTGATGGGCTCTATCCCAATCCTCGG
GAACGGTCCAGCTTCTACAGCTGTGCAGCGGGGCGGCTGTTCCAGCAAAGCTGCCCG
ACAGGCCTGGTGTTCAGCAACTCCTGCAAATGCTGCACCTGGAATTCTAGAGGGCCC
TTCGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGCGTACCGGT
CATCATCACCATCACCATTGA

Violet= pMIB/V5-HisA Vector
Gold=mellitin secretion sequence (part of vector) (cleaved)
Red=MCS+V5+His tag
Black= Hs Chit protein

Predicted expressed protein

MKFLVNVALVFMVVYISYIYAGMLSFAKLVCYFTNWAQYRQGEARFLPKDLDPSLCT
HLIYAFAGMTNHQLSTTEWNDETLYQEFNGLKKMNPKLKTLLAIGGWNFGTQKFTDM
VATANNRQTFVNSAIRFLRKYSFDGLDLDWEYPGSQGSPAVDKERFTTLVQDLANAF
QQEAQTSGKERLLLSAAVPAGQTYVDAGYEVDKIAQNLDFVNLMAYDFHGSWEKVTG
HNSPLYKRQEESGAAASLNVDAAVQQWLQKGTPASKLILGMPTYGRSFTLASSSDTR
VGAPATGSGTPGPFTKEGGMLAYYEVCSWKGATKQRIQDQKVPYIFRDNQWVGFDDV
ESFKTKVSYLKQKGLGGAMVWALDLDDFAGFSCNQGRYPLIQTLRQELSLPYLPSGT
PELEVPKPGQPSEPEHGPSPGQDTFCQGKADGLYPNPRERSSFYSCAAGRLFQQSCP
TGLVFSNSCKCCTWNSRGPFEGKPIPNPLLGLDSTRTGHHHHHH*

Vector-derived

V5 epitope

S R G P F E G K P I P N P L L G L D S
T R T G

TCTAGAGGGCCCTTCGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCT
ACGCGTACCGGT

H H H H H H *

CATCATCATCATCATTGA

His $_6$ tag

APPENDIX III

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Short communication

Chitotriosidase deficiency is not associated with human hookworm infection in a Papua New Guinean population

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Abstract

Human chitotriosidase (*CHIT1*) is a chitinolytic enzyme with suggested anti-fungal properties. Previous studies have suggested that chitotriosidase may also protect individuals against filarial nematode infections and malaria. A mutant allele, which renders chitotriosidase unstable and enzymatically inactive, is found at a frequency of >20% in Caucasians and other populations. This allele is found at much lower frequency in parts of West Africa where malarial and intestinal helminth infections are endemic. Here, we investigate whether there is a significant association between chitotriosidase genotype and the intensity of hookworm infection in 693 individuals from five villages in Papua New Guinea. Individuals were genotyped for chitotriosidase using a PCR-based assay. There was no association between *CHIT1* genotype and the intensity of hookworm infection as determined by faecal egg counts. The frequency of the mutant allele was 0.251, very similar to that found in non-endemic countries. The extent of geographical variation in allele frequencies across worldwide populations was not high (F_{st} = 0.11), and does not provide evidence for directional selection at this locus between different geographical areas. We conclude that the *CHIT1* genotype does not play a crucial role in protection against hookworm infection. This does not correlate with a previous study that linked the mutant *CHIT1* genotype to filariasis susceptibility. The possible reasons for this discrepancy are discussed.

Keywords: Chitotriosidase; *CHIT1* mutation; *Necator americanus*; Malaria; Papua New Guinea

1. Introduction

Chitotriosidase is a chitinolytic enzyme which was recently identified in man and is synthesised in large quantities by activated macrophages (Hollak et al., 1994). As humans lack endogenous chitin, a role for chitotriosidase is not fully understood. However the anti-fungal action of homologous plant chitinases has led to the suggestion that chitotriosidase may degrade chitin containing pathogens (Boot et al., 1998). The chitotriosidase gene (*CHIT1*) consists of 12 exons located on chromosome 1q31–32 (Boot et al., 1998). A 24 base pair duplication in exon 10 leads to the activation of a cryptic 3′ splice site that results in an abnormally spliced mRNA with an inframe 87 nucleotide deletion (Boot et al., 1998). The mutant protein lacks amino acids 344–372 that are required for the formation of the TIM-barrel catalytic core (Boot et al., 1998; Fusetti et al., 2002). Macrophages from chitotriosidase deficient individuals express only small amounts of mRNA and secrete virtually no chitotriosidase protein (Boot et al., 1998).

In Dutch and Ashkenazi Jewish populations 6% of individuals are homozygous for the mutant allele, whereas 35% and 34%, respectively, are heterozygous carriers (Boot et al., 1998) with a mutant allele frequency of 0.23. A similar allelic frequency is present in Portugal (0.22), Sicily (0.27) and Sardinia (0.21) (Malaguarnera et al., 2003). However in two West African countries, Benin and Burkina Faso, that are mesoendemic for *Plasmodium falciparum* malaria and endemic for gastrointestinal helminths, a total absence of the homozygous mutation and significant reduction in heterozygous individuals has been reported, with mutant allele frequency

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0.00 and 0.02, respectively (Malaguarnera et al., 2003). This observation led to the hypothesis that chitotriosidase may be involved in resistance to protozoan or helminth infections common in tropical countries (Malaguarnera et al., 2003).

Parasitic nematodes are known to contain chitin, and are thus a potential target of human chitotriosidase. Chitin is found in the egg-shell of both free-living and parasitic nematodes, and in the microfilarial sheath surrounding the first-stage larvae of filarial nematodes (Fuhrman and Piessens, 1985). More recently, chitin has been demonstrated in the pharynx of both the free-living *Caenorhabditis elegans* (Zhang et al., 2005) and the gastrointestinal parasite *Oesophagostomum dentatum* (Neuhaus et al., 1997). There are two chitin synthase genes in *C. elegans*, *chs-1* and *chs-2*, encoding eggshell and pharynx chitin, respectively, and *chs-2* knockdown leads to lack of pharyngeal function and starvation (Zhang et al., 2005). This suggests that host chitotriosidase could potentially interfere with parasitic nematode feeding. Two studies to date have investigated associations between *CHIT1* genotype and filarial nematode infection. Choi et al. (2001) found significant association between susceptibility to lymphatic filariasis (*Wuchereria bancrofti*) and homozygosity for the mutant allele in an Indian population, but in a similar study in Papua New Guinea, no association between *CHIT1* genotype and lymphatic filariasis was observed (Hise et al., 2003).

There have been no previous studies investigating *CHIT1* genotype and gastrointestinal nematode infection. Human hookworm infection is an abundant chronic gastrointestinal nematode infection in sub-tropical and tropical countries, causing significant morbidity, principally due to iron-deficiency anaemia (Brooker et al., 2004). Human hookworm burden (as assessed by faecal egg count) is known to be under host genetic control (Williams-Blangero et al., 1997), but the genes responsible have not been identified. The aims of the current study were (1) to investigate associations between *CHIT1* genotype and human hookworm infection in an endemic population from Madang Province, Papua New Guinea and (2) to investigate evidence for directional selection at the *CHIT1* locus, by comparing worldwide allele frequency variation at the *CHIT1* locus to that observed at other loci across the genome.

2. Materials and methods

2.1. Geographical location and study population

The study population consisted of five villages in lowland Madang Province, Papua New Guinea, where *Necator americanus* is the only hookworm species (Pritchard et al., 1990). Villages were censused and pedigree information collected in 1998, and sample containers for faecal collection offered to all individuals aged 4 years and above. Faecal egg counts were performed using a modified McMaster salt flotation method and results were expressed as eggs per gram (epg) of faeces (Quinnell et al., 2004). Venous blood was then collected, and all individuals were offered treatment with albendazole or pyrantel pamoate. DNA was extracted from buffy coats using phenol/chloroform. In September 2001, a second faecal sample was taken from three of the five villages and reinfection hookworm burden determined; some previously untreated individuals provided samples in 2001. Hookworm epg has been shown to be a heritable phenotype in the study population (Breitling and Quinnell, unpublished results). The study was approved by the Medical Research Advisory Committee of Papua New Guinea and informed consent was received from all subjects or their parents.

2.2. Chitotriosidase genotyping by PCR

Thermal cycling reactions consisted of Reddy Mix PCR master mix (ABgene), 4.5 pmol forward and 4.5 pmol reverse chitotriosidase primers (forward: AGCTATCTGAAGCA-GAAG; reverse: GGAGAAGCCGGAAAGTC) (Sigma) and 2 μl genomic DNA in a final volume of 15 μl. Samples were run in 96 well PCR plates (ABgene) on a Peltier PTC-200 Thermal Cycler (MJ Research). The thermal cycling protocol was: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s; and 72 °C for 5 min. Thermal cycling products with 10 base pair markers (Promega) were separated on 4% high resolution agarose (Sigma) gels in 1× TBE buffer and ethidium bromide. Gels were viewed using the Gene Genius Bio-imaging System and Gene Snap software (Syngene). The size of wild type product is 75 base pairs whereas the mutant product is 99 base pairs due to the mutant allele containing a 24 base pair duplication in exon 10 (Boot et al., 1998).

2.3. Statistical analysis

Variation in faecal egg counts by *CHIT1* genotype was analysed under both a codominant and recessive model. Faecal egg counts were highly overdispersed, so analysis was performed using a generalised linear model with a negative binomial error structure in Stata 9.1. This method has been shown to be more accurate than analysis of log-transformed parasite burden data (Wilson et al., 1996). Significant covariates included in the analysis were age, village, faecal consistency, anthelmintic treatment and the age × treatment and village × treatment interaction terms. Egg counts from both years were analysed together; since some individuals were sampled both pre- and post-treatment, standard errors were adjusted for the non-independence of samples from the same individual using the 'cluster' option. To control for non-independence of individuals due to genetic relatedness, further analysis was performed with the total test of association in a variance components framework using the programme QTDT (Abecasis et al., 2000). Finally, to control for potential population stratification, transmission disequilibrium testing was carried out using the orthogonal association model in QTDT. The phenotypic variable used for these analyses was the residuals from a negative binomial regression of egg counts against significant covariates; where two samples were taken from the same individual, the mean residual for that individual was calculated.

Table 1
Genotype and allele frequencies of the *CHIT1* mutation in different populations

Population	Subjects	(%) wt/wt	(%) wt/mut	(%) mut/mut	Wt allele frequency	Mut allele frequency	Reference
Sicily	100	51	43	6	0.73	0.27	Malaguarnera et al. (2003)
Sardinia	107	64	32	4	0.79	0.21	Malaguarnera et al. (2003)
Portugal	295	60	37	3	0.78	0.22	Rodrigues et al. (2004)
Holland	171	59	35	6	0.77	0.23	Boot et al. (1998)
Ashkenazi Jews	68	60	34	6	0.77	0.23	Boot et al. (1998)
South India	67	31	58	10	0.60	0.40	Choi et al. (2001)
Papua New Guinea	906	77	22	1	0.88	0.12	Hise et al. (2003)
Chinese Han(Taiwan)	82	15	55	30	0.42	0.58	Chien et al. (2005)
Benin	100	100	0	0	1.00	0.00	Malaguarnera et al. (2003)
Burkina Faso	100	98	2	0	0.98	0.02	Malaguarnera et al. (2003)

wt: wild-type (intact) allele; mut: mutant allele (24 bp duplication).

To quantify geographic variation in allele frequencies, F_{st} values were calculated according to Cockerham and Weir (1984) using the programme FSTAT 2.9.3 (Goudet, 1995). Allele frequencies were compared across five broad geographical areas, using the weighted mean allele frequency for each geographical area (Europe/Mediterranean, Africa, South Asia, East Asia and Papua New Guinea). To compare observed values of F_{st} to those expected for neutral markers, F_{st} was also calculated across comparable populations selected from the Human Genome Diversity Panel using published data for 210 genome-wide short insertion/deletion polymorphisms genotyped by the Mammalian Genotyping Service (data available from http://research.marshfieldclinic.org/genetics/) (Soranzo et al., 2005). The populations selected were European (French/Italian), African (Yoruba), South Asia (Sindhi), East Asia (Chinese Han) and Papuan.

3. Results

In total 693 individuals from Papua New Guinea were genotyped for the chitotriosidase mutation. The allelic frequency of the 24 bp duplication in 279 unrelated individuals, determined by PCR genotyping, was 0.251. The percentage of individuals who were homozygous mutant was 6% (16/279), heterozygous 39% (108/279) and homozygous wild type 56% (155/279). Genotype frequencies did not differ from those expected under Hardy–Weinberg equilibrium ($\chi^2 = 0.25$, d.f. = 1, $P = 0.62$).

Both egg counts and *CHIT1* genotypes were available for a total of 602 individuals (574 sampled pre-treatment and 210 sampled post-treatment). The prevalence and intensity of infection (mean egg count) in these individuals were 81% (2574 epg) pre-treatment, and 92% (1727 epg) post-treatment. Egg counts were very similar across *CHIT1* genotypes both pre-treatment and after reinfection (Table 2). Combined analysis of both years' data revealed no significant relationship between egg count and genotype under either a codominant ($\chi^2 = 0.03$, d.f. = 1, $p = 0.87$) or recessive model ($\chi^2 = 0.02$, d.f. = 1, $p = 0.90$). Further analysis using the total test of association in QTDT, controlling for non-independence of individuals due to genetic relatedness, again showed no significant association between *CHIT1* genotype and egg count ($\chi^2 = 0.39$, d.f. = 1, $p = 0.53$). Similarly, transmission disequilibrium testing, which controls for population stratification, was not significant ($\chi^2 = 0.58$, d.f. = 1, $p = 0.45$).

Published allele frequencies for *CHIT1* in different geographical areas are shown in Table 1. Using these data and data from the current study, F_{st} values were calculated to measure variation in allele frequency across five broad geographical areas (Europe, Africa, South Asia, East Asia, and Papua New Guinea). The F_{st} across all populations for *CHIT1* was 0.11. This was comparable to the median F_{st} of 0.12 for 210 indels typed in populations from similar geographical areas. Pairwise comparisons showed higher F_{st} values between Africa and East Asia ($F_{st} = 0.69$) and Africa and South Asia ($F_{st} = 0.54$), than those between other areas ($F_{st} < 0.40$). In each case the observed F_{st} was greater than that for all but 7 of 210 indels, giving an empirical $P = 0.038$ (8/210).

4. Discussion

The results from the present study in Papua New Guinea strongly suggest that chitotriosidase does not have a critical role in protection against *N. americanus*. The relative number of people who are wild type homozygous for chitotriosidase, heterozygous and homozygous mutant is similar to that seen in countries where this infection is not endemic, suggesting no advantage for the wild type allele in endemic areas. Moreover, there was no evidence for an association between *CHIT1* genotype and hookworm burden in our study population.

Table 2
Hookworm burdens according to *CHIT1* genotype in the Papua New Guinea study population

Genotype	*CHIT1*			
	Pre-treatment		Post-treatment	
	n	Hookworm epg	*n*	Hookworm epg
wt/wt	320	2495 (2109–2952)	102	1636 (1259–2127)
wt/mut	222	2675 (2131–3357)	98	1815 (1436–2295)
mut/mut	32	2374 (1377–4090)	10	1648 (882–3079)

Burdens were assessed as mean eggs per gram faeces (epg) (95% CL) before anthelmintic treatment and after reinfection. Confidence intervals were calculated assuming a negative binomial error distribution.

The mutant chitotriosidase genotype has been associated with an increased susceptibility to *W. bancrofti* infection in a study of 216 individuals from South India (Choi et al., 2001). Previously it has been demonstrated that the microfilariae, oocytes and zygotes of the filarial nematode *Brugia malayi* contain chitin (Fuhrman and Piessens, 1985; Schraermeyer et al., 1987). In the rodent filarial nematode *Acanthocheilonema vitae,* there are distinctive chito-oligomeric *N*-glycans that contain projections of up to six GlcNAc residues that may be involved in parasite-host interactions (Haslam et al., 1999). Additionally chitin synthase is required for sheath development in microfilarial progeny. Chitin has also been identified in the cuticle of mature *W. bancrofti* (Araujo et al., 1993). The presence of chitin in the sheath and cuticle of filarial nematodes and the requirement for chitin synthase in reproduction show that chitin has an important role in the lifecycle of these species. If host-derived chitotriosidase can access this chitin, thus interfering with the parasite's growth and development, then it may explain why susceptibility to *W. bancrofti* is increased in individuals with the mutant genotype. However, a second study by Hise and coauthors (Hise et al., 2003) in 906 individuals in a region endemic for bancroftian filariasis in Papua New Guinea did not find any significant correlation with infection status or disease phenotype. Thus the issue is in further need of clarification.

In contrast to filarial worms, gastrointestinal nematodes such as hookworm do not have a microfilarial stage, and the chitin-containing eggs are not accessible to host chitotriosidase. However, chitin has recently been demonstrated in the pharynx of the free-living nematode *C. elegans* (Zhang et al., 2005). It is not known whether chitin occurs in the pharynx of *N. americanus*, but it has been demonstrated in the related strongyloid parasite *O. dentatum* (Neuhaus et al., 1997). Pharyngeal chitin provides a potential target for host chitotriosidase, both in tissue-dwelling larval stages, which may be accessible for macrophage or neutrophil-derived chitotriosidase, and blood-feeding adults. Chitotriosidase levels can be elevated in serum, e.g. in acute falciparum malaria (Barone et al., 2003), and as adult hookworms are thought to ingest 0.03–0.2 ml of blood daily, they may be exposed to high levels of chitotriosidase. However, the lack of an association between *CHIT1* genotype and hookworm burden seen in the present study suggests that chitotriosidase is not an important effector mechanism against hookworm infection. Further studies would be useful to examine the bioavailability of nematode chitin, its accessibility for exogenous chitinases, or their effect on parasite viability.

The mutant allele frequency observed in the current study was higher than that in another Papuan population from East Sepik Province (Hise et al., 2003). Such variation within Papua New Guinea is not surprising, and differences between these populations have been observed at other loci (Main et al., 2001). Analysis of worldwide variation in allele frequencies showed that the degree of population differentiation at the *CHIT1* locus ($F_{st} = 0.11$) was very similar to the median value for 210 indels across the genome. Though the populations sampled were not identical, this suggests that there has not been directional selection at this locus across different geographical areas, and that the worldwide variation in allele frequencies is consistent with genetic drift (Akey et al., 2002). There was some evidence for higher than average population differentiation at the *CHIT1* locus between African and Asian populations, which may suggest some local adaptation, though this would not be significant after correction for multiple testing.

Malaguarnera et al. (2003) suggest that an intact *CHIT1* genotype may be advantageous in protection against malaria, based on the very low frequency of the mutant genotype in Burkina Faso and Benin, where malaria is *meso*-endemic. However, our results are not consistent with the hypothesis that chitotriosidase is involved in protection against malaria, or any tropical disease. There is no evidence for directional selection worldwide, and the greatest difference in allele frequencies is observed between Africa and other tropical countries, also endemic for malaria (Choi et al., 2001; Chien et al., 2005). Although Barone et al. (2003) found that chitotriosidase levels are elevated in the serum in acute *P. falciparum* malaria, there is no evidence that elevated serum chitotriosidase levels have an impact on the severity and outcome of malaria, and no study has compared genotypes with disease status. Indeed, increased serum chitotriosidase may actually increase malaria transmission, by inhibiting the formation of the peritrophic membrane in the anopheline gut (Di Luca et al., 2007). As the study area in Papua New Guinea is endemic for malaria, we have performed an association analysis which showed no significant difference between chitotriosidase genotype and the presence of *P. falciparum* and *P. vivax* malaria infection (data not shown). The caveat of this analysis is that it was based on a single time point assessment of parasitaemia, rather than repeated measurements. As parasitaemia is very variable, we cannot make a reliable statement regarding the effect of the *CHIT1* genotype on malarial infection, and further studies are needed.

Taken together, our data suggest that the chitotriosidase genotype does not play a major role in protection against hookworm infection, but this may be different in the case of lymphatic or cutaneous filariasis. However, there is no evidence for selection at this locus mediated by malaria or another tropical disease.

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Original article

Human chitotriosidase is expressed in the eye and lacrimal gland and has an antimicrobial spectrum different from lysozyme

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Abstract

Chitotriosidase is a chitinolytic enzyme expressed by maturing macrophages and preformed in neutrophil granules, suggesting a role in antimicrobial defence. Although available evidence supports a role in anti-fungal immunity, there is a lack of an obvious phenotype in humans homozygous for a mutation which renders chitotriosidase inactive. This may be explained by compensatory effects of enzymes co-expressed with chitotriosidase, such as lysozyme. We have found that chitinase is highly expressed in mouse and human eye, particularly in lacrimal glands. Chitotriosidase is the only member of the chitinase/chilectin gene cluster expressed in the murine eye. As lacrimal glands also produce lysozyme, we have asked whether chitotriosidase, in addition to its documented anti-fungal effects, has synergistic anti-bacterial properties with lysozyme. The effect of recombinant chitotriosidase on the growth of five Gram-positive (*Bacillus cereus*, *Bacillus subtilis*, *Listeria innocua*, *Staphylococcus aureus* and *Staphylococcus aureus* OatA$^{+/-}$) and two Gram-negative strains (*Escherichia coli* and *Pseudomonas aeruginosa*), were tested in a luminometric assay. Recombinant chitotriosidase did not inhibit bacterial growth and did not synergize with lysozyme. Though the expression of chitotriosidase in the eye supports a role in innate immunity, the antimicrobial spectrum appears to be complementary to lysozyme and may indeed be limited to fungi.

Keywords: Chitotriosidase; Lysozyme; Innate immunity

1. Introduction

After cellulose, chitin is the second most abundant biopolymer on Earth. It is found in insects, fungi and the egg shell of some nematodes, but it is thought not to occur in mammals. Chitin is an unbranched polymer formed by β-1,4-linked *N*-acetyl-D-glucosamine units. A high resistance of polymeric chitin to degradation is conferred by numerous inter-chain bonds, thus breakdown of chitin for growth or metabolic purposes requires the catalytic action of specialized enzymes. Catabolism of chitin is mediated by chitinases (*O*-glycosyl hydrolases (EC 3.2.1.-), which belong to Families 18 and 19 of glycohydrolases according to carbohydrate active enzyme classification (CAZy, http://afmb.cnrs-mrs.fr/CAZY/acc.html).

Chitin is not thought to be synthesised in mammals as they lack the enzymatic machinery to achieve its anabolism, thus there is *a priori* no obligatory requirement for chitin degrading enzymes in humans. Thus the gradual discovery of a conserved gene cluster encoding chitinases and related genes was unexpected. Although the existence of a chitinase activity distinct from lysozyme in goats had already been described in 1974 [1], it took another 20 years to identify the first human chitinase in human serum [2]. This chitinase was purified [3] and cloned [4] one year later and named chitotriosidase (gene name *chit1*), for its ability to cleave chitotriose, the trimeric form of β-1,4-*N*-acetyl-D-glucosamine. Another chitinase with an acidic pH optimum (acidic mammalian chitinase, AMCase) was later cloned in mice and humans [5]. Mammalian

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chitinases are all related to Family 18 chitinases, while Family 19 chitinases are mainly found in plants.

Chitotriosidase, also known as macrophage chitinase, is not expressed in monocytes but is synthesized by activated macrophages in a number of pathological settings, and also produced *in vitro* after prolonged cultivation [6]. It is dramatically elevated (>600-fold over median) in the serum of many patients with Gaucher disease, a lysosomal storage disease caused by a deficiency in glucocerebrosidase [7]. The main secreted form has a molecular weight of 50 kDa, but a fraction is processed post-translationally to a 39 kDa isoform which accumulates in lysosomes [8]. The shorter isoform lacks the C-terminal chitin-binding domain [8]. There is also constitutive chitotriosidase expression in human neutrophilic granulocytes, where it is stored in granules [9,10].

The production, storage and secretion of chitotriosidase by macrophages and neutrophils, prime components of the innate immune system, points to a role in innate immunity. Such a role is well defined in plant immunity [11], where chitinases are known as pathogenesis-related proteins [12]. The observation that chitotriosidase can digest colloidal chitin and chitin in fungal cell walls, as shown by its degradation in *Candida albicans* spheroplasts [5], lends support to this notion. Furthermore, van Eijk et al. showed that human recombinant chitotriosidase inhibited growth of *Cryptococcus neoformans* and *Mucor rouxii*, and totally inhibited switching of a *C. albicans* isolate from yeast to hyphal growth (albeit at a high concentration of 1 mg/ml) [10]. Also, chitinase activity is known to increase in blood and tissues in guinea pigs with a systemic *Aspergillus fumigatus* infection [13,14]. Van Ejick confirmed a role in anti-fungal immunity against systemic candidiasis and (to a lesser extent) aspergillosis by treating neutropenic mice with recombinant (human) chitotriosidase, leading to increased survival [10]. Thus the available evidence points to a role of chitotriosidase in anti-fungal immunity.

Here, we report that chitotriosidase is expressed in human lacrimal glands and mouse eye, as demonstrated by RT-PCR and the presence of measurable, salt-insensitive chitotriosidase activity in human tears. We have also tested the *in vitro* effects of recombinant, enzymatically active chitotriosidase on the growth of several Gram-positive and Gram-negative bacteria and the putative synergy with the known antibacterial effects of lysozyme.

2. Materials and methods

2.1. Tissue expression profiling by RT-PCR

The sequences for murine and human chitotriosidase available from the Ensembl genome database were used to design oligonucleotides primers for tissue expression screening using the Primer 3 software [15]. The primers for murine chitotriosidase span a 169 bp intron, giving 150 bp from cDNA and 319 bp from genomic DNA (gDNA). Similarly, primers for human chitotriosidase amplify 121 bp (cDNA) and 356 bp (gDNA) products. Primer sequences were: MmChit1-f, 5′-GCC CCA CAG TAG ACA AAG AG AGA-3′; MmChit1-r, 5′-CCT CGT AGC CAG CAT CCAC-3′; HsChit-1-f, 5′-CCC TGG TAC AGG ACT TGGC-3′; HsChit-1-r, 5′-ACC TCG TAT CCA GCA TCCAC-3′; HsGAP-DH-f, 5′-CGT GGA AGG ACT CAT GACC-3′; HsGAP-DH-r, 5′-GAG GCA GGG ATG ATG TTCTG-3′. All primers were manufactured by Sigma-Genosys. PCRs were carried on an MJResearch PCT200 thermocycler and 15 µl reactions using Reddymix PCR mastermix (Abgene). Cycling conditions were: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 45 s, 72 °C for 30 s, finally one cycle of 72 °C for 5 min.

2.2. Recombinant expression of human chitotriosidase

Recombinant human chitotriosidase was generated using the pMIB insect cell expression system (Invitrogen). The template used was single-stranded cDNA obtained from purified human neutrophils using Milteny Biotech's one step cDNA synthesis kit. Cloning primers (Sigma-Genosys, SDS-PAGE purified) were designed replacing the original signal sequence with the melittin signal sequence on the vector. The primers had HindIII (forward primer) and Xba1 (reverse primer) restriction sites for in-frame cloning into pMIB/V5-His (Invitrogen). Primer sequences were: HsChit1-exp-F 5′-GCC CGG GC*A AGC TTC*gcaaaactggtctgctacttca-3′ and HsChit1-WT-R2 5′-GCC CGG *TCT AGA* att cca ggt gca gca tttg-3′ (restriction sites in italic). The PCR reactions for generation of full length chitotriosidase were conducted using *Pfu* DNA polymerase (Stratagene). Cycling conditions were: 94 °C for 3 min, followed by 35 cycles of 94 °C for 90 s, 56 °C for 30 s, and 72 °C for 90 s. The reaction was terminated with a final elongation step of 72 °C for 5 min. The obtained products were purified from an agarose gel (Qiagen Gel Extraction kit), digested using the cloning enzymes and gel purified a second time before being cloned into pMIB/V5-His following the manufacturer's standard protocol. The final sequence of the recombinant plasmid was confirmed by DNA sequencing (Cogenics). Identity of the recombinant protein was confirmed unequivocally upon expression and purification by MALDI-QTOF protein sequencing (10 peptides with a 100% match of the expected sequence).

2.3. Generation of chitotriosidase active site mutant

Site-directed mutagenesis was used to generate an active site mutant of chitotriosidase. Mutagenic primers were designed in order to change the DGLDLDWE active site sequence to DGLDL*N*W*Q*. The following mutagenesis primers were designed and used with the Quickchange II site directed mutagenesis kit (Stratagene): pMIB-HsChit1-a/s-mut-F 5′-CTT TGA CGG CCT TGA CCT T*aA C*TG G*cag* TAC CCA GGA AGC-3′; pMIB-HsChit1-a/s-mut-R 5′-GCT TCC TGG GTA ctg CCA GTt AAG GTC AAG GCC GTC AAAG-3′. PCR conditions for incorporation of the mutagenic primers, using 50 ng of the previously generated wildtype chitotriosidase pMIB plasmid as template, DpnI digestion of hemimethylated template DNA and subsequent cloning steps were performed according to standard molecular biology techniques

as directed by the manufacturer. The final sequence was confirmed by sequencing of the mutated pMIB-HsChit1-a/s-mut plasmid (Cogenics).

2.4. Expression of recombinant chitotriosidase in baculovirus-free insect cell expression system

SF9 insect cells (Invitrogen) were cultured as suspension and monolayer cultures in Ex-Cell 420 medium (JRH Biosciences) supplemented with glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B (all supplements from Sigma). Cells were incubated at 27 °C. Monolayer cultures of cells grown to 60–80% confluence were transfected with 2 μg of the clone DNA (pMIB-RnChi3L1) using the GeneJammer reagent (Stratagene) as directed. After 24 h, the transfected monolayers were placed under selection with blasticidin (Cayler) at 40 μg/ml until stable transfectants were obtained. These were then grown as suspension cultures to cell densities $>10^7$ on an orbital shaker (135 rpm) at 27 °C in volumes of up to 500 ml in 1000 ml Erlenmeyer flasks in serum-free medium containing 10 μg/ml blasticidin. The cell suspension was pelleted at 3350 × *g* for 20 min. The supernatant was collected, dialysed against 20 volumes of water for 24–48 h at 4 °C using 12–14 kDa cut-off dialysis tubing. The supernatant was re-centrifuged as before, filtered through a 0.22 μm filter, then made up into a 1 × Ni-NTA binding buffer and purified using Novagen's His·Bind® Resin as directed by the manufacturer. The presence of recombinant protein was detected by Western blot following standard protocols using an anti-V5 antibody (1:5000, Invitrogen) and a secondary anti-mouse IgG-HRP (1:1000) (Pharmingen) developed in substrate buffer (1 mg/ml 4-chloro-1-naphthol, 30% ethanol and 0.06% hydrogen peroxide in TBS).

2.5. Determination of recombinant protein purity by silver staining

The purity of the recombinant human chitotriosidase was determined by silver staining using the same SDS-PAGE protocol as for Western blotting. Following electrophoresis the gel was washed three times for 20 min in 30% ethanol, 1 min in 0.02% sodium thiosulphate (Sigma) and three further washes in deionised water for 20 s. The gel was developed in 3% sodium carbonate (Sigma), 0.05% formaldehyde and 0.0004% sodium thiosulphate (Sigma) for 5 min followed by two washes in deionised water for 1 min. The reaction was stopped using 2% EDTA (Sigma) for 20 min and the gel was washed with deionised water prior to storage in 2% glycerol (Sigma).

2.6. Measurement of chitinase activity

Enzymatic activity of recombinant human chitotriosidase was determined by incubating 10 μl of enzyme with 100 μl of 22 μM substrate 4-methylumbelliferyl-β-D-*N*,*N*,*N*′,*N*″triacetylchitotriose (Sigma) with readings of relative fluorescence units (RFU) at 450 nm taken every 120 s on a MFX microtitre plate fluorometer with Revelation 4.21 software (Dynex Technologies). The rate of 4-MU production was determined by plotting a standard curve of 4-MU salt (Sigma) concentration versus relative fluorescence units (RFU) minus 4-MU and buffer alone.

The activity of the recombinant chitotriosidase was tested in different buffers: 0.1 M glycine–HCl (pH 2.7), 100 mM sodium acetate (pH 4.0), 0.1 M/0.2 M citrate-phosphate buffer (pH 5.2), phosphate buffered saline (pH 7.2) and 100 mM glycine–NaOH (pH 9.0 and 10.5). All data analyses and regression plots were done using SigmaPlot 8.0. For end point measurements, the reaction was stopped by adding 2 ml of 0.3 M glycine–NaOH buffer (pH 10.5) after 15 min incubation at 37 °C. Measurements of chitinase activity of lysozyme in the presence of 0.5 M NaCl were obtained using purified human breast milk lysozyme (Sigma).

2.7. Anti-microbial activity assessment

The effect of human recombinant chitotriosidase and lysozyme on bacterial viability was determined using the BacTiter-Glo ATP luciferase assay (Promega) and Lucy 1 multiplate reader (Anthos) with Stingray software (Dazdaq). Six Gram-positive bacterial strains (*Bacillus cereus* (ATCC11778), *Bacillus subtilis* (ATCC6633), *Listeria innocua* (ATCC33090), *Staphylococcus aureus* (ATCC29213) and *Staphylococcus aureus* OatA$^{+/-}$ (ATCC35556)) and two Gram-negative bacterial strains (*Escherichia coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC27853)) were incubated overnight in a shaking incubator at 37 °C and then diluted 1:100 in low-salt LB (pH 6.0) and incubated for a further 2 h. Cultures were then aliquoted into 96-well tissue culture plates and start point readings were taken in triplicate. Plates were then incubated for a further 4 h in the presence of lysozyme, recombinant chitotriosidase or kanamycin.

The effect of chicken egg-white lysozyme (Sigma) was tested at a range of concentrations (50 μg to 0.39 μg/ml and 3.12 μg to 25 ng/ml) on bacterial viability with the antibiotic kanamycin (50 μg/ml) as positive control and untreated bacteria in triplicates. Sub-optimal doses of lysozyme were tested alone and in combination with recombinant human chitotriosidase (25 μg/ml) using the same protocol. Statistical analyses were carried out by one-way ANOVA and post hoc LSD test (significance at $P < 0.05$) using SPSS v11.5.

3. Results

As tissue-specific expression patterns of a gene with uncertain function can give clues to its role, we have initially examined the expression of chitotriosidase mRNA in a selection of human and mouse tissues. The strongest expression of chitotriosidase mRNA was found in the stomach and eye (Fig. 1), with intermediate expression in bone marrow and uterus. Strong expression of chitotriosidase in the tongue and stomach of mice has been described before [16].

This may point to a digestive function in mice. However, the expression in the eye has not been reported before. As

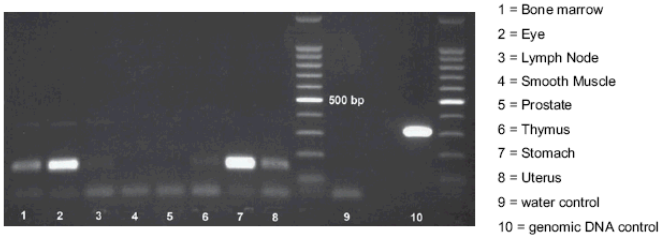

Fig. 1. Tissue expression screening of chitotriosidase mRNA in mouse tissues. cDNA was obtained from the Clontech Multiple Tissue cDNA (MTC) panel. These panels have been normalized using four different housekeeping genes, allowing a more reliable estimation of relative quantities in different tissues. We found no expression in smooth muscle and the prostate (lanes 4 and 5), weak expression in lymph node and thymus (lanes 3 and 6), intermediate expression in bone marrow and uterus (lanes 1 and 8) and strong expression in the eye (lane 2) and stomach (lane 7). Primers were designed to discriminate between genomic DNA (319 bp, lane 10) and cDNA (150 bp).

chitotriosidase is related to several chitinases and chitinase-like lectins (chi-lectins) in two clusters on chromosomes 1 and 3 in mice (both clusters are located on separate areas of chromosome 1 in humans), we have assessed expression of the other known members of the chitinase/chilectin clusters in the murine eye. As shown in Fig. 2, only chitotriosidase was shown to be expressed in the eye. The other chitinases and chilectins assayed (acidic mammalian chitinase (ChiA, a.k.a. AMCase), Chi3L1 (Brp39), Chi3L3 (Ym1), Chi3L4 (Ym2), Bclp2 and oviductin where all shown to be negative for expression. All primers used amplify products of the expected length with cDNA derived from appropriate tissues (Fig. 2).

Next, we wanted to confirm this finding in the human eye. We have tested cDNA obtained from stroma, conjuctiva, lacrimal gland, corneal endothelial and corneal epidermal cells, and whole cornea. The strongest expression was found in the lacrimal gland sample (Fig. 3).

As our RT-PCR results indicated chitotriosidase gene expression in the eye, and particularly in the lacrimal gland, we measured chitotriosidase activity in reflex tear fluid. The results obtained from 4 donors using 1, 3 or 5 μl reflex tear fluid are shown in Fig. 4.

By comparing with a standard curve generated with the 4-methylumbelliferyl salt (not shown), we obtained the mean volume activity of 128 nmol/ml per hour. This value is similar to the levels measured in human plasma by Musumeci and coworkers (101 ± 80 nmol/ml per hour for African women and 46 ± 16 nmol/ml per hour for Caucasian women [17] and 4–76 nmol/ml per hour [7]), and lower than the levels reported for colostrum (1230 ± 662 nmol/ml per hour [17]), where levels are highly increased in the first day or two. The physiological concentration is likely to be much higher in the undiluted physiological tear film, as our measurement was in reflex tear fluid.

It needs to be stressed that tear fluid, like other body fluids, will also contain lysozyme, which also (albeit with lower affinity) has the ability to cleave the chitotriosidase substrate [18,19]. Therefore, the presence of chitotriosidase activity in tear fluid, although consistent with gene expression in the lacrimal gland, cannot *a priori* be considered as sufficient evidence of its presence in tears. However, lysozyme is well known to be inactivated by salt concentrations above physiological levels (>0.15 M), an observation which was already made by Fleming [20]. Salt inactivation has been shown for chicken egg white [21–25] and human lysozyme [20,26].

To determine whether such an inhibitory effect is also found with chitotriosidase, we proceeded to produce sufficient amounts of enzymatically active recombinant chitotriosidase to assess salt-sensitivity of this enzyme.

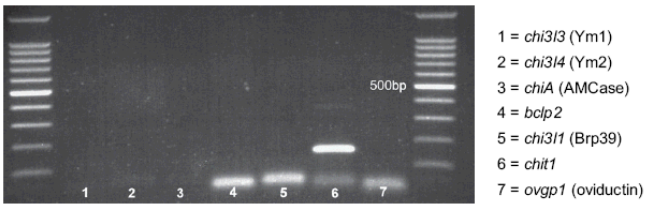

Fig. 2. Results of RT-PCR using cDNA from mouse eye showing that chitotriosidase, but not other related genes from the chitinase/chilectin gene clusters are expressed in the murine eye. Gene names are in italics, corresponding protein names in brackets. All primers amplify products with the expected sizes in appropriate tissues (not shown). Only lane 6, containing chitotriosidase primers shows strong expression. Primers for mouse chitotriosidase discriminate between genomic DNA (319 bp) and cDNA (150 bp).

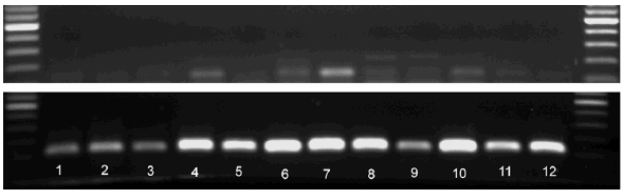

Fig. 3. Expression of chitotriosidase mRNA in different parts of the human eye. The expected size from cDNA is 121 bp for chitotriosidase and 120 bp for GAP-DH. The single lanes are: 1–3, stromal cDNA; 4–6, conjuctival cDNA; 7, lacrimal gland cDNA; 8,9, corneal endothelial cDNA; 10,11, corneal epidermal cDNA; 12, whole cornea. Strongest expression was found in the lacrimal gland sample. A direct comparison of intensities between samples is not possible due to the lack of normalization and varying cDNA samples qualities.

We generated several milligrams of pure recombinant chitotriosidase using a baculovirus-free insect cell expression system. Recombinant histidine-tagged chitotriosidase protein (wildtype and mutant) was purified using Ni-NTA affinity chromatography and checked on a silver stained SDS-PAGE gel. There was no visible contaminant (Fig. 5A) and the recombinant protein reacted strongly with an anti-V5 antibody, confirming in-frame expression (Fig. 5B) of wildtype and active site mutant chitotriosidase.

We then went on to test the enzymatic activity of our recombinant protein. Fig. 5D shows a pH dependency curve (pH 2.7–10.5) indicating an optimal pH between 5.0 and 6.0. A pH of 5.2 was used for the time course shown in the left panel (Fig. 5C), showing the kinetics of chitotrioside cleavage. The specific activity of recombinant chitotriosidase was calculated using the previously mentioned standard curve yielding a value of 7.68 nmol/h per μg. As expected, the active site mutant did not show any activity. These results also confirmed that the V5/his tag added by the pMIB expression vector does not interfere with enzymatic activity.

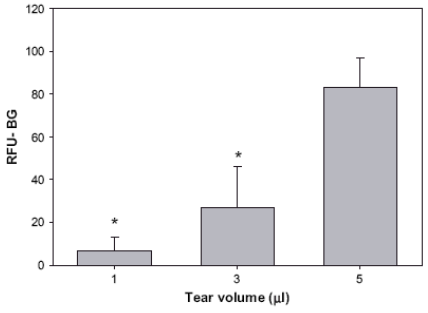

Fig. 4. Chitotriosidase activity in human tear fluid (4 independent experiments). Tears were collected and chitotriosidase activity measured as described in Section 2. Data were analysed by one-way ANOVA followed by *post hoc* LSD test using SPSS. *Significant difference, compared with the highest tear volume, at $P \leq 0.05$.

Using this recombinant chitotriosidase, we have measured chitotrioside fluorogenic substrate cleavage under optimal conditions (0.1 M/0.2 M citrate/phosphate buffer, pH 6.0) in the presence of increasing NaCl concentrations, up to 5 M (Fig. 6A). Chitotriosidase remained highly active even in the presence of 5 M NaCl, with statistically significant reduction only apparent above 500 mM NaCl. Lysozyme did not show any significant activity in the used buffer, in the presence or absence of NaCl (not shown). Fig. 6B shows chitotriosidase activity of 100 μg/ml human lysozyme (chosen as the double of the maximum expected concentration of lysozyme in tear fluid in our assay with a 1:20 dilution, based on the known value of 1 mg/ml lysozyme in human tear film [27]), 5 μl pooled (from 3 donors) reflex tear fluid or 1 μg/ml recombinant chitotriosidase. The inclusion of 0.5 M NaCl did not have any statistically significant effect on the activity of recombinant chitotriosidase or tear fluid, while lysozyme had only very low enzymatic activity in both buffers. These data clearly show that the chitotriosidase activity found in tears can be fully ascribed to salt-insensitive chitotriosidase and not to lysozyme, despite its presence and ability to cleave the used substrate.

Finally we wanted to assess whether human chitotriosidase, in addition to its documented anti-fungal effects [10], also has anti-microbial effects, and whether there are any synergisms with lysozyme. The reasons underlying this assumption will be discussed in detail in the next section. Support for an anti-microbial role of chitotriosidase comes from a recent study showing that children undergoing chemotherapy for acute myeloid leukaemia have a significantly higher risk of contracting bacterial infections [28] when they possess a mutant genotype that renders chitotriosidase enzymatically inactive [29].

Our strategy was to choose a panel of Gram-positive (more susceptible to the action of lysozyme than Gram-negative bacteria) and Gram-negative bacteria, including bacteria such as *S. aureus*, which are known to be impervious to lysozyme and to colonize the skin, and non-pathogenic bacteria such as *B. cereus* and *L. innocua* with higher sensitivity to lysozyme. All bacteria were sensitive to 50 μg/ml kanamycin, which we used as positive control in all experiments. Firstly, a lysozyme sensitivity curve was generated using serial dilutions of egg-white lysozyme for the seven bacterial strains

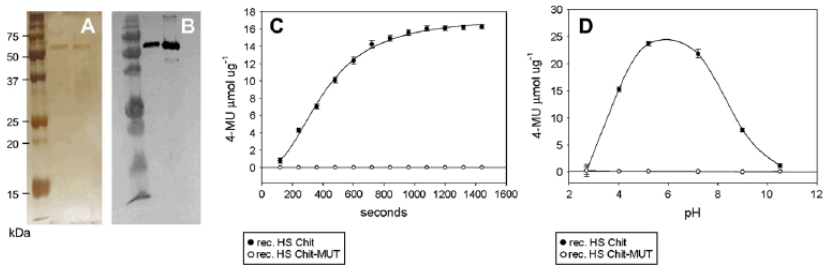

Fig. 5. (A) Silver-stained 12% SDS-PAGE of recombinant wildtype (middle lane) and mutant (right lane) chitotriosidase. There is no apparent contamination. The molecular weight of the recombinant protein is slightly larger than the 50 kDa of the native chitotriosidase, as the expression vector adds a his-tag and a V5-epitope to the C-terminus of the protein. (B) Western blot with an anti-V5 detecting antibody, confirming in-frame synthesis of recombinant wildtype (middle lane) and mutant (right lane) chitotriosidase. Sequence was also confirmed by protein sequencing of tryptic fragments as described in Section 2. (C) Enzymatic activity of recombinant human chitotriosidase (rChit) and active site mutant (rChit-MUT) obtained from stably transfected Sf9 insect cells. The results confirm that our recombinant enzyme is fully active and that the added detection/purification tag does not interfere with enzymatic activity. As expected, the active site mutant (open circles) had no enzymatic activity. The pH curve (D) indicates a broad activity curve with a pH optimum between 5.0 and 6.0.

and measuring the inhibition of growth with a luminometric ATP-based assay. As can be seen in Fig. 7A, *B. subtilis* was highly susceptible to the effects of lysozyme, with inhibition of growth at all tested dilutions. *L. innocua* (Fig. 7G) showed intermediate sensitivity to lysozyme, with partial, dose-dependent growth inhibition at all concentrations. *B. cereus*, wildtype *S. aureus* and the two tested Gram-negative strains *P. aeruginosa* and *E. coli* were completely insensitive to lysozyme (Fig. 7E,I,K,M).

The mutant *S. aureus oatA*-deficient strain, which lacks an *O*-acetyl transferase that acetylates the C6 hydroxy group of muramic acid in peptidoglycan, displayed increased sensitivity to lysozyme at the two highest concentrations (Fig. 7C), in line with previous findings [30,31].

Based on the sensitivity to lysozyme identified in our essay, we repeated the growth inhibition experiments with a suboptimal concentration of lysozyme (0.05 mg/ml for *B. subtilis* and 12.5 mg/ml for all others) in the presence of 25 µg/ml recombinant wildtype or mutant chiotriosidase. As shown in Fig. 7B,D,F,H,J,L and N, the addition of chitotriosidase to a suboptimal concentration of lysozyme did not increase growth inhibition in any of the tested bacterial strains, neither did the presence of chitotriosidase alone. Consequently, and as expected, the chitotriosidase mutant also exhibited no effect on growth inhibition. These experiments clearly show that chitotriosidase, at least in the concentration used, does not have any effect on bacterial growth, even when used in combination with lysozyme.

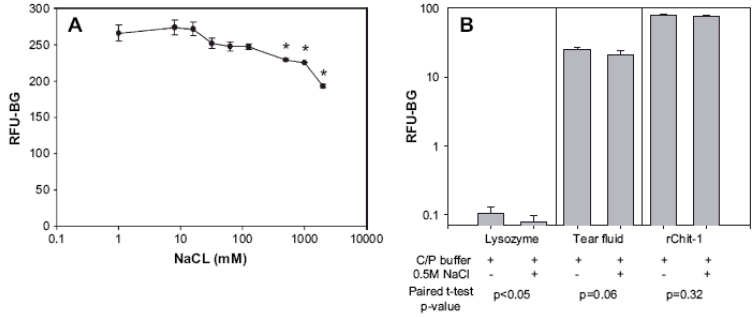

Fig. 6. (A) The effect of increasing concentrations of NaCl on the activity of recombinant chitotriosidase (mean ± SD). Data were analysed by a one-way ANOVA followed by *post hoc* LSD test using SPSS v11.5. *Significant difference, compared with chitotriosidase activity in salt-free buffer, at $P \leq 0.05$. (B) Effect of 0.5 M NaCl in 0.1 M citrate/0.2 M phosphate buffer, pH 6.0 (C/P buffer) on chitotrioside fluorescent substrate cleavage by 100 µg/ml human milk lysozyme, 5 µl pooled reflex tear fluid or 1 µg/ml recombinant chitotriosidase. Indicated paired *t*-test *P*-values are between NaCl-containing buffer and buffer alone, for all three tested enzymes/fluids. RFU-BG, relative fluorescence units-background (all experiments performed in triplicate).

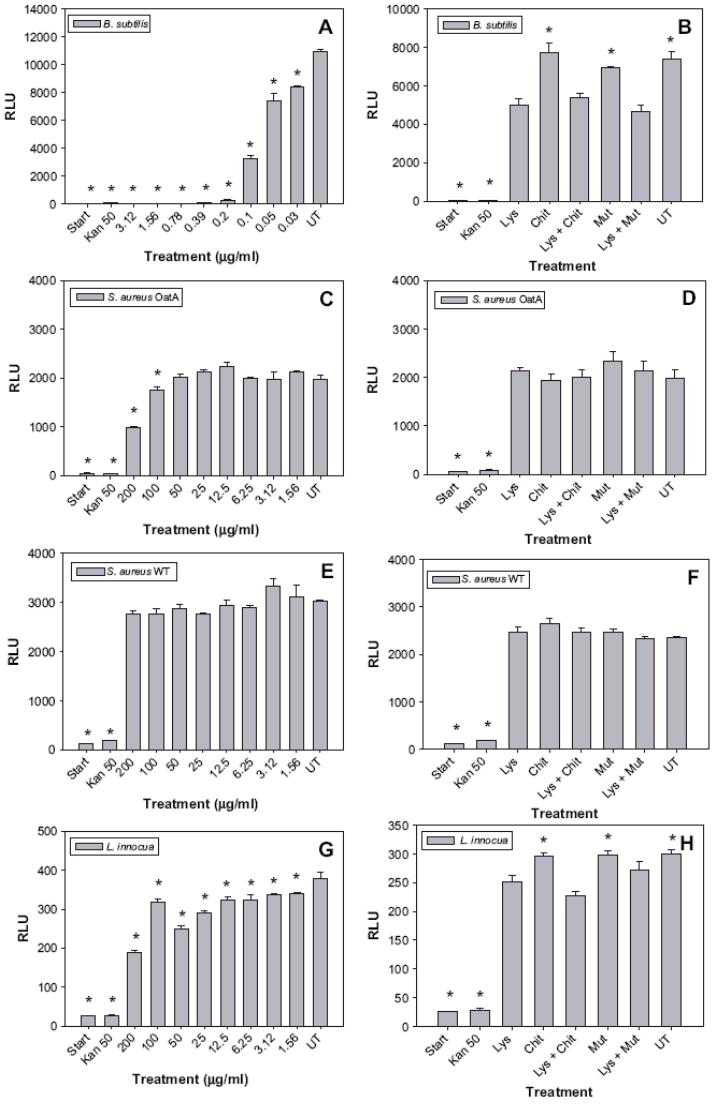

Fig. 7. Antimicrobial growth inhibition of different bacterial strains by egg-white lysozyme alone (left panels) or chiotriosidase, alone or in combination with a sub-optimal dose of lysozyme (right panels). *B. subtilis* (A), *S. aureus oatA* (C), *L. innocua* (G), but not wildtype *S. aureus* (E), *B. cereus* (I), *E. coli* (K) or *P. aeruginosa* (M), were susceptible to lysozyme. The significance for the lysozyme charts on the left is compared to the untreated control, and for the chitotriosidase assays on the right, compared to lysozyme alone. All experiments were repeated at least twice with triplicate samples with comparable results. Statistical analyses were carried out by one-way ANOVA and post hoc LSD test (*significance at $P < 0.05$) using SPSS v11.5.

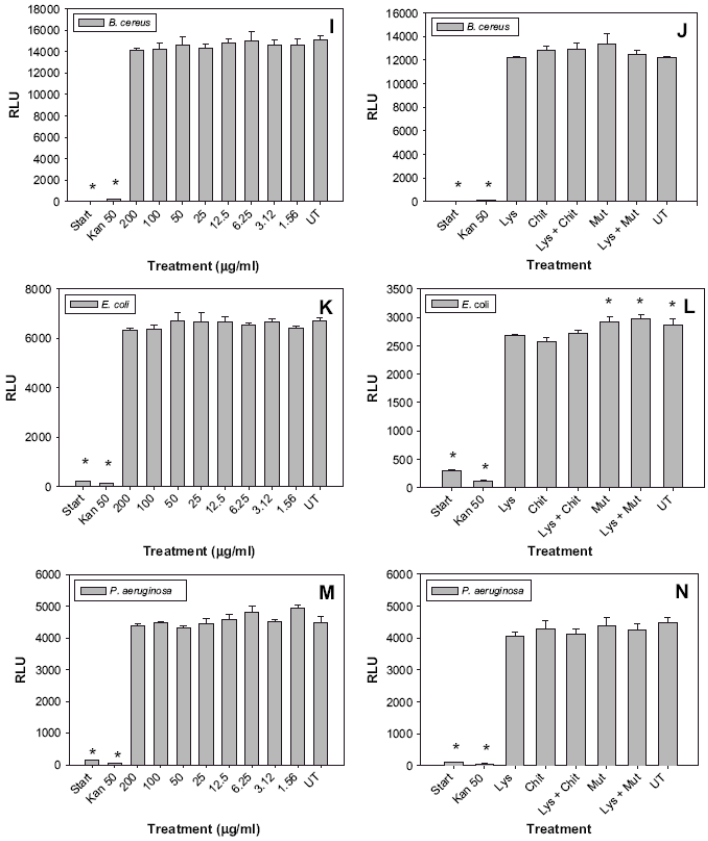

Fig. 7. (*continued*)

4. Discussion

We have shown that chitotriosidase is expressed in the human and mouse eye, particularly in lacrimal glands. In this context, it is interesting to note that human chitinases, unlike bacterial chitinases, do not appear to have any mucolytic activity [32]. This is important, as the physiological watery tear film is directly laid upon a mucoid layer, whose function is to provide a hydrophilic layer above the corneal epithelium. Any mucolytic activity of chitotriosidase would compromise the integrity of this layer.

Chitotriosidase, unlike lysozyme, remained enzymatically active even in the presence of 1–5 M NaCl concentrations. Dorschner and coauthors [33] have recently shown that human LL-37, an antimicrobial peptide that is also affected by physiological NaCl concentrations, regains bactericidal activity when *S. aureus* or *E. coli* are grown in carbonate-buffered solutions. As tear fluid is also carbonate-buffered, it is possible that lysozyme might be more active when tested against bacteria in carbonate-buffered fluids. It remains to be shown, however, whether the findings described by Dorschner also apply to other antimicrobial peptides or to antimicrobial enzymes, as the latter have an entirely different mechanism of action.

Our results support a role in innate immunity. However, we have not found any effect of chitotriosidase on bacterial growth,

even when used in combination with lysozyme. Thus our data cannot explain why children with a known chitotriosidase deficiency (see below) undergoing treatment for acute myelogenous leukaemia appear to be at higher risk of contracting serious Gram-negative infections [28]. This finding is intriguing, as bacteria are not thought to contain chitin. While lysozyme is primarily a muramidase, it also has chitinase activity. Conversely, chitotriosidase may also have additional enzymatic activities other than its chitinase activity, perhaps acting on carbohydrates with a linkage similar to chitin. For example, hevamine, a prominent chitinase produced by *Hevea brasiliensis*, has dual chitinase/muramidase activity [34].

Our data suggest, in combination with the documented antifungal effects [10], that the anti-microbial action of chitotriosidase is limited to fungi, thus displaying an anti-microbial spectrum clearly different from lysozyme. This is also in agreement with our finding that chitotriosidase gene expression is strongly up-regulated in monocyte-derived macrophages treated with fungal, but not with bacterial pathogen associated molecular patterns (PAMPs) (A.J. Hall, S. Morroll, F.H. Falcone, manuscript submitted).

Intriguing, however, is the lack of an obvious phenotype (i.e. susceptibility to fungal infection) in individuals with inherited chitotriosidase deficiency. This condition in known to occur in 5–6% of individuals of Caucasian origin, and is known to be associated with a 24 kDa duplication in the *chit1* gene, leading to the activation of a cryptic splice site, and resulting in a partial deletion in the sequence which leads to an enzymatically inactive chitotriosidase [29]. No association with the *chit1* genotype was found in survivors of *Candida* sepsis [35]. In line with this finding, the *chit1* genotype was not associated with an increased risk of chronic disseminated candidiasis in adult leukaemia patients [36]. A third, previously mentioned study, however, finds a significant association between the risk for serious Gram-negative bacterial infections and a deficient *chit1* genotype [28]. As increased levels of chitotriosidase have been described in neonates with bacterial infections [37], this may suggest that the spectrum of anti-microbial action of chitotriosidase extends to bacteria, thus playing a complementary role to lysozyme, which has a well-documented action against bacteria but also a less known anti-fungal activity [38,39]. Our data do not support such a view.

Thus chitotriosidase deficiency does not result in an obvious susceptibility to fungal or bacterial infection, and only appears to play a role in immune-compromised individuals [28]. There are two possible explanations for this: the first, is that chitotriosidase has become largely obsolete, or its functions are not related to innate immunity. This is unlikely because of the conservation throughout mammals and other higher animals, and its expression in macrophages and neutrophils, pivotal cells of innate immunity. The second, more likely explanation, is that the immune system has several tiers of redundancy making sure that organisms remain viable despite defects in innate immunity genes. Mutations in genes playing an important role in innate immunity are frequently lethal, as demonstrated by hereditary chronic granulomatous disease [40], in which a defect in the leukocyte oxidase gene results in a severe deficiency in immunity to several, especially catalase-positive, pathogens [41].

Thus, for a chitotriosidase deficiency phenotype to become apparent, there would be a requirement for another gene to mutate. As chitinases are known to act in synergy with other enzymes, e.g. with glucanases [42,43] in plants, it is *a priori* possible that only a second concurring mutation may result in a compromised phenotype. There are several mutations affecting the gene for lysozyme, such as the T70N variant, found in about 5% of the British population [44] and the lethal amyloidogenic variants [45], but none of these is associated with an increased susceptibility to microbial infection. In all currently known variants, including the amyloidogenic forms, lysozyme enzymatic activity is not affected at physiological temperatures (Mark Pepys, personal communication). However, other yet to be discovered lysozyme deficiencies could be masked by the existence of a functional chitotriosidase.

Taken together, we report the expression of chitotriosidase in the human and murine eye, supporting the currently suggested role in innate immunity. Chitotriosidase is the only member of the chitinase/chilectin gene family expressed in the murine eye. The anti-microbial spectrum appears to be distinct from lysozyme, as we saw no effects of enzymatically active recombinant chitotriosidase on the growth of Gram-positive and Gram-negative bacterial strains. In contrast to lysozyme, chitotriosidase activity was found to be unaffected by high salt concentration.

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