The Immunomodulatory effect of Pneumolysin upon CD4 T cells

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy

by

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Declaration of Authorship

This thesis is the original work of the author except where acknowledged.

Gordon R Meiklejohn[ó], University of Glasgow, August 2004

Table of Contents

Extended Table of Contents

List of Figures

Abstract

The human bacterial pathogen, *Streptococcus pneumoniae* (the pneumococcus), has been shown to modulate different parts of the innate immune response of its host, however its ability to modulate the adaptive immune response remains largely uninvestigated. Furthermore, the importance of the adaptive arm of the immune system in responding to *Streptococcus pneumoniae* has only recently begun to be elucidated. I therefore investigated a potentially novel pneumococcal immunomodulatory mechanism involving the effect of the pneumococcal toxin, pneumolysin, upon the cells at the heart of the adaptive immune response; the CD4 T cell.

I generated purified pneumolysin and a purified pneumolysin mutant called F433 to allow me to examine this potential effect. I found that pneumolysin inhibits *in vitro* antigen specific murine CD4 T cell proliferation and cytokine production and that this effect is not observed with the F433 mutant pneumolysin. Furthermore, I demonstrated that pneumolysin accomplished this inhibitory activity by inducing apoptosis of activated CD4 T cells and suggest that lipid rafts may be involved in this process since we also demonstrated that pneumolysin preferentially binds to lipid rafts. Finally I demonstrated that pneumolysin is able to inhibit the *in vivo* accumulation of T cells and also inhibits *in vivo* antibody production.

I propose that the immunomodulatory mechanism I have described may play an important role during pneumococcal infection and that this warrants further investigation. I propose that detailed *in vivo* studies are required to demonstrate that this mechanism functions during infection and to elucidate the effect this has upon the course of infection.

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Abbreviations

5-(-6-) carboxyfluorescein diacetate, succinimidyl ester	CFSE
Activated Cell Autonomous Death	ACAD
Activation Induced Cell Death	AICD
Antigen Presenting Cell	APC
base pair	bp
Bone Marrow derived Dendritic Cells	BMDC
Cerebro Spinal Fluid	CSF
Cholesterol Dependent Cytolysin	CDC
Collagen Induced Arthritis	CIA
Colony Forming Units	CFU
counts per minute	cpm
C-Reactive Protein	CRP
Dendritic cell	DC
Enzyme Linked Immuno Sorbent Assay	ELISA
Fetal Calf Serum	FCS
Fluorescence Resonanace Energy Transfer	FRET
Green Fluorescent Protein	GFP
Hanks Balanced Salt Solution	HBSS
Hydrophobic Interaction Column	HIC
Immunoglobin	Ig
Immuno-Histo-Chemistry	IHC

Interferon	IFN
Interleukin	IL
intra-peritoneal	i.p.
Lactate Dehydrogenase Assay	LDH
Laser Scanning Cytometer	LSC
Lipopolysaccharide	LPS
Listeriolysin	LLO
Major Histocompatibility Complex	MHC
Mannose Binding Lectin	MBL
Mean Fluorescence Intensity	MFI
Membrane Attack Complex	MAC
Metal Chelate	MC
Optical Density	OD
Ovalbumin	ova
ovalbumin in complete freunds adjuvant	OVA-CFA
Pattern Recognition Receptor	PRR
Perfringolysin	PFO
Phorbol Myrisate Acetate	PMA
Phosphate Buffered Saline	PBS
Pneumolysin	PLY
Polymerase Chain Reaction	PCR
Polymorhponuclear Leukocytes	PMNL
Programmed Cell Death	PCD

Propidium Iodide	PI
Repeats in Toxin	RTX
Streptolysin	SLO
subcutaneously	s.c.
T cell dependent	TD
T cell independent	TI
T cell Receptor	TcR
T helper cell type 1	TH1
T helper cell type 2	TH2
Toll Like Receptor	TLR
Transforming Growth Factor beta	TGF-β
Transgenic	Tg
Tryptophan	Trp
Tumor Necrosis Factor alpha	TNF-α
Variant Surface Glycoprotein	VSG

1 Introduction

1.1 Introduction

The existence of pathogen's is all too obvious in the world today, however the reason for the pathogenic way of life remains unclear (Granucci and Ricciardi-Castagnoli, 2003); It may result from some disregulation from 'normal' functioning or as a result of an organism's journey to becoming a commensal (Rhen *et al.*, 2003).

Regardless of the reason it is clear that central to pathogenesis is a parasitic lifestyle that involves a complex interplay of host-parasite interactions. The effectiveness of the host's immune system is central to the outcome of these interactions. As a result, the ability of the parasite to modulate the host's immune response (immunomodulation) is equally influential.

In this thesis I examine the host pathogen interaction of the bacterium *Streptococcus pneumoniae* (the pneumococcus) with its human host using murine models. I will be specifically investigating a potential mechanism that *S. pneumoniae* uses to modulate the adaptive immune response of the host. The reasons for these investigations are twofold; firstly, I am seeking to further understand the pathogenic mechanisms of the pneumococcus and secondly, I am interested in the potential therapeutic applications of 'pathogen derived' immunomodulators.

However prior to discussing my investigations it is necessary to understand the context of my work and this is the purpose of this chapter. I will first discuss the immune system and then examine some of the mechanisms that pathogen's use to modulate host immune systems. This discussion will allow an understanding of the different mechanisms that the pneumococcus potentially uses to modulate the host immune system. I will then describe some of the contexts in which immunomodulatory molecules may be applied for therapeutic benefit. Finally I will focus on the pneumococcus itself, specifically the immune response it provokes and some of the factors produced that allow it to colonise and infect the host.

1.2 Overview of the Immune System

The immune system has two parts that are distinct yet co-operate effectively. The first part is the innate immune response and the second the adaptive immune response (Figure 1-1). The innate immune response is relatively non specific and acts quickly upon encountering a pathogen while the adaptive immune response is highly specific and able to recognise individual pathogen's, however the generation of the adaptive immune response takes longer than the innate response and occurs in specific organs within the lymph tissue (Figure 1-2).

1.2.1 Innate Immune Response

Upon entering the host a pathogen encounters the innate immune response. The first component that a pathogen encounters is one or both of the alternative or mannose binding lectin (MBL) complement pathways which are part of a series of plasma proteins collectively termed 'complement' (Figure 1-3). The alternative complement pathway is activated by the surface of microbes (Winkelstein and Tomasz, 1977) while MBL binds to the sugar residues on bacterial surfaces and activates the MBL pathway (Estabrook *et al.*, 2004; Jack *et al.*, 2001). Both pathways result in a cascade of complement proteins to interact resulting in opsonisation of the pathogen by member iC3b which enhances phagocytic uptake due to the presence of CD21 on phagocyte's surface which is the receptor for an iC3b derivative (Mosser and Edelson, 1985). Alternatively, accumulation of further members of the complement cascade on the surface of the pathogen results in the formation of the Membrane Attack Complex (MAC) which causes lysis of the pathogen (Tranum-Jensen *et al.*, 1978). Although less well defined natural antibody is proposed to be an important component of the early immune response (Lutz, 1999). Natural antibody is of the IgM type and forms without specific antigen stimulation (Ochsenbein and Zinkernagel, 2000). Natural IgM is polyreactive to conserved antigenic structures on pathogen's and in this way is able to provide a degree of immediate protection to the host (Boes, 2000).

Pathogen's also encounter phagocyte's such as macrophages and neutrophils early after entry into the host since these cells are distributed throughout the body (Djaldetti *et al.*, 2002). Pathogen's are

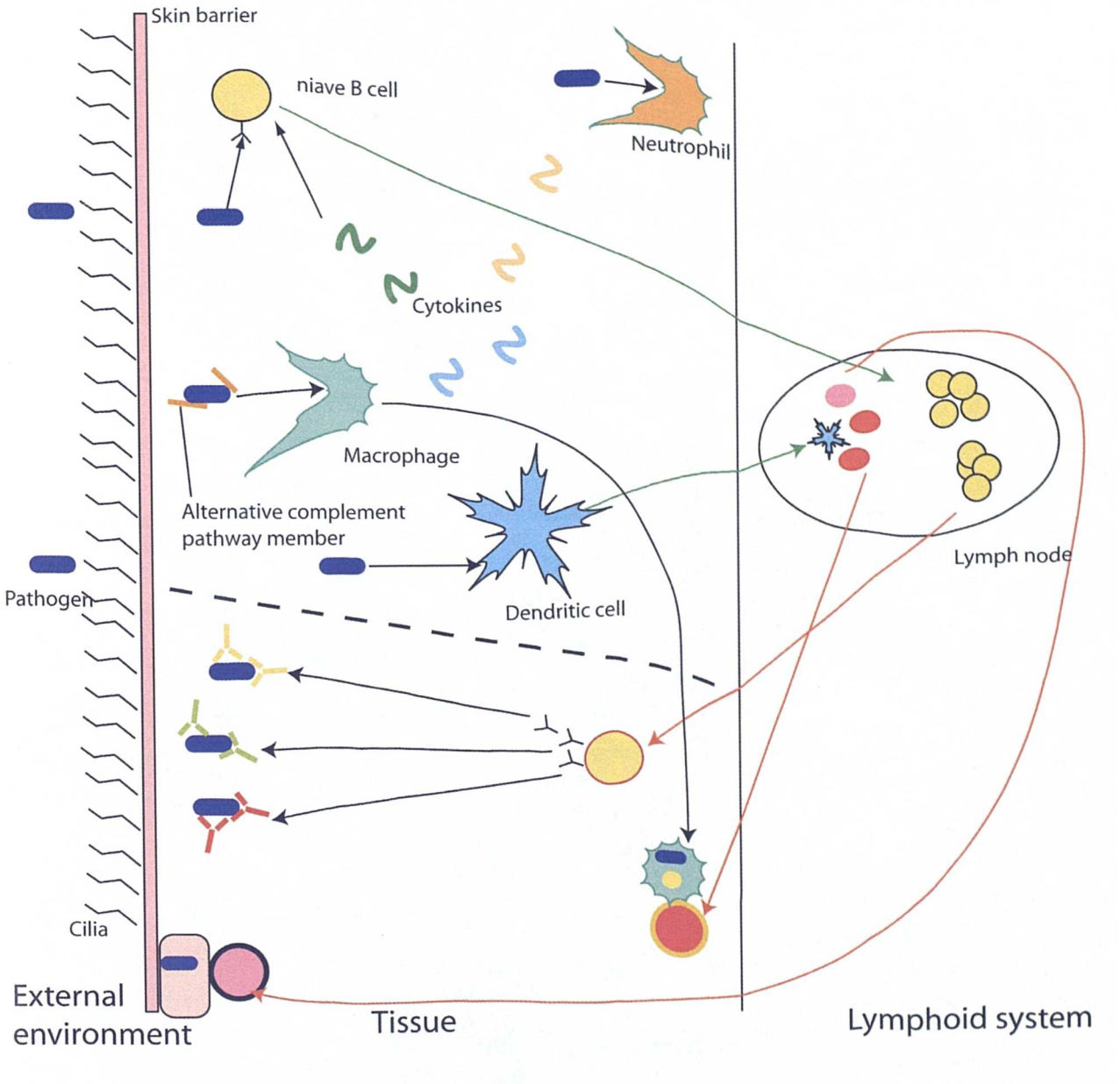


Figure 1-1

Overview of the Immune System

The immune response to a pathogen is shown above and highlights the primary components of the immune response. The innate immune system is first represented by anatomical barriers such as skin and cilia. The coating of the pathogen by alternative complement prior to macrophage phagocytosis is shown as is neutrophil phagocytosis. Release of cytokines by macrophage that have engulfed a pathogen is illustrated; these have a multitude of effects. The adaptive immune response begins with dendritic cell uptake of the pathogen at the site of infection, then it migrates to the lymph node as shown in the figure. Figure 1-4 shows the interactions that occur in the lymph node but the effects are shown here with the production of CD4 T cells that can activate macrophage, help B cells produce antibody and the production of CD8 T cells that can kill infected cells.

recognised by Pathogen Recognition Receptors (PRR) such as Toll Like Receptor (TLR) (Reis e Sousa, 2004). PRR's recognise conserved components of pathogen's such as LPS, CpG, flagellin and peptidoglycan (Dziarski, 2003; Hayashi *et al.*, 2001; Kopp and Medzhitov, 1999). Recognition results in three important events (Janeway and Medzhitov, 2002). Firstly phagocytosis (literally cell eating) of the pathogen results in the destruction of the pathogen although sometimes CD4 T cell help is required when macrophages harbour a pathogen that resists the fusion of the lysosome with the phagosome. CD4 T cell help enables full activation of the phagocyte. Secondly upon ingestion of a pathogen phagocyte's also secrete cytokines which are molecules that induce a change in another cell and in this context encourage the development of the immune response (Monton and Torres, 1998). Finally macrophages can act as antigen presenting cells for the induction of the secondary adaptive immune response.

Each of the cytokines, such as IL-1, IL-8, TNF-a, IL-6 and IL-12, released by macrophages (Monton *et al.*, 1999) have important effects on the developing host's immune response. IL-1 and TNF-a induce changes in the vascular endothelium characteristic of inflammation which increase the blood flow to infected areas and decrease the flow rate in these areas (Lamping *et al.*, 1998). Furthermore increased permeability is also induced which allows the influx of antibody, complement and cells to infected areas. Locally IL-6 causes activation of lymphocytes however systemically it causes the release of acute phase protein. Release of IL-8 causes the recruitment of neutrophils to the site of infection (Gerber *et al.*, 2004) while IL-12 causes the CD4 T cell response to become skewed towards T_H1 (Hsieh *et al.*, 1993).

The final member of the innate immune response is the acute phase proteins which includes C-reactive protein which binds to certain bacterial and fungal lipopolysaccharide and activates the classical complement pathway (Palaniyar *et al.*, 2002).

1.2.2 Adaptive Immune Response

The induction of the primary adaptive immune response (Figure 1-4) begins with the encounter of the pathogen by a dendritic cell at the site of infection which then migrates to the draining peripheral lymphoid organs such as the lymph nodes (Jenkins *et al.*, 2001). Alternatively soluble antigen

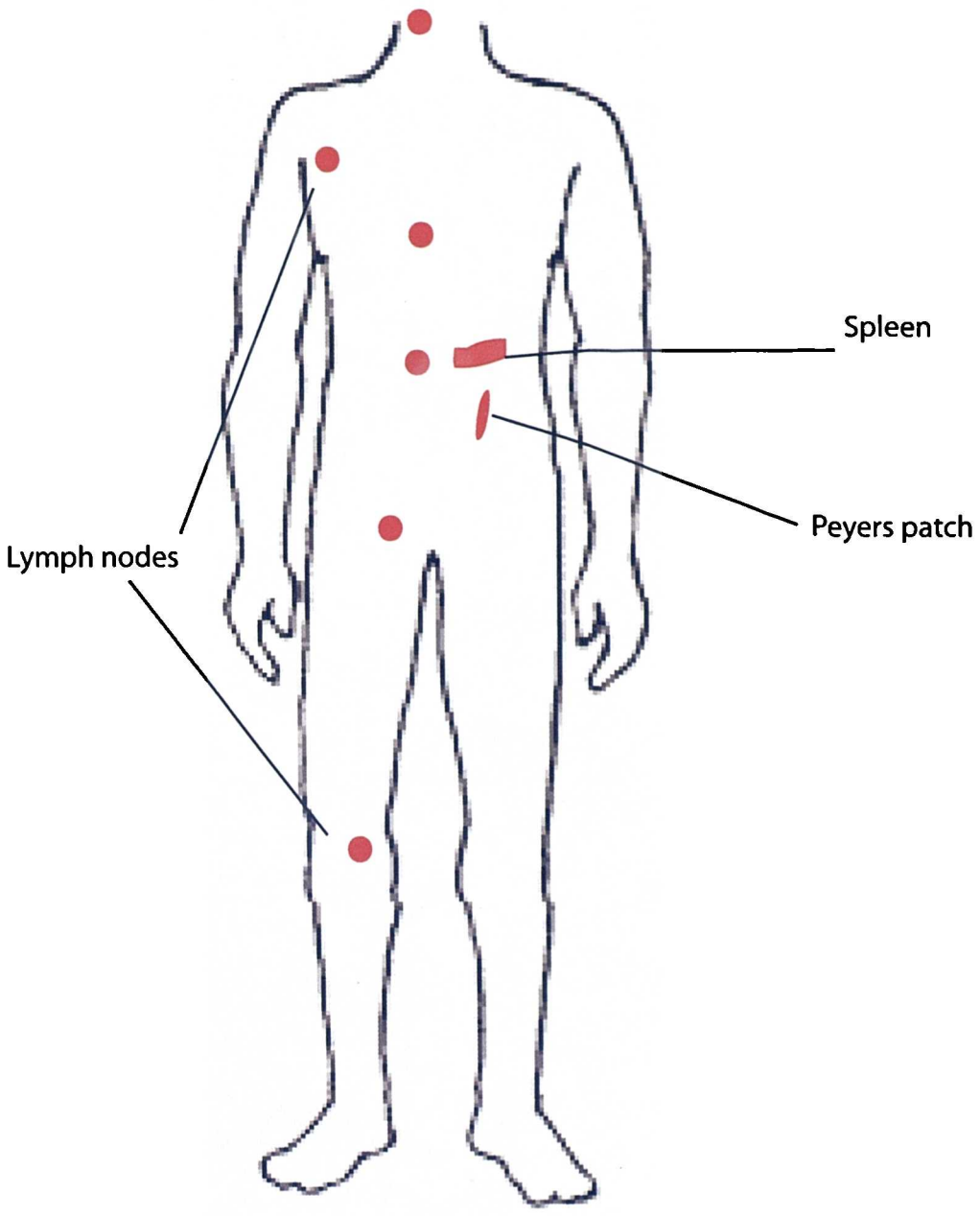


Figure 1-2

Distribution of lymphoid tissue in humans

The red circles represent lymph nodes which are distributed at the sites indicated. Distribution is symmetrical within the body and numbers are greater than shown. Other lymphoid organs are also shown.

may enter the peripheral lymph nodes independent of an APC but be ingested in the lymph organ by a dendritic cell (Smith *et al.*, 2004a). The DC processes components of the pathogen into peptides that are presented to the TcR on T cells as part of the Major Histocompatibility Complex (MHC). There are two MHC's; MHC I and MHC II. MHC I is present on the surface of cells throughout the body, however MHC II are present only on the surface of APC's. The source of the proteins dictates which complex they are presented on; protein present in the cytosol of the APC is presented on MHC I while protein present in the endocytic vesicle is presented on MHC II (Morrison *et al.*, 1986). On the surface of T cells along with the TcR is either the cell surface molecule CD4 or CD8. Cells expressing CD8 interact with MHC I while cells expressing CD4 interact with MHC II. However, MHC:TcR with CD4 or CD8 interaction is not the only interaction required for T cell activation. The APC must also provide costimulation through a number of receptor:ligand interactions including, CD40:CD40L, CD80:CD28, CD86:CD28 (Liu and Janeway, 1992). Interaction of the APC and T cell leads to activation and proliferation of T cells which produces a population of armed effector T cells. These armed effector T cells then function to eliminate the infection (Figure 1-4).

CD8 T cells migrate from the lymph node to the site of infection where they recognise pathogen peptides expressed on MHC I of infected cells. They are then able to kill the infected cell by the release of cytotoxic granules such as granzyme (Waterhouse and Trapani, 2002). When CD4 T cells are activated they differentiate into one of two subsets. The mechanism that dictates the subset is unclear however the cytokines present during activation by the APC are thought to have some effect so that cytokines such as IL-12 cause differentiation into T_H1 CD4 T cells and cytokines such as IL-4 cause differentiation into T_H2 CD4 T cells (Hsieh *et al.*, 1993). T_H1 CD4 T cells then migrate from the lymph node to the site of infection where they are able to activate macrophages (Reinhardt *et al.*, 2003; Stout and Bottomly, 1989) while T_H2 CD4 T cells express costimulatory molecules such as CD40, ICOS, OX40 that help activated B cells that have acquired antigen to make antibody and undergo affinity maturation in the lymph node. In humans four different types of antibody is then produced that function in different contexts (Mestas and Hughes, 2004). IgM antibody is produced rapidly in response to infection and performs a neutralising function in the blood and also activates the classical complement pathway. IgG has five subtypes, of which the production of each subtype is dictated by the T_H1 / T_H2 nature of the response. IgG is an effective opsoniser and complement activator and functions primarily in the blood. The classical complement pathway is activated by antibody:antigen complexes and after activation follows the same

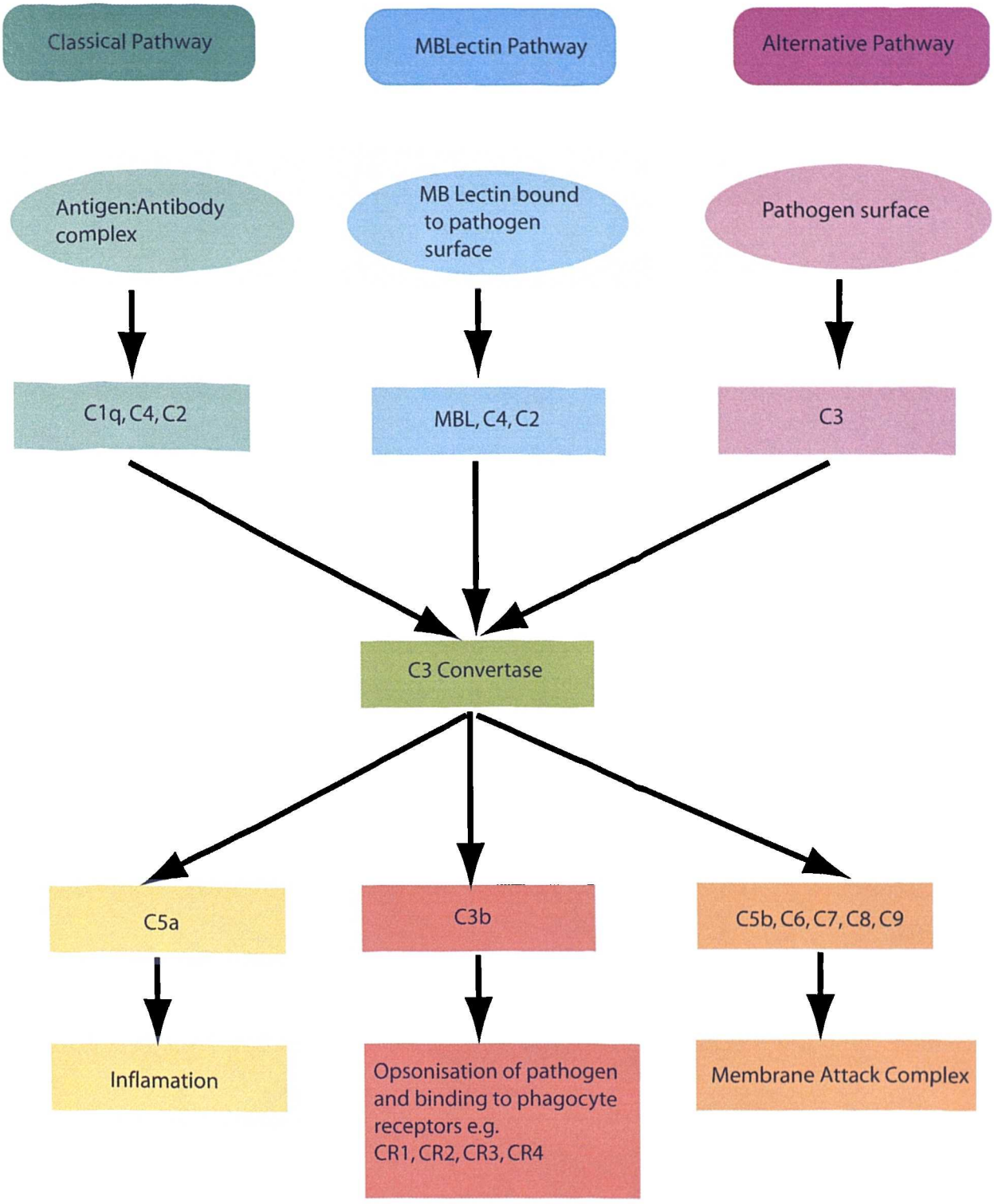


Figure 1-3

Overview of the complement pathway

The schematic shows the 3 arms of the complement pathway and their convergence on the C3 convertase and the 3 subsequent outcomes.

cascade as the alternative complement pathway to produces either opsonisation of pathogen's or the formation of the MAC on the pathogen surface (Figure 1-3). Two subtypes of IgA are produced and line the mucosal surface of the body and neutralises pathogen's and toxins entering by these routes. Finally IgE is found bound to mast cells which are involved in helminth immunity (Else and Finkelman, 1998). Some of the effector cells differentiate into memory cells that persist after the others have died and are present to enable a rapid secondary response should the same pathogen be encountered again (Crotty and Ahmed, 2004).

Antigens that stimulate antibody production from B cells fall into three categories depending on their requirement for T cell help (Mond *et al.*, 1995b). T Dependent (TD) antigens (such a peptides) require T cell help to induce effective antibody responses. T Independent (TI) antigens are able to induce antibody formation in the absence of T cells; and as a result of not recruiting T cell help for the B cells they do not induce germinal centre formation, memory generation or affinity maturation. There are two types of TI's; Type 1 are mitogenic for B cells and induce antibody by crosslinking of surface Ig. Type 2 antigens are unable to associate with the MHC and so cannot interact with the TcR, however the response they induce is able to be influenced by T cells (Mond *et al.*, 1995a; Sangster *et al.*, 2003).

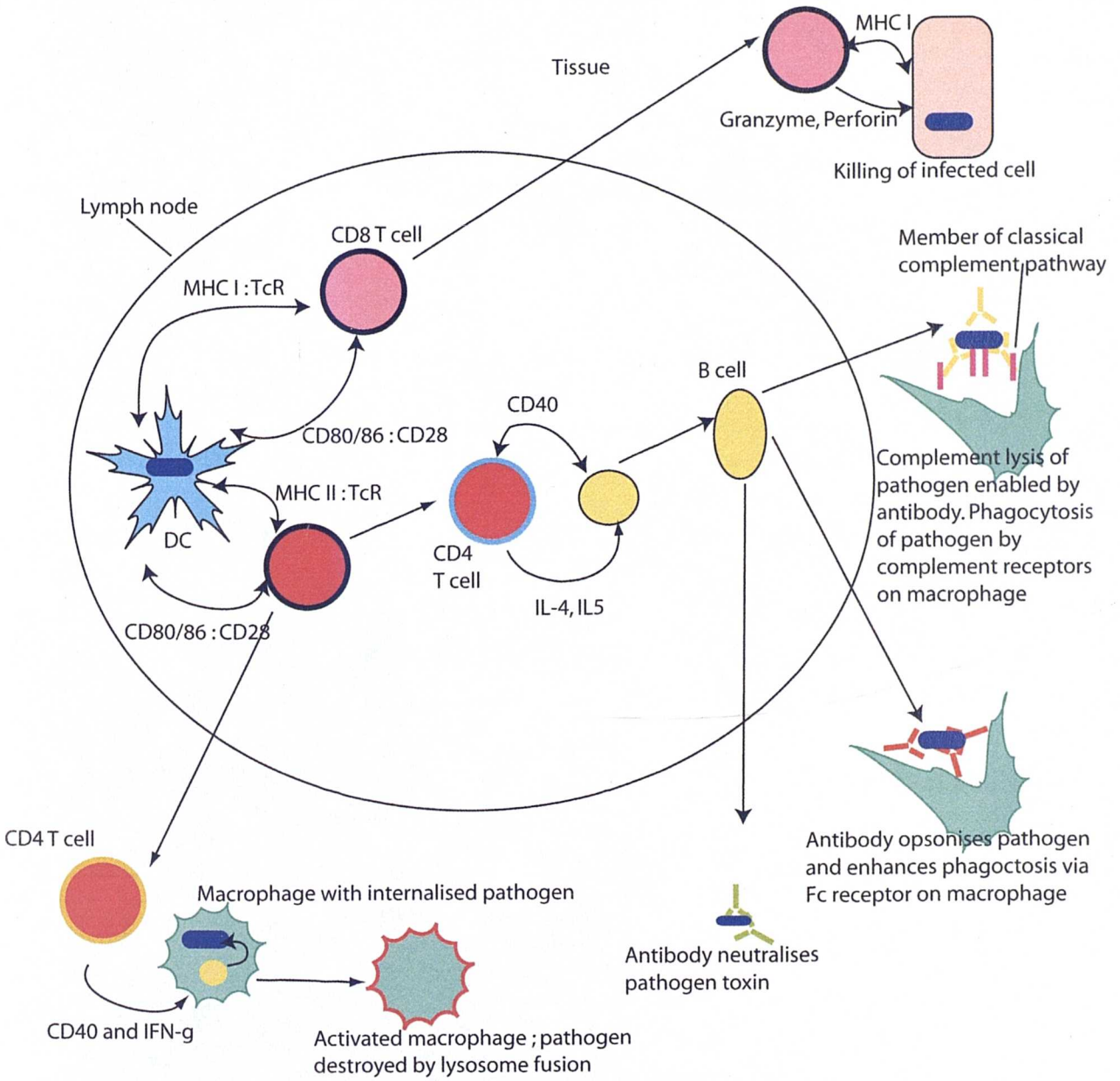


Figure 1-4

Overview of the production of effector T cells

The schematic shows the entry of antigen on a dendritic cell and presentation via the 2-signal model to the T cell. Subsequent effector function is displayed; CD8 T cell killing of an infected cell, TH1 CD4 T cell macrophage activation leading to fusion of the lysosome and phagosome and TH2 CD4 T cells providing B cell help to enable antibody production. The subsequent effects of antibody production are shown; antibody binding to pathogens can allow MAC formation on the surface of the pathogen or can lead to engulfment. Neutralisation of toxins by antibodies is also important.

1.3 Pathogen Immunomodulation

Prior to examining some of the mechanisms that pathogen's use to modulate each part of the immune system described above, it should be noted that most pathogen's target more than one of aspect of the host immune response. African trypanosomes provide a clear example of this (Mabbott *et al.*, 1998). Firstly African trypanosomes disrupt the action of the innate immune response by interfering with the action of complement through the expression of a protease that resembles the gp63 protease of Leishmania. Bloodstream trypanosomes activate the alternative complement pathway and yet this does not result in trypanosome lysis; perhaps because of the presence of this protease (El-Sayed and Donelson, 1997). Secondly the trypanosome disrupts the cell mediated adaptive immune response by inducing CD8 T cells to produce large amounts of IFN-g (Darji *et al.*, 1996). The bloodstream trypanosome produces what has been called T lymphocyte triggering factor (TLTF) which binds to T cells via the CD8 molecule. The resulting expression of IFN-g acts in a beneficial manner for the trypanosome since IFN-g stimulates a MAP kinase in the trypanosome which is suggested to enhance proliferation of the trypanosome (Olsson *et al.*, 1991). The release of IFN-g also cause a decrease in IL-2 receptor expression and IL-2 synthesis which decreases the T cell proliferative response (Darji *et al.*, 1996). Thirdly by releasing a mitogenic factor trypanosomes cause huge B cell expansion and antibody production yet the antibody produced is not parasite specific and may even be host reactive (Reina-San-Martin *et al.*, 2000). Finally avoidance of the humoral arm of the adaptive immune response comes via perpetual switching of the surface glycoprotein coat. The coat is called the Variant Surface Glycoprotein (VSG) and is sequentially expressed by each trypanosome meaning that when an immune response is mounted against one VSG type there is another present within the population that can resist immune attack and therefore the parasite proliferates unchecked (Schleifer *et al.*, 1993). From this example of the trypanosome, my point is clear; pathogen's immunomodulatory activities are multifaceted.

It is not however my intention to focus on individual pathogen's in this chapter but rather to sequentially examine different parts of the host immune response and examples of pathogen's that modulate these to enhance their survival. This will allow a survey of the breadth of immunomodulatory mechanisms that pathogen's use and will also enable recognition of convergent mechanisms of immunomodulation.

1.3.1 Colonisation

The human host's first line of defence against pathogen's is anatomical barriers (Figure 1-1). For example the bacterium *Bordetella pertussis* must overcome the cilia' beat of the epithelial cells of the respiratory tract to allow successful infection of the respiratory tract. Bordetella accomplishes this by secretion of cell wall constituents that cause nitric oxide release from non-ciliated cells which in turn inhibits the cilia beat of epithelial cells (Flak and Goldman, 1999). Furthermore, upon reaching the site of infection a pathogen is not guaranteed successful colonisation if the niche is already occupied by another microbial species. Thus some pathogen's have within their armoury not only mechanisms of host manipulation but also mechanisms to allow them to remove other organisms from the niche they are targeting. For example Pericone *et al.* (Pericone *et al.*, 2000) demonstrated that the pneumococcus releases hydrogen peroxide which inhibits the growth of other bacteria in the upper respiratory tract.

1.3.2 Complement

In order to overcome complement mediated lysis (Figure 1-3) that Group A streptococcus encounters upon invasion of the host, it produces an inhibitor of complement (Akesson *et al.*, 1996). The Streptococcal Inhibitor of Complement (SIC) does not bind strongly to individual members of the complement pathway but binds the C5b67 complex strongly, thereby preventing the formation of the full Membrane Attack Complex.

1.3.3 Phagocytosis and Antigen presentation

Some pathogen's overcome the threat of phagocytosis by preventing uptake, such as *Staphylococcus aureus* which generates an anti-phagocytic capsule (O'Riordan and Lee, 2004) or group A Streptococcus which produces a bacterial homologue of CD11b that inhibits phagocytosis (Lei *et al.*, 2001), however Leishmania encourages phagocytosis via the degradation of C3b to iC3b. This

enhances the ability of macrophage to phagocytose the Leishmania parasites (Mosser and Edelson, 1985). Leishmania then, at least initially, prevents fusion of the lysosome with the phagosome in order to avoid destruction (Spath *et al.*, 2000).

Another way to avoid phagocytosis and presentation by APC's is simply to kill these cells. While some pathogen's induce necrosis of APC's the more favourable option appears to involve the induction of apoptosis since it not only kills the APC but also avoids the release of pro-inflammatory mediators. As an extracellular bacteria Yersinia prevents uptake by APC by inducing macrophage apoptosis. *Yersinia enterocolitica* accomplishes this by injection of YopP (Mills *et al.*, 1997) via a type III secretion system. The mechanism of apoptosis induction by YopP was not initially clear since YopP was shown to activate NF-kδB. However recently it has been suggested that YopP induces apoptosis when combined with the activation of the TLR system by some unknown bacterial product such as LPS (Navarre and Zychlinsky, 2000).

Other pathogen's interfere with APC function by altering the intracellular processing and signalling pathways to prevent presentation of their peptides by the APC. One such pathogen is the Epstein Barr Virus (EBV). EBV expresses a protein called EBV nuclear antigen-1 (EBNA-1) which appears to inhibit proteosome processing (Levitskaya *et al.*, 1997). Herpes simplex virus 1 (HSV-1) utilises a different method based on expression of infected cell protein 47 (ICP47). ICP47 blocks MHC II transport (York *et al.*, 1994) by binding to TAP-1 and TAP-2, weakening the interaction and thereby inhibiting the transport of processed peptide fragments (Hill *et al.*, 1995).

Recently increasing attention has been focused upon the importance of Pattern Recognition Receptors (PRR) for host immune functioning (Medzhitov and Janeway, 2002). Unsurprisingly there has been the simultaneous discovery that pathogen's have developed avoidance mechanisms of these systems. For example *Salmonella typhimurium* has an LPS cell wall structure which is recognised by TLR4. By varying the structure of the lipid A component of the LPS it is proposed that Salmonella attempts a type of phase variation aimed at overcoming recognition by the PRR's (Guo *et al.*, 1997; Hornef *et al.*, 2002).

1.3.4 Cytokines

Understanding of the complex role of cytokines within the immune system has been enhanced through the study of the way in which different pathogen's attempt to modulate the cytokine network. For example, macrophages infected with *Mycobacteria tuberculosis* produce the cytokines IL-6, IL-10 and TGF-b . IL-6 produced by macrophages infected with *M. tuberculosis* has been shown to inhibit T cell activation (VanHeyningen *et al.*, 1997), IL-10 can inhibit macrophage production of reactive oxygen species, can inhibit inflammatory cytokine release and can inhibit expression of costimulatory molecules and MHC (Giacomini *et al.*, 2001; Moore *et al.*, 1993). Therefore by stimulating the release of these cytokines *M. tuberculosis* ensures a less vigorous immune response against it.

Rather than inducing cytokines that are advantageous to their survival, some pathogen's produce cytokine homologues or cytokine receptor homologues. For example, the intestinal helminth *Trichuris muris* produces a protein with significant homology to IFN-g that has been demonstrated to bind to the IFN-g receptor and effect changes similar to those observed with IFN-g (Grencis and Entwistle, 1997). This allows *Trichuris muris* to establish chronic infection since IFN-g has been demonstrated to prevent the development of an immune response that eliminates the nematode (Else *et al.*, 1994).

1.3.5 T cells and B cells

Immunomodulation of T cells is the particular focus of this thesis and so I examine a number of mechanisms used to inhibit T cells. Figure 1-4 shows the T cell activation pathway; any point on this pathway is open to modulation.

Yersinia pseudotuberculosis is observed to inhibit both T and B cell activation when stimulated through their antigen receptors (Yao *et al.*, 1999). Yersinia expresses YopH which is a tyrosine phosphatase and subsequent work using YopH produced by *Yersinia pestis* demonstrated that YopH inhibits tyrosine phosphorylation of the TcR early signalling events resulting in a decrease in IL-2 production along with decreased T cell activation (Alonso *et al.*, 2004).

Infection with *Neisseria gonorrhoeae* does not result in the generation of effective immunological memory. This is in part due to the expression of Opa proteins on the bacteria which results in T cell inhibition. Opa 52 can bind the CD4+ TcR carcinoembryonic antigen-cell adhesion molecule-1 (CEACAM-1) which contains a immuno-receptor tyrosine based inhibitory motif (ITIM). The result is the inhibition of T cell activation and proliferation (Boulton and Gray-Owen, 2002).

The filarial nematode *Acanthocheilonema viteae* has been demonstrated to induce T cell anergy (Harnett *et al.*, 1998) and inhibits B cell activation (Deehan *et al.*, 1997) via a phosphorylcholine containing glycoprotein called ES-62. ES-62 acts by modulating activation of tyrosine kinases central to the TcR signalling cascade and as a result disrupts TcR coupling to subsequent signalling cascades which in turn interrupts the proliferative signals following ligation of TcR and results in T cell anergy (Harnett *et al.*, 1998).

I will examine in detail the mechanism of apoptosis as a T cell inhibitory mechanism later in this chapter. However, it is worth noting at this point that some of the pathogen's use this mechanism. *Aeromonas hydrophila* produces the toxin aerolysin which causes T cells apoptosis by the formation of channels in the cell membrane (Nelson *et al.*, 1999). *Borrelia burgdorferi*, when co-cultured with T lymphocytes, induces apoptosis via a Fas dependent mechanism (Perticarari *et al.*, 2003) as does *H. pylori* (Wang *et al.*, 2001).

Other pathogen's indirectly suppress T cells by stimulating the production of a T cell inhibitory factor from another cell type. For example the yeast *Cryptococcus neoformans* produces a capsule component called Glucuronoxylomannan (GXM) that while it has no direct affect on T cell proliferation it has been demonstrated to increase the production of IL-10 which inhibits the CD4+ T cell response (Mariano Andrade *et al.*, 2003).

From this examination of various mechanisms of immunomodulation it can be seen that pathogen's modulate each part of the immune response. Apoptosis was mentioned as a mechanism of interfering with dendritic cells and T cells and a fuller examination of this will now take place. I will consider this as a distinct topic although it will become clear as we progress that it is in fact an integral part of the areas already examined.

1.4 Apoptosis

1.4.1 Introduction to Apoptosis

Apoptosis plays a crucial role in maintaining homeostasis within the immune system. During lymphocyte development self reactive T and B cells are removed by apoptosis (Sohn *et al.*, 2003) thus preventing potential autoimmune diseases. Apoptosis also functions subsequent to the expansion phase of the lymphocyte response when the contraction phase is initiated in the expanded population.

Apoptosis is centrally involved in the action of the immune system as a mechanism of inducing cell death in a target cell population by cytotoxic lymphocyte killing of the target cell (Waterhouse and Trapani, 2002). Cytotoxic T cells do this by initiating the caspase cascade within the target cell by releasing granzyme B into the target cell. Granzyme B mimics the activity of the pro-caspase proteases (Trapani *et al.*, 1998) and thereby initiates the apoptotic process. Apoptosis allows removal of lymphocytes in a non inflammatory manner which also helps to prevent autoimmune reactions (Fadok and Chimini, 2001; Savill *et al.*, 1993).

There are some universal features of apoptosis observed in cells undergoing this process. These include the classically defining morphological changes (Kerr, 1971) (including membrane disruption and formation of apoptotic bodies) in addition to chromatin condensation and cleavage (Wyllie *et al.*, 1984), mitochondrial membrane potential disruption (Kluck *et al.*, 1997) and caspase activation (Lens *et al.*, 1998). These changes are the result of the activation of a family of cysteine dependent aspartine specific proteases that are called 'caspases'. These activated proteases proceed to cleave host cell structural and regulatory proteins along with nuclear DNA (Jendro *et al.*, 2002)(Figure 1-5).

1.4.2 Apoptosis and T cells

Classically apoptosis has been described as programmed cell death (PCD) and necrosis as accidental cell death, however recent investigation (Jaattela and Tschopp, 2003) have found that necrotic death is not as random as once thought and that necrotic and apoptotic cell death should be considered as a spectrum as opposed to two mutually exclusive alternatives (Proskuryakov *et al.*, 2003). Over recent years research has increasingly pointed to the existence of a number of distinct pathways that lead to T cell apoptosis and that these different pathways function in specific situations (Figure 1-5). Initial discoveries had highlighted the role of death receptors such as Fas in the induction of apoptosis of activated T cells when re-stimulated by antigen through the TcR (Genestier *et al.*, 1999). This gave rise to Activation Induced T Cell Death (AICD). This work had been carried out in hybridoma cells (Ashwell *et al.*, 1987) however as *in vivo* research took place it became clear that pathways other than death receptor induced cell death occurred *in vivo* (Budd, 2001; Hildeman *et al.*, 2002; Lenardo *et al.*, 1999). This second type of T cell apoptosis was called Activated T Cell Autonomous Death (ACAD) and was found to have distinct characteristics from AICD (Hildeman *et al.*, 2003). AICD is driven by death receptors and requires repetitive or high dose stimulation via the TcR whereas in ACAD death receptors do not have a role and a single antigen exposure is sufficient to cause death (Hildeman *et al.*, 2002). Furthermore AICD is caspase dependent while ACAD is caspase independent (Marsden and Strasser, 2003). Finally in functional terms AICD has little involvement in T cell removal after an immune response with ACAD being important in this role and both AICD and ACAD seem to have a role in auto-reactive T cell removal (Hildeman *et al.*, 2002).

Understanding of the signalling events involved in the apoptosis process has been increasing and one potentially relevant development has been a proposal that suggests that lipid rafts may control the apoptotic process since some of the proteins required for apoptotic signalling are localised to lipid rafts (Garcia *et al.*, 2003). Lipid rafts have already been implicated in T cell signalling for membrane receptors such as the TcR (Langlet *et al.*, 2000), CTLA-4 (Chikuma *et al.*, 2003), CD3 (Hiltbold *et al.*, 2003) and signal cascade members such as Src, Lck, Fyn (Alonso and Millan, 2001). The recent demonstration that the Death Induced Signalling Complex (DISC) is recruited to lipid rafts (Scheel-Toellner *et al.*, 2002) and that lipid rafts dramatically increase in activated T cells (Tuosto *et al.*, 2001) has heightened the interest in the link between apoptosis and rafts.

Some pathogen's interfere with the apoptotic pathway and I will now discuss some of the different pathogen's that use this strategy and the mechanism by which they accomplish this.

1.4.3 The induction of apoptosis by viruses, protozoa and helminths

Viruses primarily subvert apoptosis in order to prolong the survival of the host cell (Spriggs, 1996). Protozoa and helminths have been shown to induce apoptosis for example the microfilariae of the filarial nematode *Brugia malayi* induce increased apoptosis of dendritic cells (Semnani *et al.*, 2003). However it is upon the induction of apoptosis in cells of the immune system by bacteria that I shall focus.

1.4.4 The induction of apoptosis by Bacteria

Discoveries of bacterial infections that cause apoptosis of cells of their host has been increasing over the past decade - for a review see (Gao and Abu Kwaik, 2000; Jendro *et al.*, 2002; Weinrauch and Zychlinsky, 1999), however I will examine bacteria that specifically induce apoptosis of cells of the immune system. Bacteria induce apoptosis via a number of different mechanisms. Many bacteria induce apoptosis via release of a toxin. These toxins can be classified into families which allows examination of apoptosis by the different toxin families;

- Pore Forming Toxins
- Protein synthesis inhibitors
- Type III secretion systems
- Superantigens
- Others

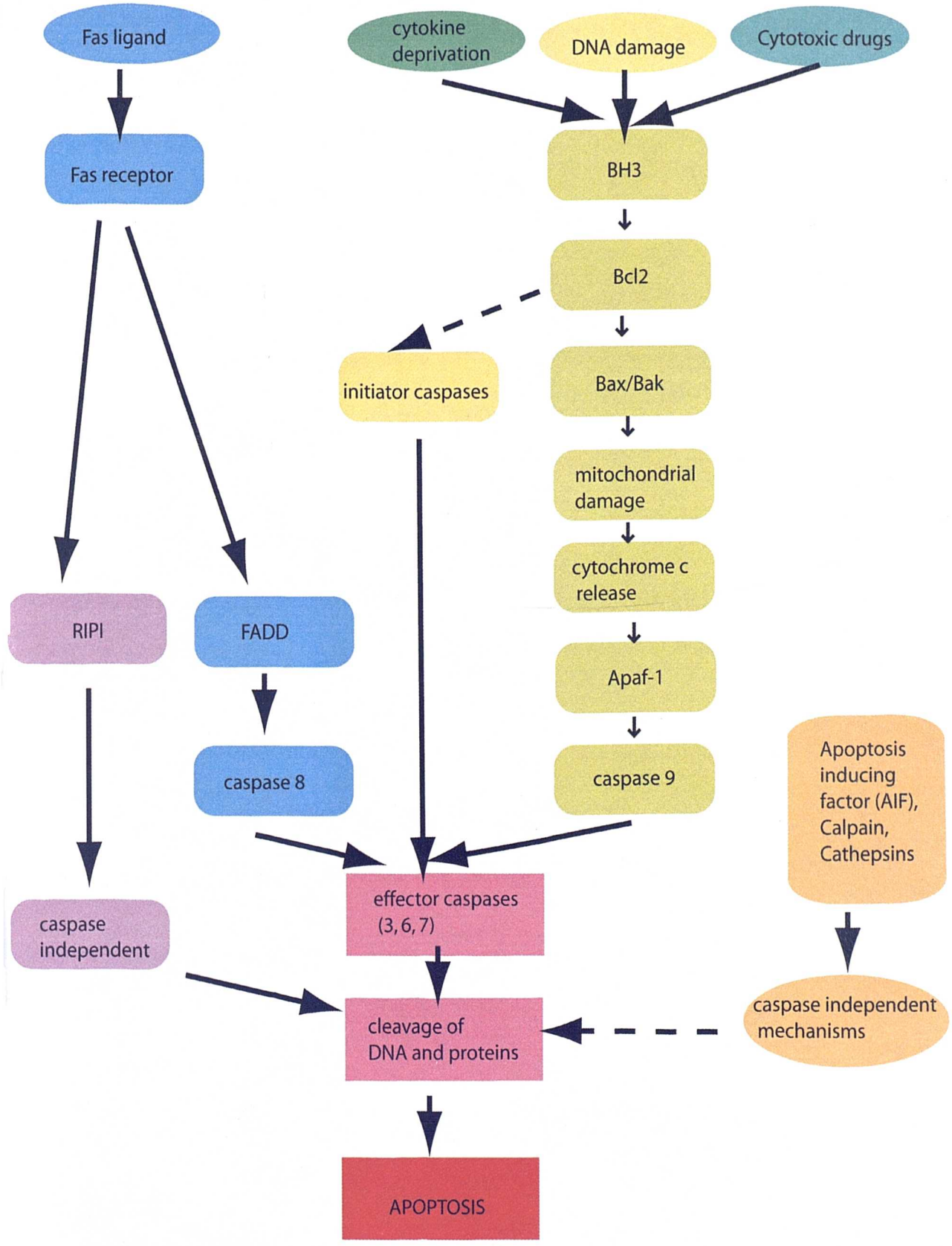


Figure 1-5

Overview of the apoptosis pathway

1.4.4.1 Pore Forming Toxins

The pore forming toxins are further classified into three sub-groups according to the type of toxin;

- α-toxin (from *S. aureas*) like toxins
- Repeats in Toxin (RTX)
- Cholesterol Dependent Cytolysins (CDCs)

1.4.4.1 a-toxin like toxins

Staphylococcus aureus secretes a-toxin which has been demonstrated to induce apoptosis in T cells (Jonas *et al.*, 1994). The exact mechanism is unclear however at low doses the toxin binds an unknown receptor and forms small ion permeable pores that allow release of monovalent cations that results in apoptosis (Hildebrand *et al.*, 1991). At higher concentrations a-toxin binds to the cell membrane inducing large pores that allow Ca^{2+} to enter and necrosis to occur.

1.4.4.2 RTX toxins

Leukotoxin from *Actinobacillus actinomycetemcomitans* induces apoptosis in leukocytes (Lally *et al.*, 1989; Taichman *et al.*, 1980), including T cells (Mangan *et al.*, 1991). Leukotoxin binds to cells via LFA-1 and then forms a pore which causes apoptosis in the host cells (Yamaguchi *et al.*, 2004). This group also contains the leukotoxin from *Pasteurella haemolytica* which causes bovine leukocytes to undergo apoptosis (Lafleur *et al.*, 2001; Leite *et al.*, 2002; Stevens and Czuprynski, 1996; Thumbikat *et al.*, 2003).

1.4.4.3 CDCs

The Cholesterol Dependent Cytolysins (CDCs) (Figure 1-6) are a group of around 20 toxins produced by Gram positive bacteria - for reviews see (Palmer, 2001; Tweten *et al.*, 2001). The toxin with which this thesis is concerned, pneumolysin, is a member of this group and so this group deserves particular attention.

Eleven of the CDC's have been sequenced and one crystal structure has been released (Rossjohn *et al.*, 1997) - another is in press(Polekhina *et al.*, 2004). Sequence comparison has demonstrated the presence of a Trp rich conserved undecapeptide region (Figure 1-11) (Tweten, 1988) that appears to be crucial for the hemolytic activity that the toxins all display.

While the role of each toxin within the context of its host pathogen interaction continues to be elucidated, recent attention has focused particularly upon the mechanism of action of these toxins (Tweten *et al.*, 2001). I will discuss this in more detail later in this chapter however a few key issues centre around the role of cholesterol in the mechanism of action and the role of each of the four domains, discovered from the crystallisation data, within the mechanism of action.

In terms of the CDC's as apoptosis inducing molecules listeriolysin produced by *Listeria monocytogenes* was discovered to induce apoptosis in DC's but not macrophages (Guzman *et al.*, 1996). The authors at the time suggested that the generation of small pores may cause apoptosis to result rather than necrosis.

1.4.4.4 A-B toxins

Corynebacterium diphtheriae, *Pseudomonas aeruginosa* and *Shigella dysenteriae* all produce a toxin with the classical structure of an A-B toxin where the B unit binds to the cell via a receptor and enables the delivery of the active A subunit into the cytoplasm. The A subunit cleaves an adenine residue on the 28S rRNA component of eukaryotic ribosome's, resulting in inhibition of protein synthesis and thereby inducing apoptosis (Nakao and Takeda, 2000). Specifically cholera toxin (Ctx) from *Vibrio cholerae* and its closely related relative, heat-labile enterotoxin (Etx) from *Escherichia coli* have been found to induce apoptosis of CD8 T cells(Nashar *et al.*, 1996; Yankelevich *et al.*, 1996). The mechanism by which the B subunit of heat-liable enterotoxin (EtxB) accom-

plishes this has been studied and shown to involve the up-regulation of the transcription factor c-myc (Soriani *et al.*, 2001) which is involved in the control of cell proliferation and death and NF-kðB (Salmond *et al.*, 2002).

1.4.4.5 Type III Secretion systems

Salmonella typhimurium, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, and *Shigella flexneri* utilise a type III secretion system - for review see (Hueck, 1998) to induce apoptosis a variety of cell types upon infection of their host. They form small membrane pores through which they inject signalling molecules into the host cell. Upon invasion, *Shigella flexneri* are phagocytosed by macrophages but escape into the cytoplasm where they induce apoptosis of the host cell (Zychlinsky *et al.*, 1992; Zychlinsky *et al.*, 1996). They accomplish this by release of IpaB which binds to and activates caspase-1 which results in apoptosis (Chen *et al.*, 1996).

1.4.4.6 Superantigens

Streptococcus pyogenes releases a number of superantigens that induce apoptosis of T cells by crosslinking the TcR which has been demonstrated to induce the apoptotic pathway (Watanabe-Ohnishi *et al.*, 1995)and in addition can cause clonal exhaustion or anergy (Xu *et al.*, 2004).

1.4.4.7 Other

Helicobacter pylori VacA serves as an example of a toxin released by a bacterium that induces apoptosis but that does not fit into one of the categories outlined above. VacA inhibits IL-2 production and prevents nuclear translocation of NFAT. It therefore inhibits the T cell response to *H. pylori* (Gebert *et al.*, 2003).

Organism	Toxin	Abbreviation
Arcanobacterium pyogenes	Pyolysin	PLO
Bacillus cereus	Cereolysin	CLY
B. anthracis	Anthrolysin O	ALO
B. thuringiensis	Thuringiolysin O	
Brevibacillus laterosporus	Laterosporolysin	
Clostridium bifermentans	Bifermentolysin	
C. botulinum	Botulinolysin	
C. chauvoei	Chauveolysin	
C. histolyticum	Histolyticolysin	
C. novyi type A	Oedematolysin	
C. perfringens	Perfringolysin O	PFO
C. septicum	Septicolysin O	
C. tetani	Tetanolysin	
Listeria ivanovi	Ivanolysin O	ILO
L. monocytogenes	Listeriolysin O	LLO
L. seeligeri	Seeligerilysin O	LSO
Paenibacillus alvei	Alveolysin	ALY
Stretococcus canis	Streptolysin O	SLO
S. equisimillis	Streptolysin O	SLO
S. intermedius	Intermedilysin	ILY
S. pneumoniae	Pneumolysin	PLY
S. pyogenes	Streptolysin O	SLO
S. suis	Suilysin	SLY

Figure 1-6

Organisms that produce a Cholesterol Dependent Cytolysin (CDC)

Twenty three organisms have been discovered to produce a CDC. Not all have an abbreviation; a reflection of the lack of investigation into some of the toxins.

The preceding section has demonstrated the variety of mechanisms that bacteria use to induce apoptosis and some of the molecules involved in this process. I have also identified different immunomodulatory molecules and their function throughout this chapter and will now proceed to discussing the potential therapeutic application context for molecules that modulate the CD4 T cell response.

1.5 Therapeutic Applications

In the context of infection, the immunomodulatory properties of pathogen's are likely to be advantageous to the pathogen and disadvantageous to the host, however it has been proposed that in understanding the mechanism of action of isolated immunomodulatory components, exploitation of these properties in a beneficial manner could occur. In particular we are interested in how immunomodulatory molecules could be applied in the area of inappropriate immune responses involving CD4+ T cells.

The mouse model of rheumatoid arthritis is collagen induced arthritis (CIA). Although the role of the CD4 T cells that infiltrate the joint synovium is unclear they are suspected to have some role in pathogenesis (Firestein, 2003). While merely killing T cells is not enough to prevent pathology (Firestein, 2004), in conjunction with other immunomodulators this may be an effective therapy (Moreland *et al.*, 1997). The B subunit of enterotoxin (EtxB) of *E. coli* has been used to effectively treat this disease in mice (Williams *et al.*, 1997) by shifting the CD4 T cell response from T_H1 to T_H2. CIA has also been effectively treated using the filarial nematode extract ES-62 (Harnett *et al.*, 2004; McInnes *et al.*, 2003) which appears to act in a similar way.

Current therapy for Crohn's disease is based upon treatment with steroids (Van Deventer, 2000) and use of compounds that prevent T cell activation (Takazoe *et al.*, 2002), however recent tests using an antibody that binds the TNF-a receptor and causes CD4 apoptosis has been identified as being successful (ten Hove *et al.*, 2002; Van Deventer, 2000) as has aziathrioprine which has been shown to induce apoptosis of CD4 T cells (Tiede *et al.*, 2003).

Similarly, the use of an anti-CD4 antibody was proposed to potentially have a similar effect in multiple sclerosis (MS) however surprisingly, studies showed that inhibition of TNF-a by a blocking antibody exacerbates MS (Group, 1999). Furthermore, global depletion of CD4 T cells failed (van Oosten *et al.*, 1997), and most antigen therapies that specifically targeted the CD4 T myelin response were inefficient or even worsened the disease. These findings have led to a reappraisal of the role of CD4 T cells in the pathology of MS (Hemmer *et al.*, 2002; Lassmann and Ransohoff, 2004).

The mechanism of pathology in Guillian-Barrè syndrome remains to be elucidated however CD4 T cells appear to be important for the production of pathology (Zhu *et al.*, 2002) (Hughes *et al.*, 1999). It is therefore possible that an anti-CD4+ effect may reduce the development or continuation of this pathology.

The chronic inflammatory disease, allergic asthma, is characterized by the presence of T_H2 cells and eosinophils in sputum and bronchoalveolar lavage. Recently IL-12 has been shown to inhibit allergic asthma by inducing apoptosis of T_H2 cells (Kodama *et al.*, 2003). Therefore induction of CD4 T cells apoptosis is, in this context, of therapeutic benefit to the host.

While it is the case that all of the components that take part in an immune response are susceptible to modulation, some examples of the potential benefit of specifically targeting CD4 T cells has been demonstrated here.

So far I have been concerned with modulation of the immune system by the pathogen and how this may be utilised in a beneficial manner. Since the topic of this thesis concerns these two themes applied to the bacterium *Streptococcus pneumoniae* I shall discuss this organism in more detail.

1.6 The pneumococcus

1.6.1 Introduction to *Streptococcus pneumoniae*

The pneumococcus is a common commensal of the upper respiratory tract of humans and usually only becomes pathogenic when the host becomes immunocompromised, a new more virulent strain colonises the host, or nasopharyngeal inflammation occurs (Bogaert *et al.*, 2004; Mizrachi-Nebenzahl *et al.*, 2003). Reported rates of carriage of the pneumococcus in children is above 50% yet in adults is only 4% (Regev-Yochay *et al.*, 2004). Non-invasive disease is represented by otitis media, sinusitis and bronchitis while invasive disease is classified as pneumonia, bacteraemia and meningitis and is responsible for 1 million deaths per annum in children under 5 years of age (Parsons and Dockrell, 2002). Incidence of invasive disease in Scotland has been reported to be 10 per 100000 (Kyaw *et al.*, 2002).

1.6.2 The Immune Response to *Streptococcus pneumoniae*

Colonisation is the first and necessary step in the development of invasive disease (Bogaert *et al.*, 2004). It remains unclear what factors mitigate the transition from commensal to pathogen for *S. pneumoniae* however a number of studies have attempted to dissect some of the possible factors.

While the strain of pneumococcus is known to influence to some degree the likelihood of invasive disease (Kadioglu *et al.*, 2002; Sandgren *et al.*, 2004), other studies have suggested that the genetic background of the host may be more influential. Studies have found that different mouse strains recruited neutrophils to differing degrees and suggested that this may be the cellular basis underlying the observed differences between host genetic backgrounds (Gingles *et al.*, 2001). Further study (Kerr *et al.*, 2002) revealed that a susceptible mouse strain had a reduced inflammatory response within the airways as judged by quantity and timing of TNF-a, IL-1b and IL-10 produc-

tion compared to a resistant strain. In addition a recently published study suggested that TLR-4 may be important in determining the level of colonisation and hence subsequent invasive disease (Malley *et al.*, 2003). However focus on this area has again switched to the strain of pneumococcus since the publication of a study that agreed that the immune response of the host was influential but suggested it is the strain of pneumococcus that determines this the degree of this response (Mizrachi-Nebenzahl *et al.*, 2003).

1.6.2.1 Antibody and Complement

Upon progressing from colonisation to invasive disease the pneumococcus faces the host's immune response. In the unimmunised host natural IgM provides the first line of defence to the pneumococcus (Boes *et al.*, 1998; Briles *et al.*, 1981). This IgM is naturally forming in pathogen free mice and is poly-reactive to common pathogenic structures (Ochsenbein *et al.*, 1999). The IgM referred to here is made in mice by B-1 cells (Boes *et al.*, 1998) which are equivalent to IgM memory B cells in humans (Kruetzmann *et al.*, 2003). IgM memory B cells are dependent on the spleen for existence and their delayed development until around 13 months of age has been shown to be responsible for the increased susceptibility to bacterial infections observed in neonates (Kruetzmann *et al.*, 2003).

C-Reactive Protein (CRP) is another factor important for defence against the pneumococcus (Briles *et al.*, 1989). CRP opsonises pneumococci to enhance phagocytosis and thereby aids bacterial removal. Both natural IgM and CRP require complement in order for their activity to protect against the pneumococcus (Mold *et al.*, 2002). Complement has been demonstrated to have a key role in pneumococcal immunity (Winkelstein, 1981). In keeping with the paradigm that the alternative pathway is activated directly by bacteria it was demonstrated that in the non-immune host the alternative pathway played a crucial role in defence against the pneumococcus (Hosea *et al.*, 1980). However as it was realised that the classical pathway activating factor C1q could bind to bacteria directly or to immune complexes formed as a result of natural IgM activity (Boes *et al.*, 1998) or to CRP bound to the pneumococcus (Mold *et al.*, 2002) the question of which complement pathway was the most important was examined. A recent study clarified the issue using a variety of knock-out mice that enabled examination of the role of the classical, alternative and mannose binding complement pathways on the innate resistance of unimmunised mice to pneumococcal infection

(Brown *et al.*, 2002). While the MBL pathway was shown to be of minimal importance despite the ability of MBL to bind pneumococcal surface polysaccharides (Zamze *et al.*, 2002), it was surprisingly reported that both the classical and alternative pathways are important. Given that the classical pathway is usually associated with the adaptive immune response as a result of the dogma that the classical pathway is activated by antibody, it seemed more likely that the alternative pathway would be prominent in unimmunised mice. However the classical pathway was shown to be of primary importance potentially through the mechanisms mentioned above involving natural IgM, CRP and also the direct binding of C1q to bacteria. Another study examined the effect of complement deficiencies on frequency of infection with different pathogen's and found that individuals with a deficiency late in the complement pathway involving C5-9 were not more susceptible to pneumococcal meningitis (Figueroa and Densen, 1991) which suggests that other complement functions other than membrane lysis are important in defence against the pneumococcus.

While the crucial role of complement in the innate immune response to the pneumococcus has been investigated and the details have begun to be worked out, the role of complement in the induction of the adaptive immune response remained unclear until a recent investigation (Haas *et al.*, 2002) using complement receptor deficient mice (CD21 and CD35; present on B cells). It was demonstrated that these mice were not any more susceptible to challenge infection with the pneumococcus than wild type mice however the mice did not develop protective antibody responses upon immunisation with the pneumococcus. The authors therefore proposed that these complement receptors on B cells play an important role in linking the innate and adaptive immune response to the pneumococcus.

1.6.2.2 Phagocyte's

Receptors for complement and Fc are present on the surface of macrophages and neutrophils and are involved in the phagocytosis of pneumococci(Ali *et al.*, 2003; Saeland *et al.*, 2003). This is reflected in the influx of neutrophils and macrophages into the lungs upon pneumococcal infection (Kadioglu *et al.*, 2000). Clearance of the pneumococci from the lungs is dependent on the phagocytosis of the bacteria (Gillespie, 1989; Knapp *et al.*, 2003). The effectors of this process are resident macrophages (Jonsson *et al.*, 1985) which act immediately, however after a few hours the influx of more effective PMNL into the alveolar space occurs (Lawrence *et al.*, 1996) and after 24 to 48

hours an influx of monocytes occurs (Goto *et al.*, 2004) which differentiate into macrophages. Subsequent to phagocytosis macrophage undergo apoptosis and this has been shown to enhance host defence to the pneumococcus by limiting the pro-inflammatory response (Ali *et al.*, 2003; DeLeo, 2004; Dockrell *et al.*, 2001) although the binding of inhibitory Fc receptor FcgRIIb by the pneumococcus has also been shown to be responsible for limiting the pro-inflammatory response (Clatworthy and Smith, 2004).

1.6.2.3 Cytokines

The myriad of cytokine interactions during pneumococcal infection are far from being elucidated however some insight has begun. The release of pro-inflammatory cytokines has been observed in response to the pneumococcus. For example the level of TNF-a in the blood was recorded after pneumococcal infection and found that it increased and as a result increased the number of neutrophils in the blood which stemmed pneumococcal growth in the blood (Takashima *et al.*, 1997). The role of TNF-a in pneumococcal infection was demonstrated by comparison of the susceptibility of BALB/c and CBA/Ca mice which revealed that the more susceptible CBA/Ca mice have a reduced ability to induced a rapid TNF-a response in the airways of the host (Kerr *et al.*, 2002). TNF-a has also been demonstrated to increase the permeability of the blood brain barrier and consequently aid the development of pneumococcal meningitis (Tsao *et al.*, 2002).

The effect of IL-1 was examined and found that mice defective in IL-1 activity had increased lung bacterial counts and a decreased ability to form inflammatory infiltrates (Rijneveld *et al.*, 2001). Furthermore they demonstrated that TNFa is more important than IL-1 in pneumococcal infection while others demonstrated that TNFa is able to compensate for IL-1 during pneumococcal pneumonia (Rijneveld *et al.*, 2001) but not during meningitis where IL-1 is observed to be essential (Zwijnenburg *et al.*, 2003b).

IL-6 levels increased in response to pneumococcal infection (van der Poll *et al.*, 1997) and in the absence of IL-6, both IL-1 and TNF-a increased. IL-6 may therefore regulate these pro-inflammatory cytokines or IL-6 may function in a similar way so that upon its removal a compensatory increase occurs. In another study while an increase in IL-6, IL-1a and TNFa occurred in the lung

early in infection this was accompanied by an IL-6 increase only in the blood. Later in infection a decrease in TNFa and IL-1a occurred and then even later an increase of TNFa and IL-6 in the blood (Bergeron *et al.*, 1998).

In 1996 it was demonstrated that upon pneumococcal infection of the lungs the level of IL-10 increased (van der Poll *et al.*, 1996b).The role of IL-10 during pneumococcal meningitis has been addressed and while the absence of IL-10 resulted in higher cytokine levels this was not observed to alter the outcome of infection in any way (Zwijnenburg *et al.*, 2003c).

The role of IL-18 and IL-12 in pneumococcal infection was examined and it was shown that IL-18 deficient mice had increased susceptibility to lung infection (Lauw *et al.*, 2002) while IL-12 deficient mice had no increased susceptibility. However in pneumococcal meningitis IL-18 has been observed to have a detrimental effect on the host since IL-18 deficient mice have prolonged survival in this context (Zwijnenburg *et al.*, 2003a). Examination of the role of IFN-g in pneumococcal infection led to the discovery that there was no difference in mortality between a IFN-g deficient strain and the wild type equivalent (Rijneveld *et al.*, 2002). However closer examination revealed that IFN-g deficient mice had lower numbers of pneumococci in their lungs and had decreased neutrophil influx. This suggests that IFN-g may be slightly detrimental to the host. This contrasts with other results that have shown a protective role of IFN-g to the host in pneumococcal infection (Rubins and Pomeroy, 1997).

The preceding paragraphs have demonstrated the importance of pro-inflammatory cytokines in the immune response to the pneumococcus. While it has been shown that pro-inflammatory cytokines are released from non-lymphoid cells (Khan *et al.*, 2002), lymphocytes are proposed to be important in releasing some of the pro-inflammatory cytokines observed during pneumococcal infection (Kadioglu *et al.*, 2004). Interestingly pneumococcal infection has been observed to be associated with a simultaneous decrease in the number of T_H1 cells and this decrease is not observed to occur in the T_H2 cell compartment (Kemp *et al.*, 2002). Related to this the pneumococcal toxin pneumolysin has been shown to stimulate the release of IL-8 from neutrophils (Cockeran *et al.*, 2002b).

While the demonstration of the importance of pro-inflammatory cytokines in the innate immune response to *S. pneumoniae* has been investigated, the role of cytokines in the adaptive immune

response has been largely ignored. However it has been demonstrated that TNF-a is required for the induction of the primary IgG response to pneumococcal proteins. Furthermore mice deficient in any of the T_H1 cytokines IL-12, IL-6 or IFN-g showed a reduced ability to form IgG to pneumococcal proteins. In contrast mice deficient in IL-4 or IL-10 had increased antibody titre to the same constituents. The effect of these cytokines upon the antibody response to phosphorylcholine (PC) was also examined and although the same effects were noticed as for the protein response, the effects were more modest (Khan *et al.*, 2002).

1.6.2.4 Pattern Recognition Receptors (PRR)

Further clarity on the role of pro and anti – inflammatory cytokines was given with the publication of a paper which demonstrated a novel way of viewing the role of anti-inflammatory cytokines such as IL-10 in the context of pneumococcal infection (Colino and Snapper, 2003b). Previously it had been observed that the pneumococcus induces release of anti-inflammatory cytokines which had been interpreted as being negative to the host (van der Poll *et al.*, 1996a). Furthermore Bone Marrow derived Dendritic Cells (BMDC) had been shown to undergo apoptosis (Colino and Snapper, 2003a) and become activated through TLR-4 (Malley *et al.*, 2003) in response to the pneumococcus. Colino *et al.* combined these two concepts by demonstrating that the release of IL-10 by DC in response to the pneumococcus provides an opposing signal to that given by TLR stimulation which induces apoptosis. In this way the release of IL-10 by *Streptococcus pneumoniae* is a positive event for the host since it prolongs the existence of DC which allows the development of an adaptive immune response by enabling presentation of antigen to lymphocytes.

The elucidation of this link between the cytokine response to the pneumococcus and the PRR response to the pneumococcus depended on prior investigations into the interactions of PRR's and the pneumococcus. The first publication investigating the effect of *Streptococcus pneumoniae* on TLR's was in 1999 by Yoshimura *et al.* who found that fibroblasts expressing TLR-2 became activated when exposed to the pneumococcus (Yoshimura *et al.*, 1999). Following on from this report TLR-2 was demonstrated to play a role in pneumococcal meningitis when it was found that TLR-2 deficient mice were more susceptible to pneumococcal meningitis than wild type counterparts (Echchannaoui *et al.*, 2002). Interestingly bacterial numbers in the blood were not higher in the TLR-2 deficient mice but were higher in the brain and coincided with higher levels of TNF-a in the cerebro-spinal fluid (CSF). The role of TLR-2 was examined during lung infection when it was

discovered that TLR-2 is crucial for the induction of TNF-a from alveolar macrophages by the pneumococcus (Knapp *et al.*, 2004). This led to the expectation that TLR-2 deficient mice would be more susceptible to pneumococcal infection. However survival of mice was not affected by the presence or absence of TLR-2 when infected with *S. pneumoniae*. The authors went on to demonstrate that although early inflammatory responses are decreased by the absence of TLR-2 this does not alter the outcome since other factors appear to compensate.

The accepted paradigm within our understanding of Toll receptors was that TLR-4 was activated by the Gram-negative bacterial product LPS while TLR-2 was activated by Gram-positive cell wall products. This understanding was revised with the publication of a paper which showed that the toxin pneumolysin produced by *S. pneumoniae* was capable of signalling through TLR-4 (Malley *et al.*, 2003). Interestingly the paper also demonstrated that a complement activating and cytolytic deficient mutant of the toxin was also able to signal through TLR-4. The authors proceeded to examine the *in vivo* effect of TLR-4 deficiency. They demonstrated that mice deficient in TLR-4 had increased susceptibility to pneumococcal infection and were more likely to have invasive disease. Since TLR-4 deficient mice have a higher level of nasopharyngeal colonisation than normal mice they proposed this discrepancy as a possible reason for the difference in invasive disease. Furthermore when comparing the infectivity of a pneumolysin deficient strain of *S. pneumoniae* with its wild type counterpart they discovered that the pneumolysin deficient strain had a comparable level of mortality to a pneumolysin sufficient strain in TLR-4 deficient mice. This finding suggests that in the absence of pneumolysin the beneficial effects to the host of the inflammation caused by TLR-4 activation by pneumolysin are not achieved. This correlates with others who have found that people who respond to pneumolysin and produce antibody to pneumolysin subsequently have more invasive disease episodes that non-responders (Cockeran *et al.*, 2002a). Subsequently a study was published which examined the effect of TLR-4 in lung infection with the pneumococcus and suggested that TLR-4 had only a minimal role in lung infection (Branger *et al.*, 2004).

1.6.2.5 T and B cells

In 1975 Winkelstein demonstrated that T cell deficient mice had the same mean lethal dose as wild type mice. From this it was concluded that T cells play an insignificant role in defence against the pneumococcus and therefore the adaptive immune response was subsequently largely ignored

(Winkelstein and Swift, 1975). While the innate immune response is of primary importance in defence against the pneumococcus, the role of the adaptive arm of the immune system has recently begun to be elucidated.

Three areas of interest have been developing as the result of investigation into the role of the adaptive immune response to the pneumococcus. The first area actually proposes a role for T cells in the innate immune response to the pneumococcus (Kadioglu and Andrew, 2004). Studies on the cellular influx into the lung of mice infected with *Streptococcus pneumoniae* showed significant lymphocyte accumulation at sites within the lung and that this coincided with the remission phase of infection (Kadioglu *et al.*, 2000). The authors suggested that the lymphocytes may be important for cytokine secretion. In a later study, by use of a mutant pneumolysin, the same group (Jounblat *et al.*, 2003) demonstrated that the complement activating properties of pneumolysin were responsible for the influx of T cells into the lung observed during pneumococcal infection. To directly assess the effect of CD4 T cells upon pneumococcal infection MHC II -/- mice were used and were infected with pneumococci since it was reported that the MHC II -/- mice were deficient in CD4 T cells but not CD8 T cells (Kadioglu *et al.*, 2004). The MHC II -/- mice had higher numbers of pneumococci in the blood and lungs than normal mice. This work extended the findings of Sestini *et al.* (Sestini *et al.*, 1987, 1988) who had earlier demonstrated a role for CD4+ T cells in the antibacterial activity against the pneumococcus in the lungs.

Studies from patients with AIDS have also demonstrated that T cells are important in pneumococcal infection. For example Janoff *et al.* (Janoff *et al.*, 1993) studied the prevalence of pneumococcal colonisation among HIV+ individuals and found no difference in colonisation levels, yet an increased frequency of invasive disease is known to exist in HIV+ individuals (Schneider and Rosen, 1999). Therefore it was suggested that the higher bacteraemia among HIV+ individuals was associated with low CD4+ T cell count rather than increased exposure to the pneumococcus as a result of increased carriage. Another study found that while HIV+ individuals may not be more frequent carriers of *S. pneumoniae*, they were more likely to carry the pneumococcus for a longer period of time (Rodriguez-Barradas *et al.*, 1997).

The third area of research comprises the cellular requirements of the adaptive immune response to the pneumococcus. The protein antigens of the pneumococcus are considered to be TD antigens

and have been demonstrated to require CD4 T cell help to enable antibody production. This help requires TcR-MHC recognition and costimulation by CD4 T cells in the form of CD40-CD40L and CD28-B71/2 (Wu *et al.*, 1999). Vaccination has demonstrated that formation of antibody to the pneumococcus polysaccharide is possible, however the role of the T cell in producing this antibody had not been elucidated until recently since polysaccharide was considered to be a TI-2 antigen. Wu *et al.* postulated (Wu *et al.*, 1999) that while purified polysaccharide does not require T cell help for antibody production (Mond *et al.*, 1995a) it may be the case that in the context of the whole pneumococcus polysaccharide, is encountered in a protein-polysaccharide conjugate fashion that means T cell help is involved in the antibody production from polysaccharide. They used an unencapsulated pneumococcal strain to examine the antibody response to the phosphorylcholine (PC) determinant of the polysaccharide cell wall component techinoic acid. By using knockout mice they were able to demonstrate the T cell independence of the IgM anti-PC response and the dependence of the IgG anti-PC response on alpha/beta CD4 and CD8 T cells. Their studies went on to demonstrate that a conjugate effect was not taking place and that CD40 costimulation is required for both the anti-PC IgM and IgG response but that only for the IgG response does the CD40 signal come from CD4 and CD8 T cells. Finally they demonstrated that there exists a B7 requirement for anti-PC antibody response but that this is different to the B7 requirement of the pneumococcal protein PspA.

These studies were followed up by examining the role of CD40L on the antibody response to encapsulated bacteria (Hwang *et al.*, 2000). It was found that CD40L was required for IgG responses to capsular polysaccharide (caps-PS) in the context of the whole bacterium but not when the caps-PS was administered in purified form. Furthermore their studies demonstrated that CD40L was not required for antibody responses to cell wall polysaccharide. Others investigated the necessity of CD40 for the antibody response to different caps-PS (Jeurissen *et al.*, 2002). They found that CD40 was required to differing degrees depending on the serotype of the caps-PS.

Wu *et al.* (Wu *et al.*, 2002) further addressed the question of costimulatory requirements of the anti-PC response and found that for both anti-PC and anti-PspA antibody B7-2 but not B7-1 was required for IgG antibody production. In addition they showed that for anti-PspA antibody CD28 costimulation is required on days 0-4 after primary immunisation, whereas anti-PC antibody CD28 costimulation is only required on day 0 after primary immunisation. They also investigated the mechanism underlying T cell help for the immune response to PC in the context of the whole

pneumococcus. Given that it was already known that polysaccharide does not associate with the MHC (Mond *et al.*, 1995a) the question of TcR recognition involving polysaccharide could be precluded, however the possibility that the T cell help mechanism in the anti-PC response depends on a protein-polysaccharide conjugate had not been fully addressed. They demonstrated, by using TcR a- mice that have a functional TcR but are unable to recognise antigen, that the antibody response to PC does not require simultaneous antigen recognition. Furthermore they demonstrated that APC processing was also not required for the anti-PC antibody response. By using DC pulsed with *Streptococcus pneumoniae in vitro* and then injected *in vivo* it was able to be shown that the dominant antibody isotype produced to PC is IgM and IgG3 (Colino *et al.*, 2002). By utilising CD40-/- DC the group went on to show that the anti-PC response is defective using these DC only in terms of IgG1 and that IgG3, IgG2b and IgM were normal.

In 2004 Khan *et al.* published a seminal paper in this area. In it they clarified many issues that have surrounded the investigations outlined above. They demonstrated that the results published for the anti-PC responses were representative of findings for capsular polysaccharide in terms of the kinetics of the antibody response, the necessity of the CD4 T cell and the requirement for CD40 costimulation. However their comparisons of anti-PC to anti-PPS responses revealed one important anomaly. Previously they had demonstrated that anti-PC IgG responses were TcR independent, however in this study while the anti-PC TcR independence was reproduced they demonstrated that the anti-PPS response was TcR dependent although this was not due to its association with a bacterial protein but more likely is due to the nature of the antigen or the type of B cells that respond to this antigen (Khan *et al.*, 2004).

In summary, in the context of the whole bacteria, pneumococcal phosphorylcholine and capsular polysaccharide has been shown to require CD4 T cell help for antibody production. This is not the result of conjugate presentation although this CD4 T cell help is distinct from that given to peptide antigens since it is TcR independent (PC only), is required only for a short period of time and is CD40 dependent.

1.6.2.6 Colonisation, antibody, T cells and pneumolysin.

The previous section has focused upon the role of the T cell in antibody production to the pneumococcus. However to understand the importance of the CD4+ T cell during pneumococcal infection requires an understanding of the importance of antibody to the pneumococcus.

Antibody is not important in the defence against primary challenge in the naive host since it has been demonstrated that the adaptive immune response has no role in host defence against this type of infection (Haas *et al.*, 2002; Winkelstein and Swift, 1975) and therefore antibody (other than natural IgM) has no role in this type of pneumococcal contact.

However most pneumococcal contacts are not of a challenge nature but rather the host becomes colonised first and then invasive disease occurs (Bogaert *et al.*, 2004). Therefore the effect of antibody in the context of colonisation is more interesting.

The effect of a normal colonisation event upon the development of antibody has been studied in humans (Rapola *et al.*, 2000; Soininen *et al.*, 2001). It was found that antibody formation to both protein and polysaccharide antigens from the pneumococcus could develop as the result of carriage although the development of antibody to polysaccharide was serotype dependent during carriage. Potentially this means that the majority of pneumococcal contacts occur in host's that have antibody to the pneumococcus.

The effect of this antibody requires careful consideration. Antibody formed as a result of colonisation has been shown to prevent re-colonisation when antibody to the PspA protein is present but antibody to the pneumococcus capsule was not protective (McCool *et al.*, 2002). Therefore the normal course of infection would be to be colonised once but be unable to be recolonised.

Interestingly, the authors of the study above (McCool *et al.*, 2002) made the discovery that despite carriage resulting in a rise in the titre of anti-PspA, this rise did not affect the current carriage but only affected subsequent colonisation. They also found a serotype specificity in their studies whereby one serotype did not cause a rise in anti-PspA antibody titre.

Other researchers have tried to elucidate the role of pneumolysin during colonisation. One group demonstrated that an increase in antibody to pneumolysin does not occur upon experimental human carriage (McCool *et al.*, 2003). Furthermore production of the toxin pneumolysin was not

found to differ between strains that colonise and those that cause invasive disease, suggesting that production levels of the toxin are not responsible for the difference in invasive capacity (Spreer *et al.*, 2004). Two groups have examined the colonising ability of pneumolysin deficient strain of the pneumococcus and found conflicting results. One group found that a pneumolysin deficient strain was no less successful in colonising the host than a pneumolysin sufficient strain (Rubins *et al.*, 1998) while another group found that a pneumolysin deficient strain was cleared rapidly from the nasopharynx (Kadioglu *et al.*, 2002).

1.6.3 Virulence Factors of *Streptococcus pneumoniae*

The complex interaction of the pneumococcus with the host immune system is further observed in the interaction of specific pneumococcal virulence factors with parts of the immune system. Virulence factors are those molecules expressed by the pneumococcus that allow *in vivo* growth but are not required for *in vitro* growth (Hava *et al.*, 2003). Interestingly in relation to the discussion in the above section a recent study demonstrated that many factors required for invasive disease were also required for colonisation (Hava and Camilli, 2002).

1.6.3.1 Cell Wall

The polysaccharide capsule (Figure 1-7) that forms the outermost layer of the pneumococcus is crucial for virulence as evidenced by the avirulence of unencapsulated pneumococci. The anti-phagocytic nature of the capsule has been demonstrated (Jonsson *et al.*, 1985) and the formation of antibodies to the capsule gave rise to the serotype classification system for the pneumococci. Currently around 90 capsular types have been identified.

The cell wall of the pneumococcus is composed of peptidoglycan (Figure 1-7). Attached to this are polysaccharide chains of teichoic acid and embedded in the peptidoglycan is lipoteichoic acid which is attached to the plasma membrane. The cell wall mediates attachment to cells of the lung (Geelen *et al.*, 1993) while the phosphorylcholine binds other protein motifs to the surface (Gosink

et al., 2000). The cell wall is partly responsible for initiating the inflammatory response (Knapp *et al.*, 2004).

1.6.3.2 Pneumococcal Enzymes

S. pneumoniae has been known to produce a neuraminidase for some time (Kelly *et al.*, 1967). Purification of the neuraminidase has been carried out (Stahl and O'Toole, 1972), and work has revealed that three distinct neuraminidase's are present in *S. pneumoniae* (Camara *et al.*, 1991; Shakhnovich *et al.*, 2002). Recently a mutant *S. pneumoniae* type has been produced by insertion-deletion that does not express the neuraminidase A gene. Upon i.p. challenge infection studies the deletion of neuraminidase A was found to have no effect on the survival time of the mice (Berry and Paton, 2000). However a different model involving intranasal instillation in chinchilla examined the persistence time in the nasopharynx of a wild type *S. pneumoniae* strain compared to that of a neuraminidase knockout strain and found that the neuraminidase knockout was more rapidly cleared from the nasopharynx (Tong *et al.*, 2000). More recently it has been shown that the neuraminidase produced by the pneumococcus deacylates the lipopolysaccharide of *Neisseria meningitidis* and *Haemophilus influenzae* thereby aiding the pneumococcus' colonisation of the upper respiratory tract (Shakhnovich *et al.*, 2002).

Streptococcus pneumoniae also produces hyaluronidase. It is proposed that hyaluronidase may be involved in the degradation of connective tissue since hyaluronic acid is a major component of this type of tissue (Hynes and Walton, 2000). In addition hyaluronidase was one of the genes identified from a signature tagged mutagenesis screen for genes important for infection (Hava and Camilli, 2002). However more recently the elucidation of a new role for hyaluronan in the DC:T interaction (Levesque and Haynes, 2001; Mummert *et al.*, 2002; Termeer *et al.*, 2003) has developed the possibility of another role for hyaluronidase as a potential immunomodulator.

1.6.3.3 Choline binding proteins

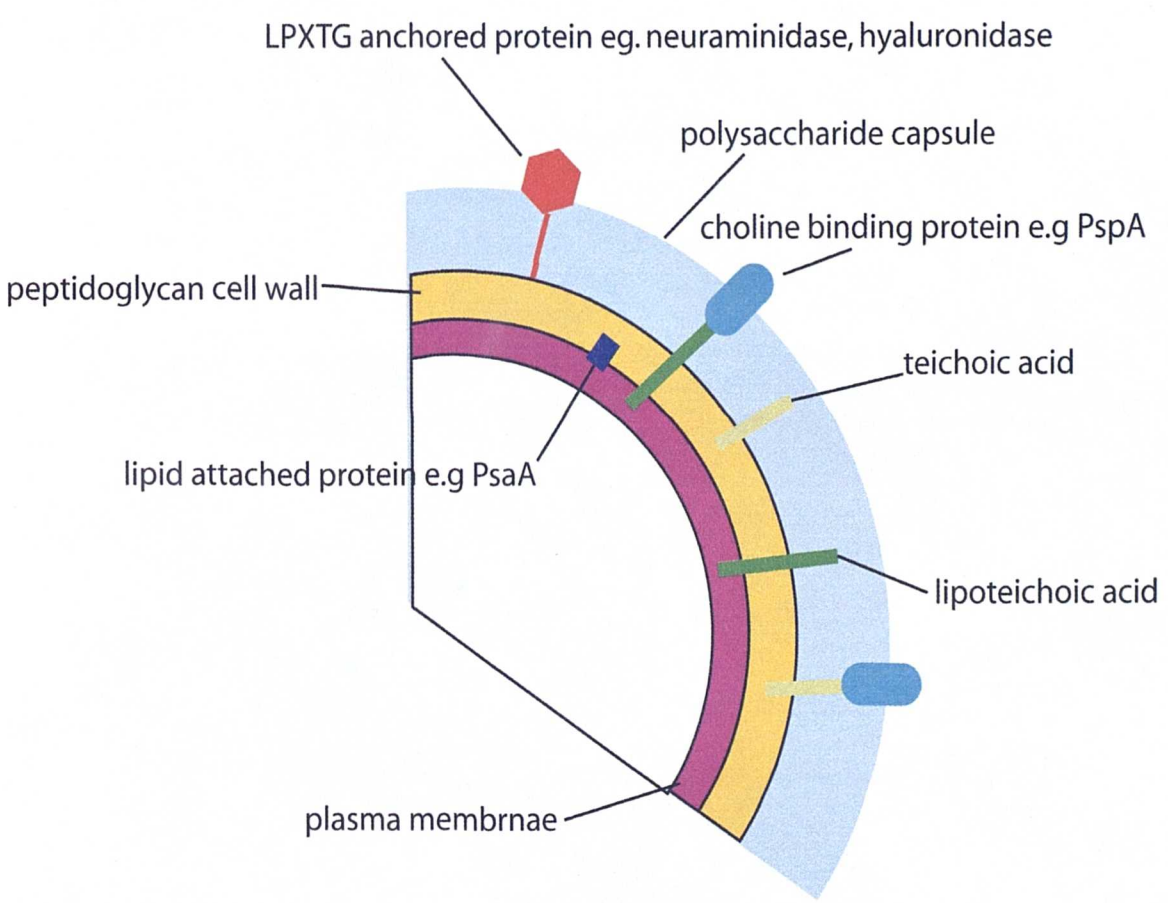


Figure 1-7

Overview of the pneumococcal cell wall

Pneumococcal surface protein A (pspA) has been studied largely as a potential vaccine candidate since it is protein found in all strains of *S. pneumoniae* (Crain *et al.*, 1990) and is sufficiently conserved between strains that protective antibodies induced to PspA are protective against all strains (McDaniel *et al.*, 1991). As such the focus on the function of PspA *in vivo* has been neglected to some degree. PsaA is a metal binding component of an ABC transporter and it has also been observed that PspA is able to inhibit complement activation (Tu *et al.*, 1999) and can act as a lactoferrin binding receptor (Hammerschmidt *et al.*, 1999).

Similar to pspA, Pneumococcal surface adhesin A (PsaA) has been studied largely as a potential vaccine candidate and has proven to be protective for both invasive disease but also carriage (Talkington *et al.*, 1996). PsaA was originally shown to be homologous to other bacterial adhesin molecules (Sampson *et al.*, 1994) and this function was later confirmed (Berry and Paton, 1996).

Choline binding protein A (CbpA) has been proposed to be involved in cell binding (Rosenow *et al.*, 1997) although knockout studies have not revealed a crucial role in virulence (Berry and Paton, 2000) but a crucial role in sepsis has been demonstrated (Iannelli *et al.*, 2004). Furthermore CbpA under the alternative name of SpsA, has been shown to bind to secretory IgA (Hammerschmidt *et al.*, 1997). PspC (CbpA, SpsA) has also been shown to inhibit complement activation on the surface of *S. pneumoniae* by binding complement inhibitory factor H (Dave *et al.*, 2004).

1.6.3.4 Others

Production of hydrogen peroxide has been shown to have a similar effect to neuraminidase in inhibiting the colonisation of the respiratory tract by other pathogen's (Pericone *et al.*, 2000) although its mechanism is distinct since hydrogen peroxide is directly toxic to the other organisms.

S. pneumoniae has been shown to produce an IgA protease (Male, 1979). This may degrade the IgA that is present on mucosal surfaces and thereby aid infection by enhancing pneumococcal attachment to the epithelium (Weiser *et al.*, 2003).

1.7 Pneumolysin

1.7.1 Introduction to Pneumolysin

Although pneumolysin is a virulence factor produced by the pneumococcus it will be considered in a separate section. Pneumolysin was identified due to its hemolytic properties (Sutliff and Zoffuto, 1968). Subsequent studies have revealed that pneumolysin is found in all virulent strains of *S. pneumoniae* and that pneumolysin is a member of a group of toxins that have sequence, structural and functional homology (Billington *et al.*, 2000). This group has had a variety of names throughout its history and debate continues over the most appropriate however here they shall be referred to as the Cholesterol Dependent Cytolysin's (CDC's) (Figure 1-8).

1.7.2 Cholesterol Dependent Cytolysins

Prior to discussing pneumolysin itself, I will give an overview of some relevant findings from research carried out using the other CDCs. While many of the CDC's have been found to be essential for pathogenesis of their host bacterium (Berry *et al.*, 1989; Jost *et al.*, 1999; Kimoto *et al.*, 2003; Limbago *et al.*, 2000; Mengaud *et al.*, 1989; Nagamune *et al.*, 2000), and many have been found to have effects on host immune cells, (Bremm *et al.*, 1984; Ito *et al.*, 2003; Kimoto *et al.*, 2003; Kohda *et al.*, 2002; Macey *et al.*, 2001; O'Brien and Melville, 2004; Stassen *et al.*, 2003) some of the toxins have been studied in specific detail. One of the most studied CDC's is perfringolysin (PFO) produced by *Clostridium perfringens.* Much of the research using this toxin has focused on the structural aspects of the toxin and the mechanism of pore formation which we will discuss later in this chapter however an area of interest to this thesis concerns the work that has investigated the interaction of perfringolysin and cholesterol. In 1988 Ohno-Iwashita *et al.* (Ohno-Iwashita *et al.*, 1988) demonstrated that perfringolysin bound in two different ways to cell membrane cholesterol; they found that there was high affinity and low affinity regions within the membrane. In 1990 the same group showed that perfringolysin derivatives could be used as probes to label membrane cholesterol (Ohno-Iwashita *et al.*, 1990) and then demonstrated that perfringolysin

preferentially binds to areas of the membrane that are cholesterol rich (Ohno-Iwashita *et al.*, 1991). The group went onto demonstrate that the cholesterol in these regions is sequestered to caveolae (Fujimoto *et al.*, 1997) and then finally to show that perfringolysin binds preferentially to lipid raft structures in cell membranes (Waheed *et al.*, 2001).

Related to this work is some investigation into the mechanism of action of the CDC, listeriolysin (LLO) produced by *Listeria monocytogenes*. It has been shown that listeriolysin is internalised via caveolae on host cells and this enables its action on intracellular processes (Coconnier *et al.*, 2000).

Streptolysin O (SLO) produced by *Streptococcus pyogenes*, *Streptococcus equisimilis* and *Streptococcus canis* has been used as a cell permeabilising agent and much of the work on SLO reflects this. For example the generation of pores in the membrane by SLO was shown to be a reversible process when SLO is used at a low dose (Walev *et al.*, 2001; Walev *et al.*, 2002) and this process was shown to be dependent on the fusion of the endomembrane with the cell membrane (McNeil *et al.*, 2003). The functional relevance of the formation of these pores during infection with *S. pyogenes* was investigated after *S. pyogenes* was shown to manipulate keratinocyte signalling (Ruiz *et al.*, 1998). Madden *et al.* (Madden *et al.*, 2001) not only demonstrated that streptolysin O was found in the detergent insoluble fraction of the cell (i.e. the lipid rafts) but also demonstrated that another protein was introduced into the cell by streptolysin O. This appeared to represent a Gram-positive equivalent to the gram negative type III secretion mechanism. Upon further investigation the second protein delivered into the cell was found to be NAD+ glycohydrolase which induced apoptosis of the target cells (Bricker *et al.*, 2002) and streptolysin O was demonstrated to play an active role in this process since substitution with perfringolysin abolished the translocation process (Meehl and Caparon, 2004).

1.7.2.1 Structure and Pore Formation of CDC's

Pneumolysin was first cloned and expressed in 1986 (Paton *et al.*, 1986) and the gene was sequenced in 1987 (Walker *et al.*, 1987). These studies revealed that pneumolysin is a 53kDa peptide. Homology studies to other members of the CDC's have shown that pneumolysin's closest relative within the family is Intermedilysin (See Figure 1-8). There is no crystal structure available for pneumolysin and so structural insights come from comparison with others CDC's for which the structure

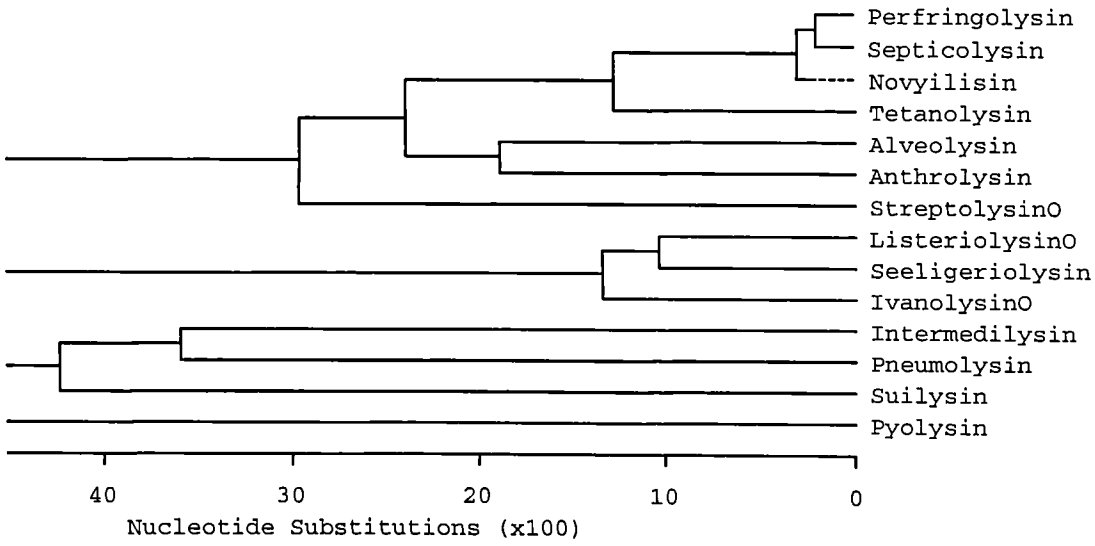


Figure 1-8

Phylogenetic tree of the CDCs

Pneumolysins closest relative can be seen from this diagram to be Intermedilysin

Diagram courtesy of Graeme Cowan, University of Glasgow

has been solved (Figure 1-9). These studies suggest that pneumolysin consists of four domains (Rossjohn *et al.*, 1998). An area of intense speculation concerns that of the mechanism of pore formation by the CDC's and how this relates to the structure (Figure 1-10).

Before specific investigations in the area of pore formation began, the mechanism of pore formation was not known, but was speculated to involve the binding of monomers to the cell surface, penetration into the membrane before lateral diffusion in the membrane led to the formation of oligomers and subsequently pores (Morgan *et al.*, 1993). Theories on the mechanism of pore formation largely were derived from analogies made with other pore forming proteins. Bhakdi *et al.* characterised the pore forming mechanism of complement (Tranum-Jensen *et al.*, 1978) and of staphylococcus alpha toxin (Fussle *et al.*, 1981) and suggested that streptolysin O used a similar membrane damaging mechanism (Bhakdi and Tranum-Jensen, 1984; Bhakdi *et al.*, 1985).

In 1996 the regions of streptolysin O that moved into the core of the cell membrane were identified (Palmer *et al.*, 1996) and were shown to be in what became known as domain 3. In 1997 the crystal structure of perfringolysin was solved which was the first CDC to be solved (Rossjohn *et al.*, 1997). The authors showed that perfringolysin is composed of four domains, each of distinct structural features. Previous experiments had demonstrated that domain four contains the cholesterol binding site but that the highly conserved undecapeptide is close to, but is not, the actual binding site for cholesterol (Vazquez-Boland *et al.*, 1989). By examining the crystal structure of perfringolysin Rossjohn *et al.* were able to propose a model of membrane insertion. They proposed that the Trp residue at point 464 functioned as a hydrophobic pocket and as such would be the cholesterol binding site. Membrane penetration was proposed to result from the ejection of the undecapeptide Trp rich loop from the pocket upon cholesterol binding to form a hydrophobic 'dagger' which would then penetrate the membrane and form the membrane spanning region. Previously it was shown that cholesterol binding results in partial insertion of this region of domain four into the membrane (Nakamura *et al.*, 1995) and so this mechanism of action was consistent with Nakamura's results but inconsistent with the results of Palmer (Palmer *et al.*, 1996) mentioned above since the region they had demonstrated to insert into the membrane lay within domain 3 of Rossjohns crystal structure of perfringolysin as opposed to domain 4. However, they further justified their model via the assertion that the conformational change that occurs during membrane insertion was not as much as would be required for domain 3 to enter the membrane.

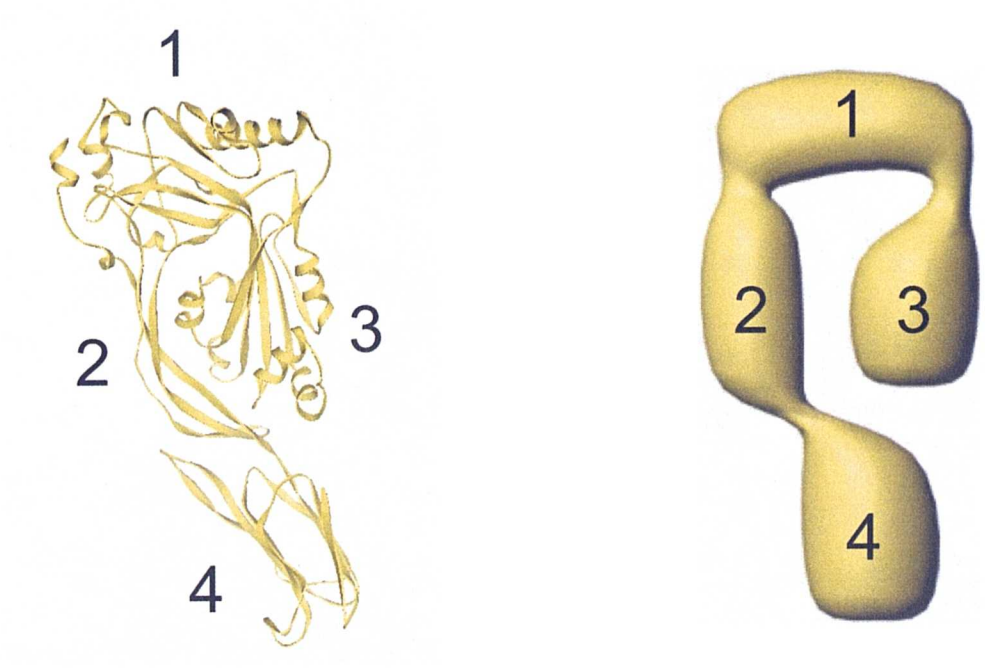


Figure 1-9

Structure of a CDC

The schematic shows a crystalisation model of a CDC and the four domains identified.

Diagram courtesy of Graeme Cowan, University of Glasgow

However, it was subsequently demonstrated that significant conformational change of domain 3 of pneumolysin does occur and that both domains 3 and 4 partially insert into the membrane(Gilbert *et al.*, 1999). The formation of two b-sheets in domain 3 was demonstrated (Shatursky *et al.*, 1999; Shepard *et al.*, 1998) and these were proposed to insert into the membrane.

Based on observations of incomplete rings of oligomers in membranes (Cowell *et al.*, 1978) Palmer *et al.* studied the formation of pores in the membranes in an effort to differentiate the order of pore formation and assess whither oligomerisation occurs prior to insertion (Palmer *et al.*, 1998). Based on their observations that incomplete rings seen as arcs could function as pores they proposed a different theory to that proposed by Rossjohn. Palmer proposed that monomers bind to the cell membrane and then insert as dimers into the membrane. Oligomerisation then occurs in the membrane to build full rings.

Despite elucidation of the parts of the molecules that function within the membrane it there remained questions over the order of the formation of these pores. The model of Rossjohn proposed the formation of a pre-pore complex prior to insertion and as such held that oligomerisation preceded insertion (Rossjohn *et al.*, 1997)while Palmer suggested that oligomerisation occurred after membrane insertion and that pores gradually enlarged over time. This was solved when it was shown that oligomerisation occurs at 4°C despite insertion not occurring at this temperature (Shepard *et al.*, 2000) which suggested oligomerisation occurs prior to insertion however they further demonstrated (Heuck *et al.*, 2003) that uniform pores form and that these are large and do not increase in size over time. The authors suggest that the role of domain 4 is in binding the cell membrane and that it remains close to the cell surface.

In summary (Figure 1-10), the current data seems to reject the 'hydrophobic dagger' hypothesis in favour of insertion of domains 3 and 4 into the membrane. Current data also suggests that oligomerisation occurs prior to membrane insertion.

1.7.2.2 Role of Cholesterol in CDC cell binding

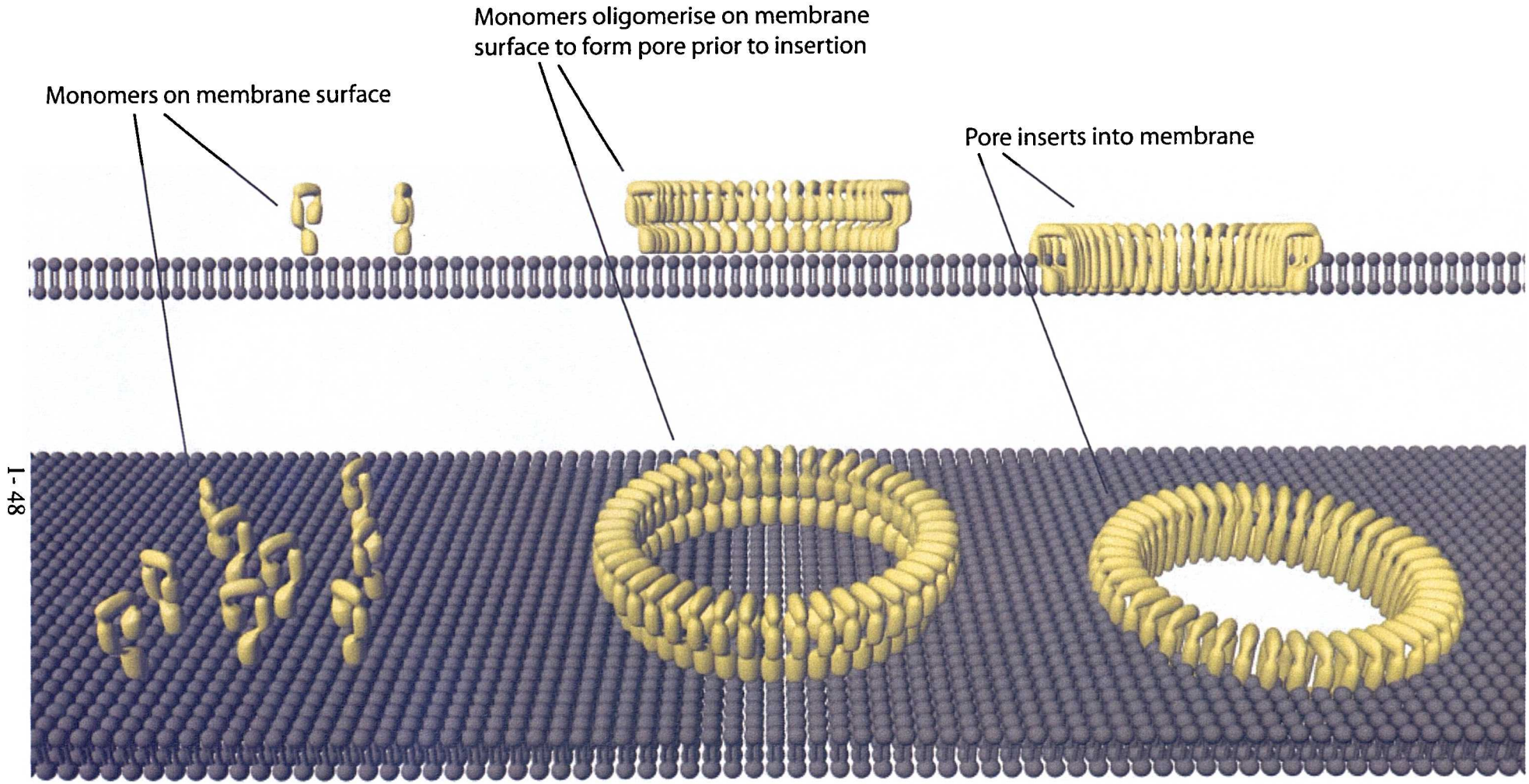


Figure 1-10

Mechanism of pore formation by a CDC

The schematic shows the binding of the monomers to the membrane followed by oligomerisation occurring prior to membrane insertion.

Diagram courtesy of Graeme Cowan, University of Glasgow

In 1991 the interaction of perfringolysin with cholesterol depleted membranes was studied and by depleting the cholesterol in membranes it was demonstrated that cholesterol was necessary for the interaction of perfringolysin with cell membranes (Ohno-Iwashita *et al.*, 1991). This confirmed data produced previously by other groups (Alving *et al.*, 1979; Johnson *et al.*, 1980) which had led to the acceptance that cholesterol was the cell membrane receptor of the CDC's. However other work questioned this established dogma by demonstrating that cholesterol inhibited listeriolysin cytolysis of cells but did not inhibit cell binding (Jacobs *et al.*, 1998). Furthermore it had previously been suggested that cholesterol binding was required prior to monomer interaction however it was shown that this is not the case (Gilbert *et al.*, 1998). Recently it was demonstrated that the kinetics of cholesterol binding do not fit the kinetics of pore formation (Giddings *et al.*, 2003) and that cholesterol depletion in a membrane does not inhibit oligomer formation. The accumulation of this data suggested that the role of cholesterol was not as a cell receptor. Further data by Giddings *et al.* (Giddings *et al.*, 2003) led them to suggest that cholesterol is essential for the transition from pre-pore to pore rather than for initial toxin binding.

1.7.2.3 Studies on the Trp rich region of the CDC's

The sequencing of pneumolysin made it possible to carry out site directed mutagenesis upon the toxin. This has provided valuable insight into the function of the toxin *in vivo* and has given information regarding the mechanism of action of the toxin via *in vitro* studies. The Trp rich undecapeptide (Figure 1-11) has been shown to be crucial for the hemolytic activity of the toxin and in particular that mutation of the Trp in the 433 position results in a hemolytic activity of less than 1% when compared to the wild type toxin (Hill *et al.*, 1994). Regarding the mechanism of pore formation studies reported that mutating the three tryptophan residues in the undecapeptide of pneumolysin did not reduce cell binding (Boulnois *et al.*, 1990). This was collaborated by the finding that mutations in the Trp residues of listeriolysin were able to bind cells (Michel *et al.*, 1990). However, this data was challenged by work that showed that mutating the Trp residues in perfringolysin did result in a loss of hemolytic activity and that a decrease in membrane binding was responsible for this (Sekino-Suzuki *et al.*, 1996). Others examined the binding capacity of the F433 in pneumolysin and found no decrease in cell binding ability (Korchev *et al.*, 1998) and this was recently consolidated when Imaizumi *et al.* published a study on pyolysin where they mutated the tryptophans in

the undecapeptide. As expected this resulted in a dramatic decrease in hemolytic activity however they also reported that these mutant's still bound to erythrocyte membranes (Imaizumi *et al.*, 2003).

By use of truncated pneumolysin constructs further examination of the role of each part of the pneumolysin molecule on cell binding has been possible. It has been demonstrated that domain 4 is capable of binding to cells and cholesterol independently of the rest of the molecule (Baba *et al.*, 2001). Furthermore that domains 1-3 require domain 4 for cell binding was demonstrated. These studies also suggested a role for the C terminal region of the toxin in oligomerisation since a construct with the C terminal removed is capable of binding to cells but is unable to cause hemolysis which may result from an inability to oligomerise.

1.7.3 Functional studies on Pneumolysin

I have reviewed the literature concerning the biophysical properties of pneumolysin, however many functional studies have also been carried out to understand the role of the toxin in the pathogenesis of *Streptococcus pneumoniae* infection. To this end the effects of purified pneumolysin *in vitro* on a variety of cell types has been studied, however most insight has come from a large number of studies that have examined the effect on pneumococcal infection of a variety of pneumolysin mutants and knockout strains.

1.7.3.1 Pneumolysin and Complement

Pneumolysin has been demonstrated to activate the classical complement pathway (Paton *et al.*, 1984). This was initially proposed to be the result of sequence similarity between PLY and acute phase protein (Mitchell *et al.*, 1991), however this proposal was later retracted when the structure of pneumolysin was examined based on a homology model with PFO (Rossjohn *et al.*, 1998). The homology model demonstrated that the similarity in sequence of acute phase protein and pneumolysin could not occur since the sequences were in remote domains. This model did suggest an alternative theory for complement activation by illustrating that pneumolysin may have a similar shape to the Fc region of antibody.

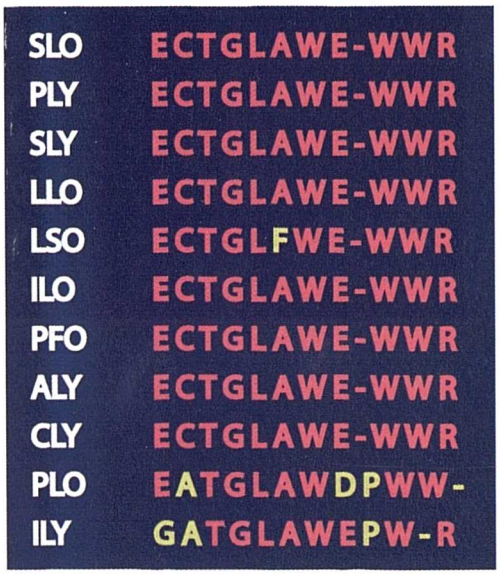


Figure 1-11

The Undecapeptide region of the CDCs

Listed is an alignment to show a comparison of the most highly conserved region of all the CDCs sequenced. This region is the 'undecapeptide'. The pneumolysin mutant 'F433' is produced by changing the first tryptophan residue into a phenylalanine.

1.7.3.2 The effect of pneumolysin on cells

The effects of pneumolysin upon human PMNL at low doses included inhibition of leukocyte migration and chemotaxis (Johnson *et al.*, 1981). A similar study later observed that pneumolysin also inhibits the respiratory burst and bacterialcidal activity of PMNL's (Paton and Ferrante, 1983). The focus of examining the effect of pneumolysin upon cells of the immune system was continued with the demonstration that pneumolysin was able to inhibit the *in vitro* proliferation of human lymphocytes (Ferrante *et al.*, 1984) and then again with the demonstration that pneumolysin was able to inhibit the respiratory burst of monocytes (Nandoskar *et al.*, 1986). It has been demonstrated that *S. pneumoniae* is able to induce apoptosis and necrosis of neutrophils (Zysk *et al.*, 2000) while more detailed studies have recently been carried out by Cockeran *et al.* who have investigated the effect of purified pneumolysin upon neutrophils. They have demonstrated that pneumolysin causes calcium ion flux in the cell membrane which results in the activation of pro-inflammatory signalling (Cockeran *et al.*, 2001b). They have identified the result of this signalling to be the increased production of prostaglandin E(2) and leukotriene B(4) (Cockeran *et al.*, 2001a) and an activation and release of IL-8 (Cockeran *et al.*, 2002b). Pneumolysin has been shown to induce the release of nitric oxide from macrophages (Braun *et al.*, 1999) while others have investigated the component of pneumolysin responsible for the induction of cytokines. They used a truncated pneumolysin that lacked hemolytic activity. The intact IFN-g inducing ability of the truncate shows this activity of pneumolysin is independent from the cytolytic component of the molecule (Baba *et al.*, 2002).

1.7.3.3 In vivo studies on pneumolysin

These *in vitro* studies have been interesting and informative up to a point, however it is *in vivo* studies that have particularly given insight into the role of pneumolysin in pneumococcal pathogenesis. In 1989 Berry *et al.* made a mutant version of the type 2 wild type *Streptococcus pneumoniae* that did not contain a functional pneumolysin gene (Berry *et al.*, 1989). When inocu-

lated with a lethal dose via the intranasal or intraperitoneal route the mutant infected mice showed an increased survival time compared to the wild type infected mice, which suggested that pneumolysin may have an important role in pathogenesis. A similar experiment (Berry *et al.*, 1992) was carried out in type 3 pneumococcus with the same results which confirmed that the decreased virulence of strains lacking pneumolysin was not strain dependent.

Following these studies the necessity of pneumolysin within each pathogenesis setting has been examined by different researchers. The effect of the different parts of pneumolysin upon infection via the i.p. route was examined by using pneumococci with point mutations in pneumolysin at Asp 385 to Asn which disables the complement activating properties of pneumolysin (Berry *et al.*, 1995). This was found to have no effect on virulence when administered by the i.p. route. Another mutation of Trp 433 to Phe abolished cytolytic ability of pneumolysin. This was found to increase the survival time of mice. This experiment was repeated in 1999 but the different roles for the cytolytic and complement activating parts had by then been elucidated for the intranasal and intratracheal routes so in this experiment they elucidated whither there was a third activity of pneumolysin apart from the cytolytic and complement activating activities. They demonstrated that pneumococci lacking both complement activating and cytolytic activities had a virulence greater than pneumococci deficient in pneumolysin thereby proving that pneumolysin has other activities besides the cytolytic and complement activating activities (Berry *et al.*, 1999). The effect of pneumolysin upon colonisation was examined by comparing the ability of a type 14 strain that lacked PLY to colonise the upper respiratory tract with the wild type strain. No difference in colonising ability or persistence over a four week period was noted between the two strains (Rubins *et al.*, 1998).

Pneumolysin has been shown to play an important role in eye infection with *S. pneumoniae* (Johnson *et al.*, 1992) (Ng *et al.*, 2002) yet appears to have no role in ear infections (Sato *et al.*, 1996). Using strains deficient in pneumolysin two different studies have shown that pneumolysin during meningitis has a role in virulence but not a crucial one (Friedland *et al.*, 1995) (Wellmer *et al.*, 2002). In an attempt to understand the role of pneumolysin during bacteraemia Benton *et al.* (Benton *et al.*, 1995) injected a pneumolysin deficient strain of pneumococci intravenously into mice. They found that pneumolysin played a crucial role in the development of acute sepsis instead of chronic bacteraemia. In an attempt to decipher which part of the pneumolysin molecule was responsible for potentially combating the host response a pneumococcal strain that contained a construct in which

pneumolysin lacked either the hemolytic or complement activating properties was used (Benton *et al.*, 1997). These studies showed that these mutated strains of the pneumococcus had a similar virulence to the wild type strain, suggesting that another part of the pneumolysin molecule was responsible for allowing the development of acute sepsis over chronic bacteraemia. In a further study the key host resistance mechanism that functions to prevent pneumolysin deficient strains progressing to acute sepsis was shown to be TNF-a (Benton *et al.*, 1998).

The effect of pneumolysin deficiency during pneumococcal infection of the lung was found to be that in mice infected with pneumolysin deficient pneumococci the pneumococci grew more slowly and did not appear in the blood as quickly (Canvin *et al.*, 1995). An examination of the histology of the lung revealed that the pathology induced by pneumolysin deficient strains was delayed and less severe compared to the wild type. Notably the neutrophil influx was less dramatic in pneumolysin deficient infected mice. These findings were extended by utilising pneumococcal strains engineered with pneumolysin that lacked either cytolytic or complement activating ability. By intratracheal installation of pneumococci the development of lobar pneumonia occurred and it was demonstrated that both the cytolytic and complement activating properties of pneumolysin contribute to virulence although an additive effect was not observed for double mutants (Rubins *et al.*, 1995). Instillation of the same bacteria by the intranasal route to induce bronchopneumonia was found to reveal that again both properties of pneumolysin were important although they are important at different stages of infection for each type of pneumonia (Alexander *et al.*, 1998). In addition the authors noticed that pneumococci possessing both cytolytic and complement deficiencies are not avirulent which led them to suggest that pneumolysin possesses some sort of 'sub-lytic' function that is important also. In 2000 Kadioglu *et al.* published further details on the processes that occur during the development of pneumococcal induced bronchopneumonia (Kadioglu *et al.*, 2000). They compared the cells present in the lungs after 24 hours infection with wild type *S. pneumoniae* or with a pneumolysin deficient strain. The wild type infection caused an increase in PMNL and a decrease in macrophages with the number of lymphocytes remaining unchanged, while the pneumolysin deficient strain caused the same changes but to a lesser extent. While the number of lymphocytes remained unchanged in the tissue as a whole, immunohistochemical analysis showed that in lungs infected with wild type pneumococci the number of T cells increased dramatically around inflamed bronchioles with the number of B cells also increasing later in the time course and not to the same degree. This change in lymphocyte distribution was also observed when lungs were in-

fected with the pneumolysin deficient strain but changes occurred later and to a much lesser extent. From their observation Kadioglu *et al.* were able to draw some novel conclusions. They observed that despite the influx of neutrophils into the lung during the first 12 hours of infection, this influx does not prevent the rapid growth of wild type pneumococci observed to occur at 16 hours yet the neutrophils do prevent the rapid growth of pneumolysin deficient strains. This means that pneumolysin is potentially responsible for the inactivation of neutrophils. Previously neutrophils had been considered to be responsible for the cessation of pneumococcal growth in the lungs but here Kadioglu was able to demonstrate that it is the influx of T cells into the inflamed areas that coincides with cessation of pneumococcal growth.

In a follow up study the group attempted to elucidate the effect of the cytolytic and complement activating components of pneumolysin upon the cellular infiltrate into the lung (Jounblat *et al.*, 2003). While difference in bacterial growth during the early hours of infection were noted, the differences did not persist, meaning that the most significant differences discovered by this study came from examining the histology during infection. Pneumococci containing a pneumolysin construct that lacked complement activating activities recruited neutrophils to the same degree as the wild type infection but did not cause any alteration of the T cell distribution. In contrast pneumococci with cytolytic deficient pneumolysin did not recruit neutrophils to the same degree as the wild type infection yet the pattern of T cell distribution was the same as the wild type.

1.8 Conclusion

In this chapter an overview of some of the mechanisms of immunomodulation used by pathogen's has been given. This has allowed the recognition of the convergent mechanisms of immunomodulation. Therefore as the pneumococcus was considered, an expectation that it may modulate complement, phagocytosis, cytokine production, B cells and T cells had formed. Some of these expectations were fulfilled as the immunomodulatory mechanisms of the pneumococcus were examined. Furthermore as the immunomodulatory effect of pneumolysin upon T cells is considered an awareness of some of the potential mechanisms has been generated. In this chapter a review of the immune response to the pneumococcus has also been given that has focused on the recently elucidated adaptive immune response. Finally the toxin pneumolysin has itself been considered with particular emphasis on its role in infection and mechanism of action.

It is the aim of this thesis to determine if pneumolysin is able to exert an immunomodulatory effect upon CD4 T cells *in vitro* and the mechanism of action used. Furthermore a demonstration of these effects *in vivo* and an elucidation of the potential consequence of these effects for pneumococcal pathogenesis will also be discussed.

1.9 Aims

The aim of this thesis is to examine a potential immunomodulatory mechanism used by the pneumococcus to modulate the adaptive immune response. I will examine the *in vitro* immunomodulatory ability of the toxin pneumolysin and will attempt to dissect the mechanism of action of pneumolysin upon T cells. I will then proceed to examine the ability of the toxin to accomplish *in vivo* what it does *in vitro*.

2 Materials and Methods

2.1 DNA preparation

Oligonucleotide primers and Polymerase Chain Reaction (PCR)

Primers used for PCR or for sequencing were obtained from Sigma Genosys. Figure 2-1 shows the sequences and names of the primers. PCRs (Rush *et al.*, 2002) were performed using an automated PCR machine (Hybaid, PCR Express). The program used for all PCRs conducted, utilised a cycle of 94°C for 30 seconds, a 55°C annealing stage for 45 seconds and finally a 90 second stage at 72°C. 10x thermopol buffer (New England Biolabs) was diluted 1:10 in the reaction. dNTP's (Promega) were used at 10mM. Primers were used at 50pmol/ml. 1unit of the polymerase Vent (NEB) was used per reaction. 100ng of the F433 template plasmid was used per reaction. The remaining volume was made up with autoclaved distilled water.

Agarose Gel

A 1% agarose gel was made by dissolving agarose (Invitrogen) in TAE (Amresco) and, after heating, Ethidium Bromide (Sigma) was added to 0.5mg/ml. The gel was cast and the samples loaded (total volume; 10ml per well) with an equal volume of DNA loading buffer (see appendix 1). A 1kb+ DNA ladder from Invitrogen was used to estimate fragment length and is suitable for sizing linear double stranded DNA from 100bp – 12kb. The ladder has twenty bands, twelve of which range from 1000 bp to 12,000 bp in 1000-bp steps and eight bands ranging in size from 100 to 1650 bp. The 1650-bp band represents 8% of the total ladder and is therefore brighter than the other bands and so can be clearly distinguished on a gel. Electrophoresis was carried out in TAE buffer at 80 volts until the ladder was sufficiently spread over the length of the gel. Gels were photographed using UV light.

Purification, digestion and amplification of DNA

Primer name	Primer sequence
4T	GTT GAT CGT GCT CCG ATG AC
4U	TAT ACA GTC AGC GTA GAC GC
4V	CAA TAC AGA AGT GAA GGC GG
9Y	CGG GAT CCG GCA AAT AAA GCA GTA AAT GAC TTT
9Z	GAC GGA GCT CGA CTA GTC ATT TTC TAC CTT ATC

Figure 2-1

Primers used for PCR and sequencing

In order to isolate PCR or digestion products from the contaminants and from the gel itself the required band was cut from the Agarose gel under ultraviolet light and purified using a Qiagen QIAquick Gel Extraction Kit following the manufacturers instructions.

Digestions of PCR products and plasmid vectors were carried out using BAM H1/Sac I restriction enzymes (Promega) at 0.5 units/ml. Multicore buffer (Promega) was used to buffer the reaction and distilled autoclaved water was used to make up the remaining volume.

Small scale plasmid DNA amplification was carried out using the Qiagen QIAprep Miniprep Kit. Manufacturers instructions were followed throughout.

Large scale plasmid DNA amplification was carried out using the Qiagen Maxiprep Kit. Manufacturers instructions were followed throughout.

Ligation and Transformation

When ligating into the pCR 4 TOPO plasmid (Invitrogen), addition of 3' A-Overhangs to the PCR products was necessary since Vent polymerase was used in the PCR does not leave these present. 3' A-Overhang addition was carried out on PCR products following the procedure of the TOPO TA Cloning Kit. This involves incubating the PCR products with Taq for 15 minutes at 72°C along with Taq buffer.

For ligations of both pCR 4 TOPO and pET33b+ (Novagen), ligating incubations were performed at room temperature for 6 hours. The ligation mixture consisted of a vector to insert molar ratio of 1:3 using 50ng of vector per ligation. 10x ligation buffer was diluted 1:10 and 3 units of DNA ligase (Promega) was used per reaction. The remaining volume was made up with autoclaved distilled water.

When transforming TOP 10 *E. coli*, 1-50ng of ligation was transformed into TOP 10 (DH5a) *E. coli* using the standard protocol of the TOPO TA Cloning Kit. After addition of the ligation, *E.coli* were incubated on ice for 5-30 minutes and then heat shocked at 42°C for 30 seconds. SOC medium (see

appendix 1) was added and the cells were left shaking for 1 hour at 37°C. The transformation was then plated on ampicillin or kanamycin agar plates and incubated overnight at 37°C.

When transforming BL21 *E. coli* (Stratagene), 1-50ng of plasmid was added to *E. coli* prepared with β-ME (Sigma). This was left on ice for 30 minutes before heat shocking the cells at 42°C for 45 seconds. The cells were added to ice for 2 minutes prior to receiving 0.9ml of SOC medium (see appendix 1) and being incubated shaking for 1 hour at 37°C. The transformation was then plated on kanamycin agar plates and incubated overnight at 37°C.

Luria Broth (LB) Agar Plates

LB (Sigma) was made according to the manufacturers instructions. 1% Agar (Duchefa) was added to this prior to autoclaving. Upon reheating kanamycin (Sigma) was added to 30mg/ml or ampicillin (Sigma) to 100mg/ml. Using aseptic technique the medium was then poured into sterile petri dishes (Bibby Sterelin) and left to set. When preparing overnight cultures a single colony was selected and grown in LB with kanamycin overnight and then purified on a small or large scale.

2.2 Protein Preparation

Growth and induction of *E. coli*

BL21 *E. coli* were streaked onto an LB agar plate containing kanamycin and incubated overnight at 37°C. A single colony was picked and grown overnight at 37°C in LB with kanamycin to ensure vector pressure was applied. A small amount (10ml) of the overnight culture was inoculated into 1 litre of LB with kanamycin which was grown at 37°C to mid log phase (Optical Density 600nm of 0.6 m, measured using a UV/Vis Spectrophotometer UV/2 [Unicam]) before being induced with 1mM isopropyl-b-d-thiogalactopyranoside (IPTG) (Melford Laboratories Ltd) for 1.5 hours. The bacteria were then harvested by centrifugation at 4°C and 5000G for 7 minutes in a Sigma Laboratory Centrifuge 4K15 centrifuge. The bacteria were resuspended at 5ml/g of bacteria in 'bugbuster' (Novagen) and lyzozyme (Sigma) added to 1mg/ml and 1ml of benzonase (Novagen) per ml of suspension. This was then put on ice for 30 minutes prior to shaking on a desktop shaker for 20 minutes at room temperature. The supernatant was harvested by centrifugation at 4°C for 30 minutes at 14000 RCF and then 0.2mm filtered using a syringe tip filter (Satorius).

XL10 *E.coli* were streaked onto an LB agar plate containing ampicillin and inoculated overnight at 37°C. A single colony was picked and grown overnight at 37°C in LB broth with ampicillin. A small amount (10ml) of the overnight culture was inoculated into 1 litre of LB with ampicillin which was grown at 37°C to mid log phase (OD600nm of 0.6) before being induced with 1mM IPTG for 1.5 hours. The bacteria were then harvested by centrifugation at 4°C at a speed of 5000G for 7 minutes in a Sigma Laboratory Centrifuge 4K15 centrifuge. The bacteria were resuspended in 20ml PBS (see Appendix 1) for every litre of original culture before being disrupted in a Constant Systems cell disrupter at a pressure of 12Kpsi. The supernatant was harvested by centrifugation at 4°C for 30 minutes at 14000 RCF.

2.2.1 Chromatography

To perform Metal Chelate Chromatography (MCC), the BioCAD 700E perfusion chromatography workstation (Perseptive Systems) was equipped with a metal chelate column to enable the extraction of the proteins tagged with six histidine residues.

The column used was 100mm in length with a diameter of 4.6mm and a volume of 1.662ml. The flow rate was set at 4ml/min throughout. The column was charged with nickel sulphate initially by the following protocol; 30 column volumes (CV) of 10% 500mM EDTA was run through the column to clean it then 20CV of 100% H^2O to wash it. The column was then charged with 10CV of 100% 100mM nickel sulphate and washed with 10CV 6.6% 3M NaCl.

The column was then equilibrated using a blend of 90% 50mM Hepes and 10% 3M NaCl for 10 CV then 5 CV of 25% 2M Imidazole, 10% 50mM Hepes and 3M NaCl. Finally the blend was reduced to 10 CV 0.1% 2M Imidazole, 89.9% Hepes, 10% 3M NaCl.

The crude preparation of sample was loaded on in 5ml amounts. A minimum of 5ml and maximum of 10ml of crude preparation was loaded at any point.

The column was then washed first with 5 CV of a blend of 0.5% 2M Imidazole, 83% 50mM Hepes, 16.5% 3M NaCl followed by 10 CV of 1.0% 2M Imidazole, 68% 50mM Hepes, 15% 3M NaCl, 15% 100% EtOH and finally with 10 CV of 2.5% 2M Imidazole, 66.5% 50mM Hepes, 15% 3M NaCl, 15% 100% EtOH.

The bound protein was then eluted by increasing concentrations of imidazole. The starting concentration was 100mM which corresponds to 5% 2M Imidazole 95% H2O. 5 CV were run of each concentration and 50mM increments were used up to 300mM before a final wash with 500mM imidazole.

To perform Hydrophobic Interaction chromatography (HIC), the BioCAD 700E perfusion chromatography workstation was equipped with a column containing POROS 20PE (Phenyl Ether) from Applied Biosystems that enable the extraction of the proteins according to their surface exposed hydrophobic groups.

The column used was 100mm in length with a diameter of 4.6mm and a volume of 1.66ml. The flow rate was set at 4ml/ml throughout.

The column was then equilibrated using a blend of 50% PBS-NaCl and 50% 1M NaCl for 14 CV. The crude preparation of sample was loaded on in 5ml amounts. A minimum of 5ml and maximum of 10ml of crude preparation was loaded at any point. The column was then washed and the protein eluted with a starting concentration of a blend of 50% PBS-NaCl and 50% 1M NaCl rising to 100% PBS-NaCl over 9 CV then 10CV of 100% H^2O.

To perform Anion Exchange Chromatography, the BioCAD 700E perfusion chromatography workstation was equipped with a HQ non porous column to enable the extraction of the proteins according to their surface charge.

The column used was 4.6mm in length with a diameter of 50mm and a volume of 0.831ml. The flow rate was set at 5ml/ml throughout.

The column was equilibrated with 3 CV of H^2O, then 3 CV of PBS-NaCl, then 3 CV of 50% PBS-NaCl and 50% 3M NaCl, then S3 CV of PBS-NaCl. The sample received from HIC was loaded on in 5ml amounts. The column was then washed with 10 CV of PBS-NaCl. Finally the protein was eluted with a gradient starting at 100% PBS-NaCl and rising to 50% PBS-NaCl and 50% 3M NaCl over 30 CV.

Where required samples were concentrated using minicom sample concentrators (Amicon, Millipore).

2.2.2 Quality Control

SDS-PAGE

To assess the purity of proteins Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out. Apparatus to cast the gel was from Biorad. The separating gel (see Appendix 1) was poured to fill ¾ of the gel space and left to set. After 1 hour the stacking gel (see Appendix 1) was carefully layered on top of the separating gel and a comb was inserted. This was left to set for 1 hour before filling the tank with running buffer (see Appendix 1). Samples were prepared by boiling an equal amount of sample and sample buffer (see Appendix 1) in an eppendorf tube for 5 minutes immediately before addition of 15ml of each sample to different wells of the gel. For every gel 6ml of Biorad Kaleidoscope marker was added to one well of every gel. The gel was run at 100V for 2 hours.

The gel was then carefully removed and left gently shaking in Coomassie stain (see Appendix 1) for ¾ hour and then left in destain (see Appendix 1) until it was clear.

Biorad Kaleidoscope Prestained standards were used in all SDS PAGE gels. These standards have a range of 200kDa – 6.5kDa.

Western Blotting

SDS PAGE gels were equilibrated in transfer buffer (see Appendix 1). Protein was transferred from the gel, using a Bio Rad transfer tank, to Hybond-C super membrane (Amersham Life Sciences). The gel was sandwiched between filter paper and fibre pads and put into the transfer tank with transfer buffer. A magnetic stirrer and ice pack in the tank ensured the temperature remained constant within the tank during the transfer which was conducted at 80 V for 2 hours.

After transfer the membrane was stained with the protein stain Ponceu S (Amersham Bioscience) to check the transfer had been successful. The membrane was then blocked in 3% skimmed milk (Marvel, Premier Brands, UK) in Tris NaCl (see Appendix 1) for 1 hour or overnight at room

temperature. The membrane was then incubated for 2-3 hours at 37°C or overnight at room temperature in 1:1000 dilution of anti-pneumolysin antibody (made in rabbits and purified by Tim Mitchell) in 3% skimmed milk Tris NaCl. After washing the membrane four times in Tris NaCl the membrane was blotted with the secondary antibody (1:1000 dilution in 3% skimmed milk Tri NaCl; anti rabbit Ig HRP-linked whole antibody from donkey[Amersham]) for 1 hour at 37°C. After washing a further 4 times with Tris NaCl the membrane was transferred to developer solution (see Appendix 1) for 15 minutes and then washed in water and left to dry.

Absorbance Assay

To assess the nucleic acid and protein content of each fraction an absorbance assay was carried out on each sample. An aliquot of each fraction was scanned on the spectrophotometer to give the 260, 280 and 320 nm reading. If the sample contained aggregates then this would be noticed in the 320 reading. Fractions suitable for pooling for further examination are those with a 280 value larger than the 260 value and with a 320 value close to 0.

Prior to use quartz cuvettes (Hellma) were cleaned using 5% Decon (Decon) and 5% Acetic acid (BDH) and then rinsed with PBS (see Appendix 1). A UV/Vis Spectrophotometer UV/2 (Unicam) was programmed to asses the sample absorbance at intervals from 220nm – 320nm. The baseline was set using PBS. 100ml of sample was added to one cuvette and the scan was carried out on this sample. The concentration of pneumolysin in the sample if it is pure pneumolysin can be determined using the extinction co-efficient which for pneumolysin is 0.75.

Hemolytic Assay

To assess the hemolytic activity of pneumolysin red blood cells were prepared by centrifuging 1ml of defibrinated sheep blood (E&O Laboratories) at 13000 RPM for 4 minutes. 200ml of the remaining blood pellet is resuspended in 10ml of PBS to make a 2% solution. 50ml PBS (see Appendix 1) was aliquoted into every well of a 96 well round bottomed plate (Nunc). 50ml of the sample

is added to the first well and doubly diluted across the row using new pipette tips for each dilution. 50ml of the blood cell suspension was added to each well and incubated at 37°C for 30 minutes and then left at room temperature until the PBS control wells become pelleted in the base of the well. The hemolytic activity of the sample was determined by recording the well in which the pellet formed was half the size of the PBS/control pellet. Hemolytic activity was then calculated according to a predefined chart (see Figure 2-2).

Bradford Assay

This assay detects the quantity of protein in a sample based on the change in absorbance that occurs when protein binds to the reagent Coomassie Brilliant Blue (Sigma). Absorbance shifts from 465 to 595nm.

Standards were made using ovalbumin protein (grade V - Sigma) from 200ug/ml to 1500ug/ml. 10ul of standard was added in triplicate to selected wells of a 96 well flat bottomed plate. 10ul of sample was also added in triplicate to the first well of the 96 well plate and doubly diluted in PBS down the plate. Absorbance was measured at 595nm using a Dynex plate reader and the concentration of protein in the samples calculated relative to the standards.

Endotoxin Assay

Endotoxin levels were measured using the Limulus Amebocyte Lysate (LAL) Kinetic QCL kit from BioWhittaker. Throughout this assay endotoxin free tubes and tips were used. Endotoxin standard (Bio Whittaker) was reconstituted in endotoxin free water and then vortexed for 15 minutes. To make the standard curve, the endotoxin standard was diluted in water to give standards of 50, 5, 0.5, 0.05, 0.005 IU/ml (international units). Vortexing for 1 minute was necessary between each dilution. Samples were diluted in a similar manner to give a range of concentrations between 1:100 and 1:1000000 dilution. Standards and samples are loaded onto a 96 well plate in duplicate and appropriate 'spikes' of 5IU/ml added to determine assay interference levels. Finally the lysate

Well Number	Titre (HU/ml)	Well Number	Titre HU/ml
1	40	13	1.6×10^5
2	80	14	3.2×10^5
3	160	15	6.4×10^5
4	320	16	1.3×10^6
5	640	17	2.6×10^7
6	1280	18	5.2×10^7
7	2560	19	1×10^8
8	5120	20	2×10^8
9	1×10^4	21	4×10^8
10	2×10^4	22	8×10^8
11	4×10^4	23	1.6×10^9
12	8×10^4	14	3.2×10^9

Figure 2-2

Calculation table for hemolytic assay

is added to the plate and the OD 405nm reading at 37°C was taken every 2 minutes for 1.5 hours using an Ultra Microplate Reader from Biotek Instruments.

2.3 Tissue Culture

Mice

Mice homozygous for the cOVA peptide 323-339/I-Ad-specific DO11.10 TcR transgenes (detected using the clonotypic mAb KJ1.26) on the BALB/c background (Murphy *et al.*, 1990) were used throughout when reference is given to DO11.10 mice. On average the percentage transgenic T cells in a lymph node preparation from these mice is 40% as measured by flow cytometry (see Figure 2-3). BALB/c mice were purchased from Harlan-Olac. All animals were specified pathogen free and were maintained under standard animal house conditions with free access to both water and standard rodent pellets at the University of Glasgow CRF in accordance with local and Home Office regulations.

Pneumococcal strains

The wild-type *S. pneumoniae* strain used was serotype 2 strain D39, NCTC 7466 (National Collection of Type Cultures, London, UK). The pneumolysin negative mutant used, PLY-, was made in the lab of Tim Mitchell (University of Glasgow). Pneumococci were grown in Brain Heart Infusion Broth (BHI – made to manufacturers specification - [Oxoid]) to log phase and then frozen at –70°C in 20% glycerol. Where required, erythromycin selection was used at 1ug/ml.

Viable counts were carried out by diluting the stock pneumococci in PBS (see Appendix 1) using ten fold dilutions and plating three 20µl aliquots of each dilution onto a sector of a blood agar plate that was divided into 6. The plates were incubated at 37°C overnight and colonies counted in each sector where the spot had between 40-250 colonies. CFU was calculated as the number of colonies multiplied by the dilution factor. The bacteria were used directly from the frozen stocks by washing them with PBS and then diluting to the required CFU/ml in PBS.

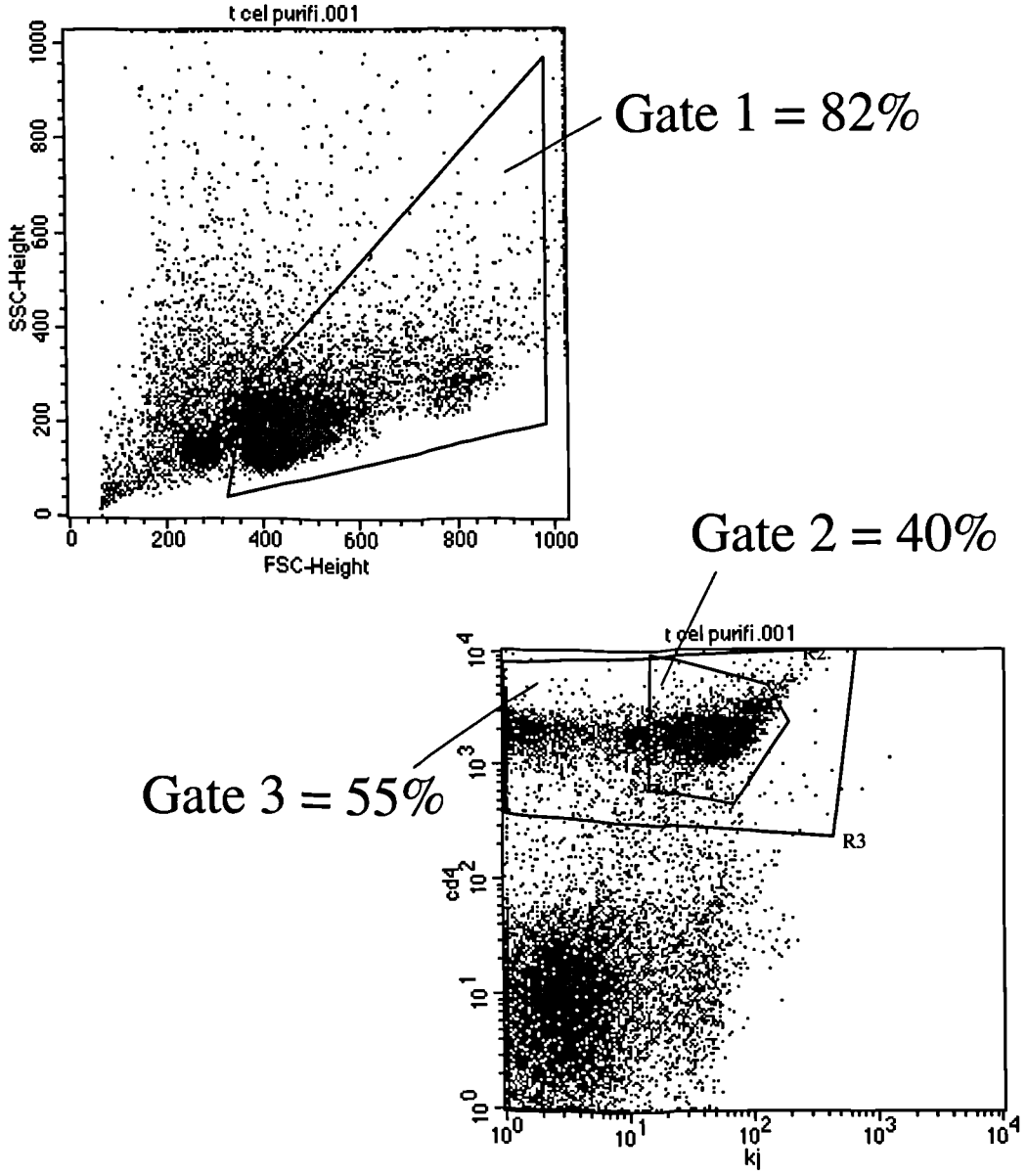


Figure 2-3

The percentage of transgenic CD4 T cells in a DO11.10 donor

The plots are of a lymph node preparation from a niave DO11.10 mouse. Gate 1 is set around the population of lymphocytes that represent 82%. Gate 2 is set around transgenic T cells (CD4+, KJ+) and represents 40% of total cells. Gate 3 is set around CD4+ T cells and represents 55% of cells.

Lymph node single cell suspension

The inguinal, mesenteric, superficial cervical, brachial and popliteal lymph nodes were removed from mice and pooled. They were made into a single cell suspension by mashing with the rubber end of a syringe and then passing through Nitex mesh (Cadisch Precision Meshes) and centrifuging at 450G for 5 minutes before resuspending in complete RPMI (see Appendix 1).

Pneumococci : lymph node suspension co-culture

Co-culture of lymph node cell suspensions and pneumococci was carried out in 6 or 24 well plates (Costar) depending upon the volume of cells required. If a proliferation assay was subsequently to be set up then 6 well plates were used. 1ml cultures of 1 x 10^6 lymphocytes were set up in 24 plates or 5ml cultures containing 5 x 10^6 lymphocytes when 6 well plates were used. Single cell lymph node suspensions were made in complete RPMI without pen/strep (Gibco). Pneumococci were inoculated into the cultures at 0 hours and incubated at 37°C , 5% CO_2.

In some experiments bacteria were killed by addition of penicillin (Gibco) to 100U/ml, streptomycin (Sigma) to 100mg/ml and gentamicin (Sigma) to 200ug/ml. At some points catalase (Sigma C3155) was used at 2000 units/ml and Hepes (Gibco) at 25mM.

Thymidine Incorporation Assay

To measure the amount of DNA replication in a cell population, 200ml of cell suspension containing 2x 10^5 lymphocytes was plated into each well of a 96 well plate (Costar) (Garside *et al.*, 1995). Cultures were incubated at 37°C , 5% CO_2. 24 hours prior to harvesting, the cultures were pulsed with 1mCi/well 3H-thymidine (Radionucleotide Dispensary, Western Infirmary, Glasgow). Plates were harvested using a Tomtec cell harvester onto filter mats (Wallac) and 3H-thymidine incorporation measured on a Trilux liquid scintillation and luminescence counter (Perkin Elmer Life Science).

Cytokine Assay and ELISA

To measure the amount of specific cytokines produced by a cell population, 1ml of cell suspension containing 1x 10^6 lymphocytes was plated into a 24 well cell culture cluster plate (Costar). Cultures were incubated at 37°C, 5% CO_2. To harvest the medium for cytokine ELISA the medium was removed from the culture cluster and centrifuged at 450G for 5 minutes in an eppendorf to allow the supernatant to be removed. Supernatant was frozen at -20°C until further analysis.

ELISAs (Eaton *et al.*, 2003) were carried out using 96 well plates (Immunolon 4 HBX, Dynex) which were coated with 50ml of anti-IL-2 (1mg/ml) or anti-IFNg (1.5mg/ml) binding antibodies (all Pharmingen) overnight at 4°C. Plates were washed with PBS/0.05% Tween, blocked with PBS/10% FCS for 1hr at 37°C, then washed and incubated with samples (50ml) and cytokine standards (R&D Systems)(50ml per well of 5ng/ml IL-2 or 50ml of 80ng/ml doubly diluted down the plate in triplicate) at 4°C overnight. Plates were washed again, incubated with 50ml biotinylated anti-IL-2 (0.5mg/ml) or anti-IFNg (1ug/ml) detection antibodies (all Pharmingen) for 1hr at 37°C, then washed and incubated with streptavidin peroxidase (1/1000) (Sigma) for 1hr at 37°C. Plates were then washed and TMB Microwell Peroxidase Substrate (Kirkegaard and Perry Laboratories) was added. Enzymatic colour changes were analysed on a Dynex plate reader at 630nm.

Ovalbumin

Whole chicken ovalbumin protein (OVA - Fraction V) was obtained from Sigma and made to the required stock concentration by dissolving in PBS (see Appendix 1) and was then 0.2mm filter sterilised (Sartorius). Ovalbumin peptide of the fragment 323-339 was obtained from Genosys.

CFSE staining

To allow detection of cell division using the flow cytometer cells were stained using CFSE dye (Lyons and Parish, 1994). Lymph node single cell suspensions were resuspended to 5 x 10^7 cells/ml

in HBSS. 5-(and-6-) carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA, or CFSE) (Molecular Probes) was added to give a concentration of 0.5mm CFDA. The CFDA was from a CFDA SE Cell Tracer kit from Molecular Probes. The cells were incubated in the CFDA for 10 minutes at 37°C and then washed three times in HBSS.

Bone Marrow Derived Dendritic Cells (BMDC)

GMCSF was made by growing the x63-GMCSF myeloma cell line for 2-3 days in complete IMDM (see Appendix 1) supplemented with 1mg/ml G418 (Sigma) and then harvesting the supernatant.

Dendritic cells were cultured from bone marrow by a method adapted from Lutz *et al.* (Lutz *et al.*, 1999). The femur and tibia were removed aseptically from BALB/c mice and sterilised with ethanol. The end of each bone were removed and the bone marrow flushed out using a 26 gauge needle (BDH Microlance) and syringe filled with RPMI 1640. After washing twice the cells were resuspended in complete RPMI (see Appendix 1) containing 5% GMCSF at 4x 10^5 cells/ml. 2ml of this was added to each well of a 6 well culture cluster (Corning Costar) and the cultures were incubated at 37°C , 5% CO_2. 3 days after setting up the cultures 2ml more of 5% GMCSF was added to each well. 5 days after setting up the cultures 2ml of medium was removed from each well and a fresh 2ml was added.

T cell purification

Lymph node single cell suspensions were resuspended to 2x 10^8 lymphocyte/ml in MACS buffer (see Appendix 1). To remove unwanted cell populations, 25ml rat anti mouse-CD8, 70ml rat anti mouse-CD19 and 50ml rat anti mouse-CD11b (all Serotec) were added per ml of the lymphocyte suspension. 60ml rat anti mouse-CD16/32 (Pharmingen) was also added (for antibody details see Appendix 1). This was placed on ice for 10 minutes before washing twice in MACS buffer and resuspending in MACS buffer at 2x 10^8 lymphocyte/ml. 200ml of goat anti rat IgG beads (Miltenyi Biotec) was added for every ml of cell suspension and placed on ice for 10 minutes. The cells were washed twice before being applied to a CS column (Miltenyi Biotec) fitted with a 23 gauge needle as a flow regulator. The T cells came off in the flow through which was collected while the other

cell types remained bound to the column. A purity of 95% or greater was routinely achieved as determined by CD4 staining and flow cytometric analysis (Smith *et al.*, 2000).

DO11-GFP Hybridoma

DO11-GFP T hybridoma cells ((Underhill *et al.*, 1999)) are based on the conventional DO11-10 T hybridoma that recognises amino acids 323–339 of OVA independent of co-stimulation. The DO11-GFP hybridoma had a plasmid introduced that contained the gene encoding GFP. This GFP gene is regulated by an IL-2 promoter, which is activated by the binding of nuclear factor of activated T cells (NFAT).

2.4 Cell Viability/Apoptosis Assays and Lipid Raft detection

Trypan Blue Exclusion Assay

To test for cell viability the trypan blue exclusion assay was used since trypan blue is excluded from viable cells. 30ml of cell suspension was incubated with 30ml of 0.4% trypan blue (Sigma). The number of lymphocytes excluding trypan blue was assessed using an Improved Neubauer Hemocytometer (Hawksley & Sons)

Annexin V/PI Assay

The Annexin V / Propidium Iodide assay was used to detect cell death and apoptosis. Annexin V binds to phosphatidylserine which is exposed to the outer membrane of the cell as one of the early signs of apoptosis. Viable cells exclude propidium iodide. An Annexin V-FITC apoptosis detection kit was purchased from Pharmingen and used as directed in the manufacturer's instructions. After culturing cells were washed twice with cold PBS prior to resuspending at 1x 10^6 cells/ml in 'binding buffer'. 1x 10^5 of these cells were then incubated with 5ml of both Annexin V and Propidium Iodide at room temperature for 15 minutes prior to analysis by flow cytometry.

Lactate Dehydrogenase (LDH)

The LDH assay was used to detect loss of cell membrane integrity and therefore viability. A 'Cytotox 96 non radioactive cytotoxicity Assay' kit was purchased from Promega and used according to the manufacturers instructions. The 'Cytotox 96' kit measures lactate dehydrogenase (LDH) presence in medium by utilising the ability of LDH to catalyse the transformation of a tetrazolium salt into a red formazan material that can be detected using a plate reader set to detect at 490nm.

Lipid rafts

To detect lipid rafts on the surface of cells, 13mm round cover slips (BDH) were coated with polylysine and placed into a 24 well tissue culture cluster (Corning Costar). Cells were cultured on top of the cover-slips in the cluster for 24 hours at 37°C, 5% CO^2 prior to the addition of CtxB-AF647 (Molecular Probes) to a final concentration of 0.5mg/ml. After 20 minutes incubation at 37°C the medium was removed and replaced with fresh medium. The cover slip was then removed and washed in PBS and then sealed onto a microscope slide with nail varnish (Rimmel). The cells were viewed and photographed on a Zeiss Axiovert 200 microscope with the appropriate filter set.

2.5 Flow Cytometry

A Becton Dickinson FACSCalibur Flow Cytometer equipped with CellQuest software was used for all flow cytometry applications.

Where staining was required it was carried out by taking 1x 10^6 of the cells to be stained and washing them with FACS buffer (see Appendix 1) and resuspending them in 100ml Fc block (see Appendix 1) before incubating at 4°C for 10 minutes prior to addition of the primary antibodies. 1ml of primary antibody was added and incubated at 4°C for 20 minutes. Cells were washed with FACS buffer and resuspended in a 1:50 dilution of secondary antibody in FACS buffer and incubated for 10 minutes at 4°C before washing in FACS buffer and resuspending in 500ml FACS flow (Smith *et al.*, 2002a). For antibody details see appendix 1.

Prior to analysing stained cells, forward scatter and side scatter were adjusted so the cells of interest could be gated on the screen. Unstained samples were used as auto-fluorescence controls allowing settings to be adjusted so that auto-fluorescence background was within the first decade of the log scale of the fluorescence intensity histogram. Unstained samples were compared with isotype and positively stained controls to confirm the correct scale. Compensation settings were set such that on an FL1 vs. FL2 dot plot, an FL1 positive population was vertically aligned with negative population, and an FL2 positive population was horizontally aligned with the FL2 negative population. A similar process was used to compensate FL2 vs. FL3.

2.6 Laser Scanning Cytometer (LSC)

A Laser Scanning Cytometer from Compucyte was used for all LSC applications. To stain the cells, 1x 10^5 cells were cytospun at 600RPM for 4 minutes using a Shandon Cytospin 3 onto Super Frost Plus microscope slides (Menzel Glaser) using Thermoshandon filter cards, cytofunnels and sample chambers. Cells were then fixed for 15 minutes at room temperature in 1% paraformaldehyde before being washed twice with PBS. 100ml TNB (see Appendix 1) was applied for 10 minutes prior to the addition of 100ml of the primary antibody biotinylated KJ1.26 at a 1:250 dilution in TNB (see Appendix 1) for 40 minutes. The slide was washed three times with TNT (see Appendix 1) prior to incubating with 50ml of streptavidin-HRP (Sigma) at a 1:100 dilution for 30mins. The slide was washed three times in TNT (see Appendix 1) before biotinylated tyrimide (Perkin Elmer) at a 1:50 dilution was added to the cells for 10 minutes. The slide was washed again three time in TNT (see Appendix 1) and 100ml of streptavidin AF647 (Molecular Probes) diluted 1:500 in TNB (see Appendix 1) was added for 30 minutes. The slide was then washed three times in TNT (see Appendix 1) and left to dry before mounting in vectasheild mounting medium containing DAPI (Vector Laboratories) and a cover slip (Adams *et al.*, 2004a).

2.7 In vivo

Adoptive Transfer

A lymph node single cell suspension was prepared from naive DO11.10 mouse lymph nodes (inguinal, popliteal, brachial, superficial cervical, mesenteric) and spleen (Smith *et al.*, 2002b). The proportion of CD4+ KJ+ T cells was assessed by staining for CD4 and using the clonotypic antibody KJ1.26 in flow cytometry. Cells were then resuspended in RPMI to either 1 x 10^7 KJ+ cells/ml or 1.5 x 10^7 KJ+ cells/ml. Using a 26 gauge needle (BDH) and 1ml syringe 200ml was injected via the tail vein into 6-8 week old age and sex matched BALB/c recipients.

Injections

100ml PBS (Gibco) or Pneumolysin was injected intraperitonealy into mice using a 26 gauge needle (BDH). The pneumolysin used *in vivo* was made using the hydrophobic interaction column and gel filtration column.

Ovalbumin (Sigma) was dissolved in PBS to 2mg/ml and filter sterilised. This was then made into an emulsion by combining it with an equal quantity of Complete Freunds Adjuvant (CFA) (Sigma) and vortexing the mixture until it remained aggregated when placed on water. 100ml (100mg) was injected subcutaneously into the back using a 21 gauge needle and 1ml syringe.

Antibody ELISA

ELISA's (Eaton *et al.*, 2003) were carried out using 96 well plates (Immunolon 2 HBX, Dynex) which were coated with 20mg/ml Ovalbumin (Sigma) overnight at 4°C. Plates were washed with PBS/0.05% Tween, blocked with 200ml PBS/10% FCS for 1hr at 37°C, then washed and incubated with samples at 4°C overnight. Samples were diluted according to this procedure; IgG1 diluted 1/

200 (unimmunised) or 1/3000 (immunised). Plates were washed again, incubated with biotinylated anti-IgG1 (0.25mg/ml – A85-1)) detection antibody (all Pharmingen) for 1hr at 37°C, then washed and incubated with streptavidin peroxidase (1/1000) (Sigma) for 1hr at 37°C. Plates were then washed and TMB Microwell Peroxidase Substrate (Kirkegaard and Perry Laboratories) was added. Enzymatic colour changes were analysed on a Dynex plate reader at 630nm.

Immunohistochemistry

An inguinal lymph node was removed from the mouse and embedded in OCT embedding medium (Sakura) and a cryomould (Sakura). This was snap frozen in liquid nitrogen before further freezing at -20°C until further use. Tissue sections were cut 6mm thick on a cryostat microtome (ThermoShandon) and then fixed with acetone and left to dry. Tg T and B cells were detected on sections (Smith *et al.*, 2002b) by blocking in 2% goats serum/PBS for 15 minutes before being incubated with 100ml of biotinylated KJ1.26 (at 1:500 dilution in 2% Goats serum) for 30 minutes. Sections were washed with PBS and then incubated for 30 minutes in 100ml ABC-AP (Vector Labs) and then washed again in PBS prior to incubating in the alkaline phosphatase substrate BCIP/NBT/Tris-HCl pH 9.5 for 45 minutes in the dark. After PBS washing, 100ml of B220 (at 1:400 dilution in 2% Goats serum) was added for 30 minutes and then sections were washed again with PBS. Section were then incubated with ABC-HRP (Vector) for 30 minutes. After further PBS washing, the HRP substrate 3,3'-diamminobenzidine was added for 10 minutes before washing in H^2O. Subsequent exposure to acetone/2% acetic acid, 70% ethanol, 95% ethanol, 95% ethanol then 100% ethanol dehydrated the tissues, before clearing in Histoclear and immediate mounting in Histomount.

Statistics

Results are expressed as mean ± SEM. To test significance Student's unpaired t-tests were performed. A p value of < 0.05 was regarded as significant.

3 Production of pneumolysin

3.1 Introduction

To allow the investigation of the immunomodulatory properties of pneumolysin with a high degree of accuracy and also to allow the utilisation of a variety of experimental systems, I purified pneumolysin. I used a pneumolysin mutant called F433 which has a Tryptophan (TGG) changed to a phenylalanine (TTC) at position 433 and as a result is not hemolytic.

The purification process is essentially biomolecule separation and depends on recognition of some unique, and therefore identifiable property, of the target molecule that enables it to be isolated from the contaminating material. Features such as molecular weight, charge, hydrophobicity and solubility may be used, however particular affinity properties such as binding specificity for a substrate can be exploited to allow extraction of the target molecule. Furthermore, if the molecule of interest can be cloned then it can be engineered to contain a tag of some sort that allows the target molecule to be purified using the affinity of the adjoining tag. The most common example of this is the His-tag system.

Pneumolysin was first purified in 1971 by Shumway *et al.* (Shumway and Klebanoff, 1971) by isolating the toxin from the whole pneumococcus. This was the practice, albeit with modifications, until 1986 when Paton *et al.* (Paton *et al.*, 1986) cloned the pneumolysin gene into *E. coli* and purified the toxin from the supernatant of crude extracts. Since then *E. coli* has become the standard expression vector for preparation of purified pneumolysin.

For my studies I chose to use two different methods to isolate pneumolysin. Both methods have been used previously and both utilise pneumolysin expression in an *E. coli* vector. The first method used a His tag purification method for obtaining both wild type and F433 type pneumolysin for use *in vitro*. The second method purified pneumolysin based on its hydrophobicity and charge. Both these methods require the pneumolysin gene to be in a suitable expression vector. The wild type pneumolysin gene was available in the pET33b+ expression vector which is suitable for use with the His tag / Metal Chelate (MC) purification method since pET33b+ adds 6 histidines to the peptide thereby allowing the protein to bind to the nickel ions. The F433 pneumolysin mutant was not available in pET33b+ and therefore was transferred into this expression vector system to make it compatible with the MC purification method. The wild type pneumolysin gene had previously

been cloned into the pKK expression vector which was suitable for use with the hydrophobic interaction and anion exchange purification method.

3.2 Results

3.2.1 Cloning of F433 pneumolysin into pET33b+

PCR amplification of F433 gene from template

I amplified the F433 pneumolysin gene from a template plasmid using primer 9Z and 9Y. The products were analysed on a gel. The pneumolysin gene is 1416bp (Walker *et al.*, 1987) and therefore would be expected to show up as a 1.4kb fragment when run on a gel. A 1.4kb fragment was evident and no contaminants were present.

Cloning of F433 into TOPO

To allow me to further amplify the F433 gene I ligated the PCR product into the PCR4 TOPO plasmid and transformed it into TOP 10 cells which were grown with antibiotic selection. To check if this had been successful I then extracted and digested the plasmid (with BAM H1 and SAC I) containing the PCR product before running it on a gel. Two bands were observed at the correct sizes (4kb [TOPO is 3.9kb] and 1.4kb).

Ligation into pET33b+ and transformation into TOP 10

My data suggested that I had successfully cloned the F433 gene into pCR4 TOPO plasmid. To allow me to purify pneumolysin using metal chelate chromatography I had to transfer the F433 gene from the pCR4 TOPO plasmid to the pET33b+ plasmid. I therefore prepared a large amount of the pCR4 TOPO plasmid containing the F433 gene by large scale DNA preparation and then extracted the F433 gene by restriction digestion. I prepared the pET33b+ plasmid in a similar way then carried out a digestion on it. Prior to ligation I wanted to confirm that the fragments of vector and insert I had prepared were correct. I therefore ran a sample of each on a gel (Figure 3-1). In Figure 3-3 the 1 kb+ ladder is shown in lane 1. In lane 3 the F433 insert was run and a band appears

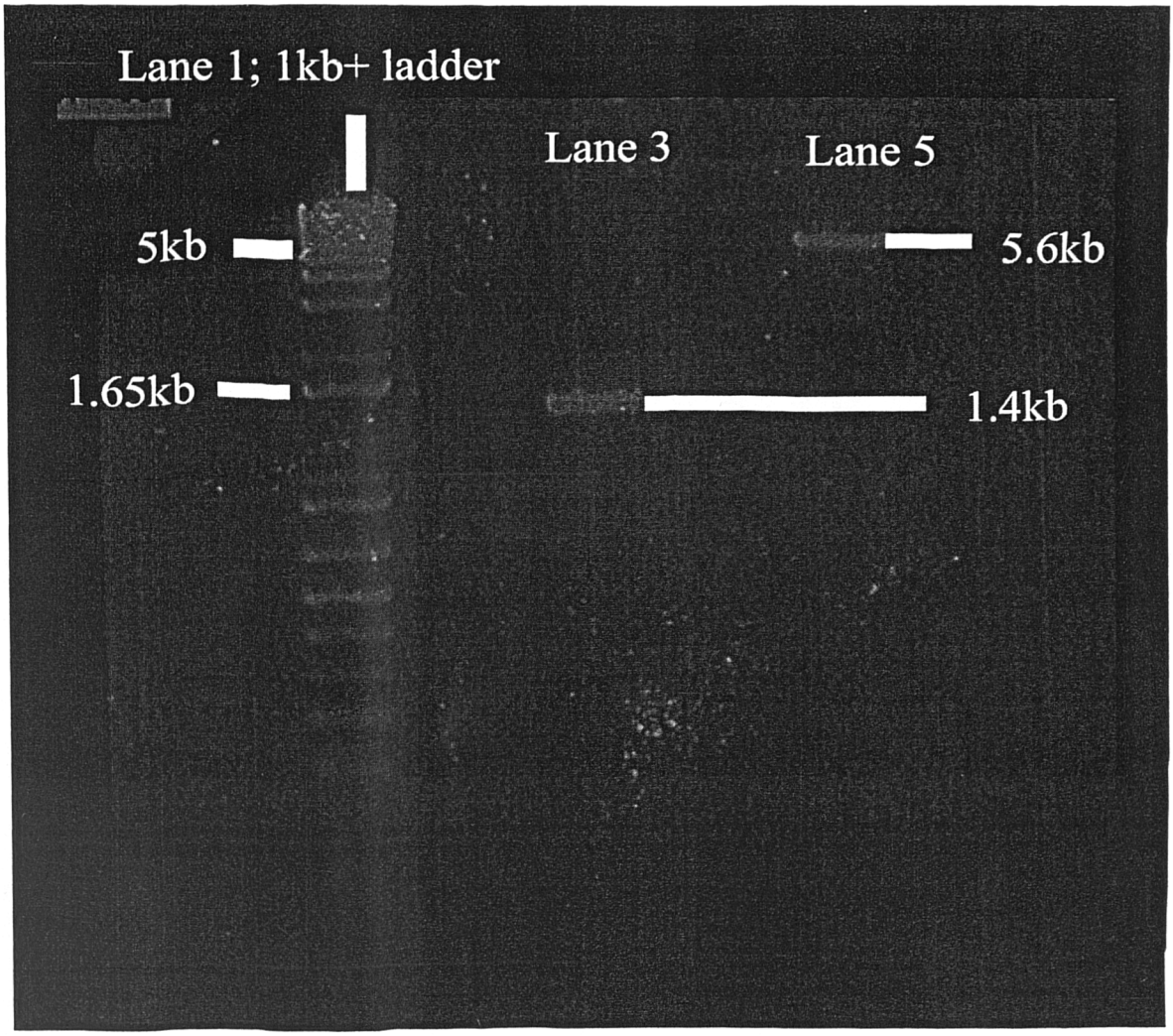


Figure 3-1

pET33b+ Plasmid and F433 Insert

The TOP 10 strain containing the pCR4 TOPO plasmid with the F433 gene insert was maxipreped. A maxiprep of the strain containing the empty pET33b+ plasmid was also carried out. Both maxipreps were carried out by isolating a single colony of each strain and growing an overnight culture. Both preparations was subsequently digested using BAM and SAC restriction cnzymes. The digested F433 gene was run on an agarose gel and then gel purified. We also ran a sample of each preparation on the agarose gel shown above.

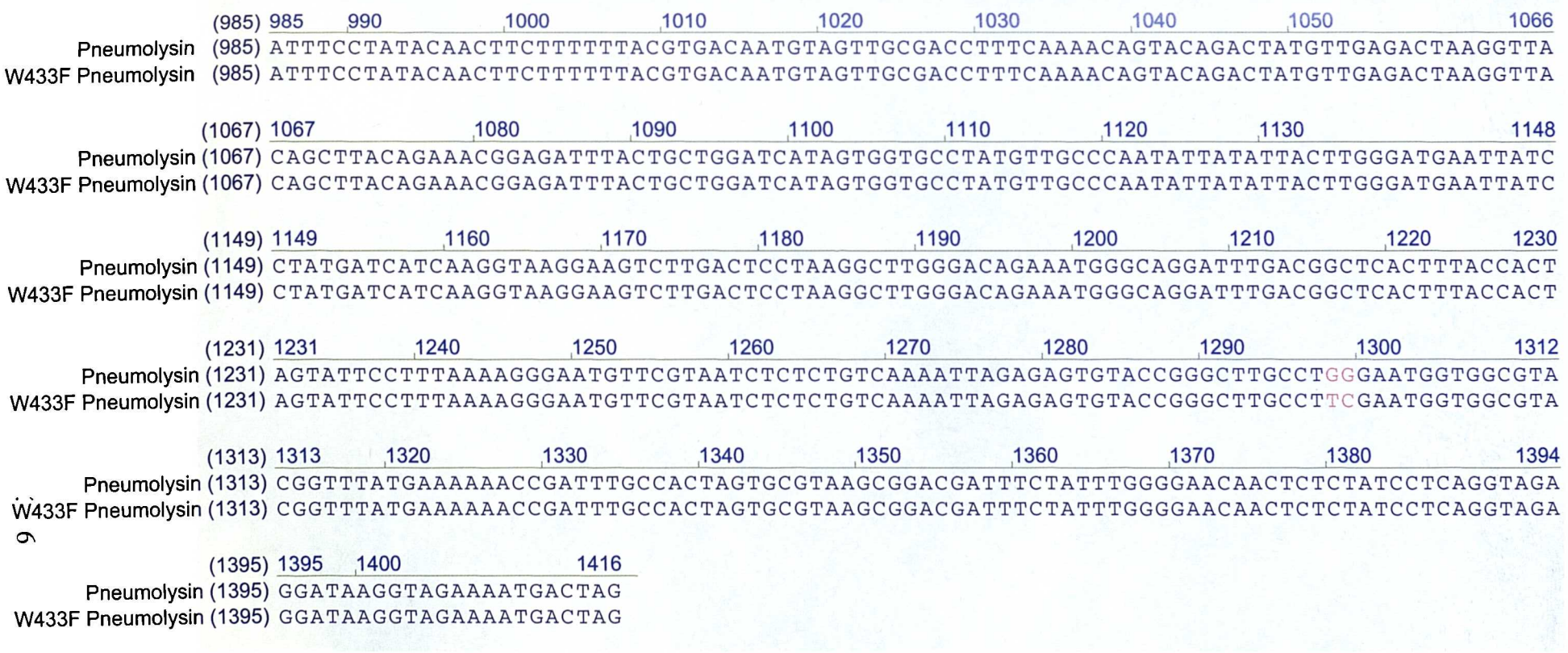


Figure 3-2

Sequence data from F433

Primers T7, 4U, 4T and 4V were used to sequence the pET33b+ plasmid containing the F433 pneumolysin gene at the MBSU in the University of Glasgow.

at 1.4kb which is the expected size of the F433 gene. In lane 5 a band is evident that correlates to between the 5 and 6 kb markers on the ladder. The pET vector is known to be 5.4kb. This gel confirmed that I had isolated the correct fragments so I ligated the pET33b+ vector and the F433 insert and then transformed them into TOP10 cells. To check the correct vector and insert had been ligated a digestion of the ligation was analysed on a gel and found to contain the correct size of products (pET33b+ at 5.4kb plus F433 at 1.4kb gives an expected size of 6.8kb).

Transformation of BL21 *E. coli*

From the results above it seemed highly probable that I had cloned the F433 gene into pET33b+ in TOP 10 cells. I therefore transferred some of the plasmid from the miniprep of the TOP10 cells into BL21 cells for protein expression (see next section) and used some of the plasmid for sequencing (carried out by MBSU, University of Glasgow). The sequence data confirmed that I had correctly cloned the F433 gene into the pET33b+ plasmid. Figure 3-2 illustrates the alignment of the F433 pneumolysin and wild type pneumolysin. Figure 3-3 contains a schematic representation of the pET33b+ plasmid containing the F433 pneumolysin gene.

3.2.2 Production and purification of wild and F433 pneumolysin using Metal Chelate Chromatography

3.2.2.1 Process Optimisation

I used the pET33b+ plasmid containing the wild pneumolysin gene (previously prepared) and the F433 pneumolysin gene (prepared in the above section) in the BL21 host to generate pneumolysin protein. During expression in this system some aggregates of pneumolysin can form and so in order to obtain the maximum amount of pneumolysin in the soluble fraction, I determined the optimum time of protein induction.

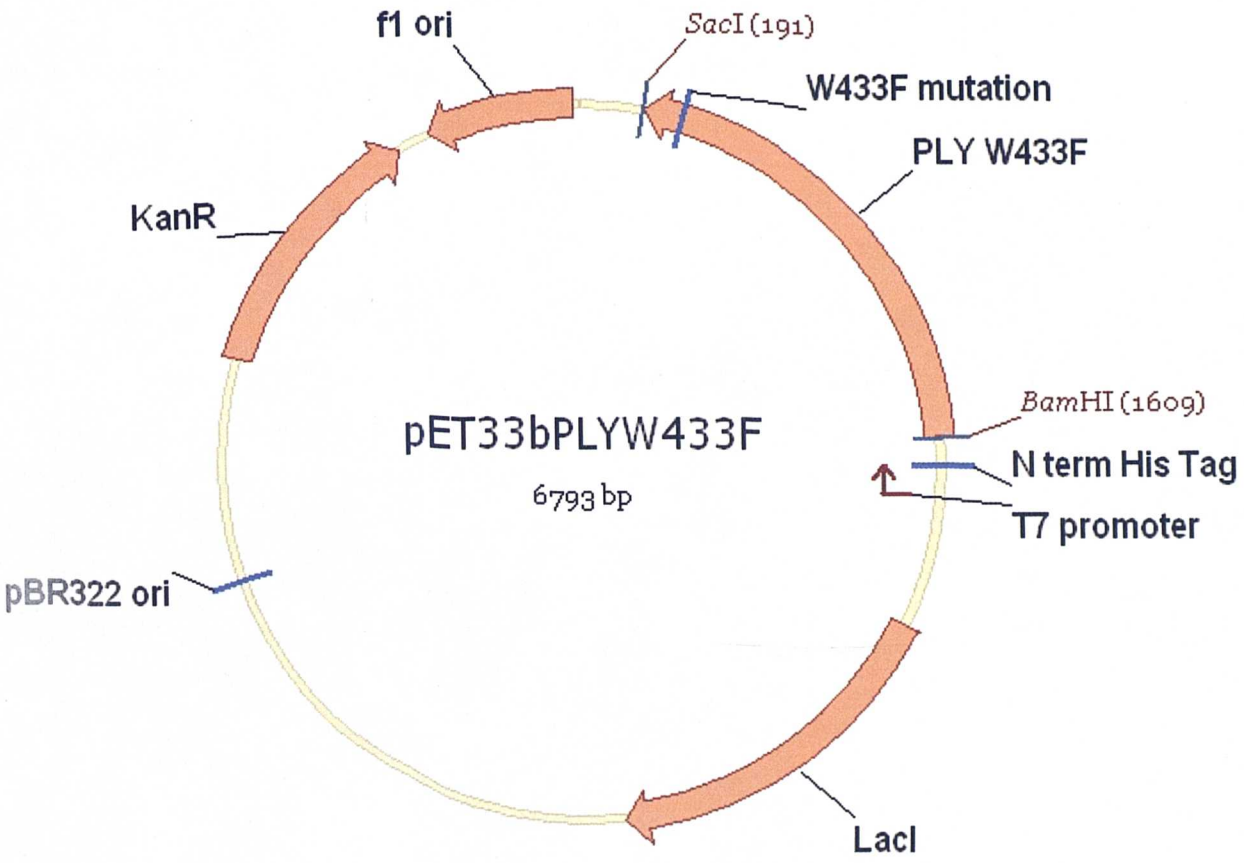


Figure 3-3

pET33b+ with F433 pneumolysin gene

The schematic shows the pET33b+ plasmid containing the F433 pneumolysin gene inserted between the Bam and Sac restriction sites. The ampicillin and kanamycin resistance genes are also shown.

Diagram courtesy of Graeme Cowan, University of Glasgow

I measured the amount of pneumolysin in each fraction over a 4 hour induction period. Figure 3-4 shows the gel produced from the soluble fraction of wild type expression and in lane 1 is the ladder. Pneumolysin is 53kDa in size and so would be expected to correlate with a position between the violet band at 41kDa and the green band at 85kDa on the ladder.

In lane 2 the soluble fraction from the 0 minutes sample is present. There is no band where pneumolysin would be expected to be observed in this lane indicating that production of pneumolysin occurs only when the bacteria are induced with IPTG. In lane 3 the soluble 30 minute sample is present and a band is evident where pneumolysin would be expected. After 60 minutes lane 4 shows us that the amount of pneumolysin has increased and by 90mins lane 5 demonstrates that this has reached a maximum. Subsequently in lanes 6 to 11 the amount of pneumolysin remains the same or decreases.

Insoluble samples from the wild type expression are shown in Figure 3-5. By 90 minutes the insoluble amount is at a maximum as seen in lane 4 and although the amount fluctuates in subsequent samples (Gel 1 - lanes 5 and 6 and Gel 2 - lanes 2-5) the amount of insoluble pneumolysin remains high.

The gel of the soluble production for F433 mutant type pneumolysin in BL21 is shown in Figure 3-6. The fractions show similar expression levels over the same time course as the wild type pneumolysin containing BL21 *E. coli.* Lane 9 (gel 1) contains previously purified pneumolysin to act as a positive control. As shown in lane 5, peak pneumolysin production appears to occur 90 minutes after induction.

From this data I concluded that 90 minutes was the optimum induction period that would yield the highest amount of soluble wild and F433 type pneumolysin.

3.2.2.2 Metal Chelate Chromatography

After identifying the optimal induction period I proceeded to produce purified pneumolysin from crude extracts using perfusion chromatography. The results of the perfusion chromatography are

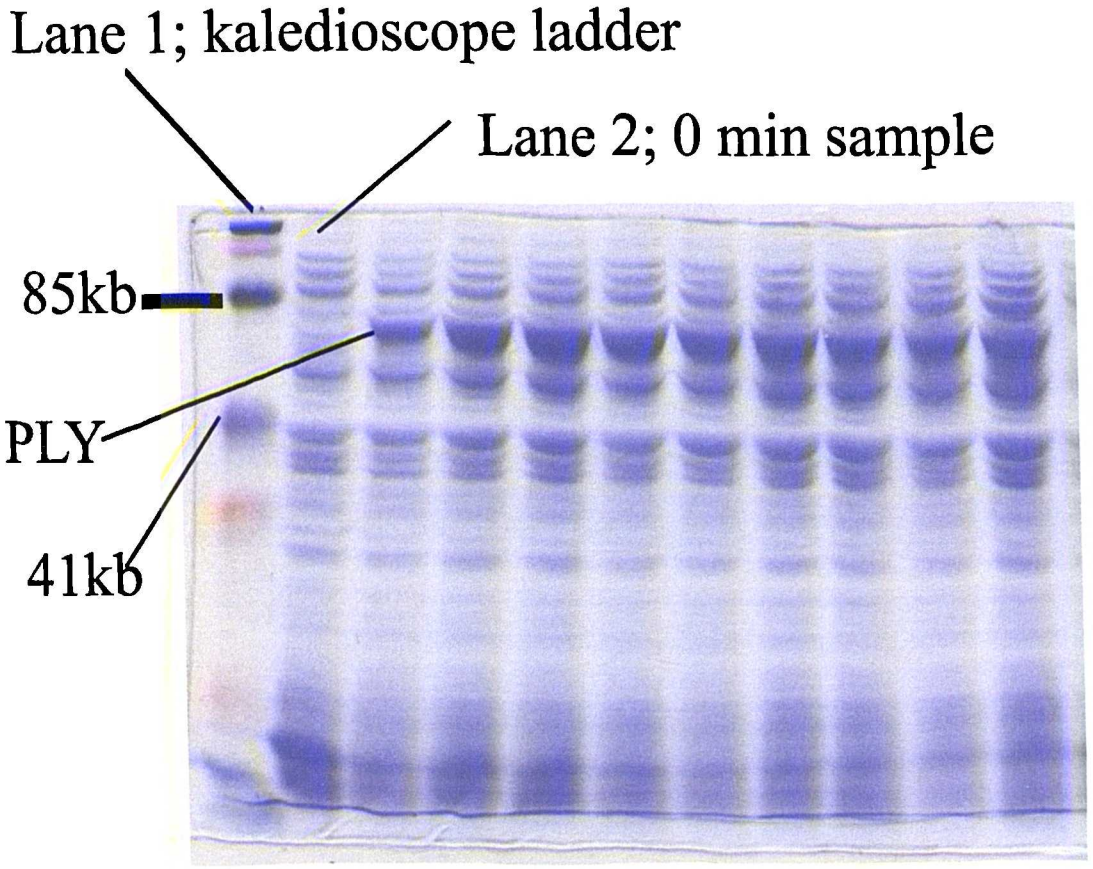


Figure 3-4

Soluble fractions of wild type pneumolysin

The pET33b+ containing wild or F433 pneumolysin in BL21 *E. coli* were grown and induced. For 4 hours after induction with IPTG, a sample of the culture was taken every 30 minutes. After disrupting with bug buster and centrifugation, the proportion of pneumolysin in the soluble and insoluble fractions was determined by running SDS PAGE gels of the samples. Figure 3-4, 3-5 and 3-6 show the results. We used the kaleidoscope ladder as the standard for each gel.

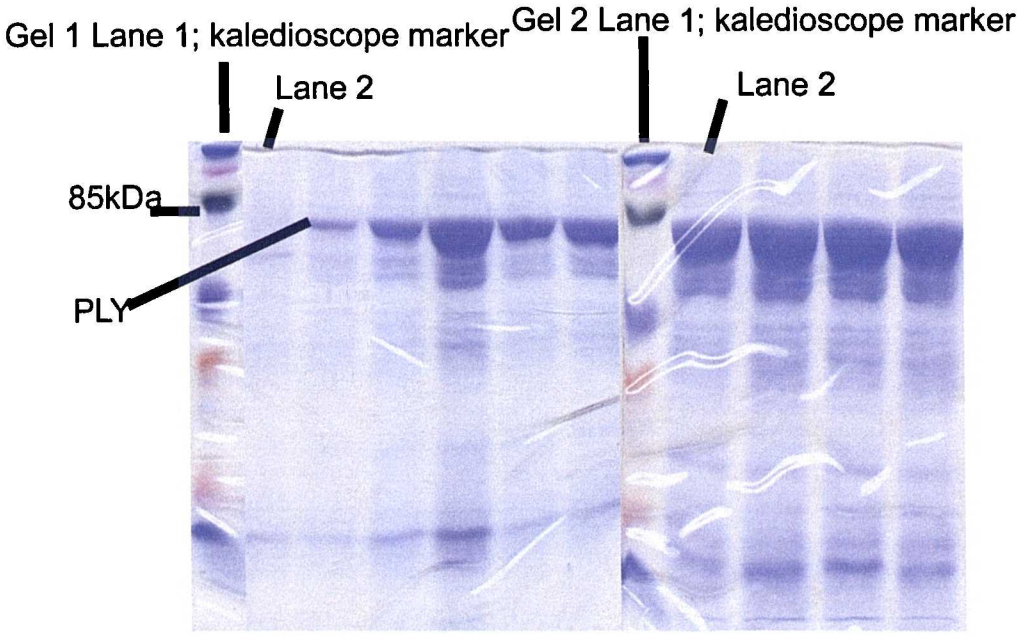


Figure 3-5

Insoluble fractions of wild type pneumolysin

Samples were prepared as described in Figure 3-4 and separated over two gels and have been aligned here.

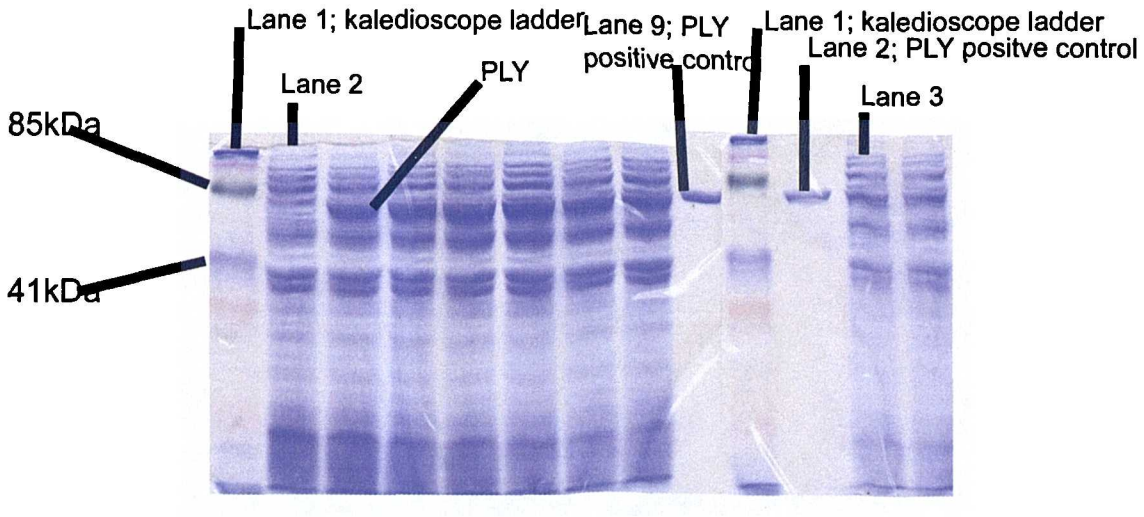


Figure 3-6

Soluble F433 pneumolysin fractions

Samples were prepared as described in Figure 3-4. The fractions were separated over two gels and have been aligned.

shown in Figure 3-7 and 3-8. The plots for both wild and F433 pneumolysin are very similar. In fraction 1 and 2 the increase and then decrease in 280 and 260 nm reading are the result of the column being loaded with sample and then being washed to remove any non binding debris. In fraction 4 the concentration of salt increases in the column. This causes the release of some material which results in the rise in 280 and 260 nm value. In fraction 6-9 50mM imidazole is introduced to the column and this results in more material being removed from the column and a distinct peak resulting. In fractions 10 and 11 the imidazole concentration is increased to 100mM and this results in more protein being released from the column. The remaining fractions do not yield any further material despite the increasing concentration of imidazole.

While some pneumolysin is likely to be present in all fractions, this data suggested that purified pneumolysin would be present in fractions 8-11. I proposed that pooling these fractions would yield a highly purified preparation of pneumolysin, however prior to pooling these fractions I conducted a number of test to confirm this hypothesis.

3.2.2.3 Quality Controls

Absorbance assay

One of the key indications of a purified fraction is the presence of a higher level of protein than nucleic acid. An absorbance assay showed that only in fractions 8-11 was the 260 reading was lower than the 280 reading and there were no aggregates as indicated by the low 320nm reading.

This data confirmed what I had suggested from the chromatography plot; that for both wild and F433 fractions 8-11 were potential candidates for further examination since fractions 1-7 contained too much nucleic acid contaminants and fractions 12-15 contained aggregates.

Hemolytic activity

Since pneumolysin is a hemolytic protein, the amount of pneumolysin present in any sample can be estimated by the amount of hemolysis it causes. I therefore carried out a hemolytic assay on each

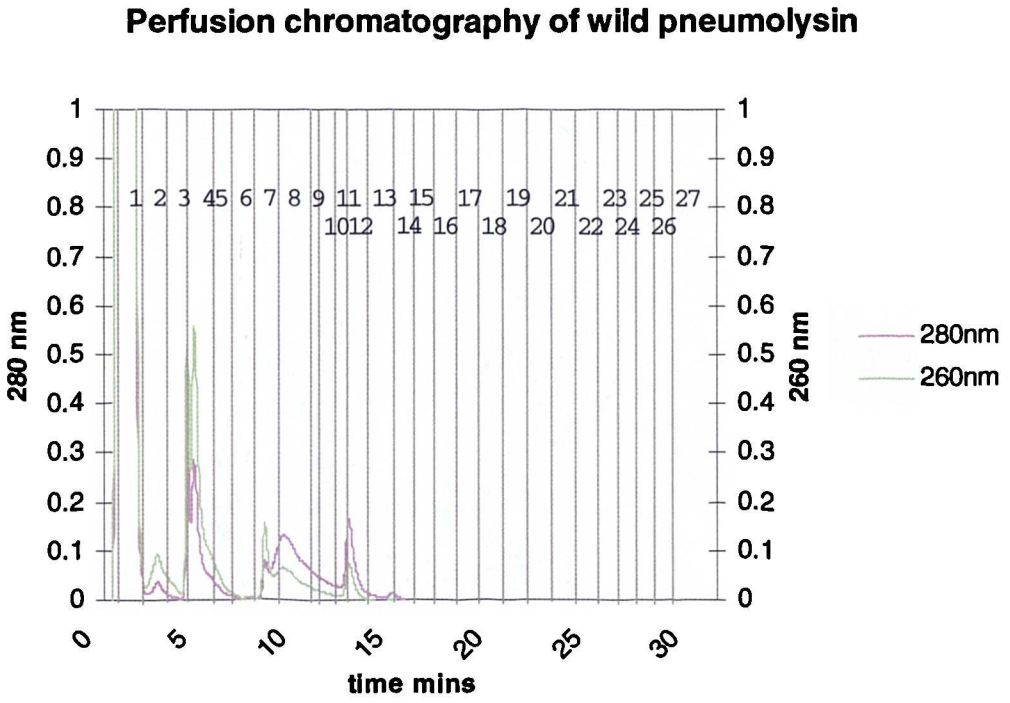


Figure 3-7

Purification of wild type pneumolysin using the BioCAD workstation and Metal Chelate Chromatography

BL21 *E. coli* containing the wild or F433 pneumolysin gene in pET33b+ were separately grown and induced. After induction the bacteria were resuspended in bugbuster and then centrifuged and the supernatant collected and loaded onto the metal chelate column on the BioCAD workstation. The plot above and in Figure 3-8 graphically displays the purification process. The red line correlates with the right hand axis and represents the 280nm reading which is representative of the amount of protein in the sample. The green line correlates with the left hand axis and represents the 260nm reading which is representative of the amount of nucleic acid in the sample. The plot is divided into 27 sections, each one correlating to a fraction from the BioCAD column.

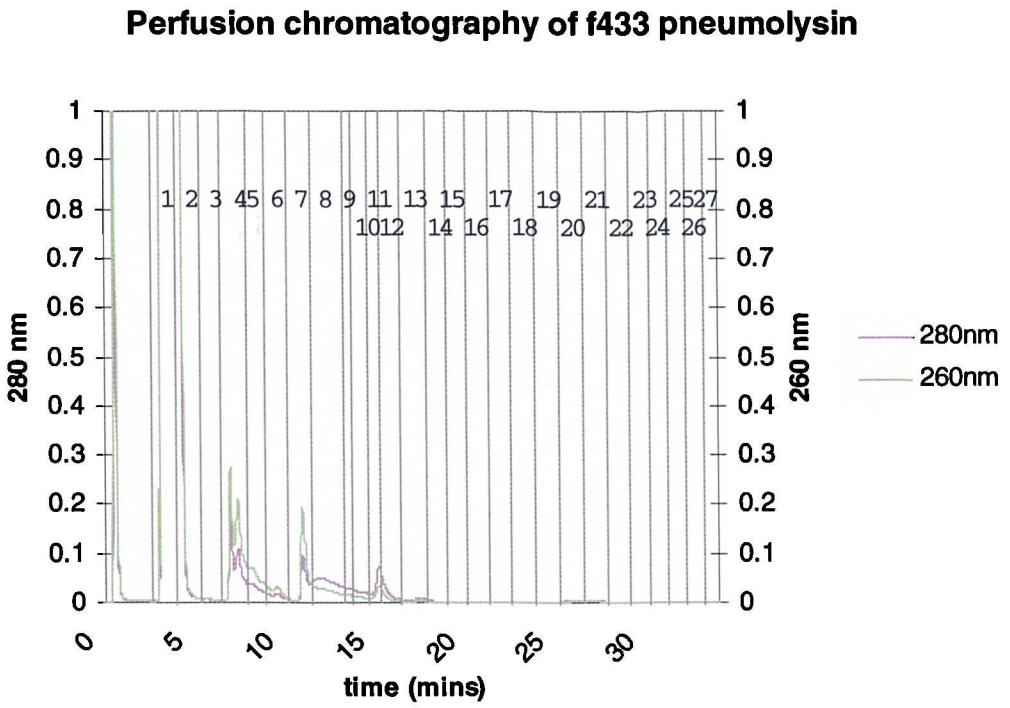


Figure 3-8

Purification of F433 pneumolysin using the BioCAD workstation and Metal Chelate Chromatography

Chromatography was carried out as described in Figure 3-7

fraction as a second test. Fractions 7-11 contained a significant amount of hemolytic activity of 1000-20 000 HU/ml.

Although the F433 mutant has greatly reduced hemolytic ability, the presence of pneumolysin can still be detected using this assay. The results are similar to those for the wild type.

This data, for both the wild and F433 type pneumolysin, confirmed the data obtained from the absorbance assay; that fractions 8-11 contained pneumolysin and were the fractions of highest purity of pneumolysin to be included when pooling the fractions.

SDS PAGE

It was important to determine if the fractions to be pooled were pure and so as a third test I measured the purity of each fraction by SDS PAGE. My data from the previous two tests indicated that fractions 1-7 and 12-15 would be unsuitable for inclusion in the final pooling and so I ran only fractions 8-11 from both wild and F433 on SDS PAGE. In figures 3-9 and 3-10 the ladder is present in lane 1. In Figure 3-12 lanes 2-5 contain fractions 8-11 from the wild type preparation and in Figure 3-13 lanes 2-5 contain fractions 8-11 from the F433 type preparation. Each lane contains a single band at the expected size of 53kDa and no other bands are observed indicating that all fractions are free of contaminants. As a result of observing that all fractions contain a highly purified single protein I concluded that all of the fractions were suitable for pooling.

Western Blot

I carried out a fourth and final test to confirm that the protein we had expressed and found to be hemolytic and highly purified was in fact pneumolysin. To do this I carried out a Western blot for pneumolysin. In Figure 3-11 and 3-12 the ladder is clearly visible in lane 1. In lane 2-6 fractions 8-11 are present respectively. In every lane a single band is observed which indicates that each fraction contains pneumolysin

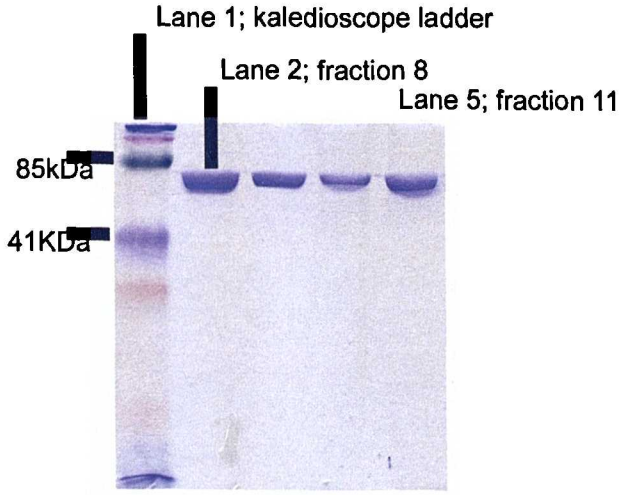


Figure 3-9

SDS PAGE gel of fractions 8-11 from wild pneumolysin purification

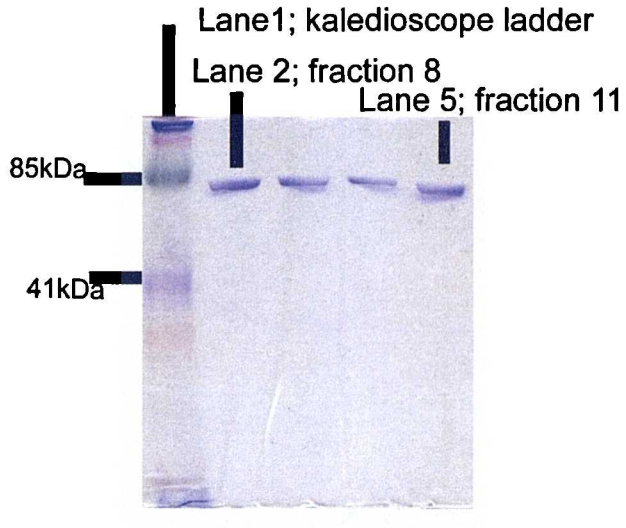


Figure 3-10

SDS PAGE gel of fractions 8-11 from F433 pneumolysin purification

3.2.2.4 Pooling and subsequent analysis

In the previous four tests I had concluded that fractions 8-11 contained less nucleic acid than protein and had significant hemolytic activity which indicated not only the presence of protein but that the protein is functionally active. Furthermore I had observed that fractions 8-11 contained no contaminants and confirmed the protein present was pneumolysin. Fractions 1-7 and 12-15 had been shown to be unsuitable based on the same tests. I therefore concluded that each of the fractions 8-11 of both wild and F433 type pneumolysin were suitable for pooling into the final preparation. I then performed some further quality controls on both stock pneumolysin types.

Absorbance assay

To confirm that the pooled fractions continued to have a higher 280 reading than 260 and that the protein had not aggregated upon pooling a further wavelength scan was conducted. The 260 value remained lower than the 280 value for both types of pneumolysin and the 320 reading remained negligible.

In order to determine the final concentration of pneumolysin in each sample the 280nm reading can also be used to estimate the concentration of pneumolysin by using the extinction co-efficient. Using this method wild type pneumolysin was estimated to be at a concentration of 0.129mg/ml and F433 type pneumolysin at 0.054mg/ml.

Bradford Assay

To confirm the estimated concentration obtained by using the extinction co-efficient, a Bradford assay was conducted on each sample. Wild type pneumolysin was found to have a concentration of 0.137mg/ml by this method while F433 pneumolysin was found to have a concentration of 0.065mg/ml. By averaging the concentration value obtained from the extinction coefficient with that from the bradford assay, I concluded that wild type pneumolysin had a concentration of 0.133mg/ml while the F433 pneumolysin had a concentration of 0.059mg/ml.

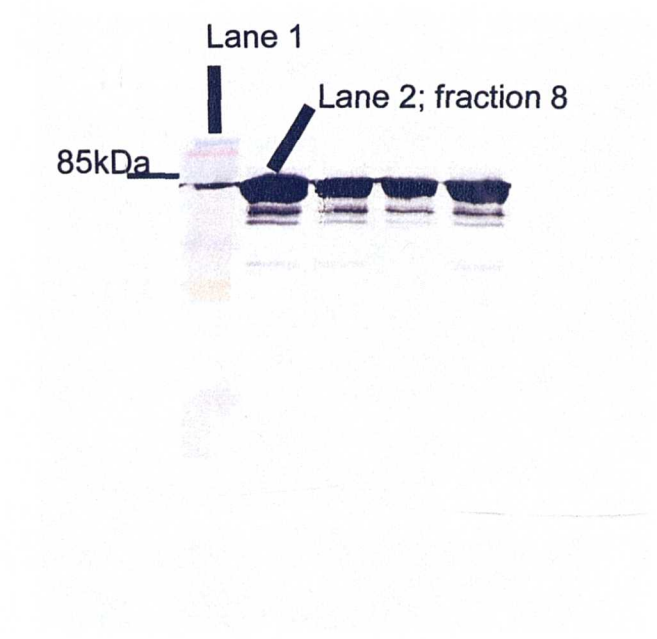


Figure 3-11

Western blot of gel of figure 3-9 (wild type pneumolysin)

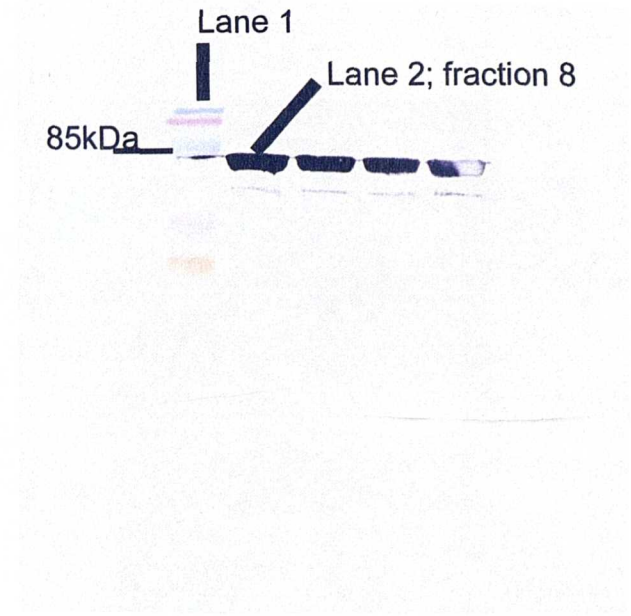


Figure 3-12

Western blot of gel of figure 3-10 (F433 pneumolysin)

Unit	EU/ml	Ng/ml	EU/mg	EU/100ng PLY	Pg LPS/100ng PLY
Wild PLY 0.133mg/ml	237	23.7	1781	0.17	17
F433 PLY 0.059mg/ml	419	41.9	7112	0.71	71

Figure 3-13

LPS levels in pneumolysin

The amount of endotoxin in each sample was measured using the LAL assay. The table above presents the average of three measurements using the LAL assay.

Hemolytic assay

In order to determine the hemolytic activity of the final pneumolysin preparations, hemolytic assays were performed. Wild type pneumolysin caused hemolysis to well 8 which corresponds to a hemolytic activity of 5120 HU/ml and correlates to an activity of 3.8×10^4 HU/mg of protein. The F433 type pneumolysin was found to cause hemolysis up to well 2 which corresponds to an activity of 80 HU/ml and correlates to an activity of 1.3×10^3 HU/mg.

Endotoxin Assay

Endotoxin levels were measured in pneumolysin samples. Figure 3-13 shows that wild type pneumolysin was found to have 237 EU/ml which corresponds to 17pg LPS per 100ng of pneumolysin. F433 pneumolysin had 419 EU/ml which corresponds to 71pg LPS per 100ng of pneumolysin.

3.2.3 Production and purification of wild type pneumolysin by Hydrophobic Interaction and Anion Exchange Chromatography

3.2.3.1 Chromatography

Hydrophobic Interaction Chromatography

In order to obtain purified wild type pneumolysin using the HIC and Anion Exchange system I prepared crude extracts of cells containing the pneumolysin protein. The optimal induction period had previously been ascertained in our laboratory and so I was not required to determine this. The results of the perfusion chromatography are shown in Figure 3-14. Fractions 1-4 contain the initial washes from the column as evidenced by the high 280 and 260 readings in these fractions. Fraction

5-8 contain a 'shoulder' of these washes and produce lower 280 and 260 readings. I selected fractions 5-8 for further purification.

Anion Exchange Chromatography

In order to remove the salt present in fraction 5-8 obtained from the HIC we dialysed the fractions overnight against PBS-NaCl prior to loading onto the anion exchange column for purification. The process of purification is displayed graphically in Figure 3-15. Fraction 9 clearly contains the only peak of protein. Previous work in my lab had demonstrated the reliability and predictability that this fraction would contain pneumolysin. The ninth fraction from each run of the BioCAD was pooled and concentrated using the minicom concentrators.

3.2.3.2 Quality controls

Wavelength Scan

In order to ensure that the 280nm reading was higher than the 260nm reading and that aggregates were minimal, a wavelength scan was performed upon the pneumolysin from the Anion Exchange column. The 260 reading was 0.161 while the 280 reading was 0.162. The 320 reading 0.067.

Using the 280 reading I was able to calculate the concentration of pneumolysin to be 1.23mg/ml.

SDS PAGE

To assess the purity of the preparation I ran a sample on an SDS PAGE gel. Figure 3-16 shows this gel and the previously prepared pneumolysin which acts as a positive control value and can be seen to run at a value of approximately 53kDa in lane 1 as judged by the purple (41kDa) and green bands (85kDa) of the ladder in lane 3. Within lanes 4 and 5 a single band is clearly visible that correlates

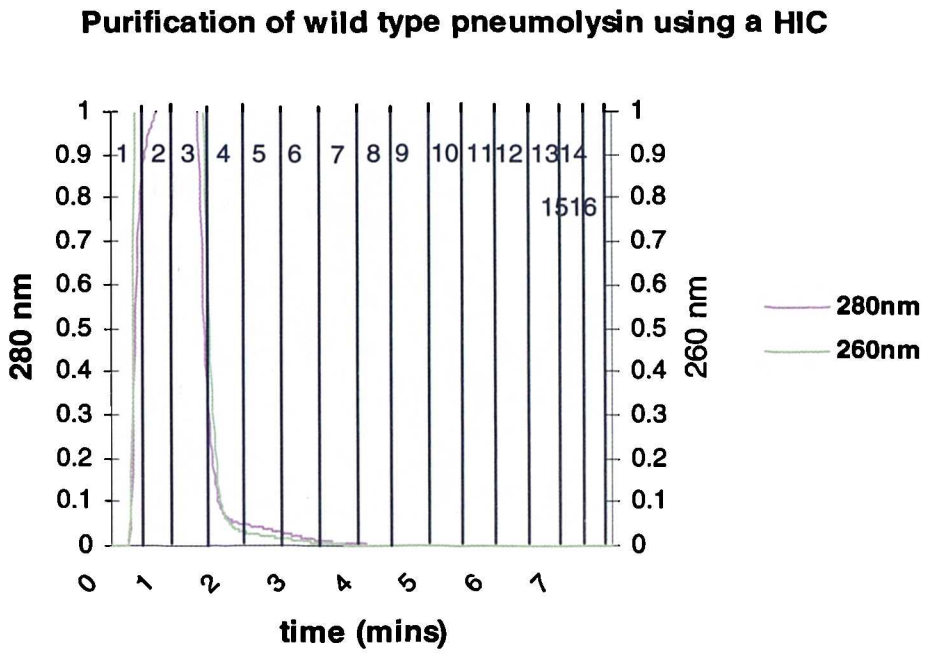


Figure 3-14

Purification of wild type pneumolysin using a Hydrophobic Interaction Column (HIC)

The E. coli strain XL10 containing the plasmid pKK2332 with the wild type pneumolysin gene was grown, induced and resuspended in PBS prior to disrupting with the cell disrupter. After centrifugation, the supernatant was loaded onto the hydrophobic interaction column on the BioCAD workstation. The process of purification is graphically displayed above; The red line correlates with the right hand axis and represents the 280nm reading which is representative of the amount of protein in the sample. The green line correlates with the left hand axis and represents the 260nm reading which is representative of the amount of nucleic acid in the sample. The plot is divided into 16 sections, each one correlating to a fraction from the BioCAD column.

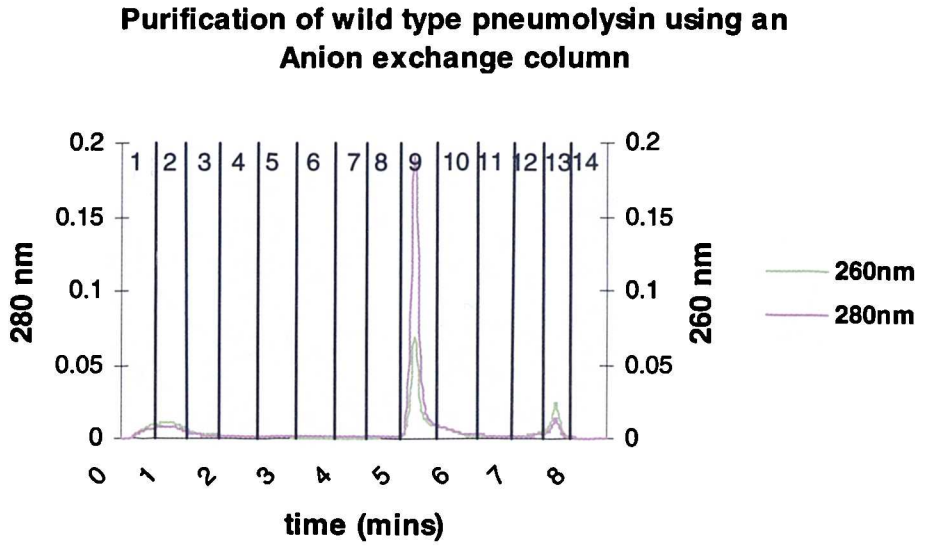


Figure 3-15

Purification of wild type pneumolysin using Anion Exchange Chromatography

Fraction 5-8 from the HIC purification were dialysed overnight at 4oC against PB-S and then loaded onto the Anion Exchange column on the BioCAD workstation. The process of purification is graphically displayed above; The red line correlates with the right hand axis and represents the 280nm reading which is representative of the amount of protein in the sample. The green line correlates with the left hand axis and represents the 260nm reading which is representative of the amount of nucleic acid in the sample. The plot is divided into 14 sections, each one correlating to a fraction from the BioCAD column.

with the position of a 53kDa protein on the ladder and is in exactly the same position as the pneumolysin control. This confirms the purity and identity of the protein prepared.

Hemolytic Assay

In order to determine the hemolytic activity of the final pneumolysin preparation a hemolytic assay was performed. Wild type pneumolysin caused hemolysis up to well 14 which correlates with an activity of 3.2×10^5 HU/ml which is equivalent to an activity of 2.6×10^5 HU/mg of protein.

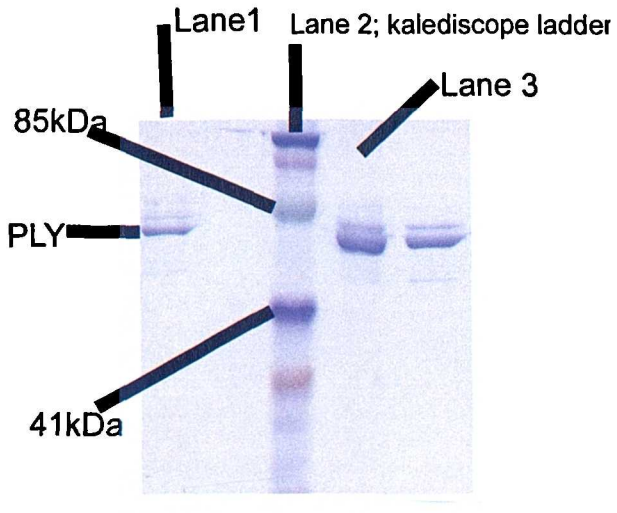


Figure 3-16

SDS PAGE gel of fraction 9 from Anion Exchange column

Lane 1 contains a positive control of pneumolysin. Lane 2 is blank. Lane 3 contains the ladder (kaleidoscope). Lanes 4 and 5 contain a different amount of the pneumolysin sample.

3.3 Discussion

In this chapter I cloned the F433 pneumolysin gene into the pET33b+ vector. I then expressed and purified wild and F433 type pneumolysin protein using the pET33b+ vector and metal chelate chromatography for use *in vitro*. Finally I used a pKK232 expression vector containing the wild type pneumolysin gene in conjunction with hydrophobic interaction and anion exchange chromatography to produce purified wild type pneumolysin for use *in vivo*.

The PCR reaction used to amplify the F433 gene from the plasmid stock utilised the primers 9Z and 9Y (see Figure 2-1). These primers were designed to include the BamH1 restriction site on the forward primer and the Sac1 restriction site on the reverse primer. The primers therefore added a BamH1 site to the beginning of the cloned gene and a Sac1 site to the end of the gene during PCR amplification and so Bam/Sac digestion was used throughout the cloning work. When I was cloning the F433 gene into pET33b+ I used the TOPO plasmid as an intermediate transfer plasmid to allow me to increase the success of cloning the PCR product. Insertion into the TOPO plasmid avoids the use of restriction sites and so I could insert blunt ended PCR product which increased the efficiency of the TOPO plasmid in taking up PCR product made it an attractive intermediate in this situation where limited PCR product was available.

When deciding which purification method to use to purify pneumolysin I examined the traditional method of producing pneumolysin directly from the whole pneumococcus by the method of Shumway *et al.* (Shumway and Klebanoff, 1971) who used an anion exchange purification column. This method has been modified including successful attempts to increase the yield (Kanclerski and Mollby, 1987). However production of pneumolysin by plasmid expression in *E. coli* has become the standard procedure due to the high yield this method affords. Purification of pneumolysin from *E. coli* was first carried out in 1986 by Paton *et al.* (Paton *et al.*, 1986) who continued to use a anion exchange column to purify the crude extract. In 1989 Mitchell *et al.* (Mitchell *et al.*, 1989)increased the yield and speed of the purification process by bringing a hydrophobic interaction column into the purification process. Purification of pneumolysin from *Bacillus subtilis* was attempted by Taira *et al.* (Taira *et al.*, 1989) however this method has not been developed further.

Furthermore, previous studies in my laboratory (J. Search, PhD Thesis, University of Glasgow, 2000) which had demonstrated that purification methods using a standard *E. coli* expression system and hydrophobic interaction and anion exchange purification columns yielded pneumolysin that had considerable endotoxin contamination. Therefore in light of all these previous studies, and given that my *in vitro* studies were potentially highly sensitive to endotoxin (due to the sensitivity of BMDC to LPS), it was necessary to identify a more rigorous purification method. I chose the His-tag purification method for purifying pneumolysin since we considered it to be the most appropriate to enable me to produce highly purified pneumolysin. Braun *et al.* (Braun *et al.*, 1999) had previously used the His tag purification method to purify pneumolysin and had demonstrated that the method yields pneumolysin of high purity. These authors used a similar pET vector to the one we used but they purified pneumolysin under denaturing conditions to avoid the problems of the formation of insoluble fractions. I chose not to use denaturing conditions with the pET vector since I considered ample protein to be present in the soluble phase. Braun *et al.* (Braun *et al.*, 1999)demonstrated that the pET purification method results in low endotoxin contamination.

After successfully cloning the F433 pneumolysin gene into the pET33b+ plasmid I carried out an optimisation experiment to determine the induction period that would utilise the maximum amount of pneumolysin in the soluble fraction. My data shows that the amount of pneumolysin in solution does reach a plateau (Figures 3-4 and 3-6) and my data also shows an increasing amount of pneumolysin in the insoluble fraction however the gels for the insoluble fractions do not portray a consistent amount of pneumolysin indicating that the boiling procedure used to liberate the insoluble pneumolysin into solution may not have been entirely successful for all fractions.

After determining the optimal conditions for inducing pneumolysin production I proceeded to purifying pneumolysin. The clear peak on the anion exchange chromatogram indicate the presence of the pneumolysin as expected. I chose fractions that had a higher protein content than nucleic acid content since fractions with high nucleic acid content may interfere with future *in vitro* studies. The western blots (see Figure 3-11 and 3-12) appeared to have a faint band below the main band of protein. The degradation is minimal and may be caused by enzymatic digestion of the crude preparation prior to purification by *E. coli* enzymes.

After pooling the fractions I analysed the final preparation. The differing concentrations of the final preparations of wild and F433 pneumolysin are the results of differing concentration of crude

protein loaded onto the column. The hemolytic activity of the final preparation of wild type pneumolysin is 3.8x 10^4 HU/mg. This is a surprisingly low value for the wild type toxin compared to other reports in the literature which range from 4x 10^4 HU/mg to 2x 10^6 HU/mg (Baba *et al.*, 2001; Kelly and Jedrzejas, 2000; Mitchell *et al.*, 1989; Paton and Ferrante, 1983). While it may be the case that the toxin prepared here does have a low hemolytic activity, I am also aware that the blood used within my lab may have some effect on the low hemolytic activity reported here since other results from my lab have yielded surprisingly low hemolytic activities. In addition the only other reported hemolytic activity of His tagged pneumolysin in the literature had a low hemolytic activity of 4x 10^4 HU/mg (Baba *et al.*, 2001).

Previous results in my laboratory (J. Search, PhD Thesis, University of Glasgow, 2000) had demonstrated that pneumolysin produced by isolation from *E. coli* and purification using HIC chromatography gave endotoxin levels of 200pg endotoxin per 100ng PLY. The endotoxin levels within my samples using metal chelate chromatography are lower than these. The difference in endotoxin levels present in the wild and F433 preparations is possibly the result of differing concentration of toxin. Two previous reports of purifying a CDC using metal chelate chromatography and measuring endotoxin contamination yielded endotoxin levels lower than mine. Braun *et al.* (Braun *et al.*, 1999) produced pneumolysin with 0.2pg endotoxin/100ng pneumolysin. Ito *et al.* (Ito *et al.*, 2003) produced seeligerilysin O (LSO) with endotoxin contamination of 0.01pg endotoxin/100ng of LSO. Both experimenters used subsequent purification procedures after metal chelate chromatography and this may account for the significantly lower endotoxin levels obtained compared to my values. Ito *et al.* (Ito *et al.*, 2003) used a Detoxi-Gel Endotoxin removing column and this would be a possible additional purification step to add to my procedure. Pneumolysin purified using the HIC and Anion Exchange column was found to have a hemolytic activity of 2.6x 10^5 HU/mg. This is ten fold higher than that obtained for the his-tagged toxin and compares with previous findings although remains lower than expected and further suggests a problem with the blood used in the hemolytic assay.

In this chapter the preparation of pneumolysin for use in future experiments was successful.

4 Assessing the T cell inhibitory ability of pneumolysin *in vitro.*

4.1 Introduction

Streptococcus pneumoniae is a pathogen which can cause a number of important diseases including pneumonia and meningitis. Like other pathogens (Banks and Rouse, 1992; Donelson *et al.*, 1998; Gebert *et al.*, 2003; Harnett and Harnett, 2001), *S. pneumoniae* may attempt to inhibit the immune response against it in order to allow colonisation of its host, to prolong host colonisation, or to enhance dissemination within the host. In the introduction I discussed the ways in which different pathogens manipulate aspects of the host response and the evidence that *S. pneumoniae* affects the innate immune response. Natural antibody, complement and CRP were all observed to be important in the defence against the pneumococcus (Boes *et al.*, 1998; Mold *et al.*, 2002). Furthermore, it was observed that *S. pneumoniae* was able to modulate components of the innate immune response such as complement, neutrophils and macrophages(Braun *et al.*, 1999; Cockeran *et al.*, 2002b).

The role of the adaptive immune response in defence against the pneumococcus was also discussed in the introduction. There the antibody produced by memory B cells (natural antibody) was shown to be important in initial defence against the pneumococcus and that this antibody was produced in a T cell independent manner (Kruetzmann *et al.*, 2003; Wu *et al.*, 2002). It was also observed that specific antibody is made to both polysaccharide and protein elements of the pneumococcus and is produced as a result of both colonisation and invasive disease (Rapola *et al.*, 2000). While this antibody does not apparently aid the resolution of current colonisation, it is observed to prevent re-infection (McCool *et al.*, 2002). Production of antibody to both polysaccharide and protein elements of the pneumococcus was shown to be T cell dependent (Khan *et al.*, 2004). Studies in nude mice demonstrated that the T cell has no role in defence against high dose pneumococcal infections (Winkelstein and Swift, 1975), however this model is potentially misleading since colonisation generally precedes infection.

Studies by Darji *et al.* (Darji *et al.*, 1997) had demonstrated that the pneumolysin related toxin, listeriolysin, was able to induce T cell anergy. Additionally, the study of Ferrante *et al.* (Ferrante *et al.*, 1984) demonstrated that pneumolysin was able to inhibit mitogen induced proliferation of human B and T cells. I therefore decided to further examine the effects of pneumolysin upon the

prime controller of the adaptive immune response, the CD4 T cell. I was interested to investigate the effect of pneumolysin upon antigen specific proliferation of murine T cells.

4.2 Results

4.2.1 The role of pneumolysin in inhibiting T cell proliferation in the context of the whole pneumococcus.

Examination of the *in vitro* effect of pneumolysin upon T cells in the context of the whole bacterium is a physiologically relevant model of T cell exposure to the pneumococcus. Given the recruitment of lymphocytes to the lung observed during pneumococcal infection (Kadioglu *et al.*, 2000), it is likely that the whole pneumococcus potentially encounters lymphocytes within the spleen and within the lung. I started to address the question of the effect of pneumolysin upon lymphocytes during pneumococcal infection by co-culturing *S. pneumoniae* expressing wild type pneumolysin, or an *S. pneumoniae* mutant that does not express pneumolysin (called PLY-), with lymphocytes. Initially it was necessary to optimise the culture conditions.

4.2.1.1 Optimisation of culture conditions

In co-culture systems, the lymphocytes may be exposed to a variety of toxic bacterial derived immunomodulatory factors. I therefore wanted to determine a ratio of bacteria to lymphocytes, and culture period, which would allow me to compare the differential effect of the presence or absence of pneumolysin in the bacteria.

Optimising the concentration of bacteria and duration of culture.

I cultured D39 and PLY- *S. pneumoniae* with lymphocytes for 24 hours and measured lymphocyte viability in order to determine if a 24 hour incubation time resulted in an effect on lymphocyte viability. I found that after 24 hours bacterial cultures were in decline for both D39 and PLY- as evidenced by declining numbers of bacteria (Figure 4-1 & 4-2). At 24 hours lymphocyte viability was assessed by trypan blue exclusion (Figure 4-3). Lymphocytes that had not been incubated with bacteria were the positive control and the number of viable lymphocytes in these cultures after 24 hours was taken as 100% viability. Lymphocytes incubated with any of the three concentrations of

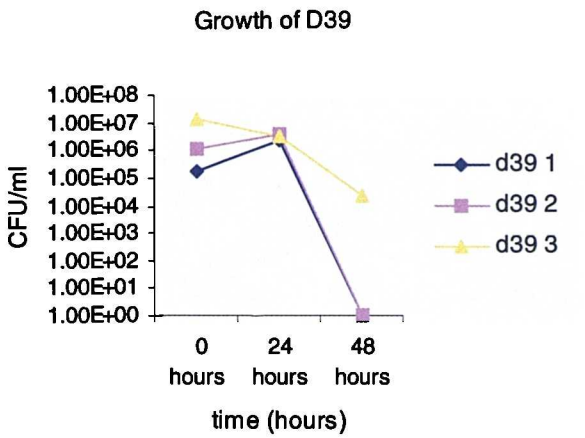


Figure 4-1

Growth of D39 when incubated with lymphocytes.

At 0 hours D39 *S. pneumoniae* was inoculated at a final concentration of 2.5 x 10^5 (blue line - d39 1), 1 x 10^6 (pink line - d39 2) and 1 x 10^7 (yellow line - d39 3) CFU/ ml into DO11.10 lymph node single cell suspension cultures. Bacteria viability was measured by viability counting at 24 and 48 hours.

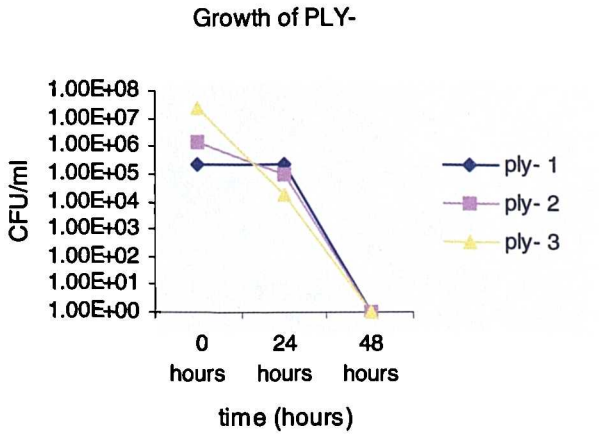


Figure 4-2

Growth of PLY- when incubated with lymphocytes.

At 0 hours the *S. pneumoniae,* pneumolysin knockout, PLY-, was inoculated at a final concentration of 2.5 x 10^5 (blue line - ply- 1), 1 x 10^6 (pink line - ply- 2) and 1 x 10^7 (yellow line - ply- 3) CFU/ml into DO11.10 lymph node single cell suspension cultures. Bacteria viability was measured by viability counting at 24 and 48 hours.

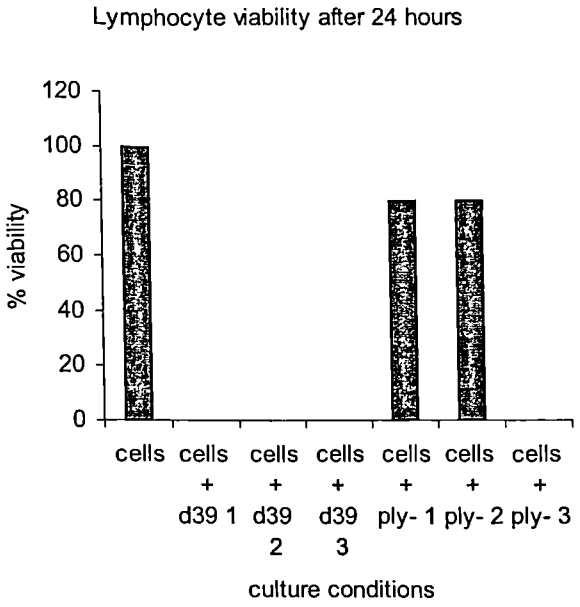


Figure 4-3

Viability of lymphocytes incubated with *S. pneumoniae* for 24 hours

Lymph node single cell suspensions from DO11.10 mice were incubated with D39 and PLY- *S. pneumoniae* as outlined in Figure 4-1 & 4-2. Lymphocyte viability was measured by trypan blue exclusion after 24 hours. The control of cells that were incubated without bacteria were taken to be 100% viable and the cells incubated with bacteria were compared with this. Cultures initially inoculated with 2.5 x 10^5 CFU/ml bacteria are given the suffix '1' in the chart above. Cultures initially inoculated with 1 x 10^6 CFU/ml bacteria are given the suffix '2' in the chart above. Cultures initially inoculated with 1 x 10^7 CFU/ml bacteria are given the suffix '3' in the chart above.

D39 were found to be dead while lymphocytes incubated with the lower two inoculums of the PLY- were found to be 80% viable however the higher concentration of PLY- bacteria had killed 100% of the lymphocytes.

I therefore concluded that 24 hours is too long a period of time to incubate live *S. pneumoniae* with lymphocytes since the lymphocyte viability decreases dramatically within this period even with bacteria which do not express pneumolysin.

In order to determine a suitable length of time for bacterial growth that did not alter lymphocyte viability, I measured the effect of growing *S. pneumoniae* on lymphocyte viability over a 24 hour period. (Figures 4-4, 4-5, 4-6). I measured bacteria numbers by viability counting at each time point and found that the growth curves of D39 and PLY- were very similar as demonstrated in Figures 4-4 and 4-5. The lower starting concentration of bacteria for both D39 and PLY- (starting at 5×10^4 CFU/ml) peaked after 8 hours at a value of ~1×10^8 CFU/ml (Figure 4-4). The higher starting concentrations of bacteria (starting at 1×10^6 CFU/ml) peaked after 4 hours at 1×10^8 CFU/ml and then rapidly declined (Figure 4-5).

Figure 4-6(A) demonstrates that after four hours, lymphocytes incubated with the lowest concentration of either D39 or PLY- had not decreased in viability compared to the control lymphocytes in medium alone. After the same four hour period, however, the lymphocytes incubated with the higher concentration of D39 or PLY- had decreased dramatically in viability with almost 100% dead. By the eight hour time point (Figure 4-6B) a 50% decrease in lymphocyte viability was evident relative to the control for the lymphocytes incubated with the lower concentration of D39. A similar decrease was not observed for the lymphocytes incubated with the lower PLY- concentration of bacteria which maintained nearly 100% viable cells. Unsurprisingly, the higher concentration of both bacteria strains still had 100% dead cells. As shown in Figure 4-6(C) and 4-6(D) at the later time points of 18 and 24 hours the trend of decreasing lymphocyte viability continues for all the cell cultures where bacteria are present. These results suggested that the higher initial concentration of bacteria was not useful for further studies since lymphocytes die within the first four hours of being cultured with bacteria at this initial concentration. After 4 hours lymphocytes incubated with the lower concentration of either bacteria are still alive yet, after 8 hours lymphocyte viability decreases. I concluded that 4 hours was the optimal time for bacterial co-culture with the

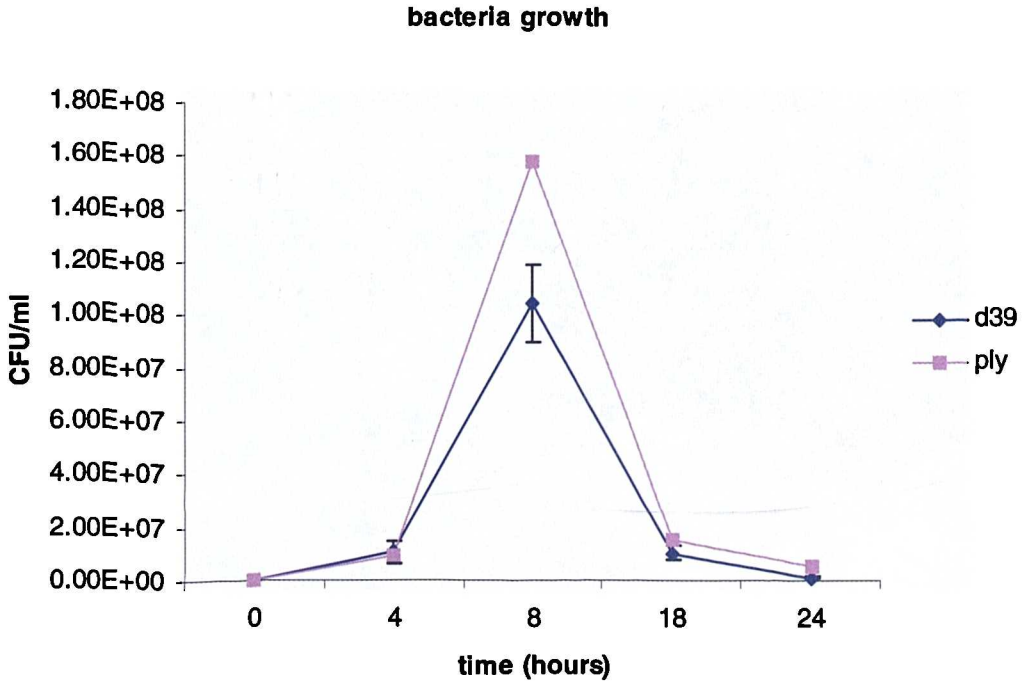


Figure 4-4

Growth of *S. pneumoniae* strains in the presence of lymph node cells

At 0 hours D39 *S. pneumoniae* and the *S. pneumoniae* mutant PLY- were inoculated at a final concentration of 5 x 10^4 CFU/ml into DO11.10 lymph node single cell suspension cultures. At 0, 4, 8, 18 and 24 hours the number of bacteria were measured by bacteria viable counting. The blue line represents the growth curve of D39 while the pink line illustrates the growth of PLY-.

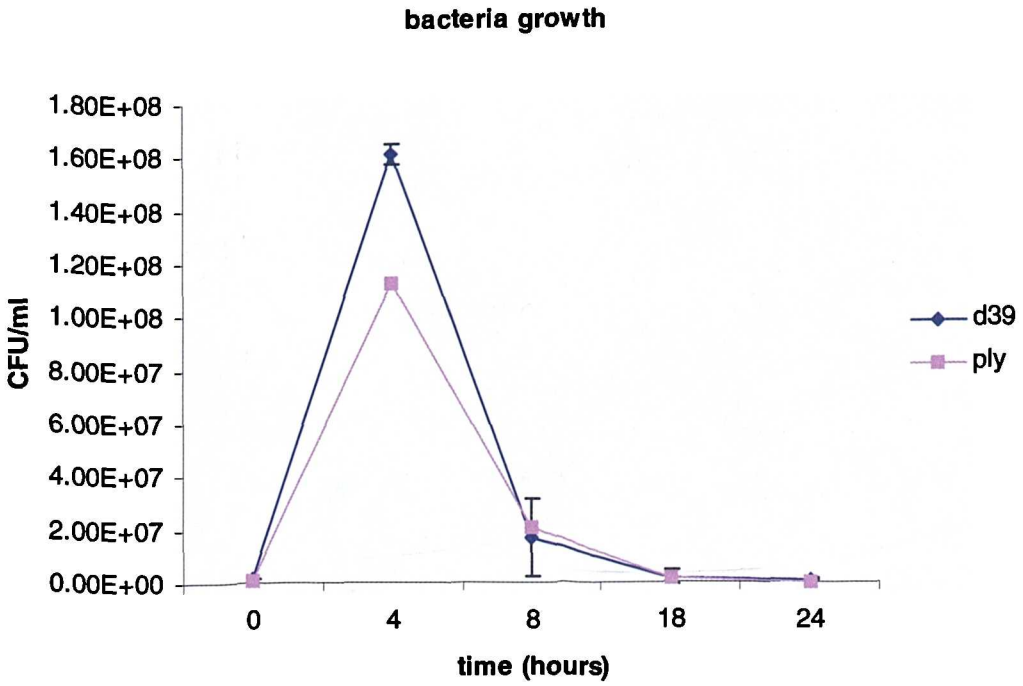


Figure 4- 5

Growth of *S. pneumoniae* strains in the presence of lymph node cells

At 0 hours D39 *S. pneumoniae* and the *S. pneumoniae* mutant PLY- were inoculated at a final concentration of 1 x 10^6 CFU/ml into DO11.10 lymph node single cell suspension cultures. At 0, 4, 8, 18 and 24 hours the number of bacteria were measured by bacteria viable counting. The blue line represents the growth curve of D39 while the pink line illustrates the growth of PLY-.

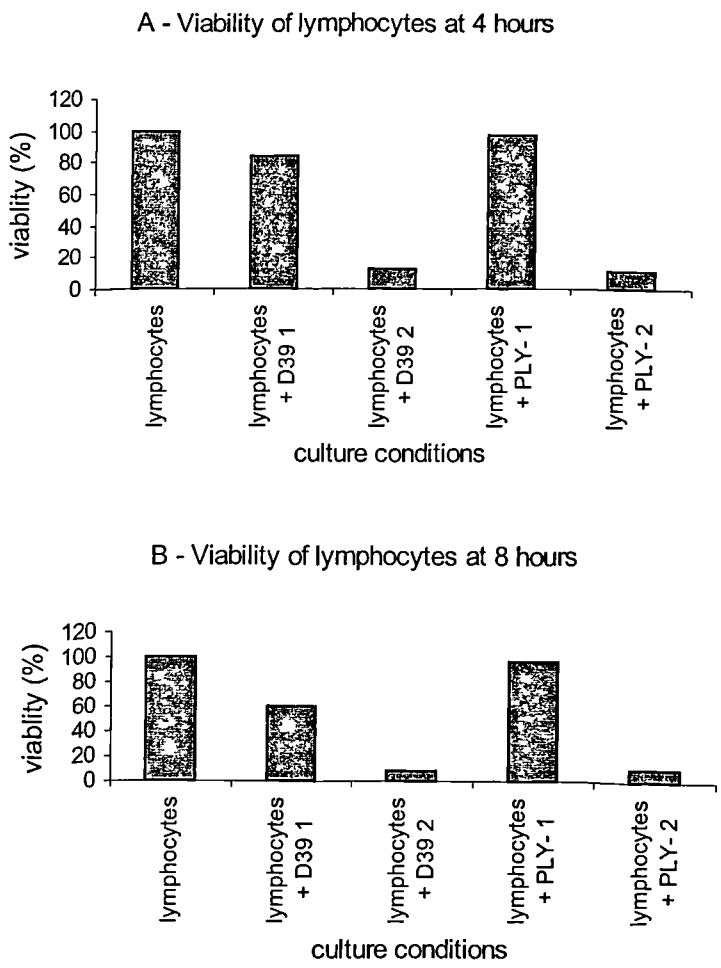
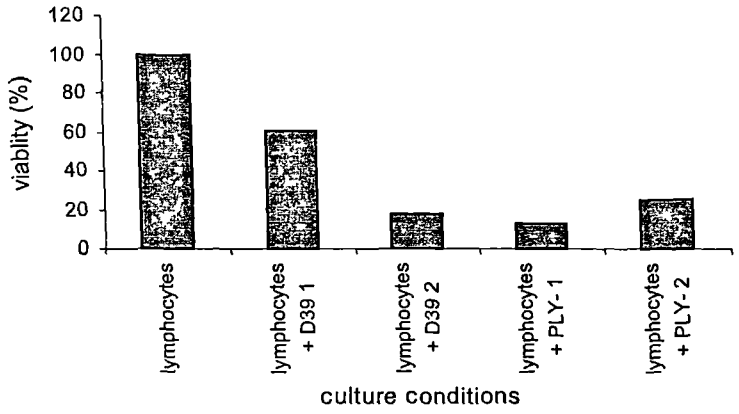


Figure 4-6

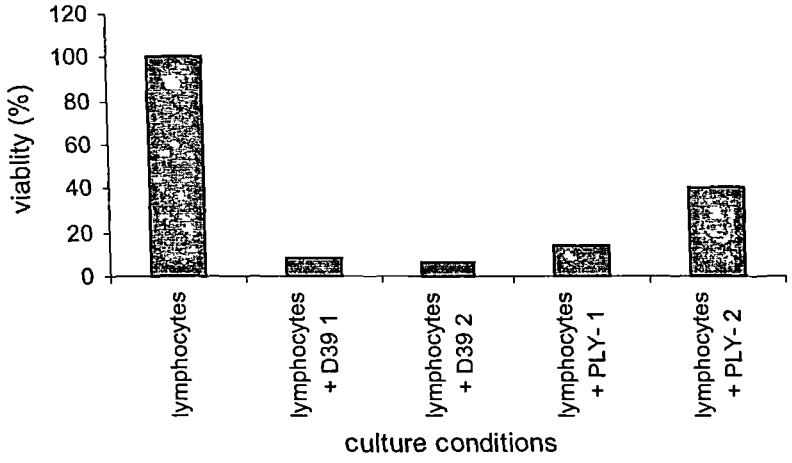
Viability of lymphocytes cultured with *S. pneumoniae*

At 0 hours D39 *S. pneumoniae* and the *S. pneumoniae* mutant PLY- were inoculated into DO11.10 lymph node single cell suspension cultures. At 4 (A), 8 (B), 18 (C) and 24 (D) hours the viability of the lymphocytes was assessed by trypan blue exclusion. Cultures initially inoculated with 5 x 10^4 CFU/ml bacteria are given the suffix '1' in the charts i.e. "D39 1" indicates inoculation with 5 x 10^4 CFU/ml. Cultures initially inoculated with 1 x 10^6 CFU/ml bacteria are given the suffix '2' in the charts.

C -Viability of lymphocytes at 18 hours



D - Viability of lymphocytes at 24 hours



lymphocytes before lymphocyte viability began to decrease as a result of non-pneumolysin mediated effects.

4.2.1.2 Determining the effect of pneumolysin upon lymphocyte proliferation in the context of the whole pneumococcus

Having established that an initial inoculum of 5 x 10^4 CFU/ml of *S. pneumoniae,* incubated for 4 hours in cultures of lymph node cells did not decrease lymphocyte viability I was able to proceed in testing the effect of the presence or absence of pneumolysin in this context upon the proliferation of antigen specific T cells. I did this by utilising the Tg mouse strain DO11.10 (for details see Material and Methods) in conjunction with the wild type D39 *S. pneumoniae* and the pneumolysin knockout mutant of *S. pneumoniae,* PLY-.

S. pneumoniae does not affect T cell proliferation.

I inoculated lymph node cultures prepared from the DO11.10 mice with D39 and PLY-. After 4 hours growth, bacteria were killed using antibiotics (see Material and Methods for details). This prevented any further bacterial growth, and thereby prevented the toxic effects associated with bacterial growth beyond 4 hours. Bacteria had reached a CFU/ml of 1x 10^6 at the time of adding antibiotics (data not shown). The cultures were incubated for a further 6 hours prior to lymphocyte viability being assessed by trypan blue exclusion immediately prior to setting up proliferation assays. As can be seen from Figure 4-7 at the end of the incubation period there is little appreciable difference in viability between the lymphocytes cultured with bacteria and those that were not. The proliferation assay (Figure 4-8) illustrates that there was no significant difference in the degree of thymidine incorporation between the control of untreated lymph node cells stimulated with ovalbumin compared to lymph node cells that had been incubated with D39 or PLY- strains of *S. pneumoniae* and then stimulated with ovalbumin. Therefore pre-incubation with or without pneumolysin in the context of the whole bacteria, in these cultures, did not affect the ability of T cells to proliferate in response to subsequent antigen stimulation. The results presented are representative of three experiments.

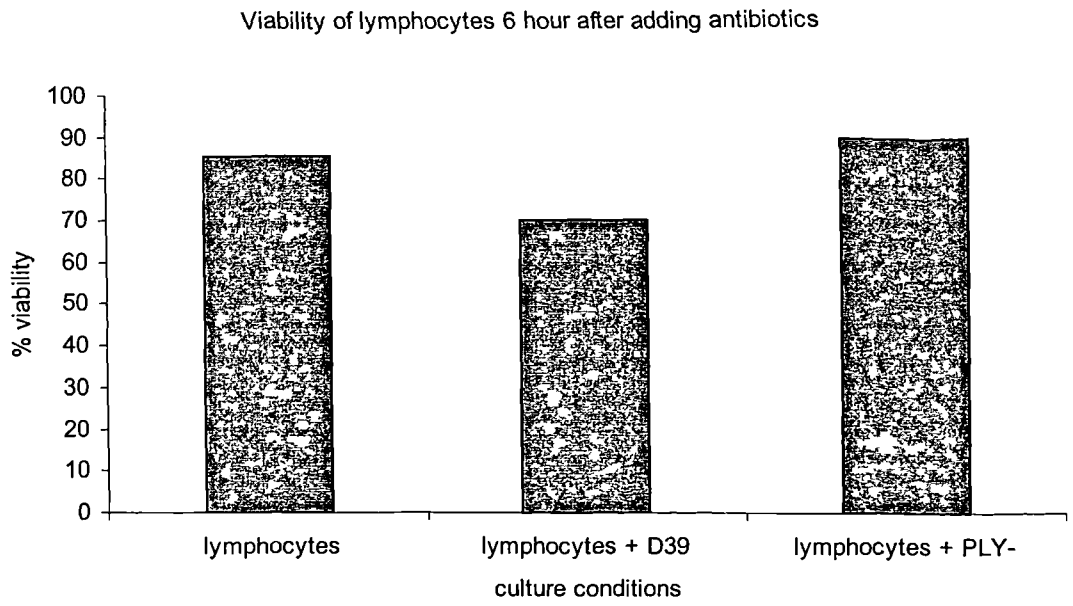


Figure 4-7

The viability of lymphocytes immediately before setting up proliferation assay

Lymph node single cell suspensions were prepared from DO11.10 mice and were inoculated with 1 x 10^5 CFU/ml of D39 or PLY-. After four hours incubation antibiotics were added to the cultures to kill the bacteria. After a further 6 hours the viability of the lymphocytes was assessed by trypan blue exclusion. The results are shown in the chart above. Lymphocytes that were cultured without bacteria are labelled as 'lymphocytes'. Lymphocytes that were cultured with D39 or PLY- are labelled accordingly. Lymphocytes alone were taken as 100% viable and used as the baseline to compare the other culture conditions to.

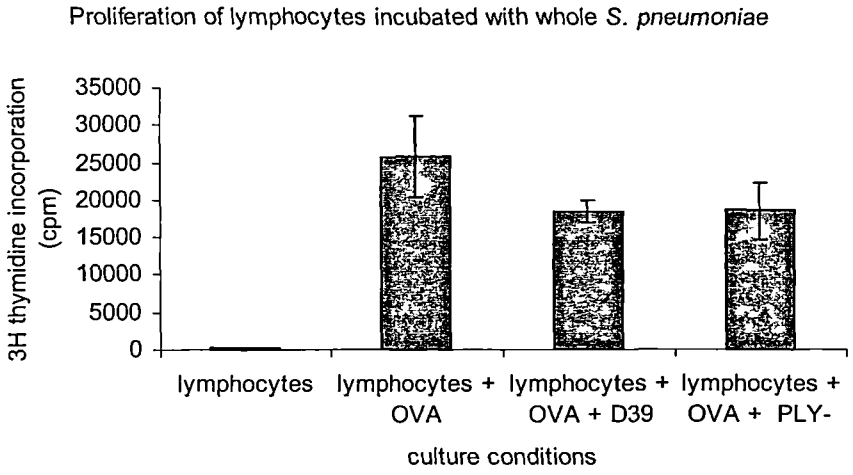


Figure 4-8

Proliferation of lymphocytes cultured with *S. pneumoniae*

After incubation with *S. pneumoniae* (see Figure 4-7), lymphocytes were washed and put into thymidine incorporation assays with 1mg/ml ovalbumin and thymidine incorporation was measured at 72 hours. Lymphocytes that had not been exposed to bacteria are labelled as 'lymphocytes' or 'lymphocytes + OVA' and act as controls. Lymphocytes cultured with D39 prior to the proliferation assay are labelled 'lymphocytes + OVA + D39'. Lymphocytes cultured with PLY- prior to the proliferation assay are labelled 'lymphocytes + OVA + PLY-'.

Hydrogen peroxide is released by *S. pneumoniae* and is responsible for lymphocyte death.

There remained the possibility that pneumolysin was not present at a high enough concentration in the above system and therefore did not exert any effect. Therefore, to allow an increase in the pneumolysin concentration without affecting lymphocyte viability, I attempted to neutralise any other toxic components that kill lymphocytes when the bacteria were used at higher concentrations or longer culture periods. I had observed a change in pH of the medium when bacteria were grown with lymphocytes and I therefore supplemented the medium with hepes buffer. Furthermore, I proposed that hydrogen peroxide may be being released by *S. pneumoniae* which may adversely affect lymphocyte viability. In order to assess the effects of pH change and hydrogen peroxide release upon lymphocyte viability, I cultured lymphocytes in medium supplemented with hepes buffer, catalase, or both. I used catalase to neutralise hydrogen peroxide.

In this experiment I used a starting concentration of bacteria of $3x\ 10^7$ CFU/ml of D39 and $5x\ 10^5$ CFU/ml for PLY-. After 4 hours both D39 and PLY- recorded a CFU/ml of $5x\ 10^7$ CFU/ml. Clearly this was a surprising result given the difference in initial bacterial concentrations between D39 and PLY-. Bacteria were killed at this 4 hour time point and after a further 12 hours lymphocyte trypan blue exclusion (Figure 4-9) indicated that in cultures of medium alone, 80% of lymphocytes were viable. Lymphocytes exposed to D39 or PLY- in un-supplemented medium had no viable cells. In cultures supplemented with catalase, lymphocytes alone did not show any decrease in viability compared to the control of medium alone. For lymphocytes exposed to D39 in medium supplemented with catalase 30% of cells were viable. For lymphocytes exposed to PLY- in medium supplemented with catalase 60% of cells were viable. Therefore catalase is able to neutralise some, but not all, of the toxic products from *S. pneumoniae* produced in co-culture. In cultures exposed to D39 or PLY- in medium supplemented with catalase and hepes, 80% of cells were viable. Therefore catalase and hepes used in combination are able to fully neutralise all of the toxic components released upon co–culture of lymphocytes and *S. pneumoniae*.

To confirm the results from trypan blue exclusion and to utilise a more rapid method for assessing lymphocyte viability I used annexinV / propidium iodide staining and flow cytometry. I used this on the samples from the experiment above (Figure 4-10). The data obtained by this method was

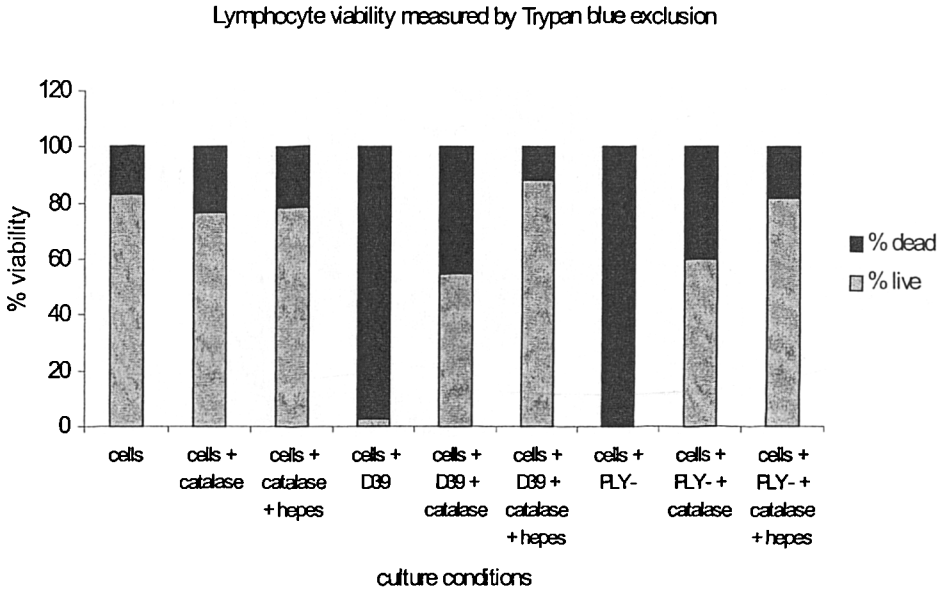


Figure 4-9

The effect of hydrogen peroxide on lymphocytes cultured with *S. pneumoniae*

Cultures of lymph node cells prepared from BALB/c mice in complete RPMI were inoculated with 3 x 10^7 CFU/ml D39 or 5 x 10^5 CFU/ml PLY-. Into additional identical cultures, catalase alone, or catalase and hepes, was added. The bacteria were incubated for 4 hours then antibiotics were added to kill the bacteria. After a further 12 hours lymphocyte viability was measured by trypan blue exclusion and the results are shown above.

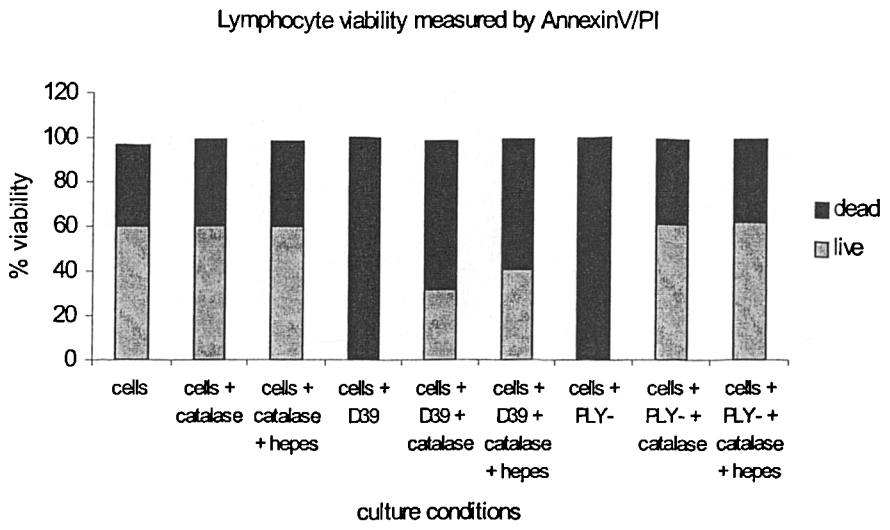


Figure 4-10

The effect of hydrogen peroxide on lymphocytes cultured with *S. pneumoniae*

Cultures of lymph node cells prepared from BALB/c mice in complete RPMI were inoculated with 3 x 10^7 CFU/ml D39 or 5 x 10^5 CFU/ml PLY-. Into additional identical cultures catalase alone, or catalase and hepes, was added. The bacteria were incubated for 4 hours then antibiotics were added to kill the bacteria. After a further 12 hours lymphocyte viability was measured by annexinV/PI.

identical in pattern compared to the trypan blue data although the actual percentage of live cells in each sample was generally 20% less than when using trypan blue.

The results above indicated that hydrogen peroxide was being released by the bacteria and was toxic to the lymphocytes. The results also demonstrated that the pH change in the medium was toxic to the lymphocytes. By neutralising the hydrogen peroxide and buffering the medium, a higher concentration of *S. pneumoniae* could be used before any decrease in lymphocyte viability occurred. Increasing the bacteria concentration may increase the concentration of pneumolysin. The results of these experiments are representative of two experiments

Pneumolysin does not appear to affect T cell proliferation when present as a component of the whole pneumococcus and examined within these experimental systems.

As my previous data demonstrated that bacterially derived hydrogen peroxide was toxic for lymphocytes but that catalase in the medium could neutralise this, I again examined the effect of pneumolysin upon T cell proliferation. I utilised a higher concentration of bacteria than had been used in the experiment of Figure 4-7 and 4-8.

I cultured lymph node cells in the presence of *S. pneumoniae* and measured the growth of the bacteria. After 4 hours bacteria were counted and found to be at a CFU/ml of $5x\ 10^7$. Lymphocyte viability was then assessed (Figure 4-11). In medium alone lymph node cultures, viability was 55%. Culturing lymphocytes with bacteria did not decrease lymphocyte viability since 55% of lymphocytes were alive in cultures containing bacteria.

I then used the treated lymphocytes in a proliferation assay to compare the effects of pretreating the lymphocytes with bacteria that contained or lacked pneumolysin (Figure 4-12). As expected, the unstimulated cells did not proliferate throughout the 72 hour time period examined. Untreated cells stimulated with ovalbumin proliferated to 4×10^4 cpm within the first 48 hours rising to 2×10^5 cpm after 72 hours. Cells pre-treated with D39 proliferated to 1.7×10^5 over 72 hours while those pre-treated with PLY- proliferated to 1.8×10^5. There is little difference between treated and untreated

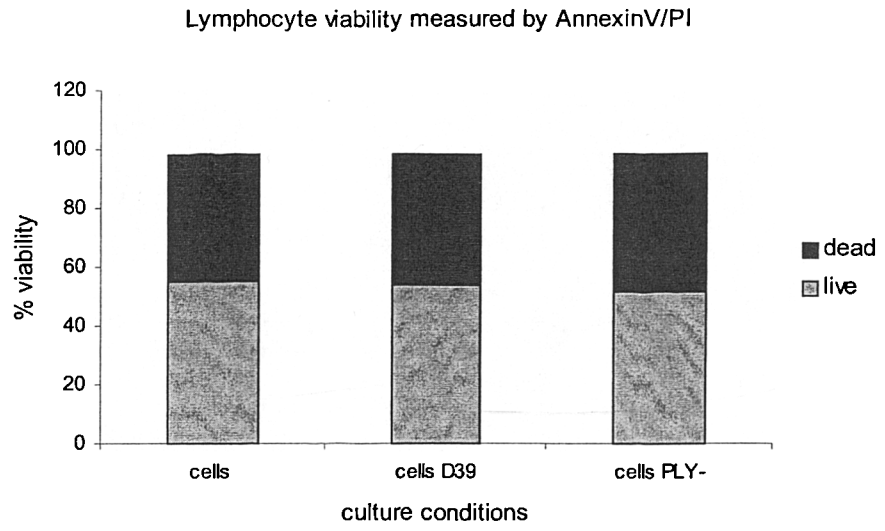


Figure 4-11

The effect on lymphocyte viability of growing *S. pneumoniae* with lymphocytes in the presence of catalase and hepes.

D39 and PLY– were inoculated to a CFU/ml of 5 x 10^4 in cultures of single cell suspensions made from DO11.10 lymph nodes containing catalase and hepes. After 4 hours bacteria were killed using antibiotics and after a further 12 hours the viability of the lymphocytes was assessed using the Annexin V/PI assay.

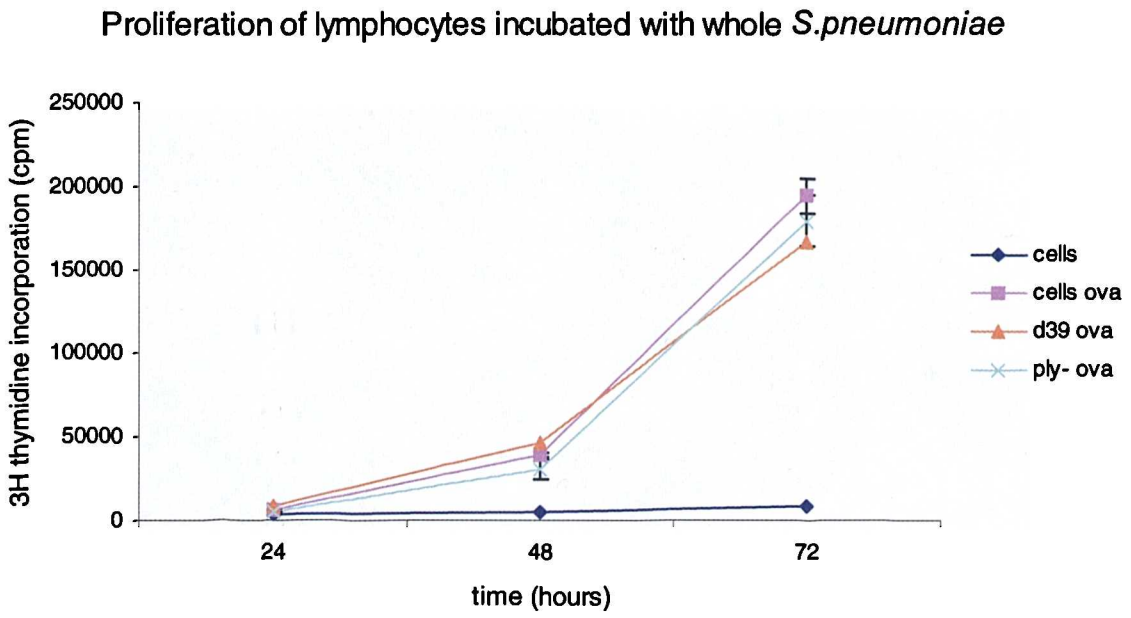


Figure 4-12

Proliferation of lymphocytes cultured with *S. pneumoniae*.

Lymph node cultures from DO11.10 mice were set up and treated as outlined in figure 4-11. After washing the cells in RPMI, they were set up in a proliferation assay and stimulated with 1mg/ml ovalbumin and proliferation monitored over 24, 48 and 72 hours by thymidine incorporation. Unstimulated cultures are marked as 'cells' and are represented by a blue line. Cultures stimulated with ovalbumin are marked as 'cells ova' and are represented by a pink line. Cultures of cells previously treated with D39 and now stimulated with ovalbumin are denoted as 'D39 ova' and represented as a yellow line. Cultures of cells previously treated with PLY- and now stimulated with ovalbumin are denoted as 'PLY- ova' and represented as a turquoise line.

lymphocytes upon stimulation and certainly very little difference between the lymphocytes pre-treated with D39 and those pre-treated with PLY-. The results presented are representative of two experiments.

Therefore, despite the increased concentration of bacteria present in the cultures, no observable effect upon proliferation, resulting from the presence of pneumolysin, was observed using this system. This is potentially due to the experimental system used however this point will be discussed further later.

4.2.2 The effect of purified pneumolysin on the T cell response

Although pneumolysin had not been observed to inhibit lymphocyte proliferation in the context of the whole bacterium, I had concluded that the intrinsic difficulties involved in conducting these assays meant that, while my results were accurate in terms of the experiments conducted they had not yielded a conclusive result regarding the effect of pneumolysin upon murine T cell proliferation. Ferrante *et al.* (Ferrante *et al.*, 1984) had utilised purified pneumolysin toxin to investigate the effects of pneumolysin on lymphocytes and so I decided to pursue this line of enquiry.

4.2.2.1 The effect of pneumolysin upon T cell proliferation and cytokine production.

As outlined in the introduction, proliferation and cytokine production are useful indicators of T cell function.

Purified wild type pneumolysin inhibits antigen driven proliferation and F433 mutant pneumolysin does not.

I first of all measured the effect of purified wild type pneumolysin and the non lytic mutant F433 pneumolysin upon murine derived antigen specific T cell proliferation using lymph node single cell suspensions from naive DO11.10 mice.

Lymph node cells that did not receive antigen did not proliferate throughout the 72 hour period. Figure 4-13A illustrates that lymph node cells that were stimulated with ovalbumin had a detectable increase in the level of thymidine incorporation relative to unstimulated cells by 24 hours however this difference becomes more evident by 48 hours when the stimulated cells have a cpm value of 3.7×10^4 while the unstimulated cells have a cpm under 1000. By 72 hours the stimulated cells have a cpm of 1.3×10^5 while the unstimulated cells remain under 1000 cpm. It is therefore clear that antigen driven proliferation of the lymphocytes is taking place.

As can be clearly seen in Figure 4-13A pneumolysin inhibited antigen driven proliferation. Inhibition of proliferation was achieved most effectively with the highest dose of wild type pneumolysin and was first evident after 48 hours when lymph node cells stimulated with ovalbumin and treated with 100ng/ml of wild type pneumolysin have a cpm of 4000 compared to control cells stimulated with ovalbumin alone that have a cpm of 3.7×10^4 at the same time point. However, the inhibitory effect of 100ng/ml wild type pneumolysin was most evident after 72 hours when the pneumolysin treated cells stimulated with ovalbumin have a cpm of 6000 while the untreated cells stimulated with ovalbumin have a value of 1.3×10^5 cpm; this represents a 95% inhibition. 70ng/ml and 40ng/ml wild type pneumolysin had a similar, but not quite as dramatic, inhibitory effect as 100ng/ml resulting in values of 1.4×10^4 and 2.5×10^4 cpm after 72 hours respectively. 10ng/ml was the lowest concentration of pneumolysin used and resulted in the least inhibition; after 72 hours lymph node cells stimulated with ovalbumin in the presence of 10ng/ml pneumolysin proliferated to a cpm of 1×10^5 cpm.

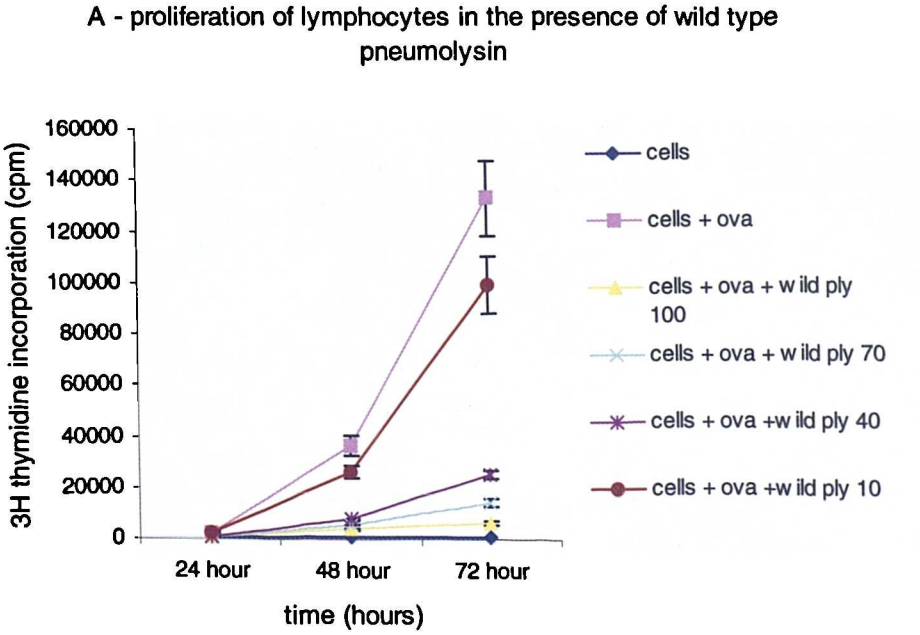
From this data it can be observed that wild type pneumolysin inhibits the proliferation of antigen driven T cell proliferation in a dose dependent manner.

I also tested the effect of the pneumolysin mutant 'F433' upon T cell proliferation within the same assay. Therefore the stimulated and unstimulated controls were the same as outlined above. Figure 4-13B shows that in the presence of 100, 70, 40 and 10 ng/ml F433 pneumolysin ovalbumin stimulated lymph node cells proliferated to a similar level as the stimulated control cells.

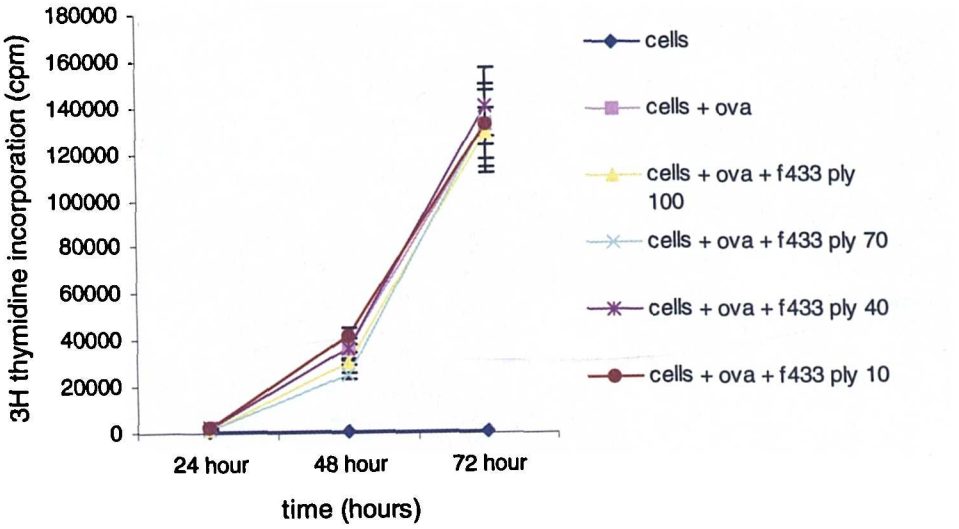
Figure 4-13

Proliferation of murine antigen specific T cells in the presence of pneumolysin

Cultures of single cell suspensions from DO11.10 mice were set up in a proliferation assay and were stimulated with 1mg/ml ovalbumin. Pneumolysin was added simultaneously with the ovalbumin at a final concentration of 10 (brown line), 40 (purple line), 70 (turquoise line) or 100 (yellow line) ng/ml. Proliferation was measured by thymidine incorporation at 24, 48, and 72. Unstimulated cells are indicated by a blue line. Stimulated but untreated cells are indicated by a pink line. Graph A represents the proliferation of lymphocytes in the presence of wild type pneumolysin. Graph B represents the proliferation of lymphocytes in the presence of F433 pneumolysin.



B - proliferation of lymphocytes in the presence of F433 pneumolysin



This data shows that the F433 mutant pneumolysin does not inhibit antigen specific T cell proliferation.

Cells that did not receive antigen did not proliferate in the presence of any dose of either wild type or f433 pneumolysin and therefore I could conclude that pneumolysin was not exhibiting a mitogenic effect within these experiments. Furthermore upon repetition of this experiment 3 times, similar results were obtained every time.

From all the data presented above we were able to conclude that wild type pneumolysin inhibits murine antigen driven T cell proliferation and F433 pneumolysin does not.

Pneumolysin inhibits cytokine production by T cells.

Having observed that pneumolysin was able to inhibit T cell proliferation I was interested to discover whither pneumolysin was able to inhibit the production of cytokines from T cells. I measured the production of a variety of T cell cytokines from antigen specific lymphocytes stimulated with ovalbumin in the presence or absence of pneumolysin. IL-2 is the major T cell growth factor and so we measured it to allow me to determine if there was a correlation between inhibition of proliferation and IL-2 production. As further indicators of CD4 T cell function I also measured IFN-g since this is produced as part of the T cell response to antigen.

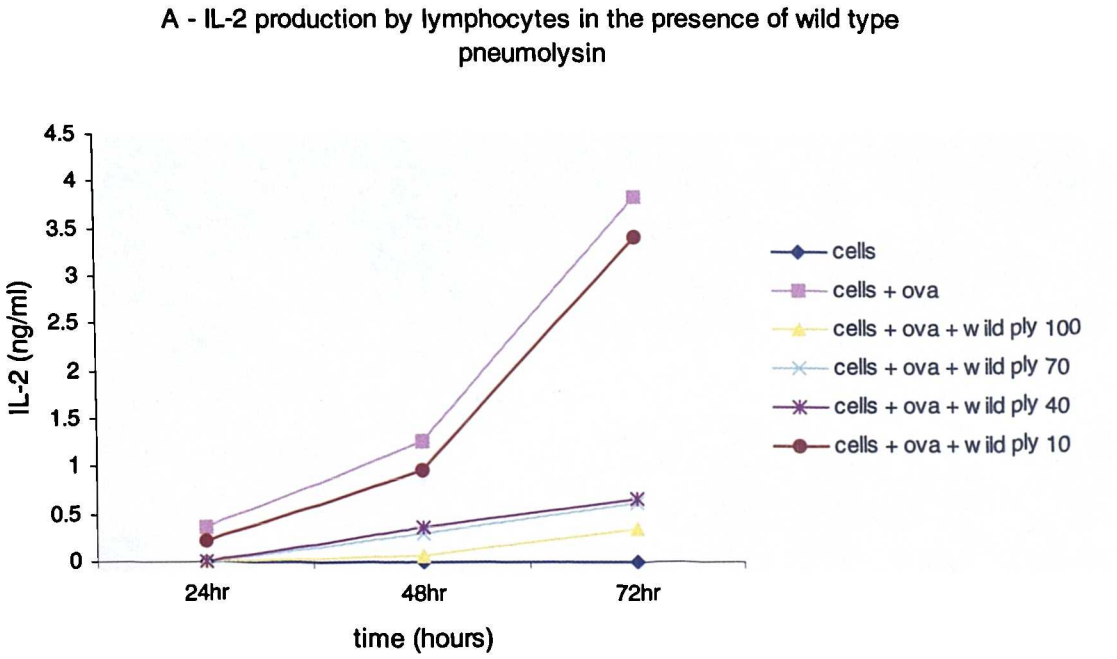
Pneumolysin inhibits IL-2 production

Figure 4-14 shows the effects of pneumolysin upon IL-2 production. Unstimulated lymph node cells did not produce any IL-2 over the 72 hour time period examined. After 24 hours cells stimulated with ovalbumin produced a detectable level of IL-2 of 0.3ng/ml. By 48 hours this has increased to 1.2ng/ml and by 72 hours it is at 3.8ng/ml. Inhibition of IL-2 production is most evident using the highest concentration of wild type pneumolysin and is first detected after 24 hours when cells stimulated with ovalbumin in the presence of 100ng/ml of wild type pneumolysin have no detectable IL-2 in the medium. After 48 hours there is a small amount of IL-2 detectable at 0.06ng/

Figure 4-14

The effect of pneumolysin on IL-2 production by lymphocytes

Lymph node single cell suspensions were made from naive DO11.10 mice and were set up in a cytokine assay. Where appropriate 1mg/ml ovalbumin was used and doses of wild and F433 pneumolysin at 100 (yellow line), 70 (turquoise line), 40 (purple line), 10 (brown line) ng/ml. At 24, 48 and 72 hours the supernatants were harvested and ELISA' s were carried out to detect IL-2. The effect of wild type pneumolysin is displayed in A and the effect of F433 type pneumolysin is displayed in B. Unstimulated controls are shown as a blue line and stimulated controls as a pink line.



ml. By 72 hours 0.3ng/ml IL-2 is present, compared with the stimulated control which has 3.8ng/ml by the same time. This represents a 92% inhibition. This pattern is repeated with 70 and 40 ng/ml pneumolysin although the degree of inhibition is not quite as large since 0.6ng/ml of IL-2 is produced by 72 hours in both cases. 10ng/ml pneumolysin inhibits IL-2 production to a lesser degree than the higher concentrations of pneumolysin since after 72 hours 3.4ng/ml IL-2 is found in the medium which is not significantly less than the 3.8ng/nl produced by the stimulated cells alone.

I concluded that pneumolysin was inhibiting the antigen driven production of IL-2 by T cells in a dose dependent manner within this system.

I was also interested to note whether F433 pneumolysin exerted any effect upon IL-2 production. The same controls were used for this part of the assay as those used for the wild type so the unstimulated cells produced no IL-2 over the 72 hour time course but the stimulated cells produced IL-2 detectable after 24 hours and rising to a final level of 3.8ng/ml in the medium after 72 hours. Stimulating the cells with ovalbumin in the presence of 100, 70, 40 or 10 ng/ml F433 pneumolysin did not alter the amount of IL-2 produced relative to the untreated stimulated control.

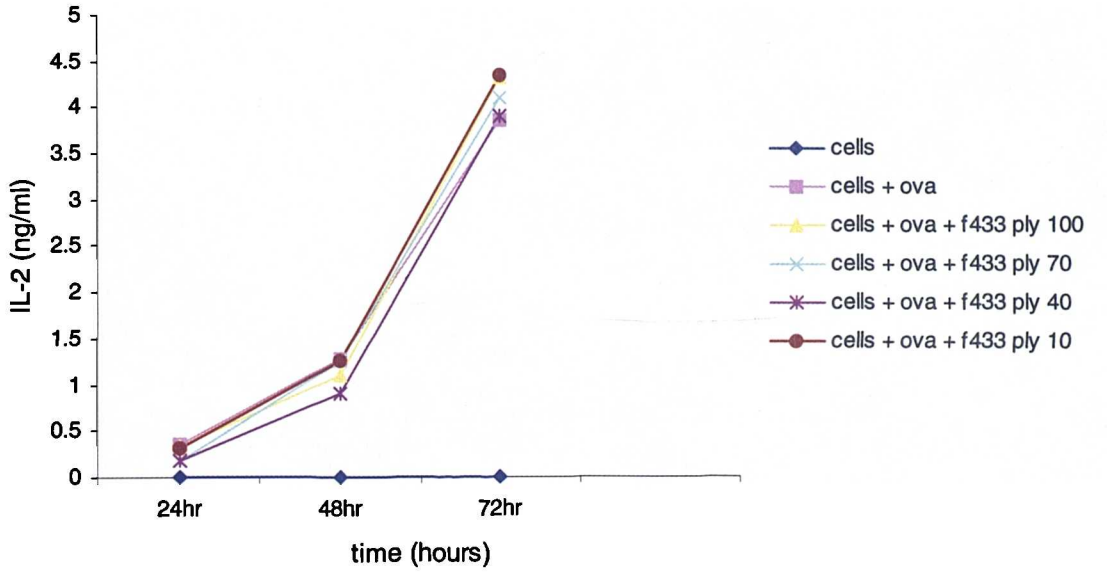
I concluded that F433 pneumolysin did not inhibit the production of IL-2 by antigen stimulated T cells.

Pneumolysin inhibits IFN-g production

Figure 4-15 shows the results of the ELISA carried out to determine the effect of pneumolysin upon lymphocyte production of IFN-g. After 24 hours unstimulated lymph node cells had produced IFN-g to a level of 1ng/ml in the medium. In the following 48 hours this decreased so that by 72 hours there was no detectable amount of IFN-g in the lymph node culture. After 24 hours the lymph node cultures stimulated with ovalbumin had produced IFN-g to a level in the medium of 4.8ng/ml, rising to 30ng/ml by 48 hours and finally 40ng/nl after 72 hours.

Lymph node cells stimulated with ovalbumin in the presence of 40ng/ml wild type pneumolysin produced less IFN-g over the 72 hour time period examined compared with the untreated stimulated control. This was first detected after 24 hours when pneumolysin treated cells had produced

B - IL-2 production by lymphocytes in the presence of F433 pneumolysin



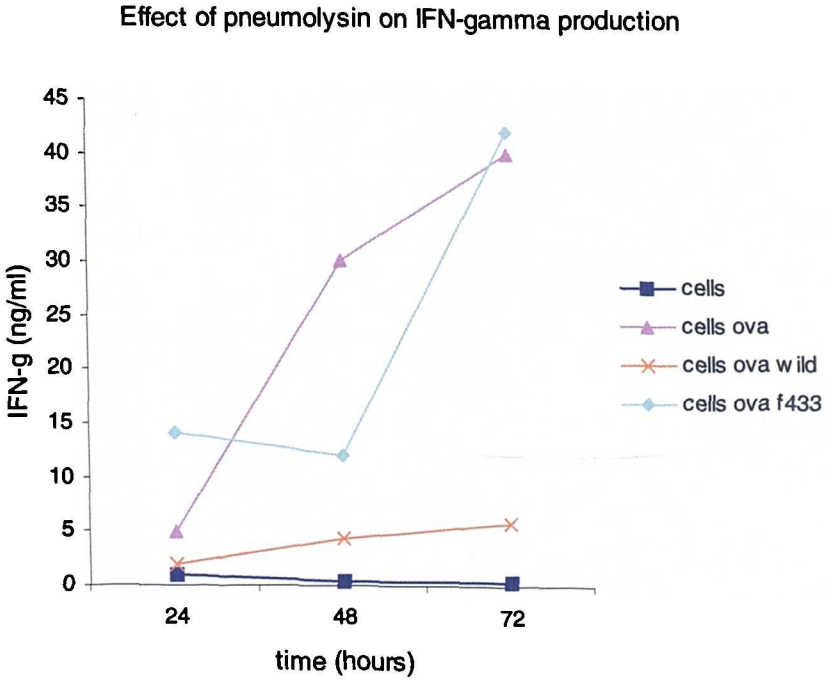


Figure 4-15

The effect of pneumolysin on IFN-gamma production by lymphocytes

Lymph node single cell suspensions made from naive DO11.10 mice and were set up in a cytokine assay. Where appropriate 1mg/ml ovalbumin was used and doses of wild (yellow line) and F433 (turquoise line) pneumolysin at 40 ng/ml. At 24, 48 and 72 hours the supernatants were harvested and ELISA' s were carried out to detect IFN-gamma. Stimulated controls are shown as a pink line. Unstimulated controls are shown as a blue line.

IFN-g to 1.9ng/ml compared to 4.8ng/ml by the untreated control. This difference was more noticeable at 48 and 72 hours when the pneumolysin treated cells produced IFN-g to 4.4 and 5.9ng/ml, respectively, compared to the stimulated control of 30 and 40ng/ml at the same time points.

These results mean that pneumolysin markedly inhibits IFN-g production by antigen stimulated T cells.

Lymph node cells stimulated with ovalbumin in the presence of 40ng/ml F433 pneumolysin produced IFN-g to a higher level over the first 24 hours than the cells stimulated with ovalbumin alone; 14.1ng/ml compared to 4.8ng/ml for the ovalbumin alone. However, by 72 hours the cells stimulated with ovalbumin in the presence of the F433 pneumolysin had produced an equivalent amount of IFN-g compared to cells stimulated with ovalbumin alone of 40ng/ml. From this I concluded that the F433 mutant pneumolysin does not alter IFN-g production

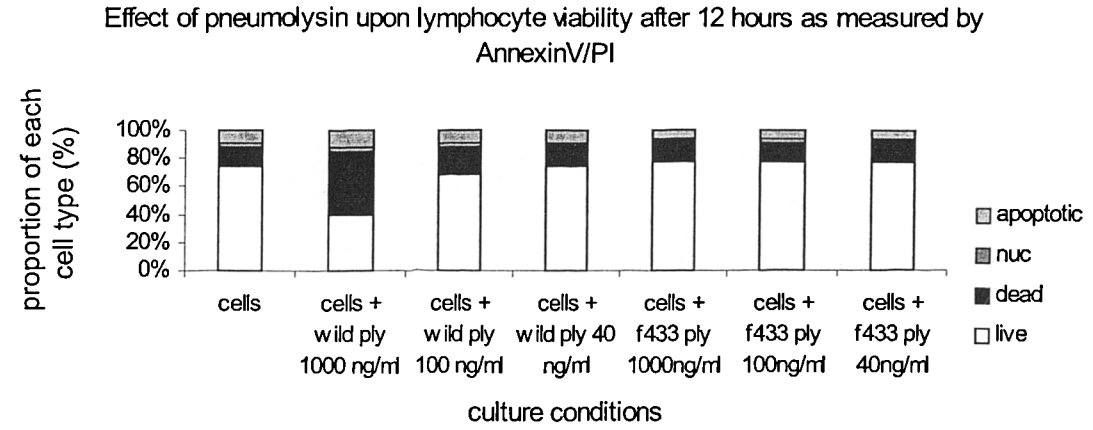
These findings led me to conclude that wild type pneumolysin inhibits CD4 T cell cytokine production and that F433 pneumolysin does not. I had not, however measured cytokine production on a 'per cell' basis and so we could not conclude that pneumolysin actually prevented individual cell cytokine production. It therefore remained unclear if the inhibition in cytokine production was the function of inhibition of proliferation or if it was a separate inhibitory mechanism. Taken together the above data demonstrates that wild type pneumolysin is able to inhibit T cell function.

4.2.2.2 The effect of pneumolysin on resting lymphocyte viability

There remained the possibility that wild type pneumolysin was causing the inhibitory effects outlined above by simply lysing the resting lymphocytes. I therefore wanted to confirm that the effects I was observing were not the result of resting cell lysis but were due to some other mechanism and that the concentration of toxin we were using was non-lytic.

AnnexinV/PI

I measured the effect of pneumolysin upon resting lymphocyte viability by assessing their viability after 12 and 24 hours using the AnnexinV/PI assay. Figure 4-16 illustrates that after 12 hours 75% of untreated cells are alive. Comparably only 40% of cells treated with 1000ng/ml wild type pneu-



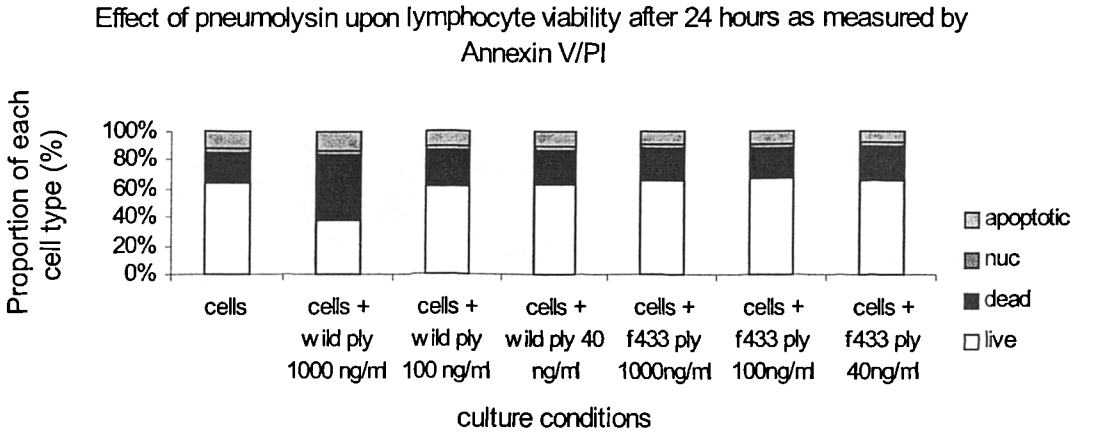
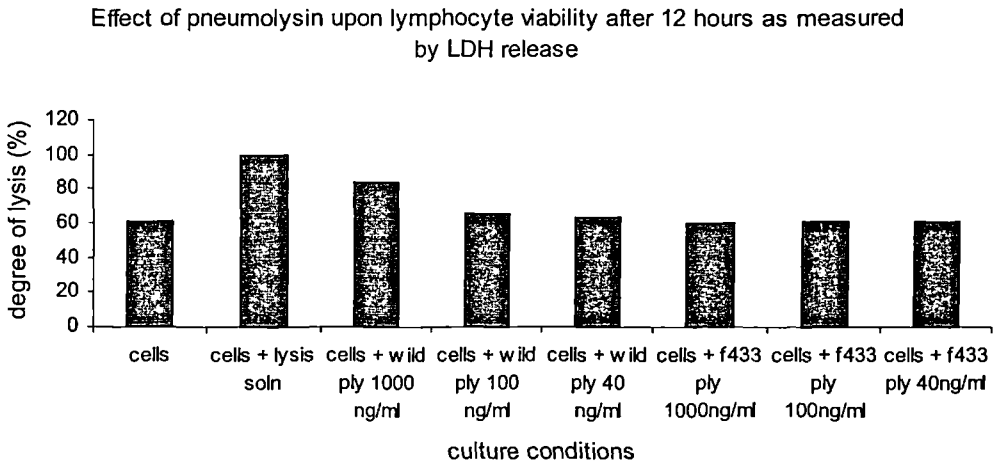


Figure 4-16

The effect of pneumolysin on lymphocyte viability as assessed using AnnexinV/PI

Lymph node single cell preparations were made from BALB/c mice and treated with 1000, 100 or 40 ng/ml of wild or F433 type pneumolysin. Cell death was measured using the Annexin V/PI assay at 12 (A) and 24 (B) hours. Cells were classified as being either apoptotic, nucleated (nuc), dead or alive depending on their AnnexinV/PI characteristics; An+ PI+ = dead. An+ PI- = apoptotic. An- PI+ = nucleated. An- PI- = live.



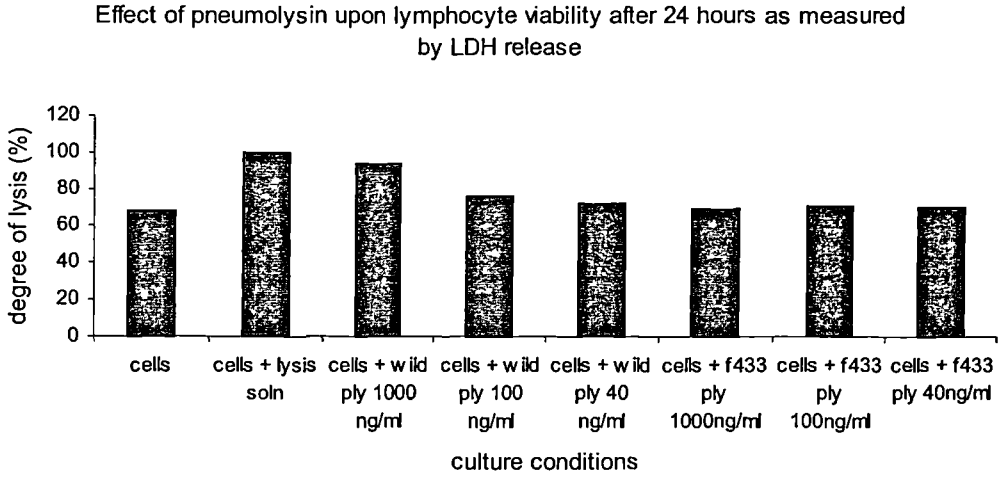


Figure 4-17

The effect of pneumolysin on lymphocyte viability as assessed using AnnexinV/PI

Lymph node single cell preparations were made from BALB/c mice and treated with 1000, 100 or 40 ng/ml of wild or F433 type pneumolysin. Cell death was measured using the LDH assay at 12 (A) and 24 (B) hours. Cells treated with lysis solution were taken to be 100% lysed and used as the baseline to calculate the other data.

molysin are alive while 68% of cells treated with 100ng/ml of wild pneumolysin are alive. 75% of cells treated with 40ng/ml of wild type pneumolysin are alive after 12 hours. After 24 hours the number of live cells within the untreated group of lymph node cells had decreased to 65%. The same pattern as that observed at 12 hours was evident; 1000ng/ml wild type pneumolysin induced 50% decrease in viability while 100ng/ml wild type pneumolysin caused a small decrease in viability and 40ng/ml wild type pneumolysin did not cause any decrease in viability compared with the untreated control group.

Throughout the two time points examined treating the cells with F433 pneumolysin of any concentration did not alter the viability of the resting cells when compared with the untreated control group. Also of note is that there was no changes in the way cells were dying between groups in that the proportions of apoptotic or nucleated cells did not increase within any group.

From this I can conclude that 40ng/ml of wild type pneumolysin does not cause lysis of the resting lymphocytes whereas it does cause significant inhibition of proliferation, and IL-2 and IFN-g production.

LDH

In order to confirm the data reported in Figure 4-16, I used the LDH assay as a further measure of cell lysis. The results (Figure 4-17) demonstrate that 40ng/ml of wild type pneumolysin does not cause lysis of resting lymphocytes.

Taking both the Annexin V /PI data and the LDH data together I concluded that 40ng/ml pneumolysin was a non lytic dose of pneumolysin. Therefore experiments where pneumolysin was observed to inhibit T cell function was not due to cell lysis but must represent another mechanism. Both experiments were repeated twice and the results presented above are representative of all the experiments

4.2.2.3 Pneumolysin appears to inhibit T cell division

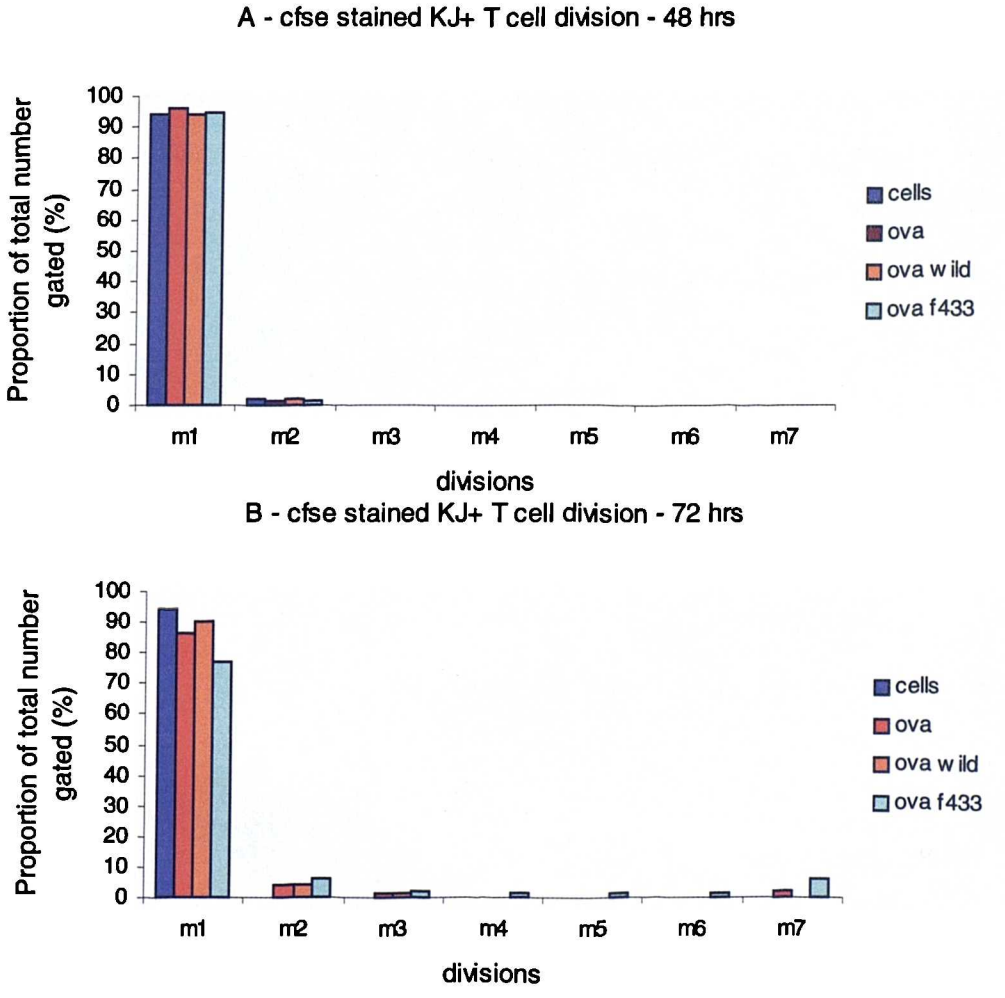
It seemed most likely that pneumolysin was inhibiting the proliferation of ovalbumin specific T cells in Figure 4-13 since the antigen used was specific for the T cells. There remained the possibility, however, that thymidine incorporation was recording the proliferation of both antigen specific T cells and other cell types present in the lymph node preparation such as B cells. This then opened the possibility that pneumolysin was not acting upon the T cells but upon another cell type. In addition thymidine incorporation used in Figure 4-13 does not necessarily measure cell division, but rather measures DNA replication. To clarify and confirm that my conclusions from Figure 4-13 were correct; i.e. that pneumolysin was inhibiting the proliferation of antigen specific T cells I wanted to measure directly the effect of pneumolysin upon the T cell and upon cell division. The fluorescent cell dye CFSE in conjunction with flow cytometric analysis allowed us to do this.

I measured the fluorescence intensity of transgenic T cells stained with CFSE over a period of 144 hours in order to assess the number of cell divisions that had taken place. I also assessed the effect of pneumolysin upon the number of cell divisions. Figure 4-18 shows that unstimulated cultures did not divide throughout the time period examined. Figure 4-18 also demonstrates that in cultures that received ovalbumin only a small amount of cell division took place during the first 72 hours since 95% of cells remain undivided (CFSE high) while only 1% of cells have divided once. However in the succeeding 24 hours from the 72 hour time point, cell division of the stimulated cells begins to occur. After 96 hours only 32% of cells stimulated with ovalbumin remain undivided. A further 4% have made one division while 52% have divided up to 7 times or more. Stimulating with ovalbumin and treating the cells with wild type pneumolysin alters the cell division cycle compared to untreated cells since after 96 hours 73% remain undivided while 11% have divided 7 times or more. Treating the cells with F433 pneumolysin and ovalbumin does not affect the cells since after 96hrs, 19% of the cells remain undivided and 66% are divided 7 times or more. The inhibitory effects of wild type pneumolysin are observed at 120 hours and 144 hours also. Of the cells stimulated with ovalbumin alone, 9% remain undivided after 120 hours and 82% have divided 7 times or more. At the same time point 34% of cells treated with wild type pneumolysin remain undivided and 46% have divided 7 times or more. After 144 hours 7% of cells stimulated with ovalbumin alone remain undivided compared to the wild type pneumolysin treated cells for which 18% are undivided. 83% have divided 7 times or more when stimulated with ovalbumin compared to 66% for those treated with pneumolysin. At both time points T cells treated with F433 pneumolysin had a comparable number of divided cells to the T cell stimulated with ovalbumin alone.

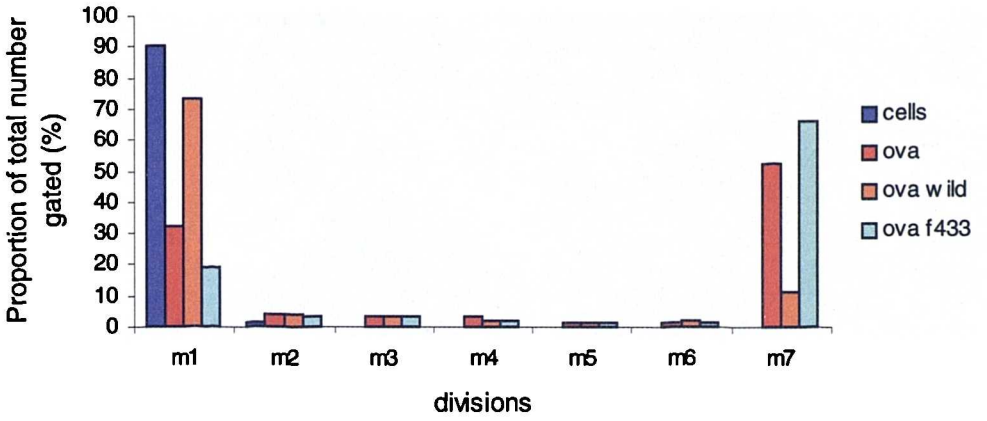
Figure 4-18

The effect of pneumolysin upon lymphocyte cell division as assessed by CFSE staining

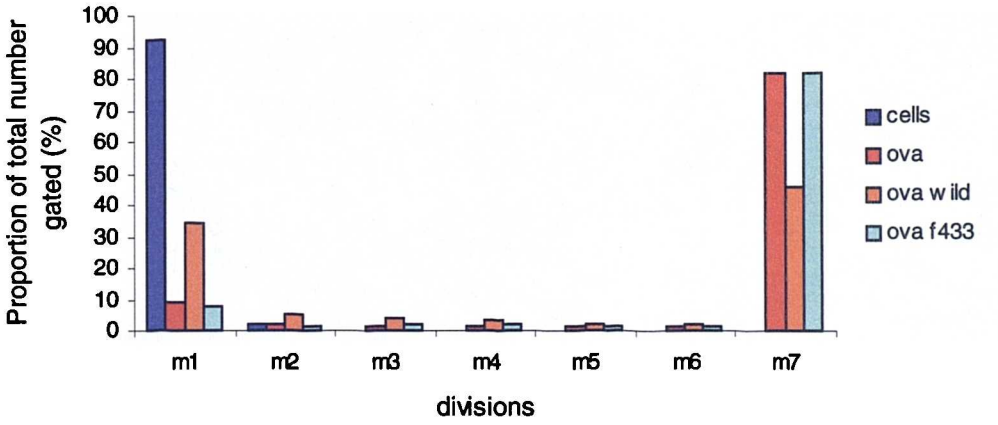
Lymph node cell suspensions were prepared from DO11.10 mice and stained with CFSE. These cells were then cultured with 1mg/ml ovalbumin and 40 ng/ml of wild or F433 type pneumolysin over a 144 hr time period. By staining for CD4-PerCP and biotynilated KJ1.26 conjugated to Streptavidin-PE we were able to measure the fluorescence intensity of the transgenic T cells using the flow cytometer. The cell division status of each group we measured at 48 (A), 72 (B), 96 (C), 120 (D), and 144 (E) hours. Unstimulated cells are presented as blue bars. Stimulated cells are red bars. Cells stimulated in the presence of wild type pneumolysin are presented as yellow bars and those stimulated in the presence of f433 pneumolysin are turquoise bars



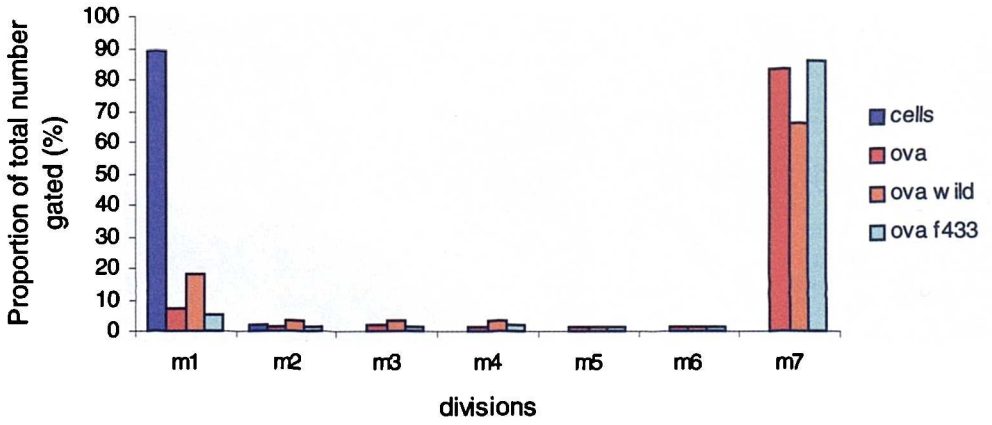
C - cfse stained KJ+ T cell division - 96 hr



D - cfse stained KJ+ T cell division - 120hr



E - cfse stained KJ+ T cell division - 144hr



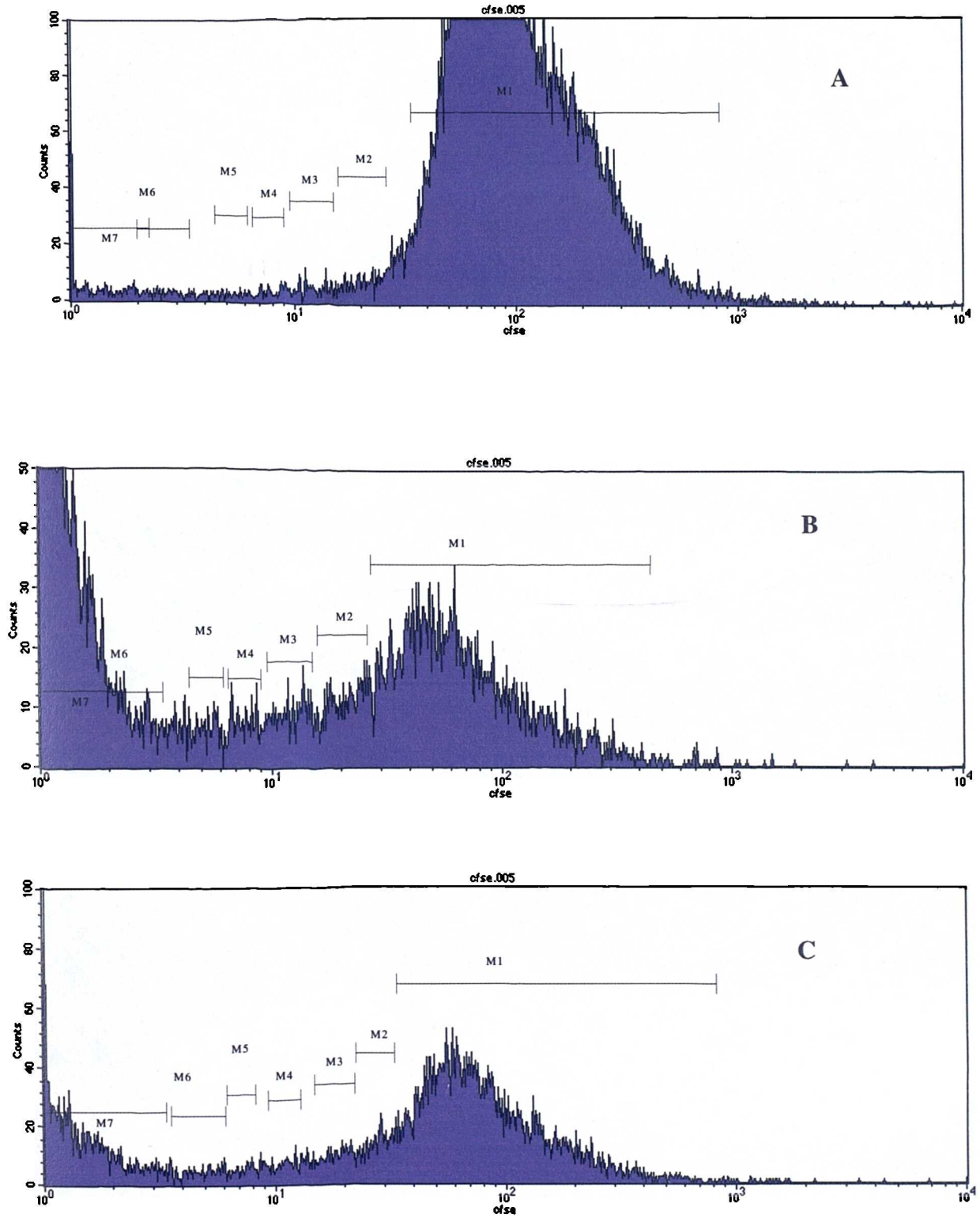


Figure 4-19

Data obtained from Figure 4-18

Some of the histograms obtained from the flow cytometry carried out in Figure 4-18 are shown. (A) shows the data obtained at 72 hours from cells stimulated with ovalbumin. (B) and (C) show the same group at 96 and 120 hours respectively.

From these observations I concluded that our presumptions based on our observations in Figure 4-13 were correct and that pneumolysin was inhibiting antigen specific T cell division. Furthermore, repetition of this experiment confirmed these results to be accurate and repeatable.

4.3 Discussion

In this chapter I have demonstrated that the toxin pneumolysin produced by *Streptococcus pneumoniae* is able to inhibit the proliferation and cytokine production of murine T cells. This occurs by a mechanism distinct from direct cell lysis by the toxin. In the context of the whole bacteria pneumolysin was not observed to have the same ability.

In this chapter I used *ex vivo* DO11.10 cells to study the effect of a potential immunomodulator on the T cell response to bystander antigen. This is a system that has been used before (Matsue *et al.*, 2002) to assess the impact of immunomodulators upon the T cell response.

The discrepancy between my findings using the whole bacteria (Figure 4-12) and those using the purified toxin (Figure 4-13) has a number of explanations. Other researchers have found the toxicity of the whole bacteria problematic in these sorts of systems and have resorted to using heat killed bacteria (e.g. (Madsen *et al.*, 2000) (Arva and Andersson, 1999a, b; Colino and Snapper, 2003a)). I chose to use live bacteria to enable pneumolysin production and release however this introduced unknown variables that were difficult to control for. The variables included; variance of the inoculum dose between strains within an experiment and also inoculum variance between experiments. Related to this, but distinct, is the potential variation in strain growth rate *in vitro*. Any factor released or produced by the pneumococcus may affect the experiment and thereby introduce an unknown variable that cannot be controlled for. Furthermore the synergy or compensation of released factors with each other could introduce further variables into the experiments. Finally, within my experiments the concentration of pneumolysin was unknown. I attempted to address some of these variables within our experiments. I tried to ensure inoculum consistency at all points. I addressed the releases of hydrogen peroxide by the pneumococcus. I also tried to address the concentration of pneumolysin.

I succeeded in ensuring inoculum consistency between strains in all experiments except one (Figure 4-9) however the difference in this experiment between D39 and PLY- seems likely to be the result of an error of measuring the initial inoculams considering the consistency later in the experiment. There is more variation when considering inoculum consistency between experiments. For example in Figure 4-7 we had hoped to add 5x 10^4 CFU/ml but the resulting CFU/ml was 1x 10^5,

however all of this variation was recorded and none of it would have had significant impact upon the experiments. Previous work has demonstrated that the PLY- mutant shows no *in vitro* growth defect when compared to the wild type pneumococcus ((Berry *et al.*, 1989))and so I could reasonably assume that this potential variable could be dismissed.

In identifying hydrogen peroxide as a toxic factor I succeeded in removing the toxic effect of released factors from the pneumococcus. This however does not exclude the possibility that another non toxic released factor may act in these experiments. My identification of hydrogen peroxide as a toxic factor is in keeping with studies carried out on the pneumococcus that show that hydrogen peroxide is produced by the bacteria and in quantities sufficient enough to exert a toxic effect on other bacteria (Pericone *et al.*, 2000).

I succeeded in my attempt to increase the final concentration of bacteria despite the similarity in starting inoculum between the experiments of Figure 4-7 and Figure 4-11 (starting inoculum of 1x 10^5 CFU/ml and 5x 10^4 CFU/ml respectively). In Figure 4-7 the final concentration was 1x 10^6 CFU/ml while in Figure 4-11 the final concentration of bacteria is 5x 10^7 CFU/ml. At the time of conducting these experiments I was unaware of the concentration of pneumolysin required to exert an inhibitory effect of lymphocytes and I therefore did not measure the concentration of pneumolysin. In retrospect this would have been a good idea as it would have allowed me to compare my data more directly with the work done using the purified toxin and thereby assess whether inhibitory concentrations of pneumolysin in the medium were reached using the co-culture system. Therefore despite my attempts to increase the concentration of pneumolysin there remains the possibility that concentrations within my experiments was insufficient. The only indication on this matter comes from the data of Houldsworth *et al.* (Houldsworth *et al.*, 1994) who estimated 1x 10^3 pneumococci to be equivalent to 10pg of pneumolysin. I would estimate therefore that in our experiments a final concentration of 5x 10^7 CFU/ml pneumococci would give 500ng/ml of pneumolysin. From retrospective data using the purified toxin I can suggest that this is a more than adequate concentration of toxin to observe an inhibitory effect.

Despite the recent studies by Balachandran *et al.* (Balachandran *et al.*, 2001) which demonstrated that the release of pneumolysin is not dependent on the production of autolysin for the strain WU2, the data for other strains seems to indicate that pneumolysin release is autolysin dependent (Lock *et al.*, 1992). It is therefore a possibility that in my cell culture experiments, the conditions present

did not meet the requirements for the release of pneumolysin into the medium. Recently it has been demonstrated that release of pneumolysin is inhibited by the presence of erthromycin and this may also be a factor in our experiments (Lagrou *et al.*, 2000). Furthermore despite another demonstration that penicillin enhances the release of pro-inflammatory products from the pneumococcus (Moore *et al.*, 2003), this effect was autolysin dependent.

I considered the possibility that the experimental procedure may also be responsible for the lack of inhibitory effect observed using the whole bacteria. Walev *et al.* (Walev *et al.*, 2002)found that pores formed in the membrane by streptolysin O were resealable. Furthermore Walev *et al.* (Walev *et al.*, 1995)found that streptolysin is shed from the membrane over time. The experimental procedure involving washing cells after exposure to the toxin may allow resealing or increased shedding of toxin from the membrane which may result in lack of inhibitory effect. In conflict with this proposal is the data of Ferrante *et al.* (Ferrante *et al.*, 1984) who washed the cells after toxin exposure yet still observed toxin activity. Another possibility is that of thiol activation. For many years thiol activation was thought to be required for the function of the CDC's, hence the reason for them being called the Thiol Activated Cytolysins, however it was repeatedly demonstrated that thiol activation is not a requirement for toxin activity (Pinkney *et al.*, 1989; Saunders *et al.*, 1989) when the toxin is purified. However thiol activation may be required when the toxin is not in a purified form and this could be a reason for the lack of effect since the toxin is not purified.

Further investigation is required to elucidate which of the variables outlined is finally responsible for the discrepancy between the effect of pneumolysin in the context of the whole pneumococcus and in purified form. My data suggests that inoculum and growth variance is not responsible as does previous work on the growth rate of PLY-. My data also excludes the possibility of a toxic factor being the responsible variable. My work on the concentration and release of pneumolysin is inconclusive and therefore these remain potential variables responsible for the difference between results using the whole bacteria and the purified toxin. Further work is required in this area.

As observed in the introduction to this thesis, studies using pneumolysin strains in which the gene has been deleted have yielded much information regarding the role of pneumolysin in the pathogenicity of the pneumococcus. To a much lesser degree pneumolysin deficient strains of the pneumococcus have also been used *in vitro* to study the effect of pneumolysin. The effect of pneumolysin upon brain cells has been examined in this way (Hirst *et al.*, 2000; Zysk *et al.*, 2001), as has

the effect on endothelial cells (Rubins *et al.*, 1992). Perhaps most relevantly, Zysk *et al.* (Zysk *et al.*, 2000) used a pneumolysin deficient strain to examine the effect of pneumolysin upon the induction of apoptosis of neutrophils.

My results (Figure 4-13), showing the inhibitory effect of purified wild type pneumolysin upon lymphocytes are consistent with the data of Ferrante *et al.*, (Ferrante *et al.*, 1984), however my results extend these findings. These studies demonstrated that pneumolysin could inhibit the mitogenic induced proliferation of human lymphocytes; here I have demonstrated that antigen specific proliferation can be inhibited and that murine T cells are susceptible. The human specificity of a member of the CDC's (e.g. Intermedilysin (Nagamune *et al.*, 1996)) is currently attracting interest and it is therefore pertinent that this ability of pneumolysin to inhibit lymphocyte proliferation is not confined to human lymphocytes but reflects the species non-specificity of its other functions.

I had hoped that the use of the F433 mutant pneumolysin would give me insight into the mechanism of pneumolysins inhibitory activity. The controversial nature of the effects of mutations in the Trp rich region of the CDC's was outlined in the introduction to this thesis. Here the work of Boulnois *et al.* was examined (Boulnois *et al.*, 1990) and others (Imaizumi *et al.*, 2003; Korchev *et al.*, 1998; Michel *et al.*, 1990) who produced data that showed that the F433 mutant or the equivalent in other CDC's still bound to cells but was dysfunctional in pore formation. This data was challenged by Sekino-Suzuki *et al.* (Sekino-Suzuki *et al.*, 1996) who showed that in perfringolysin mutations of the Trp's in the undecapeptide region resulted in decreased cell binding. I have not examined cell binding and so my data does not enlighten this controversy however the interpretation of my studies using the F433 pneumolysin mutant is clearly dependent on the work mentioned above. If the work of Boulnois *et al.* (Boulnois *et al.*, 1990) and others, who have suggested that the F433 mutant retains full binding capacity is correct, then I would conclude that the lymphocyte inhibitory activity of pneumolysin that I have observed is dependent on the ability to form functional membrane pores. In contrast, if the work of Sekino-Suzuki *et al.* is accurate then I would simply conclude that cell binding is required for this activity of pneumolysin. As discussed in the introduction a mutant strain of *S. pneumoniae* containing the F433 mutant pneumolysin has been made and was shown to cause reduced virulence *in vivo* compared to the wild type strain but its virulence was intermediate when comparing the wild type and PLY- strains (Berry *et al.*, 1995).

In Figures 4-14 and 4-15 data is displayed regarding the effect of wild type pneumolysin upon the production of cytokines by antigen driven T cells. I demonstrated that wild type pneumolysin is able to inhibit T cell cytokine production as evidenced by the decreased production of IL-2 and IFN-g. My method did not measure the absolute production of cytokines per cell but rather as a population of cells, therefore I cannot conclude from our data that pneumolysin inhibits the production of cytokines from each cell but rather that pneumolysin inhibits the production of cytokines from the whole population. This represents a distinct difference in mechanism since the former would represent a direct activity of pneumolysin upon cytokine production whereas the latter would mean that decreased cytokine production was simply a function of the effect on proliferation.

My data in Figure 4-16 and 4-17 showing that pneumolysin is not lytic for lymphocytes at 40ng/ml is consistent with the data of Walev et al (Walev *et al.*, 2001). These researchers found that treating the THP cell line with concentrations of 100 - 20ng of streptolysin O resulted in cells remaining viable. They made the interesting observation simultaneously that at these concentration pore formation occurs yet does not alter cell viability. 40ng/ml pneumolysin may therefore be forming membrane pores despite there being no change in cell viability. This may be pertinent to the mechanism of action

In the introduction to this thesis it was observed that pneumolysin affects different cell types to a differing degree. Each cell type has a different sensitivity to pneumolysin (Hirst *et al.*, 2002). For example, while we found lymphocytes to be sensitive to 100ng/ml pneumolysin, it was discovered that the effect of pneumolysin upon alveolar epithelial cells (Rubins *et al.*, 1993) and found that 150ng/ml was toxic to these cells. Studies on the effect of pneumolysin on human neutrophils have found that in calcium free medium, cell lysis measured by LDH, was 40% when pneumolysin was present at 40ng/ml (Cockeran *et al.*, 2001b). Comparing this data with my data where I observed concentrations of pneumolysin greater than 40ng/ml to affect lymphocyte viability, I can conclude that neutrophils are more sensitive than lymphocytes to lysis by pneumolysin.

Some have used the LDH assay to measure the lytic effects of pneumolysin upon spleen cells (Baba *et al.*, 2002). They found that around 300ng/ml of pneumolysin caused 35% lysis of spleen cells while 2600ng/ml of pneumolysin caused 75% lysis. This would suggest that spleen cells are

not as sensitive to pneumolysin as lymph node cells when taking our data into comparison. Others found that 0.1mg – 10mg/ml of pneumolysin induced microgilial cell apoptosis but did not make a study of the lytic ability in this situation (Braun *et al.*, 2002). The ability to induce lysis of monocyte and epithelial cell lines was studied and it was found that concentrations of around 7-70ng/ml of pneumolysin induced significant lysis of both cell lines (Hirst *et al.*, 2002). Feldman *et al.* found that 100ng/nl pneumolysin caused significant damage to epithelial cells *in vitro* (Feldman *et al.*, 2002) while Johnson *et al.* found that at around 90ng/ml pneumolysin, 50% of polymorphonuclear leukocytes are lysed. (Johnson *et al.*, 1981) .My results add to the data on the differing sensitivity of cells to pneumolysin. I note the differing hemolytic activity per mg of pneumolysin between researchers and therefore suggest that only when using the same preparation of toxin can a true comparison be made.

The data I have obtained using *in vitro* CFSE analysis shows few intermediate cell divisions. For example in Figure 4-19 C around 30% of stimulated cells are in m1 and 50% in m7 yet only around 5% are in m2-6. The reason for this may be due to the nature of *in vitro* cell division which has been noticed to be asynchronous. The asynchrony is the result of different entry times into the cell division cycle as opposed to differences in the length of the cell division cycle (Hasbold *et al.*, 1999; Lyons, 2000). It is therefore possible that the lack of intermediate divisions observed in my data results from asynchronous entry into cell division but rapid progress through the cell division cycle once it has been initiated. Furthermore my data shows that not all of the percentages within each group add up to 100%. This is because some death occurs with each round of cell division (Usherwood *et al.*, 1999) but they clump together to form CFSE "high" particles that remain within the gated areas.

My data in this chapter has clearly demonstrated that pneumolysin is able to inhibit lymphocyte function however the mechanism by which pneumolysin acts remains unclear. As I saw in introduction to this thesis, the inhibition of lymphocyte function is a common pathogen immunomodulatory mechanism. I noted that *Yersinia pseudotuberculosis*, *Nesseria gonorrhoeae*, *Acanthocheilonema viteae* and *Staphylococcus aureus* are all examples of pathogens that target T cell function. I also examined the different mechanism by which pathogens accomplish this alteration of T cell function. I noticed that some pathogens alter cell signalling to cause decreased T cell activation, while others induce T cell anergy or apoptosis. Of particular interest at this point in my

investigation into the inhibitory effect of pneumolysin is the studies carried out by Darji *et al.* (Darji *et al.*, 1997) who observed that listeriolysin induced T cell anergy via alteration of DC's. I therefore went on to investigate the mechanism by which pneumolysin may be accomplishing the inhibitory effect on T cell function observed in this chapter.

5 Dissecting the T cell inhibitory mechanism of pneumolysin *in vitro*.

5.1 Introduction

Pathogens have been observed to utilise a number of distinct mechanisms to inhibit T cells. Pneumolysin, produced by *Streptococcus pneumoniae,* modulates T cell function. The mechanism pneumolysin uses to accomplish this remains unclear and there exists a number of possible options. From my studies presented so far it is not clear if pneumolysin acts directly upon the T cell or in an indirect way. Acting in an indirect manner, pneumolysin may affect accessory cells found in the lymph node preparation which may then act upon the T cell or the accessory cells may release a factor which acts on the T cell. Alternatively pneumolysin may act directly upon the T cell to cause the inhibitory mechanism. The points on the T cell activation pathway upon which pneumolysin could act are shown in Figure 5-1.

The effects of pneumolysin upon cells such as macrophages and neutrophils have been outlined earlier in this thesis. These cell types are contained within a lymph node preparation such as that used in Figure 4-13. It is possible that pneumolysin could stimulate the release of a factor from one of these cells that subsequently acts on the T cell. For example nitric oxide could be released by macrophages (Braun *et al.*, 1999) which could result in T cell inhibition. A similar situation was observed with the nematode worm *Brugia malayi* which inhibits the T cell response *in vitro* (Jenson *et al.*, 2002) through stimulation of nitric oxide from macrophages which in turn inhibits T cell proliferation.

Acting on the APC, pneumolysin could alter antigen uptake, antigen processing, antigen presentation or costimulatory molecule upregulation. An effect on any one of these could cause an inhibitory effect on the T cell. *Listeria monocytogenes* releases the CDC, listeriolysin, which indirectly inhibits T cell proliferation by acting upon the APC (Darji *et al.*, 1997).

Finally, pneumolysin may act directly upon the T cell and may alter T cell upregulation of activation markers such as CD25, which is part of the IL-2 receptor, or may inhibit cytokine release or finally may induce anergy or apoptosis of the T cell. Vac A toxin from *Helicobacter pylori* inhibits the T cell response by inhibiting the production of IL-2 by preventing the translocation of NFAT within the T cell (Gebert *et al.*, 2003). Also, the introduction the effect of the filarial nematode

product ES-62 upon T cells is to induce T cell anergy (Harnett *et al.*, 1998). Furthermore aerolysin causes the induction of T cell apoptosis (Nelson *et al.*, 1999).

The examples given for each mechanism serve to highlight the fact that all of the possible inhibitory mechanisms are used by at least one pathogen and therefore could be used by pneumolysin. Given the range of potential mechanism by which pneumolysin may be acting, I decided to sequentially analyse each possibility (see Figure 5-1).

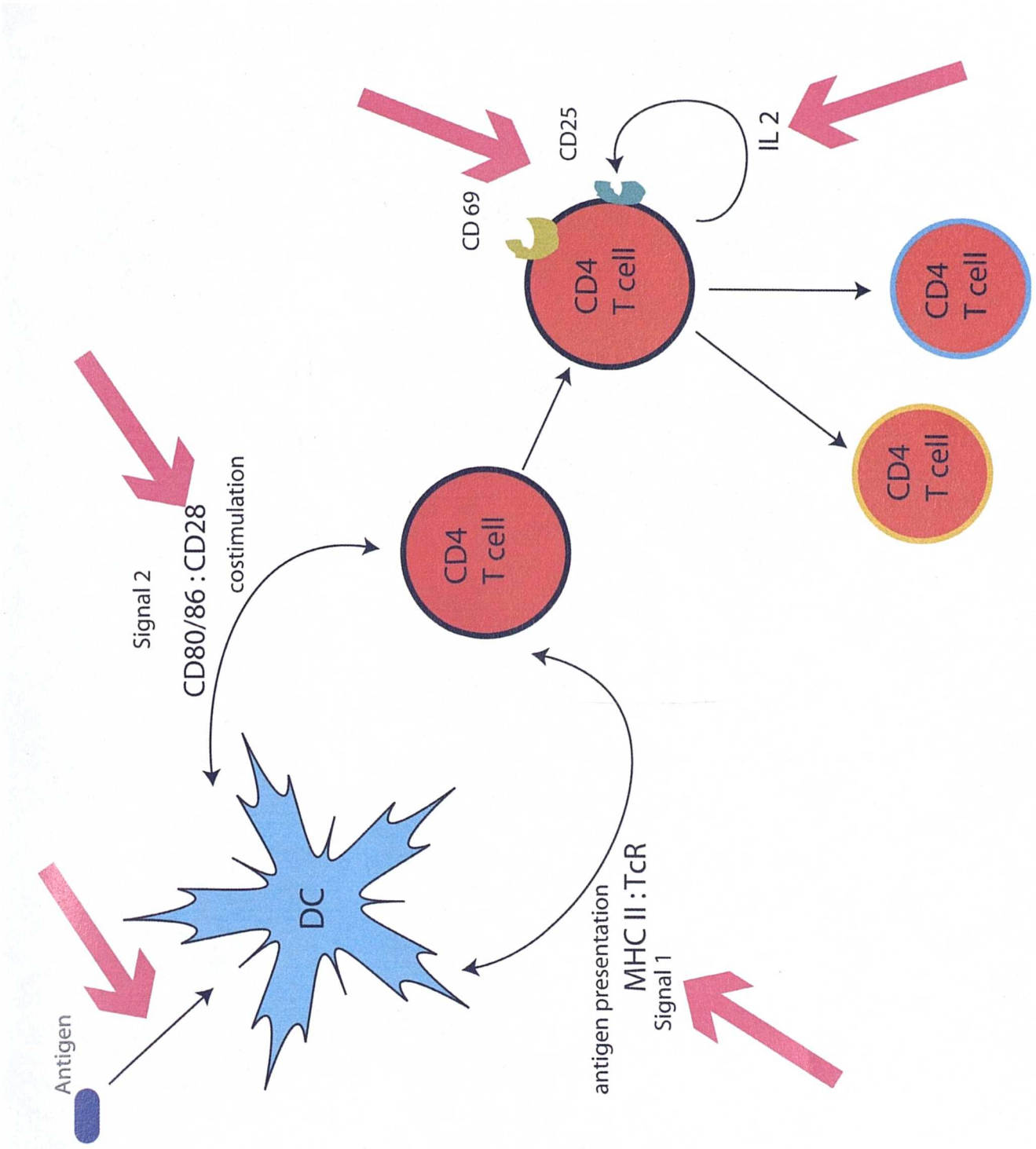


Figure 5-1

The T cell activation pathway.

The points at which an immunomodulator could act on the T cell activation pathway are indicated by a pink arrow.

5.2 Results

5.2.1 The role of accessory cells in the inhibitory activity of pneumolysin

Inhibition of T cell proliferation had been observed in experiments using mixed cell populations (Figure 4-13) where dendritic cells, macrophage, T cells and B cells are all present. The possibility remained that pneumolysin's inhibitory effect upon the T cell was via an indirect mechanism involving another cell type such as the macrophage. To address this problem I purified T cells and thereby removed all other cell types.

5.2.1.1 The requirement of accessory cells for the T cell inhibitory activity of pneumolysin

The first step in examining the inhibitory mechanism of pneumolysin was to remove accessory cells from the environment where T cell inhibition by pneumolysin had previously been observed. I selected the proliferation assay of Figure 4-13 as the assay to mimic. By purifying the T cells and culturing them with BMDC, I addressed the question of the necessity of accessory cells.

Accessory cells other than DC's are not required for inhibition

I measured the effect of accessory cells upon pneumolysin's inhibitory activity by using purified BMDC and purified Tg T cells within a proliferation assay. Figure 5-2 demonstrates that in cultures containing DC and T cells alone, T cells did not proliferate throughout the 72 hour period examined. Cultures of DC and T cells with ovalbumin had minimal thymidine uptake within the first 24 hours, however by 48 hours thymidine incorporation had increased to give a cpm of 6×10^4 and by 72 hours, 1.8×10^5 cpm. In comparison, cultures of DC and T cells with ovalbumin and wild type pneumolysin had a cpm at 48 hours of 1.5×10^4 and 5×10^4 at 72 hours. This represents

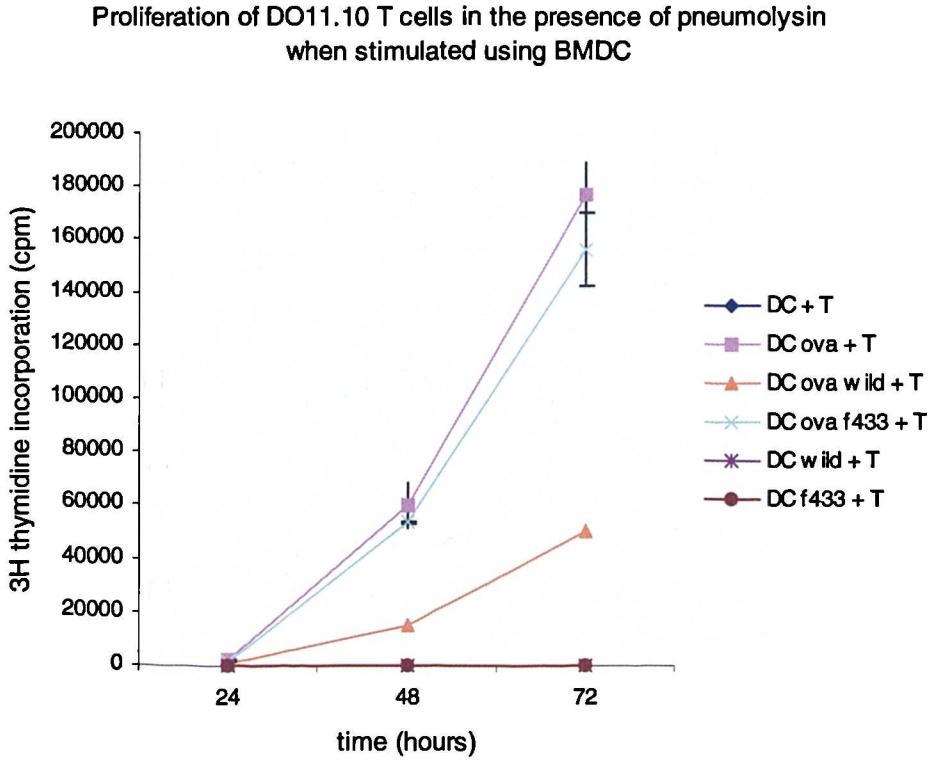


Figure 5 - 2

The effect of pneumolysin upon T cell proliferation in the absence of accessory cells

BMDC were used on day 7 of culture as APC and T cells were isolated from DO11.10 transgenic mice and purified. A proliferation assay was set up using 1mg/ml ovalbumin and 40ng/ml of wild or F433 pneumolysin where appropriate. The ratio of DC to T cells was 1:40 to give a final number of cells per well of a standard 96 well proliferation plate of $2x\ 10^5$. Proliferation was measured by thymidine incorporation at 24, 48, 72 hours. Cultures containing no antigen but both cell types are represented as a blue line. Cultures stimulated with ovalbumin are presented as a pink line. Cultures stimulated with ovalbumin in the presence of wild or f433 pneumolysin are presented as a yellow and turquoise line respectively. Unstimulated cultures with wild or f433 type pneumolysin are presented as a purple and brown line respectively.

a 75% inhibition of the control when wild type pneumolysin is present. In this system F433 pneumolysin did not alter T cell proliferation compared to the control. The data presented in Figure 5-2 are representative of at least 3 experiments. (Purified T cells alone did not proliferate with antigen. Data not shown.) I also measured the effect of pneumolysin on the viability of BMDC and observed no effect of pneumolysin upon BMDC (data not shown). This ensured that in this system pneumolysin was not acting by killing the BMDC and thereby reducing proliferation.

Overall, the data demonstrated that pneumolysin must be acting directly upon the APC or the T cell and was not causing the release of an accessory cell factor that was subsequently causing T cell inhibition.

5.2.1.2 The effect of pneumolysin upon APC independent proliferation

There remained the possibility that pneumolysin could be inducing the release of a factor by the BMDC that caused T cell inhibition. I therefore measured the effect of pneumolysin upon APC independent proliferation by stimulating purified T cells with Ionomycin and PMA.

The data in Figure 5-3 shows that only in the presence of both Ionomycin and PMA do T cells proliferate. T cells stimulated with Ionomycin and PMA proliferated by 48 hours to give a cpm of $1.2x\ 10^5$ cpm and by 72 hours, $2x\ 10^5$ cpm. In comparison T cells stimulated with Ionomycin and PMA in the presence of wild type pneumolysin proliferate to $9x\ 10^4$ cpm after 48 hours and $1.5x\ 10^5$ cpm after 72 hours. This represents a 25% inhibition of proliferation which clearly illustrates that pneumolysin is inhibiting the APC independent proliferation of T cells. This data is representative of at least two experiments.

The data from the above two experiments (Figure 5-2 and 5-3) led me to deduce that pneumolysin was able to inhibit T cell proliferation directly and did not require the presence of any accessory cells and therefore was not acting by inducing the release of a factor from an accessory cell. In addition I was able to conclude that pneumolysin did not require the presence of the APC's for T cell inhibition to be observed. However the data presented in Figure 5-3 was not as convincing as that which was presented in Figure 5-2 and so I examined some of the factors associated with antigen presentation that pneumolysin may interfere with.

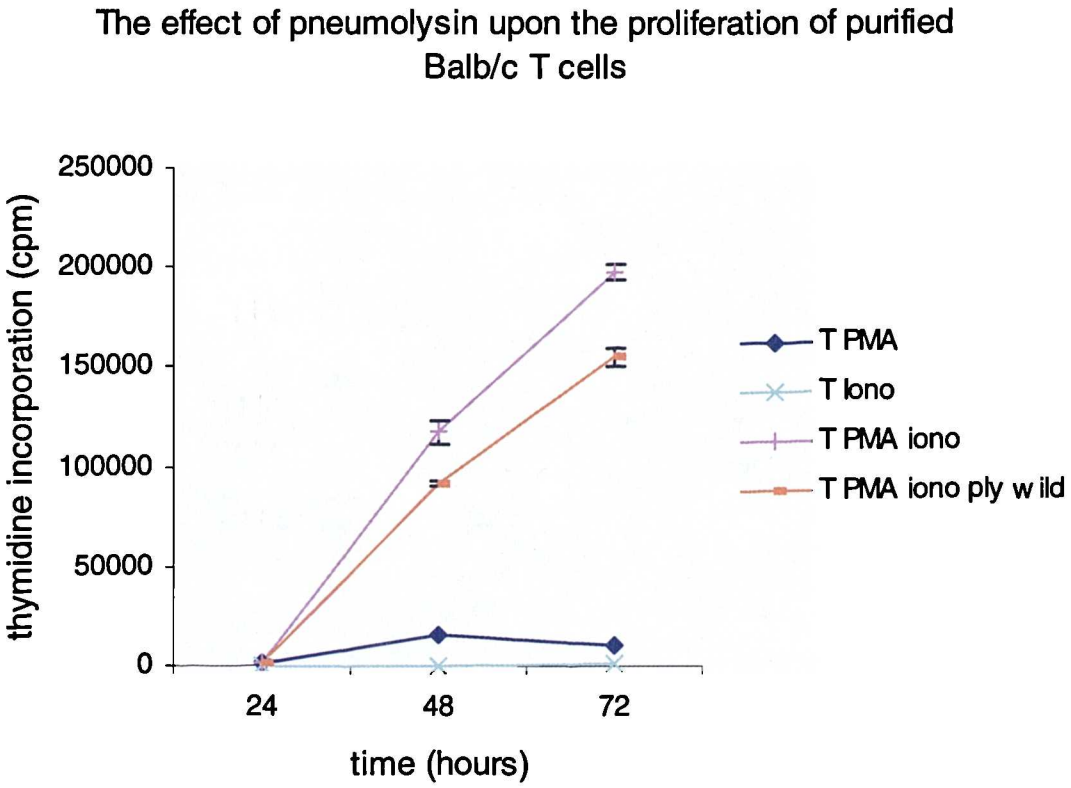


Figure 5-3

The effect of pneumolysin upon APC independent proliferation

Purified T cells were isolated from BALB/c mice. A proliferation assay was set up using 50ng/ml PMA and 1uM Ionomycin along with 40ng/ml of wild pneumolysin where appropriate. 1x 10^5 T cells per well were used. Proliferation was measured by thymidine incorporation at 24, 48, 72 hours. Cells stimulated with only one of Ionomycin or PMA are represented by turquoise and blue lines respectively. Stimulated cells are represented by the pink line while cells stimulated in the presence of wild type pneumolysin are represented as a yellow line.

5.2.1.3 Pneumolysin does not affect the antigen processing ability of the APC.

Although my work using the APC independent system had demonstrated that pneumolysin was capable of acting directly upon the T cell I wanted to assess whether in this system pneumolysin was acting exclusively on the T cell or was exerting a synergistic effect by acting on the T cell and APC. In order to address the question of whether pneumolysin was acting on the APC and how pneumolysin may be acting upon the T cell, I began to investigate the sequential steps in the T cell activation pathway.

Figure 5-1 illustrates the T cell activation pathway. The first step in the pathway is antigen processing. By using peptide fragments as antigen, the requirement for antigen processing prior to presentation was avoided. I used ovalbumin peptide as antigen within whole DO11.10 lymph node preparation cultures since I had demonstrated the irrelevance of other cells present within the whole lymph node preparation.

Figure 5-4 shows that unstimulated cells did not proliferate throughout the time period examined however cells stimulated with ovalbumin peptide began to proliferate within the first 24 hours to record a cpm of 6×10^4. By 48 hours this had increased to 3×10^5 cpm and by 72 hours, 7×10^5 cpm. Stimulated cells incubated with wild type pneumolysin proliferated less than the control; At 48 hours the cpm was 1.7×10^5 compared to the untreated value of 3×10^5, and after 72 hours cells incubated with wild type pneumolysin record a cpm of 3×10^5 compared to the untreated control value of 7×10^5 cpm. This means that, where present, wild type pneumolysin is causing a 42% inhibition of peptide stimulated proliferation. Cells stimulated with ovalbumin peptide and treated with F433 pneumolysin recorded similar values compared to the control of cells and peptide alone. The data in Figure 5-4 is representative of at least 3 experiments.

From this data I were able to conclude that pneumolysin does not act by interfering with antigen processing since it is still able to exert an inhibitory effect even when antigen processing is required.

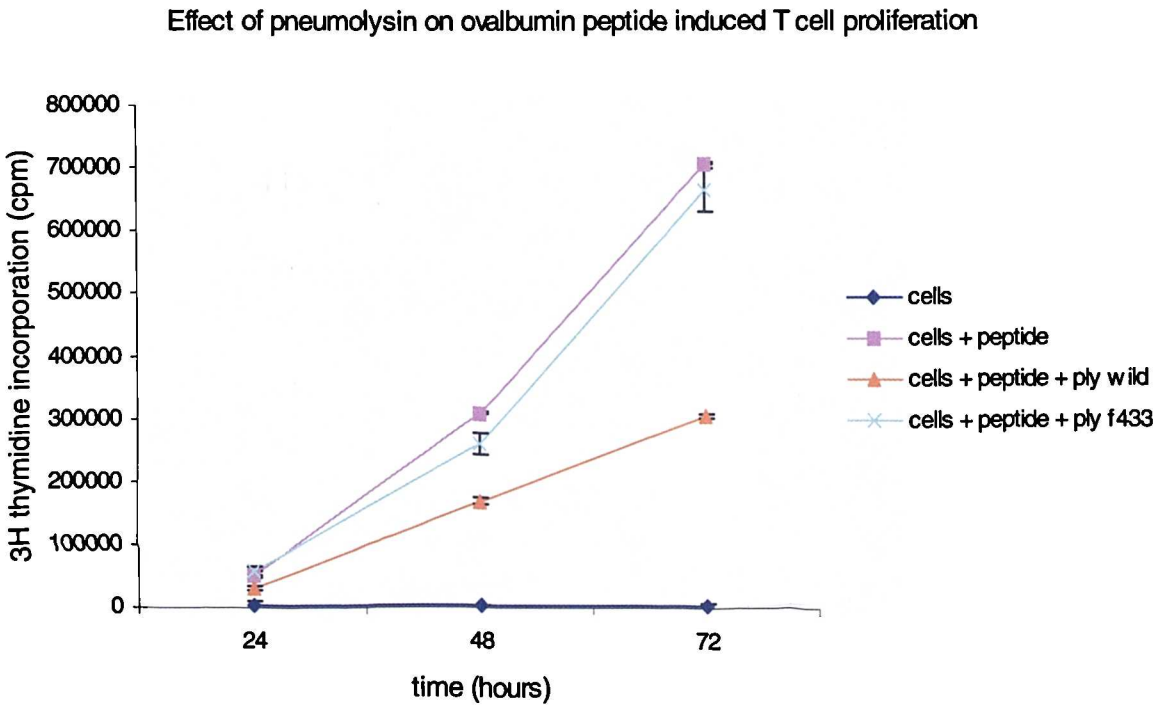


Figure 5 - 4

The effect of pneumolysin upon antigen processing by APC

Lymph node single cell suspensions were prepared from DO11.10 mice. Proliferation assays were set up using 0.5 μg/ml ovalbumin peptide and 40ng/ml wild or F433 pneumolysin. Proliferation was measured by ^{3H} thymidine incorporation 24, 48 and 72 hours. Unstimulated cells are represented by a blue line and stimulated cells by a pink line. Cells stimulated in the presence of wild or f433 pneumolysin are represented by a yellow or turquoise line respectively

5.2.1.4 Pneumolysin does not alter antigen presentation

The next step in the T cell activation pathway after antigen processing is antigen presentation. I therefore wanted to examine if pneumolysin was inhibiting the T cell response by decreasing the amount of antigen being presented by the APC to the T cell.

Antigen presentation measured by DO11.10 GFP T cell hybridoma

The DO11.10 GFP T cell hybridoma can be used to measure antigen presentation. GFP expression is under the control of the NFAT promoter within the hybridoma T cell. The hybridoma T cell is activated when ovalbumin is presented to it and the resultant GFP expression can be detected and quantified by measuring fluorescence. The fluorescence produced is proportionate to the amount of GFP which is in turn proportionate to the amount of T cell activation which is proportionate to the amount of antigen presentation. I measured fluorescence using flow cytometry (see Figure 5-5) and found that after 24 hours, cultures containing DC and GFP only had a MFI of 4. With ovalbumin present the MFI increased to a value of 13. When wild type pneumolysin was present (along with ovalbumin, DC's and T cells), the MFI decreased to a value of 9 while F433 type pneumolysin did not alter the MFI relative to the positive control. After 48 hours the stimulated cells have an MFI of 12 and the cell incubated with wild type pneumolysin having a MFI of 10. The difference in MFI between cells stimulated with ovalbumin and those stimulated in the presence of wild type pneumolysin persists to 48 hours. By 72 hours the difference between the stimulated groups is minimal. Only when each of; DC's, T cell hybridoma and ovalbumin were present did GFP expression increase. This experement is representative of two experiments.

From this data it appears as though the presence of wild type pneumolysin is inhibiting the presentation of antigen by the DC since the MFI of GFP T cells in cultures with wild type pneumolysin is lower than in cultures lacking wild type pneumolysin. However, this not the only interpretation; the GFP T cells are not an inert readout. Wild type pneumolysin may be acting on the T cells to alter the readout. It is possible therefore that equal amounts of presentation by the APC could be occurring yet a difference in fluorescence is observed. This result therefore required further investigation prior to drawing definitive conclusions.

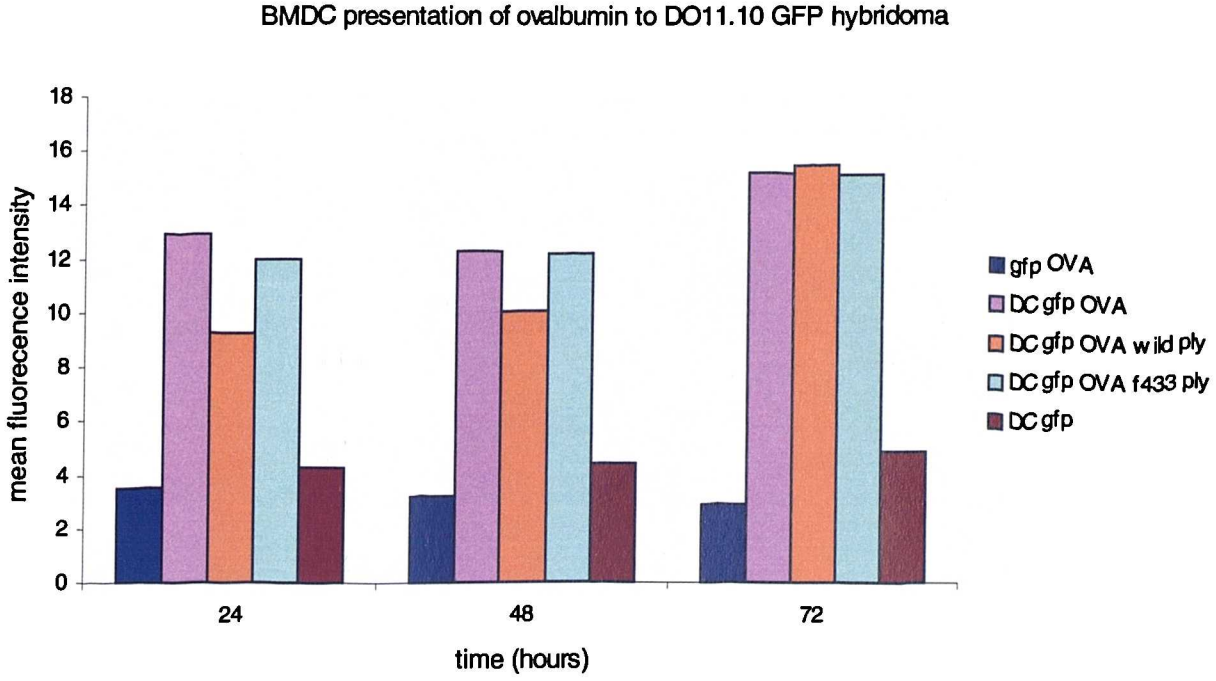


Figure 5 - 5

The effect of pneumolysin upon antigen presentation

BMDC were used on day 7 of culture as APC to the DO11.10 GFP T cell hybridoma at a ratio of 9:1. Ovalbumin was used to stimulate the cultures at 1mg/ml and 40ng/ml pneumolysin was used where appropriate. At 24, 48 and 72 hours the cultures were harvested and the mean fluorescence intensity (MFI) of the GFP T cells was measured by *flow cytometry*. DO11.10 GFP T cells that received only ovalbumin are marked as 'gfp OVA' and are presented as a dark blue bar. Where DC's were incubated with DO11.10 GFP T cells and ovalbumin only they are marked as 'DC gfp OVA' and are presented as a pink bar. Where DC's were incubated with DO11.10 GFP T cells, ovalbumin and wild type pneumolysin they are marked as 'DC gfp OVA wild ply' and are presented as a yellow bar. Where DC's were incubated with DO11.10 GFP T cells, ovalbumin and f433 type pneumolysin they are marked as 'DC gfp OVA f433 ply' and are presented as a turquoise bar. Where DC's were incubated with DO11.10 GFP T cells only they are marked as 'DC gfp' and are presented as a brown bar.

Pneumolysin does not alter T cell activation

The next step after antigen presentation in the T cell activation pathway is the upregulation of activation markers on the surface of the T cell. I wanted to determine if pneumolysin was inhibiting upregulation of these markers and thereby inhibiting T cell activation. Furthermore, this experiment served as a supplementary experiment to the GFP hybridoma presentation assay since the amount of T cell activation in a population is dependent on the amount of antigen being presented by the APC. I measured the level of T cell activation (by assessing CD69 and CD25 expression) occurring within the lymph node culture to both inform as to the interpretation of the above data but also as an assessment of the next step in the T cell activation pathway.

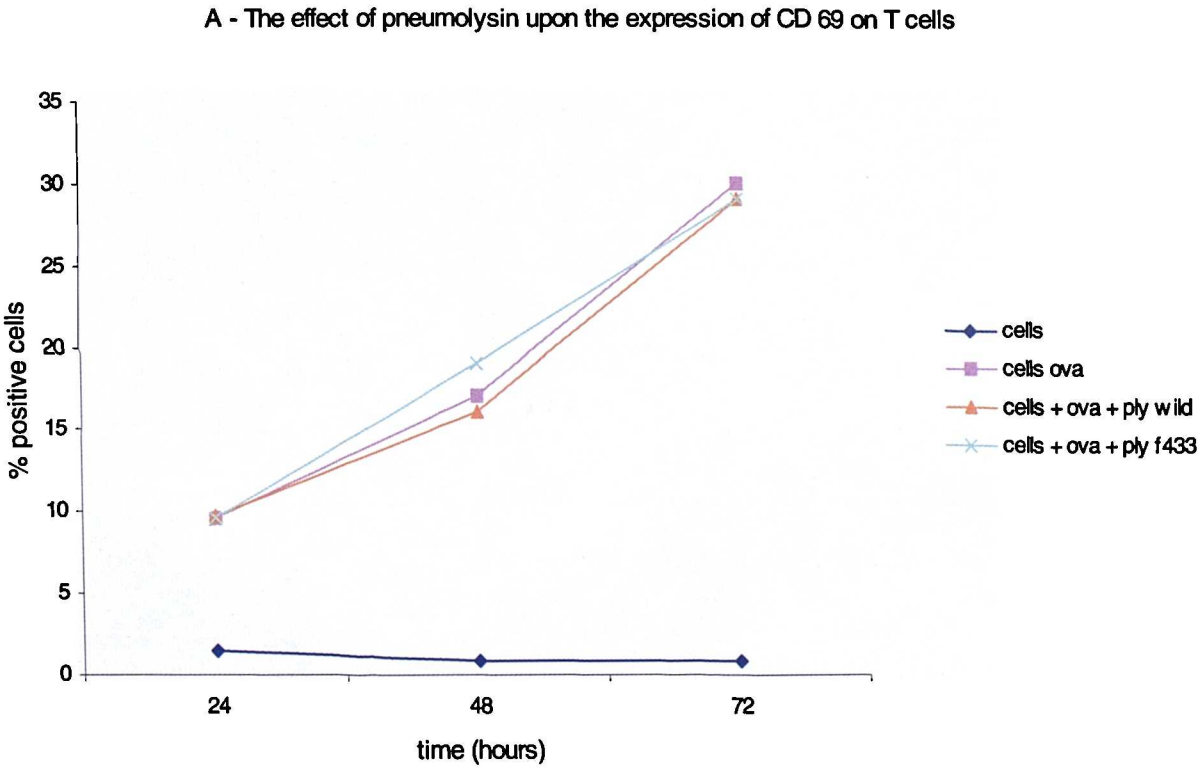
Figure 5-6 (A) shows that cells that did not receive antigen were considered unactivated and therefore CD69 'low'. Using this to set the gating on the flow cytometer, I was able to measure the percentage of CD69 'high' cells in cultures that had received antigen. After 24 hours 10% of KJ+ CD4+ T cells incubated with ovalbumin had become CD69 positive. Cells incubated with wild or F433 type pneumolysin and ovalbumin had a similar value. By 48 hours the number of activated cells had increased as evidenced by the increase in the percent of CD69 positive CD4+ KJ+ T cells to 17%. Cells incubated with either type of pneumolysin had a similar value. After 72 hours the highest amount of activation is observed; 30% of KJ+ CD4+ T cells are CD69 positive when incubated with ovalbumin alone. Cells incubated with either type of pneumolysin and ovalbumin had a similar value.

Simultaneous to the measurements above of CD69 expression I measured CD25 expression (see Figure 5-6[B]). T cells from cultures without antigen did not upregulate CD25 over the 72 hour time period. In cultures stimulated with ovalbumin 8% of CD4+ KJ+ T cells had become CD25 positive by 24 hours. Cells incubated with wild type or F433 pneumolysin and ovalbumin had the same value. After 48 hours the percentage of positive CD4+ KJ+ cells had doubled to 15% and by 72 hours the percentage of CD25 positive CD4+ KJ+ cells had doubled again to 30%. At both time points wild or F433 type pneumolysin did not alter the level of CD25 surface expression on the

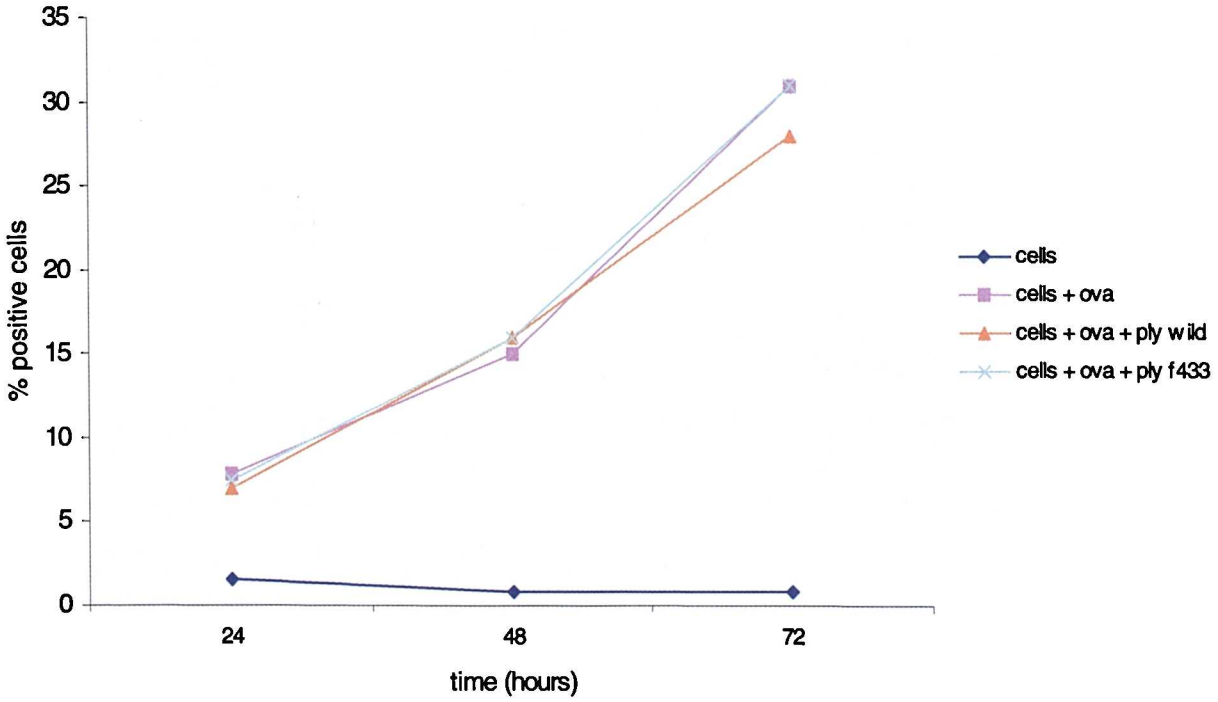
Figure 5 -6

The effect of pneumolysin upon T cell upregulation of activation markers

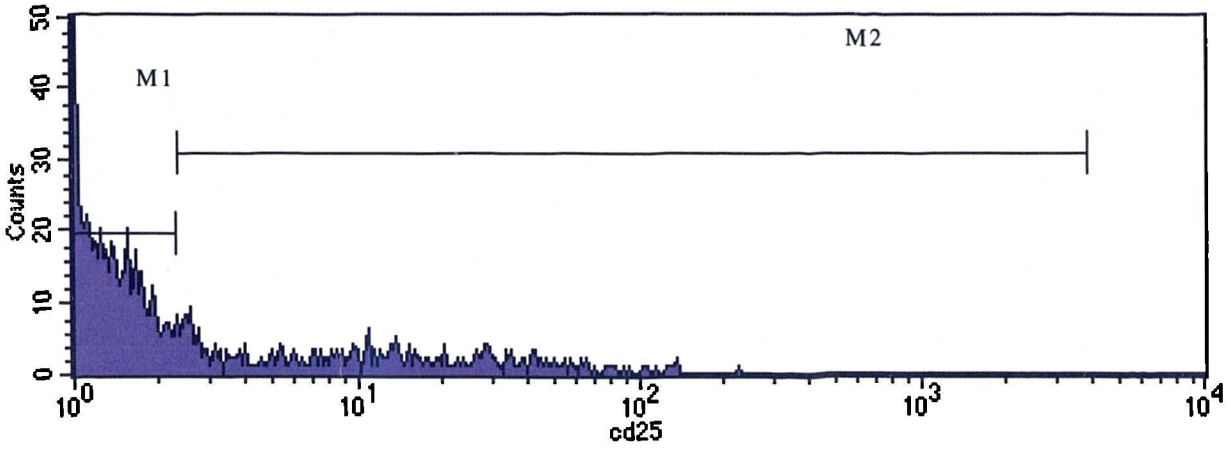
Lymph node single cell suspensions were prepared from DO11.10 mice and set up as for a cytokine assay with wild or F433 type pneumolysin at 40ng/ml where appropriate and 1mg/ml ovalbumin. At 24, 48 and 72 hours the expression level of CD25 and CD69 on transgenic T cells was measured by flow cytometry using CD4-PerCP, biotynilated KJ1.26, streptavidin-PE, and CD25-FITC or CD69-FITC. The data is presented as percent positive cells, however the Mean Fluorescence Intensity data is identical when compared. Isotype controls were used; Rat IgM FITC and Hamster IgG FITC. Unstimulated cells are represented by a blue line and stimulated cells by a pink line. Cells stimulated in the presence of wild or f433 pneumolysin are represented by a yellow or turquoise line respectively. The data obtained from flow cytometry is shown in C-N. The similarity between stimulated and stimulated in the presence of Wild type pneumolysin is evident.



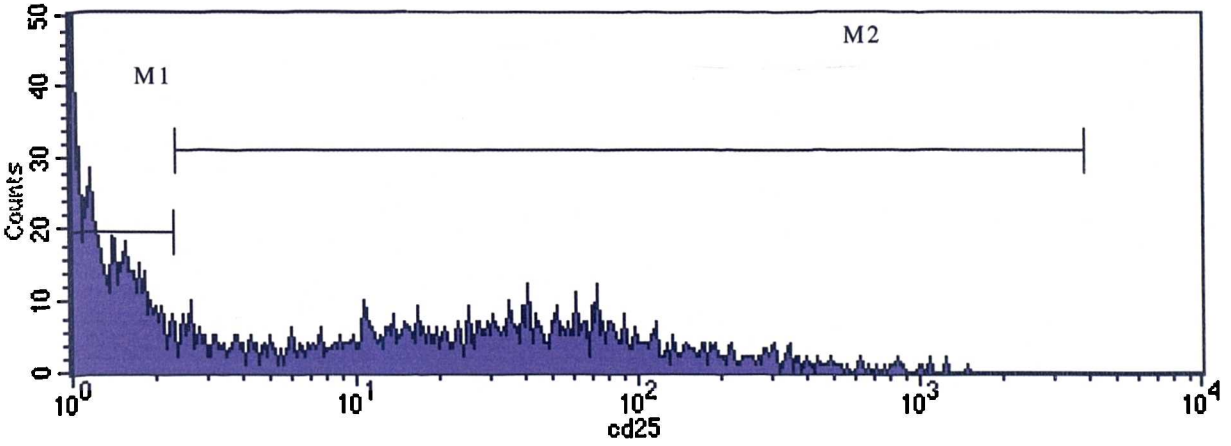
B - The effect of pneumolysin upon the expression of CD 25 on T cells



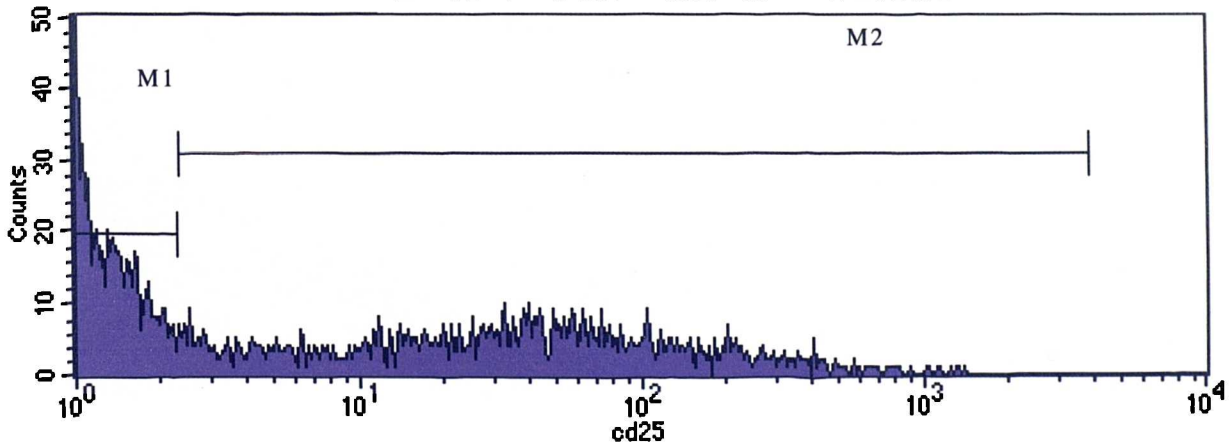
C - CD25 – 24hrs -Unstimulated



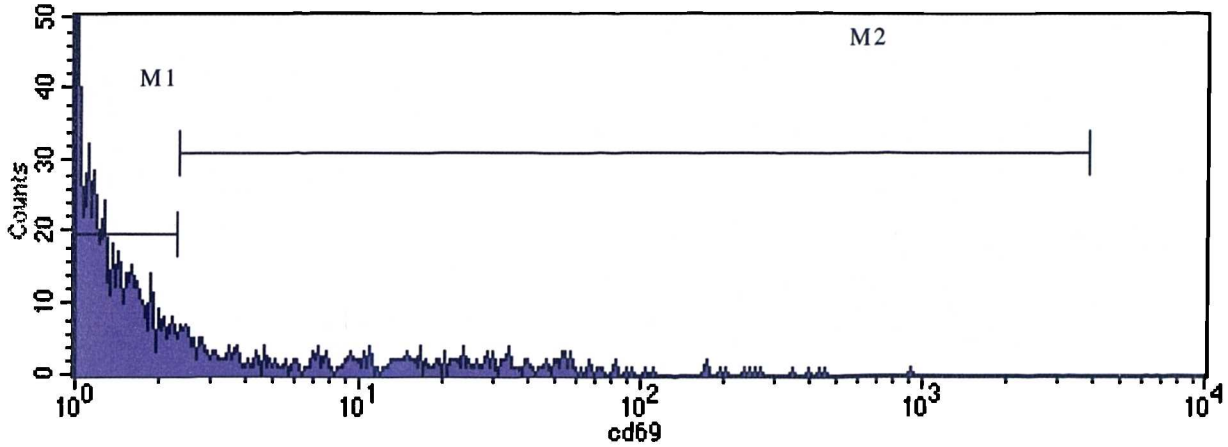
D - CD25 – 24hrs -Stimulated



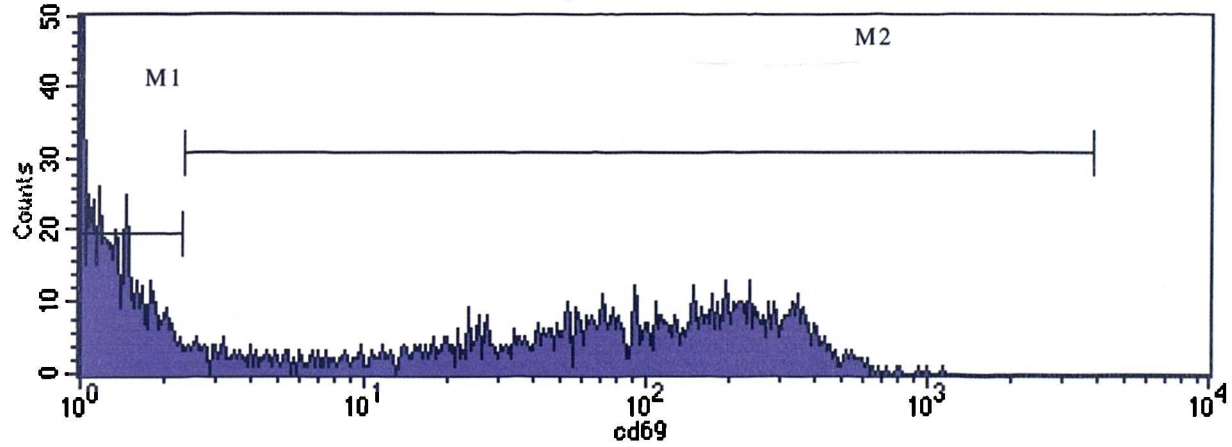
E - CD25 – 24hrs –Stimulated + Wild PLY



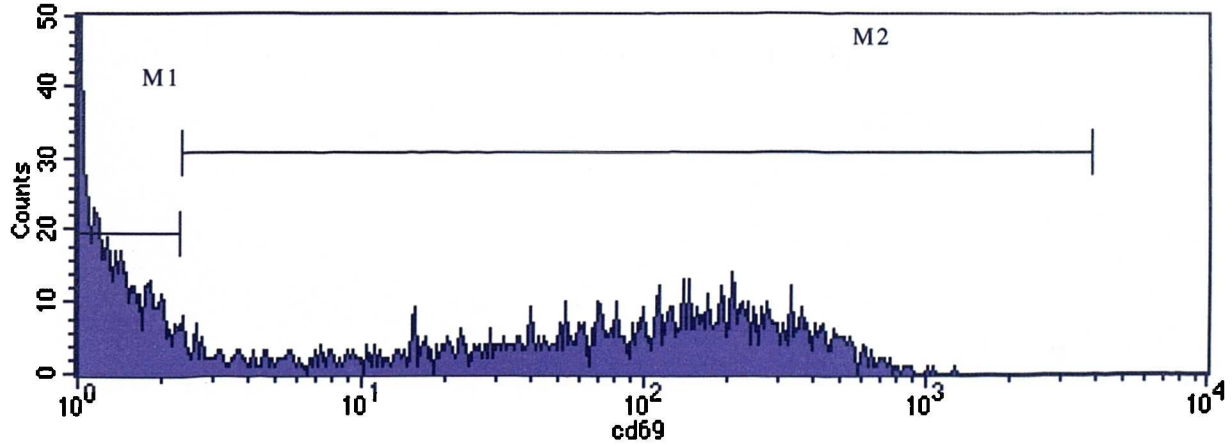
F - CD69 – 24hrs -Unstimulated



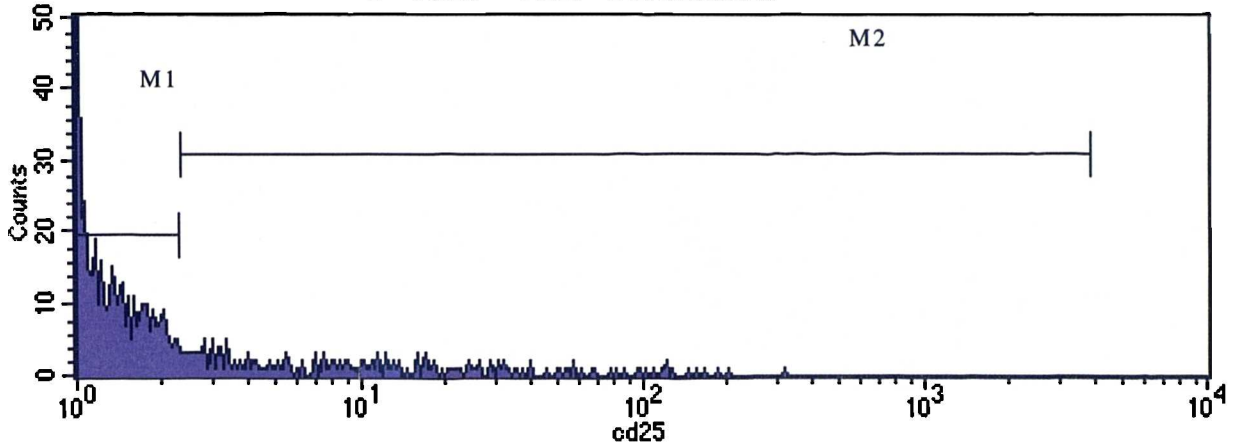
G - CD69 – 24hrs -Stimulated



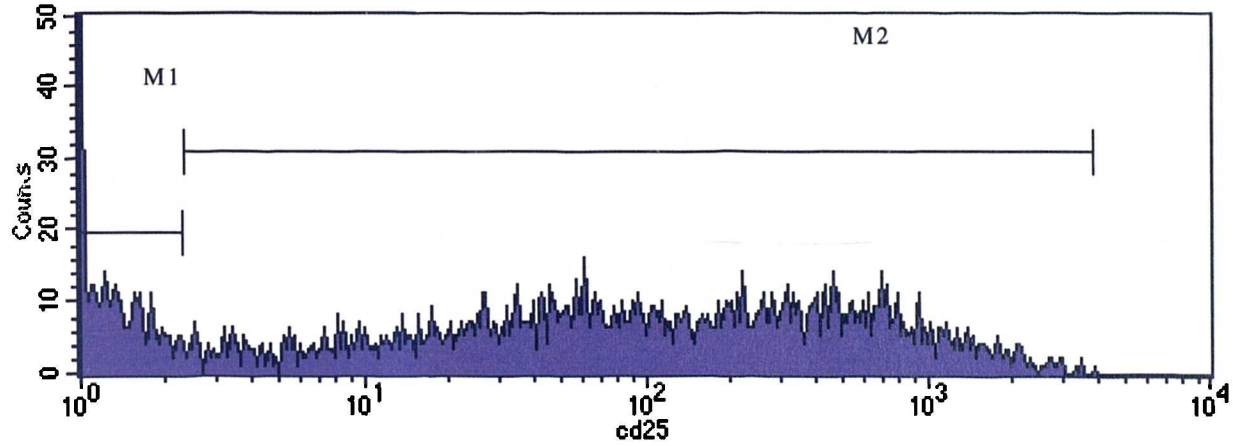
H - CD69 – 24hrs –Stimulated + Wild PLY



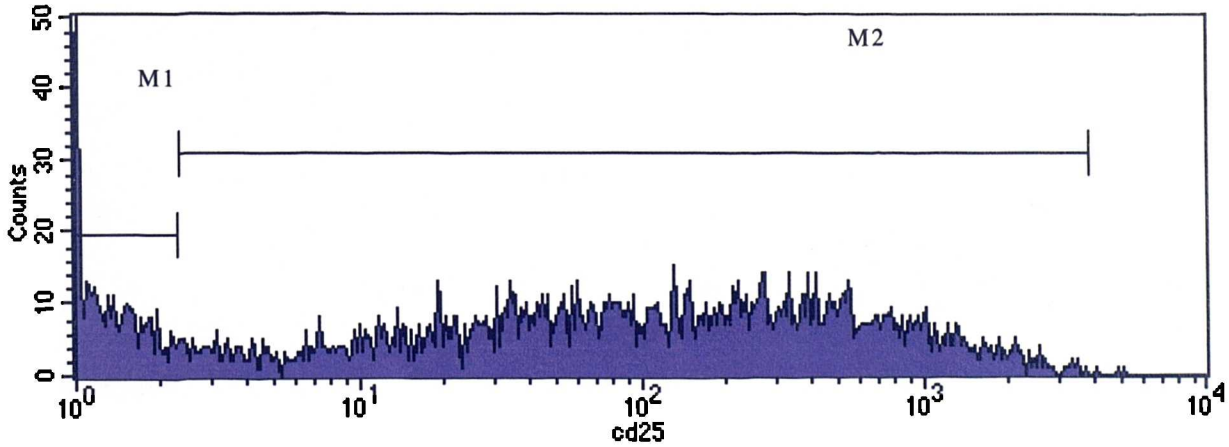
I - CD25 – 48hrs -Unstimulated



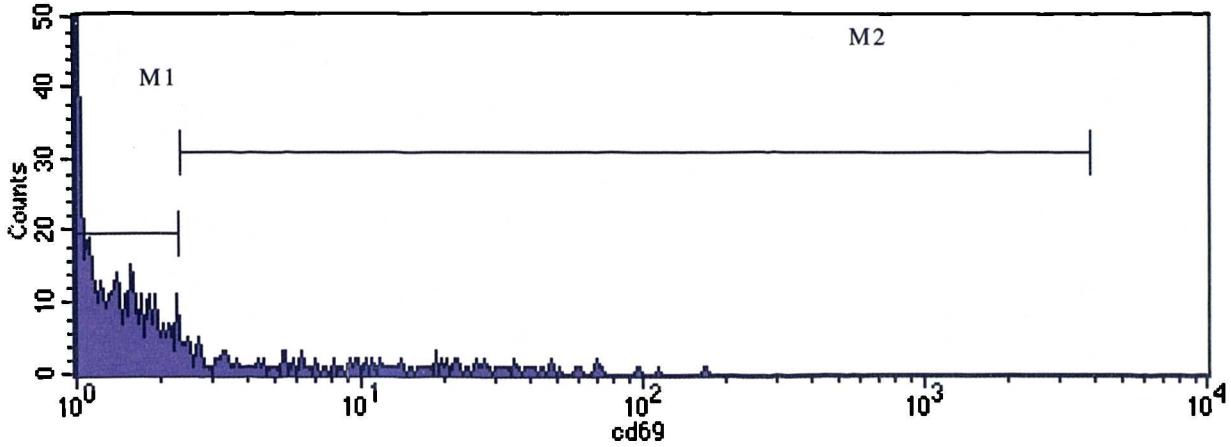
J - CD25 – 48hrs -Stimulated



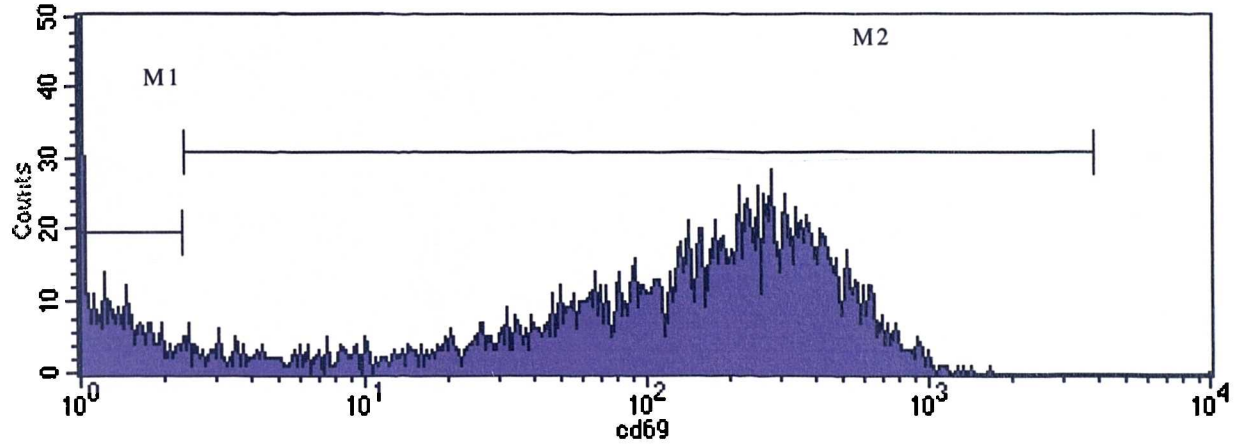
K - CD25 – 48hrs –Stimulated + Wild PLY



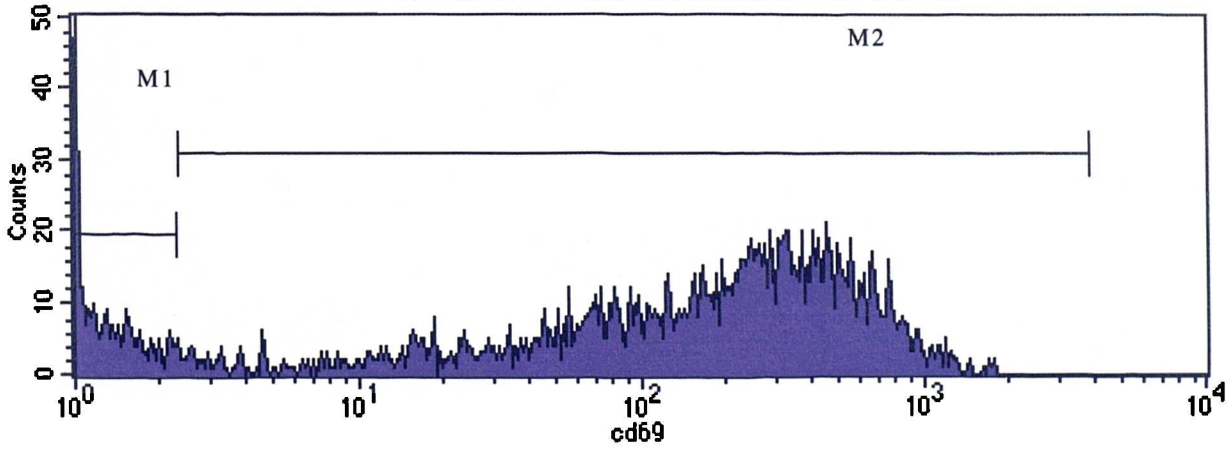
L - CD69 – 48hrs -Unstimulated



M - CD69 – 48hrs -Stimulated



N - CD69 – 48hrs –Stimulated + Wild PLY



transgenic T cells. The data presented above is representative of at least three experiments. Isotype controls showed no increased staining (data not shown).

From this data I observed that the Tg T cells all become activated to the same degree regardless of the presence or absence of pneumolysin. I concluded that pneumolysin's inhibitory activity was not the result of its interference with the T cell upregulation of activation markers. This data also allowed me to interpret the previous data from the GFP presentation assay. I now suggest that the most consistent interpretation of the presentation data is that pneumolysin is causing some alteration in the nature of the GFP T cell as opposed to inhibiting the presentation of antigen since if the latter were the case then I would expect to see a difference in the number of T cells becoming activated within this experiment.

5.2.2 The induction of T cell anergy, apoptosis and death by pneumolysin.

My data up to this point led me to conclude that pneumolysin was acting directly on the T cell and was acting later in the pathway of T cell activation to cause inhibition of proliferation. Given that the T cells are becoming fully activated yet are not proliferating I proposed two alternatives; either pneumolysin was inducing T cell anergy or pneumolysin was inducing T cell apoptosis subsequent to activation.

5.2.2.1 Pneumolysin does not induce T cell anergy.

My results indicated that T cells incubated with wild type pneumolysin and ovalbumin shared characteristics with anergic cells; both become activated and divide a few times then stop dividing and become unresponsive to antigenic stimulation. The unresponsiveness of anergic cells is broken by the addition of exogenous IL-2 which restores responsiveness, enabling them to proliferate.

While I had not demonstrated the unresponsiveness of the T cells treated with pneumolysin I had observed that the cells become activated yet do not proliferate to the same degree as untreated cells.

I had also demonstrated that APC's treated with pneumolysin process and present antigen to the same degree. Yet studies using LLO had found that LLO inhibits the T cell response not by altering the APC quantitatively by altering processing or presentation but rather by altering antigen presentation in such a way as to induce T cell anergy rather than proliferation (Darji *et al.*, 1997).

Exogenous IL-2 does not break the inhibitory effect of pneumolysin

I added exogenous IL-2 to stimulated whole lymph node cultures containing wild type pneumolysin and measured proliferation by thymidine incorporation to test the hypothesis that pneumolysin was inducing T cell anergy.

Figure 5-7 shows that in cultures without antigen cells proliferated little throughout the time period examined even with 1, 5 or 10 ng/ml of IL-2 present. Cells stimulated with ovalbumin proliferated above the unstimulated level by 48 hours reaching a cpm of 1 x 10^4 and by 72 hours 5.5 x 10^4 cpm. In comparison cells stimulated with ovalbumin in the presence of wild type pneumolysin while having similar cpm values at 24 and 48 hours to the untreated cells, by 72 hours had a cpm of 3.5 x 10^4. Therefore pneumolysin is inhibiting proliferation in this system as would be expected.

Cells stimulated with ovalbumin in the presence of 1 ng of IL-2 gave a cpm of 5.6 x 10^4 which is slightly greater than that observed for cells with ovalbumin alone. 5ng/ml of IL-2 gave a cpm of 6.8 x 10^4 after 72 hours however 10 ng/ml IL-2 recorded the highest cpm of 7.5 x 10^4. This data shows that addition of IL-2 to cultures stimulated with ovalbumin caused an increase in proliferation as the concentration of exogenous IL-2 increased.

Cells stimulated with ovalbumin and incubated with wild type pneumolysin in the presence of 1 or 5ng/ml of IL-2 did not differ at any time point in the amount of thymidine incorporated compared to cells stimulated with ovalbumin in the presence of wild type pneumolysin that lacked exogenous IL-2; all groups proliferated to give a cpm of 3.5 x 10^4 after 72 hours. In comparison, stimulated cells incubated with wild type pneumolysin in the presence of 10ng/ml of IL-2 proliferated slightly more so that after 72 hours they recorded a cpm of 4 x 10^4. The data from this experiment is representative of three experiments.

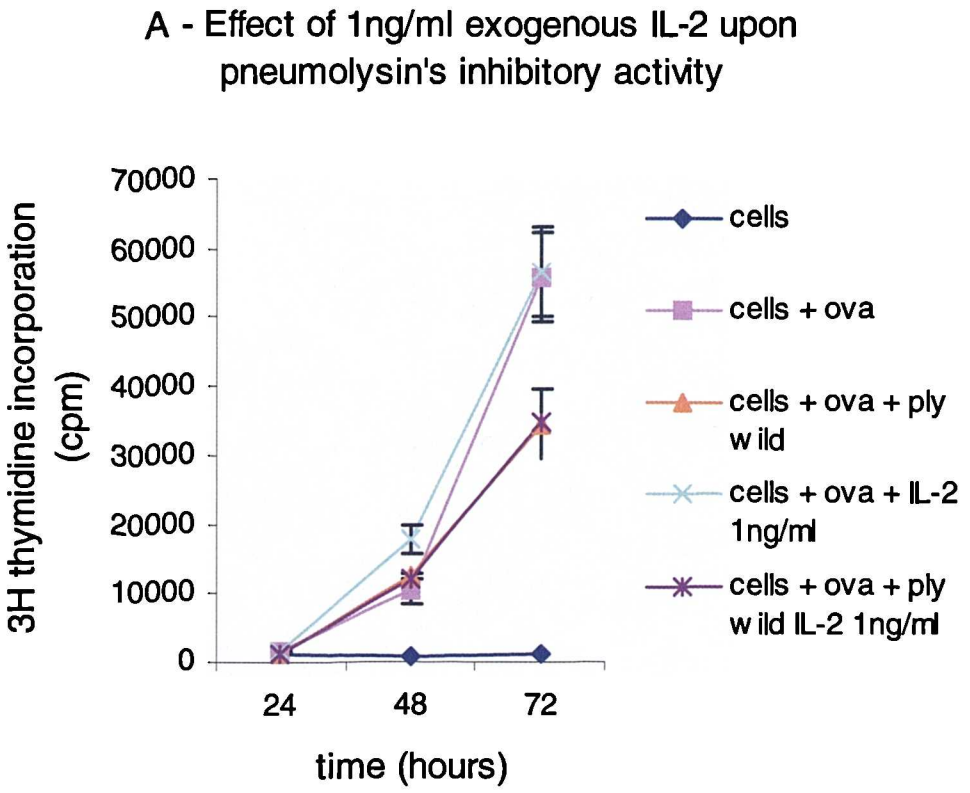
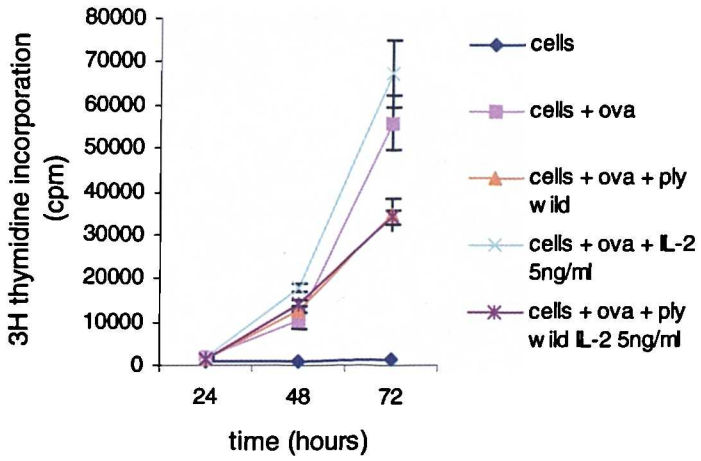


Figure 5 - 7

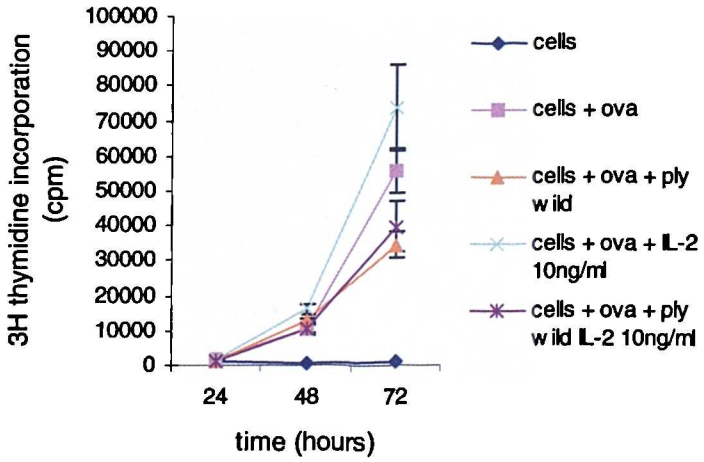
The effect of exogenous IL-2 upon the inhibitory ability of pneumolysin

Proliferation assays were set up using lymph node single cell suspension made from DO11.10 mice. 1mg/ml ovalbumin was added and 40ng/ml wild or F433 pneumolysin where necessary. IL-2 was added exogenously to some of the cultures during the set up procedure at concentrations of 1,5 and 10 ng/ml. These concentrations were chosen in light of the amount of IL-2 detected in a standard cytokine assay. Proliferation was measured by ^{3H} thymidine incorporation at 24, 48 and 72 hours. Unstimulated cells are represented by a blue line and stimulated cells by a pink line. Cells stimulated in the presence of wild type pneumolysin are shown as a yellow line. Stimulated cells with exogenous IL-2 are denoted by a turquoise line. Stimulated cells with exogenous IL-2 and wild type pneumolysin are shown as a purple line.

B - Effect of 5ng/ml exogenous IL-2 upon pneumolysin's inhibitory activity



C - Effect of 10ng/ml exogenous IL-2 upon pneumolysin's inhibitory activity



This data produced clear findings. Firstly, pneumolysin caused inhibition within this system. Secondly exogenous IL-2 clearly caused cells stimulated with ovalbumin alone to proliferate more than when exogenous IL-2 is not added. Thirdly, exogenous IL-2 does not break the inhibitory effect of pneumolysin and therefore pneumolysin does not induce T cell anergy.

5.2.2.2 Pneumolysin treated cells do not arrest in G0/G1 but progress to apoptosis.

Anergic cells arrest in the cell cycle at G0/G1. Therefore I would expect to observe an accumulation of cells at this stage of the cycle if anergy was induced within a population. In order to confirm the results above about anergy and begin to examine the question of apoptosis, I measured the cell cycle stages within a population of T cells stimulated with ovalbumin and incubated with pneumolysin. In order to assess individual cells rather than looking at a whole population I used laser scanning cytometry (LSC)

Figure 5-8 demonstrates that, after 24 hours, 85% of unstimulated cells are within either the G0 or G1-S gate as would be expected of resting cells. Some cells are found in the daughter cell gate however these are most likely to be pre-apoptotic rather than newly divided. By 48 hours 35% of unstimulated cells are apoptotic and 10% are in G0 with a further 20% in each of the G1-S and daughter gates. Cell cycle stages are similarly distributed at 72 hours. Thus, unstimulated cells, over the 72 hour time period, have the characteristics of a population progressing from resting to dying.

Cells stimulated with whole ovalbumin show the expected pattern of a proliferating cell culture where the cells move from being in G0 and G1-S at 24 hours through to being predominantly in G1- S or newly divided after 72 hours. Thus I observe that 90% of cells for the first 48 hours are in G0 or G1-S and then after 72 hours only 10% are in G0, 60% are in G1-S and 20% are newly divided daughter cells.

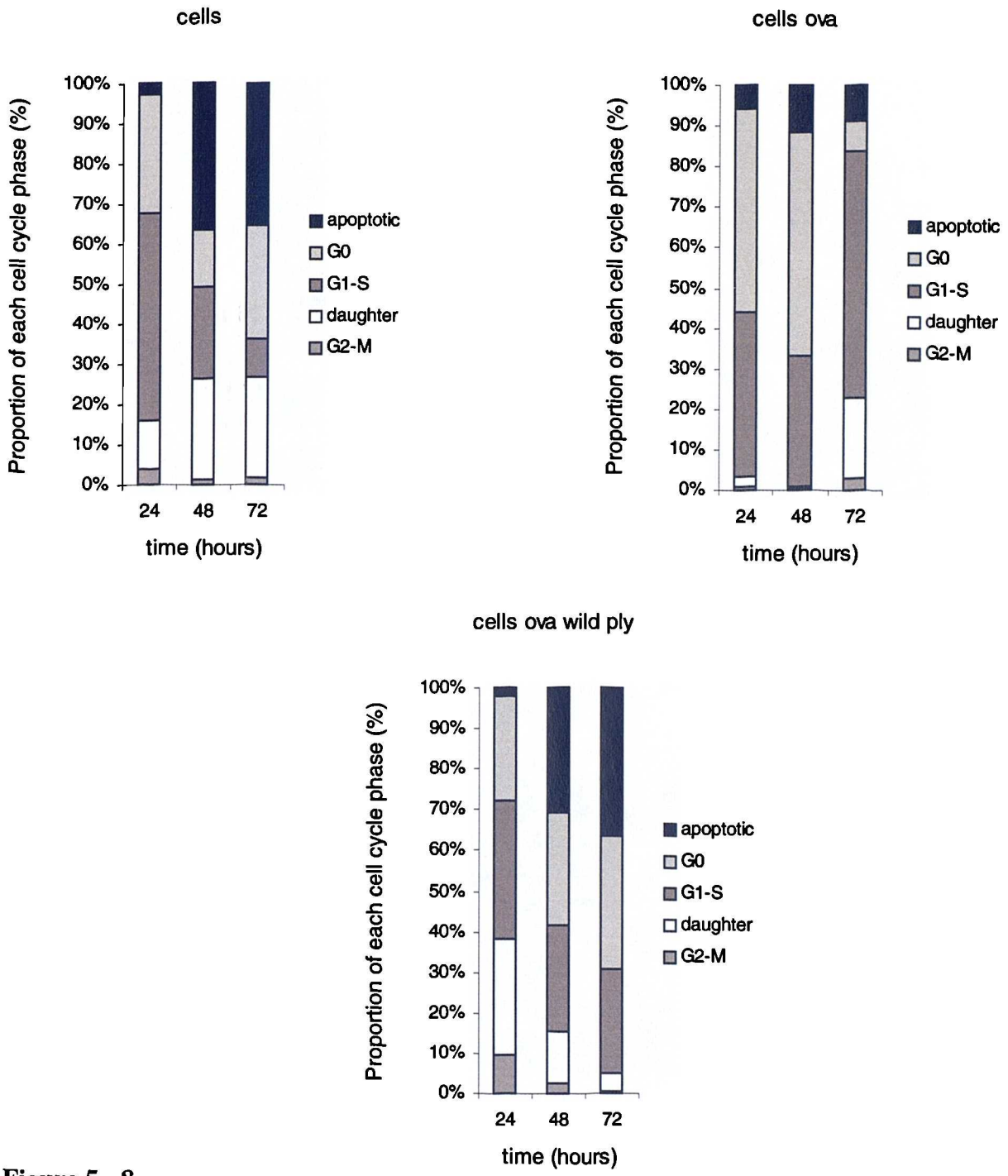


Figure 5 - 8

The effect of pneumolysin upon Tg T cell cycle progression

Single cell suspensions were prepared from DO11.10 mice and assays set up as for cytokine assays with 1mg/ml ovalbumin and 40ng/ml wild pneumolysin where appropriate. At 24, 48 and 72 hours cultures were harvested and prepared for analysis on the LSC by staining with KJ1.26 and DAPI. Cell cycle analysis of transgenic T cells was then carried out.

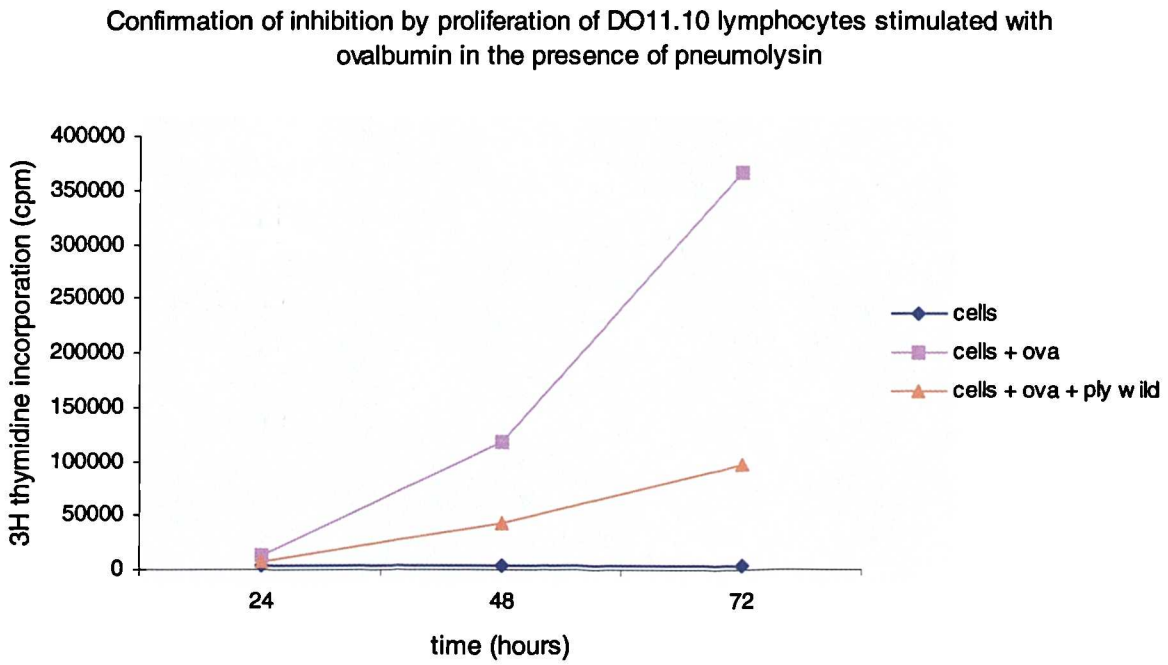


Figure 5-9

Proliferation of murine antigen specific T cells in the presence of pneumolysin

Cultures of single cell suspensions from DO11.10 mice were set up to measure proliferation and were stimulated with 1mg/ml ovalbumin. Pneumolysin was added simultaneously with the ovalbumin at a final concentration 40 ng/ml (yellow line). Proliferation was measured by thymidine incorporation at 24, 48, and 72. Unstimulated cells are indicated by a blue line. Stimulated but untreated cells are indicated by a pink line.

Stimulated cells treated with wild type pneumolysin while having 60% of cells in G0 or G1-S after 24 hours also have 30% of cells in the daughter cell gate. Those cells in the 'daughter cell' gate at 24 hours possibly progress by 48 hours to become apoptotic so that at 48 hours 30% of cells are apoptotic. There remains 60% of cells in either G0 or G1-S after 48 and 72 hours with the apoptotic group increasing to 35% of cells by 72 hours. The data for this experiment is representative of two experiments.

From this I can observe that pneumolysin does not cause an accumulation of cells in the G0 gate but rather appears to induce apoptosis of stimulated cells.

Figure 5-9 shows the proliferation data obtained from the same cells used for the LSC work presented above. This data shows that pneumolysin was acting in an inhibitory way as I had demonstrated previously and that the work using the LSC was representative of and relevant to earlier work such as Figure 4-13.

Pneumolysin induces apoptosis of activated T cells

In order to confirm the observations of the data presented above that pneumolysin induces apoptosis of activated T cells, I measured the level of apoptosis occurring in cultures of cells stimulated with ovalbumin in the presence of pneumolysin. I used the Annexin V / PI assay that utilises flow cytometry to measure apoptosis (see Figure 5-10).

AnnexinV/PI

As would be expected of unstimulated cells, after 72 hours 80% are dead or dying and 20% remain alive. After 96 hours the dead cells increase further to 85% and the live cells decrease to 15%. In comparison, after 72 hours 35% of cells stimulated with ovalbumin are alive and 65% dead and after 96 hours 50% of stimulated cells are alive and 50% dead. 25% of cells stimulated with ovalbumin in the presence of wild type pneumolysin are alive after 72 hours and after 96 hours 40% of

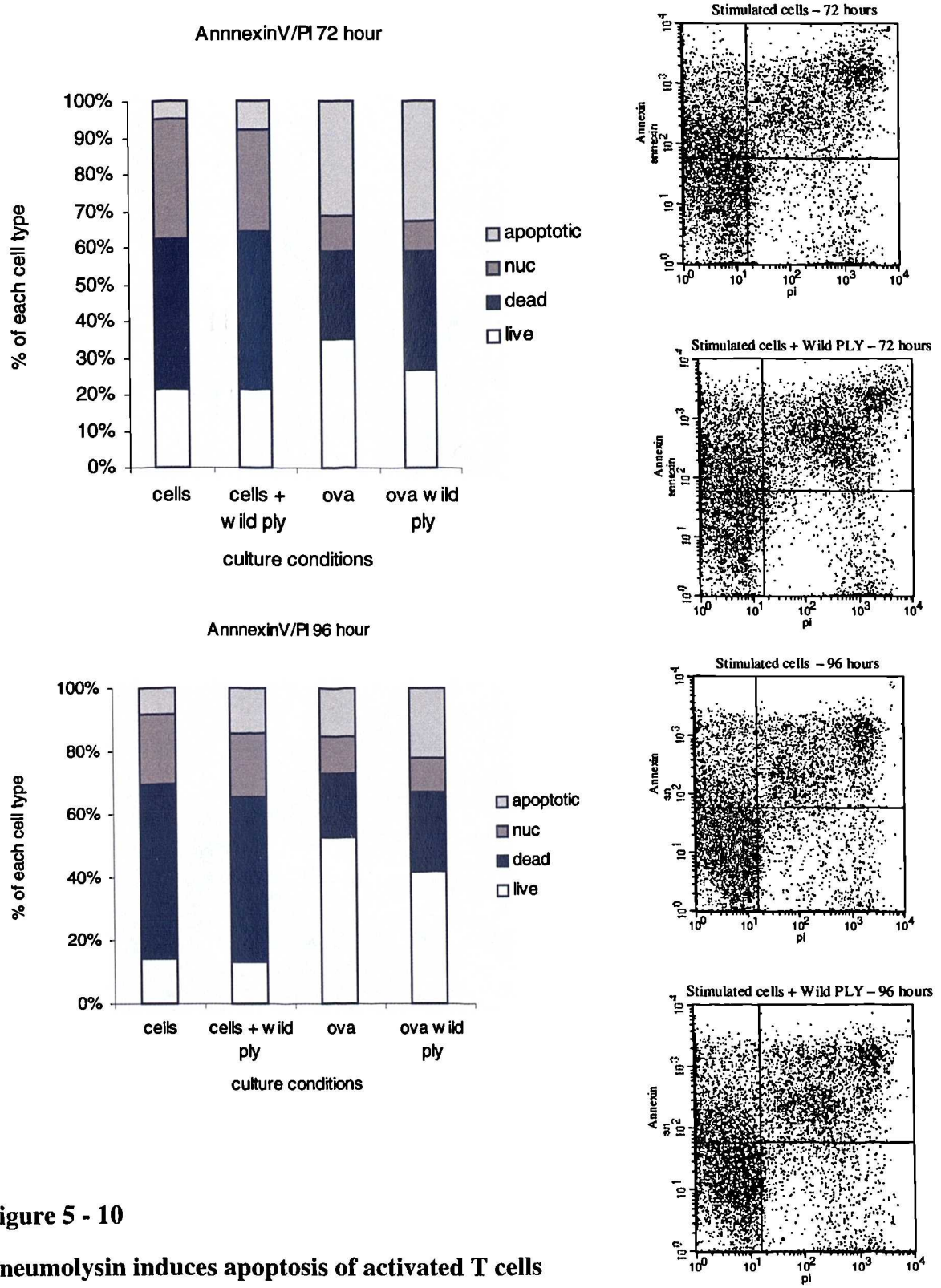


Figure 5 - 10

Pneumolysin induces apoptosis of activated T cells

Single cell suspensions were prepared from DO11.10 mice and were set up as for cytokine assays with 1mg/ml ovalbumin and 40ng/ml wild pneumolysin where appropriate. At 72 and 96 hours we measured the percentage of apoptotic cells within each culture condition using the AnnexinV/PI assay. Some of the flow cytometric data is shown. Quadrants; LL=Live cells, UL=Apoptotic, UR=Dead, LR=Nucleated.

cells are alive. This represents 10% less live cells at each time point for the cells stimulated with ovalbumin in the presence of pneumolysin compared to stimulated cells not exposed to pneumolysin. In terms of the actual number of apoptotic cells, at 72 hours there is no difference between cells stimulated with ovalbumin and those stimulated in the presence of pneumolysin. The overall difference between the two groups at this time point is observed in number of dead cells. In contrast at 96 hours, there is almost 10% more apoptosis occurring when cells are stimulated in the presence of pneumolysin than when cells are stimulated with ovalbumin alone.

It is clear that stimulated cultures increase in live cells over time whereas cells in unstimulated cultures die. Furthermore, it is also clear that stimulating cells in the presence of pneumolysin causes the induction of apoptosis.

5.2.3 Pneumolysin co-localises to lipid rafts.

The work presented in this section on lipid rafts and in Figure 5-11 was carried out in collaboration with Graeme Cowan, University of Glasgow. The work in this section is purely preliminary.

The data generated thus far pointed to the changes to the T cell upon activation as being crucial to the inhibitory effects of pneumolysin. One of the key changes upon T cell activation is the formation of lipid rafts which preferentially sequester cholesterol into their structure (Garcia *et al.*, 2003). While the exact role of cholesterol within the binding of pneumolysin to cells remains unclear it is obvious that cholesterol is vital to the binding activity (Giddings *et al.*, 2003). I therefore proposed that the inhibitory effect I was observing of pneumolysin upon activated T cells may be the result of accumulation of pneumolysin within the lipid rafts of activated cells. This would explain the difference in effect of pneumolysin upon stimulated and resting T cells since resting cells would have far

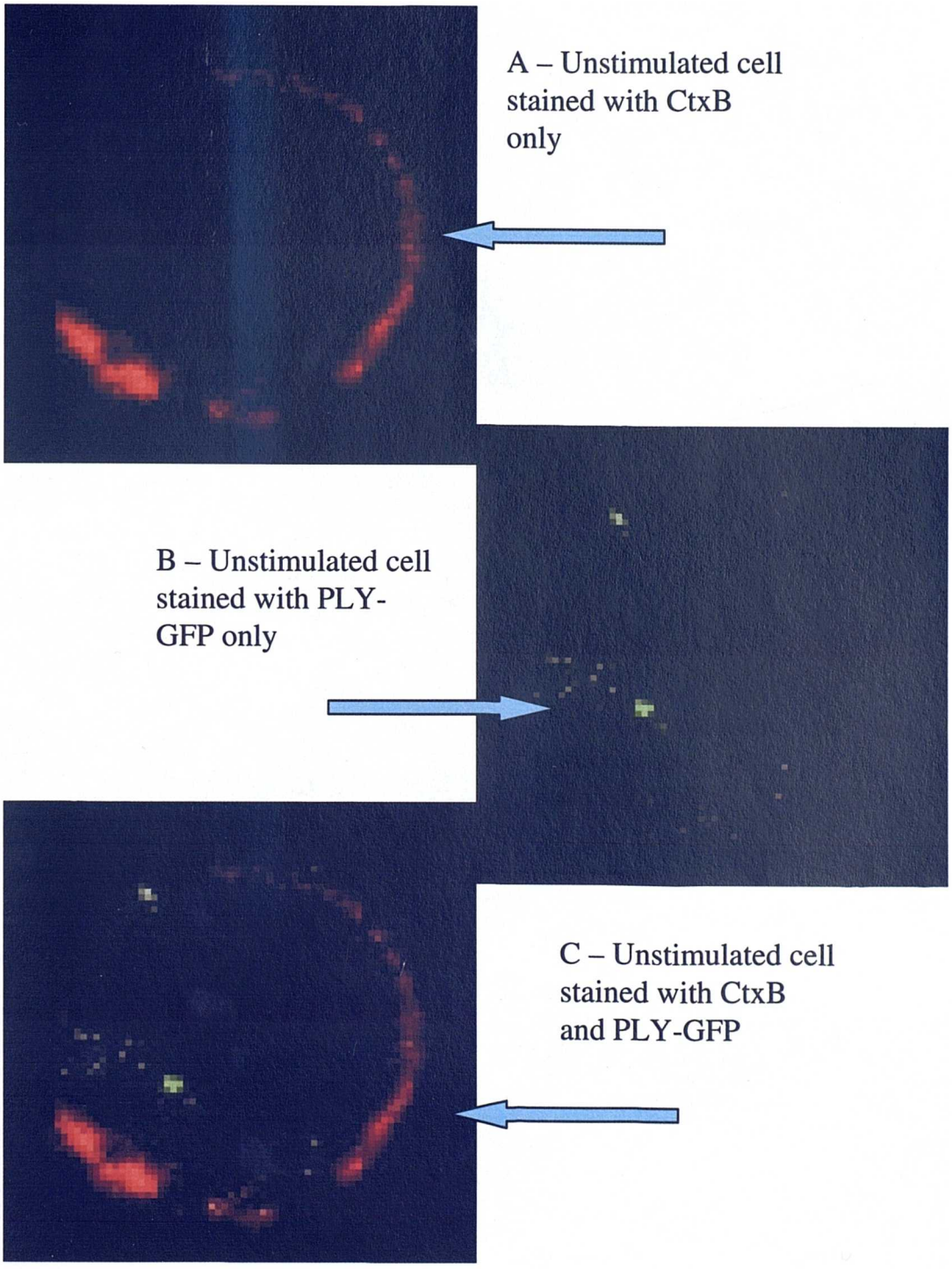
less lipid rafts. I therefore assessed the binding of pneumolysin to lipid rafts on resting and activated cells.

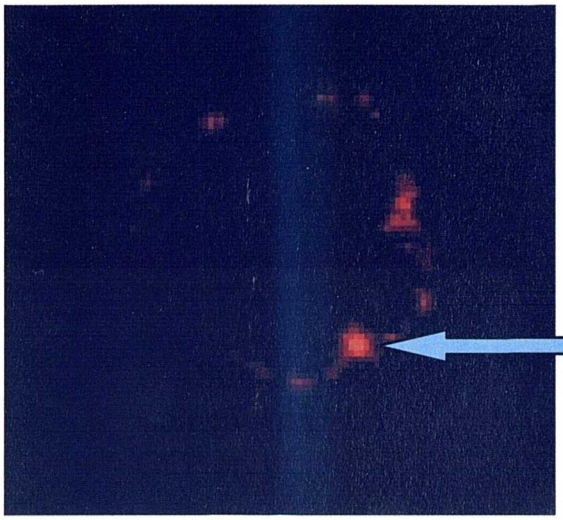
As observed in Figure 5-11, unstimulated cells (A) have a diffuse pattern of GM-1 distribution indicating the absence of lipid rafts while pneumolysin (B) is not localised to a particular region. Furthermore no co-localisation of the cholera toxin and pneumolysin is observed in C. In comparison, stimulated cells (D) show a punctate pattern of GM-1 distribution that is characteristic of lipid raft accumulation in the cell surface membrane and co-localisation is observed of the lipid rafts with pneumolysin in E. This data suggests that pneumolysin does indeed concentrate within the lipid rafts of activated T cells.

Figure 5 - 11

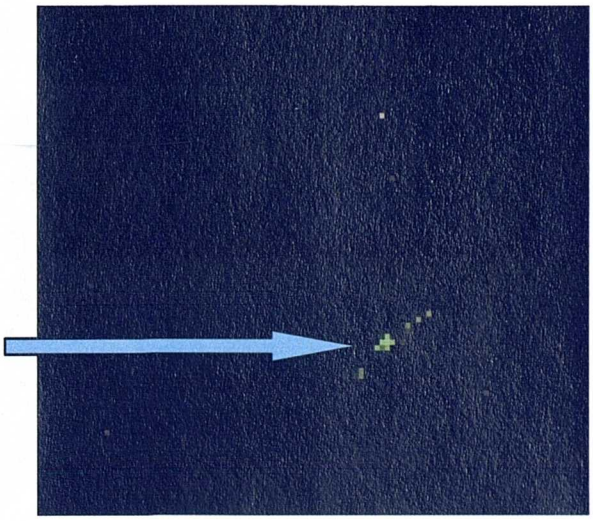
Pneumolysin co-localises to lipid rafts

We used lymph node preparations from DO11.10 mice and where appropriate stimulated them using ovalbumin. We compared the localisation of GFP-PLY6 with the raft marker CtxB- on activated and unactivated cells.

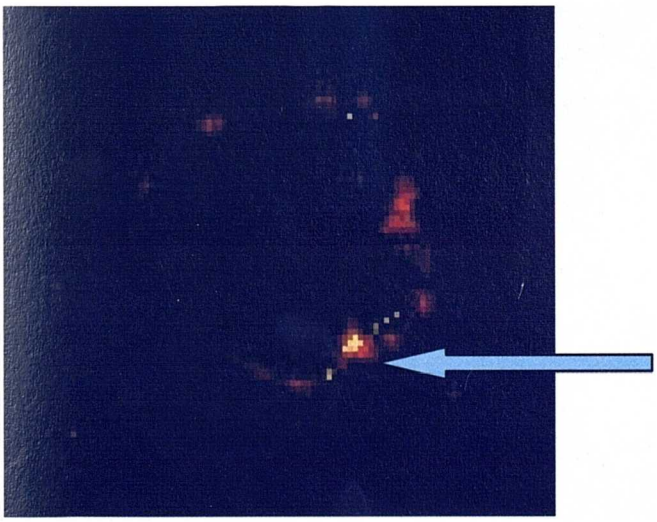




D – Stimulated cell stained with CtxB only



E – Stimulated cell stained with PLY-GFP only



F – Stimulated cell stained with CtxB and PLY-GFP

5.3 Discussion

In this chapter I demonstrated that pneumolysin acts directly upon the T cell, inducing apoptosis by a mechanism potentially involving preferential binding to lipid rafts. By sequential examination of the possible sites of pneumolysins action, I demonstrated that the mechanism of T cell inhibition by pneumolysin does not involve inducing a secondary messenger from accessory cells within the lymph node preparation. Furthermore I demonstrated that pneumolysin does not alter antigen processing and presentation by dendritic cells and that pneumolysin acts directly upon the T cell. I demonstrated that pneumolysin does not alter the upregulation of T cell activation markers nor does it induce T cell anergy but that pneumolysin induces apoptosis of activated T cells.

To examine if pneumolysin was acting indirectly upon the T cell via accessory cells I was required to use T cells purified from naive mice along with BMDC. Since studies using these cells were to be compared to my earlier work using whole lymph node preparations, it was important to keep the number of variables to a minimum. It has been shown that DC's are the primary APC responsible for presenting antigen to naive T cells (Jenkins *et al.*, 2001) and so I wanted to use purified DC's as APC within my proposed system. At the time of my studies it was impractical to use DC's isolated directly from mice. I therefore used BMDC. Clearly the use of BMDC introduces a variable when comparing this experiment to my earlier studies using the whole lymph node preparation. However it has been shown that BMDC function similarly to splenic isolated DC *in vitro* (Garrigan *et al.*, 1996). I was therefore confident that my results using BMDC would be a true representation of the situation when using lymph node preparations. The work of Colino *et al.* (Colino and Snapper, 2003a) demonstrated that pneumolysin is able to induce apoptosis of dendritic cells. I therefore checked that at the concentration of pneumolysin I was using dendritic cells were remaining viable (data not shown). Colino *et al.* had demonstrated this effect in experimental systems different to ours particularly in the use of high concentrations of whole bacteria.

As I discussed in the introduction to this thesis, pneumolysin has many effects on different cell types. One of those effects is inducing macrophage to release nitric oxide (Braun *et al.*, 1999). However the results of Figure 5-2 show that accessory cells such as macrophages are not required for the T cell inhibitory activity of pneumolysin. This means that despite the range of effects of pneumolysin on cells none of these effects are critical to mediate the effect of inhibiting the T cell

response. It should be noted however that the possibility of a synergistic effect between a released factor and pneumolysins inhibitory action cannot be ruled out.

Despite these findings there remained the possibility that pneumolysin was causing the BMDC to release a T cell inhibitory factor. Reports in the literature had suggested that BMDC may be able to release nitric oxide to a greater extent than *ex vivo* DC (Powell *et al.*, 2003) and the inhibitory effect of nitric oxide release by BMDC on T cell proliferation had been confirmed (Bonham *et al.*, 1996). We demonstrated that the inhibitory effect was not dependent on BMDC by utilising APC independent proliferation. This was a critical result to enable us to conclude that BMDC are not releasing an inhibitory factor.

In order to examine the question of the effect of pneumolysin on antigen processing by APC I chose to revert to using the whole lymph node system. This allowed me to make a direct comparison of the effect of pneumolysin upon antigen processing requirements for the inhibitory effect of pneumolysin since the only variable between my earlier experiments and these experiments was the change in using whole ovalbumin as antigen to using ovalbumin peptide. I found that pneumolysin was still able to inhibit T cell proliferation even when peptide was used as antigen and therefore was able to conclude that pneumolysin did not act by inhibiting the processing of antigen by the APC. My results are in contrast to the findings of Darji *et al.* (Darji *et al.*, 1997),who found that LLO inhibited T cells by altering antigen processing so that antigen was presented in a way that induced T cell anergy. In further contrast to my results they demonstrated that peptide-induced T cell activation is not affected at all by LLO since the inhibitory mechanism is by-passed.

For my studies on antigen presentation I used the GFP expressing T cell hybridoma. This system has previously been used to measure the amount of antigen presented by dendritic cells (Sun *et al.*, 2003) (Rush *et al.*, 2002). Similar systems using different reporter genes have also been used (Storozynsky *et al.*, 1999) (Hartmann and McCoy, 1996; McCoy *et al.*, 1995) to measure antigen presentation, however use of the DO11.10 GFP hybridoma is a rapid and quantitative measure of antigen presentation (Underhill *et al.*, 1999). We found that pneumolysin appeared to inhibit the expression of GFP in hybridoma T cells which suggests a decreased amount of antigen was being presented to the cell. In hindsight the fact that I was examining the effect of a toxin upon T cells makes the choice of a non-T cell readout more attractive since the potential complication is evident; i.e. it is equally reasonable to suppose that the toxin may affect the readout T cells as it does

the normal T cells in my previous experiments. In recognition of this I suggest the detection of antigen bound to the MHC of the APC would be a better method. At the time of experimenting such a method was available (Kobets *et al.*, 2003; Manickasingham and Reis e Sousa, 2000; Reis e Sousa and Germain, 1999; Zhong *et al.*, 1997) however this method does not yield particularly clear data and so was not used. Subsequently a better method has been developed that could be used (Itano *et al.*, 2003). Earlier it was noted that Vac A of Helicobacter pylori inhibits T cells by preventing the translocation of NFAT (Gebert *et al.*, 2003). This work on Vac A meant that my proposal, that pneumolysin may be interfering with the T cell and specifically with NFAT, was not unprecedented.

I went on to measure the degree of T cell activation within the system in which I had observed pneumolysin inhibiting the T cell response. I found that pneumolysin did not alter the expression level of the activation markers that I measured. It is common practice to measure CD69 and CD25 when examining the effects of a potential immunomodulator upon T cells (e.g. (Holcombe *et al.*, 2002)). CD25 is part of the IL-2 receptor while the function of CD69 is unknown but its expression level on the T cell surface is observed to increase rapidly upon activation (Ziegler *et al.*, 1994). Not only does CD25 inform about the activation state of the T cell but it also informs about the ability of the T cell to utilise IL-2. Some toxins produced by pathogens have been shown to alter IL 2 receptor expression; either increasing it (Bone *et al.*, 2002) or decreasing it (Szamel *et al.*, 1997) yet in our study pneumolysin did not alter expression level at. A previous study examined the effect of radiation killed *Streptococcus pneumoniae* on the CD25 and CD69 expression levels on T cells isolated from humans (Arva and Andersson, 1999a). The researcher found that incubating *Streptococcus pneumoniae* with the T cells caused an increase in expression of CD69 and CD25 within the first 24 hours when compared to unstimulated cells. However compared to T cells stimulated with PHA this increase was significantly smaller. The study did not compare the effect of incubating the pneumococcus with a bystander antigen and so the results are not directly relevant to my work. Additionally, the use of the whole pneumococcus, as I saw earlier, introduces a range of variable that were absent from my studies using the purified toxin.

The apparent incongruence in my results between measuring NFAT expression via the GFP hybridoma cells and measuring activation markers on *ex vivo* T cells requires explanation. It may be expected that these two experiments would yield the same result yet the GFP system suggested pneumolysin was inhibiting presentation while the activation marker system suggested there was

no difference in presentation. The difference in susceptibility of different cell types to pneumolysin is my primary hypothesis for this incongruence since it is probable that the hybridoma will have a different susceptibility compared to *ex vivo* T cells. However it has also been demonstrated that hybridoma T cells and primary T cells have a different susceptibility to death due to different biochemical features that make hybridoma T cells initially more prone to die after TcR stimulation (Hildeman *et al.*, 2002).

Darji *et al.* (Darji *et al.*, 1997) had found that LLO inhibits T cell altering APC processing of antigen in such a way that causes the presented antigen to induce T cell anergy. As I mentioned above they had found that LLO was unable to inhibit T cells when using peptide antigen. My work contrasts with this since I found that pneumolysin exerted no effect on the APC in terms of antigen processing or presentation. None the less I recognised an important point from their work; that despite the APC appearing to present antigen to the same degree, it may be the case that the antigen is presented in a qualitatively different manner that causes the induction of T cell anergy. I therefore were concerned to use exogenous IL-2 to examine if anergy was being induced by pneumolysin. I showed that anergy was not being induced since the addition of exogenous IL-2 did not restore T cell proliferation and so concluded that pneumolysin was utilising a mechanism distinct from that used by LLO in the reports by Darji *et al.* of T cell inhibitory activity (Darji *et al.*, 1997).

My observation that addition of exogenous IL-2 to proliferation cultures did not alter the inhibitory activity of pneumolysin and therefore was not inducing anergy *in vitro* (DeSilva *et al.*, 1996), led me to measure apoptosis. The work of Li *et al.* (Li and Darzynkiewicz, 1995) helped me to interpret my results from Laser Scanning Cytometry which initially indicated that cells treated with pneumolysin had a rapid increase in the number of daughter cells. Li *et al.* showed that this cell population with the phenotype of daughter cells is in fact a pre apoptotic group that has condensed chromatin not as the result of being newly divided but rather as the result of being apoptotic. My data from the LSC (see Figure 5-8) showed that pneumolysin was inducing the formation of an apoptotic group of cells by 72 hours. I subsequently measured apoptosis using the Annexin V / PI method (see Figure 5-10). Overall my results from this method were consistent with those from the LSC; that pneumolysin is causing increased death of activated cells, however the actual percentages between the methods are not the same. Using the Annexin V method revealed that by 72 hours 75% of cells stimulated with ovalbumin and pneumolysin are dead or dying while using the

LSC only 35% of cells are dead or dying. This discrepancy is more likely accounted for by the difference in technique. Highly fragmented DNA is unlikely to be picked up by the LSC and so cells that are already dead may not be recorded thereby giving an unusually low record when compared to a method such as Annexin V / PI which recorded dead cells. Additionally the Annexin V / PI method measures cell permeability and phosphatidylserine exposure which are early indicators of cell death, whereas the LSC technique measure DNA fragmentation which is later in the cell death pathway. These difference in technique are likely to be responsible for the difference in percentage between the experiments.

After establishing that the T cell inhibitory mechanism of pneumolysin is the induction of apoptosis the natural progression was to examine the mechanism of inducing apoptosis. A number of findings made me consider the role that lipid rafts may have in the mechanism of induction of apoptosis by pneumolysin. Shimada *et al.* (Shimada *et al.*, 2002) had previously demonstrated that the pneumolysin related toxin, perfringolysin, preferentially bound to the cholesterol in lipid rafts. In addition Tuosto *et al.* (Tuosto *et al.*, 2001) had shown that activated T cells have more lipid rafts than resting T cells. I had observed a difference in susceptibility to apoptosis between resting and activated T cells and I therefore postulated that this difference may be the result of pneumolysin interacting differently with cells containing high numbers of lipid rafts.

My studies on the colocalisation of pneumolysin to lipid rafts are preliminary. I found that pneumolysin does co-localise to the lipid raft however I used a small group size and identified the cells as being lymphocytes only by light morphological examination. Further investigations would need to use a larger study group and identify the cells examined as being activated Tg T cells. It would also be informative to examine whether activated T cells bind more pneumolysin than unactivated T cells. The answer to this possibility would help to determine whether pneumolysin causes apoptosis of activated T cells as a result of increased toxin binding per cell, or because of unequal distribution of toxin on the cell membrane. Finally it would be informative to examine the effect of the F433 pneumolysin mutant upon lipid rafts. The popularity of FRET analysis to examine colocalisation more definitively would also be an advantage to further studies (Kenworthy *et al.*, 2000; Willhite *et al.*, 2002).

My tentative findings that pneumolysin interacts with lipid rafts on activated T cells to induce apoptosis are consistent with other studies on the mechanism of apoptosis induction by pathogen toxins (Manes *et al.*, 2003; Nusrat *et al.*, 2001; Rohde *et al.*, 2003; Wu *et al.*, 2003). For example, studies on shiga toxin have shown that one of the mechanisms the toxin uses to induce apoptosis of it target cell is via preferential binding to its receptor located in lipid rafts (Cherla *et al.*, 2003). This enhances the uptake of the toxin into the cell where it then stimulates an apoptotic signalling cascade (Kovbasnjuk *et al.*, 2001). Tetanus toxin also requires the presence of lipid rafts to allow it to bind to its GPI anchored protein receptor (Munro *et al.*, 2001). Williams *et al.* (Nashar *et al.*, 1996) demonstrated that the non-toxic B subunit oligomer (EtxB) of *Escherichia coli* heat-labile enterotoxin (Etx) induces apoptosis of CD8 T cells by cross linking the GM-1 receptor on the T cell surface. The signalling pathways involved have recently been elucidated (Salmond *et al.*, 2002). Vac A of Helicobacter pylori has been shown to bind preferentially to lipid rafts (Kuo and Wang, 2003; Patel *et al.*, 2002; Ricci *et al.*, 2000; Schraw *et al.*, 2002). Finally the pore forming toxin, aerolysin, has been shown to induce T cell apoptosis. There is some debate over whether aerolysin preferentially binds to lipid rafts (Nelson and Buckley, 2000) but the evidence is in favour of it doing so (Abrami and van Der Goot, 1999; Abrami *et al.*, 2000). This leads to selective permeabilisation of the membrane which causes the cell to take on the characteristics of apoptotic cells (Abrami *et al.*, 1998). The authors have subsequently shown that the preferential association of the toxin with lipid rafts effectively results in areas of the membrane that have a high toxin concentration. This allows the toxin to oligomerise and therefore form pores in the membrane (Abrami and van Der Goot, 1999). Clearly the similarities between aerolysin and pneumolysin make the theories proposed above for aerolysin a potential staring point for further examination of the mechanism of pneumolysin.

During the writing of this thesis Zenewicz *et al.* (Zenewicz *et al.*, 2004) published a seminal paper. Previously it had been demonstrated that during listeria infection lymphocyte apoptosis occurs (Merrick *et al.*, 1997). However Zenewicz *et al.* (Zenewicz *et al.*, 2004) found that listeriolysin induces the upregulation of Fas L on T cells yet does not alter the expression of CD25 or CD69 (or CD44, CD28, CD128). These findings regarding CD25 and CD69 are consistent with my data. Shortly after the publication of the paper by Zenewicz *et al.* (Zenewicz *et al.*, 2004), Carrero *et al.* (Carrero *et al.*, 2004) published data that demonstrated that *in vitro* LLO induces apoptosis of activated T cells at a dose of 25ng/ml via a caspase dependent mechanism. Clearly the consistency of all these findings with my data consolidates my finding that pneumolysin induces T cell apoptosis.

One of the key issues to address after my findings was the type of apoptosis induced by pneumolysin. The recent recognition of multiple apoptotic pathways not restricted to death receptor mediated pathways (Davidson *et al.*, 2002; Jaattela and Tschopp, 2003; Proskuryakov *et al.*, 2003) (Figure 1-5) and the different implications of each (Hildeman *et al.*, 2002) means that the elucidation of the pathway induced is critical. However the work by Zenewicz *et al.* appears to give me a starting place by implicating Fas in the mechanism of apoptosis. The work of Scheel-Toellner *et al.* (Scheel-Toellner *et al.*, 2002) recently demonstrated the necessity of lipid rafts for the induction of apoptosis by Fas while rafts have been proposed as central to the control of apoptosis due to their ability to recruit signalling molecules, thereby allowing their interaction (Garcia *et al.*, 2003). In addition to this resting T cells are generally resistant to Fas mediated death (Budd, 2001). All of these studies seem to be consistent with my findings on the mechanism of inducing apoptosis by pneumolysin. As a result of these I speculate that Fas mediated apoptosis is likely to be the mechanism of apoptosis used by pneumolysin and that the preferential binding of pneumolysin to lipid rafts will be an important part of this mechanism. Interestingly *S. pneumoniae* has been found to induce apoptosis of dendritic cells by a pneumolysin dependent but caspase independent mechanism (Colino and Snapper, 2003a) and in neuronal cells by a caspase independent mechanism (Braun *et al.*, 2002). An examination of the caspase dependency and Fas involvement of the apoptosis of T cells by pneumolysin would therefore be informative.

My studies here suggest that pneumolysin and thereby *Streptococcus pneumoniae* could join a growing list of toxins / pathogens that induce apoptosis of T cells. I recognised that the importance of pneumolysin's induction of apoptosis to the knowledge of pneumococcal infection was dependent upon proving that pneumolysin induces T cell apoptosis *in vivo* during pneumococcal infection. Similarly the importance of pneumolysins induction of apoptosis to its usefulness as an immunomodulator is also dependent on proving it functions in this way *in vivo*.

I start to address these issues in the following chapter however it is pertinent at this point to mention the work of Kemp *et al.* (Kemp *et al.*, 2002) who studied T cells in human patients with pneumococcal infections. In their studies they noticed that pneumococcal infections were associated with an increase in the number of apoptotic T cells and soluble Fas levels and a decrease in the

number of activated T cells. The number of resting T cells remained constant. Furthermore they they found that the CD4 T cell count returned to normal after infection and the decrease in CD4 T cell numbers was found in the T_H1 T cell compartment. These results are remarkably consistent with the predictions that would be drawn from my data. The authors did not suggest a mechanism for the decline in T cell numbers however I propose, as a result of my studies, that pneumolysin is a likely cause of this finding.

6 Assessing the T cell inhibitory ability of pneumolysin *in vivo.*

6.1 Introduction

The importance of my findings regarding the induction of T cell apoptosis by pneumolysin is dependent on proving that pneumolysin functions in this way *in vivo*. This is for two reasons; firstly the relevance of pneumolysin inducing T cell apoptosis *in vivo* may contribute to the understanding of pneumococcal infection but only if this is actually what occurs during infection. Furthermore, the potential usefulness of pneumolysin as a therapeutic molecule clearly depends on it being able to function *in vivo*.

My studies on the interaction of pneumolysin and the T cell would, until recently, have been regarded as irrelevant since the T cell was regarded to have no role in pneumococcal infection (Winkelstein and Swift, 1975). However, there has been renewed interest in the role of the T cell in pneumococcal infection and this interest appears set to increase (Kadioglu and Andrew, 2004). As seen in the introduction to this thesis, recent work suggests that the T cell may be involved in two aspects of pneumococcal infection. Some investigators have suggested a possible role for T cells as part of the innate response to pneumococcal lung infection (Kadioglu *et al.*, 2000) while the work of a number of groups have identified an essential role for CD4 T cells in the antibody response to pneumococcal protein and polysaccharide antigens (Baxendale *et al.*, 2000; Hwang *et al.*, 2000; Jeurissen *et al.*, 2002; Khan *et al.*, 2004). Despite these recent advances it remains unclear what the role of pneumolysin is in relation to these findings.

My studies in this chapter represent initial investigations into the significance of pneumolysins ability to induce T cell apoptosis. My work *in vitro* allowed me to orientate my *in vivo* investigation. I had demonstrated *in vitro* that pneumolysin inhibits the T cell response by acting directly on the T cell and that this inhibitory mechanism is mediated by the induction of apoptosis. I therefore wanted to begin my studies *in vivo* by examining the same parameters.

6.2 Results

6.2.1 Optimising the pneumolysin dose *in vivo*

In vitro I had observed that pneumolysin was able to inhibit the proliferation of antigen-stimulated CD4 T cells by inducing cell death after the cells became activated. Pneumolysin did not induce death of unstimulated T cells. I wanted to be able to examine the same parameters *in vivo* as I had examined *in vitro*. The DO11.10 adoptive transfer system would allow me to examine the parameters such as T cell proliferation, T cell activation and T cell death (for review see (Adams *et al.*, 2004b; Adams *et al.*, 2004c)). I therefore considered it a good system in which to begin my *in vivo* studies.

6.2.1.1 Optimisation process

Throughout my *in vitro* studies both wild and F433 type pneumolysin purified using the His tag/ metal chelate column method had been used. Due to the large amounts of toxin required for the *in vivo* experiments, I used only the wild type toxin prepared using the HIC/Anion Exchange method.

I wanted first of all to assess the effect of pneumolysin on *in vivo* T cell proliferation. In order to do so I had to determine the concentration of pneumolysin that I could inject into the mice that did not alter the resting T cell population. After obtaining this concentration I would then be able to progress to examining the effects of pneumolysin upon antigen stimulated lymphocytes.

10ug Pneumolysin i.p. does not alter basal lymphocyte population

To establish a concentration of pneumolysin that could be administered without altering resting lymphocytes I measured the percent of Tg T cells in mice that received various concentrations of pneumolysin. Figure 6-1 shows that mice that received only PBS had a basal level of transgenic cells of 0.8%. Mice that received 20ug of pneumolysin had 0.4% Tg T cells which represent 50% less transgenic T cells, compared to the PBS control and although this is not significantly different

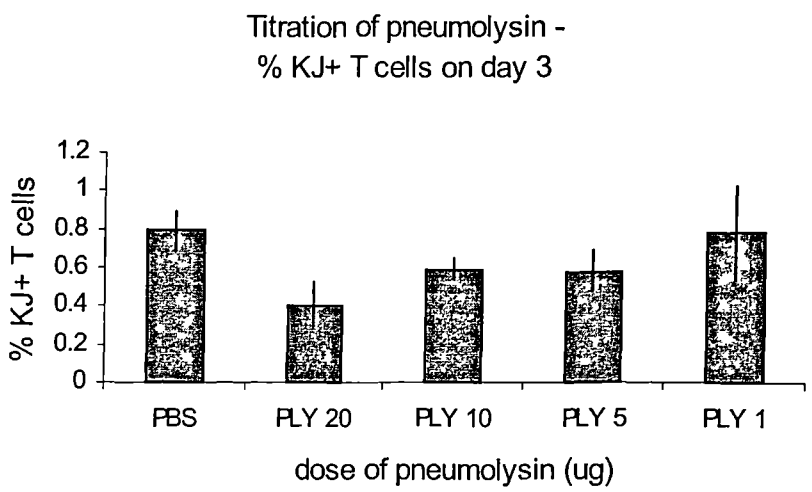


Figure 6-1

The effect of pneumolysin upon resting T cells in vivo

BALB/c mice were adoptively transferred with cells from DO11.10 mice on day -1. On day 0 and day 1 groups of three mice were injected with 20, 10, 5, or 1 ug of pneumolysin or PBS i.p. On day 3 mice were sacrificed and the lymph nodes (inguinal, brachial, cervical) removed and stained with CD4-PE and biotinylated KJ1.26/ streptavidin FITC to enable the determination of the percent of transgenic T cells within the lymph nodes by flow cytometry. None of the results were statistically significant to $p <= 0.05$.

it is considerable. In mice receiving 10ug or 5ug of pneumolysin, transgenic cells represented 0.6% of cells. Finally, mice receiving 1ug of pneumolysin had 0.8% transgenic cells which is the same as the control.

From this I concluded that 20ug pneumolysin may potentially decrease the basal level of resting T cells in transferred animals and therefore was deemed unsuitable for further study. 10 and 5ug, despite causing a decrease in basal T cells, did not significantly alter this level and were therefore considered for further study. 1ug pneumolysin did not alter the resting cell level and was a candidate dose for further study.

10, 5 and 1 mg pneumolysin do not alter T cell accumulation.

On the basis of the data from the above experiment I used 10, 5 and 1 mg pneumolysin to examine the hypothesis that pneumolysin would cause apoptosis of T cells *in vivo*. By adoptively transferring T cells Tg for ovalbumin and administering ovalbumin to transferred mice, I proposed that pneumolysin would inhibit T cell accumulation within the lymph nodes of mice that received ovalbumin but would not affect the resting T cell population in mice that did not receive ovalbumin.

In order to examine the effect of pneumolysin upon T cell accumulation I measured the accumulation of Tg T cells in mice that received ovalbumin along with pneumolysin. In mice injected with PBS i.p. and PBS s.c., the number of Tg cells was constant at 0.5% on days 3,5 and 7 (see Figure 6-2) while in mice injected with PBS i.p. and OVA-CFA s.c. the percentage of transgenic T cells found in the lymph nodes changed over the 7 day period examined; On day 3 transgenic cells constituted 1.3% of the lymph node cells increasing to 2.5% by day 5 and then decreasing by day 7 to 0.9%.

Figure 6-2 (A) demonstrates that in mice that received 1ug pneumolysin i.p. and PBS s.c. the percentage of transgenic cells was 0.6% over days 3, 5 and 7, which is slightly higher than the control mice that received PBS only. However, the difference is not significant. In mice that received 1ug pneumolysin i.p. and OVA-CFA s.c. the percentage of transgenic cells was 1.4% on day 3, 3% on day 5 and 1.8% on day 7. This is slightly greater than that for control mice receiving PBS

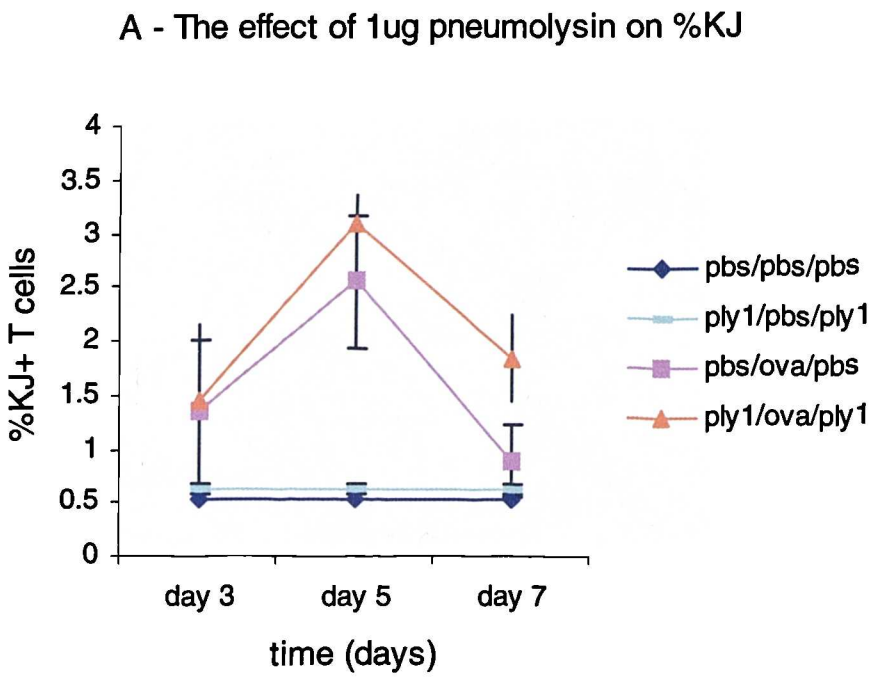
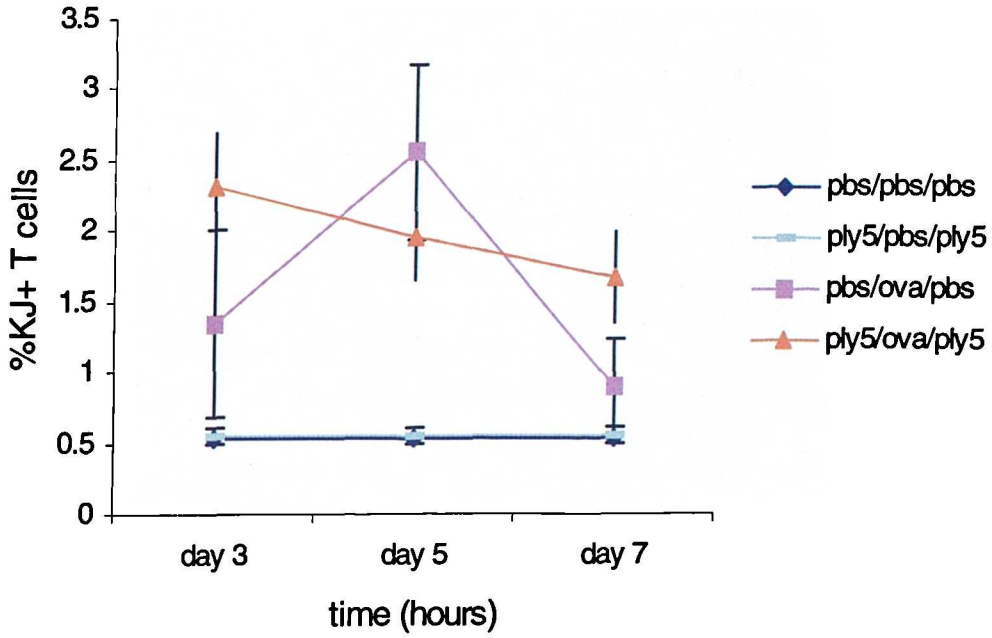


Figure 6-2

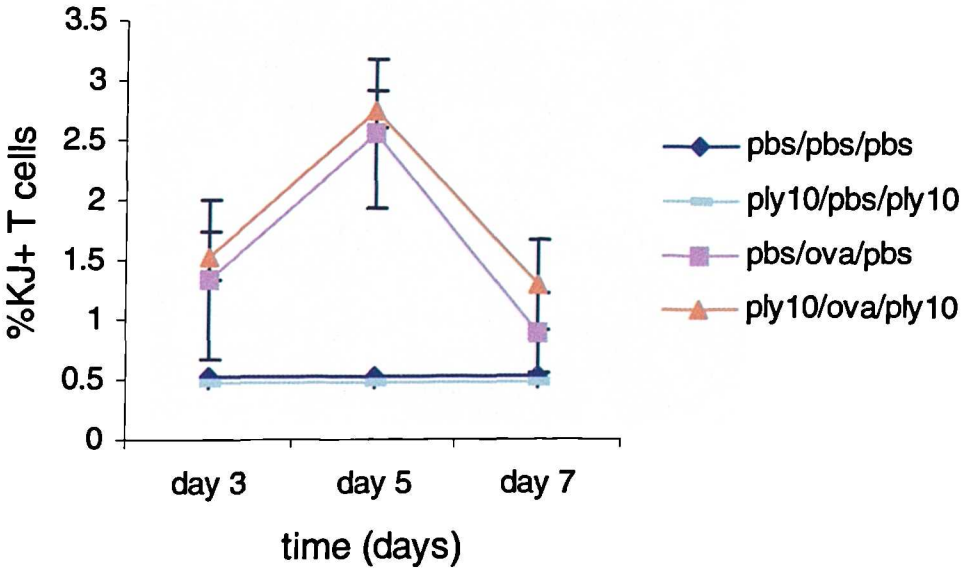
The effect of 10, 5, 1 ug pneumolysin upon T cell accumulation in vivo

We transferred DO11.10 cells from transgenic donor mice into BALB/c recipients on day –1. On day 0 groups of three mice were injected with PBS or 1ug (see A), 5ug (see B) or 10ug (see C) of pneumolysin i.p, a few hours prior to receiving either PBS or OVA-CFA subcutaneously. The following day (day 1) mice were again injected with PBS or the same dose of pneumolysin as they had received the previous day. Mice were sacrificed on day 3,5 and 7 and the percent of transgenic T cells was measured in the lymph nodes (inguinal, brachial, cervical) by flow cytometry using CD4 antibody and the clonotypic antibody KJ1.26. The graphs A, B and C illustrate the positive control of PBS/OVA/PBS as a pink line. The negative control of PBS/PBS/PBS is represented as a blue line. Mice that received ovalbumin and were treated with pneumolysin (PLY/OVA/PLY) are represented on each graph as a yellow line while mice treated with pneumolysin but received no ovalbumin (PLY/PBS/PLY) are shown as turquoise line. On day 7 no statistical difference was observed between any of the groups as judged by $p <= 0.05$.

B - The effect of 5ug pneumolysin on %KJ



C - The effect of 10ug pneumolysin on %KJ



and OVA-CFA. Again, as for the mice that did not receive ovalbumin, this difference was not significant on day 7.

In mice that received 5ug of pneumolysin i.p. and PBS s.c. the percentage of transgenic T cells is exactly the same as for the control mice that received PBS i.p. and PBS s.c. over the 7 day period (see Figure 6-2 B). Mice that received 5ug of pneumolysin i.p. and OVA-CFA s.c. have an unusually high percentage of transgenic cells on day 3 of 2.3% and this declines to 2% on day 5 and 1.6% on day 7. This is an unexpected pattern of transgenic T cell distribution when compared to the control mice that received PBS i.p. and OVA-CFA s.c., however the error bars on this data are large and the difference is not significant on day 7.

Mice that received 10ug of pneumolysin i.p. and PBS s.c. had the same percentage of transgenic cells over the 7 day period examined as the control mice that received PBS i.p. and PBS s.c. as shown in Figure 6-2 C. Mice that received 10mg pneumolysin i.p. and OVA-CFA s.c. had 1.6%, 3.2% and 2% transgenic cells on days 3,5 and 7 respectively. As can be seen in Figure 6-2 C, this represents 0.2% more transgenic T cells at each time point relative to the PBS/OVA-CFA control value at the same time points. This difference is not significant on day 7.

My conclusions were; As expected, mice that did not receive ovalbumin did not accumulate T cells whereas mice that received OVA/CFA did. This peaks on day 5. 10, 5 and 1 ug pneumolysin did not alter the number of T cells in mice that did not receive ovalbumin. None of these doses of pneumolysin caused an inhibition of T cell accumulation since pneumolysin treated mice that received ovalbumin did not have significantly different levels of transgenic T cells compared to the control mice that received only ovalbumin. I knew from Figure 6-1 that a 20mg dose was too high since it altered the resting T cell population and from Figure 6-2 I knew that 10mg had no effect on antigen stimulated T cells. In order to obtain a dose of pneumolysin that altered antigen stimulated T cells but did not alter resting levels, I proposed to use a higher dose of pneumolysin of 15mg and also of 20mg as a positive control.

15ug and 20ug pneumolysin inhibit T cell accumulation

I did not know if 15ug or 20ug pneumolysin would inhibit T cell accumulation after antigen stimulation. Therefore in order to assess the value of a full scale experiment using these doses, a

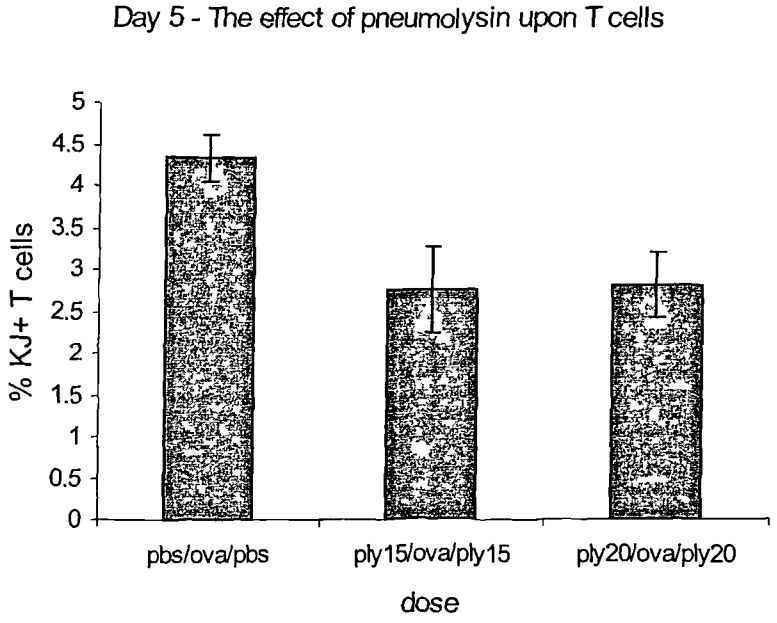


Figure 6-3

The effect of 15ug or 20ug pneumolysin upon T cell accumulation

BALB/c mice were transferred with DO11.10 cells on day –1 and then injected on day 0 with pneumolysin (15ug or 20ug) or PBS i.p then a few hours later with OVA-CFA and finally injected with pneumolysin (15ug or 20ug) or PBS i.p on day1. Three mice were used in each group. On day 5 mice were sacrificed and the proportion of transgenic T cells measured in the lymph nodes (inguinal, brachial, cervical) using flow cytometry. As this was a preliminary experiment there was no negative control group; all mice received OVA-CFA s.c. Mice that received 15ug pneumolysin i.p. followed by ovalbumin s.c. followed by 15ug pneumolysin i.p. are marked as PLY15/OVA/PLY15. Mice that received 20ug pneumolysin i.p. followed by ovalbumin s.c. followed by 20ug pneumolysin i.p. are marked as PLY20/OVA/PLY20. The difference between PLY20/OVA/PLY20 and PBS/OVA/PBS is statistically significant to $p <= 0.05$.

pilot experiment was first carried out. A full scale experiment would use many mice and take up much time; a pilot experiment would ensure I was not wasting resources. I assessed, the effect of 15mg and 20mg of pneumolysin on T cell accumulation in mice that did not receive ovalbumin at one time point.

Figure 6-3 demonstrates that 4.3% of cells in mice that received PBS i.p. and OVA-CFA s.c. were transgenic on day 5. In comparison in mice that received 15 or 20mg of pneumolysin i.p. and OVA-CFA s.c. 2.8% of cells were transgenic on day 5 for 20mg This is a significantly lower value than that of the control indicating that 20mg of pneumolysin is sufficient to inhibit the accumulation of T cells in the lymph nodes of adoptively transferred mice that received ovalbumin. 15mg was not quite statistically significant but the decrease was considerable.

Since both doses of pneumolysin had a very similar effect it was considered best to proceed with 15mg for further study since 20mg was known to alter resting T cell numbers.

6.2.2 Pneumolysin inhibits T cells *in vivo*

My previous work had established that 15mg of pneumolysin was a potentially suitable dose to use for my *in vivo* studies since it had an observable inhibitory effect upon T cell accumulation after administration of ovalbumin. I therefore wanted to determine that this dose had no adverse effect on the basal lymphocyte population and then to further investigate the parameters of the inhibitory effect.

6.2.2.1 Pneumolysin inhibits T cell accumulation

I measured the effect of pneumolysin upon T cells by adoptively transferring the Tg cells from DO11.10 mice and measuring the effect of pneumolysin upon the resting cell population and the effect of pneumolysin upon mice that received ovalbumin (see Figure 6-4).

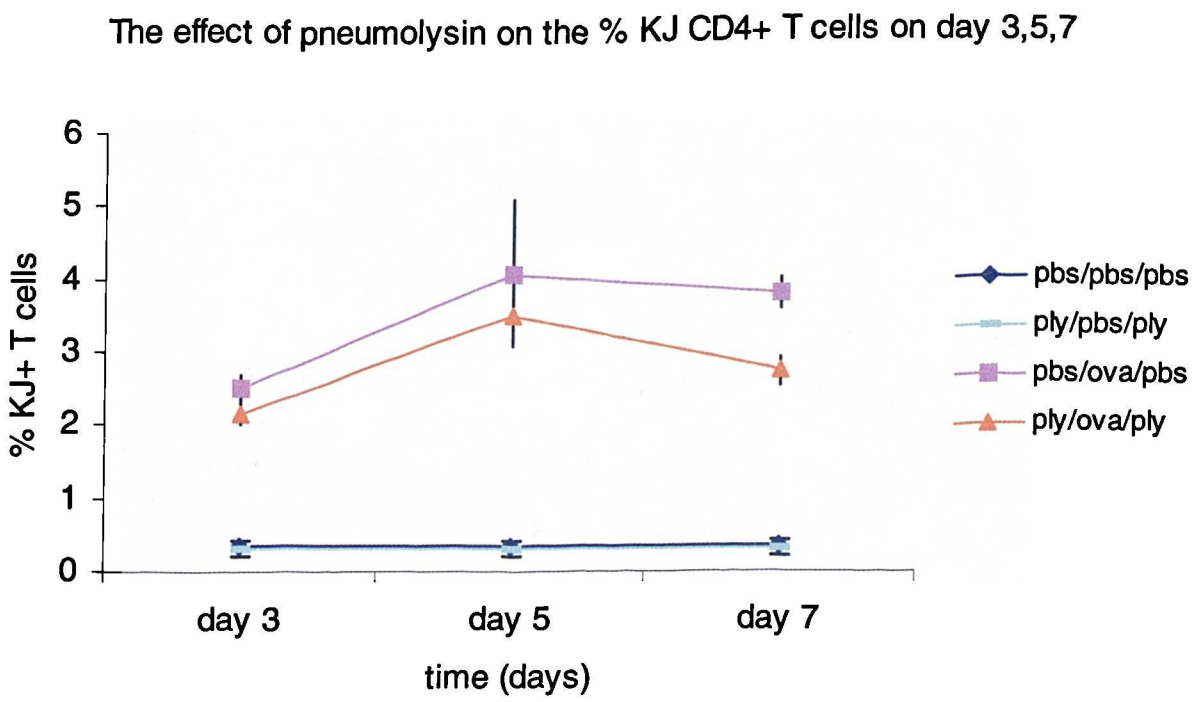


Figure 6-4

The effect of 15ug of pneumolysin upon T cells in vivo

We transferred DO11.10 cells from transgenic donor mice into BALB/c recipients on day –1. On day 0 mice were injected with PBS or 15υg of pneumolysin i.p a few hours prior to receiving either PBS or OVA-CFA subcutaneously. The following day (day 1) mice were again injected with PBS or the same dose of pneumolysin as they had received the previous day. Mice were sacrificed on day 3,5 and 7 and the percent of transgenic T cells was measured in the lymph nodes (inguinal, brachial, cervical) by flow cytometry using CD4 antibody and the clonotypic antibody KJ1.26. The group called PBS/PBS/PBS (blue line) received PBS i.p and PBS s.c and had a total of three mice in the group. The group labelled PLY/ PBS/PLY (turquoise line) received 15ug pneumolysin i.p twice and PBS s.c and had three mice in total in the group. The group labelled PBS/OVA/PBS (pink line) received PBS i.p twice and OVA/CFA s.c and had 9 mice in the group. The group labelled PLY/OVA/PLY (yellow line) received 15ug pneumolysin i.p twice and OVA/CFA s.c and had 9 mice in the group. The difference on day 7 between PLY/ OVA and PBS/OVA is statistically significant to $p <= 0.05$.

Figure 6-4 demonstrates that in mice injected with PBS i.p. and PBS s.c. transgenic cells represented 0.35%. Similarly, mice that received PLY i.p. and PBS s.c. transgenic cells represented 0.35% throughout the 7 day period examined. Thus mice that did not receive ovalbumin showed no difference in the proportion of KJ+ cells, nor was there any effect on the basal level of lymphocytes attributable to pneumolysin throughout the period examined.

On day 3 mice that had received PBS i.p. and OVA-CFA s.c. had increased T cell percentage (see Figure 6-4) compared to the control of mice that received PBS only so that transgenic cells represented 2.5% of cells in the lymph nodes. By day 5 this had increased to 4.1% and then decreased to day 7 where 3.8% of cells were KJ+. At all three time points, mice that had received 15mg pneumolysin i.p. and OVA-CFA s.c., had a lower percentage of KJ+ cells than the control of PBS i.p. and OVA-CFA. On day 3 the difference is minimal; pneumolysin treated mice have 2.1% KJ+ cells compared to the control of 2.5%. On day 5 the difference is larger; pneumolysin treated mice have 3.5% KJ+ cells compared to the control of 4.1%. On day 7 the difference is largest; pneumolysin treated mice have 2.7% KJ+ cells and the control has 3.8% KJ+ cells. This difference is significant at this time point.

From the data presented in Figure 6-4, I was able to conclude two things. The fact that on day 7, in mice that received ovalbumin, those treated with pneumolysin had less KJ+ cells than those that did not receive pneumolysin led me to conclude that pneumolysin is able to inhibit T cell accumulation *in vivo*. Furthermore the fact that, in mice that did not receive ovalbumin, those treated with pneumolysin did not have any difference in the number of KJ+ cells compared to those mice that did not receive ovalbumin led me to conclude that pneumolysin does not rely on the removal of a percentage of the basal lymphocyte population to accomplish its T cell inhibitory effect *in vivo*.

6.2.2.2 Pneumolysin inhibits the number of KJ+ cells in the lymph nodes

Simultaneous to the measurements of Figure 6-4, I further assessed the proportion of transgenic T cells within the groups. I measured the actual number of KJ+ cells isolated when the lymph nodes of each mouse were pooled. Figure 6-5 illustrates that the mice injected with PBS i.p. and PBS s.c. had 1.1×10^4 KJ+ transgenic cells while mice that received PLY i.p. and PBS s.c. had 1.2×10^4 KJ+ transgenic cells throughout the 7 day period examined.

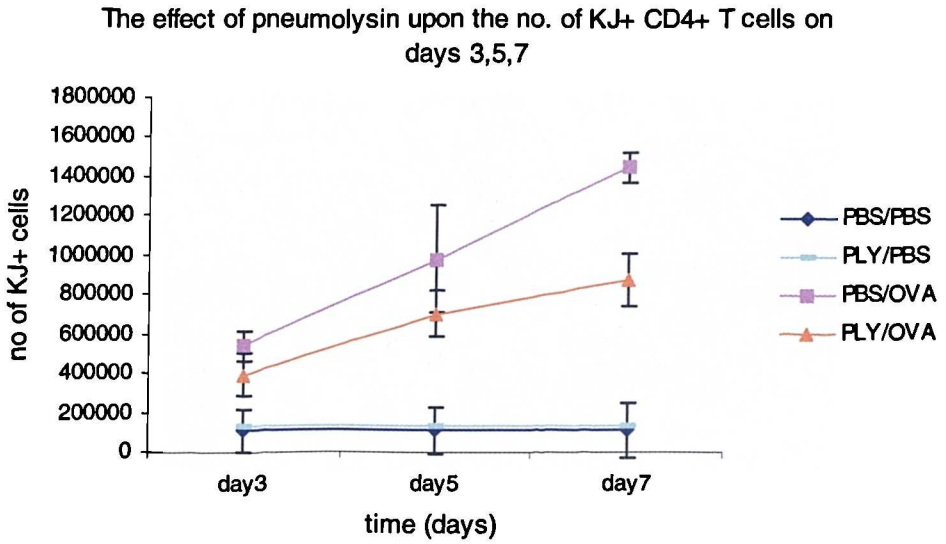


Figure 6- 5

The effect of pneumolysin on the number of KJ+ CD4+ T cells in the lymph nodes of mice that recieved ovalbumin

The experiment was set up as for figure 6-4. On each day the number of lymphocytes was counted by trypan blue exclusion within the single cell suspensions made from the lymph nodes of each mouse prior to flow cytometric analysis. Using the percent KJ+ cells measured by flow cytometry the number of KJ+ cells was calculated and the results presented above are the averages from each group. Lymphocytes from mice that received PBS i.p and PBS s.c are represented as a blue line. Those from mice that received PLY i.p and PBS s.c are shown as a turquoise line. Lymphocytes from mice that received PBS i.p and OVA s.c are represented as a pink line. Those from mice that received PLY i.p and OVA s.c are shown as a yellow line.

On day 3 mice that had received PBS/OVA had 5.5x 10^5 KJ+ cells rising to 9.8x 10^5 cells on day 5 and finally 1.5x 10^6 KJ+ cells on day 7. Comparison of this with mice that had received ovalbumin and had been treated with pneumolysin demonstrates that pneumolysin inhibits real number of KJ+ cells since the treated group has 4x 10^5 KJ+ cells on day 3, 7x 10^5 cells on day and 5 and 8.7x 10^5 cells on day 7.

This data confirms the observations of Figure 6-4; that there are less KJ+ T cells in mice that received ovalbumin and were treated with pneumolysin.

6.2.2.3 *Ex vivo* stimulation of lymph node cells from pneumolysin treated mice proliferate less than those from PBS treated mice.

In addition to measuring the percentage of KJ+ T cells and the actual number of KJ+ cells, I utilised a further measure of T-cell proportion within the lymph nodes by re stimulating lymph node preparations *ex vivo* using ovalbumin.

The proliferation data from each individual mouse was pooled to create a group data set (see Figure 6-6). Lymphocytes from the PBS/PBS group or the PLY/PBS group proliferated little over the 72 period examined. After 24 hours lymphocytes from the PBS/OVA group that had been stimulated with ovalbumin had proliferated to 1.3 x 10^4 cpm. By 48 hours this had increased to 1 x 10^5 cpm and by 72 hours 1.8 x 10^5 cpm. In comparison lymphocytes from the PLY/OVA group proliferated after 24 hours to 7000 cpm rising to 6 x 10^4 cpm after 48 hours and 1.2 x 10^5 cpm after 72 hours. Therefore PLY/OVA treated mice are shown to contain less cells that proliferate in response to ovalbumin than PBS/OVA treated mice.

Since, in the data presented in Figure 6-6, the amount of thymidine incorporation is simply a reflection of the number of T cells present in each mouse, this data does not inform about the ability of the T cells from each mouse to proliferate. However this data provided further evidence that the KJ+ T-cells accumulated less in the pneumolysin treated mice.

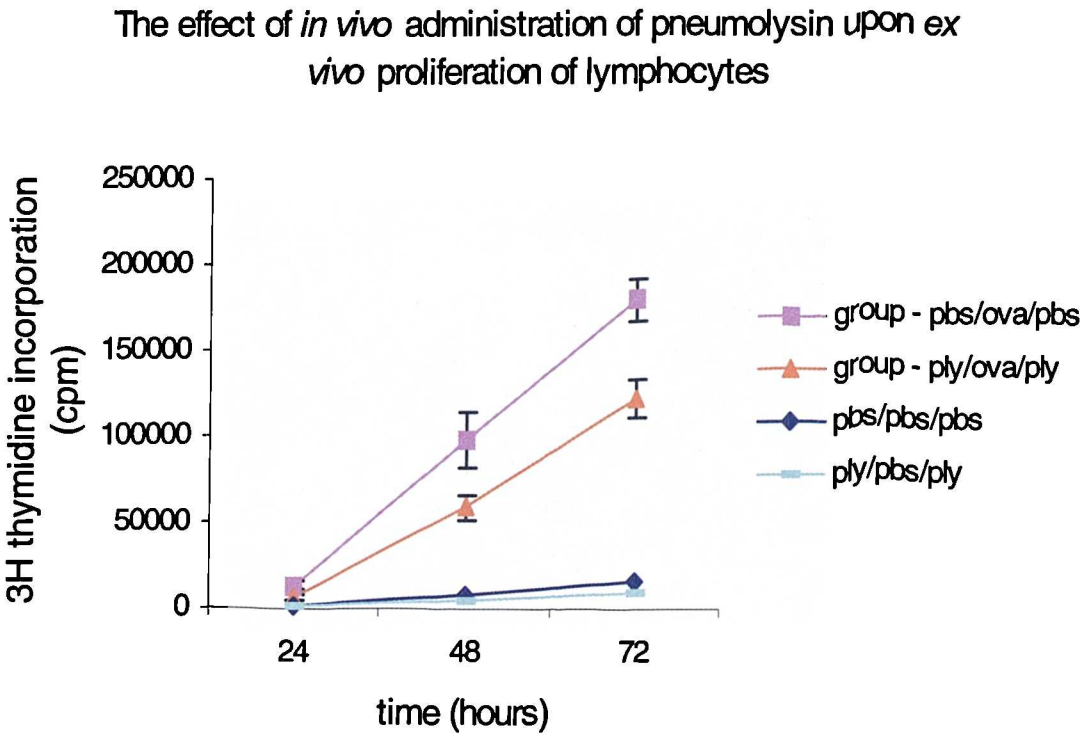


Figure 6-6

The effect of pneumolysin upon T cell accumulation as measured by the ex vivo proliferation of transgenic cells.

On day 7 a proliferation assay was set up using lymph node single cell suspensions from each mouse. The number of lymphocytes was equalised so that each well contained 2 x 10^5 lymphocytes. Cultures were stimulated with 1mg/ml ovalbumin and proliferation was measured by 3^H thymidine incorporation at 24, 48 and 72 hours. Proliferation of lymphocytes from mice that received PBS i.p and s.c is shown as a blue line and represents a group of one mouse. Proliferation of lymphocytes from mice that received PLY i.p and PBS s.c is shown as a turquoise line and represents a group of one mouse. Proliferation of lymphocytes from mice that received PBS i.p and OVA s.c is shown as a pink line and represents a group of three mice. Proliferation of lymphocytes from mice that received PLY i.p and OVA s.c is shown as a pink line and represents a group of three mice. For groups of mice, the proliferation of each individual mouse was recorded and then an average calculated and plotted on the graph above.

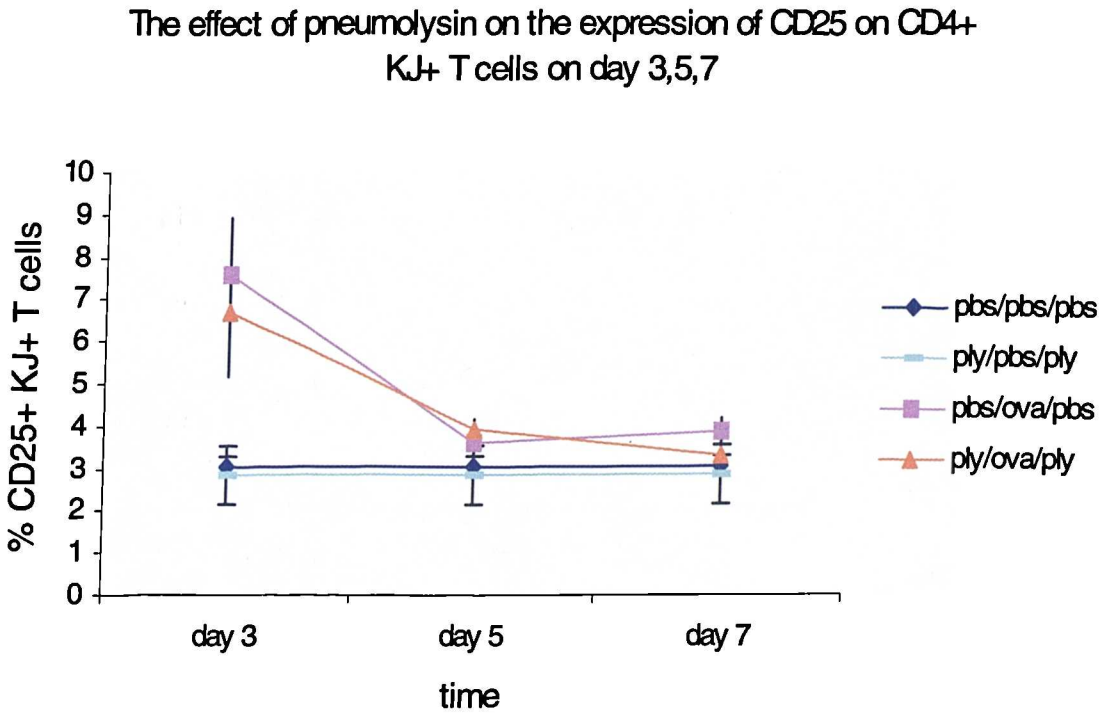


Figure 6-7

The effect of pneumolysin administration upon the activation of T cells.

In the experiment outlined in figure 6-4 we simultaneously measured the activation state of the T cells by measuring the amount of CD25 expressed on the surface of the KJ+, CD4+ T cells using an antibody to CD25 and flow cytometry. The group called PBS/PBS/PBS (blue line) received PBS i.p and PBS s.c. The group labelled PLY/PBS/PLY (turquoise line) received pneumolysin i.p twice and PBS s.c. The group labelled PBS/OVA/PBS (pink line) received PBS i.p twice and OVA/CFA s.c. The group labelled PLY/OVA/PLY (yellow line) received pneumolysin i.p twice and OVA/CFA s.c. Rat IgM FITC was used as the isotype control (data not shown).

6.2.2.4 Pneumolysin does not affect T cell activation

In vitro I had observed that pneumolysin did not alter the proportion of T cells to become activated yet the subsequent proliferation of those T cells was altered by the presence of pneumolysin. I was therefore interested to see if the same effect could be observed *in vivo*. Simultaneous to measuring the proportion of KJ+ cells within the lymph node (see Figure 6-4) I also measured the activation state of the T cells using an anti-CD25 antibody (see Figure 6-7). In mice that received either PBS i.p. and PBS s.c. or PLY i.p. and PBS s.c. the number of CD25+ cells remained constant at 3% of transgenic T cells. Mice that received PBS i.p. and OVA-CFA s.c. or PLY i.p. and OVA-CFA s.c. had the same percentage of activated cells at each time point. On day 3 this was 7%, day 5, 4% and by day 7 it had decreased to 3%. No significant difference between mice receiving PBS/OVA-CFA and those receiving PLY/OVA-CFA was observed at any time point. The isotype control showed no increase at any time point (data not shown). I concluded that in mice receiving ovalbumin and treated with pneumolysin, T cells become activated to the same degree as those in mice receiving ovalbumin and treated with PBS.

This data coincides with the data I obtained from my *in vitro* studies where T cells became activated to the same degree when pneumolysin was present but the subsequent expansion after activation differs.

6.2.2.5 Pneumolysin inhibits antibody production *in vivo*

CD4 T-cell function can be further assessed by measuring antigen-specific antibody levels 21 days after ovalbumin administration. Ovalbumin specific anti IgG1 was measured by antibody ELISA using blood harvested from mice on day 21. As can be seen from Figure 6-8 no anti Ovalbumin specific IgG1 is produced in response to injections of PBS or pneumolysin alone. However groups receiving PBS/OVA a titre of 10000 is achieved after day 21 yet a titre of only 3500 is achieved for groups that received ovalbumin and were treated with pneumolysin. Mice that received PLY/OVA therefore produce significantly less IgG1 than mice that received PBS/OVA.

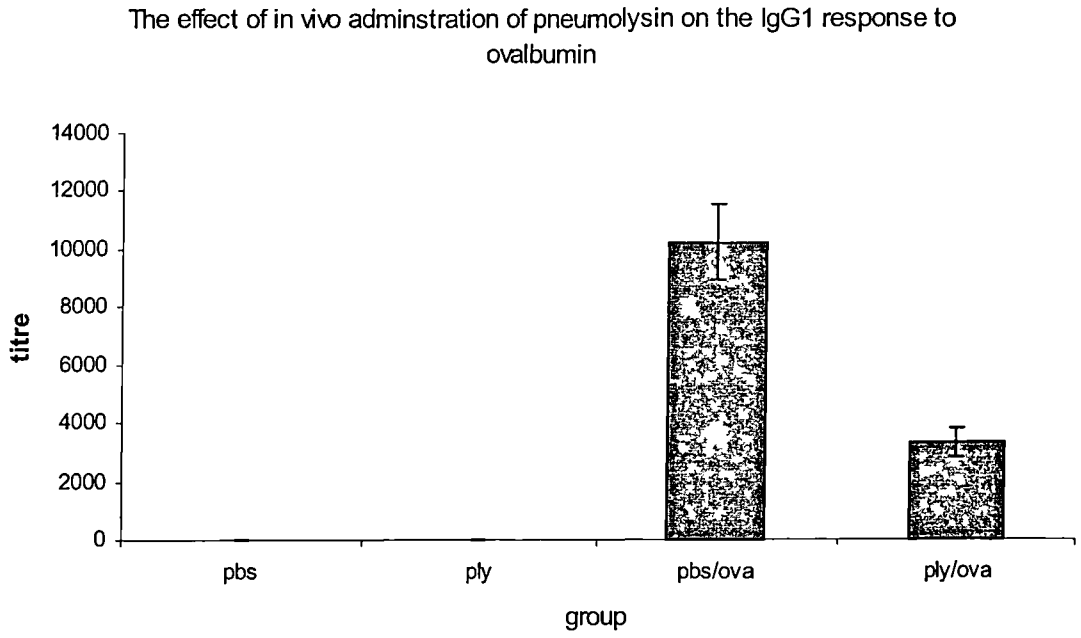


Figure 6-8

The effect of in vivo pneumolysin administration upon antibody response to ovalbumin

On day 21 of the experiment described in figure 6-4, mice were sacrificed and blood collected from the portal vein and antibody ELISA's were performed for anti-ova IgG1. The Pbs group and the Ply group had 1 mouse per group while the Pbs/ova and the Ply/ova had three mice per group. Titres for each mouse were calculated and averaged. The difference between pbs/ova and ply/ova is statistically significant to 0.05.

6.2.2.6 Immunohistochemical anaylsis of the effect of pneumolysin *in vivo*

Differences in the number of antigen-specific CD4 T-cells within a lymph node can sometimes be more clearly evident using immunohistochemistry. While this method is not quantitative it can be informative since decreased antibody production may be the result not only of less T cells being present but also the result of a lack of T cell migration to the B cell follicles. I performed immuno-histochemistry on lymph nodes frozen on day 7 by staining for B220 and KJ1.26.

Figure 6-9 shows the images. Image A demonstrates the lack of KJ+ T cells within an animal that did not receive ovalbumin. The high degree of blue staining in the next three images (B, C, D) illustrates the increase in KJ+ T cells in animals that received ovalbumin. The final three images (E, F, G) show that in animals that received ovalbumin and were treated with pneumolysin the amount of KJ+ T cells noticeably decreases when compared to mice that have received ovalbumin since in these images the amount of blue dots is noticeably less than in images B, C and D. This data corresponds with the data from Figure 6-4 where the percentage of KJ+ T cells was shown to be less in the lymph nodes of mice that had received ovalbumin and been treated with pneumolysin compared to those of the mice that received ovalbumin but did not receive pneumolysin.

Figure 6-9

Immunohistochemistry

On day 7 in the experiment outlined in figure 6-4 in addition to measuring %KJ and %CD25+ T cells, one lymph node was frozen and later used for IHC where staining for KJ1.26 (blue) and B220 (brown) was carried out. Image A is from a mouse that received PBS only. Images B, C and D are sections from mice treated with PBS/ OVA. Images E, F and G are from mice treated with pneumolysin/OVA.

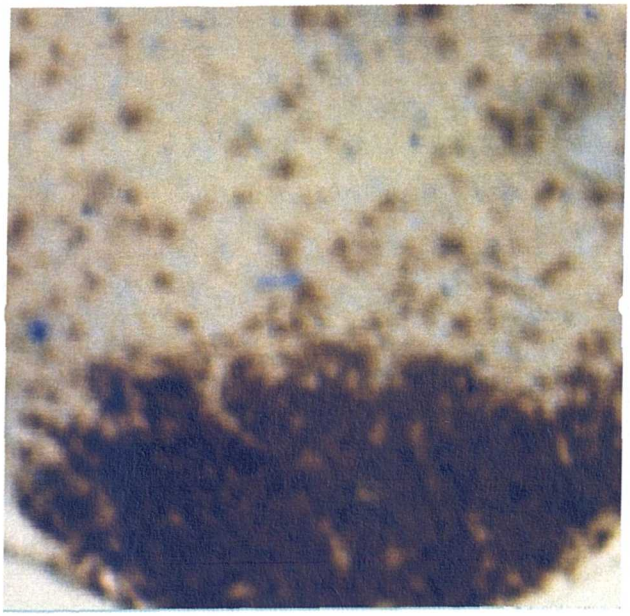
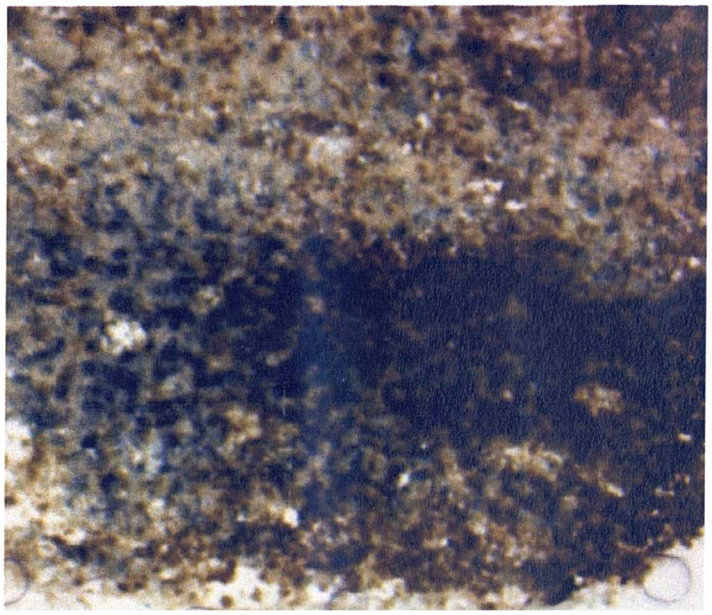


Image A



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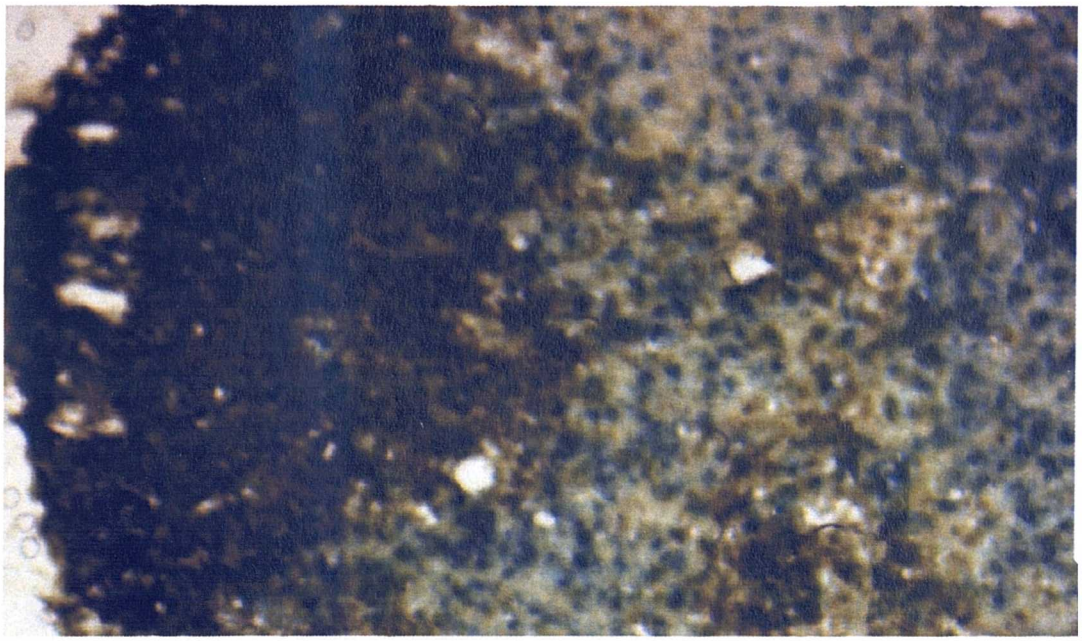

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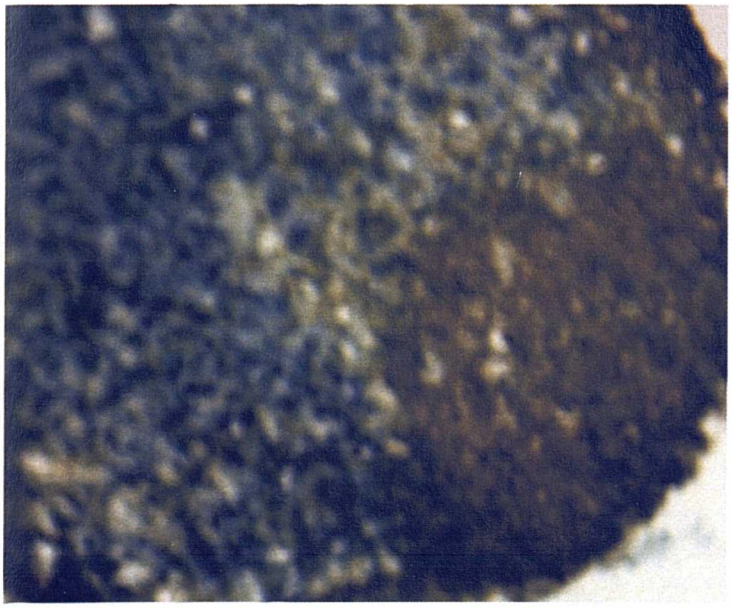
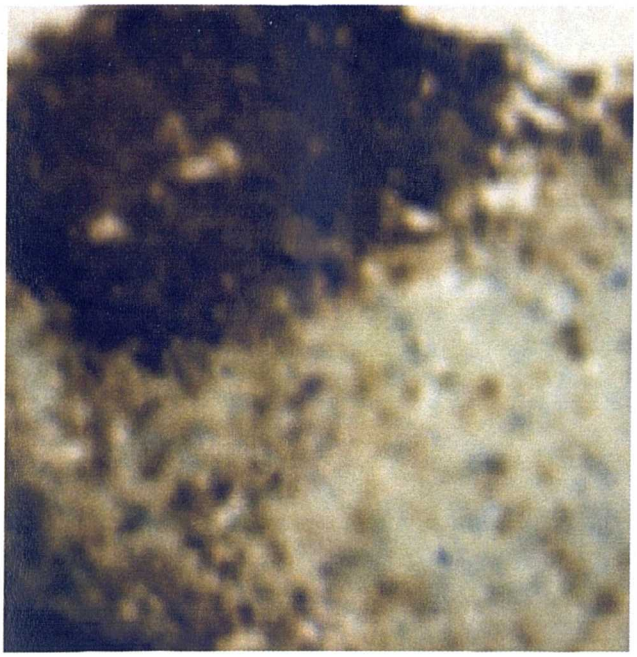


Image D



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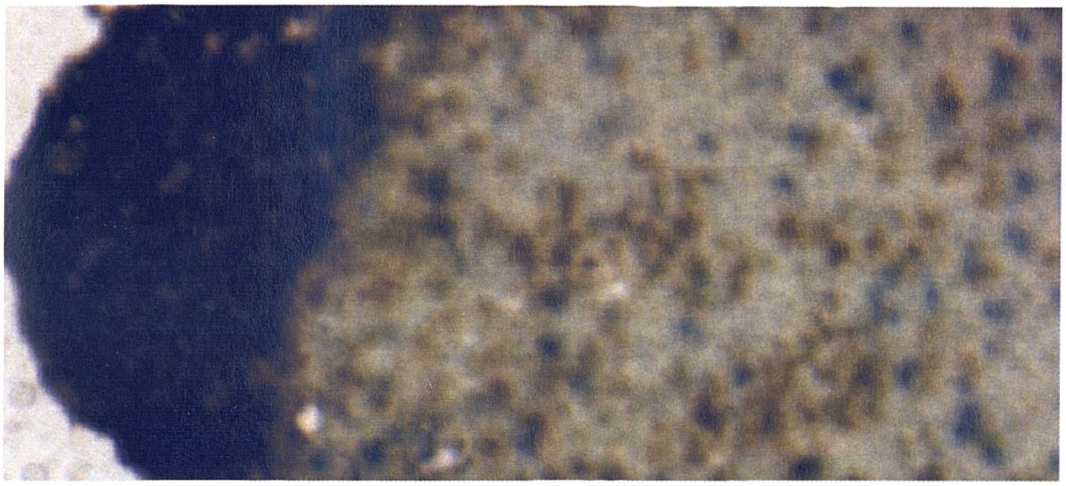

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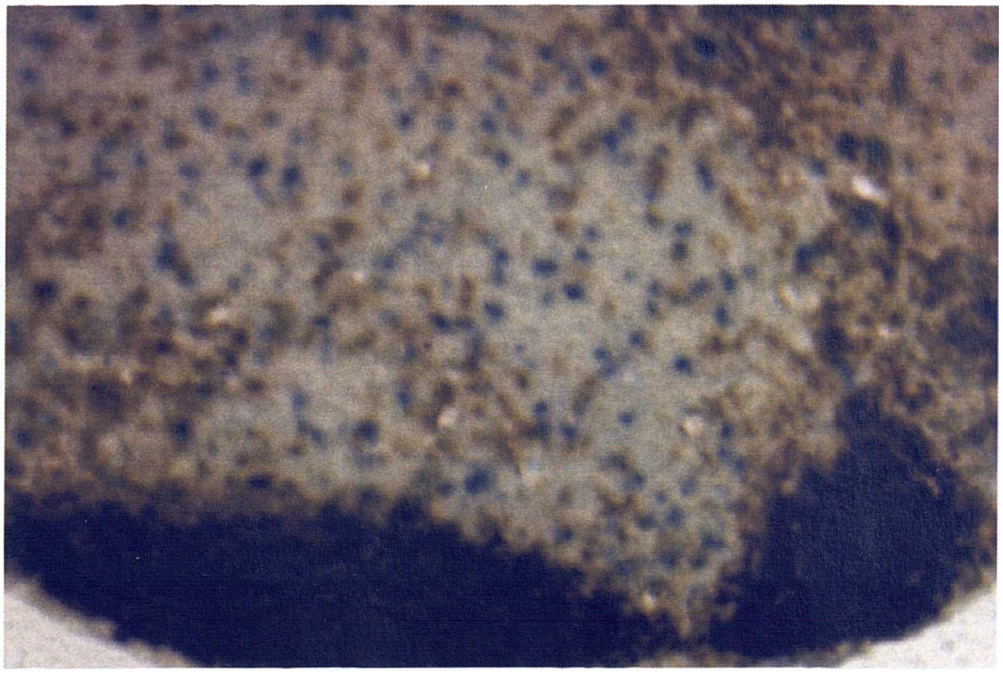

Image G

6.3 Discussion

In this chapter I have demonstrated that wild type pneumolysin is able to inhibit the accumulation of transgenic T cells in mice that received ovalbumin. I have demonstrated this by direct flow cytometric measurement and by *ex vivo* restimulation of lymph node cells. Furthermore the evidence from antibody production and immunohistochemistry confirms this data. I have also shown that the number of T cells that become activated by day 3 after receiving ovalbumin is not altered by pneumolysin yet there are less T cells within the lymph nodes on day 7.

In 1994 Kearney *et al.* first used the DO11.10 adoptive transfer system to track transgenic T cells *in vivo* (Kearney *et al.*, 1994). Since then this system has been used to study a range of immune responses *in vivo* including the activation and development of T cell phenotypes following the induction of immunity, autoimmunity, allergy and tolerance and the trafficking of effector lymphocyte populations (Chen and Jenkins, 1998; Garside *et al.*, 1998; Iqbal *et al.*, 2002; Jenkins *et al.*, 2001; Khoruts *et al.*, 1998; McSorley *et al.*, 2002; Pape *et al.*, 1998; Rush *et al.*, 2002; Smith *et al.*, 2004b). There have been few groups who have used this system to assess the effect of potential immunomodulators on the T cell response despite it being ideally suited to this purpose. In 2000 Shepherd *et al.* used the transfer system to assess the effects of TCDD on the activation of transgenic T cells *in vivo* (Shepherd *et al.*, 2000). However the work most closely related to my studies was that of Boitelle *et al.* (Boitelle *et al.*, 2003) and Plant *et al.* (Plant *et al.*, 2003) who, used the system to assess the effects of a pathogen produced molecule (Ascaris body fluid and EtxB, respectively) on the T cell response. These studies confirm the validity of using the DO11.10 system as I have done while other work using the system has helped me with the design an interpretation of my results.

The data I presented showing expansion of antigen specific T cells on day 3, peaking on day 5 and then declining to day 7 is consistent with other groups findings when using this system (Kearney *et al.*, 1994). While my data clearly shows that pneumolysin results in less transgenic T cells on day 7, the situation on day 5 is less evident. The decreased number of T cells observed on day 5 is not significant which suggests two possible interpretations of the data. If the number of T cells on day 5 was essentially the same between untreated mice and mice treated with pneumolysin, yet the number of T cells in treated mice was less on day 7, then I would interpret the data to show that

pneumolysin does not inhibit T cell expansion but rather sensitises T cells to a more rapid death. If however a difference in number of T cells was observed on day 5 then this would demonstrate that pneumolysin does inhibit T cell expansion. Further work is necessary to decide between these two alternatives although my *in vitro* data suggests that the latter is more likely.

The data presented in this chapter is remarkably consistent with the data I provided from my studies *in vitro*. *In vitro* I demonstrated that pneumolysin inhibited antigen specific T cell expansion and I have shown this *in vivo*. However it is essential to recognise the differences between the *in vitro* and *in vivo* systems. The *in vitro* system is closed, whereas the lymph nodes *in vivo* are not. T cell accumulation is measured *in vivo* as opposed to proliferation since when the percentage of transgenic T cells is measured *in vivo* this is a product of proliferation in the lymph node but also of recruitment of transgenic cells and other cell types, the migration of cells out of the lymph nodes and cell death. It is possible therefore that a decrease in the percentage of transgenic T cells observed *in vivo* could be the result of a migration of non transgenic T cells into the lymph node which effectively dilutes the concentration of transgenic cells. Where this effect was occurring, the percentage and number of transgenic cells would appear less as measured by flow cytometry, *ex vivo* proliferation and lymphocyte counting. A functional measure of T cells, such as antibody production, would be able to discriminate between a real decrease and a perceived decrease in T cell numbers. My data measuring the antibody response to ovalbumin is therefore informative in this regard as it gives some indication that a real decrease in T cell function is occurring in the lymph nodes, although an effect of pneumolysin upon B cells cannot be excluded.

Direct injection of pneumolysin has been used to mimic a number of pneumococcal induced pathologies (Feldman *et al.*, 1991; Harrison *et al.*, 1982) and immunisation with pneumolysin has been used to induce protective antibody against pneumococcal infection (Alexander *et al.*, 1994; Briles *et al.*, 2000; Kuo *et al.*, 1995), however using pneumolysin as I have done is, to my knowledge, unprecedented. I was therefore unsure about the initial dose of toxin to use and chose a dose as had been used for immunisation. The distribution of pneumolysin after it has been injected has never been examined. Given the propensity of pneumolysin to bind to cells it seems likely that much toxin will have bound to cells at the injection site and whether any free toxin reached the site of antigen presentation to T cells is unknown. What does seem likely is that the effective dose of pneumolysin at the site of antigen presentation to T cells is likely to be significantly less than that injected. The distribution of toxin and the concentration of free toxin along with the delivery of cell

bound toxin to lymph nodes are all un-investigated parameters of this system that require further examination. The fact that pneumolysin is immunogenic and requires no adjuvant means that it seems likely that antibodies to pneumolysin would have formed and therefore repeated administration for therapeutic purposes may be problematic.

In vitro I had examined the early activation markers CD69 and CD25 on transgenic T cells and found no decrease in their upregulation in the presence of pneumolysin. This demonstrated that T cells were becoming activated as normal and that the inhibitory effect I observed was not the result of a defect in T cell activation. *In vivo* I chose to examine CD25 only since CD69 peaks within the first 24 hours of T cell activation and therefore would be an invalid measure on day 3. The *in vivo* data was consistent with the *in vitro* data since no difference in T cell activation was observed in the presence of pneumolysin which suggests that the same inhibitory mechanism functions *in vivo* as does *in vitro*.

I have mentioned the usefulness of my studies on antibody production to enable me to functionally measure the effect of pneumolysin upon the T cell which helps me to interpret the questions that arise as a result of the system being open. My examination of lymph node sections by immunohistochemistry is also useful in this regard since they clearly display a real difference in the number of T cells within the whole lymph node. One further area to investigate via immunohistochemistry is whether the T cells migrate to the B cell follicles to the same degree in mice treated with pneumolysin as in controls. This would allow us to observe if pneumolysin is also inducing anergy in any T cells since these would be observed not to migrate. Consolidation of the data obtained by IHC is required since while the images shown are representative of those obtained, it would be good practice to obtain images from a larger sample group.

Overall my data in this chapter clearly demonstrates that pneumolysin can inhibit the T cell response *in vivo* in the purified form. To further address this question it would be interesting to further mimic my *in vitro* findings *in vivo* by using CFSE labelled T cells and transferring them to examine cell division in this way. It would also be interesting to examine if T cell apoptosis is being induced by administration of the purified toxin *in vivo*. The work I mentioned previously of Kemp *et al.* (Kemp *et al.*, 2002) suggests that it may.

I have demonstrated in this chapter that pneumolysin is able to inhibit the expansion of antigen specific T cells *in vivo* and have proposed on the basis of my *in vitro* studies that pneumolysin accomplishes this by inducing apoptosis although this has yet to be demonstrated *in vivo*. It is important to set this work in the wider context of the pneumococcal infection.

I mentioned in the introduction to this chapter that the role of the T cell in the immune response to the pneumococcus is still being elucidated. Initial work by Winkelstein (Winkelstein and Swift, 1975) has suggested that T cells were unimportant for the immune response to the pneumococcus. These studies were based on the i.p. injection of lethal doses of pneumococci and resulted in the conclusion that T cells have no role in defence against the pneumococcus since no difference in lethal dose is observed in the presence or absence of T cells. The validity of these studies was limited by two key points; the i.p. injection of pneumococci is not the standard route of host encounter with pneumococci, and secondly, since T cells are primarily mediators of adaptive immunity, use of a challenge model does not examine the functional role of the T cell correctly.

In 1987 Sestini *et al.* (Sestini *et al.*, 1987, 1988) found that T cells in the lung mediate an antibacterial effect against the pneumococcus. More recently Kadioglu *et al.* (Kadioglu *et al.*, 2000) have implicated the T cell in the immune response to the pneumococcus in the lung since they discovered that the decline in pneumococcal numbers within the lung coincided with the influx of B and T cells into certain parts of the lung tissue. They were careful to point out that this appears to be the result of redistribution within the lung tissue as opposed to influx of cells from outside of the lung. Recently the same group have communicated that CD4 deficient mice are more susceptible to bronchopneumonia and septicaemia than mice with intact CD4 T cell functioning (Kadioglu and Andrew, 2004) and they have demonstrated this using MHC II -/- mice (Kadioglu *et al.*, 2004). Taken together this work suggests that T cells may have a role in the innate immune response to the pneumococcus since the time period in these studies is too short for any adaptive response to be involved.

In the introduction to this thesis I made extensive reference to the studies that have elucidated the role of the T cell in the production of antibody to pneumococcal proteins and polysaccharides. The studies of Wu *et al.* (Wu *et al.*, 1999) have demonstrated the necessity of CD4 T cells for an effective antibody response to protein antigens such as PspA to be generated. Investigations into the T cell requirements for an antibody response to pneumococcal polysaccharide antigen remains

controversial (Snapper, 2004) however the consensus of results suggest that an effective antibody response to the phosphorylcholine (PC) determinant of the polysaccharide cell wall component, techinoic acid, and capsular polysaccharide requires CD4 T cells to provide a CD40 and CD28 signal (Hwang *et al.*, 2000; Wu *et al.*, 1999) (Wu *et al.*, 2000). Wu also demonstrated that the despite being T cell dependent, the anti-PC response is TcR independent (Wu *et al.*, 2000). In 2004 Khan *et al.* (Khan *et al.*, 2004) demonstrated that these findings for PC are consistent for capsular polysaccharide however they discovered that capsular polysaccharide is TcR dependent.

To summarise the role of the T cell in the immune response to the pneumococcus Kadioglu *et al.* suggest that T cells may have some undefined role during the innate phase of the immune response and that T cells have a defined role in enabling the production of antibody to protein and polysaccharide antigens of the pneumococcus. My work on the induction of T cell apoptosis may be relevant with respect to these two T cell activities since it implies that wherever the pneumococcus encounters an activated T cell, apoptosis may be induced. This may occur during a range of pneumococcal pathologies, however my work is only really of interest where a defined and important role has been elucidated for the T cell since it is in then that the induction of T cell apoptosis may have an effect on the outcome of pneumococcal infection.

I therefore hypothesise that pneumolysin may induce T cell apoptosis during the innate immune response in the lung or during the formation of antibody during invasive disease or colonisation. This hypothesis has a number of testable implications. As soon as these implications are considered however the complications imposed by the multifunctional nature of pneumolysin are immediately clear. It has been repeatedly demonstrated that pneumolysin has a number of important functions *in vivo* and therefore the study of pneumolysin deficient strains is complicated by the way they differ from the wild type strain. None the less my hypothesis in relation to the lung activities of T cells would be that if one of the functions of pneumolysin is to induce apoptosis of T cells in the lung then I would expect to see fewer T cells when pneumolysin sufficient pneumococci are present compared to a pneumolysin deficient strain. When using a pneumolysin deficient strain the opposite is actually discovered (Kadioglu *et al.*, 2000) but this is because pneumolysin is actually required for recruitment of the T cells. No other studies have attempted to examine this question and so further insightful studies are required in this area.

Studies have shown that carriage and invasive disease can result in antibody formation to pneumococcal antigens including PspA, polysaccharide and pneumolysin (Rapola *et al.*, 2000) however this is strain dependent for polysaccharide (Soininen *et al.*, 2001) and not always observed for pneumolysin (McCool *et al.*, 2003). According to the summary of the role of the CD4 T cell in antibody formation to the pneumococcus presented in the introduction to this thesis, this antibody will be CD4 T cell dependent. If apoptosis of the CD4 T cells involved in the production of antibody to the pneumococcus is occurring then a number of hypothesises result;

I would predict that antibody formed during invasive disease would be greater in the absence of pneumolysin. Studies using the pneumolysin deficient strain of pneumolysin have not examined this issue. Once again these studies are complicated by the differing course of infection of pneumolysin deficient strains. I would also predict that the formation of antibody as a result of carriage would also be greater in the absence of pneumolysin. I suggest that pneumolysin functions during colonisation not to enable current colonisation but rather to enable future colonisation by inhibiting the development of antibodies to the pneumococcus during colonisation. Again no studies have directly addressed this question however a number of studies have produced some interesting data in this area.

While the development of antibodies during colonisation has been demonstrated this has been found to vary between strains. Therefore it is possible that pneumolysin is the reason for this variation. Furthermore in 1998 Rubins *et al.* (Rubins *et al.*, 1998) studied the effect of pneumolysin upon colonisation. They found that a pneumolysin deficient strain of *S. pneumoniae* was able to colonise and persist in the nasopharynx equally as well as the wild type strain. This data is in contrast with other data that found that pneumolysin deficient strains did not colonise the nasopharynx with the same success as pneumolysin sufficient strains (Kadioglu *et al.*, 2002) although these studies used different strains and so some strain variation seems to be a factor.

Neither study addressed the effect of pneumolysin upon antibody formation since the work of McCool *et al.* demonstrated that during colonisation antibody to PspA increased, yet it had very little effect upon the carriage of the current strain but did inhibit re-colonisation (McCool *et al.*, 2002). Therefore measuring current colonisation does not correlate with the development of antibody. Therefore since we are suggesting that during colonisation pneumolysin may be inducing

apoptosis of CD4 T cells and thereby inhibiting antibody production, the effect of it doing this will not be observed on current colonisation but only upon attempted recolonisation.

My survey of the current literature suggests that the hypothesis presented in this chapter remains largely un-addressed and is worthy of a fuller examination. I made suggestions earlier in this chapter of possible future directions of enquiry using the purified toxin and I now suggest that a line of enquiry be pursued using a pneumolysin deficient strain of *Streptococcus pneumoniae* into the effect of pneumolysin upon the T cell in the context of pneumococcal infection.

7 Conclusion

7.1 Conclusion

In this thesis I have demonstrated that pneumolysin inhibits *in vitro* antigen specific murine CD4 T cell proliferation and cytokine production. Furthermore, I have shown that this effect is not observed with the non-hemolytic pneumolysin mutant F433. I have demonstrated that pneumolysin accomplished this inhibitory activity by inducing apoptosis of activated CD4 T cells and suggest that lipid rafts may be involved in this process since I also demonstrated that pneumolysin preferentially binds to lipid rafts.

In order to further understand the mechanism of action of pneumolysin it would be interesting to elucidate a more detailed description of the role lipid rafts, caspases, active cell death and anergy have in the apoptosis induction process by pneumolysin. Furthermore given the role lipid rafts have in controlling T cells and that lipid rafts have been identified as being potential therapeutic targets (Van Laethem and Leo, 2002) it is possible that pneumolysin could be a highly functional therapeutic molecule and therefore understanding its interaction with lipid rafts could prove critical. The studies conducted on listeriolysin which have demonstrated that listeriolysin induces apoptosis of T cells by a Fas dependent mechanism (Zenewicz *et al.*, 2004) when considered along with the work which demonstrated elevated Fas ligand levels during pneumococcal infection (Kemp *et al.*, 2002) suggests that further *in vitro* investigation into the role of Fas in the apoptosis inducing process of pneumolysin would be worthwhile.

In this thesis I have also demonstrated that pneumolysin is able to inhibit the *in vivo* accumulation of T cells and also inhibits *in vivo* antibody production. I have suggested that these results may be important in terms of pneumococcal pathogenesis whenever pneumolysin encounters CD4 T cells. Some recent data has focused on the encounter between the pneumococcus and T cells during lung infection (Kadioglu and Andrew, 2004) and has suggested that T cells are essential for pneumococcal clearance. Furthermore pneumolysin sufficient strains have been demonstrated to persist for longer within the host lung (Kadioglu *et al.*, 2002). While it has been shown that pneumolysin is involved in T cell recruitment in the lung, there have been no studies that have examined whether pneumolysin induces T cell apoptosis and whether this is responsible for the increased virulence observed in pneumolysin sufficient strains of pneumococci. I specifically therefore suggest that

pneumolysin may be involved in the pathogenesis of the pneumococcus by inducing apoptosis of activated T cells in the lung.

In order to further elucidate the role of apoptosis in the pathogenesis of the pneumococcus, I suggest that demonstration that purified pneumolysin induces apoptosis of T cells *in vivo* should be followed up by the measurement of T cell apoptosis during pneumococcal infection. While measuring the amount of apoptosis during pneumococcal infection such as was carried out in humans (Kemp *et al.*, 2002) is relatively straightforward, demonstration of pneumolysin to be the causative agent is more difficult. This is because pneumolysin deficient pneumococcal strains do not follow the same course of infection and therefore differences are hard to measure. One possibility would be to use a pneumococcal strain containing the F433 mutant since T cell recruitment into the lung is the same for this mutant and yet according to our studies the pneumolysin would not induce apoptosis.

Other studies have focused upon the T cell requirement of the antibody response to the pneumococcus (Khan *et al.*, 2004; Snapper *et al.*, 2001; Wu *et al.*, 2001; Wu *et al.*, 2002) and have shown a T cell dependency for antibody produced to pneumococcal protein and polysaccharide. I therefore also specifically have suggested that pneumolysins induction of T cell apoptosis may therefore have an effect on antibody production to the pneumococcus.

Given the limited role antibody development has on the clearance of current infection (McCool *et al.*, 2002) the effect of pneumolysin upon antibody development is of interest with regard to future infection since antibody developed during previous exposure to the pneumococcus would prevent colonisation upon re-exposure. While it has been shown that pneumolysin deficient strains of the pneumococcus are carried for the same length of time as pneumolysin sufficient strains it would be interesting to measure the development of antibody in these situations.

Finally the use of pneumolysin as a potential therapeutic molecule is also of interest. In demonstrating that pneumolysin is able to inhibit the *in vivo* function of CD4 T cells it would be interesting to examine the effects administering the toxin (or some derivative of it) on CD4 T cell mediated diseases such as rheumatoid arthritis. Experiments similar to those carried out using ES-62 on the collagen induced arthritis model (McInnes *et al.*, 2003) would be ideal for examining this therapeutic potential.

Appendix

Appendix 1

DNA loading buffer (6x)

0.25% Bromophenol blue
0.25% Xylene cyanol FF
40% (w/v) sucrose in water

SOC

Make SOB first;
950ml dH20
20g Bacto Tryptone
5g Bacto Yeast Extract
0.5g NaCl
10ml 250nM KCl
5ml 2M MgCl

Then make into SOC by adding 20ml of 1M glucose

PBS

2.4g KH^2PO^4
2g KCL
14.4g NaH^2PO^4
80g NaCl
in 1 litre water

1.5M Tris HCL

24.2g Tris base (trizma)
add to 50ml dH2O
add HCl to pH 8.8 or pH 6.8
make to 100ml

Bromophenol Blue

100mg Bromophenol Blue in 10ml water

Running buffer

3g Tris (25mM)
14.4g glycine (192mM)
1g SDS (0.1%)
H^20 to 1L

Sample buffer

Tris HCl (pH 6.8) – 60mM
5ml 50% glycerol (25%)
2ml 10% SDS (2%)
0.5ml 2-ME (14.4mM)
1ml Bromophenol blue (0.1%)
0.9ml of dH2O

Coomassie stain

1g coomassie blue (0.1%)

1L destain

Destain

40% methanol

10% acetic acid

50% dH2O

	Separating gel	Stacking gel
dH2O	4.05ml	3.05ml
1.5M Tris HCl	pH 8.8 – 2.5ml	pH 6.8 – 1.25ml
10% SDS	100ul	50ul
30% Acyrilamide bis	3.3ml	665ul
10% APS	50ul	25ul
Temed	5ul	5ul

Transfer buffer

25mm Tris base 3.03g

192mM Glycine 14.4g

20%v/v methanol 200ml

Add dH2O to 1L,

Tris NaCl

Tris base 1.2g

NaCl 8.7g

Conc HCl 800ul

Make to 1L

Developer

30mg of 4-chloro-1-naphtol (freezer)

dissolve in 10mls of methanol

add to 50mls Tris NaCl

also 30ul H2O2 (30% v/v)

MACS Buffer

500ml PBS

2ml 0.5M EDTA

25ml 10% sterile BSA

FACS buffer

500ml PBS

2% FCS

0.2% Na Azide

Fc block

anti-CD16/32 hybridoma supernatant

10% mouse serum (Diagnostic Scotland, Edinburgh, UK)

0.1% sodium azide (Sigma)

Complete RPMI

RPMI 1640 (Gibco BRL, Paisley, UK)
10% foetal calf serum
glutamine
pen/strep (100U/ml penicillin, 100mg/ml streptomycin)
50μM 2-mercaptoethanol (all Gibco BRL, Paisley, Scotland, UK).

Complete IMDM

Pen/Strep
2mM L-Glutamine
50μM 2-Mercaptoethanol
10%FCS

TNB

0.1M Tris-HCl pH7.5, 0.15M NaCl, 0.5% blocking reagent (NEN Life Science)

TNT

0.1M Tris-HCl pH7.5, 0.15M NaCl, 0.05% Tween

Ionomycin – Ca^{2+} salt - Sigma

PMA – Phorbol 12 Myristate 13-Acetate – Sigma

Recombinant murine IL-2 was purchased from R&D Systems.

FACS Antibodies

Biotinylated clonotypic anti-TcR antibody, KJ1.26 (produced from the original hybridoma (Haskins *et al.*, 1983))

	Antigen	Conjugate	Details	Clone	Supplier
Flow cytometry					
	CD4	FITC	rat anti-mouse 0.5mg/ml	L3T4	Pharmingen
	CD4	PE	rat anti-mouse 0.2mg/ml	L3T4 GK1.5	Pharmingen
	CD4	PerCP	rat anti-mouse 0.2mg/ml	RM4-5	Pharmingen
	CD25	FITC			Pharmingen
	CD69	FITC	rat anti-mouse 0.5mg/ml	H1.2F3	Pharmingen
	Isotype	FITC	rat IgM kappa isotype R4-22 0.5mg/ml		Pharmingen
		FITC	hamster IgG1 isotype alpha-TNP 0.5mg/ml		Pharmingen
		FITC	rat IgG2a kappa		Pharmingen
Cell purification	CD8	None	rat anti-mouse	YTS105.18	Serotec
	CD19	None	rat anti-mouse	6D5	Serotec
	CD11b	None	rat anti-mouse	5C6	Serotec
	CD16/32	None	rat anti-mouse	FcY III/II Receptor	Pharmingen
IHC					
	B220	Biotin	rat anti-mouse 0.5mg/ml	RA3-6BZ	Pharmingen

Appendix 2

AMRESCO Inc.
30175 Solon Industrial Parkway
Solon, Ohio

Amico Millipore
Millipore (U.K.) Limited
Units 3&5 The Courtyards
Hatters Lane
Watford, WD18 8YH
England

Becton Dickinson/Pharmingen
BD Biosciences
21 Between Towns Road
Cowley
Oxford, UK

Bibby Sterelin
Lindon House
Heeley Road
Birmingham
UK

Biorad Labs Ltd
Bio-Rad House
Maylands Ave
Hemel Hempstead
Hertfordshire

UK

Biotek Instruments
via Fisher Scientific UK
Bishop Meadow Road
Loughborough
Leicestershire LE11 5RG
United Kingdom

BioWhittaker Ltd
BioWhittaker House
1 Ashville Way
Berkshire
UK

Cadisch Precision Meshes, London, UK

Costar, Corning, N.Y., USA

CompuCyte Corporation
12 Emily Street
Cambridge, MA 02139

Constant Systems Ltd
Cell Disrupting Equipment
Unit 3
Amherst Business Centre
Budbrooke Road
Warwick

Decon Labs Ltd
Conway Street, Hove, East Sussex, BN3 3LY UK

Duchefa
PO Box 2281
2002 CG Haarlem
Netherlands

Dynex Technologies, Vancouver, USA

Gibco/Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley
UK

Harlan-Olac
Shaw's Farm
Blackthorn, Bicester
Oxon OX25 1TP
England

Hawksley & Sons
Marlborough Road
Lancing Business Park
Lancing
Sussex

Hellma
Hellma GmbH & Co KG
Klosterrunsstrasse 5
D-79371 Müllheim

Hybaid
Action Ct

Ashford Rd
Middlesex
UK

Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA

Melford Laboratories Ltd
Suffolk
UK

Menzel Glaser, Germany

Molecular Probes
Molecular Probes, Inc.
PO Box 22010
Eugene,

Miltenyi Biotec
Miltenyi Biotec Ltd.
Almac House
Church Lane, Bisley
Surrey GU24 9DR

New England Biolabs
32 Tozer Road
Beverly, MA 01915-5599

Novagen
Merck Biosciences Ltd.
Boulevard Industrial Park
Padge Road
Beeston
NOTTINGHAM

NG9 2JR

Nunc
Nalge Limited
Unit 1a, Thorn Business Park
Hereford UK

Oxoid Ltd, Basingstoke, Hampshire, England

Perseptive Systems
850 Lincoln Centre Drive
Foster City, CA 94404 USA

Promega
Southampton
UK

QIAGEN Ltd.
QIAGEN House
Fleming Way
Crawley
West Sussex,

R&D Systems, Minneapolis, MN, USA

Rimmel
via Boots UK

Serotec Ltd, 22 Bankside, Station Approach, Kidlington, Oxford, OX5 1JE

Sartorius Ltd.
Longmead Business Park
Blenheim Road

Epsom Surrey
UK

Sakura
Hoge Rijndijk 48a
2382 AT Zoeterwoude
The Netherlands

Sigma-Aldrich Company Ltd
Second Avenue
Heatherhouse Industrial Estate
Irvine
KA12 8NB
Scotland

Sigma-Genosys Ltd.
London Road, Pampisford
Cambridge CB2 4EF, UK

Stratagene
11011 N. Torrey Pines Road
La Jolla, CA 92037

ThermoShandon
Pittsburgh
PA, USA

Unicam
PO Box 206
York Street
Cambridge
UK

Vector Laboratories
Burlingame
CA, USA

Wallac/Perkin Elmer Life Science
549 Albany Street
Boston, MA USA

Zeiss
Woodfield Road
Welwyn Garden City
Herts
UK

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