THE UNIVERSITY OF HULL

STREPTAVIDIN-CYTOKINE FUSION PROTEINS FOR USE AS ADJUVANTS IN CANCER VACCINES

Being a Thesis Submitted for the Degree of Doctor of Philosophy

In the Division of Cancer, University of Hull

By

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Abstract

The work presented in this thesis describes the production and characterisation of recombinant streptavidin-cytokine fusion proteins. Immunoadjuvants can augment the weak or non-existent antitumour response of a patient with cancer to immunotherapy. The response of breast cancer patients to cancer vaccines directed against tumour associated antigens, such as HER2/neu, is often weak and it has been reported that additional immunostimulation, such as that provided by an immunoadjuvant, is required in order to achieve effective therapeutic results. One approach to the development of novel immunoadjuvants is the production of fusion proteins which are designed to perform the dual functions of general activation of the immune system as well as directing the immune response specifically towards a TH1-type response, which is widely regarded as the optimal patient response for cancer immunotherapy.

Streptavidin is a bacterial protein and an immunogenic molecule that induces an unspecific immune response. The cytokines IL-2 and IL-18 both promote TH1-type responses; IL-2 is already in clinical use as a cancer therapy, while investigation of the role of IL-18 as an immunotherapy is ongoing.

A strategy was devised for the production of novel recombinant fusion proteins designed for use as immunoadjuvants; these proteins comprised an N-terminal truncated streptavidin core protein sequence and a C-terminal cytokine, specifically either IL-2 or IL-18, separated by a short polypeptide linker region. Molecular cloning techniques were used to generate DNA expression constructs encoding the recombinant fusion proteins which were expressed in an inducible bacterial system using plasmid expression vector pCR®T7/NT-TOPO®. The expressed recombinant proteins were found to accumulate within insoluble bacterial inclusion bodies and protocols were developed and optimised for the isolation and solubilization of these proteins. Protein solubilization required the use of buffers at high pH (pH 12.5) which resulted in disrupting protein structural integrity; a pulsed dilution method was subsequently employed to achieve refolding of the proteins prior to further analysis.

Characterization of fusion proteins STV/IL-2 (streptavidin-IL-2) and STV/IL-18 (streptavidin-IL-18) was conducted using native and dissociating PAGE and Western analysis. Antibody binding studies provided preliminary confirmation of the identity of the STV/IL-2 fusion protein. Similar studies to characterize STV/IL-18 were initially encouraging but proved inconclusive and further analysis of this protein is required.

These initial investigations have validated this approach using the expression systems established here for the production of recombinant streptavidin-cytokine fusion proteins. A number of issues to be addressed have been highlighted regarding problems encountered with protein yields, solubilisation and maintenance of structural integrity. It is therefore concluded that further modification and optimisation of the expression system and protein isolation procedures employed is necessary to provide an appropriate system for the production of these fusion proteins; this will subsequently permit the investigation of their potential for use in therapeutic applications.

Table of Contents

Chapter 2

Materials and Methods

Chapter 3

Cloning of Streptavidin-Cytokine Fusion Proteins IL-2/STV and IL-18/STV

List of Figures

List of Tables

Abbreviations

$^{[3H]}$TdR	Tritiated Thymidine
APC	Antigen Presenting Cell
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CEA	Carcinoembyonic Antigen
CNS	Central Nervous System
CTL	Cytotoxic Lymphocytes
dH_2O	Deionised Water
DMSO	Dimethyl Sulphoxide
dNTPs	Deoxyribonucleotide Triphosphates
EASIA	Enzyme Amplified Sensitivity Immunoassay
EDTA	Ethylenediaminetetraacetic Acid
F(ab')	Antibody Fragment
FCA	Freund's Complete Adjuvant
FDA	U.S. Food and Drug Administration
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HIFBS	Heat Inactivated Foetal Bovine Serum
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma virus
HRP	Horseradish Peroxidase
hTERT	human Telomerase Reverse Transcriptase
ICE	IL-1β Converting Enzyme
IGIF	Interferon-gamma Inducing Factor
IFNα	Interferon-alpha
IFNγ	Interferon-gamma
IL	Interleukin
IL-2	Interluekin-2
IL-2R	Interleukin-2 Receptor
IL-18	Interleukin-18
IL-18BP	Interleukin-18 Binding Protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside

LAK	Lymphokine Activated Killer
LB	Luria Bertani
LPS	Lipopolysaccharide
m	murine
MHC	Major Histocompatability Complex
MUC	Mucin
NHS	National Health Service
NK	Natural Killer
NSCLC	Non Small Cell Lung Carcinoma
PBMC	Peripheral Blood Mononucleocyte Cells
PMA	Phorbol Myristyl Acetate
r	recombinant
rmtp	Room Temperature
RT	Reverse Transcriptase
SAC	*Staphylococcus aureus*
SCID	Severe-Combined Immunodeficiency
SCLC	Small Cell Lung Carcinomas
SDM	Site Directed Mutagenesis
SLE	Systemic Lupus Erythematosus
STAT	Signal Transducers and Activator of Transcription
TAA	Tumour Associated Antigen
TC	Cytotoxic T cells
TCR	T cell Receptor
TEMED	N,N,N',N' – Tetraethylethylene Diamine
TH	T Helper
TMB	Tetramethylbenzide
TNF	Tumour Necrosis Factor
TSG	Tumour Suppressor Gene
γc	Common γ Chain

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This Thesis is dedicated to my husband Carl M^{c}Cabe who has supported me both emotionally and financially and without whom there is no doubt this thesis would have never been completed.

Declaration

I hereby declare that the thesis entitled “Streptavidin-Cytokine Fusion Proteins for Use as Adjuvants in Cancer Vaccines” has not been submitted for a degree, diploma or any other qualification at any other university. The work presented in this thesis was done solely by the author except where stated.

Rosalind Henderson

Publications

Conference Abstracts

Henderson R., Maraveyas A., Greenman J., Dyer C.E. (2005) "Streptavidin/Cytokine Fusion Proteins as Immunoadjuvants in Cancer Therapy". *Clincal Bioscience Graduate Research Conference.*

Chapter 1

General Introduction

1.1 Cancer

1.1.1 Cancer Statistics

Cancer is a very prevalent disease with more than 1 in 3 people developing some form during their lifetime. In 2004, there were 356,992 incidences of cancer in the UK, with 153,491 deaths from cancer in 2005 (Cancer Research UK 2007). In the UK the most commonly diagnosed cancers are breast, lung, large bowel and prostate cancer.

1.1.2 Tumourigenesis of Solid Tumours

There are over 100 types of cancer which have been described, most of which form solid tumours. A tumour is a neoplastic cell mass which forms due to the abnormal proliferation of cells. Tumours can be benign or malignant. Benign tumours do not transform into cancer, and are not discussed further here. Malignant tumours are formed from groups of cells which divide in an uncontrolled manner and invade and damage surrounding tissues. Malignant tumours become metastatic when cells from the primary tumour spread to other body tissues via direct migration through the body, via the blood or lymphatic systems (reviewed by Pantel *et al* 2008).

Malignant tumours arise from mutations in DNA which cause the loss of function of tumour suppressor genes (TSGs), which usually act to inhibit mechanisms such as cell proliferation, or the gain of function of oncogenes which act via mechanisms such as the stimulation of cell proliferation. Proto-oncogenes have expression which is essential for normal cell functions, so knocking out proto-oncogenes to avoid gain of function mutations is not a viable cancer prevention strategy. An example of one such oncogene that can be involved in the tumourigenesis of breast cancer is the gene which codes for HER2/neu, the activation of which can be seen in about 30% of primary breast cancer cases (Olayioye 2001). The overall effect of these mutations is to remove cells from the

normal controls of cell cycle regulation and allow uncontrolled cell proliferation and resistance to apoptosis. These tumourigenic mutations may occur initially in just one cell. The Knudson hypothesis of retinoblastoma (Knudson 1971) and the Fearon and Vogelstein colorectal cancer model (Fearon and Vogelstein 1990) postulate that one mutation alone is not enough to cause a cell to become cancerous, and suggest instead that several mutations in independent pathways (at least 3-5) must occur. Studies of colorectal cancers have shown that individual tumours may contain as many as 100 different mutations (Sjöblom *et al* 2006).

Somatic mutation of DNA can be caused by several factors. Carcinogens are environmental factors (such as UV, food additives and pesticides) that promote cancer. Carcinogenic mutagens cause direct mutation of a cell's DNA, and other carcinogens (such as alcohol) which do not cause mutagenesis promote cell proliferation and therefore may cause the promotion of tumour proliferation (Report on Carcinogens, Eleventh Edition; U.S. Department of Health and Human Services, Public Health Service, National Toxicology Program). Inheritance may also play a role in tumourigenesis; in some cases a mutation of one copy of a TSG (heterozygosity) can be inherited. Only mutation of the other allele would then be needed for a loss of function of the TSG in question.

The p53 gene is a well studied tumour suppressor gene which is mutated in up to half of all human cancers, including 20-30% of human breast cancers. Under normal conditions p53 acts as a regulating mechanism for cell division (Figure 1.1) (Osborne *et al* 2004).

Some cases of tumourigenesis may be caused by viral infection, e.g. HPV, the most common cause of cervical cancer (Schiffman *et al* 2007).

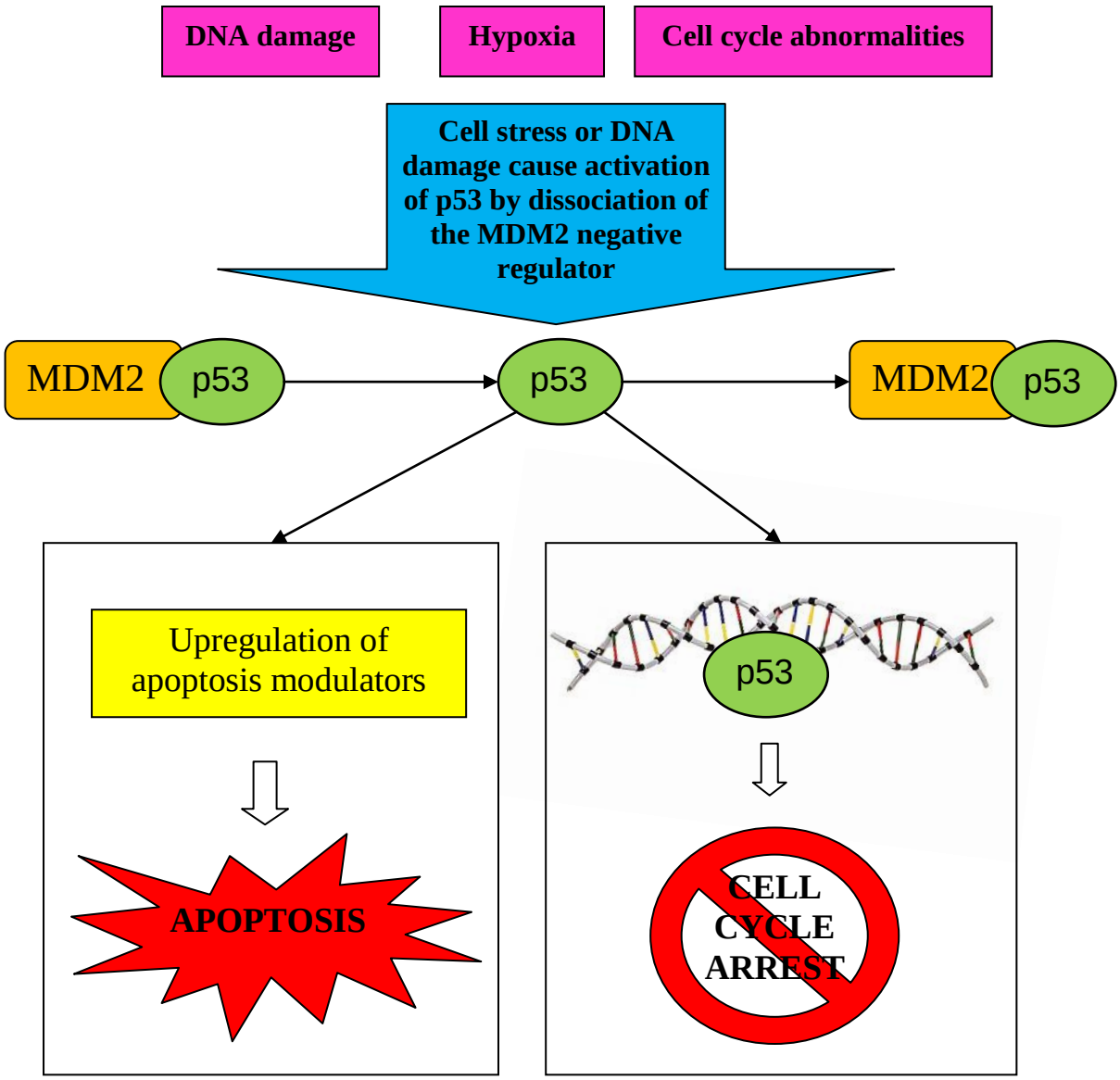

Figure 1.1 p53 function.

Cell stress causes the dissociation of the MDM2/p53 complex activation p53. Once activated p53 will bind to damaged DNA arresting the cell cycle and therefore further cell proliferation, or upregulate apoptosis modulators leading to cell death.

After one or more cells have acquired the mutations required for tumourigenesis, rapid clonal expansion takes place, and as a result of genetic instability more mutations subsequently occur throughout the tumour. Those cells which have gained mutations that are advantageous to the proliferation of the tumour (such as enhanced replication or resistance to apoptosis) will further drive the tumour development (Beerenwinkel *et al* 2007).

Another model of tumourigenesis is the transformation of adult stem cells which was first observed by Bonnet and Dick (1997). These cells exist as a sub-population of cells within the tumour, and retain key stem cell properties such as the ability to differentiate and self-renew. Cancer stem cells give rise to a large population of differentiated cells which make up the bulk of the tumour. These differentiated cells lack the ability to self-renew, so, since cancer stem cells are the only tumour cell type not destined to die, they continue to proliferate and differentate and are the driving force behind ongoing tumour growth. Cancer stem cells also have a role in the relapse and the emergence of metastasis (Ailles and Weissman 2007). This model of tumourigenesis is complementary to the Knudson and Fearon and Vogelstein tumourigenesis models, placing cancer stem cells as the site where the tumourigenesis process begins. The cancer stem cell model is reviewed in depth by Shipitsin and Polyak (2008)

1.1.3 Tumour Evasion

Tumour evasion is defined as the failure of the immune system to respond to tumour-associated antigens, whilst normal immune responses are preserved. The immune system is able to control or destroy the majority of tumours early in their development. Patients on immunosuppressive therapy or those whose immune systems are otherwise compromised are more susceptible to the development of tumours. However, not all

tumours are immunogenic. Some tumours are recognised by immune cells as 'self' because, having developed from the body's own cells, they express self antigens. Thus these tumours will not provoke an immune response. Other tumours may downregulate or lose expression of co-stimulatory molecules such as major histocompatibility complex (MHC) class I which allows the cells to escape recognition by the immune system. The loss of MHC class I molecules has been reported in a significant proportion of primary breast cancers (Palmisano *et al* 2001). Even tumours which are immunogenic may proliferate faster than the immune system can destroy them, or may suppress the immune response which is generated against their antigens by producing immunosuppressive factors such as interleukin-10, transforming growth factor-β and vascular endothelial growth factor (Tenderich *et al* 2001, Kim *et al* 2006).

1.1.4 Breast Cancer

There are numerous different forms of solid tumours that can occur depending upon the cell and tissue type of origin and the specific underlying causative (and subsequently accumulated) mutations. As a result, there is a huge variation between tumour types in terms of the clinical presentation and progression of the disease and the associated patient prognosis following intervention. Many clinical cancer therapies are, therefore, only relevant to certain specific tumour types, often depending on the tissue of origin. For the purpose of the studies described here, which aim to explore the potential of novel therapeutic recombinant proteins as immunoadjuvants to boost naturally occurring anti-tumour immune responses in cancer patients, it was, therefore, decided to focus on breast cancer as a general tumour model.

The type of immunotherapeutic approach for the treatment of cancer proposed here relies on the patient retaining an appropriate level of immune function and is not suitable for patients who are severely immunocompromised. Breast cancer was therefore selected as an appropriate target for this investigation since it is more commonly occurring in younger women than other cancer types such as colorectal cancer (Cancer Research UK 2008) and is often identified at earlier stages of progression due to breast cancer screening programmes, factors which are associated with patients who are less likely to have compromised immune systems. As such, breast tumours potentially offer a very relevant target for the possibility of using immunotherapeutic interventions for the treatment of cancer.

Breast cancer is the third most prevalent cancer behind lung and colorectal cancer, and account for 16% of all cancers. Each year in the UK approximately 41,000 new cases of breast cancer are diagnosed. In 2004 there were 44,345 new cases of breast cancer diagnosed in women and 324 new cases in men (Cancer Research UK 2007). Women have a 1 in 9 chance of developing breast cancer during their lifetime and 1 in 3 of all malignancies diagnosed in women is breast cancer. Over 80% of these cases are diagnosed in individuals between 50 and 64 years of age. In women under 35, breast cancer is the most commonly diagnosed cancer, with the incidence increasing with age. The incidence of recorded breast cancer cases increased considerably (41%) between 1982 and 2001, especially in woman aged over 50. This increase is not entirely due to increased detection following the introduction of breast screening by the NHS, as the rising trend predates this introduction. The introduction of screening did skew the figures for the first 4-7 years of introduction; however, this increase continues to date (Coleman 2000). Other causes of incidence increase include an increased use of hormone replacement therapy, an increase in obesity in postmenopausal women as well

as lower levels of physical activity, earlier menarche, delayed childbearing, and having fewer children (American Cancer Society 2008). Breast screening continues to be used in the UK despite controversy over the benefits and risks of breast screening programmes (Gray *et al* 2008).

As well as an increase in breast cancer incidence, there has been an improvement in survival rates for patients with breast cancer over the past 20 years. For women diagnosed with breast cancer between 1971 and 1975, the 5 year survival rate was 52%, compared to 77% for women diagnosed between 1996 and 1999 (Cancer Research UK 2008). This is due in part to increased detection rates and earlier detection of tumours via breast screening programmes, and in part to better treatment such as the widespread use of tamoxifen and hormone therapies and the introduction of new treatments such as trastuzumab which targets tumours over-expressing HER2/neu (Nicolini *et al* 2006).

1.1.5 Breast Cancer Treatment

Current clinical treatment of breast cancer uses a multi-disciplinary approach including surgery to remove the tumour mass, chemotherapy which can be delivered prior to (neoadjuvant) or following (adjuvant) surgery and radiotherapy. Although effective in certain cases, all of these treatments are associated with limitations (Amar *et al* 2008), such as the development of tumour resistance to chemotherapy and radiotherapy and the promotion of metastasis caused by surgical intervention (Ben-Eliyahu 2003, Goldfarb and Ben-Eliyahu 2006-2007). Furthermore, radiotherapy and chemotherapy elicit numerous negative side effects which reduce the patient's quality of life whilst receiving these treatments (Nicolini *et al* 2006).

Due to the limitations of conventional cancer therapy there is much interest in the development of alternative therapeutic strategies, one of the most promising of these being immunotherapy in which the patient's own immune system is stimulated to bring about the destruction of tumour cells (Lucas and Keisari 2006). Recent advances in the understanding of the role of the immune system in cancer have lead to a large amount of research in this area with promising results (Curagliano *et al* 2007, Emens *et al* 2005, Bhardwaj 2007).

1.2 The Immune System

The immune system is the body's defence against pathogens and tumours. It can be split into the innate and the adaptive immune responses. Both immune responses require the identification of foreign bodies as non-'self. A brief review of the immune system is included here (Figure 1.2).

1.2.1 The Innate Immune Response

The innate immune response is a fast, relatively non-specific response against invading pathogens. This response includes inflammation, activation of cascade proteins, production of cytokines, phagocytosis and activation of the adaptive immune responses by antigen presentation on dendritic cells. Antigen presentation by dendritic cells occurs in combination with both MHC class I and MHC class II molecules, whereas most other cells only produce MHC class I molecules, so dendritic cells are termed professional antigen presenting cells (APCs). The cells involved in the innate immune response are mast cells, phagocytes, macrophages, neutrophils, dendritic cells, basophils, eosinophils and natural killer cells (Müller *et al* 2008**,** Zänker 2008)

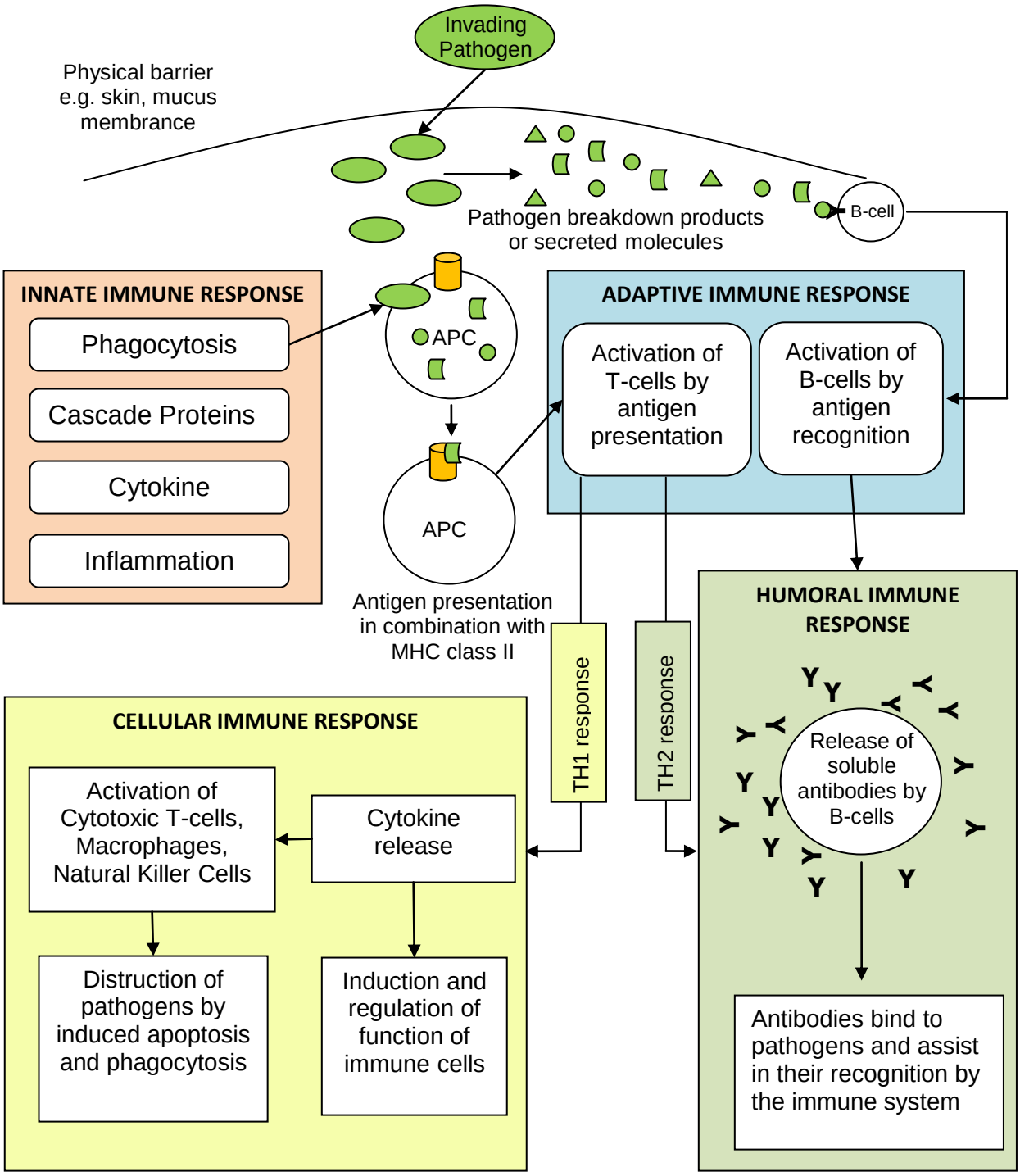

Figure 1.2 Overview of the immune response to invading pathogens.

Antigen Presenting Cells are denoted by APC

1.2.2 The Adaptive Immune Response

The adaptive immune response is a slower but highly specific response which leads to immunological memory, allowing a faster response time against further challenges by the pathogen. The predominant cell types involved with the adaptive immune response include lymphocytes, B cells and T cells (Fietta 2007**,** Parish and O'Neill 1997).

1.2.3 Cells of the Immune System

(i) B cells

B cells are produced and matured in bone marrow. Like dendritic cells, they are professional APCs which present antigens in combination with both MHC class I and MHC class II molecules. Each B cell expresses an antibody against a specific antigen and, having recognised that antigen, proliferates into plasma cells which secrete large amounts of soluble antibody and memory B cells, allowing a fast response upon repeated exposure to the antigen. Soluble antibodies assist in the destruction of pathogens by binding to them and making them easier targets for the innate and adaptive immune responses.

(ii) T cells

T cells, like all leukocytes, are also produced in bone marrow, but are matured in the thymus and stimulated to differentiate into several different subsets, including T helper cells and cytotoxic T cells. T helper (TH) cells express the unique cell surface marker CD4, leading them to be named $CD4^+$ cells, and cytotoxic T cells (TC) express the CD8 marker, leading them to be named $CD8^+$ cells.

$CD8^+$ cells are responsible for the apoptosis of a target pathogen or infected cell. They are stimulated by interaction with antigens presented by MHC class I molecules expressed on APC to interact directly with infected host cells or to release cytotoxins which signal to other cells. Stimulation of $CD8^+$ cells also leads to clonal expansion of the $CD8^+$ subset of T-cells cells (Street and Mosmann 1991).

$CD4^+$ cells are activated by interaction with antigens presented by MHC class II molecules. The activation of a resting $CD4^+$ cell causes it to release cytokines that influence the activity of many cell types. Cytokine signals produced by $CD4^+$ cells enhance the microbicidal function of macrophages and the activity of other T cells and APCs. In addition, TH cell activation causes an upregulation of molecules expressed on the T cell surface which provide extra stimulatory signals typically required to activate antibody-producing B cells (Street and Mosmann 1991).

1.2.4 T Helper Cell Responses

Two types of T helper cell responses can be triggered by APC interaction with $CD4^+$ cells; these are known as the TH1 (cellular) and TH2 (humoural) immune responses (Reviewed by Kidd 2003). The TH1 response is characterized by the production of a specific subset of cytokines including interferon-γ and interleukin-2, which activate macrophages and induce B cells to secrete antibodies. The TH1 immune response leads to cell-mediated immunity and is effective against intracellular viruses and invading bacteria. The production of TH1 cytokines is known to correlate with an increase in anti-tumour responses (Dredge *et al* 2002).

The TH2 response is characterized by the production and release of cytokines including interleukin-4 and interleukin-10, which result in the activation of B cells to secrete

specific antibodies against antigens on the pathogen or infected host cell, leading to humoural immunity which is effective against parasites and toxins.

The innate and adaptive immune responses do not work in isolation from each other. In any infection both the innate and adaptive responses are important. The innate immune response provides a fast response against invading pathogens, and activates the slower adaptive response which is pathogen-specific and results in immunological memory. Thus, the contributions of both the innate and the adaptive immune responses are essential to the immune system.

1.3 Cancer Immunotherapy

As discussed in Section 1.1.3, tumours often evade or downregulate the immune system. Cancer immunotherapy aims to enhance and modify the immune response against tumour cells to destroy tumour cells (Bremers *et al* 2000). Since a cellular TH1 immune response is beneficial in the treatment of cancer, many therapeutic cancer vaccines have been developed to encourage the immune system towards a TH1-type immune response against the tumour cells (Section 1.5).

Whole tumour cells and cell lysates (Morton *et al* 1992, Hanna *et al* 2001), recombinant proteins (Samanci *et al* 1998), synthetic peptides (reviewed in Curigliano *et al* 2007), naked DNA (reviewed in Haupt *et al* 2002) and dendritic cell based vaccines (Steinman and Pope 2002) are among the types of vaccines that have been developed and are currently, or have been, in clinical trials for the immunotherapy of breast cancer (Whelan *et al* 2003, Mocellin *et al* 2004, Slingluff and Speiser 2005, Curigliano *et al* 2007). None of these vaccines, however, are as yet commercially available, with many vaccines past phase II trials but failing in phase III trials for reasons such as poorly

defined patient groups and changes in protocol (Dalgleish 2006, Ward *et al* 2008). Other challenges facing the development of cancer vaccines are technical practicalities such as large scale design and production (Ward *et al* 2008).

1.4 Immunoadjuvants

An immunoadjuvant can be defined as an agent which, when added to a vaccine, acts to accelerate, prolong or enhance specific immune responses to the vaccine antigen (Vogol 2000). The use of immunoadjuvants with vaccines allows the potential for a lower dose of antigen or less frequent immunisation to achieve the desired immune response. The advantages of using lower or less frequent doses of antigen include the reduction of toxicity and/or side effects of the antigen, as well as potential reduction of treatment costs. Immunoadjuvants may also enhance immune responses to weaker antigens, such as highly purified or recombinant antigens, which might otherwise elicit little or no response. Additionally, immunoadjuvants can act to enhance immune responses in patients with weakened or suppressed immune systems, such as elderly or new-born patients.

Many adjuvants such as those containing bacterial components, synthetic peptides or oil emulsions are foreign to the body and therefore may cause adverse reactions. Consequently, adjuvants must undergo extensive evaluation before they can enter clinical trials (Gupta *et al* 1993, Vogol 2000). The development of safe and effective novel adjuvants is an important area of vaccine research and development (Kwissa 2007, Hauguel and Hackett 2008).

Immunoadjuvants can be designed to drive the immune system towards the development of either a TH1- or a TH2-type of response to the vaccine antigen;

therefore, adjuvants must be selected for use with specific vaccines on the basis of the type of immune response required, as well as other considerations. TH2-type responses are preferred for antibacterial responses but TH1-type responses are desired for protection against viruses and parasites, as well for the treatment of cancers. Several types of adjuvants are known to boost or augment the immune response, including oil emulsion (suspensions of killed bacteria in mineral oil) (Freund *et al* 1937), mineral compounds (aluminium phosphate and aluminium hydroxide are common adjuvants for human vaccines) (Gupta *et al* 1993), bacterial products (whole killed bacteria, bacterial components, or bacterial metabolic products, although these are too toxic for use in human vaccines) (reviewed by Gupta *et al* 1993 and Volgel 2000) and liposomes (Richards *et al* 1998) (may help stimulate cell-mediated immunity and supply antigen to APCs and in addition may increase the half-life of an antigen and prevent its clearance).

1.5 Cancer Vaccines

Vaccines against tumour cells differ from vaccines against microbial pathogens since they need to target cells derived from the patient's own tissue, which therefore carry many of the self-antigens present in normal cells. However, cancer cells often also express cell surface proteins which are not expressed in normal cells, or over-express normal or mutated proteins (Curigliano *et al* 2007). It is these proteins which may be used as potential antigens in any anti-cancer vaccine design strategy. In addition, antigens loaded onto MHC class I molecules may be derived from any protein expressed by a tumour as long as they fit the MHC I binding groove, and are not limited only to those proteins expressed on the tumour cell surface. These expressed proteins, which are unique to tumour cells, are known as tumour associated antigens (TAA). Accordingly, cancer vaccine design has tended to focus on the identification of TAA and the induction and development of a TH1-type immune response including

activation and regulation of cytotoxic T lymphocytes (CTL) and TH cells in order to overcome self-tolerance to tumour cells (Dredge *et al* 2002, Mocellin *et al* 2004, Curigliano *et al* 2007).

1.5.1 Tumour Associated Antigens

There have been several different approaches to the design of cancer vaccines, many of which involve loading TAA onto MHC class I molecules (Nencioni and Brossart 2004, Vari and Hart 2004), either deriving TAA from autologous tumour cells or allogeneic cancer cell lines or using synthetically generated TAA epitopes. Vaccines developed from autologous tumours are, in principle, highly specific to each patient, containing a complete set of tumour associated antigens for the tumour of interest; however, there are major logistical challenges in manufacturing such specific vaccines from a limited tumour sample. In contrast, allogeneic tumour vaccines can be mass produced, although they lack the specificity of autogenic tumour vaccines (Copier *et al* 2007). Viral and bacterial vectors and injection of naked DNA have all been investigated for the delivery of TAA and TAA epitopes to MHC class I molecules (Mocellin *et al* 2004). Another approach is to use whole dendritic cells presenting MHC molecules loaded with TAA epitopes (Koido *et al* 2007, Ferrantini *et al* 2008).

There are several TAA that have been associated with breast cancer. Some of the most well-studied TAA for use in stimulating the immune system for cancer vaccines are discussed below. Interestingly, studies so far indicate the requirement for further immune stimulation by some additional mechanism in therapies using most of these TAA emphasising the critical importance of the development of immunodjuvants for use in association with vaccines based on TAA.

(i) HER2/neu

HER2/neu is a protein receptor which is a member of the epidermal growth factor receptor family. It is normally involved in cell proliferation and differentiation (Olayioye 2001). HER2/neu is a TAA which is expressed in epithelial tumours and over-expressed in 30% of breast cancers where its expression is associated with poor prognosis. The role HER/2 neu in breast cancer is reviewed in depth by Ferretti et al (2007)

HER2/neu has an extracellular domain (Figure 1.3) that can be targeted by antibodies produced by B cells as well as targeting HER2/neu peptides presented by MHC class I or II. Vaccination studies in rats and humans have shown that HER2/neu-derived peptides provoke a $CD8^+$ immune response (Disis and Cheever 1998, Disis *et al* 1998, Baxevanis *et al* 2004), although this immune response is not long-lasting (Knutson *et al* 2002). Several vaccine strategies have been trialled including monthly HER2/neu-derived peptide injections and vaccination in combination with an immunoadjuvant such as GM-CSF (Murray *et al* 2000), which showed increased duration of immune response compared with HER2/neu vaccines containing no immunoadjuvant.

(ii) Mucin (MUC)-1

MUC-1 is a membrane associated glycoprotein which is expressed by the ductal epithelia of many tissue types, including pancreas, breast and gastrointestinal tract. MUC-1 is over-expressed and incorrectly glycosylated by malignant cells in cancers of these tissues (Singh and Bandyopadhyay 2007).

MUC-1 is multi-functional and has a role in the protection of mucus membranes, signal transduction and modulation of the immune system. More than 70% of cancers

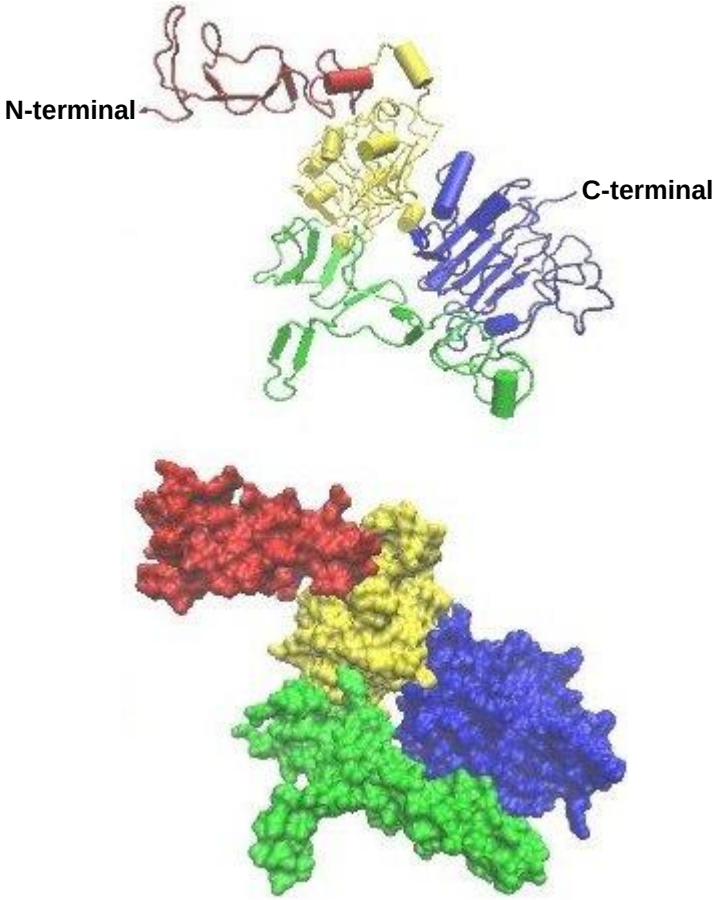

Figure 1.3 HER2/neu extracellular domain.
The structure of the HER2/neu extracellular domain as published by Franklin *et al* (2004). Data accessed from the Protein Data Bank (PDB: 1S78), rendered using VMD and released into the public domain by K.Murphey (2007).

over-express MUC-1, and over-expression of MUC-1 in breast cancer is linked to poor prognosis (Kohlgraf *et al* 2004). MUC-1 is immunogenic but elicits a humoural TH2 immune response as a tumour antigen without provoking a cellular TH1 response (Gilewski *et al* 2000). Several strategies have been tried in order to improve the TH1 response against MUC-1, including the administeration of a co-stimulatory molecule, conjugation of MUC-1 to a protein carrier and fusion of MUC-1 positive tumour cells with APC (reviewed by Singh and Bandyopadhyay 2007).

(iii) Carcinoembryonic antigen (CEA)

CEA is an adhesion molecule which is over-expressed in many cancers, including those of breast origin. In cancer cells CEA promotes adhesion and the metastatic process (Marshall 2003). Since CEA is normally expressed in many cells, the immune system is tolerant to it. Vaccines against CEA must, therefore, be designed to break tolerance by containing co- stimulatory molecules, or immunoadjuvants. Clinical trials have shown mixed results, with only a few patients responding to CEA vaccines (Curigliano *et al* 2007).

(iv) Human telomerase reverse transcriptase (hTERT)

hTERT normally functions to maintain chromosomal integrity by protecting telomeric DNA that is susceptible to loss in successive rounds of cell division (Blackburn *et al* 1989). It shows widespread expression in human cancer cells, being expressed in about 85% of all carcinomas (Vonderheide 2002). hTERT vaccines have shown the ability to elicit an immune response in many tumours in clinical trials (Nair *et al* 2000, Vonderheide *et al* 2004). A comprehensive review of the role of hTERT in breast cancer and breast cancer immunotherapy is provided by Carpenter and Vonderheide (2006)

1.5.2 Immunoadjuvants for Cancer Vaccines

Because of the weak or non-existent immune response to tumours which occurs because of the reasons discussed in Section 1.1.3, tumour vaccines alone are often not enough to stimulate an appropriate immune response to elicit tumour eradication. Incorporating an immunoadjuvant can, however, improve the anti-tumour response to cancer vaccines by manipulating immune pathways. Immunoadjuvants seek to provide a stimulatory signal which may otherwise be lacking in a cancer vaccine (Dredge *et al* 2002). For the treatment of cancer, recombinant TH1 cytokines such as IL-2, IL-12 and IFN-α, which manipulate the immune system to activate $CD8^+$ TC, have been suggested as possible immunoadjuvants (Dredge *et al* 2002, Berinstein 2007).

Interleukin-2 (IL-2) is a TH1 cytokine which has been well characterised, and is currently in clinical use as a cancer therapy. Several clinical trials have been undertaken using IL-2 as a combination treatment with cancer vaccines. Except for one study by Rosenburg *et al* (1998), none as yet have shown an increase in the immune response to the combined vaccine. Interleukin-18 (IL-18) is another cytokine which has more recently also been shown to be important in the development of a TH1-response where it stimulates NK and T cells, which help to eliminate cancer cells. However, the role of IL-18 as a therapy for cancer treatment is controversial, as it has also been found to promote tumour progression (reviewed by Park *et al* 2007). Because of the contradictory effects of IL-18, it is an interesting and novel molecule for further investigation, especially in the role of a potential cancer vaccine immunoadjuvant. Both IL-2 and IL-18 are discussed further in the following sections.

1.6 Interleukin-2

IL-2, first identified by Morgan *et al* (1976) and originally known as T cell growth factor, is a cytokine which is mainly produced by activated T cells. It has, however, been shown that activated B cells and dendritic cells can also produce IL-2, although in smaller amounts (Walker *et al* 1988, Granucci *et al* 2003). As well as numerous effects on T cells, IL-2 also has effects on the growth and differentiation of B cells, natural killer (NK) cells, monocytes, macrophages and oligodendrocytes (Malek 2008). The effects of IL-2 are mediated through receptors on the cell surface, specifically the IL-2 receptor (IL-2R) (Section 1.6.5) (Waldmann *et al* 1998, Smith 1992). The full array of IL-2 function *in vivo* is, as yet, unknown.

1.6.1 The Function of IL-2 in Immune Regulation

(i) T cells

Activation of naïve T cells by interaction of APC with the T cell receptor (TCR) leads to the *de novo* synthesis and secretion of IL-2 as well as expression of the IL-2Rα subunit of the IL-2 receptor and surface display of the high affinity IL-2R complex (Smith 1992). Secreted IL-2 has both autocrine and paracrine effects on T cells, including the clonal expansion of activated T helper subsets and induction of CTL, as well as proliferative effects on a number of other lymphocytes including B cells and NK cells. The T cell response to APC is terminated when the expression of IL-2 and IL-2Rα diminishes. As IL-2 production is stimulated by antigen presentation to T cells, activated T cells die due to withdrawal of IL-2 when antigenic stimulation ceases (Gaffen and Liu 2004) (see Figure 1.4).

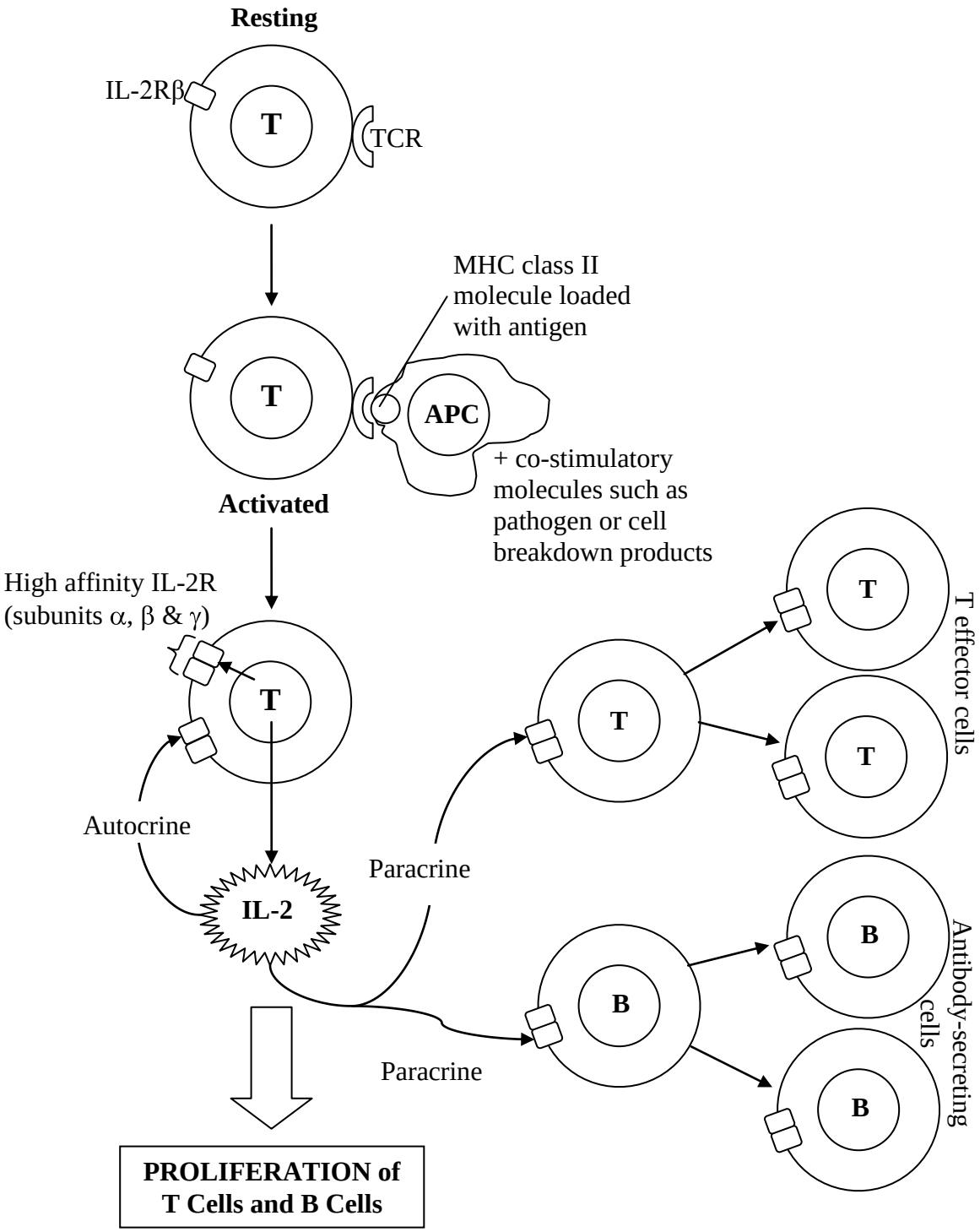

Figure 1.4 The main effects of IL-2 in T and B cells.

The main effects of the stimulation of T cells (T) and B cells (B) by Antigen presenting cell APC to produce IL-2 . T cell receptors are denoted by (TCR).

IL-2 may also have a role in the early stages of T cell development in the thymus. T cell progenitors constitutively expressing IL-2Rβ and secreting low levels of IL-2 have been identified in the human thymus. It is possible that an autocrine pathway promotes growth of these immature thymocytes (de la Hera *et al* 1986), although it remains uncertain what the exact role of IL-2 is in the development of thymocytes, it has been demonstrated by using gene knockout models and bone marrow transplant studies that IL-2 has a definite role in thymic function (Wrenshall *et al* 2007).

The action of IL-2 on the growth of thymocytes also acts to prevent autoimmunity by influencing the development of T regulatory ($CD4^+CD25^+$) cells which suppress the immune response and maintain immune homeostasis (Malek *et al* 2002). Studies in IL-2 knockout mice have shown that IL-2 works to down-regulate immune responses to prevent autoimmunity; $IL\text{-}2^{-/-}$ and $IL\text{-}2R^{-/-}$ mice show an increase in activated T helper cells and a decrease in naïve T cells, leading to massive activation of B cells and CTL and hyperplasia of lymph nodes and spleen (Schorle *et al* 1991). *In vivo* IL-2 is thought to mediate self tolerance by maintenance of high affinity IL-2 receptors on T regulatory ($CD4^+$ $CD25^+$) cells, which results in the survival of the T regulatory subset of cells and therefore tolerance to self. The absence of T regulatory cells causes increased activation of T helper cells which compete with T regulatory cells for binding of IL-2. This increase in the activation of T helper cells can cause autoimmunity, providing further evidence for the role of IL-2 in the prevention of autoimmunity (Antony *et al* 2006).

(ii) B cells

Activated B cells also have high affinity IL-2R at their surface, although in much smaller amounts than T cells (5 or 10 times lower) (Waldmann *et al* 1984). Resting B cells express receptor subunits IL-2Rα, β and γ. Expression of IL-2Rβ is upregulated

by IL-2 or IL-4, and expression of IL2Rα is upregulated by anti-IgM (Nakanishi *et al* 1992) or IL-5 (Loughnan and Nossal 1989). IL-2 facilitates the growth of B cells that have been prestimulated with an IgM-specific antibody or CD40, and promotes their differentiation into antibody secreting cells (Nakanishi *et al* 1992) (Figure 1.1).

(iii) NK cells

NK cells can be split into two main categories: those expressing high levels of CD56 (known as $CD56^{bright}$) and those expressing low levels of CD56 (known as $CD56^{dim}$). $CD56^{bright}$ NK cells express the high affinity IL-2R composed of the three IL-2R subunits at their cell surface, whereas $CD56^{dim}$ NK cells express the medium affinity IL-2R composed of the IL2Rβ and IL-2Rγ subunits (Section 1.6.5). Both these receptors can bind IL-15 as well as IL-2 (Farag and Caligiri 2006). *In vitro* and *in vivo* stimulation of $CD56^{bright}$ NK cells result in expansion of this NK subset at picomolar concentrations. These cells are immunoregulatory and produce several cytokines in response to monokine stimulation. Cytotoxic $CD56^{dim}$ NK cells proliferate little in response to nanomolar concentrations of IL-2 (Nagler *et al* 1990, Robertson *et al* 1992). IL-2 may also be important in the development of NK in the bone marrow. *In vitro* addition of IL-2 to bone marrow cultures lead to the differentiation of $CD34^{+}$ haematopoietic progenitors into NK cells (Shibuya *et al* 1995). *In vivo* doses of IL-2 result in the expansion of NK cells; however, as IL-2 is produced by activated T cells in the periphery, it is not IL-2 but IL-15 that is the major promoter of NK development in bone marrow (Williams *et al* 1998).

1.6.2 The IL-2 Gene

The IL-2 gene (IL2) is 5040 base pairs in length and exists in a single copy per haploid genome, located on the long arm of chromosome 4 (4q26-q27) (Devos *et al* 1983,

Seigel *et al* 1984). This suggests that any heterogeneity of the human IL-2 molecule is due to post-transcriptional modifications (Fujita *et al* 1983, Seigel *et al* 1984). The IL-2 gene is arranged into 4 exons and 3 introns (Holbrook *et al* 1984) (see Figure 1.5). A TATAAA promoter is present 77 base pairs (bp) upstream from the translation initiation site, and a CAT regulatory region occurs 104bp upstream from the translation initiation site (Figure 1.2).

1.6.3 Expression

IL-2 is an approximately 15kDa glycoprotein which is initially synthesised as a 153 amino acid precursor polypeptide that undergoes post-translational cleavage of a 20 amino acid peptide to produce the mature 133 amino acid protein. Prior to secretion, a carbohydrate moiety is added to the threonine at position 3 by O-linked glycosylation and a disulphide bond is formed between the cystine residues at position 58 and 105 (Robb *et al* 1984, Robb and Greene 1987). The glycosylation of Thr-3 has no effect on the bioactivity of secreted IL-2. The extent and nature of the glycosylation may, however, affect the rate of clearance of the molecule *in vivo* (Robb and Greene 1987). Variations in this glycosylation appear to be responsible for structural heterogeneity seen in IL-2 when separated by immunoaffinity chromatography and chromatofocusing (Robb *et al* 1984).

1.6.4 The Structure of the IL-2 Protein

A theoretical 3D model of the secondary and tertiary structure of IL-2, based on x-ray crystallography (Brandhuber *et al* 1987) and comparison of the IL-2 amino acid sequence with those of related cytokine sequences, suggests that IL-2 contains four core α-helices and two crossover β-strands (Bazan 1992) (Figure 1.6). Mutation and deletion studies have demonstrated that the first 20 N-terminal amino acids and the last

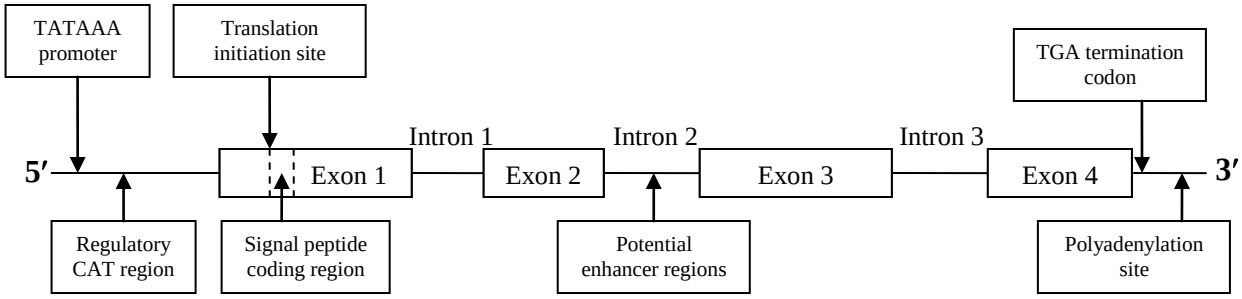

Figure 1.5 Structure of the IL-2 gene.

The IL-2 gene as published by Holbrook *et al* (1984) showing the positions of introns, exons and gene regulatory elements.

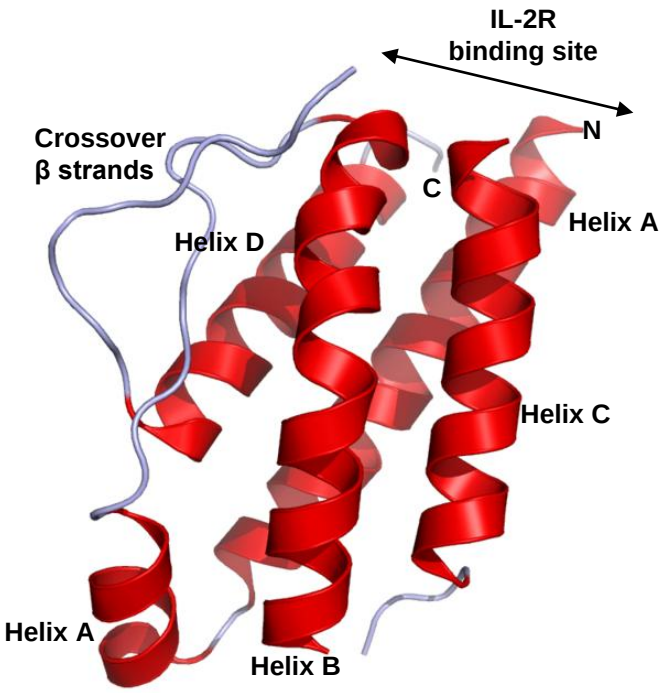

Figure 1.6 Crystalline Structure of Human IL-2.

The crystal structure of human IL-2 as published by Arkin *et al* (2003). Data accessed from the Protein Data Bank (PDB: 1M47), rendered using Pymol and released into the public domain by Ramin Herati (2006). Helix (A-D) represents the four α-helices. The N-terminal and C-terminal are labelled (N) and (C) respectively.

10 C-terminal amino acids of the mature protein are critical for full biological activity, as deletion of either causes a 99% loss of bioactivity and binding. Similarly, antibody blocking of residues 7-8 prevents binding to the IL-2 high-affinity receptor (Kuo and Robb1986), whilst four residues in the first α-helix (Leucine 31, Aspartic acid 34, Leucine 35 and Leucine 38) were found to be crucial for bioactivity and binding (Zurawski and Zurawski 1989). IL-2 is folded with the N-terminal, the C-terminal and the internal residues from 30 to 60 forming a binding site recognised by the IL-2 receptor. Human IL-2 preparations are active on human, rat and mouse IL-2-dependent cells (Ruscetti and Gallo 1981).

1.6.5 Interleukin-2 Receptor

The interleukin-2 receptor is composed of three subunits and exists in multiple functional affinity forms. The high-affinity receptor (K_d 10-80pM) contains one of each of three subunits: α,β and γ. The intermediate form (K_d 500pM to 2nM) of the receptor contains only one each of the β and γ subunits, and the α subunit alone constitutes the low affinity receptor form (K_d 10-20nm) (Waldmann 1991).

(i) IL-2Rα

The α subunit (CD25) is 55kDa and contains 251 amino acids including 11 cysteines, some of which are involved in the formation of disulphide bonds and determining the tertiary structure of the molecule. It is mostly extracellular with a single 19 amino acid hydrophobic transmembrane domain and a 13 amino acid intracellular C-terminal domain. The 163 amino acids contained in the N-terminal extracellular portion of the molecule contain all the necessary information for IL-2 binding. Residues 158 to 160 may be important for interaction with the β and γ subunits. IL-2Rα is not constitutively

expressed, but in some T cells, B cells and NK cells is induced upon activation (Sharon *et al* 1986, Waldmann 1991).

(ii) IL-2Rβ

The β subunit (CD122) is 75kDa and contains 524 amino acids including 8 cysteine residues. It has an intracellular domain of 286 amino acids containing two key signalling elements, Box 1 and Box 2, six tyrosine residues which are potential phosphorylation sites and an extracellular domain of 214 amino acids. The 212 N-terminal amino acids are sufficient for the binding of IL-2 in the absence of any other subunit, but at a much lower affinity (K_d 10-20nm). IL-2Rβ is constitutively expressed at the cell surface of many cell types including resting lymphocytes, monocytes, macrophages and neutrophils and is upregulated upon T cell activation. Its expression in B cells is upregulated under some conditions by IL-2 or IL-4. The β chain also forms part of the IL-15 receptor molecule (Sharon *et al* 1986, Tsudo *et al* 1990).

(iii) IL-2Rγ (γc)

The γ subunit (CD132) is 64kDa and contains 347 amino acids. It has a 255 amino acid N-terminal extracellular component, a 29 amino acid hydrophobic transmembrane region and an 86 amino acid C-terminal intracellular domain which is integral to the signalling of IL-2R. It is constitutively expressed on lymphocytes, monocytes and neutrophils. The γ chain of the IL-2R complex also forms part of the IL-4, IL-7, IL-9 and IL-15 receptor complexes and is sometimes referred to as the common γ chain (γc) (Tsudo *et al* 1987, Waldmann 1991).

1.6.6 Role of IL-2 in Immune Disease and Disorders

The loss of IL-2 secretion or function often leads to auto-immune disorders due to the central role of IL-2 in the regulation of the immune response to self, or self tolerance, by T regulatory cells. Two diseases in which a deficiency of IL-2 alone can be seen to have a profound effect are severe-combined immunodeficiency and systemic lupus erythematosus.

(i) Severe-combined immunodeficiency

A small minority of cases of severe-combined immunodeficiency (SCID) are specifically due to a deficiency in IL-2 gene expression. While most SCID patients have an abnormal number of T and B cells, IL-2 deficient patients have normal numbers of T and B cells, but low serum Ig. Conventional SCID bone marrow transplant treatment was rejected by these patients (Smith 1992). A similar condition to SCID can be reproduced in mice in which the normal IL-2 gene has been replaced with a defective sequence (Schorle *et al* 1991). IL-2 knockout mice have normal levels of T and B cells and also show normal T cell maturation in the thymus, although they show reduced *in vitro* T cell proliferation in response to polyclonal mitogens. T cells from these mice are unable to stimulate B cells to secrete IgM. Whilst IgM serum levels remain normal, IgG and IgE levels are increased, suggesting an effect on B cell differentiation.

In vivo, many immune responses of IL-2 knockout mice appear to proceed normally during the first 4-6 weeks of life. T and B cell responses to some viruses remain intact, while NK cell activity is reduced but inducible and T helper cell responses are delayed but functional (Kundig *et al* 1993). Despite this relatively normal immune response, many (approximately 50%) IL-2 knockout mice die between 4 and 9 weeks and all that survive develop an ulcerative colitis-like disease (Sadlack *et al* 1993), suggesting that

IL-2 may have a role in the aetiology of this disease. Overall, knockout mice and SCID observations suggests that while IL-2 has an important role in the immune response, it is not, or not wholly, responsible for T and B cell development.

(ii) Systemic lupus erythematosus

T cells from human patients or mice with systemic lupus erythematosus (SLE) produce reduced amounts of IL-2 compared to normal, which causes an increased susceptibility to infection and a decrease in activation-induced cell death as well as the survival of autoreactive lymphocytes (Tsokos and Liossis 1999). The central role of IL-2 in the development and function of T regulatory cells may also contribute to this disorder. The decreased secretion of IL-2 may be due to a decrease in the expression of transcriptional factors which leads to a repression of the transcription of IL-2 (Katsiari and Tsokos 2006).

1.6.7 Clinical and Therapeutic Use of IL-2

(i) Treatment of cancers

IL-2 has been used since the 1990s in the treatment of metastatic renal cell carcinoma. High dose IL-2 therapy given by intravenous bolus has resulted in a significant clinical response but with very significant toxicity (Macfarlane *et al* 1995, Smith 1997). IL-2 activates the immune response against tumour cells by inducing the proliferation of activated T cells and CTL (Fehniger *et al* 2002). Administration of high dose IL-2 causes activation of the intermediate affinity IL-2R (IL-2Rβγ) which is expressed on many types of immune cell, causing a secondary proinflammatory release which is likely to be responsible for the toxicity associated with the therapy (Macfarlane *et al* 1995, Fehniger *et al* 2002). Treatment regimes for renal cell carcinomas involving lower doses of IL-2 used alone or in combination lead to lower remission rates, and so

some studies have concentrated on the identification of factors which may predict the responsiveness of tumours to high dose IL-2 therapies (Kammula *et al* 1997).

Concentrations of IL-2 that are of a level able to saturate the high-affinity IL-2 receptor (IL-2Rαβγ) with only limited activation of the lower affinity βγ receptor, by continuous low doses of IL-2 for extended periods of time, are termed low dose IL-2 therapy. This low dose IL-2 therapy allows selective NK ($CD56^{bright}$) cell proliferation without significant toxicity. There is no change in the number of peripheral blood granulocytes, macrophages, T or B cells with this therapy (Fehniger *et al* 2002). Low dose IL-2 therapy causes proliferation of NK cell populations, but these NK cells are not activated and require higher amounts of IL-2 *in vitro* to lyse NK resistant tumour cells (Fehniger *et al* 2002). Several studies have combined low, NK-activating doses with intermediate bolus pulses to provide a greater response rate with lower toxicity (Meropol *et al* 1998, Schriber *et al* 1998, Farag *et al* 2002).

IL-2 has been trialled as a monotherapy, in combination or as an adjuvant treatment following surgery for solid gastric tumours, pancreatic cancer, ovarian cancer, breast cancer, soft tissue and bone sarcomas, hepatocarcinomas, mesotheliomas, hepatic neoplasm, brain tumours, urological neoplasm and head and neck cancers, with several patients responding to the treatment in some cases (reviewed in Grande *et al* 2006).

Suppression of IL-2 expression is a prognostic marker only in some tumours. Accordingly, only some tumours that have been treated with anti-IL-2 therapies show a response. For example, a decrease in IL-2 correlates with non-small cell lung carcinoma (NSCLC) tumour progression and development of metastasis. IL-2 was found to be a negative predictor for response to chemotherapy and survival in this

disease. Several treatment regimes using IL-2 have been tested in clinical trial with varying success, but responses and increased survival have been seen with IL-2 administered interpleurally, used in combination with chemotherapy, and as a post-surgery adjuvant therapy (reviewed in Grande *et al* 2006).

As in NSCLC, IL-2 suppression is a predictor of response and survival in small cell lung carcinoma (SCLC). Treatment with IL-2 has not been effective in many studies. However, treatment with a continuous infusion of IL-2 in a small group of patients who had not responded to chemotherapy showed a complete or partial response in 12% of the patients with a median survival of 12 months (Clamon *et al* 1993).

IL-2 is a useful component of combination immunotherapy for cancer vaccines. IL-2 has been administered to cancer patients in combination with cyclophosphamide, a biological response modifier. This combination therapy can be administered subcutaneously, allowing self-administration and reduced intensity of adverse effects. Responsive tumours include liver metastases, lung metastases, renal cell carcinomas and breast cancer (Mitchell 2003, Spicer *et al* 1992). This therapy can provide a tumour response which is maintained for several years without regression or progression. It has been commented that ‘it can be more valuable to have a sustained lack of progression than a short profound shrinkage of the tumour’ (Mitchell 2003). The method of action of this therapy appears not to be associated with an increase in NK cell activity. Increases in response may be because the doses of IL-2 used in this therapy may be sufficient to stimulate proliferation of T cells (Mitchell 2003).

(ii) Other uses for IL-2 therapy

IL-2 production is reduced in Human Immunodeficiency Virus (HIV) patients, leading to progressive immunodeficiency characterized by reduced numbers of $CD4^+$ cells. Treatment with IL-2 leads to expansion of the $CD4^+$ T cell population and is currently in clinical use (Natarajan *et al* 2002). As well as its application for treatment of many cancers, IL-2 is also undergoing clinical trial for use in the treatment of non-Hodgkins lymphoma, and clinical trials for use in hepatitis B infection and Epstein-Barr virus (Gaffen and Liu 2004).

(iii) IL-2 Toxicity

IL-2 has shown significant systemic toxicity in early clinical trials with symptoms including hypotension, nausea, vomiting, diarrhoea, confusion, shortness of breath, pulmonary oedema, abnormal liver function, renal failure, pancytopenia, rash, fever, chills, malaise and infection. Reduced doses of IL-2 as well as prophylactic therapy for prevention of infection have resulted in reduced toxicity with no change in response to therapy (Kammula *et al* 1997).

1.6.8 Recombinant IL-2 Fusion Proteins

Several antibody-interleukin-2 fusion proteins have been produced to target IL-2 particularly for the treatment of cancer, wherein IL-2 is targeted to the tumour site and stimulates an immune response to lyse the tumour cells (Xu *et al* 2000, Cruz *et al* 2002, Gillies *et al* 2002, Zhang *et al* 2004). The use of antibody-cytokine fusion proteins in cancer was reviewed by Dela Cruz *et al* (2004). A search of Pubmed (*http://www.ncbi.nlm.nih.gov/sites/entrez*) was performed using the search term "antibody IL-2 fusion protein". Relevant studies of antibody IL-2 fusion proteins of interest in cancer therapy are summarised in Table 1.1.

1.7 Interleukin-18

Interleukin-18 (IL-18) is produced predominantly in the liver, but is also expressed in many, other human tissues (Okamura *et al* 1995a). IL-18 is an important regulator of the immune system, and has roles in both innate and acquired immunity. Biological activities of IL-18 include the enhancement of NK cell cytotoxicity (Ushio *et al* 1996) and proliferation of naïve T cells (Kohno *et al* 1997).

IL-18 was originally identified as interferon gamma-inducing factor (IGIF) (Okamura *et al* 1982), due to its ability to induce interferon gamma (IFN-γ) production by activated NK cells. This phenomenon was first observed in mice, following endotoxic shock. Subsequently it was purified from Kuppfer cells of mouse liver origin primed with *Propionibacterium acnes* and challenged with lipopolysaccharide (LPS) (Okamura *et al* 1995a). This treatment resulted in high serum IFN- γ levels (Okamura *et al* 1995b). Originally described as a member of the IL-1 family because of a high structural similarity with IL-1, it is now accepted as an independent member of the interleukin family (Bazan *et al* 1996).

1.7.1 The Function of IL-18 in Immune Regulation

IL-18 is a proinflammatory cytokine that plays a central role in systemic and local inflammation and in the development of the adaptive immune response. It is particularly important for the development of TH1-type immune responses because it promotes expression of TH1 cytokines including IFN-γ, IL-2, TNF-α and GM-CSF (Micallef *et al* 1996). IL-18 was first described in activated macrophages and Kupffer cells (Okamura *et al* 1995a). It has since been described in other cells types including dendritic cells, Langerhans cells, myelomonocytic cells and B cell lines (as well as non-

Table 1.1 Antibody-IL-2 fusion proteins and their targets.

NAME	TARGET OF ANTIBODY	REFERENCE
IL2-B7.1	T cells (CD28)	Kong *et al* 2007
mSCH-IL-2	Murine Ep-CAM	Schanzer *et al* 2006
IL-2-183B2scFv	Ovarian carcinoma TAA	Zhang *et al* 2006
anti-CD3/IL-2	CD3	Lee *et al* 2006
sFv/IL-2	HER2/neu	Shen *et al* 2006
anti-HER2/neu IgG3-(IL-2)	HER2/neu	Helguera *et al* 2006
MK2-23-IL-2	HMW-MAA	Wang *et al* 2005
anti-Her-2/neu-IL-2	HER2/neu	Lustgarten 2003
FUscFv/IL-2	MK-1	Matsumoto *et al* 2002
IL-2-IgG2b	CD8	Budgian *et al* 2002
L19-IL-2	ED-B	Carnemolla *et al* 2002
IgG3-(IL-2)	HER2/neu	Penichet *et al* 2001
IL-2-FuP (several IL-2-antibody fusion proteins)	hCD4, hCD8, human monocytes, human NK cells, HLA class I, HLA-A2.1, human DR/DQ, mouse monocytes	Christ *et al* 2001
scFvFc.IL-2	CEA	Xu *et al* 2000
L6 sFV-hIL	Human carcinoma antibody L6	Zhao *et al* 1999
C215Fab-IL-2	*Unspecified carcinoma cell target*	Sogaard *et al* 1999
IL-2/MOV19 scFv	Alpha-folate receptor	Melani *et al* 1998
chS5A8-IL-2 scFvS5A8-IL-2	Murine B cell lymphoma	Liu *et al* 1998
IgG3-IL-2	Murine B cell lymphoma	Penichet *et al* 1998
SCIg-IL-2	Tumour-associated glycoprotein 72	Shu *et al* 1995
IL2 immunocytokine	Mouse transferring receptor	Dreier *et al* 1998
huKS1/4-IL-2	Colon cancer metastases	Xiang *et al* 1998
chCLL-1/IL-2	B cell malignancies	Hornick *et al* 1997
ch225-IL2	EGF receptor	Becker *et al* 1996
ch14.18-IL-2	GD2	Gillies *et al* 1992

haematopoietic cells such as keratinocytes, astrocytes, microglial cells, osteoblasts, melanocytes, endothelial cells, fibroblasts, cardiomyocytes and many epithelial cells) (Okamura *et al* 1995a, Conti *et al* 1997, Stoll *et al* 1997).

IL-18 in the presence of IL-12 modulates the activity of many cell types. IL-18 and IL-12 are endogenously released through the activation of TH1 cells by spleen cell APCs (Kohno *et al* 1997). *In vitro* studies with PBMC showed that IL-18 was pivotal in the induction of IL-2, GM-CSF and IFN-γ expression and production. However, IL-18 alone was not enough to induce significant amounts of IFN-γ. A combination of IL-18 and IL-12 were seen to work synergistically to induce IFN-γ production by naïve T cells and NK cells and induce proliferation of naïve T cells (Okamura *et al* 1995a, Micallef *et al* 1996, Ahn *et al* 1997). IL-12 is secreted by APCs, and is important in the induction of macrophages to produce IFN-γ during intracellular infection, generation of TH1 cells and CTL, suppression of IgG1 and IgE production, and resistance to bacterial and parasitic infections (Trinchieri 1995).

It has been shown *in vitro* that recombinant IL-18 increases not only the production of IFN-γ, but also GM-CSF, Tumour Necrosis Factor (TNF)-alpha and IL-2 production. These effects are associated with a TH1-type immune response, and in fact IL-18 has a crucial role in the development of the TH1-type immune response (Micallef *et al* 1996). In a study by Kohno *et al* (1997), recombinant IL-18 alone had no effect on the activation of TH1 cells; however, it increased the IFN-γ production by antigen-stimulated T cell lines and acted synergistically with IL-12 to augment further IFN-γ differentiation of naïve T cells into TH1 cells and to induce the expression of the IL-2Rα subunit expression on TH1 cells. Even in the presence of saturating amounts of IL-12, IL-18 still increased the production of IFN-γ, demonstrating that the IL-18 receptor

and signal transduction pathways are distinct from those of IL-12. In the same study IL-18 induced IL-2 production by activated TH1 clones, independently of IL-12. Stimulation by IL-18 also enhanced the production of IL-2 and GM-CSF in T cell cultures, whereas IL-12 did not. However, IL-18 had no effect on the production of IL-4 and IL-10, the differentiation of naïve T cells into TH2 cells or the proliferation of TH2 cells (Kohno *et al* 1997).

Naïve T cells and B cells do not express IL-18R. IL-12 induces the production and expression of IL-18R. Pre-treatment with IL-12 renders them responsive to IL-18, inducing cell proliferation and IFN-γ production (Yoshimoto *et al* 1998). Likewise IL-18 sustains the expression of the IL-12R complex in TH1 cells. The synergistic effects of IL-18 and IL-12 may therefore be due in some part to the reciprocal upregulation of their respective receptor complexes. Anti-IL-18R antibody inhibits IL-18-induced IFN-γ production, shifting the immune response from a TH1- to a TH2-type response (decreased proinflammatory cytokines, increased IL-4 and IL-5 – TH2 cytokines) and decreasing local inflammation (Xu *et al* 1998).

IL-18 has an important role in host defence against bacterial infection. Infection by bacteria causes a primary proinflammatory production of IL-18 and IL-12, followed by INF-γ, TNF-α and FasL production. This invokes a TH1-type response in the host. Exposure to IL-18 enhances host defence against bacterial infection; however, an excess of IL-18 may be detrimental, by inducing injury in the host such as septicaemia or liver damage (Okamura *et al* 1998, Dinarello and Fantuzzi 2003).

As well as inducing proinflammatory and TH1 cytokine production, IL-18 has many other biological functions including the induction of nitric oxide, allowing macrophages

to kill target cells, roles in homeostasis, CNS local immune responses, roles in the skeletal system including catabolism of articular cartilage, suppression of osteoclastic bone-reabsorption and induction of TH1 cytokine production by synovial fibroblasts and leukocyte recruitment (Vidal-Vanaclocha *et al* 2006). IL-18 levels are high in patients with chronic inflammatory diseases such as insulin-dependent diabetes, mellitus and Crohn's disease, and advanced infectious disease where it promotes the development of and sustains inflammation (Dinarello 2007).

1.7.2 The Interleukin-18 Receptor

The interleukin-18 receptor belongs to the toll-like receptor/IL-1R super-family. It is expressed widely, occurring on a variety of cells including macrophages, neutrophils, NK cells, endothelial and smooth muscle cells (Hyodo *et al* 1999, Leung *et al* 2001, Afkarian *et al* 2002 and Gerdes *et al* 2002). It is composed of two subunits; IL-18Rα, which binds IL-18 with low affinity ($K_d \approx$ 20-50nM), and IL-18Rβ, which does not bind IL-18. Together the two subunits form a high affinity ($K_d \approx$ 0.3nM) receptor which binds IL-18 and signals via pathways shared with the IL-1R. Binding of IL-18 to IL-18Rα recruits IL-18Rβ. Signal transduction is via the toll-like intracellular domain, and the downstream signalling pathway is shared by other members of IL-1R super-family. Expression of IL-18R is upregulated by IL-12 and IL-2 and inhibited by IL-4 (Torigoe *et al* 1997, Yoshimoto *et al* 1998).

1.7.3 Interleukin-18 Binding Protein

IL-18 is regulated by IL-18 binding protein (IL-18BP), which is a member of the immunoglobulin super-family (Novick *et al* 1999). IL-18BP is a soluble, secreted, constitutively expressed molecule that binds IL-18 with a high specificity and affinity for IL-18 but with no affinity for proIL-18. It is mainly expressed by the spleen but is

also found in thymus and resting PBMC (Novick *et al* 1999). Binding of IL-18 with IL-18BP neutralizes IL-18 by preventing its interaction with IL-18Rα (Novick *et al* 1999 and 2001), which leads to a reduction in IFN-γ production and hence a decrease in the TH1 immune response.

In humans, four isoforms of IL-18BP exist, formed by alternate mRNA splicing; of these, two bind IL-18 (IL-18BPa and IL-18BPc, while two do not (IL-18BPb and IL-18BPd). As yet it is unclear what the function of these two isoforms is, but they may bind another cytokine, possibly a member of the structurally similar IL-1 family (Kim *et al* 2000a). The level of IL-18BP in the circulation is dependent on renal function. Impaired renal function correlates with an increase in IL-18BP concentration, which results in a reduction in the activity of IL-18 (Lonnemann *et al* 2003). IL-18BP levels are raised in correlation with IL-18 levels in many infectious and inflammatory diseases (Möller *et al* 2003). IL-18BP expression is induced by IFN-γ in a feedback mechanism which controls IL-18 activity. In a similar way, TNF-α has been shown to induce IL-18BP expression leading to a feedback mechanism. Some poxviruses secrete proteins which are structurally similar to IL-18BP, thereby interfering with the IL-18-mediated immune response and therefore avoiding the CTL action on the viruses (Novick *et al* 1999).

1.7.4 The IL-18 Gene

IL-18 was initially extracted from mice livers treated with *Propionibacterium acnes* and then challenged with LPS to induce toxic shock (Okamura *et al* 1995b). Digestion of the purified protein with trypsin produced a number of peptide fragments which were then sequenced. Using degenerate primers based on the amino acid sequence of these fragments, partial cDNA fragments were generated by RT-PCR from mRNA extracted

from the mice livers. A full-length (0.9kb) clone was obtained from a cDNA library prepared from *P. acnes*-primed LPS-challenged mice livers which predicted a previously unknown 192 amino acid precursor polypeptide, including a 35 amino acid leader sequence similar to that of the IL-1β precursor.

Subsequently human IL-18 was cloned and expressed (Ushio *et al* 1996). The human IL-18 gene encodes a 193 amino acid precursor protein which shares 65% homology with the murine IL-18 precursor protein, including an IL-1-like signature sequence which is conserved between the mouse, rat, porcine and human IL-18 amino acid sequences (Okamura *et al* 1995b, Ushio *et al* 1996, Gillespie and Horwood 1998).

1.7.5 Expression

The human mRNA encoding IL-18 is 1.1kb long (Ushio *et al* 1996) and is abundant in pancreas, kidney, liver, lung, osteoblasts, keratinocytes and skeletal muscle tissues. It is also found in unstimulated peripheral blood mononuclear cells (PBMC). Since IL-18 was originally cloned from liver Kuppfer cells in association with a pathogen response, it was presumed that these cells were the source of IL-18. Subsequently Conti *et al* (1997) showed that IL-18 is produced in the adrenal gland after cold stress or treatment with reserpine, a sedative drug which resembles the biochemistry of stress (Conti *et al* 1997). This suggests an additional role for IL-18 as a neuromodulator. The same group went on to show IL-18 mRNA localisation in the zona reticularis and fasciculata of the adrenal cortex. However, because of the need for correct processing, evidence of IL-18 expression does not presuppose biological activity of IL-18 in these tissues.

1.7.6 The Structure of the IL-18 Protein

On purification, mature murine IL-18 was discovered to be an 18.3kDa, 157 amino acid cytokine (Okamura *et al* 1995a). In humans the encoded protein is produced as a 22.3kDa precursor polypeptide that is 192 amino acids in length, of which a leader sequence of 35 amino acids is cleaved to form the mature protein (Figure 1.7). The human IL-18 precursor protein is cleaved after Asp36 by IL-1β converting enzyme (ICE), also known as caspase-1, at a processing site that is similar to that of IL-1β. This cleavage activates IL-18 and facilitates its export from the cell (Gayer *et al* 1997, Gu *et al* 1997). In a study by Li *et al* (1997), Kuppfer cells from LPS-challenged ICE-deficient mice were able to synthesise the IL-18 precursor but could not process it into the biologically active form. As expected, the serum of these mice showed no IL-18 activity and decreased IFN-γ activity. In addition co-expression of pro-IL-18 with a biologically inactive form of ICE failed to produce any IL-18 activity. Mature IL-18 is cleaved and inactivated by caspase-3 (CPP32) (Akita *et al* 1997).

Recombinant IL-18 expressed as a mature protein lacking the propeptide leader sequence has low biological activity due to aberrant folding (Liu *et al* 2000). Correct folding of human IL-18 takes place at the pro-IL-18 level. Liu *et al* (2000) produced an active IL-18 molecule in *E. coli* that was structurally stable by first expressing the precursor of IL-18 and then converting it to the mature peptide. The ICE cleavage site was replaced with a factor Xa recognition sequence, and the precursor IL-18 was cleaved by Xa rather than by ICE. The mature 157 amino acid IL-18 molecule is biologically active as a monomer, and does not contain any disulphide bonds (Liu *et al* 2000). IL-18 shares 12% sequence homology with IL-1α and IL-1β, which led to IL-18 originally being described as a member of the IL-1 family, IL-1γ (Bazan *et al* 1996). IL-18 and IL-1β both have a β-sheet structure, but IL-18 does not share significant

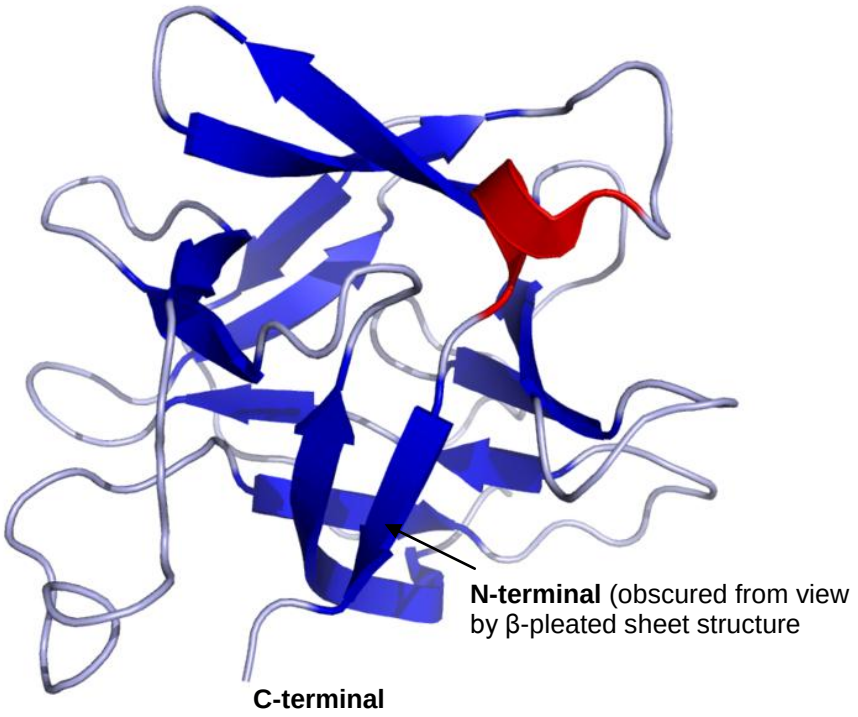

Figure 1.7 Structure of human IL-18 in solution.

The structure of human interleukin-18 in solution as published by Kato *et al* (2003). Data accessed from the Protein Data Bank (PDB: 1J0S), rendered using Pymol and released into the public domain by Ramin Herati (2006).

homology with the regions of IL-1β which interact with the IL-1 receptor (IL-1R).

1.7.7 Clinical and Therapeutic Use of IL-18

(i) The anti-tumour properties of IL-18

The role of IL-18 in the progression and metastasis of tumours and its reported antineoplastic properties, are controversial. IL-18 is produced by many cancers, and elevated IL-18 levels have been reported in oesophageal, gastric, pancreatic, colon, head and neck, lung, breast, ovarian, bladder, myeloma, renal and prostate cancers. In these tumours its presence is an indication of poor prognosis and increased risk of metastasis (except in bladder cancer where an increase in IL-18 expression correlates with a reduced risk of tumour recurrence) (reviewed by Vidal-Vanaclocha *et al* 2006). IL-18 is an important marker of breast cancer; high IL-18 levels have been detected in metastatic breast cancer patients compared to healthy volunteers and patients with non-metastatic tumours (Merendino *et al* 2001, Elissa *et al* 2005).

Several studies have used enzyme linked immunosorbent assays investigate serum levels of IL-18 in patients suffering with malignant tumours and the relationship between these levels, clinical prognosis and survival. The first study to link cancer with an increase in serum IL-18 levels reported on a number of tumour types and found an overall increase in IL-18 levels in patients with metastases compared to healthy controls and cancer patients with non-metastatic tumours (Lissoni *et al* 2000). Several other studies back up these findings, having found that a decrease in the level of IL-18 levels correlates with improved survival (Vidal-Vanaclocha *et al* 2006).

Cancer cells can escape immune recognition, increase vascular wall adhesion and induce production of angiogenic/growth factors via IL-18 dependant pathways. IL-18 downregulates the expression of Fas ligand, thereby promoting tumour escape, and

directly promotes tumour proliferation and angiogenesis. It also stimulates the generation of reactive oxygen intermediates which promote tumour cell migration (reviewed by Park *et al* 2007).

Conversely, however, artificially or constitutively increased IL-18 levels have been shown to enhance antitumour response, decrease tumour angiogenesis and increase survival (Vidal-Vanaclocha *et al* 2006). The ability of IL-18 (in the presence of IL-12) to stimulate NK cell activity, induce IFN-γ and GM-CSF production by T cells, stimulate T cell proliferation and induce TH1 cell responses (Dinarello 1990) is essential to the immune response against cancer (reviewed by Park *et al* 2007). Administration of IL-18 to cancer patients has been reported as having antineoplastic effects due to its immune-stimulating effects in a number of studies (Jonak *et al* 2002, Yamashita *et al* 2002, Okamoto *et al* 2004).

(ii) IL-18 as an immunoadjuvant

IL-18, either independently or in combination with IL-12, can be used as an adjuvant for reducing tumour growth and metastasis in models of immunogenic tumour (Coughlin *et al* 1998). In primates, there are problems with systemic toxicity when recombinant human IL-18 is used for *in vivo* therapy at high doses of 75mg/kg/day. However, repeat intravenous administrations at lower doses (below 10mg/kg/day) were well tolerated (Golab and Stoklosa 2005). Another problem related to using IL-18 as an adjuvant is overcoming neutralization by constitutively expressed and circulating IL-18 binding proteins which keep the TH1-type immune response low after infections and aim to avoid autoimmune diseases. Unfortunately this may also result in a depressed TH1 response to tumours (Kim *et al* 2000b).

(iii) IL-18 toxicity

Herzyk *et al* (2003) investigated the toxicity of recombinant human IL-18 in primates. Repeated high doses (75mg/kg/day) of recombinant human IL-18 by intravenous administration led to renal toxicity and dysfunction. A similar effect was observed in mice treated with high doses (30mg/kg/day) of recombinant murine IL-18. Lower doses (below 30mg/kg/day) of rhIL-18 in primates do not indicate any serious adverse effects (Herzyk *et al* 2002 and 2003). Herzyk *et al* concluded that recombinant human IL-18 was well tolerated at pharmacologically active doses in monkeys and mice. The intended clinical dose of IL-18 for cancer immunotherapy in humans is 1mg/kg/day.

1.8 Streptavidin

Streptavidin is a bacterial protein that was originally isolated from the culture medium of *Streptomyces avidinii* by Chaiet and Wolf (1964). It takes its name from this organism and from its similarity to avidin, a hen egg white protein, which shares much structural and functional similarity with streptavidin (Chaiet and Wolf 1964). Like avidin, streptavidin has the ability to bind up to four molecules of biotin with high affinity. Streptavidin has been well characterized because of its exceptional biotin binding ability, which makes it useful as an affinity tag for a number of biological applications (reviewed by Sano *et al* 1997, Laitinen *et al* 2006 and 2007). The *in vivo* function of streptavidin is still unknown.

1.8.1 Streptavidin Protein Structure and Biotin Binding

Mature streptavidin forms tetramers of four identical monomeric subunits with a molecular weight of 15kDa each (Green 1975). Each subunit binds one molecule of biotin with extremely high affinity for a non-covalent interaction, with a K_d of approximately 1×10^{-14}. There is a high dissociation constant for streptavidin-biotin

binding which is stable over a wide range of temperatures and pH (Chait *et al* 1963, Chait and Wolf 1964).

Streptavidin subunits are organised as eight-stranded, sequentially connected, antiparallel β sheets (Figure 1.8). Staggered hydrogen bonds between the β sheets produce a cyclically hydrogen-bonded barrel core, with several hairpin loops that extend above the surface of the subunit core. The streptavidin barrels bind together via hydrogen bonds between their free loops, producing symmetrical dimers. Inter-linking of two streptavidin dimers along their complimentary curved faces produces the naturally occurring streptavidin tetramer, which is stabilised by extensive van der Waals interaction between the subunit core barrels (Chait and Wolf 1964, Weber *et al* 1989).

Biotin binds in pockets at the ends of the streptavidin β barrels. Hydrogen bond interactions between polar and aromatic amino acid residues lining these pockets form between the streptavidin and the biotin, causing the β sheet structure of the subunit to twist slightly, ordering the free hairpin loops at the surface and effectively burying the biotin within the streptavidin binding pocket. As the streptavidin dimer is connected via the β barrel core, the slight twist caused by the binding of biotin to the subunit is repeated in the β core of the corresponding dimer subunit. A change in the structure of the streptavidin tetramer then occurs to accommodate the change in dimer structure, preserving the symmetry of the protein, which is important for preservation of biotin binding ability. No evidence has been published indicating that biotin binding by one subunit induces further biotin binding in the tetramer; however, the structural changes to the tetramer which occur as a consequence of the initial biotin binding suggest a mechanism of subunit communication (Weber *et al* 1989). It has been observed that single streptavidin units have a much lower affinity for binding biotin than tetrameric

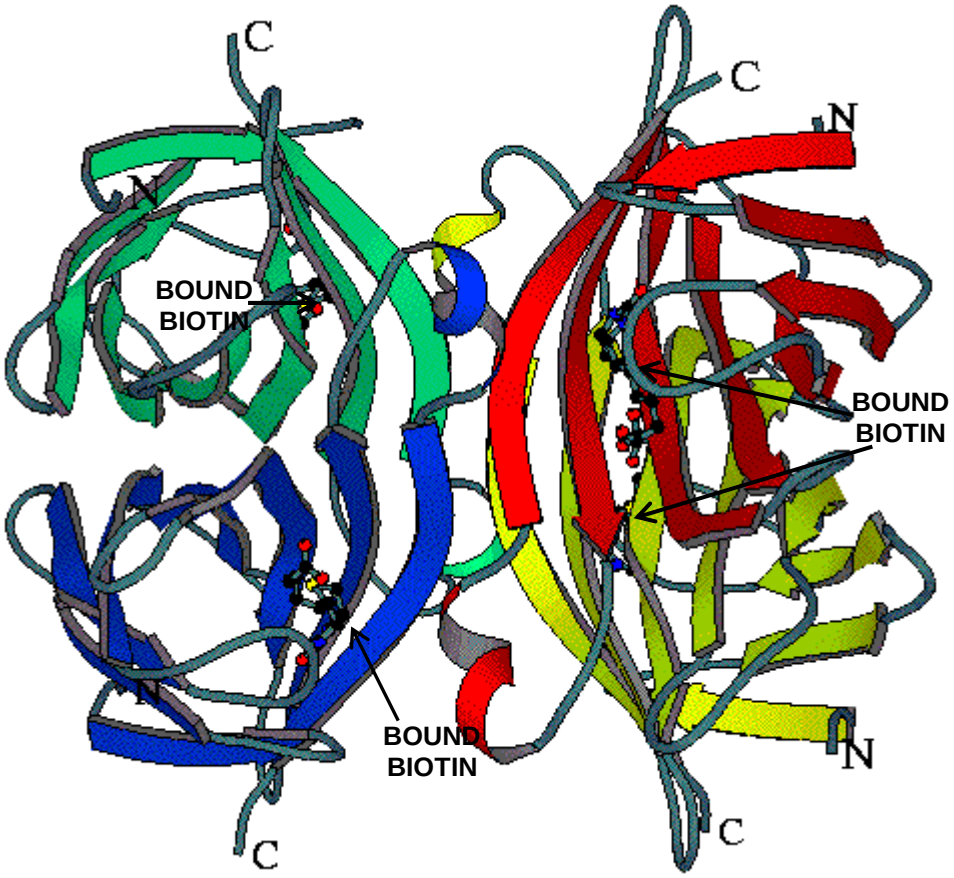

Figure 1.8 The streptavidin tetramer, showing four molecules of bound biotin. The structure of the streptavidin tetramer, including the β barrel structure of each monomer; coloured arrows represent the β sheets, with each monomer shown in a different colour. Also shown are four molecules of bound biotin. Modified from a diagram by Laitinen *et al* (2006).

streptavidin and that the dissociation constant for tetrameric streptavidin-biotin binding is higher and stable over a wide range of temperatures and pH. Both of these observations can be explained by the structural changes that occur to the whole tetramer upon binding biotin (Pahler *et al* 1987).

1.8.2 Streptavidin Protein Expression

The nucleotide sequence encoding full-length streptavidin and the corresponding amino acid sequence has been described (Argarana *et al* 1986). Post-translational processing removes 24 amino acid residues from the N-terminal of the 183 amino acid immature protein to yield the mature protein. This 24 amino acid sequence shares common characteristics with those signal peptides present in the genes of most transmembrane and secreted proteins. After amino-terminal processing, the mature protein contains 159 amino acids (Argarana *et al* 1986).

1.8.3 Core Streptavidin

In addition to the beta-barrel structure at its core, streptavidin has disordered flexible terminal regions that are highly susceptible to post-secretory proteinases. As a result the mature 159 amino acid mature streptavidin rarely survives the conditions generally used for the culture of *Streptomyces avidinii* (Weber *et al* 1989). This means that the streptavidin isolated from *S. avidinii* culture medium has often undergone post-secretory cleavage of both N- and C-terminal regions. Streptavidin that is naturally truncated in this manner is known as natural core streptavidin. Although natural core streptavidin has been reduced in size from the mature form of translated streptavidin, it shows enhanced biotin-binding activity, and is more soluble than full-length streptavidin as well as being less likely to form aggregates. Aggregates are suggested to form due to intermolecular cross-linking of terminal regions (Bayer *et al* 1989). The biological

reason why *S. avidinii* produces streptavidin with poor solubility and high aggregation is unknown.

Natural core streptavidin commonly comprises amino acids 13 – 139 of the mature streptavidin protein sequence, although a heterogeneous mix with cleavage at amino acids 14 and 138 is also naturally observed. Even a single tetrameric streptavidin molecule may have monomers of different lengths (Sano *et al* 1995). Interestingly, the cleavage sites used to produce core streptavidin from mature streptavidin are closely aligned with the beginning and end of the avidin molecule, suggesting strong evolutionary conservation. Both cleavage sites have the same amino acid sequence Serine Alanine Alanine, which suggests that both cuts may be made by the same enzyme (Pahler *et al* 1987).

Due to the heterogeneity found in preparations of core streptavidin isolated from the culture medium of *S. avidinii*, many commercial sources provide streptavidin that has been treated with proteinases to further truncate the terminal sequences. This maximises the homogeneity of the streptavidin preparations (Sano *et al* 1995).

Sano *et al* (1995) designed two recombinant core streptavidins, Stv-25 (amino acids 14-138) and Stv-13 (amino acids 16-133), produced by genetic modification. Stv-25 has an amino acid sequence that is very similar to that of natural core streptavidin, whereas Stv-13 is further truncated and consists of only the beta-barrel section of the protein. A schematic illustration of Stv-25 and Stv-13 compared to mature, full length and natural core streptavidin is shown in Figure 1.9. All of these core streptavidins show similar solubility, but Stv-13 was shown to be most stable and expressed more efficiently than either Stv-25 or natural core streptavidin. The biotin binding ability of core

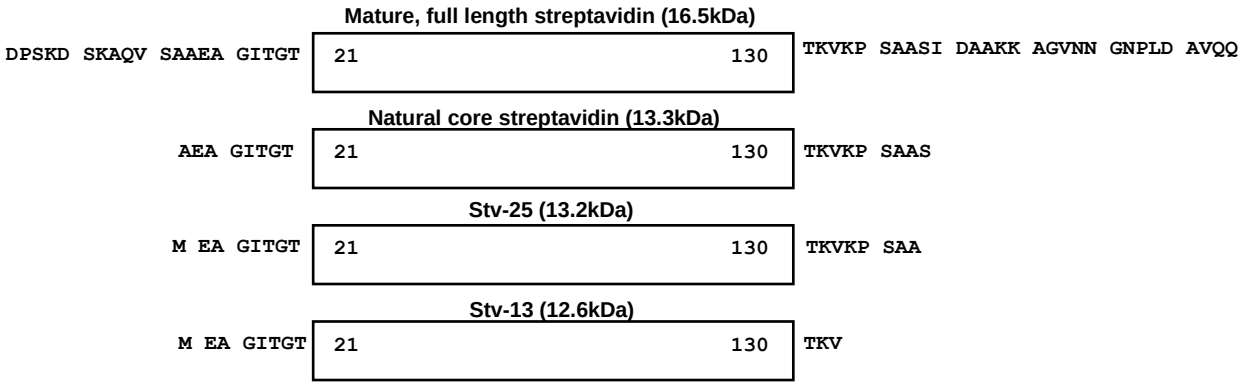

Figure 1.9 Schematic illustration of the primary amino acid sequence of recombinant streptavidin molecules Stv-25 and Stv-13 compared to native and naturally occurring truncated streptavidin.

streptavidins is not compromised by this further truncation; in fact, full length and partially truncated streptavidins have a lower affinity for biotinylated macromolecules due to steric hindrance caused by the terminal regions. The disordered terminal regions of the full-length streptavidin molecule hinder the folding of the biotin binding site, as well as obstructing the initial entry of biotinylated macromolecules approaching the site. Other recombinant core streptavidins have also been investigated, including Stv-21 (amino acids 16-133), which has successfully been incorporated into recombinant fusion proteins (Sano *et al* 1995).

1.8.4 Applications of the Streptavidin-Biotin Binding System

The streptavidin-biotin binding system has been exploited by the biotechnology industry as a versatile and commonly used tool, primarily in bioanalytical applications such as ELISA, DNA probes and immunolabelling. Most of these tools rely on the streptavidin-biotin binding system as a detection tool. It is also used *in vivo* to target drugs and radionucleotides, e.g. to cancer tumour sites for drug delivery and imaging (e.g Wang *et al* 2007).

It has been proposed that streptavidin provokes an immune response in patients. The immunogenic properties of streptavidin, as reported by Dr A. Maraveyas, Department of Oncology, University of Hull (personal communication), has the potential to be beneficial in cancer treatments since it is hypothesised that the non-specific immune response provoked by streptavidin can be directed towards a TH1-type immune response by fusion with TH1 cytokines.

1.8.5 Recombinant Streptavidin Fusion Proteins

A number of recombinant fusion proteins incorporating streptavidin have been successfully generated using a variety of approaches. To facilitate the production of N-terminal streptavidin-containing fusion proteins, two expression vectors, pTSA-18F and pTSA-19F, containing the DNA sequence encoding core streptavidin, and several unique cloning sites which facilitate construction of gene fusions of core streptavidin with a target protein, were produced for use in a T7 *E. coli* expression system (Sano and Cantor 1991). Mouse metallothionein I cDNA was inserted into the pTSA-18F expression vector to produce an expression vector, pTSAMT-2, encoding N-terminal streptavidin and C-terminal metallothionein with a 10 amino acid linker region between the two moieties (Sano *et al* 1992). The streptavidin-metallothionein fusion protein was expressed using the T7 expression system. The streptavidin-metallothionein chimeric protein formed tetramers containing four identical subunits which retain their biotin binding function, allowing the molecule to bind four biotin molecules.

Streptavidin fused to single chain antibodies (Dubel *et al* 1995, Koo *et al* 1998, Schultz *et al* 2000) has been produced for use in a variety of applications including targeted cancer therapy, detection of antigens in ELISA and Western blots and removal of bacterial contaminants in foodstuffs, using the biotin binding function of streptavidin. These fusion proteins all contain N-terminal single chain antibodies and C-terminal streptavidin.

Other fusion proteins containing streptavidin include streptavidin-β-galactosidase (which contains N-terminal streptavidin, modified from the full length streptavidin sequence contained in plasmid pUC8-SZ (Walsh and Swasigood 1994)), streptavidin-lipase (Lee and Swasigood 1998), trypsin-streptavidin (which contains C-terminal streptavidin (Clare *et al* 2001)), streptavidin-luciferase (Nakamura *et al* 2004) (which

contains modified N-terminal streptavidin) and streptavidin-protein A (a cell wall constituent of *Staphylococcus aureus* containing N-terminal streptavidin) (Sano and Cantor 1991).

1.9 Aims

It is hypothesised that the application of fusion proteins containing the immunogenic molecule streptavidin fused to a TH1 cytokine, either IL-2 or IL-18, to a patient with either an immune deficiency or compromised immune system (such as that seen in cancer patients), or with malignancies unresponsive to immunotherapy, would provoke a TH1 directed immune response. This non specific TH1 response may be beneficial in addition to enhance a specific immune response against an antigen such as that provoked by a cancer vaccine containing a TAA, which often provokes only weak immune responses in cancer patients. The argument for the use of these novel immunoadjuvants for the treatment of solid tumours such as breast cancer is compelling.

The specific aims of this thesis are:

- to design streptavidin-IL-2 and streptavidin-IL-18 fusion constructs;
- to produce suitable expression vectors for the expression of the recombinant fusion proteins;
- to optimise expression of the fusion proteins in *E. coli*;
- to analyse the structure and function of the fusion proteins;
- to investigate the potential therapeutic application of the fusion proteins.

Chapter 2

Materials and Methods

2.1 Suppliers

All general chemicals were supplied by Sigma and all cell culture reagents were supplied by Invitrogen, unless otherwise stated.

Amersham Pharmacia, Bucks, UK

Bio-Rad, Hercules, California, USA

Biosource, Nivelles, Belgium

Dako, Glostrup, Denmark

ECACC, Porton Down, Salisbury, UK

Fuji, London, UK

Ilford Imaging UK Limited, Cheshire, UK

Invitrogen, Paisley, UK

Kodak, Hemel Hempstead, UK

Millipore, Watford, Hertfordshire, UK

MWG Biotechnology, Ebersberg, Germany

Nalgene, Hereford, UK

New England Biolabs, Hertfordshire, UK

Novagen, Darmstadt, Germany

Pierce Biotechnology, Cramlington, Northumberland, UK

Qiagen, Crawley, West Sussex, UK

Sigma, Sigma-Aldrich Company Ltd, Poole, Dorset, UK

Statagene, Amsterdam, the Netherlands

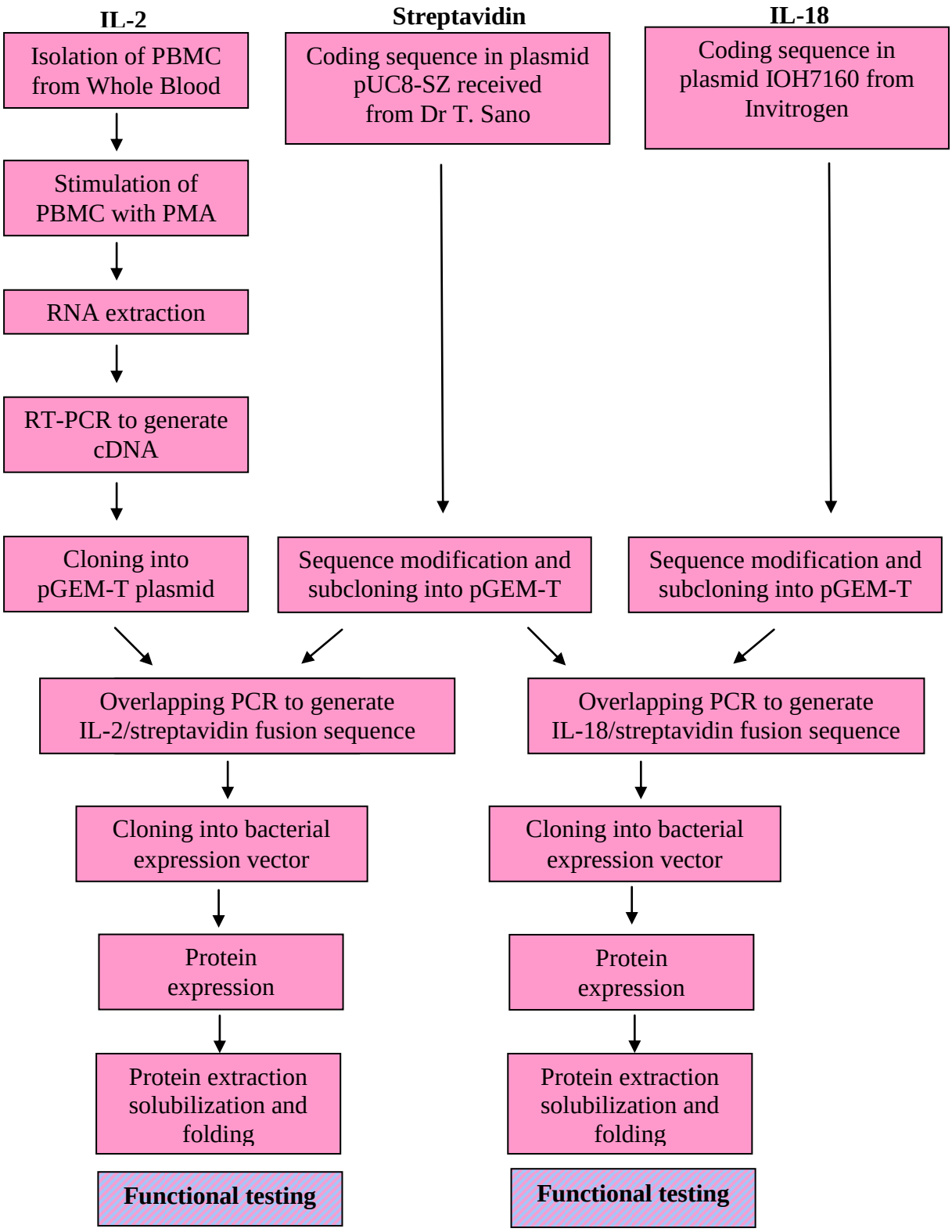

Figure 2.1 A schematic flow diagram showing the stages involved in the production of recombinant streptavidin-cytokine fusion proteins.

2.2 Isolation of Peripheral Blood Mononuclear Cells from Whole Blood

50ml of whole blood was taken from a healthy volunteer after obtaining informed consent (Ethics approval was obtained from Hull and East Riding Local Research Ethics Committee (April 2003) – LREC/04/03/079). Under sterile conditions and using aseptic technique, the blood was mixed with an equal volume of sterile phosphate buffered saline (PBS), and 20ml of blood/PBS mixture was layered on top of 20ml of Histopaque® (Invitrogen) by slowly pipetting the mixture down the side of a 50ml polypropylene tube. The tubes were centrifuged at 400g and 4ºC for 30 min. After centrifugation, three discrete layers were visible in the tube, the bottom red layer containing red blood cells, the middle clear layer containing PBMC and the top yellow layer containing plasma. The clear middle layer and white blood cells between this layer and the top yellow layer were carefully extracted by pipette, mixed with an equal volume of PBS and centrifuged at 400g for 10 min at 4ºC. The supernatant from each tube was removed and discarded and the remaining cells were resuspended in 10ml PBS in a 50ml falcon tube. The cell suspension was then centrifuged for 10 min at 400g and 4ºC. Following centrifugation the supernatant was removed and discarded and the cell pellet was resuspended in 5ml of cell freezing medium (filtered heat inactivated foetal bovine serum (HIFBS) with 10% dimethyl sulphoxide (DMSO)). 1ml of the cell suspension was pipetted into each of 5 cryovials which were then stored in liquid nitrogen followed by slow cooling to -80˚C at a rate of 1˚C/min using a Mr. Frosty (Nalgene®).

2.2.1 Phorbol Myristate Acetate (PMA) Stimulation of PBMC

Complete medium	**Activation medium**
RPMI 1640	15ml complete medium
10% HIFBS	2µl ionomycin (5mg/ml) (Sigma)
100U/ml penicillin-100µg streptomycin	10µl brefeldin A (10mg/ml) (Sigma)
	10µl PMA (10µl/ml) (Sigma)

0.5ml of PBMC suspended in cell freezing medium (Section 2.2), thawed from an aliquot stored in liquid nitrogen, were stimulated by incubation in activation medium (complete medium was used as a control) for 4.5 hours at 37°C and 5% CO_2.

2.3 Total RNA Extraction

Total RNA was extracted from PBMC stimulated by PMA (Section 2.2.1). PBMC were centrifuged at 400g for 3 minutes to pellet the cells. 1ml of Trizol® RNA extraction reagent (Invitrogen) was added to the cells and mixed up and down using a 1ml pipette tip to lyse the cells, then incubated for 10 minutes at room temperature. The cell lysate was transferred into a 1.5ml microcentrifuge tube and 200µl of chloroform:isoamyl alcohol (24:1) was added. The tube was vortexed for 1 min, making sure that the top of the tube was covered with parafilm, and then centrifuged at 12,000g for 15 min at room temperature to produce two discrete phases: a clear aqueous phase at the top and an organic layer at the bottom. The aqueous layer was transferred into a clean microcentrifuge tube and retained, whilst the remaining organic layer was centrifuged again to obtain any remaining aqueous layer which was then pooled with the first aqueous layer extract. 500µl of 100% (v/v) ethanol was added to the aqueous layer sample, and RNA was precipitated by gently shaking the tube and incubating at room temperature for 10 minutes. The tube was centrifuged at 12,000g for 10 min in order to

pellet the RNA. The supernatant was removed and discarded, and the RNA was washed by vortexing with 1ml of 75% (v/v) ethanol in UltraPure™ RNase-free water (Invitrogen). The RNA was re-pelleted by centrifuging for 5 minutes at full speed, and the supernatant was removed and discarded. The pellet was left to air dry before being resuspended in 100µl of UltraPure™ RNase-free water and incubated at 60ºC for 10 min to completely dissolve the RNA. The RNA was stored at -80ºC.

The total RNA concentration was quantified using a GeneQuant™ Pro Spectrophotometer (Amersham Pharmacia), and the integrity of the RNA was checked by taking the $OD_{260/280}$ and checking that the ratio was between 1.8 and 2. A ratio which is lower than this may indicate protein contamination of the sample.

2.4 Cloning of cDNA Coding Sequence

2.4.1 cDNA synthesis

Total RNA extracted from stimulated PBMC was reverse transcribed into cDNA using either Sensiscript® or Omniscript® Reverse Transcriptase Kit (Qiagen). Sensiscript® reverse transcriptase is of particular use in cases where the concentration of the target RNA transcript is low. For use with Sensiscript® reverse transcriptase the RNA was diluted to 50ng/µl in UltraPure™ RNase-free water. No dilution was required for use with the Omniscript® reverse transcriptase. RNA was denatured by incubation at 90ºC for 10 minutes. After denaturation the RNA was kept on ice to prevent reconstitution of secondary (2º) structure. 1µl of denatured RNA was then added to a mixture containing 2µl of 10x Buffer RT (Qiagen), 2µl of 5nM dNTPs (Invitrogen), 2µl of 10µM reverse primer (Table 2.1) (Invitrogen), 1µl RNase inhibitor (10units/µl) (Invitrogen),

Table 2.1 Oligonucleotide primers used for cloning.

PCR Primers (desalted purification) were ordered from the Custom Primer Synthesis Service, Invitrogen. Primers were resuspended to a stock solution of 200μM in UltraPure™ water (Invitrogen), and diluted to a working solution of 10 μM.

Primer Name	Sequence
IL-2F (Section 3.2)	5′ CCTCAACTCCTGCCACAATGTAC 3′
IL-2R (Section 3.2)	5′ TCAAGTTAGTGTTGAGATGATGC 3′
T7 (sequencing primer)	5′ GACCTGATTACGCCAACTATTTAGG
SP6(sequencing primer)	5′ GTGAATTGTAATACGACTCACTATAGG G 3′
IL-2LinkF (Section 3.2)	5′ GTTCTGGTGGAGGTGGAGCACCTACTTCAAGTTC 3′
STV F (Section 3.4)	5′ GGCATCACCGGCACCTGGTA 3′
StvLinkR (Section 3.4)	5′ CCACCTCCACCAGAACTCACCTTGGTGAAGGTG 3′
IL-18F (Section 3.3)	5′ GTCGCAGGAATAAAGATGGC 3′
IL-18 R (Section 3.3)	5′ GCTAGTCTTCGTTTTGAACAG 3′
IL-18LinkF (Section 3.3)	5′ GTTCTGGTGGAGGTGGATACTTTGGCAAGCTTGAATC 3′
Streptavidin F (Section 3.4)	5′ ATG CGCAAGGTCGTCGTTGCAG 3′
Streptavidin R (Section 3.4)	5′ CTACTGCTGAACGGCGTCGAGC 3′
STV vector F (Section 3.5)	5′ CCACGGCATCACCGGCACCTG 3′
IL-2 vector R (Section 3.5)	5′ CCAGTCAGTGTTGAGATGATGC 3′
IL-18 vector R (Section 3.2)	5′ GTCTTCGTTTTGAACAGTGAAC 3′

1µl Sensiscript® Reverse Transcriptase or Omniscript® Reverse Transcriptase and 11µl RNase-free water. The reverse transcription reaction was performed in a thermal cycler using a temperature of 37ºC, which is the optimum temperature for the activity of these enzymes, for 60 min, then 93ºC for 5 min to denature the enzyme, followed by rapid cooling on ice.

2.4.2 PCR Amplification

(i) PCR amplification using ProofStart™ DNA polymerase

DNA produced by cDNA synthesis from RNA was PCR amplified using the ProofStart™ PCR kit (Qiagen). This kit uses the high fidelity enzyme ProofStart™ DNA polymerase in order to reduce the number of sequence errors incorporated during multiple rounds of PCR amplification as compared to using *Taq* DNA polymerase (ProofStart™ has an error rate of less than 1 in 5×10^6 compared to an error rate of approximately in 1×10^4 of *Taq* DNA polymerase (Qiagen 2008)). 5µl of cDNA template was added to a reaction mixture containing 5µl of 10× PCR buffer (Qiagen), 3µl of 2.5mM dNTPs

(Qiagen), 2.5µl of 10µM forward primer (Table 2.1), 2.5µl of 10µM reverse primer (Table 2.1), 1µl of ProofStart™ DNA polymerase (2.5 units) and 31µl RNase-free water, making a final reaction volume of 50µl. The PCR amplification reaction was performed using a thermal cycler according to the reaction conditions described below (Table 2.2). Products of PCR amplification were analysed by agarose gel electrophoresis, as described in Section 2.4.3.

Table 2.2 Reaction conditions of PCR amplification with ProofStart™ DNA polymerase.

	Temperature (°C)	**Time (minutes)**	**Cycles**
Initial "hot start" activation of DNA polymerase	95	5	1
DNA denaturation	94	1	25-35
Primer annealing	55-60 (Varied according to primers)	1	
Extension	72	1	
Final hold	4	Hold	

(ii) PCR amplification using Taq *DNA polymerase*

cDNA or DNA produced by PCR amplification with Proofstart™ DNA polymerase was amplified using *Taq* polymerase (Invitrogen). 1µl of DNA template was added to a reaction mixture containing 5µl of 10× PCR buffer (Invitrogen), 1.5µl of Mg^{2+} (Invitrogen), 3µl of 2.5mM dNTPs, 2.5µl of 10µM forward primer (Table 2.1), 2.5µl of 10µM reverse primer (Table 2.1), 1µl of *Taq* DNA polymerase (5U/µl) and 33.5µl RNase-free water, making a final reaction volume of 50µl. The amplification reaction was performed using a thermal cycler according to the reaction conditions described below (Table 2.3). Products were analysed by agarose gel electrophoresis gel, as described in Section 2.4.3

(i) PCR amplification using AccuPrime™ Taq DNA polymerase

DNA produced by cDNA synthesis from RNA was PCR amplified using *AccuPrime*™ (Invitrogen). AccumPrime *Taq* DNA polymerase improves PCR specificity and fidelity compared with standard using *Taq* DNA polymerase (Accuprime *Taq* has twice the fidelity of standard *Taq* DNA polymerase). 5µl of cDNA template was added to a reaction mixture containing 5µl 10X AccuPrime™ PCR Buffer (Invitrogen), 2.5µl of 10µM forward primer, 2.5µl of 10µM reverse primer, 1µl of Accuprime™ *Taq* DNA polymerase (2.5 units) and 34µl RNase-free water, making a final reaction volume of 50µl. The PCR amplification reaction was performed using a thermal cycler according to the reaction conditions described below (Table 2.4). Products of PCR amplification were analysed by agarose gel, as described in Section 2.4.3.

Table 2.3 Reaction conditions of PCR amplification with *Taq* DNA polymerase.

	Temperature (ºC)	Time (minutes)	Cycles
Initial "hot start" activation of DNA polymerase	95	5	1
DNA denaturation	94	1	25-35
Primer annealing	55-60 (Varied according to primers)	1	
Extension	72	1	
Final hold	4	Hold	

Table 2.4 Reaction conditions of PCR amplification with AccuPrime™ *Taq* DNA polymerase.

	Temperature (ºC)	Time (minutes)	Cycles
Initial "hot start" activation of DNA polymerase	94	2	1
DNA denaturation	94	0.5	30
Primer annealing	55-60 (Varied according to primers)	0.5	
Extension	72	1	
Final hold	4	Hold	

2.4.3 Analysis of PCR Products by Agarose Gel Electrophoresis

1.3g of agarose was added to 50ml of TAE buffer (40mM tris(hydroxymethyl)methylamine (tris), 40mM acetic acid, 1mM ethylenediaminetetraacetic acid (EDTA) in distilled water) and heated until the agarose had dissolved. The agarose was cooled to approximately 40ºC and 1µl of 10mg/ml ethidium bromide was added to the flask and swirled to mix. The gel was poured into a gel rig (Bio-Rad), combs inserted, and left to set. After setting, the gel was placed into a gel electrophoresis tank and covered with TAE buffer. The sample was loaded with 5:1 sample to 6× DNA loading dye (0.25% (w/v) bromophenol blue, 0.25% (v/v) xylene cyanol FF, 30% (v/v) glycerol) and run against a DNA $1kb^{+}$ molecular weight marker ladder (Invitrogen) for 1 hour at 100 volts (V). DNA products were visualised on the agarose gel using a UV transilluminator (302nm).

2.4.4 Isolation of DNA Products by Gel Extraction

PCR products prepared as according to Section 2.4.2 were separated and visualised by agarose gel electrophoresis as described in Section 2.4.3. To isolate and extract the PCR product of interest, the observed band of DNA corresponding approximately to the expected size of the required product was excised from the gel using a clean sterile scalpel, removing as much excess agarose as possible. The DNA was then extracted from the gel using a QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer's recommended protocol, including an optional step to remove all traces of agarose and eluting in 50µl of UltraPure™ RNase-free water.

2.5 Cloning of PCR Products into Plasmid Vectors

2.5.1 Ligation of PCR Products into Plasmid Vectors

(i) pGEM®-T

A ligation reaction was set up in a 0.5ml microcentrifuge tube (see Table 2.5). The ligation buffer was vortexed prior to addition to the reaction mix. Reagents were mixed by pipetting, and the reaction was incubated at room temperature for 1 hour and then placed on ice.

(ii) pCR®T7/CT-TOPO®

A ligation reaction was set up in a 0.5ml microcentrifuge tube (see Table 2.6). Reagents were mixed by pipetting, and the reaction was incubated at room temperature for 5 minutes and then placed on ice.

2.5.2 Transformation of *E. coli* XL-1 Blue or One Shot® TOP10F′

(i) Preparation of Luria Bertani (LB) Agar plates

40g of Luria agar mix was made up to 1L in double distilled water and autoclaved at 121ºC for 16 min at 1.05 bar to sterilize. After sterilization the agar was left to cool to below 50ºC before addition of 50mg/ml ampicillin (final concentration 100µg/ml). The agar was poured into Petri dishes and left to set.

(ii) Luria Bertani (LB) broth

25g of LB broth mix was made up to 1L in double distilled water and autoclaved at 121ºC for 16 min at 1.05 bar to sterilize.

Table 2.5 Reagent mixture for pGEM®-T vector ligation reactions.

Reagent	Quantity
2x Rapid Ligation Buffer (Promega)	5µl
pGEM®-T Vector (50ng/µl) (Promega)	1µl
PCR product (32µg/ml)	0.25µl
T4 DNA Ligase (3 Weiss units/µl) (Promega)	1µl
UltraPure™ water to a final volume of 10µl	1µl

Table 2.6 Reagent mixture for TOPO® vector ligation reactions.

Reagent	Quantity
TOPO® Vector (10ng/µl) (Promega)	1µl
PCR product (32µg/ml)	4µl
UltraPure™ water to a final volume of 5µl	1µl

2µl of ligated sample (Section 2.5.1) was added to a sterile 1.5ml microcentrifuge tube and kept on ice. One 50µl aliquot of XL1-Blue supercompetent cells (Stratagene) or One Shot® TOP10F′ chemically competent cells (Invitrogen) was thawed on ice and mixed by gently flicking the tube. All 50µl of cells were added to the tube containing the ligated sample, and mixed by gentle flicking. The tube was left on ice for 20 minutes and then heat shocked for 45 seconds in a 42ºC water bath before returning to ice. 950µl of room temperature LB broth was added to the tube, which was then incubated in an orbital incubator at 37ºC for 90 minutes. 100µl of the transformed bacteria was spread onto an agar plate and 100µl of a 1:10 dilution of the transformed bacteria in LB broth was spread onto a second agar plate. Both plates were incubated for 16 hours at 37ºC. Agar plates spread with cultures of transformed bacteria showed growth of discrete colonies.

2.5.3 Overnight Cultivation of Transformed Bacteria

Discrete isolated colonies from each plate prepared as in Section 2.4.2 were picked using a pipette tip and each added to 5ml of LB broth containing 100µg/ml ampicillin in a sterile 30ml polypropylene tube. The tubes were incubated in an orbital incubator at 37ºC for 16 hours.

2.5.4 Plasmid Extraction and Purification

1.5ml of overnight culture (Section 2.5.3) was centrifuged for 10 minutes at 13,000g to pellet the bacterial cells. The supernatant was then removed and the pellet was treated with a QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions, including all optional wash steps and eluting in 50µl of UltraPure™ RNase-free water. The eluted DNA was stored at -20ºC.

2.5.5 Restriction Enzyme Digestion of Plasmid DNA

DNA from the plasmid extraction described in Section 2.4.2 was quantified using a GeneQuant™ Pro Spectrophotometer (Amersham Pharmacia), diluted to 1µg/µl and added to the reaction mixtures (Table 2.7, 2.8 and 2.9) prepared in 0.2ml microcentrifuge tubes. The reaction mixture was then incubated at 37ºC (*Vsp*I and *Hind*III) or 60ºC (*Bst*YI) for 1 hour before inactivating the appropriate enzyme by heating at 65ºC (*Vsp*I and *Hind*III) or 60ºC (*Bst*YI) for 15 minutes. The digestion reaction mixture was stored at -20ºC. The resulting products from restriction enzyme digestion reactions were separated by electrophoresis on a 1.3% agarose gel and visualised using a UV transilluminator (302nm).

2.5.6 Direct Colony PCR

Colonies were picked from agar plates spread with the products of the transformation of *E. coli* (Section 2.5.2) under sterile conditions using a pipette tip. The pipette tip was then dipped in 30µl of PCR mix containing reagents as shown (Table 2.10) (Invitrogen). The reaction was performed using a thermal cycler according to the programme described (Table 2.11)

Following completion of the PCR amplification reaction, DNA products were separated by gel electrophoresis using a 1.3% agarose gel and visualised using a UV transilluminator at 302nm wavelength as described in Section 2.4.2.

Table 2.7 Reagent mixture for *VspI* digestion reactions.

Reagent	Quantity (µl)
pGEM-T +/- Insert	1
*Vsp*I Enzyme	0.5
10x Reaction Buffer (Buffer D – Promega)	2
Bovine Serum Albumin (BSA) (10mg/ml) (New England Biolabs)	0.2
UltraPure™ RNase-free water	16.3

Table 2.8 Reagent mixture for *Bst*Y1 digestion reactions.

Reagent	Quantity (µl)
pGEM-T +/- Insert	1
*Bst*YI Enzyme (New England Biolabs)	0.5
10x Reaction Buffer (NEBuffer 2 – New England Biolabs)	2
BSA (10mg/ml) (New England Biolabs)	0.2
UltraPure™ RNase-free water	16.3

Table 2.9 Reagent mixture for *Hind*III digestion reactions.

Reagent	Quantity (µl)
Vector +/- Insert	1
*Hind*III Enzyme (New England Biolabs)	0.5
10x Reaction Buffer (NEBuffer 2 – New England Biolabs)	2
BSA (10mg/ml) (New England Biolabs)	0.2
UltraPure™ RNase-free water	16.3

Table 2.10 Reagent mixture for colony PCR reactions.

Reagent	Per Tube (µl)
10x PCR buffer	5
50mM Mg^{2+}	1.5
2.5mM dNTPs	3
10µM Forward Primer (T7)	2.5
10µM Reverse Primer (SP6)	2.5
Taq DNA polymerase 5U/µl	1
UltraPure™ water	34.5
Total	50

Table 2.11 Reaction conditions of colony PCR amplification with *Taq* DNA polymerase.

	Temperature (ºC)	Time (minutes)	Cycles
Initial "hot start" activation of DNA polymerase	95	5	1
DNA denaturation	94	1	35
Primer annealing	57	1	
Extension	72	1	
Final extension	4	Hold	

2.5.7 Preparation of Bacterial Glycerol Stocks

60% glycerol was made by adding 60ml of sterile glycerol to 40ml of sterile distilled water. The liquids were mixed by shaking and then autoclaved at 121ºC and 1.05 bar for 16 minutes. The 60% glycerol solution was stored at room temperature.

For preparation of bacterial glycerol stocks, 750µl of bacterial culture was added to a 1.5ml centrifuge tube. 250µl of ambient temperature 60% glycerol was added to each tube and then vortexed to mix giving a final concentration of 15% glycerol. The stocks were then stored at -80ºC.

2.5.8 Ethanol Precipitation of DNA

20µl of plasmid DNA prepared by plasmid extraction and purification (Section 2.5.4) was pipetted into a 1.5ml microcentrifuge tube. 20µl of 5M ammonium acetate and 80µl of 100% ethanol (molecular grade) were added to the tube and vortexed briefly. The tube was placed on ice for 20 minutes and then centrifuged for 15 minutes at 16,000g. The supernatant was decanted and discarded. To wash the pelleted DNA, 1ml of 70% ethanol (molecular grade) was added, and the tube was centrifuged at 16,000g for 5 minutes to repellet the DNA. The supernatant was decanted and discarded. The DNA pellet was left to air dry by covering the tube with parafilm and piercing small holes in it. After drying the DNA was stored at -20ºC.

2.5.9 DNA Sequence Analysis

Analysis of sequences of inserts cloned into plasmid vectors was performed on samples of DNA prepared by ethanol precipitation (Section 2.5.8) (MWG Biotech). Sequence analysis was carried out using the forward T7 primer (5′ GTG AAT TGT AAT ACG

ACT CAC TAT AGG G 3′) (Invitrogen) which occurs before the insertion site in the pGEM-T, pCR T7-NT and pCR T7-CT vectors

2.5.10 Mutation of DNA sequences by Site-Directed Mutagenesis

Site-directed mutagenesis can be applied to mutate a single base pair in a DNA vector. The plasmid to be mutated is denatured and oligonucleotide primers containing the desired mutation are annealed to the corresponding DNA sequence. Extension of the primers by *PfuUltra*® High Fidelity DNA polymerase is used to amplify the original plasmid creating mutated copies of the containing staggered nicks. Following amplification the product is treated with *Dpn*I, which is an endonuclease specific for the parental *dam*-methylated DNA, to digest the original non-mutated plasmid.

NZY Broth

21g of NZY broth mix (Gibco) was made up to 1l in double distilled water and autoclaved at 121ºC for 16 min at 1.05 bar. Site-directed mutagenesis was carried out using the QuickChange® II Site-Directed Mutagenesis (SDM) Kit (Stratagene).

(i) Amplification reaction

DNA prepared using the plasmid extraction and purification procedure (Section 2.5.4) was diluted to a concentration of 5ng/µl. An amplification reaction was prepared (Table 2.12).

Table 2.12 Reagent mix for DNA amplification reactions.

Reagent	Quantities (µl)	
	Tube 1 (5ng DNA)	**Tube 2 (50ng DNA)**
10× Reaction Buffer (Stratagene)	5	5
Miniprep Plasmid DNA (5ng/µl)	1	10
Forward primer (0.125µg/µl)	10	10
Reverse primer (0.125µg/µl)	10	10
dNTP mix	1	1
UltraPure™ RNase-free water	23	14
Total	**50**	**50**

After preparation of the reaction mixture, 1µl of *PfuUltra*® High Fidelity DNA polymerase (2.5U/µl) (Stratagene) was added to the mixture, and the reaction was performed using a thermal cycler according to the reaction conditions (Table 2.13). Samples were then stored at -20ºC.

(ii) Digestion of amplification product

1µl of *Dpn*I restriction enzyme (Stratagene) was added to each amplification reaction tube and pipetted up and down to mix. Samples were centrifuged for 1 minute at 16,000g and then incubated in a heat block at 37ºC for 1 hour.

(iii) Site-directed mutagenesis transformation reaction

The transformation of XL1-Blue supercompetent cells with the product of the site-directed mutagenesis reaction was performed following the protocol for the QuickChange® II Site-Directed Mutagenesis Kit (Stratagene). Briefly, XL1-Blue supercompetent cells were thawed on ice, and 50µl of cells were then aliquoted into each of three 1.5ml microcentrifuge tubes pre-chilled on ice. 1µl of *Dpn*I-treated DNA was added to each tube and swirled to mix. Samples were incubated on ice for 30 minutes before being heat pulsed for 45 seconds in a 42ºC water bath and then immediately placed on ice for 2 minutes. 0.5ml of NZY broth preheated to 42ºC was added to each tube and the tubes were incubated for 1 hour at 37ºC in an orbital incubator. 250µl of transformation reaction was spread on each of 2 agar plates per tube and the agar plates were incubated (as described in Section 2.5.2). Colonies from

Table 2.13 Reagent mix for SDM reaction mix.

	Temperature (ºC)	Time (seconds)	Cycles
Initial "hot start" activation of DNA polymerase	95	30	1
DNA denaturation	95	30	12
Primer annealing	55	60	
Extension	68	210	
Final extension	4	Hold	

each plate were selected and cultured overnight (Section 2.5.3). Plasmid DNA was prepared from the overnight cultures by plasmid extraction and purification (Section 2.4.2). Glycerol stocks of each bacterial culture were prepared (Section 2.5.7) for sequence analysis using the T7 forward primer as described in Section 2.5.9)

2.6 Cloning of Expression Constructs into the pCR®T7/NT-TOPO® Expression Vector

The full length expression construct coding sequences STV/IL-2 and STV/IL-18 encoding recombinant fusion proteins STV/IL-2 and STV/IL-18 were amplified from pGEM-T STV/IL-2 and pGEM-T STV/IL-18. Amplification was carried out using AccuPrime™ *Taq* (Invitrogen) according to the manufacturer's protocol using the IL-2 vector F or IL-18 vector F and STV vector R primers (Table 2.1). The amplified product was ligated into the pCR®T7/NT-TOPO® expression vector using the cloning techniques outlined in Section 2.5.

2.7 Expression of Recombinant Proteins Using a Bacterial Expression System

2.7.1 Transformation of BL21(DE3)pLysS with pCR®T7/NT-TOPO containing the coding sequence for STV/IL-18 or STV/IL-2

50µl of BL21(DE3)pLysS cells were thawed on ice. 1µl of expression plasmid DNA (pCR®T7/NT-TOPO containing the coding sequence for STV/IL-2 or STV/IL-18) was added to the cells and mixed by gently flicking the tube. The tube was left on ice for 30 minutes and then heat shocked for 30 seconds in a 42ºC water bath before returning to the ice. 250µl of room temperature LB broth, containing no additives, was added to the tube which was then incubated in an orbital incubator at 37ºC for 90 minutes.

2.7.2 Expression of STV/IL-2 and STV/IL-18 in BL21(DE3)pLysS cells

The transformation reaction (2.7.1) was added to 10ml LB broth containing 100µg/ml ampicillin and 34µg/ml chloramphenicol and grown at 37^{o}C with shaking overnight. A 1:200 dilution of overnight culture was made in LB broth containing 100µg/ml ampicillin and 34µg/ml chloramphenicol (e.g 500µl of overnight culture was diluted into 10ml of LB broth) and grown at 37^{o}C. After 2 hours 0.8M isopropyl β-D-1-thiogalactopyranoside (IPTG) (final concentration) was added to the culture medium and incubated at 37^{o}C with orbital shaking for the required time to achieve expression of the recombinant protein, usually overnight. Following pilot expression studies the culture medium was thereafter supplemented with 1% (w/v) glucose which appeared to increase the expression of the recombinant protein.

2.7.3 Extraction of the Recombinant Protein from BL21(DE3)pLysS byCell Lysis with BugBuster®

Soluble and insoluble proteins were extracted from bacterial pellets using BugBuster® protein extraction reagent (Novagen). BugBuster® is a detergent mix which disrupts the cell wall of *E. coli*, allowing the release of expressed bacterial proteins. The manufacture's instructions state that BugBuster® is especially efficient when used with the BL21 strain of *E. coli*, and with cells containing the pLysS plasmid, due to the activity of T7 lysozyme. Adding Benzonase® Nuclease (Novagen) degrades DNA which reduces the viscosity of the cell pellet/BugBuster® mix. Soluble and insoluble proteins in inclusion bodies can be separated by centrifugation.

50ml of overnight bacterial culture was centrifuged at 2,000g for 20 minutes. The supernatant was removed and the pellets were stored at -20ºC. Prior to performing cell lysis, cell pellets were defrosted to room temperature, removing any excess liquid, the

cell pellet was then resuspended in 3ml of BugBuster® Extraction Reagent containing 1µl/ml Benzonase® Nuclease by pipetting and vortexing. The cell suspension was incubated with shaking for 20 minutes at ambient temperature and then centrifuged for 20 minutes at 5,000g. After this centrifugation the supernatant contained soluble bacterial proteins. The soluble fraction was stored at -80ºC and the insoluble fraction was stored at -20ºC. As the insoluble fraction is comprised of inclusion bodies the protein in this fraction is more stable than that contained within the soluble fraction and does not require storage at -80ºC.

2.7.4 Protein purification by His-tag affinity binding

Wash Buffer (pH 8.0)	**Elution Buffer (pH 8.0)**
1.4ml 0.2M NaH_2PO_4	238µl 0.2M NaH_2PO_4
24ml 0.2M $NaH_2PO_4.2H_2O$	4.3ml 0.2M $NaH_2PO_4.2H_2O$
17ml 3M NaCl, 3.4ml	3ml 3M NaCl,
3.4ml 500mM imidazole	15ml 500mM imidazole
Total Volume 45.8ml	Total Volume 22.538ml

Soluble protein fractions were prepared using Bugbuster® protein extraction reagent (Section 2.7.3). His-Select™ Cobalt Affinity Gel (Sigma) resin was centrifuged in a 50ml tube at 5,000g for 20 minutes. The supernatant was removed from the pelleted resin and discarded. The resin was then washed in 10 volumes of wash buffer to initial gel volume by resuspending and centrifuging at 5,000g for 20 minutes before removing and discarding the supernatant. Soluble protein fraction was added to the resin (Soluble protein from 1ml of cell culture per 1µl resin) and mixed on an orbital shaker at 175rpm for 5 minutes. The resin/protein mixture was then centrifuged at 5,000g for 20 minutes and the supernatant removed and discarded. The resin was washed 3 times in 10

volumes of wash buffer (as previously) and the supernatant removed and discarded. 2 volumes of Elution buffer to initial gel volume was added to the resin and mixed on an orbital shaker at 175rpm for 30 minutes. The resin was then centrifuged at 5,000g for 20 minutes and the supernatant removed and retained. The elution step was repeated twice and the supernatant pooled (for a total of 6 volumes of elution buffer).

2.7.5 Inclusion Body Purification

BugBuster® with the addition of rLysozyme™ (Novagen) was used to purify the insoluble fraction of the protein, removing any membrane bound or other contaminating proteins. rLysozyme hydrolyses N-acetylmuramide linkages in bacterial cell walls, digesting the cell membranes and leaving the purified inclusion bodies which are then harvested by centrifugation.

Inclusion bodies were isolated and purified from the insoluble fraction prepared by the bacterial cell lysis procedure (Section 2.7.3). The insoluble pellet was resuspended in 3ml BugBuster® Protein Extraction Reagent containing 1kU/ml rLysozyme and vortexed for 5 minutes to mix. A 1:10 dilution of BugBuster® reagent: distilled water was prepared and 18ml of this dilution was added to the suspension and vortexed for 1 minute. The suspension was centrifuged at 5,000g for 20 minutes at 4ºC to pellet the inclusion bodies, and the supernatant was discarded. The inclusion bodies were resuspended in 25ml of diluted BugBuster® reagent, mixed by vortexing for 1 minute and centrifuged for at 5,000g for 20 minutes at 4ºC to pellet the inclusion bodies. Once again the supernatant was discarded. The resuspension of inclusion bodies in diluted BugBuster® reagent was repeated twice. Inclusion bodies were then resuspended in diluted BugBuster® reagent, transferred to a microcentrifuge tube and centrifuged at

16,000g for 5 minutes to removing any excess liquid. The supernatant was removed and discarded. Purified inclusion bodies were stored at -20ºC.

2.7.6 Solubilization of Purified Inclusion Bodies

Solubilization buffers containing 100mM tris, 2M urea at pH 8.5, 9.5, 10.5, 11.5, 12.5 were prepared. 1ml of the required solubilization buffer was added to 0.8mg of inclusion bodies and incubated at room temperature for 30 minutes. After incubation the samples were centrifuged at 16,000g for 30 minutes to collect any soluble debris. The supernatant containing the solubilized protein was transferred into a clean microcentrifuge tube and stored at -80ºC.

2.7.7 Refolding of Solubilized Recombinant Protein in PBS or dH_2O

Soluble protein in 100mM tris, 2M urea was added drop-wise to 20 volumes of PBS or dH_20 in pulses spaced 5 seconds apart, to allow protein folding of each drop, with constant stirring of the diluent. The resulting protein buffer contained 5mM tris, 100mM urea. 0.5 ml of refolded protein solution was concentrated approximately 5 times using a Microcon Centrifugal Filter Device (10,000 MW cut off) (Millipore). The sample was added to the device and centrifuged for 30 minutes at 14,000g and 25˚C. After centrifugation the sample reservoir was inverted into a 1.5ml microcentrifuge collecting tube and again centrifuged for 3 minutes at 1000g to transfer the contents of the filter device to the collection tube.

2.8 Analysis of Recombinant Protein

2.8.1 Bio-Rad Bradford Protein Assay

Protein extracted from bacterial inclusion bodies (Section 1.7.4) solubilized (Section 2.7.5) and diluted in PBS was assayed using the Bio-Rad protein assay (Bio-Rad),

which is based on the standard Bradford protein assay. Binding of an acidic Coomassie™ blue dye to basic and aromatic amino acid residues shifts the dye from 465nm to 595nm giving a measure of protein concentration. 20µl of protein sample or BSA protein standard was transferred into each well of a 96 well plate. 200µl of protein reagent (diluted 1 in 5 stock solution in water) was dispensed into each well and the colour change from brown(465nm) to blue(595nm) was analysed using a spectrophotometer (PowerWave$_x$™ - Bio-Tek Instruments, Inc) at a wavelength of 595nm. A standard concentration curve was constructed from the readings for BSA standards, and the protein concentration of recombinant protein samples was estimated from this curve. An example standard curve is shown in Figure 2.2

2.8.2 Polyacrylamide Gel Electrophoresis

Native separating gel

3.3ml acrylamide/bisacrylamide (40%w/v)
2.5ml 1.5M tris-HCl (pH 8.8)
4.1ml distilled water
50µl ammonium persulphate (10%w/v)
10µl N,N,N',N' - tetraethylethylene diamine (TEMED).

Denaturing separating gel

3.3ml acrylamide/bisacrylamide (40%w/v)
2.5ml 1.5M tris-HCl (pH 8.8)
4.0ml distilled water
50µl ammonium persulphate (10%w/v)
10µl TEMED
0.1ml 10% sodium dodecyl sulphate (SDS)

Stacking gel

1.3ml acrylamide/bisacrylamide (40%w/v)
1.25ml 1.0M tris-HCl (pH 6.8)
7.4ml distilled water
50µl ammonium persulphate (10%w/v)
20µl TEMED

Native running buffer (pH 8.8)

25mM tris base
19.2mM glycine
in distilled water

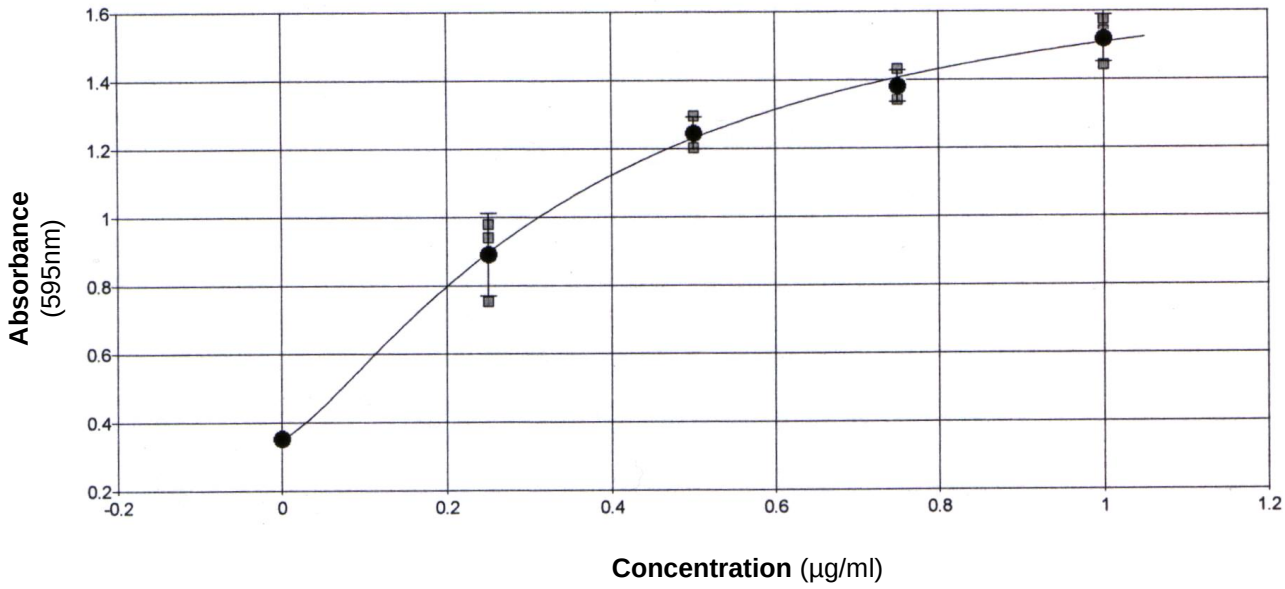

Figure 2.2 Concentration curve of BSA protein standards against absorbance at 595nm.

The results are expressed as the mean +/- the standard mean of three sample triplicates from one experiment.

Denaturing running buffer (pH 8.8)	**Transfer buffer (pH 8.8)**
25mM tris base	25mM tris base
19.2mM glycine	19.2mM glycine
0.1% SDS	20% methanol
in distilled water	in distilled water

A 10% (w/v) polyacrylamide gel was prepared using miniProtean II apparatus (Bio-Rad). The glass plates used to make the gel were cleaned with ethanol and assembled using a one piece clamp and spacers to form a mould. This was fitted into a gel casting stand, and water was transferred into the mould and left for 2-3 minutes to test the apparatus for leaks, before being removed. A separating gel solution was prepared in a fume cupboard, taking care to add the TEMED to the solution last, and then immediately pipetted between the glass plates leaving a 1.5 cm gap at the top. A thin layer of butanol was pipetted onto the top of the gel to ensure an even surface. The gel was left for 20-30 minutes to polymerize. After the gel had set, the butanol was carefully removed by blotting using a piece of filter paper. A stacking gel solution was prepared in a fume cupboard and pipetted onto the surface of the separating gel. A 10 well comb was inserted between the glass plates to form wells in the stacking gel, and the gel was left for 20-30 minutes to polymerize. The required running buffer was poured into the tank to cover the gel.

Protein samples for analysis were mixed with gel loading buffer (5× Non-Reducing Lane Marker Sample Buffer (Pierce)) and loaded into the wells of the stacking gel along with a pre-stained protein marker ladder (Invitrogen). Proteins were separated by electrophoresis at 180V until the dye front was a small gap (approximately 10mm) from the bottom of the gel (approximately 90 minutes).

In addition to the above polyacrylamide gels, precast polyacrylamide gels (Pierce) were also used on some occasions. Precast polyacrylamide gels require different buffers to those gels described above.

Running buffer (pH 8.0)

100mM tris base
100mM 4-(2-hydroxyethyl)-1
-piperazineethanesulfonic acid (HEPES)
0.1% SDS
in distilled water

Transfer buffer (pH 8.0)

25mM tris base
25mM bicine
10% Methanol
in distilled water

2.8.3 Detection of Proteins on PAGE gel using Coomassie™ Blue Stain

polyacrylamide gels containing protein separated by gel electrophoresis (Section 2.8.2) were incubated for 2-4 hours with agitation, in Coomassie blue staining solution (0.1% (w/v) Coomassie™ brilliant blue, 50% (v/v) methanol, 10% (v/v) glacial acetic acid in distilled water). The gels were then destained in destaining solution (50% methanol, 10% glacial acetic acid in distilled water) 3 times for 1-2 hours, with agitation, until background staining had been removed and the gel bands appeared as blue bands.

2.8.4 Western Transfer

Proteins separated by gel electrophoresis (Section 2.8.2) were transferred from the gel onto nitrocellulose membrane by Western transfer to permit detection and analysis of the protein. Protein gels were placed in a transfer cassette in the order shown below:

Fibre pad

4 sheets of filter paper

0.45µm nitrocellulose membrane (Bio-Rad)

polyacrylamide gel

4 sheets of filter paper

Fibre pad

The cassette was then placed into a Bio-Rad transfer tank filled with ice-cold transfer buffer, a magnetic stirrer and ice pack. The transfer of protein from the gel to the nitrocellulose membrane was conducted at 100V for 1 hour at 4ºC or at 30V overnight at 4ºC.

2.8.5 Immunodetection of Recombinant Proteins

Once Western transfer of the protein was completed, the nitrocellulose membrane was placed in blocking buffer comprising 10% (w/v) milk powder (Marvel) in PBS for at least 60 minutes at room temperature, to block further protein binding to the membrane. The primary antibody was then added to the blocking buffer and incubated for an hour at room temperature. The membrane was again washed 3 times with PBS containing 0.5% TWEEN before being incubated with the secondary antibody in blocking buffer at room temperature and again washed 3 times in 0.5% TWEEN in PBS. After washing, the membrane was incubated in Enhanced chemiluminescence (ECL) ™ Western blotting detection reagent (Amersham Pharmacia) for 5 minutes.

(i) Antibody concentrations

Antibodies used in experiments for immunodetection of proteins following Western transfer (section 2.8.5) were used the concentrations described below (diluted in blocking buffer):

Primary antibodies:

Polyclonal mouse anti histidine tag (Serotec) 1/500

Polyclonal goat anti-human IL-18 (Santa Cruz) 1/1,000

Polyclonal goat anti-human IL-2 (R&D) 1/500

Polyclonal rabbit anti-streptavidin 1/500

Secondary antibodies:

Rabbit F(ab')2 anti-mouse IgG- horseradish peroxidase (HRP) (Serotec) 1/2,000

Donkey anti-goat IgG-HRP (R&D) 1/1,000

Sheep anti-rabbit IgG-HRP (Serotec) 1/10,000

(ii) Detection on x-ray film

In a dark room the nitrocelluose membrane was placed against x-ray film (Fuji) in a developing cassette (Kodak) and exposed for 3 minutes. For development, the film was placed in three solutions: firstly, the film was placed in Dektol developer (Kodak) (1:5 dilution in water); secondly, the film was placed in 2% acetic acid to neutralise the developing solution; finally, the film was placed in Ilford Hypam fixer (Ilford) (1:5 dilution in water) and incubated until the film had become clear. The film was washed with tap water and allowed to dry.

(iii) Detection of chemoluminescence with a gel imaging system

Visualization of the proteins with a UVP EC3™ Imaging System took place for 20 minutes (1 picture was taken every 5 minutes and the results were summed onto the most recent image) according to the manufacturer's instructions, and the picture showing the clearest bands was chosen for analysis.

2.8.6 Estimation of the Molecular Mass of the STV/IL-2 and STV/IL-18 Fusion Proteins

To allow identification of the expressed recombinant fusion proteins via western blotting or Coomassie blue staining, it is important to estimate their molecular masses (including histidine tag). This was achieved using the bioinformatics tool Expasy Translate (*http://www.expasy.ch/tools/dna.html http://www.expasy.ch/tools/dna.html*) to translate the DNA coding sequence of the expression construct into an amino acid sequence. This deduced amino acid sequence was then used to calculate an estimated molecular mass for the recombinant protein using Encor Biotechnology's 'Free Protein and Peptide Molecular Mass Calculator' (*http://www.encorbio.com/protocols/Prot-MW.htm*). Estimation of the molecular masses of the recombinant proteins in this way gave a result of 32.22kDa for STV/IL-2 and 35.08kDa for STV/IL-18.

2.8.7 Streptavidin ELISA

An ELISA was used to assess the biotin binding abilities of the recombinant fusion proteins in comparison to recombinant streptavidin (Sigma). In order to identify the most effective primary and secondary antibodies to be used in this ELISA it was first necessary to optimize the method.

Antibody optimization

A 96 well plate was incubated overnight at 4ºC with 100µl of 10µg/ml streptavidin in PBS per well and then washed 3 times with PBS. Each well was then blocked with either 200µl of 2% BSA in PBS (w/v), 200µl 2% powdered milk solution in PBS (w/v) or 200µl PBS and incubated for 1 hour at room temperature. 100µl of primary antibody diluted in PBS at the concentrations described below was then added to each well and the plate was incubated for 2 hours at room temperature. The assay was conducted using either the primary antibody linked to biotin or the primary antibody without biotin to permit an estimation of non-specific binding of the antibody to the streptavidin or the ELISA plate.

Primary antibody dilutions:

- Goat anti-rabbit IgG-biotin 1:1500 (A)
- Goat anti-mouse IgG-biotin 1:1000 (B)
- Rabbit anti-goat IgG-biotin 1:1500 (C)
- Rabbit anti-mouse IgG 1:1000 (D)
- Goat anti-mouse IgG 1:100 (E)
- Rabbit anti-goat IgG 1:1000 (F)

The ELISA plate was then washed 3 times with PBS, and 100µl of an appropriate secondary antibody, diluted in PBS at the concentrations described below was added to each well, and incubated for 1 hour at room temperature. The combinations of primary and secondary antibodies used in the ELISA are given in Table 2.14

Secondary antibody dilutions:

Donkey anti-goat IgG-HRP 1:2000 (1) Sheep anti-rabbit IgG-HRP 1:2000 (2)

The plate was washed 3 times with PBS and then 100µl of tetramethylbenzide (TMB) was added to each well. TMB forms a yellow product (370nm) when it reacts with horseradish peroxidase, which turns blue (450nm) on addition of an acidic stop soloution. The plate was incubated in the dark for 5 minutes at room temperature to

allow the HRP colour reaction to develop; the reaction was then stopped by addition of 100µl of 1N sulphuric acid (H_2SO_4). The plate was then analysed using an Anthos 2010 absorbance plate reader at 450nm. Antibody pair A was chosen for the fusion protein ELISA (Section 5.2.6)

2.8.8 Streptavidin-Cytokine Fusion Protein Biotin Binding Assay

Using the optimized streptavidin ELISA developed as described above a streptavidin-biotin binding assay was performed to assess the biotin binding activity of the recombinant fusion proteins STV/IL-2 and STV/IL-18 (Figure 2.3). Recombinant streptavidin (Sigma) standards of known concentrations and recombinant fusion proteins STV/IL-2 and STV/IL-18 were diluted in PBS, and 100µl was pipetted into each well of a 96 ELISA plate, making sure each sample was repeated in triplicate. The plate was covered in parafilm, left to incubate overnight at 4ºC. Each well was then washed 3 times with PBS and then blocked with 200µl of 2% powdered milk solution in PBS (w/v). 100µl of rabbit anti-goat IgG-Biotin (Dako) diluted 1:1500 in 2% powdered milk in PBS (w/v) was added to each well, and the plate was incubated for 2 hours at room temperature. After washing 3 times with PBS, 100µl of sheep anti-rabbit IgG-HRP diluted 1:2000 in 2% powdered milk in PBS (w/v) was added to each well and incubated for 1 hour at room temperature. The plate was washed 3 times with PBS, and then 100µl of tetramethylbenzide was added to each well. The plate was then incubated in the dark for 5 minutes at room temperature, then the reaction was stopped by addition of 100µl of 1N sulphuric acid (H_2SO_4). The plate was then analysed using an Anthos 2010 absorbance reader at 450nm. A standard concentration curve was constructed from the readings for recombinant streptavidin (Figure 5.11).

Table 2.14 Antibody pairs used in streptavidin ELISA.

1º antibody + 2º antibody
A. Goat anti-rabbit IgG-Biotin + donkey anti-goat IgG-HRP
B. Goat anti-mouse IgG-Biotin + donkey anti-goat IgG-HRP
C. Rabbit anti-goat IgG-Biotin + sheep anti-rabbit IgG-HRP
D. Rat anti-mouse IgG-Biotin + sheep anti-rabbit IgG-HRP
E. Goat anti-mouse IgG + donkey anti-goat IgG-HRP
F. Rabbit anti-goat IgG + sheep anti-rabbit IgG-HRP

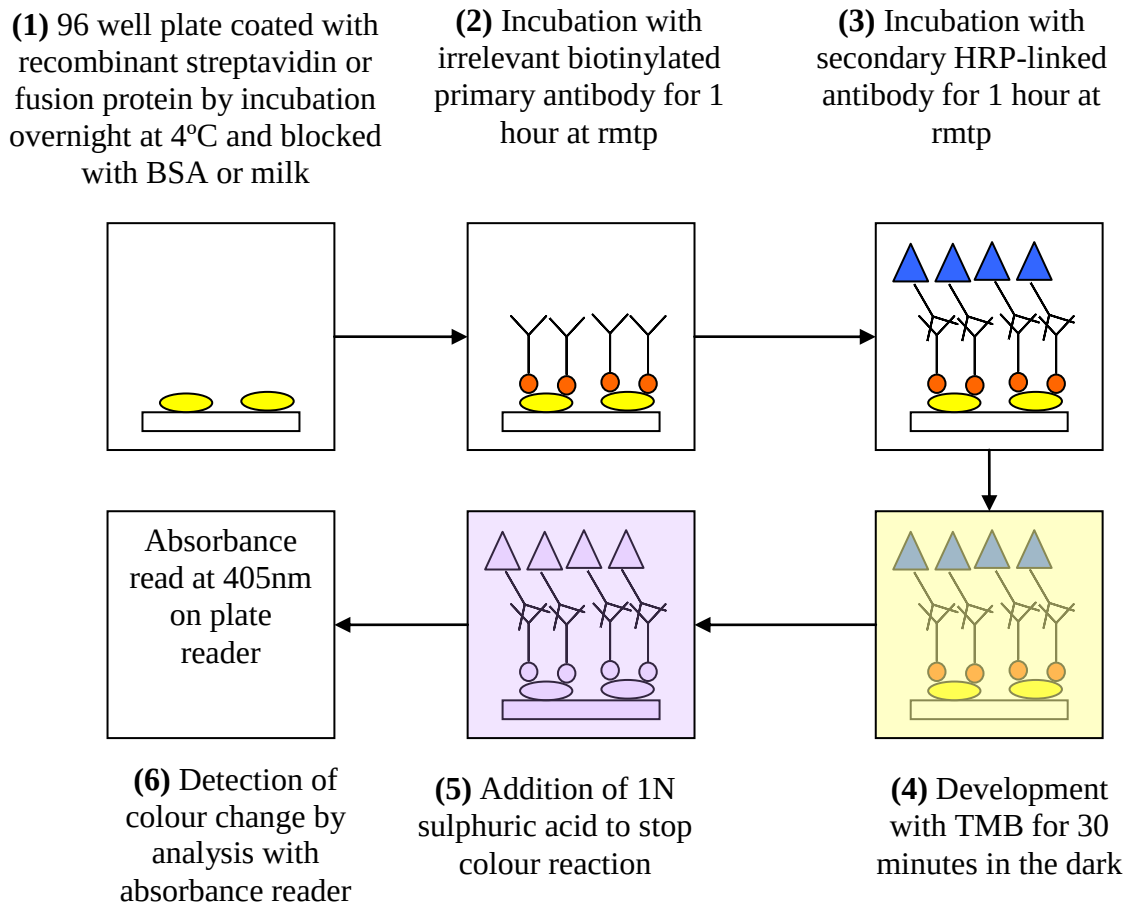

Figure 2.3 Schematic diagram of Streptavidin-Cytokine Fusion Protein Biotin Binding Assay.

2.8.9 IL-2 Enzyme Amplified Sensitivity Immunoassay (EASIA)

An EASIA assay (BioSource) was performed to assess the concentration of STV/IL-2 fusion protein that was produced using the methods outlined in this thesis. Microtitre wells coated with a blend of monoclonal antibodies (Mab 1), directed against human IL-2 epitopes, were incubated with 100µl of serial dilutions of STV/IL-2 fusion protein and 100µl of human plasma, or human IL-2 standards in human plasma and 50µl of an anti-human IL-2 monoclonal antibody labelled with HRP (Mab 2), for 2 hours at room temperature with horizontal shaking at 700rpm. After washing the wells 3 times with washing solution, 100µl of chromatogen solution was pipetted into each well and incubated for 15 minutes at room temperature with horizontal shaking at 700rpm wrapped in foil to avoid sunlight. 200µl of stop solution was added to the chromatogen in each well, and the wells were analysed using an Anthos 2010 absorbance reader at 450nm.

Chapter 3

Cloning of Streptavidin-Cytokine Fusion Proteins IL-2/STV and IL-18/STV

3.1 Introduction

3.1.1 Cloning Strategy

The aim of this study was the production of novel recombinant streptavidin-cytokine fusion proteins for use as immunoadjuvants in the treatment of cancer. These fusion proteins consist of a truncated core streptavidin sequence fused to a cytokine known to promote an anti-tumour, TH1 immune response; specifically interleukin-2 or interleukin-18, via a peptide linker region.

We are particularly interested in the application of these adjuvants to breast cancer vaccines in support of current treatment regimes. Immunotherapy of breast cancer in addition to other treatment strategies has decreased morbidity and mortality and reduced disease recurrence (Viani *et al* 2007).

Preliminary studies have investigated the potential of cytokines as immunoadjuvants to direct the immune response to cancer vaccines towards a TH1-type response (Chang *et al* 2004, Nohria and Rubin 1994). Both IL-2 and IL-18 are important cytokines which are known to have roles in the promotion of the TH1-type immune response. IL-2 has previously been used in studies as a potential cancer therapy (Macfarlane *et al* 1995, Smith 1997) and is FDA approved for use in clinical therapy of renal cell carcinoma (Schmidinger *et al* 2004). IL-18, either independently or in combination with IL-12, has been investigated as a vaccine for reducing tumour growth and metastasis in models of immunogenic tumour (Coughlin *et al* 1998, Lissoni *et al* 2000, Merendino *et al* 2001, Elissa *et al* 2005, Vidal-Vanaclocha *et al* 2006). Both of these cytokines are therefore considered to be promising candidates for incorporation into novel immunoadjuvants for use with cancer vaccines.

Streptavidin has been shown to be immunogenic in humans, with little toxicity when given as an intradermal or subcutaneous vaccine (Dr A. Maraveyas, personal communication), and it is hypothesised that this may be used to boost a general immune response. Streptavidin has been previously used in fusion proteins for the targeting and delivery of drugs.

In order to produce the recombinant fusion proteins, the initial phase of this work focused on generating DNA expression constructs encoding the proteins using standard cloning and recombinant DNA techniques. These constructs were later used for production of the proteins in bacterial expression systems.

3.1.2 N-Terminal Truncated Streptavidin Core Protein Sequence

The truncated streptavidin core protein sequence (described in Section 1.8.3), rather than the full length native streptavidin sequence, was used for construction of the recombinant proteins since it is more stable and is expressed more efficiently *in vitro* than full length streptavidin (Sano *et al* 1995). The core streptavidin sequence forms the N-terminal component of the recombinant fusion proteins. Streptavidin has been previously been successfully incorporated at either the C-terminal or N-terminal of fusion proteins, suggesting that for these novel streptavidin-cytokine fusion proteins the streptavidin could be placed in either position allowing the flexibility of choice of position to the system. These recombinant proteins described, which have streptavidin fused to the C-terminal, include streptavidin-protein A (Ohno *et al* 1996) and streptavidin-metallothionein (Sano *et al* 1992). There have been many fusion proteins including streptavidin fused to the N-terminal of single chain antibodies (Dubel *et al* 1994, Schultz *et al* 2000).

Since the sequence encoding core streptavidin does not include a native signal peptide region and hence no start codon, this was compensated for by using an expression vector containing a sequence encoding an upstream signal peptide with integral start codon. Additionally, the sequence encoding core streptavidin does not contain the native streptavidin stop codon, so the use of this sequence allowed continuous translation of the core streptavidin followed by the linker and C-terminal fusion protein.

The core streptavidin coding sequence would be cloned from the full length streptavidin cDNA sequence contained in cloning plasmid pUC8-SZ, which comprises the streptavidin coding sequence ligated into cloning vector pUC8 (Argarana *et al* 1986).

3.1.3 C-Terminal Cytokine Sequence (IL-2 or IL-18)

IL-2 (cloned by RT-PCR from mRNA isolated from PBMCs) or IL-18 (cloned from full length IL-18 clone in plasmid IOH7160) was placed at the C-terminal of the proposed recombinant fusion protein. Both native IL-2 and native IL-18 contain additional amino acids towards the N-terminus of the immature peptide, which are not found in the mature protein. In particular, production of correctly folded IL-18 depends on its prior synthesis as Pro-IL-18 which is then processed to mature IL-18 by cleavage of the pro-peptide (Section 1.7.3). The streptavidin-cytokine fusion proteins described here were designed to incorporate IL-18 or IL-2 at the C-terminal to promote correct folding of the protein. Previous studies have shown that IL-18 can be efficiently produced as a C-terminal fusion molecule without requiring the pro-peptide sequence (Kim *et al* 2001).

3.1.4 Linker Region

The core streptavidin and cytokine regions of the fusion protein will be separated by a short polypeptide linker region to allow space and flexibility for the fused protein

sequences to fold correctly. The linker; Serine, Serine, Glycine, Glycine, Glycine, Gycine (SSGGGG) has been used previously to link mature IL-18 cDNA to ovalbumin cDNA, which was expressed as a biologically active fusion protein in HeLa cells (Kim *et al* 2001).

The linker region is generated by annealing overlapping complementary synthetic oligonucleotides. The 5′ overhanging end of the linker region of the construct will be ligated to the 3′ end of the streptavidin sequence and the 3′ overhanging ends will be ligated to the 5′ end of the IL-2 sequence or IL-18 sequence (see Figure 3.1).

3.1.5 Expression Vector

Expression of the fusion proteins was carried out in One Shot®BL21(DE3)pLysS Chemically Competent *E. coli* cells (Invitrogen) using the pCR®T7/NT-TOPO® bacterial expression systems. This system has been chosen for its ease of use and potential to generate large quantities of protein The pLysS plasmid contained in the One Shot®BL21(DE3)pLysS cells reduces basal levels of expression of the gene of interest, which is useful when the expressed protein is toxic to *E. coli* as is the case with streptavidin (Studier and Moffat 1987, Sano amd Cantor 1990).

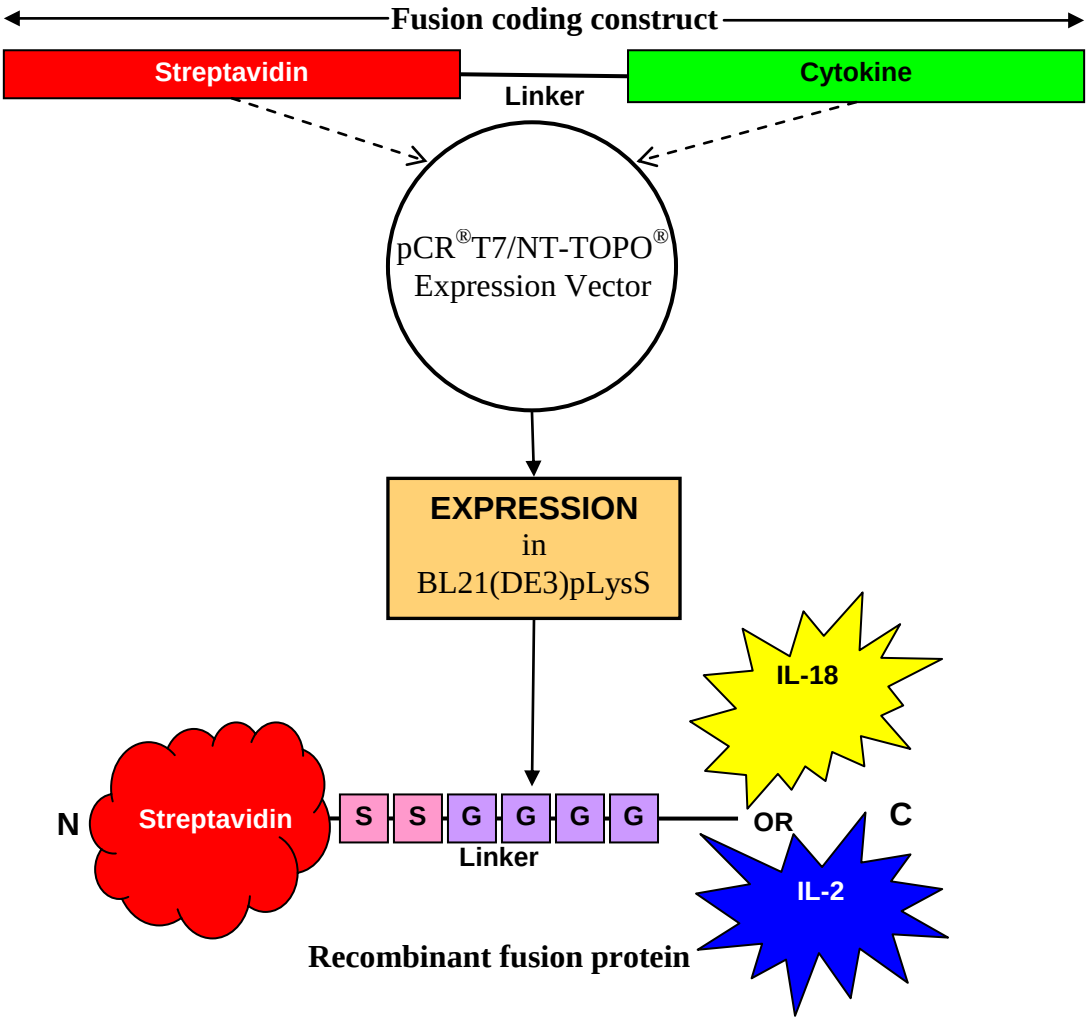

Figure 3.1 Schematic diagram showing the design of the recombinant streptavidin-cytokine fusion proteins.

3.2 Cloning of IL-2 DNA Coding Sequence into Cloning Vector pGEM®-T

3.2.1 RT-PCR Amplification of IL-2 Coding Sequence from Unstimulated PBMC and Tonsil RNA

In order to transcribe and amplify the IL-2 coding sequence, cDNA synthesis and RT-PCR (Section 2.4) was attempted using total RNA extracted from unstimulated PBMC and unstimulated tonsil (provided as a kind gift from Dr Karen Smith, Medical Research Laboratory, University of Hull) as a template. Oligonucleotide primers were designed to amplify the coding sequence of IL-2; primer target sites were chosen to flank the start (forward primer) and stop (reverse primer) codons of IL-2 (Entrez accession: X01586) (Figure 3.2). The primers were designed such that both the sense and antisense primers had similar melting temperatures and where possible were designed with a C or G base at the end of the primer in order increase the stability of annealing between the primer and the template DNA (Innes *et al* 1990). Attempted amplification of the coding sequence for IL-2 from cDNA reverse transcribed from unstimulated PBMC total RNA or unstimulated tonsil total RNA using Sensiscript® or Omniscript® Reverse Transcriptase (Section 2.4.1) and ProofStart™ DNA polymerase (Section 2.4.2) using various annealing temperatures and magnesium concentrations did not provide a product which could be visualized following separation by agarose gel electrophoresis (Section 2.4.3); therefore isolation of PBMC from whole blood (Section 2.2) and stimulation of the production of IL-2 mRNA by the isolated PBMC using treatment with PMA was undertaken (Section 2.2.1), in order to provide a higher concentration of the IL-2 coding sequence template.

(A) IL-2F (Sense Primer): 5' CCTCAACTCCTGCCACAATGTAC 3'

IL-2R (Antisense Primer): 5' TCAAGTTAGTGTTGAGATGATGC 3'

(B) Amplified IL-2 Sequence:

5' CCTCAACTCCTGCCACAATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTC
TTGCACTTGTCACAAACAGTGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAA
CTGGAGCATTTACTGCTGGATTTACAGATGATTTTGAATGGAATTAATAATTACAAGAA
TCCCAAACTCACCAGGATGCTCACATTTAAGTTTTACATGCCCAAGAAGGCCACAGAAC
TGAAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTA
GCTCAAAGCAAAAACTTTCACTTAAGACCCAGGGACTTAATCAGCAATATCAACGTAAT
AGTTCTGGAACTAAAGGGATCTGAAACAACATTCATGTGTGAATATGCTGATGAGACAG
CAACCATTGTAGAATTTCTGAACAGATGGATTACCTTTTGTCAAAGCATCATCTCAACA
CTGACTTGA 3'

Figure 3.2 IL-2 Oligonucleotide Primers (A) and DNA coding sequence (B) showing the target of the forward and reverse primers for amplification.

(A) Sense primer shown in green, antisense primer shown in blue. (B) Sense primer target site shown in green with the start codon shown underlined twice in red and antisense primer target site shown in blue with stop codon shown underlined once in red. The expected product of amplification using the primers described in (A) is 479 bp.

3.2.2 RT-PCR Amplification of IL-2 Coding Sequence from PMA-Stimulated PBMC RNA

(i) Amplification using ProofStart™ DNA Polymerase

Fresh RNA samples were prepared from PMA-stimulated PBMC (Section 2.3) and cDNA was generated from this RNA using either Sensiscript® or Omniscript® Reverse Transcriptase (RT) (Section 2.4.1). Sensiscript® RT is a high sensitivity RT that can produce cDNA from nanograms of template RNA. PCR amplification was then carried out using ProofStart™ DNA Polymerase (Section 2.4.2) with IL-2F sense PCR primer and IL-2R antisense PCR primer, and thermal cycling conditions with an annealing temperature of 55ºC for 35 cycles. ProofStart™ is a high fidelity thermostable DNA polymerase which has a 10 times lower error rate of copying than standard *Taq* polymerases (ProofStart™ PCR Handbook, Qiagen 2005).

Products amplified from cDNA produced using Omniscript® RT showed a band of approximately 500bp when visualized following separation on a 1.3% w/v agarose gel (Figure 3.3). This corresponds approximately to the expected size of the IL-2 coding sequence, which was predicted to be 479bp. Additional products seen as bands on the gel at lower molecular weight were also visible, suggesting that some unspecific annealing of the primers to the DNA may have occurred. Products amplified from cDNA produced using Sensiscript® RT showed a single discrete band of approximately 500bp, which corresponds well to the predicted size of the required IL-2 coding sequence. No additional bands were observed suggesting that little or no unspecific annealing of the primers to the DNA had occurred (Figure 3.3). The product generated by RT-PCR using Sensiscript® RT was therefore used for further cloning steps.

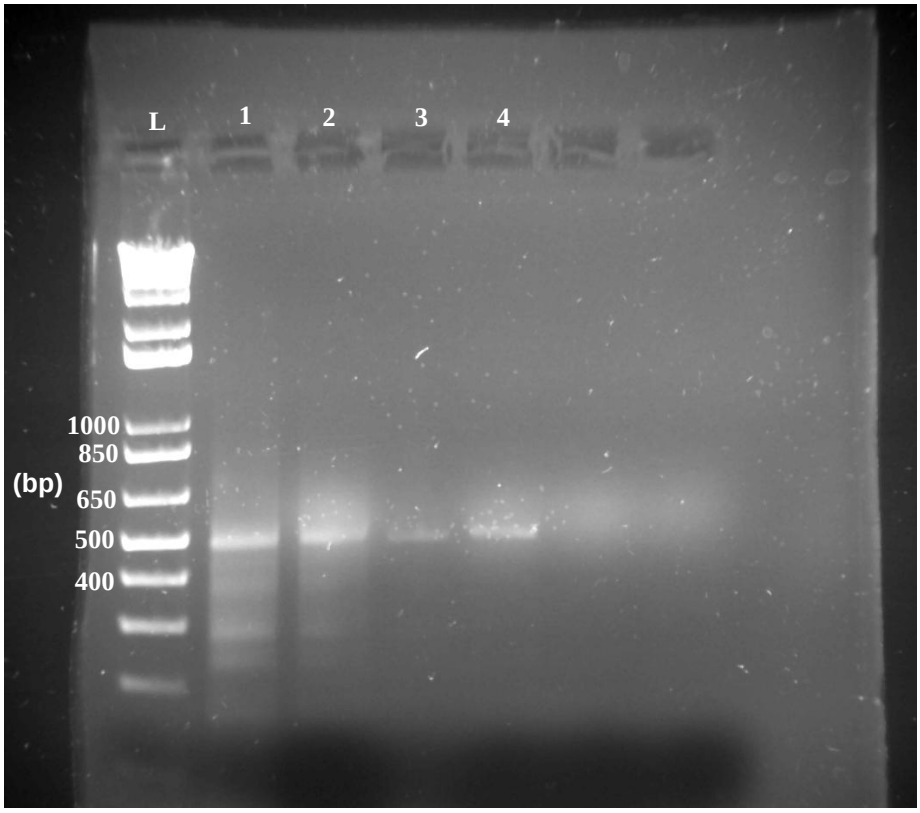

Figure 3.3 PCR amplification of IL-2 coding sequence with ProofStart™ DNA Polymerase using a cDNA template generated from PMA-stimulated PBMC RNA.

A 1.3% w/v agarose gel showing the products of PCR amplification of IL-2 coding sequence using ProofStart™ DNA Polymerase and IL-2 sense and antisense PCR primers on cDNA generated from mRNA extracted from stimulated PBMC. PCR products were separated by electrophoresis at 120V for 60 minutes and visualized using a UV transilluminator.

Lanes: (L) $1Kb^{+}$ DNA molecular weight marker (Invitrogen); (1,2) 20µl of PCR products generated from cDNA prepared from PBMC RNA using Omniscript® RT; (3,4) 20µl of PCR products generated from cDNA prepared from PBMC RNA using Sensiscript® RT. Numbers on the left hand side correspond to size in base pairs (bp).

(i) Amplification using Taq DNA polymerase

Amplification of the IL-2 product from PCR using ProofStart™ DNA polymerase with *Taq* DNA polymerase (Section 2.4.2) was carried out in order to provide sufficient IL-2 cDNA for cloning into vector pGEM®-T, and to provide A-tail overhangs for ligation of the IL-2 coding sequence into the pGEM®-T vector. Analysis of the products by electrophoretic separation on a 1.3% agarose gel showed a clear band visible at approximately 500bp, corresponding well to the predicted size of the IL-2 coding sequence (479bp) (Figure 3.4).

3.2.3 Ligation of PCR-Amplified IL-2 Coding Sequence into Cloning Vector pGEM®-T

In order to store the PCR-amplified IL-2 coding sequence, and for ease of its sub-cloning at a later date, the PCR products generated by amplification with *Taq* DNA polymerase, using sense primer IL-2F and antisense primer IL-2R, (Section 3.2.3) were ligated into the pGEM®-T Vector (Section 2.5.1). A circle vector map of the pGEM®-T vector is provided in the appendix (Figure A.1) detailing the sequence reference points and the multiple cloning site (Figure A.2).

The resulting ligation reaction products were then used to transform *E. coli* by heat shock (Section 2.5.2), and the transformed bacteria were cultivated overnight on agar plates. Nine individually selected discrete isolated colonies (named colony 1 to colony 9) were picked and cultured overnight in LB broth (Section 2.5.3). Following overnight culture the plasmids were extracted and purified (Section 2.5.4). Each plasmid preparation was PCR amplified with *Taq* DNA polymerase using sense and antisense IL-2 primers IL-2F and IL-2R to screen for preparations containing the pGEM®-T

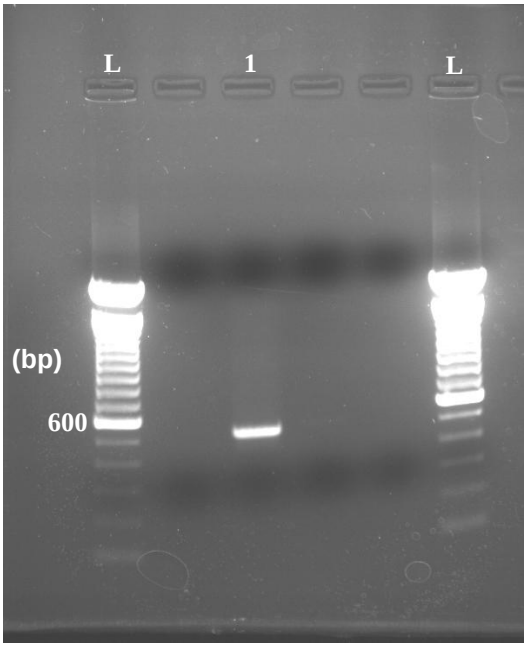

Figure 3.4 PCR amplification of IL-2 coding sequence with *Taq* DNA Polymerase using PCR products generated by PCR amplification of IL-2 coding sequence with ProofStart DNA polymerase (Figure 3.3).

1.3% w/v agarose gel showing the products of PCR amplification of IL-2 coding sequence generated using ProofStart DNA polymerase (see Figure 3.3) and further amplified using *Taq* DNA Polymerase**.** PCR products were generated using IL-2F sense and IL-2R antisense PCR primers. The products were separated on a 1.3% w/v agarose gel by electrophoresis at 120V for 60 minutes and visualized using a UV transilluminator.

Lanes: (L) 100bp DNA molecular weight marker (Invitrogen); (1) 20µl IL-2 PCR products. Numbers on the left hand side correspond to base pairs (bp).

plasmid with the IL-2 coding sequence insert (IL-2/pGEM®-T). Visualization of the PCR products from this reaction on agarose gel after separation by elecrophoresis, showed the plasmid DNA in supercoiled (S) and linear (L) state at molecular weight exceeding the highest band on the molecular weight marker ladder. One of the clones (Lane 3) showed an additional band at around the 500bp mark, corresponding approximately to the desired IL-2 sequence insert of predicted size 479bp (Figure 3.5).

3.2.4 Digestion of pGEM®-T Plasmid Clones 1 and 3 with Restriction Enzymes *Vsp*1 and BstY1

In order to gain further evidence that plasmid clone 3 (Section 3.2.4) was carrying the IL-2 coding sequence insert, digestion of the plasmid preparations of clone 3 and clone 1 was conducted using restriction enzymes *Vsp*I and *Bst*YI as described in Section 2.3.5. Digested and undigested plasmid samples were seperated by electrophoresis on agarose gel (Figure 3.6).

The expected products of digestion of pGEM-T containing the IL-2 insert with *Vsp*I are 59bp, 541bp, 1235bp and 1706bp. The expected products of digestion of pGEM-T containing no insert with *Vsp*I are 59bp, 371bp, 1235bp and 1805bp. Six fragments are expected from the digestion of p-GEM-T with *Bst*YI, with the largest fragment expected to be 2576bp with IL-2 inserted compared to 2106bp without an insert.

The digestion of the plasmid preparation of clone 3 (thought to contain the IL-2 coding sequence insert) by *Vsp*I resulted in two bands which are visible at approximately 2000 and 1400bp. The digestion of the plasmid preparation of clone 1 (not thought to contain

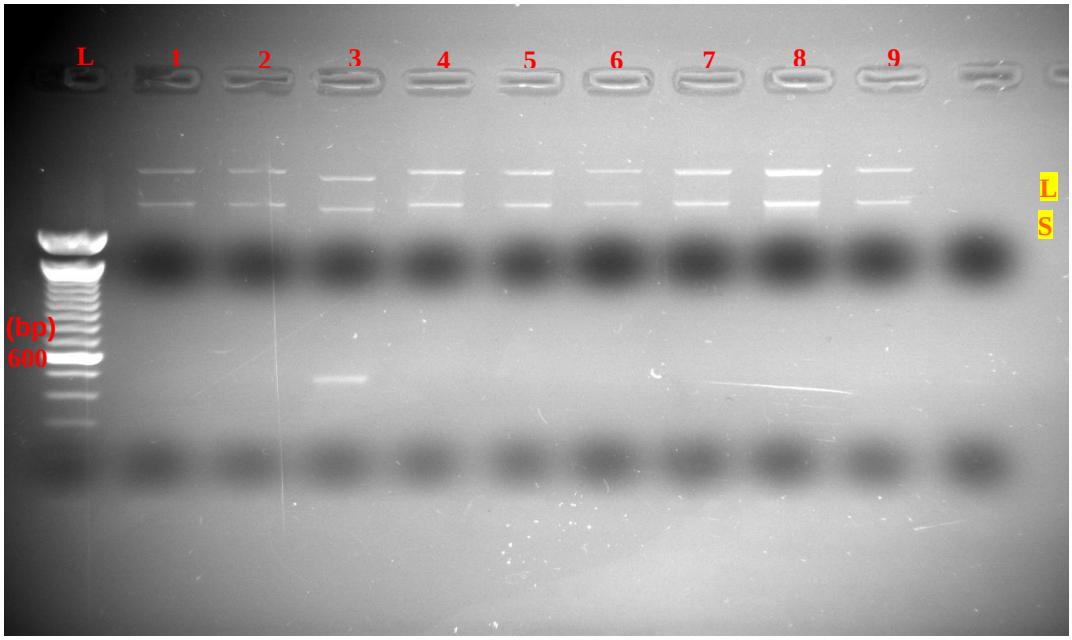

Figure 3.5 PCR products generated from the screening of nine independently selected discrete isolated colonies.

PCR was conducted on plasmid preparations from overnight cultures of single colonies from the transformation of *E. coli* using pGEM-T ligated with the PCR-amplified IL-2 coding sequence. PCR amplification was carried out using *Taq* DNA polymerase with primers IL-2F and IL-2R. The PCR products were separated on a 1.3% w/v agarose gel by electrophoresis at 120V for 60 minutes and visualized using a UV transilluminator. Lanes: (L) 100bp DNA molecular weight marker; (1-9) 10µl of PCR product amplified from plasmid preparations of overnight cultures grown from single clone colonies. Numbers on the left hand side correspond to base pairs (bp). L and S on the right hand side correspond to linear and supercoiled DNA.

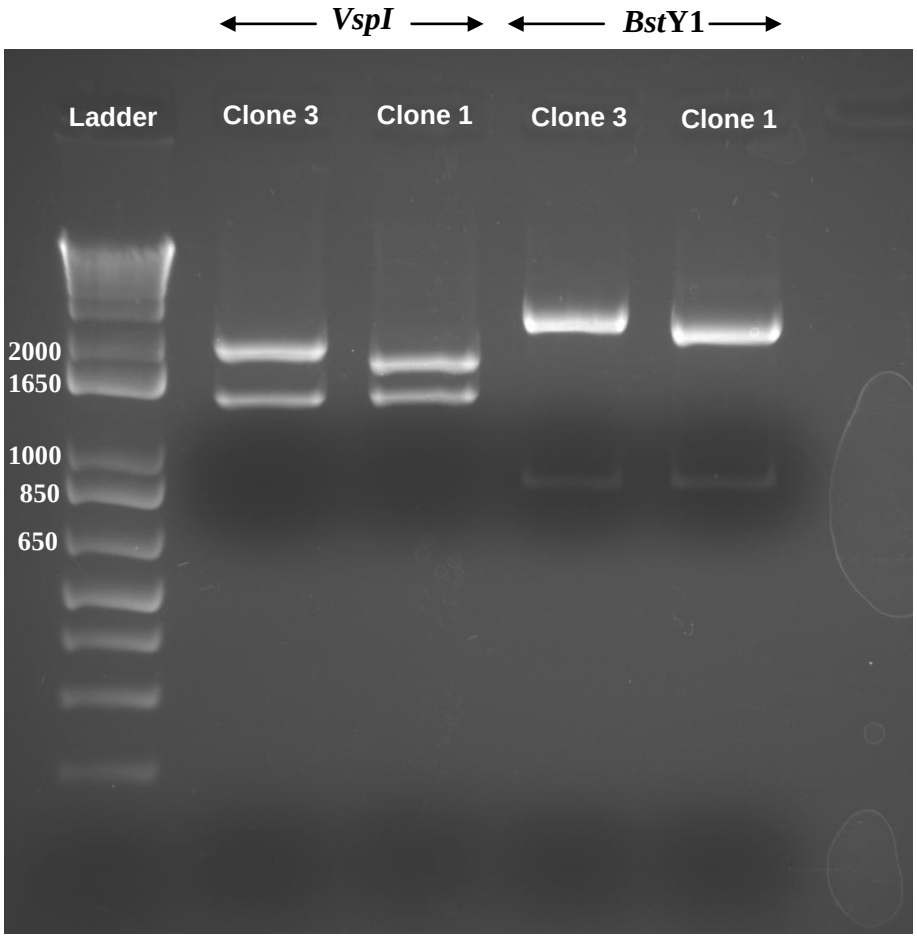

Figure 3.6 1.3 Digestion products from the restriction digestion of clone 1 and 3 with *Vsp*I or *Bst*YI.

Digestion products were separated by electrophoresis at 120V for 1 hour on a 1.3% agarose gel and visualized using a UV transilluminator.

Lanes: (L) 1Kb^{+} DNA Ladder; (Clone 3) 20µl of *Vsp*I or *BstYI* restriction digest product from the digestion of plasmid from clone 3; (Clone 1) 20µl *Vsp*I or *BstYI* restriction digest product from the digestion of plasmid from clone 1.

the IL-2 coding sequence insert) resulted in two bands which are visible at approximately 1700bp and 1500bp. These bands show larger than expected digestion products in the samples from both clone 1 and clone, however, as larger sized bands are visible in the sample containing the digestion products of clone 3, the IL-2 coding sequence insert is more likely to be present in this clone.

The digestion of the plasmid preparation of clone 3 by *Bst*YI resulted in two visible bands; one above 2000bp and another 850bp. The digestion of the plasmid preparation of clone 1 by *Bst*YI also resulted in two visible bands; one at around 850bp and a higher band which is visible at about 2000bp but at a lower size than the corresponding band seen in clone 3. As visualization of the digestion of clone 3 revealed a larger sized band above 2000bp compared to clone 1, it was suggested that this clone, as expected, may contain the required IL-2 coding sequence insert. Due to the evidence presented here, clone 3 (named pGEM-T/IL-2_3**)** was selected for sequencing.

3.2.5 DNA Sequence Analysis of Clone pGEM®-T/IL-2_3

To confirm the plasmid sequence of plasmid pGEM®-T/IL-2_3 from clone 3 (3.2.3) an ethanol precipitated plasmid preparation (Section 2.5.8) from an overnight culture of this clone was subjected to DNA sequence analysis (Section 2.5.9) (MWG Biotec) using the T7 forward promoter primer corresponding to the T7 promoter sequence contained in the pGEM®-T vector (Figure A.1 and A.2). DNA Sequencing confirmed the required IL-2 coding sequence had been successfully inserted in to the pGEM®-T vector, but showed that a single point mutation of the original IL-2 sequence(Entrez accession: X01586) had occurred at nucleotide 163 coding for amino acid 55 Lysine (AAA) resulting in the substitution of this amino acid by Glycine (GAA). The presence of this point mutation was confirmed by reverse sequencing of the same plasmid clone

using the vector SP6 promoter primer (Figure A.1 and A.2). The introduction of this mutation is not surprising as the IL-2 coding sequence has undergone 2 rounds of PCR amplification, increasing the chance of a copy error.

3.2.6 Site-Directed Mutagenesis of Clone pGEM-T/IL-2_3

In order to correct the point mutation which was identified in the IL-2 coding sequence in clone pGEM-T/IL-2_3, site-directed mutagenesis was carried (Section 2.5.10) out using the primers described below (The base corresponding to the site of mutation is shown in blue).

g204a GAATTAATAATTACAAGAATCCCAAACTCACCAGGATGCTCACA

antisense g204a GTGAGCATCCTGGTGAGTTTGGGATTCTTGTAATATTAATTC

Five clones were selected from agar plates which had been cultivated overnight with XL1-Blue supercompetent *E. coli* which had been transformed by heat shock with the plasmid resulting from the site-directed mutagenesis of plasmid pGEM-T/IL-2_3. These clones were cultured overnight in LB broth and plasmids were extracted and purified from the bacterial cultures. Ethanol precipitated samples of these plasmid preparations (Section 2.5.8) were analysed by DNA sequencing using the T7 promoter primer (Section 2.5.9). Sequencing results compared against the desired IL-2 DNA sequence using the NCBI BLAST bl2seq engine (*http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi*) (Tatusova et al 1999). Results of this comparison showed that the one clone from the site directed mutagenesis of plasmid pGEM-T/IL-2_3 gave a 100% match confirming that the base mutation present in this clone had been successfully mutated back to the original IL-2 coding sequence.

3.2.7 Production and Cloning of IL-2 with Modified Ends for Overlapping PCR

In order to produce the streptavidin-linker-IL-2 coding sequence construct , an IL-2 coding sequence containing a 5' engineered coding region for the linker peptide (SSGGGG) was designed. This was intended to link the modified end of the IL-2 coding sequence to the modified end of the streptavidin coding sequence (Section 3.4.2) by overlapping PCR (Section 3.5). The IL-2+linker coding sequence was produced by PCR amplification using the sense IL-2LinkF1 and antisense IL-2R primers (Section 2.4.2) and *Taq* DNA polymerase. The sense primer IL-2LinkF was designed to engineer the linker region coding sequence onto the 5' region of the mature IL-2 coding sequence by PCR amplification. This region will then overlap with a similar region engineered onto the end of the core streptavidin coding sequence in an overlapping PCR

The products generated by this PCR were separated by gel electrophoresis and visualised using a UV transilluminator. A visible band corresponding to the expected product size of 496 was excised from the gel, extracted and purified (Section 2.4.4) using a QIAquick® Gel Extraction Kit. These purified PCR products were ligated into the pGEM®-T cloning vector for storage and ease of sub-cloning as described previously (Section 3.2.3).

In order to select clones containing the IL-2+linker coding sequence insert in the pGEM®-T vector, Direct colony PCR (2.5.6) using *Taq* polymerase and the sense T7 and antisense SP6 promoter primers on 10 discrete isolated *E. coli* colonies from the ligation of the IL-2+linker coding sequence into the pGEM®-T vector was undertaken to determine which colonies possessed the required insert.

The expected product size of the amplification of the pGEM®-T vector with primers T7 and SP6, as described above, is 622bp when the amplified vector contains the required insert. A band of approximately this size was observed in 3 lanes (lanes 5, 6 and 7) when the PCR products were separated by electrophoresis on agarose gel (Figure 3.7). Lanes 3 and 10 showed a band at a low molecular weight (~300bp) bands and may contain DNA fragments. Other lanes each contained one high intensity band of less than 200bp which corresponds approximately to an expected product of 152bp between the primers should the plasmid contained no inserted DNA. DNA sequence analysis of the clones from lanes 5, 6 and 7 (Section 2.5.9) (MWG Biotech) showed 100% match with the IL-2+linker coding sequence when analysed with the NCBI BLAST bl2seq engine (as described in Section 3.2.6)

3.3 Cloning of IL-18 DNA Coding Sequence into Cloning Vector pCR®T7/CT-TOPO®

3.3.1 RT-PCR Amplification of IL-18 Coding Sequence from Unstimulated Tonsil and PBMC total RNA

As with the cloning of IL-2 into the pGEM®-T cloning vector (Section 3.2) cDNA synthesis and RT-PCR of total RNA extracted from tonsil and whole blood was performed using either Omniscript® or Sensiscript® RT, and ProofStart™ DNA polymerase with IL-18F sense and IL-2R antisense primers (Section 2.4). IL-18 primers were designed flanking the start (forward primer) and stop (reverse primer) codons of IL-18 (Entrez accession number: NM_001562) (Figure 3.8).

A number of RT-PCR amplifications were carried out using a variety of annealing temperatures, cycle numbers and magnesium concentrations in order to optimise the PCR conditions. Using an annealing temperature of 60ºC for 35 cycles, with no added

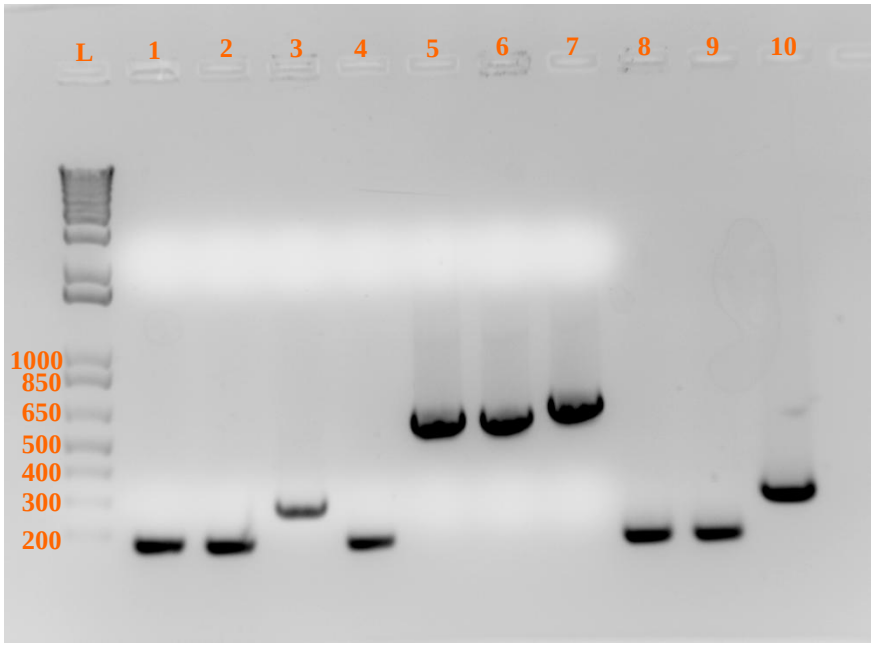

Figure 3.7 1 IL-2+ linker coding sequence PCR products generated from colony PCR.

1.3% w/v agarose gel showing the products obtained from PCR with *Taq* DNA Polymerase using the T7 sense and SP6 antisense primers on single colonies from the transformation of *E. coli* with the products of the IL-2+linker ligation into pGEM®-T. The products were separated by electrophoresis at 120V for 1 hour on a 1.3% w/v agarose gel and visualized using a UV transilluminator.

Lanes: (L) $1Kb^{+}$ molecular weight marker; (1-10) 40µl of PCR product from an independently selected discrete isolated single colony.

(A) IL-18 Forward Primer: 5′GTCGCAGGAATAAAGATGGC 3′

IL-18 Reverse Primer: 5′GCTAGTCTTCGTTTTGAACAG 3′

(B) Amplified IL-18 DNA coding Sequence

GTCGCAGGAATAAAGATGGCTGCTGAACCAGTAGAAGACAATTGCATCAACTTTGTGGCAATGAAATTTA
TTGACAATACGCTTTACTTTATAGCTGAAGATGATGAAAACCTGGAATCAGATTACTTTGGCAAGCTTGA
ATCTAAATTATCAGTCATAAGAAATTTGAATGACCAAGTTCTCTTCATTGACCAAGGAAATCGGCCTCTA
TTTGAAGATATGACTGATTCTGACTGTAGAGATAATGCACCCCGGACCATATTTATTATAAGTATGTATA
AAGATAGCCAGCCTAGAGGTATGGCTGTAACTATCTCTGTGAAGTGTGAGAAAATTTCAACTCTCTCCTG
TGAGAACAAAATTATTTCCTTTAAGGAAATGAATCCTCCTGATAACATCAAGGATACAAAAAGTGACATC
ATATTCTTTCAGAGAAGTGTCCCAGGACATGATAATAAGATGCAATTTGAATCTTCATCATACGAAGGAT
ACTTTCTAGCTTGTGAAAAAGAGAGAGACCTTTTTAAACTCATTTTGAAAAAAGAGGATGAATTGGGGGA
TAGATCTATAATGTTCACTGTTCAAAACGAAGACTAGC

Figure 3.8 IL-18 Oligonucleotide Primers (A) and DNA coding sequence (B) showing the target of the forward and reverse primers for amplification.

(A) Sense primer shown in green, antisense primer shown in blue. (B) Sense primer target site shown in green with the start codon shown underlined twice in red and antisense primer target site shown in blue with stop codon shown underlined once in red. The expected product of amplification using the primers described in (A) is 598 bp.

magnesium, faint bands could be observed when the PCR products were visualized on a 1.3% w/v agarose gel, suggesting that the primers were annealing to the cDNA; however, this result was not successfully reproduced (*data not shown*).

3.3.2 RT-PCR Amplification of IL-18 Coding Sequence from SAC-Stimulated PBMC

In order to provide a higher concentration of the IL-18 coding sequence template, *Staphylococcus aureus* (SAC)-stimulated PBMC were obtained (provided as a gift by Katherine Oliver, Medical Research Laboratory, University of Hull). RT-PCR of total RNA extracted from SAC-stimulated PBMC (Section 2.3) was performed as previously (Section 3.3.1). Visualization on a 1.3% w/v agarose gel after separation by electrophoresis (Section 2.4.3) showed no bands (*data not shown*), suggesting that IL-18 coding sequence template from RNA extracted from SAC-stimulated PBMC was too low to amplify using RT-PCR.

Because IL-18 was hard to obtain reproducibly from human RNA, a plasmid containing the full IL-18 coding sequence (plasmid IOH7160) was obtained from Invitrogen for use as a template to amplify the IL-18 coding sequence with RT-PCR.

3.3.3 Production and Cloning of IL-18 with Modified Ends for Overlapping PCR

As when designing the IL-2+linker region coding sequence (Section 3.2.7) a IL-18 coding sequence containing a 5' engineered coding region for the linker peptide (SSGGGG) was designed. This IL-18+linker coding sequence was produced by RT-PCR amplification using the sense primers IL-18LinkF and the antisense primer IL-18R.

In order to create the IL-18+linker coding sequence RT-PCR was conducting using plasmid IOH7160 and either ProofStart™ or *Taq DNA* polymerase with the IL-18linkF and IL-18R primers. Thermocycling was carried out using an annealing temperature of 60ºC for 25 cycles (Section 2.4.2). Visualization of PCR amplification products on 1.3% agarose gel showed a band at approximately 500bp in the sample amplified with *Taq* DNA polymerase, corresponding approximately to the expected product size of 492bp. This DNA band was excised from the agarose gel and the DNA was then extracted and purified (Section 2.4.4) as previously (Section 3.2.7) for ligation into pGEM®-T (*data not shown*).

3.3.4 Cloning of IL-18 into pCR®T7/CT-TOPO®

The products of RT-PCR with *Taq* DNA polymerase using primers IL-18/linker F and IL-18R were ligated into cloning vector pCR®T7/CT-TOPO® (Section 2.5.1) for storage and sub-cloning, and used to transform One Shot® TOP10F′ *E. coli*. The transformation reaction mixture was spread onto agar plates and cultivated overnight (Section 2.5.2). A circular map of the pCR®T7/CT-TOPO vector (Figure A.3) and the multiple cloning site (Figure A.4) is provided in the appendix.

Plasmid DNA extracted and purified (Section 2.5.4) from the overnight culture of discrete isolated colonies from these agar plates (Section 2.5.3) were digested using *Hind*III (Section 2.5.5), which cuts once within IL-18+linker coding sequence and once within the pCR®T7/CT-TOPO® vector sequence, to give a 478bp fragment.

Visualization of the digested products on agarose gel following separation by electrophoresis showed a band at approximately 500bp in two of the randomly selected clones (Lanes 1C and 4C) which corresponds approximately with the digestion product

size of 478bp (Figure 3.9) as discussed above. Samples 1 and 4 (clones named IL18pCRT7.01 and IL18pCRT7.04) were ethanol precipitated (Section 2.5.8) and subjected to DNA sequence analysis (Section 2.5.9) (MWG Biotech) using the T7 promoter primer. Sequencing results of clone IL18pCRT7.01 showed 100% match with the desired IL-18+linker coding sequence insert.

3.4 Cloning of Streptavidin into pCR®T7/CT-TOPO®

3.4.1 RT-PCR Amplification of Full length and Core Streptavidin (STV)

Cloning plasmid pUC8-SZ containing the full length streptavidin coding squence (provided as a kind gift from Takeshi Sano, Centre for Molecular Imaging Diagnosis and Therapy, Harvard Medical School) was obtained and used to transform XL1-Blue cells (Section 2.4.1). The transformation reaction was spread onto agar plates and cultured overnight. Discrete isolated cultures were selected from the agar plates and cultivated overnight in LB broth (Section 2.4.2). Plasmid DNA was extracted from the overnight culture (Section 2.5.4), then separated by electrophoresis and visualized on a 1.3% agarose gel to confirm the presence of the plasmid. Each culture analysed showed bands indicating the presence of the DNA plasmid (*data not shown*).

Primers were designed to amplify the full length streptavidin DNA coding sequence (Figure 3.10A) and the modified core streptavidin coding sequence with linker peptide (Figure 3.10B) PCR amplification of the full length and core streptavidin (STV)+linker peptide coding sequences from the plasmid pUC8-SZ using *Taq* DNA polymerase and full length streptavidin primers; Streptavidin F and Streptavidin R, or core streptavidin primers STVF and STVlinkR was carried out by (Section) using *Taq* DNA polymerase at the conditions describes previously (Section 3.3.1). Visualization on 1.3% w/v agarose gel after separation by electrophoresis (Section 2.4.3) showed a band at

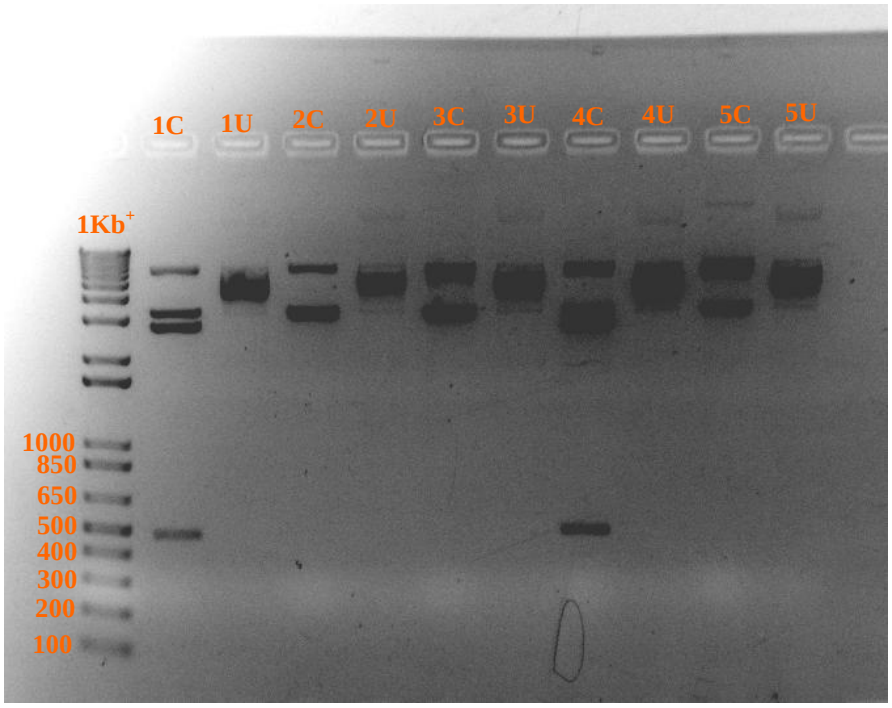

Figure 3.9 Products of the restriction digestion of pCR®T7/CT-TOPO® +/- IL-18+linker coding sequence insert with *Hind*III.

1.3% w/v agarose gel showing the products obtained from the restriction digestion of plasmid DNA from the ligation of IL-18+linker coding sequence into pCR®T7/CT-TOPO® with the restriction enzyme *Hind*III. The products were separated by electrophoresis at 120V for 1 hour on a 1.3% w/v agarose gel and visualized using a UV transilluminator. Lanes denoted with a C contain the products cut with the restriction enzyme *Hind*III and those denoted with a U contain the corresponding U uncut plasmid. Lanes**:** (Ladder) 1Kb$^+$ DNA molecular weight marker; (1-5C) 20µl of digested plasmid from an independently selected discrete isolated overnight culture grown from a single clone colony; (1-5U) 20µl of uncut plasmid from an independently selected discrete isolated overnight culture from a single clone colony.

(A) Primers for the amplification of full length streptavidin:

sense primer (Streptavidin F): 5′ATGCGCAAGATCGTCGTTGCAG 3′

antisense primer streptavidin R: 5′CTACTGCTGAACGGCGTCGAGC 3′

(B) Primers for the amplification of core streptavidin (STV) + linker peptide (SSGGGG)

sense primer (STV F): 5′ GGCATCACCGGCACCTGGTA 3′

antisense primer (STVlinkR) 5′CCACCTCCACCAGAACTCACCTTGGTGAAGGTG 3′

(C) Amplified full length streptavidin sequence

ATGCGCAAGATCGTCGTTGCAGCCATCGCCGTTTCCCTGACCACGGTCTCGATTACGGCCAGCGCTTCGG
CAGACCCCTCCAAGGACTCGAAGGCCCAGGTCTCGGCCGCCGAGGCCGGCATCACCGGCACCTGGTACAA
CCAGCTCGGCTCGACCTTCATCGTGACCGCGGGCGCCGACGGCGCCCTGACCGGAACCTACGAGTCGGCC
GTCGGCAACGCCGAGAGCCGCTACGTCCTGACCGGTCGTTACGACAGCGCCCCGGCCACCGACGGCAGCG
GCACCGCCCTCGGTTGGACGGTGGCCTGGAAGAATAACTACCGCAACGCCCACTCCGCGACCACGTGGAG
CGGCCAGTACGTCGGCGGCGCCGAGGCGAGGATCAACACCCAGTGGCTGCTGACCTCCGGCACCACCGAG
GCCAACGCCTGGAAGTCCACGCTGGTCGGCCACGACACCTTCACCAAGGTGAAGCCGTCCGCCGCCTCCA
TCGACGCGGCGAAGAAGGCCGGCGTCAACAACGGCAACCCGCTCGACGCCGTTCAGCAGTAG

Figure 3.10 – Oligonucleotide primers for the amplification of the full length streptavidin coding sequence (A) and core streptavidin (STV) + peptide linker coding sequence (B), and full length streptavidin DNA coding sequence showing the primer target sites for amplification.

(A) Sense primer shown in green, antisense primer shown in blue (B) Sense primer shown in green, antisense primer shown in blue, with linker peptide shown in red (C) Amplifed full length streptavidin sequence(552bp) (highlighted in yellow) with core streptavidin (354bp) highlighted in yellow.

approximately 500bp in the full length streptavidin coding sequence sample and 400bp in the STV+linker peptide coding sequence sample, corresponding approximately to the expected product sizes of 552bp and 371bp respectively. The bands were excised from the agarose gel and the DNA extracted and purified (Section 2.4.4) in preparation for ligation into pCR®T7/CT-TOPO® cloning vector (Section 3.4.2).

3.4.2 Cloning of Full Length and STV + Linker Coding Sequences into pCR®T7/CT-TOPO®

The products of RT-PCR amplification of the full length streptavidin and STV+linker coding sequences with *Taq* DNA polymerase (Section 3.4.2) were ligated into plasmid vector pCR®T7/CT-TOPO® and used to transform One Shot® TOP10F′ chemically competent *E. coli*. As previously (Section 3.4.2) plasmid DNA was extracted and purified from several overnight cultures that had been cultivated from discrete isolated colonies selected from agar plates cultured overnight with the transformation reaction. The extracted plasmid DNA was ethanol precipitated (Section 2.5.8) and subjected to DNA sequence analysis using the T7 promoter forward primer (Section 2.5.9) (MWG Biotech).

3.4.3 Site Directed Mutagenesis of the STV + Linker in pCR®T7/CT-TOPO®

A comparison of the DNA sequence analysis results (performed as described in Section 3.2.6) of the full length streptavidin coding sequence in pCR®T7/CT-TOPO® against the expected sequence confirmed that the full length streptavidin coding sequence was a 100% match to the expected sequence. The results of DNA sequence analysis of the STV+linker coding sequence in pCR®T7/CT-TOPO® showed a point mutation of A to C at nucleotide 370 of the STV+linker coding sequence which corresponds to an amino acid change of aspartic acid to alanine at amino acid 112. In order to correct the point

mutation in STV+linker, site-directed mutagenesis was conducted as described previously (Section 3.2.6) using the following primers (The base corresponding to the site of mutation is shown in blue):

c370a GTCGGCCACGCAACCTTCACCAAGGTGAGTTC

antisense c370a GAACTCACCTTGGTGAAGGTTGCGTGGCCGAC

A comparison of this mutated sequence (performed as described in Section 3.2.6) against the expected STV+linker coding sequence showed a 100% match, confirming that the site directed mutagenesis was successful. The STV+linker coding sequence was used in all following work, to produce the sequences coding for the STV/IL-2 and STV/IL-18 recombinant fusion proteins.

3.5 Overlapping RT-PCR to Produce Core Streptavidin-IL-2 (coding for recombinant fusion protein STV/IL-2) and Core Streptavidin-IL-18 (coding for recombinant fusion protein STV/IL-18)

In order to construct the full coding sequences for the STV/IL-2 and STV/IL-18 recombinant fusion proteins an overlapping PCR, in which overlapping complimentary fragments of DNA (in this case the linker peptide coding sequence) anneal to each other and act as primers, was performed which utilized the modified ends of the IL-2, IL-18 and STV coding sequences containing the linker peptide coding sequence

Overlapping RT-PCR fragments were amplified using Accuprime™ *Taq* DNA polymerase (Invitrogen) (Section 2.4.2) at an annealing temperature of 60^{o}C for 30 cycles AccuPrime™ *Taq* DNA polymerase is a blend of high fidelity DNA polymerase with *Taq* DNA polymerase, providing a higher fidelity copy rate while still leaving

overhanging ends on the RT-PCR product. This allows easier ligation into vectors designed for use with RT-PCR products from *Taq* polymerase amplification.

The amplified RT-PCR fragments were:

- **(A)** core streptavidin(STV) + linker peptide coding sequence, amplified using STV F sense and STVlinkR antisense primers (Section 3.4);
- **(B)** IL-2 + linker peptide coding sequence, amplified using IL-2LinkF and IL-2R primers (Section 3.2);
- **(C)** IL-18 + linker peptide coding sequence, amplified using IL-18LinkF and IL-18R primers (Section 3.3).

The amplification reactions were separated by agarose gel electrophoration, and the resulting DNA bands were excised, extracted from the gel and purified (Section 2.4.4).

Overlapping RT-PCR using AccuPrime™ *Taq* at an annealing temperature of 60^{o}C for 30 cycles (with no added primers) was carried out on the:

- **(C)** 5µl IL-18+linker coding sequence RT-PCR product and **(A)** 5µl STV+linker coding sequence RT-PCR product to produce the STV/IL-18 coding sequence.
- **(B)** 5µl IL-2+linker coding sequence RT-PCR product and **(C)** 5µl STV+linker coding sequence RT-PCR product to produce the STV/IL-2 coding sequence.

The products of this overlapping RT-PCR were separated by agarose gel electrophoresis, and the resulting DNA bands were excised, extracted from the gel and purified (Section 2.4.4).

3.6 Cloning of STV/IL-2 and STV/IL-18 coding sequences into pCR®T7/NT-TOPO®

The products of overlapping RT-PCR amplification of IL-2+linker coding sequence or IL-18+linker and STV+linker coding sequence to produce the STV/IL-2 and STV/IL-18 coding sequence were ligated into plasmid pCR®T7/NT-TOPO® for storage and ease of sub-culturing, and used to transform Top10F′ *E. coli* (as previously described in Section 3.4.2).

In order to identify clones of E. coli containing pCR®T7/NT-TOPO® vector with the required STV/IL-2 coding sequence insert, products of a plasmid preparation (Section 2.5.4) from overnight cultures of colonies (Section 2.5.3) selected from agar plates cultivated with the transformation reaction were digested using *Vsp*I. *Vsp*I digestions expected to produce fragments of 25bp, 640bp, 1235bp and 2340bp in vectors containing the STV/IL-2 coding sequence insert in the correct orientation (Figure 3.11). Samples containing plasmids from clones 5, 7 and 9 appear to have a band profile similar to that predicted.

3.7 DNA Sequence Analysis of the STV/IL-2 Coding Sequence in pCR®T7/NT-TOPO®

Clones 5 and 7 (Figure 3.11) were analysed by DNA sequence analysis (Section 3.5.9) (MWG biotech) using the T7 forward promoter primer. The result of this analysis showed one clone (clone 5) which matched 100% when compared with the desired STV/IL-2 coding sequence insert.

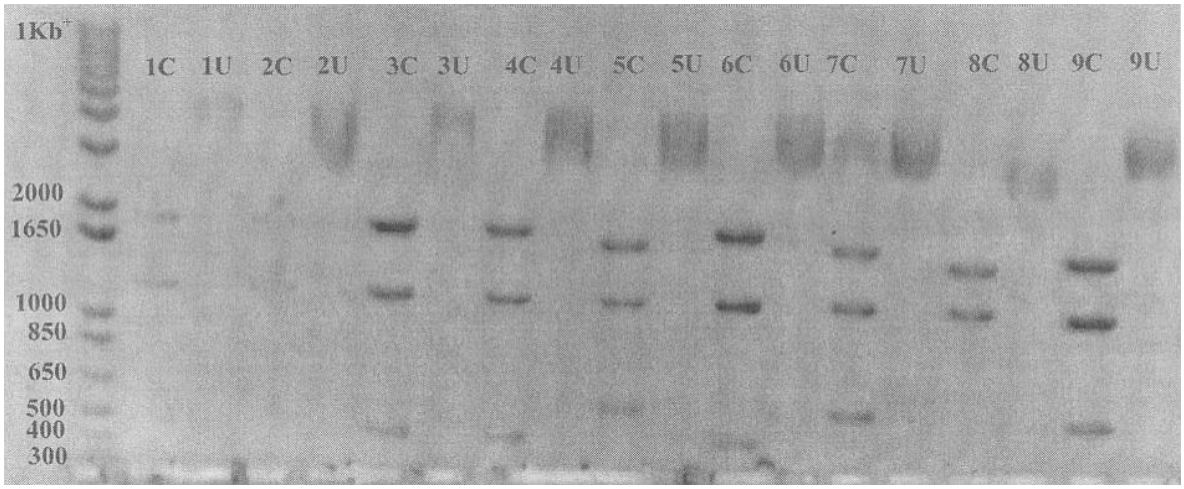

Figure 3.11 Products of the restriction digestion of pCR®T7/CT-TOPO® +/- STV/IL-2 coding sequence insert with *Hind*III.

1.3% w/v agarose gel showing the products obtained from the restriction digestion of plasmid DNA from the ligation of the STV/IL-2 coding sequence in pCR®T7/NT-TOPO® with the restriction enzyme *Vsp*I. The restriction digestion products were separated by electrophoresis at 120V for 1 hour on a 1.3% w/v agarose gel and visualized using a UV transilluminator. Lanes denoted with a C contain the products cut with restriction enzyme *Vsp*I and those denoted with a U contain uncut plasmid. Lanes**:** (Ladder) $1Kb^+$ DNA molecular weight marker; (1-9C) 20µl of digested plasmid from of an independent randomly selected overnight culture grown from a single clone colony; (1-9U) 20µl of uncut plasmid from an overnight culture grown from a single clone colony.

3.8 DNA Sequence Analysis of the STV/IL-18 coding sequence in pCR®T7/NT-TOPO®

Ten clones were subjected to DNA sequence analysis (Section 2.5.9) (MWG Biotech) using the T7 forward promoter primer. Sequence analysis results for one of the clones showed a close match with the desired STV/IL-18 coding sequence insert. On examination of the sequence it was discovered that a point mutation had occurred at nucleotide 753 which corresponds to amino acid 251 (leucine) in the section of the construct corresponding to the IL-18 coding sequence. However this mutation did not result in a change of coding for the amino acid sequence, therefore site-directed mutagenesis was not undertaken.

3.9 Discussion

Proteins which do not require post-translational modification can be successfully produced in bacterial systems; indeed there is a long history using *E. coli* to express recombinant proteins intended for human therapeutic use. The first protein intended for therapeutic use to be expressed in *E. coli* was human insulin which was produced using a protocol developed at City of Hope in collaboration with Genentech in 1979. In 1982 Eli Lilly gained approval for the general medical use of an *E. coli* expressed recombinant insulin protein 'Humulin' (the history of the research, development, production of human insulin is reviewed by Chance and Frank 1993). Since then many therapeutic proteins have been produced using this technology including Proleukin, a human recombinant interleukin-2 approved and in use for the treatment of metastatic renal cell carcinoma and metastatic melanoma (*http://www.proleukin.com/hcp/tools/pi-pharmacology.jsp*).

Although many expression systems are available for the production of recombinant proteins for therapeutic use, *E. coli* continues to be used for the expression of proteins which do not require post-translational modification. The advantages and disadvantages of bacterial expression systems are discussed further in section 4.1. This previous expression of recombinant proteins intended for therapeutic use in *E. coli* expression systems provides support for the choice of E. coli as the expression system for the production of recombinant STV/IL-2 and STV/IL-18.

3.9.1 Cloning of IL-2

RT-PCR amplification of the IL-2 coding sequence using total human RNA was attempted using unstimulated tonsil and whole blood total RNA as a source of the IL-2. IL-2 protein has previously been isolated from these sources (McGuire and Rothenberg 1987). RT-PCR amplification of IL-2 from these samples, using various parameters, was unsuccessful and no products were identified. It is possible that the levels of IL-2 mRNA in these samples were too low to be amplified by RT-PCR under the conditions used here. Sullivan *et al* (2000) showed that cytokine production by PBMC is low in unstimulated samples. In order to produce samples with higher concentration of IL-2 RNA, PMA was used to stimulate PBMC prior to isolation from whole blood samples (Sullivan *et al* 2000). RT-PCR using ProofStart™ DNA polymerase was performed on RNA extracted from PMA-stimulated PBMCs. ProofStart™ DNA polymerase is a high fidelity *Taq* which results in a lower error rate in RT-PCR reactions (Qiagen 2003 - ProofStart™ PCR Handbook). The yield of amplification products retrieved from this protocol was low but corresponded in size to the desired target product. In order to increase the amount of product available for cloning, these amplification products were subject to further rounds of RT-PCR using *Taq* DNA polymerase to provide A-tail overhang ‘sticky ends’, and the products obtained were ligated into vector pGEM®-T, a

vector designed for use with RT-PCR products containing A-tail overhang 'sticky ends'. Sequencing confirmed that the cDNA was the desired IL-2 coding sequence but revealed a point mutation in the sequence which was corrected using site-directed mutagenesis.

The cloned IL-2 coding sequence was further modified by RT-PCR amplification using primers designed to incorporate a linker region sequence which encodes the protein linker or hinge region which acts to separate the N-terminal streptavidin from the C-terminal IL-2 in the fusion protein. Incorporation of the same sequence encoding the protein linker onto the 3′ end of the core streptavidin coding sequence by amplification using appropriate primers will permit the generation of a DNA construct encoding streptavidin at the 5' end and IL-2 at the 3' end by overlapping RT-PCR.

3.9.2 Cloning of IL-18

Attempts to RT-PCR amplify the IL-18 coding sequence from tonsil and whole blood mRNA were unsuccessful; it is possible that the levels of IL-18 mRNA in these samples was low and could not be efficiently amplified using the RT-PCR conditions applied. Low intensity bands were visualized on agarose gel, but attempts to extract the bands were unsuccessful. Total RNA was extracted from cells which had been stimulated with SAC to increase the levels of expressed IL-18 mRNA (Manigold *et al* 2000). Amplification of IL-18 using primers IL-18F and IL-18R from this source, however, also proved unsuccessful. The primers have similar melting temperatures and G/C content and do not appear to be unevenly matched, which suggests that the IL-18 message may still have been low, even after SAC stimulation. In order to obtain the IL-18 coding sequence a vector containing cloned IL-18 was purchased from a commercial supplier (Invitrogen) and ultimately the IL-18 sequence was amplified from this vector

using primers containing the designed linker region for overlapping RT-PCR. This amplification product was ligated into a cloning vector into pCR®T7/CT-TOPO®. Like pGEM-T, pCR®T7/CT-TOPO® allows for the ligation of RT-PCR products containing overlapping ends (pCR®T7 TOPO® TA Expression Kits, Invitrogen 2004).

3.9.3 Cloning of Streptavidin

Both full length and core streptavidin were cloned from pUC8-SZ into pCR®T7/CT-TOPO®. As the complete sequence for pUC8-SZ was unknown, it was felt that it would be beneficial to have both the full length and core sequences in a known plasmid. Sequencing of the full length and core streptavidin clones confirmed the gene to be the correct sequence.

3.9.4 Creation of STV/IL-2 and STV/IL-18 Fusion Constructs

Fusion constructs were produced by overlapping RT-PCR (as described by Casimiro *et al* 1997) of the IL-2 or IL-18 coding sequence and core streptavidin coding sequence. The fusion coding construct was subsequently ligated into expression vector pCR®T7/NT-TOPO® for expression in One Shot®BL21(DE3)pLysS Chemically Competent *E. coli* cells (Chapter 4).

Chapter 4

Expression of Recombinant STV/IL-2 and STV/IL-18 Fusion Proteins in *E. coli*

4.1 Introduction

The production of a recombinant protein in a bacterial expression system requires a plasmid expression vector containing the DNA coding sequence for the desired recombinant protein to be introduced into a host cell. Growth of the host cell is followed by induction of expression of the protein by the cell, which is then either secreted into the culture medium or, more often, contained within the host cell (Baneyx 1999, Swartz 2001).

An *E. coli* bacterial expression system was chosen in preference to mammalian, yeast or baculovirus expression systems due to the many advantages of bacterial expression systems, including low cost, ease of manipulation of the host and vector, high expression levels and productivity, rapid protein production due to the fast growth cycle of bacteria and avoidance of the introduction of contaminating eukaryotic material associated with the use of mammalian expression systems. Furthermore, *E. coli* cells are easily lysed to release the recombinant protein, facilitating the isolation of the protein from this system. The disadvantages of bacterial expression systems include the accumulation of many recombinant proteins as insoluble aggregates, or 'inclusion bodies', lacking functional activity and the lack of post-translational modifications such as glycosylation and phosphorylation. However, the ease of use and manipulation of the *E. coli* bacterial expression systems compared with mammalian, yeast or baculovirus expression systems made it the most appealing choice of expression system for this work (reviewed by Saïda 2007)

The recombinant fusion proteins expressed in this study comprised hybrids of bacterial streptavidin and mammalian cytokines. As streptavidin is a bacterial protein, no complications were foreseen with the lack of post-translational modifications when

expressing this protein in *E. coli.* The IL-2 and IL-18 protein sequences expressed were both human in origin, but neither requires glycosylation in order to retain their function so post-translation modification is less of an issue.

4.1.1 Expression of Streptavidin as a Fusion Protein Partner

Numerous studies describe the expression of recombinant streptavidin in *E. coli*, both on its own and as a fusion protein partner (as discussed in Section 1.8.5). Sano and Cantor (1991) produced an expression vector designed for the production of streptavidin-containing chimeric proteins in *E. coli*. Expression of protein using it's vector can be controlled by induction under the T7 expression system which is permits the control of expression of proteins that may otherwise be toxic to the cell.

4.1.2 Expression of Recombinant IL-2 as a Fusion Protein Partner

Recombinant IL-2 fusion proteins have been produced in several expression systems, including bacterial, yeast and mammalian systems. IL-2 fusion proteins produced using *E. coli* expression systems include the following examples:

Xiang *et al* (1994) fused N-terminal Fv (single-chain antibody) to C-terminal human IL-2 and expressed this recombinant fusion protein gene in *E. coli* strain BL21 (DE3) pLysS using the pT7-7 plasmid vector. Recombinant protein was recovered in inclusion bodies which were resolubilized and refolded by buffer dialysis. IL-2 function was assayed using an IL-2 dependent CTLL-2 cell proliferation assay and an IL-2 binding ELISA, and was shown to be biologically active.

Castellanos-Serra *et al* (1996) fused N terminal modified human IL-2 to C terminal proinsulin and expressed this recombinant fusion protein gene in *E. coli* K12 strain

W3110(F-) using the pISL-31 plasmid vector. Recombinant protein was recovered in inclusion bodies which were resolubilized and refolded by the sulphonate exchange reaction with thiol reagent. Correct folding of the protein was assessed with reverse phase HPLC and showed the protein had folded correctly.

Jordan *et al* (2003) expressed a biotinylated recombinant human IL-2 gene in *E. coli* strain JM109 using the PinPoint Xa3 expression vector. Recombinant protein was recovered by cell lysis (in the soluble fraction) and IL-2 function was confirmed using a CTLL-2 cell proliferation assay.

Gao *et al* (1996a) expressed several recombinant fusion proteins genes containing N-terminal IL-2 linked to C- terminal *Pseudomonas* exotoxin in *E. coli*. The fusion protein was isolated from inclusion bodies and resolubilized in buffer containing 4M urea and renatured (Gao *et al* 1996b).

Zhao *et al* (1994) expressed fused N-terminal IL-6 to C-terminal IL-2. The IL-6/IL-2 protein was expressed in *E. coli* and IL-2 function was confirmed using a CTLL-2 assay.

4.1.3 Expression of Recombinant IL-18 Fusion Partners

IL-18 has been incorporated into fewer fusion proteins than either streptavidin or IL-18, and the majority of those have been produced in mammalian expression systems. Although recombinant IL-18 has been expressed and purified in *E. coli* (Ushio *et al* 1996), few IL-18 fusion proteins have been produced this way. Green fluorescent protein is an example of one such protein that has been expressed in *E. coli*. Green fluorescent protein was fused to the N-terminal of IL-18 and expressed in *E. coli*

(Omoya *et al* 2004). The fusion protein was isolated from the soluble fraction of cell lysis and IL-18 function was confirmed using a KG-1 cell assay which produces INF-γ in response to IL-18. A Fab antibody fragment was fused to the N-terminal of IL-18 and expressed in *E. coli* (Hölzer *et al* 1996). The fusion protein was isolated from the cell periplasm and shown to bind to IL-18R and induce neutrophil activation confirming that the IL-18 moiety was biologically active.

4.1.4 Aims

Following the generation of DNA expression constructs encoding novel recombinant streptavidin-cytokine fusion proteins, and the cloning of these expression constructs into the pCR®T7/NT-TOPO® bacterial expression vector (Chapter 3), the next aim of this project was to produce functional proteins by expression of the recombinant proteins in a relevant bacterial expression system. Purification of the proteins then permits their use in assays for interleukin-2, interleukin-18 and streptavidin function. Expression of fusion proteins was undertaken in One Shot®BL21(DE3)pLysS Chemically Competent *E. coli* cells using the PCR®T7 TOPO® bacterial expression system.

Protein expression by One Shot®BL21(DE3)pLysS cells is regulated by the PLysS plasmid containing a T7 promoter which produces T7 RNA polymerase. Expression of T7 RNA polymerase allows expression of proteins encoded in the expression vector (Studier and Moffatt 1987). This regulation of protein expression is especially useful in situations where the protein expressed by the gene of interest is toxic to *E. coli*. One Shot®BL21(DE3)pLysS cells require the addition of IPTG for induction of expression of the gene of interest by the induction of the T7 promoter. As genes encoding very toxic proteins may become unstable due to basal levels of T7 RNA polymerase expression, BL21(DE3)pLysS cells contain the pLysS plasmid which expresses T7

lysozyme. T7 lysozyme binds to T7 RNA polymerase, inactivating it and adding a further level of control over the protein expression. Recombinant streptavidin has been found to be toxic to *E. coli* and must be expressed under the suppression of the pLysS or pLysE plasmid (Sano and Cantor 1990). The pLysS plasmid expresses a moderate amount of T7 lysozyme which, while being enough to reduce the activity of T7 lysozyme, does not affect growth rates. For this reason it is prudent to express recombinant streptavidin-containing protein under the pLysS repression system.

The expression vector pCR®T7/NT-TOPO® contains the coding sequence for a N-terminal polyhistidine (6xHis) tag which facilitates the purification of expressed proteins by binding of the 6x histidine region to divalent metal gels such as nickel or cobalt affinity gels. The histidine tag also facilitates identification and visualization of the expressed protein using an anti-histidine tag antibody. Such antibodies designed for this application are readily available from commercial sources. As the identification of other portions of the proteins may be dependent on their correct folding, the presence of an anti-histidine tag protein is especially useful in identifying STV/IL-2 and STV/IL-18 in either their soluble or insoluble forms. After purification the histidine tag can be cleaved using an enterokinase such as EKMax™ which recognizes the specific amino acid sequence Asp-Asp-Asp-Asp-Lys, which occurs between the N-terminal histidine tag and the C-terminal recombinant protein, and cleaves following the lysine residue. The EKMax™ enzyme can then be removed by affinity chromatography on a soybean trypsin inhibitor resin such as EK-Away™ Resin (Invitrogen).

4.2 Expression of Streptavidin – Interleukin 2 (STV/IL-2) Fusion Protein in *E. coli*

4.2.1 Transformation of BL21(DE3)PLysS with pCR®T7/NT-STV/IL-2

E. coli strain BL21(DE3)PLysS was transformed with pCR®T7/NT-STV/IL-2 (pCR®T7/NT-TOPO® containing the STV/IL-2 insert) by heat shocking the cells at 42°C (Section 2.7.1). The transformed cells were added to 10ml LB liquid medium containing 100µg/ml ampicillin and 34µg/ml chloramphenicol, and grown overnight at 37˚C with shaking. Expression control plasmid pCR®T7/NT-E3 was also used to transform BL21(DE3)PLysS cells by heat shock, following the same expression protocol as the pCR®T7/NT-STV/IL-2 transformation. Following the transformation reactions, expression of the protein encoded by the pCR®T7/NT-STV/IL-2 plasmid was investigated.

4.2.2 Pilot Protein Expression Study

For both the culture containing the cells transformed with the expression control plasmid, and the culture containing cells transformed with the pCR®T7/NT-STV/IL-2 plasmid, 10ml of LB liquid medium containing 100µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with 500µl of the overnight culture (1:20 dilution) and grown at 37˚C with shaking for 2 hours after which each of the cultures was split into two 5ml cultures. One of each pair of cultures was induced with 0.8mM IPTG, and all cultures were left to grow at 37˚C with shaking. Zero time point samples of each culture were removed and the *E. coli* cells pelleted by centrifugation. Subsequently 500µl samples of each culture were taken at 2 hours, 3 hours, 4 hours, and following overnight incubation. Cells from these samples, pelleted by centrifugation at 16,000g for 1 minute, were reduced by resuspension and boiling in gel loading buffer supplemented with ß-mercaptoethanol, and the proteins separated by electrophoresis on

SDS-PAGE gel. The PAGE gel was stained for protein using Coomassie blue stain (Figures 4.1 and 4.2).

Staining of the samples containing the pCR®T7/NT-STV/IL-2 plasmid (Figure 4.1) showed no bands of the expected molecular mass of the streptavidin-interleukin-2 fusion protein in either the induced or uninduced samples at any time period, suggesting no or little expression of the recombinant protein had occurred. Staining of the samples containing the expression control plasmid pCR®T7/NT-E3 (Invitrogen), which encodes for a human kinase of approximately 58 kDa (pCR®T7 TOPO® TA Expression Kits, Invitrogen 2004), showed a discrete band of between 50 and 60 kDa molecular mass in both the induced and uninduced samples (Figure 4.2). Comparison of the intensity of the protein bands in induced samples with those seen in uninduced samples suggests that induction has not caused a large increase in expression and protein yield, although interestingly the induced samples showed fewer other protein bands, indicating that background expression may have been lower in the induced culture.

4.2.3 Pilot Protein Expression Study using LB Supplemented with Glucose

The pilot expression study described above was repeated using culture media supplemented with glucose. BL21(DE3)PLysS cells transformed with pCR®T7/NT-STV/IL-2 were added to 10ml of LB liquid medium containing 100µg/ml ampicillin and 34µg/ml chloramphenicol, supplemented with 1% w/v glucose and grown overnight at 37˚C with shaking. From this overnight culture 500µl of cell suspension were used to innculate10ml of LB liquid medium containing 100µg/ml ampicillin and 34µg/ml chloramphenicol, supplemented with 1% w/v glucose, and grown for 2 hours at 37˚C with shaking, as before. The culture was then split into two 5ml cultures, one of which was induced with IPTG and one of which was not induced. 500µl samples of each

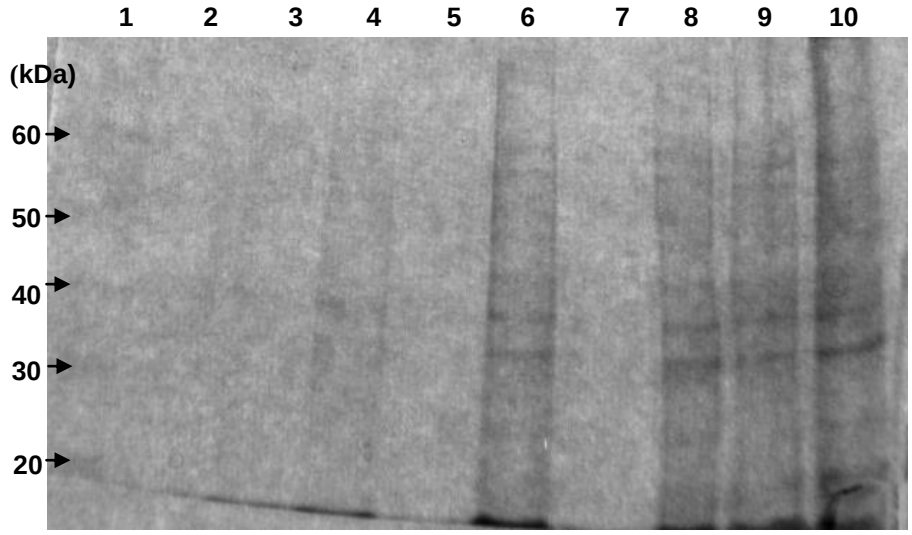

Figure 4.1 Analysis of recombinant STV/IL-2 expressed in BL21(DE3)PLysS cells.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, visualized by Coomassie blue staining.
Lanes: (1) MagicMark™ Protein standard; (2) zero time point sample; (3, 5, 7, 9) culture samples subjected to IPTG induction taken after 2, 3, 4 hours and overnight culture respectively; (4, 6, 8, 10) culture samples (no induction) taken after 2, 3, 4 hours and overnight culture respectively. Numbers on the left hand side correspond to molecular mass (kDa) markers.

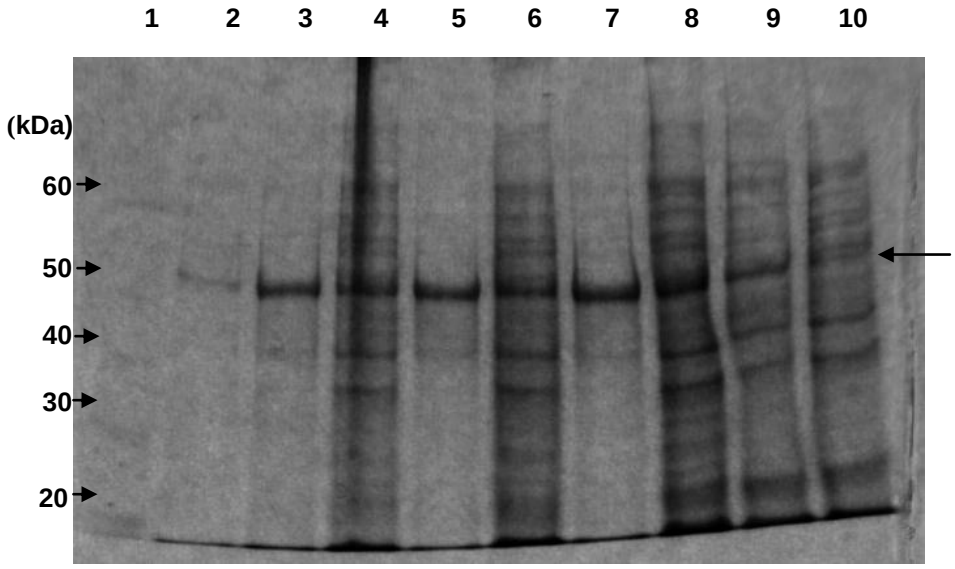

Figure 4.2 Analysis of a human kinase positive control protein expressed in BL21(DE3)PLysS cells.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-E3 expression control plasmid, visualized by Coomassie blue staining. Lanes: (1) MagicMark™ Protein standard; (2) zero time point sample; (3, 5, 7, 9) culture samples subjected to IPTG induction taken after 1, 2, 3, 4 hours and overnight culture respectively; (4, 6, 8, 10) culture samples (no induction) taken after 1, 2, 3, 4 hours and overnight culture respectively. Numbers on the left hand side correspond to molecular mass (kDa) markers. The arrow on the right hand side highlights the protein bands which correspond to the expected protein mass of the human kinase encoded by the control vector (approximately 58kDa).

culture were taken after 0 hours, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours and following overnight incubation. As previously (Section 4.2.2), pelleted cell samples were reduced by resuspension and boiled in gel loading buffer supplemented with ß-mercaptoethanol which was then analysed by SDS-PAGE separation and visualization by Western blotting and Coomassie blue staining (Figures 4.3 and 4.4). Staining with Coomassie blue revealed a protein band at approximately 30kDa that appears more intensely in the induced (Figure 4.3) than the uninduced (Figure 4.4) samples. This corresponds to the estimated molecular mass of recombinant STV/IL-2 of 32.22kDa (Section 2.8.6). The band can be seen in the induced samples from all time points from 1 hour culture time to overnight culture. Comparisons of the samples grown in medium supplemented with glucose and those grown in medium without glucose suggest that addition of glucose to the culture medium promotes the expression of the desired recombinant protein.

Western blotting of the protein samples separated by SDS-PAGE with a mouse anti-histidine tag antibody (Figure 4.5) and staining of the same samples with Coomassie blue (Figure 4.6) showed that the strong band appearing in the induced culture, as seen following Coomassie blue staining of the gel, binds this antibody on a western blot. This suggests that the observed bands are the desired recombinant protein, which has been engineered to contain an N-terminal 6x Histidine tag. Since the pilot expression study indicated that STV/IL-2 expression had been successfully induced, the system was scaled up in order to increase the total protein yield.

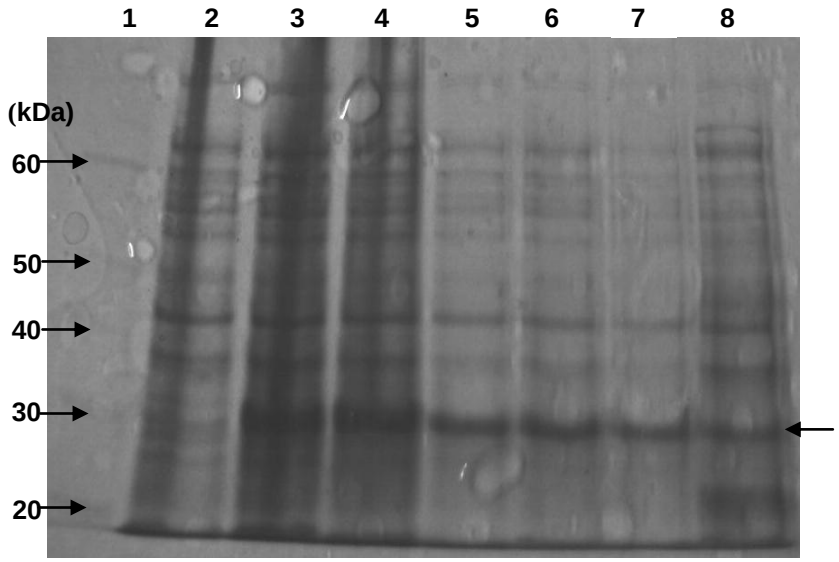

Figure 4.3 Analysis of recombinant STV/IL-2 expressed in *E. coli* supplemented with 1% glucose.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, grown in culture medium containing 1% w/v glucose, visualized by Coomassie blue staining.

Lanes: (1) MagicMark™ Protein standard; (2) zero time point sample; (3-8) culture samples after IPTG induction taken after 1, 2, 3, 4, 5 hours and overnight culture respectively. Numbers on the left hand side correspond to molecular mass (kDa) markers. The arrow on the right hand side highlights the protein bands which correspond to the expected protein mass.

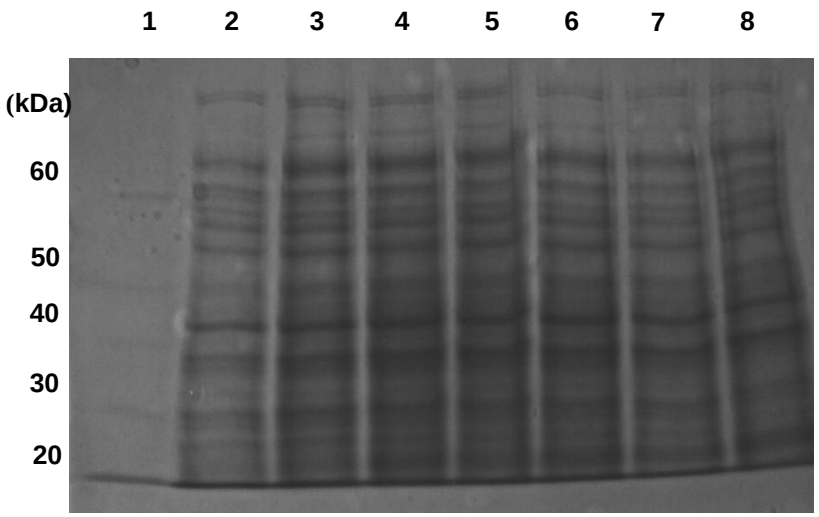

Figure 4.4 Analysis of recombinant STV/IL-2 expressed in BL21(DE3)PLysS cells not supplemented with glucose.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, grown in medium containing no glucose, visualized by Coomassie blue staining.

Lanes: (1) MagicMark™ Protein standard; (2) zero time point sample; (3-8) culture samples (no induction) taken after 1, 2, 3, 4, 5 hours and overnight culture respectively. Numbers on the left hand side correspond to molecular mass (kDa).

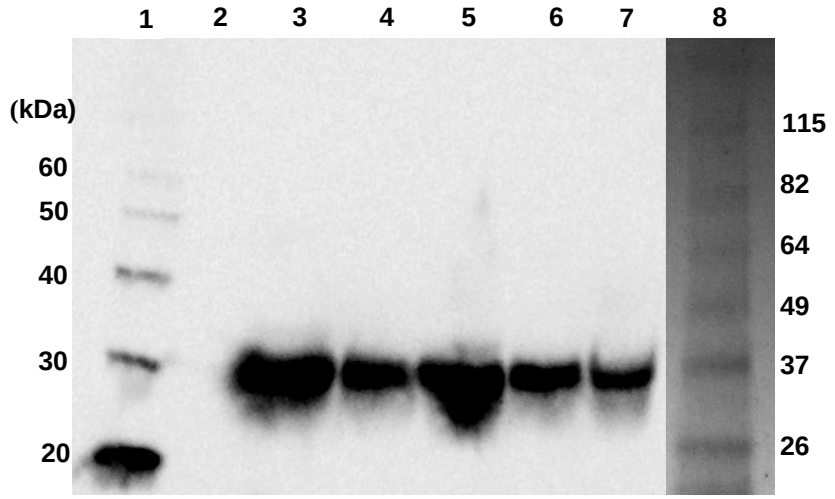

Figure 4.5 Western blot analysis of recombinant STV/IL-2 expressed in *E. coli* supplemented with 1% w/v glucose.

Western Blot analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, grown in medium containing 1% w/v glucose, separated by 10% SDS-PAGE and visualized by probing with a mouse anti-histidine tag antibody.

Lanes: (1) MagicMark™ Protein standard; (2) zero time point; (3-7) culture samples induced with IPTG taken after 1, 2, 3, 4 hours and overnight culture respectively; (8) BenchMark™ Pre-Stained Protein Ladder. Numbers on the left and right hand sides correspond to molecular mass (kDa) markers.

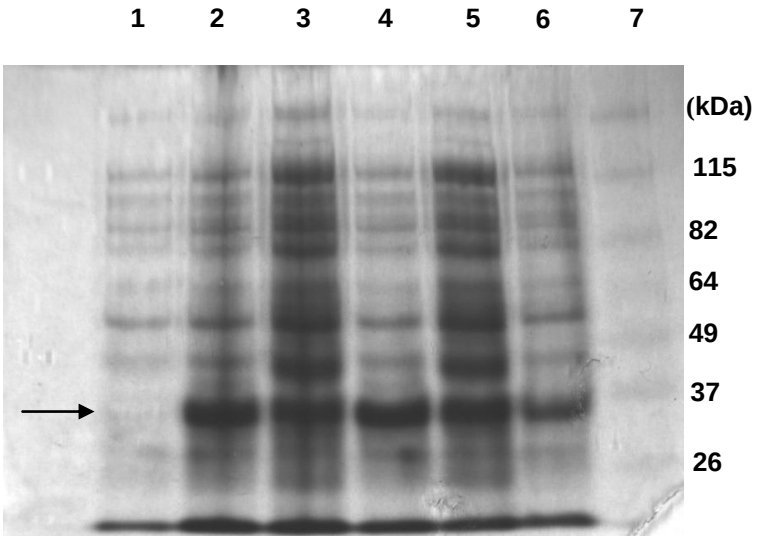

Figure 4.6 Analysis of recombinant STV/IL-2 expressed in *E. coli* supplemented with 1% w/v glucose.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, grown in medium containing 1% w/v glucose, visualized by Coomassie blue staining**.**

Lanes: (1) zero time point sample; (2-6) culture samples after IPTG induction taken after 1, 2, 3, 4 hours and overnight culture respectively; (7) BenchMark™ Pre-Stained Protein Ladder. Numbers on the right hand side correspond to molecular mass (kDa). The arrow on the left hand side highlights the protein bands which correspond approximately to the estimated recombinant STV/IL-2 mass.

4.3 Purification of STV/IL-2 by His-tag Affinity Binding

In order to obtain purified STV/IL-2 for further analysis, the soluble fraction of the lysis of bacterial cell cuture induced by IPTG to express STV/IL-2 was subjected to purification on a cobalt gel utilizing the binding affinity of the poly-hisitidine tag for divalent metal ions.

4.3.1 Cell Lysis with BugBuster® Protein Extraction Reagent

Cell pellets prepared from expression cultures supplemented with 1% glucose (Section 4.2.3) were lysed by incubation at room temperature with BugBuster® Protein Extraction Reagent supplemented with Benzonase® Nuclease (Section 2.7.3). The insoluble fraction was removed by centrifugation and stored at -80˚C. The supernatant was retained for purification.

4.3.2 Purification of Recombinant STV/IL-2

Purification of STV/IL-2 by His-tag affinity binding on His-Select™ Cobalt Affinity Gel (Sigma) (Section 2.7.4). The soluble fraction prepared by cell lysis in BugBuster® Protein Extraction Reagent (Section 4.3.1) was incubated with the His-Select™ Cobalt Affinity Gel for 30 minutes with shaking at room temperature. The affinity gel was washed and incubated with elution buffer for 20 minutes with shaking at room temperature. Samples of the soluble fraction were taken before and after incubation with the affinity gel; samples of each wash step and the elution step were also taken.

The His-tag affinity binding purified samples and a control bacterial cell pellet positive for the pCR®T7/NT-STV/IL-2 vector (prepared as in Section 4.2.3) were separated by SDS-PAGE, and subjected to Western blot analysis with a mouse anti-histidine tag

purified antibody (*results not shown*). No bands were visible in the his-tag affinity binding purified samples, but a band could be seen in the positive control sample, suggesting either that the affinity gel purified samples contained no his-tagged proteins or that the proteins were too dilute to be detected by this visualization method. Repeated attempts were made to purify the STV/IL-2 proteins by means of his-tag affinity binding, including visualization with anti-streptavidin and anti-IL2 antibodies showed no bands in purified samples.

Further efforts to purify STV/IL-2 from samples by using an alternative binding method were attempted. STV/IL-2 was subjected to column chromatography using Ni-NTA His-Bind® Resin (Novagen), according to the manufactures protocol, but failed to give any positive result. As soluble STV/IL-2 protein could not be purified using the methods described above, an investigation to determine whether the recombinant protein was being in expressed in a soluble form or in inclusion bodies was undertaken.

4.3.3 Analysis of Insoluble Protein Fraction

Insoluble protein fraction (from 1ml of culture) (prepared using BugBuster® as described in Section 4.3.1), was compared to BugBuster® lysed soluble fraction by separation by SDS-PAGE and visualization by Coomassie blue staining (Figure 4.7a) and Western blot analysis using a mouse anti-histidine tag purified antibody (Figure 4.7b). A bacterial pellet from a 50ml culture (Section 2.7.2) was lysed in a small amount of BugBuster® (500µl). When stained with Coomassie blue stain, the solubilized fraction showed similar bands to those seen in the total protein sample. Western blotting with a mouse anti-histidine tag antibody showed much unspecific binding in the total protein sample. However, a faint band can be seen in the soluble

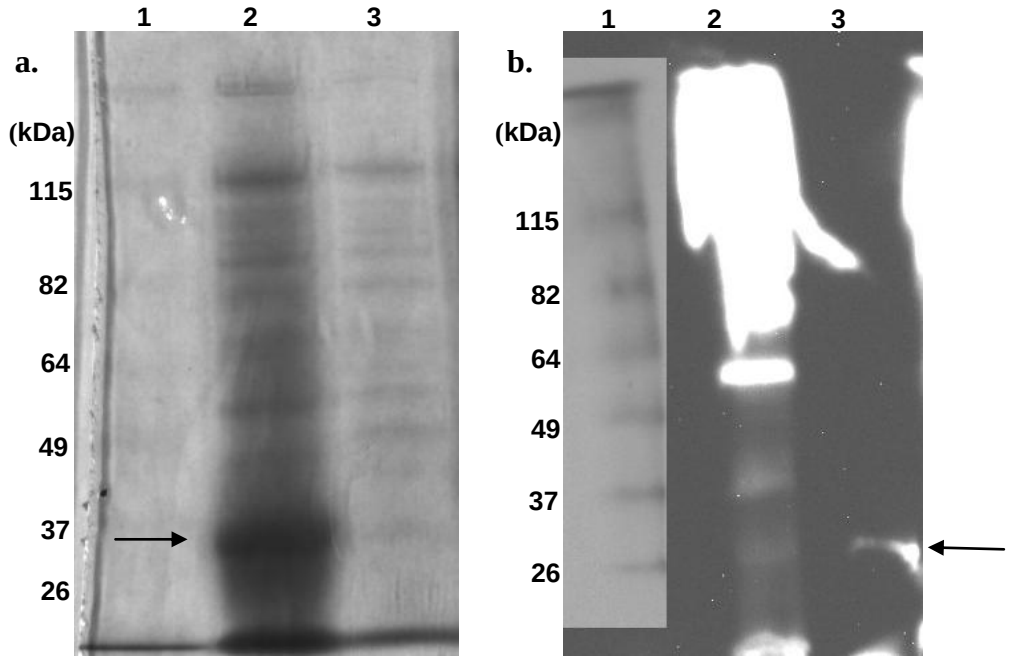

Figure 4.7 Analysis of the soluble and insoluble fractions prepared from *E. coli* cells expressing STV/IL-2.

a) 10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, visualized by Coomassie blue staining.

b) 10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, visualized by Western blot with a mouse anti-histidine tag antibody

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) complete bacterial pellet from 4 hour incubation following induction of expression with IPTG; (3) clarified soluble protein from bacterial pellet lysed with BugBuster®. Numbers on the left hand sides correspond to molecular mass (kDa) markers. The arrows on the left and right hand sides highlight the protein bands which correspond approximately to the estimated recombinant STV/IL-2 mass

fraction at approximately the size estimated for STV/IL-2. As a high concentration of protein was visualised by western blot analysis on samples prepared from whole cell pellets (Section 4.2.3) using an anti-histidine tag antibody, a more intense band would be expected in the Bugbuster ® lysed soluble fraction if the protein were fully soluble. In order to locate the STV/IL-2 protein, samples from all stages of the purification process (total protein pellet, soluble protein fraction, insoluble protein fraction (inclusion bodies) and elution after purification on His-Select™ Cobolt Affinity Gel) were analyzed by separation by SDS-PAGE and visualization by western blotting with a mouse anti-histidine tag purified antibody (Figure 4.8). Very intense bands were seen in the total protein sample and in the insoluble fraction, suggesting that the STV/IL-2 protein is insoluble, and needs solubilizing before any purification can be attempted.

Further suggestion that the STV/IL-2 protein is insoluble is shown by repeat analysis of inclusion bodies compared to whole bacterial cell cultures (Figure 4.9); inclusion bodies were isolated from the insoluble fraction by incubation of a 10ml culture with BugBuster® plus rLysozyme™, and then washed several times with 1:10 BugBuster®:dH_2O to isolate and purify the inclusion bodies (Section 2.7.5). The inclusion bodies were resuspended in gel loading buffer and separated, along with a positive control whole cell total protein sample from 1ml culture (as prepared in Section 4.2.3), by SDS-PAGE and Western blot analysis with a mouse anti-histidine tag purified antibody. Both samples showed bands of between 26 and 37kDa molecular mass, which corresponds to the estimated protein size of 32.22kDa. Another band is also visible at approximately twice this molecular weight. Figure 4.7b also shows smearing at a high molecular weight which may be due to an overloading of protein sample, or a large amount of insoluble protein aggregation.

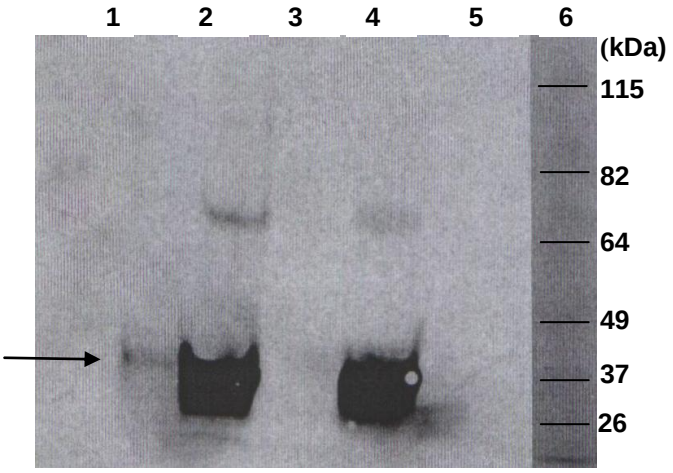

Figure 4.8 Analysis of the insoluble and soluble fractions prepared from *E. coli* cells expressing STV/IL-2 along with a positive control.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid.

Lanes: (1) positive control bacterial pellet sample from 4 hour incubation following induction of expression with IPTG, previously shown to be positive against mouse anti-histidine tag purified antibodies; (2 complete bacterial pellet from 4 hour incubation following induction of expression with IPTG; (3) soluble protein sample after lysis with BugBuster®; (4) bacterial pellet insoluble fraction (inclusion bodies); (5) elution of sample taken following purification on cobolt affinity gel; (6) BenchMark™ Pre-Stained Protein Ladder. Numbers on the right hand side correspond to molecular mass (kDa) markers. The arrow on the left hand side highlights the protein bands which correspond approximately to the estimated recombinant STV/IL-2 mass.

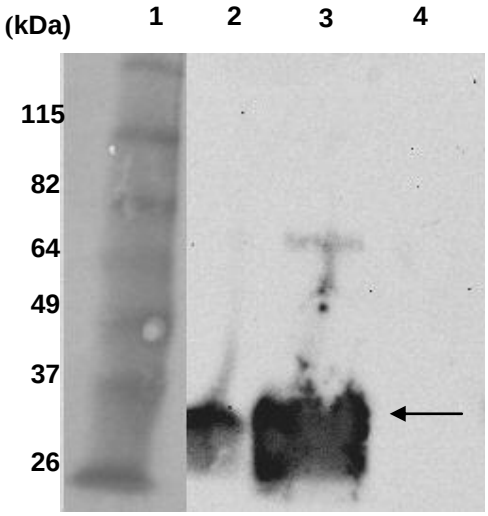

Figure 4.9 Analysis of the insoluble soluble fractions prepared from *E. coli* cells compared to a complete bacterial pellet.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid visualized by western blotting with a mouse anti-histidine tag purified antibody.

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) complete bacterial pellet from 4 hour incubation following induction of expression with IPTG; (3) purified inclusion bodies; (4) soluble protein sample after lysis with BugBuster®; (5) sample of elution from attempted purification with cobalt affinity gel. Numbers on the left hand side correspond to molecular mass (kDa) markers. The arrow on the right hand side highlights the protein bands which correspond approximately to the estimated recombinant STV/IL-2 mass.

4.4 Solubilization of STV/IL-2

4.4.1 Optimization of Culture Induction to Produce Soluble STV/IL-2

Reducing the level of expression of recombinant protein by *E. coli* can sometimes lead to a reduction in aggregation of the protein, and therefore yield more soluble protein. An experiment was designed to see if different culture conditions could provide more soluble STV/IL-2. The length of induction and the amount of IPTG used to induce expression in cultures was varied. After induction with 0, 0.01, 0.1 or 1mM IPTG, cultures were incubated at 37˚C or room temperature with shaking for 3 hours (Figures 4.10 and 11) and overnight (*results not shown*). Separation by SDS-PAGE and visualization by western blot analysis with a mouse anti-histidine tag antibody did not reveal any protein in any of the soluble fraction samples. Bands were seen in all of the insoluble fractions, although there also appears to be unspecific binding of the antibody in these samples. There appears to be little difference between samples incubated at room temperature versus those incubated at 37˚C. As these experiments did not show any benefit to altering the length of induction or concentration of IPTG used to induce expression, it was decided to attempt solubilization of the insoluble fraction.

4.4.2 Solubilization of Purified Inclusion Bodies (with His-Bind® Binding Buffer and 6M Urea)

Attempts were made to solubilize STV/IL-2 purified inclusion bodies in His-Bind® binding buffer by adding urea to the binding buffer, to a final concentration of 6M. Purified inclusion bodies (Section 2.7.6) were resuspended using 5ml His-Bind® binding buffer per 100ml dH_20 (as recommended in the His-Bind® protocol, Novagen). Analysis by SDS-PAGE and visualization by Coomassie blue and western blotting with

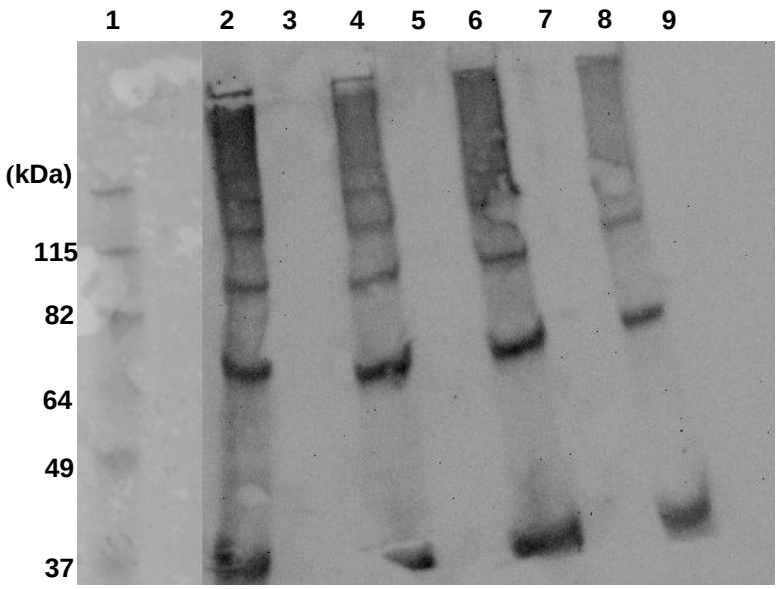

Figure 4.10 Analysis of STV/IL-2 expressed after induction with varying amounts of IPTG and incubated at room temperature.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid induced and incubated for 3 hours at room temperature, visualized by western blotting with a mouse anti-histidine tag purified antibody.

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2, 4, 6, 8) insoluble protein fraction induced with 0, 0.01, 0.1, 1mM IPTG respectively; (3, 5, 7, 9) soluble protein fraction induced with 0, 0.01, 0.1, 1mM IPTG respectively. Numbers on the left hand side correspond to molecular mass (kDa) markers.

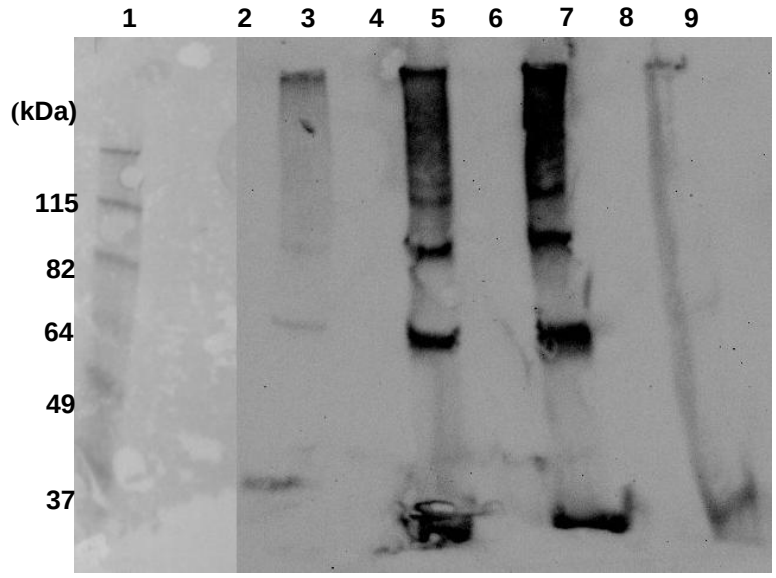

Figure 4.11 Analysis of STV/IL-2 expressed after induction with varying amounts of IPTG and incubated at 37˚C.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, induced and incubated incubated for 3 hours at 37˚C, visualized by western blotting with a mouse anti-histidine tag purified antibody. Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2,4,6,8) insoluble protein fraction induced with 0,0.01,0.1,1mM IPTG respectively; (3,5,7,9) soluble protein fraction induced with 0,0.01,0.1,1mM IPTG respectively. Numbers on the left hand side indicates molecular mass (kDa) markers.

a mouse anti-histidine tag antibody did not reveal any protein in the solubilized samples (*data not shown*).

Further attempts were made to solubilize the inclusion bodies reduced volumes of buffer to try and produce a more concentrated solubilized solution. Briefly, inclusion bodies isolated from 50ml culture pellets (Section 2.7.5) prepared as described in section 4.2.3 were resuspended in 100, 200 and 500µl of buffer. These were analysed by SDS-PAGE and visualized by western blotting with a mouse anti-histidine tag antibody (Figure 4.12). The Western blot membrane was then stripped of the antibody by incubating with Restore™ Western Blot Stripping Buffer (Pierce Biotechnology) for 15 minutes at room temperature and subjected to Western blot analysis with an anti-streptavidin antibody (Figure 4.13). Bands were seen in the positive control (total cell pellet as described in section 4.2.3) and insoluble fraction samples (Section 4.3.1) with both antibodies. No bands were observed in the solubilized inclusion body samples (or in the BugBuster® soluble protein fraction) with the mouse anti-histidine tag antibody; however, a low intensity band could be seen in the sample solubilized in 500µl of buffer at the same molecular mass as the band in the positive control sample (between 26 and 37kDa) with the anti-streptavidin antibody (Figure 4.13). Additional protein bands are also apparent in the solubilized samples possibly due to unspecific binding of the streptavidin antibody. The presence of a protein band in the inclusion body sample solubilized in 500µl of buffer, which could be observed with an anti-streptavidin antibody, suggests some solubilization of the recombinant protein is taking place.

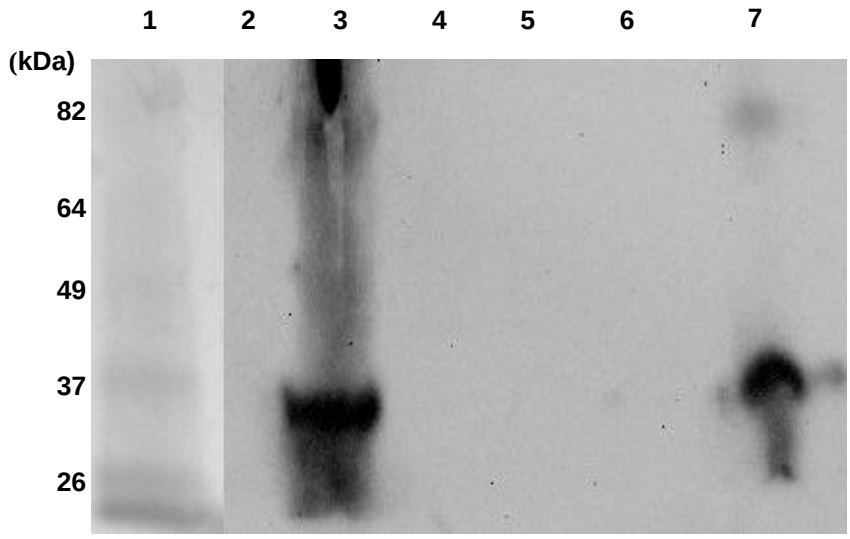

Figure 4.12 Analysis of STV/IL-2 solubilization in His-Bind® buffer with 6M urea.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid visualized by Western blotting with a mouse anti-histidine tag purified antibody.

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) BugBuster® soluble fraction; (3) insoluble pellet resuspended in gel loading buffer (4, 5, 6) solubilized protein in His-Bind® buffer with 6M urea. 50ml culture pellets solubilized in 100, 200 and 500µl respectively; (7) positive control – complete bacterial pellet from 4 hour incubation following induction of expression with IPTG. Numbers on the left hand side correspond to molecular mass (kDa) markers.

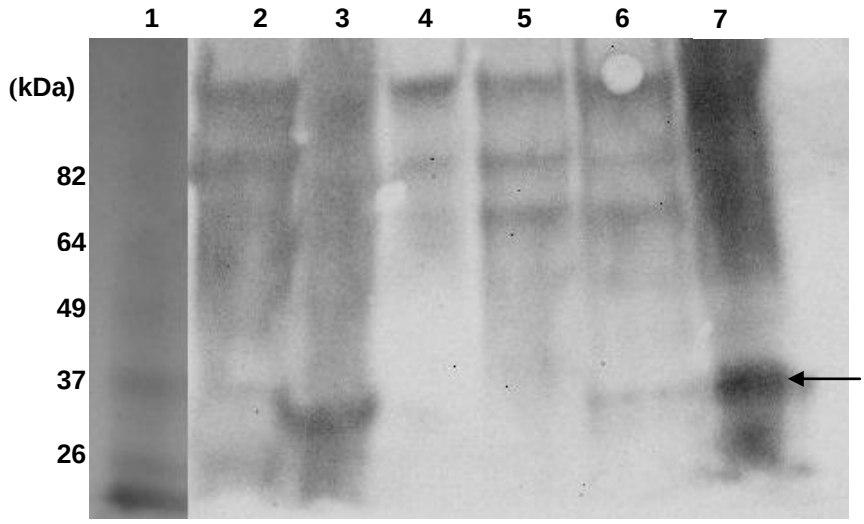

Figure 4.13 Analysis of STV/IL-2 solubilization in varying amounts of His-Bind® buffer with 6M urea.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, visualized by western analysis with an anti-streptavidin antibody.

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) BugBuster® soluble fraction; (3) insoluble pellet resuspended in gel loading buffer; (4,5,6) solubilized protein in His-Bind buffer with 6M urea. 50ml culture pellets solubilized in 100, 200 and 500µl respectively; (7) positive control - complete bacterial pellet from 4 hour incubation following induction of expression with IPTG. Numbers on the left hand side correspond to molecular mass (kDa) markers. The arrow on the right hand side highlights the protein bands which correspond approximately to the estimated recombinant STV/IL-2 mass.

4.4.3 Solubilization of Purified Inclusion Bodies with 100mM Tris, 2M Urea at Variable pHs

Since solubilzation with His-Bind® buffer containing 6M Urea showed very little soluble protein upon analysis by Western blot (Section 4.4.2), an alternative method described by Patra *et al* (2000) was undertaken.

Purified STV/IL-2 inclusion bodies were incubated with 100mM tris and 2M urea (solubilization buffer) at various pHs (Section 2.7.6). 1ml of solubilization buffer was used to resuspend 0.8mg of inclusion bodies. After incubation at room temperature for 30 minutes the samples were centrifuged and a 20µl sample of the supernatant was loaded onto an SDS-PAGE gel. The samples were separated by electrophoresis and visualized by Coomassie blue staining (Figure 4.14) and western blot analysis with a mouse anti-histidine tag antibody (Figure 4.15). Coomassie blue staining showed discrete bands between 26 and 37kDa molecular mass in all of the solubilized samples becoming more intense as the pH increased, with the band in the sample solubilized at pH 12.5 being the most intense. Western blotting with a mouse anti-histidine tag antibody showed that the antibody was binding to a protein of between 26 and 37kDa solubilized at pH 12.5. These bands correspond approximately to the estimated protein molecular mass of STV/IL-2 of 32.22kDa, and to protein bands seen in positive control - total culture pellets (Figure 4.14), and total culture pellets and inclusion body pellets (Figure 4.15). The presence of bands in samples of inclusion bodies solubilized with 100mM tris and 2M urea, especially at pH 12.5, when analysed by Western blotting and Coomassie blue staining suggests that solubilization of the recombinant protein has been successful.

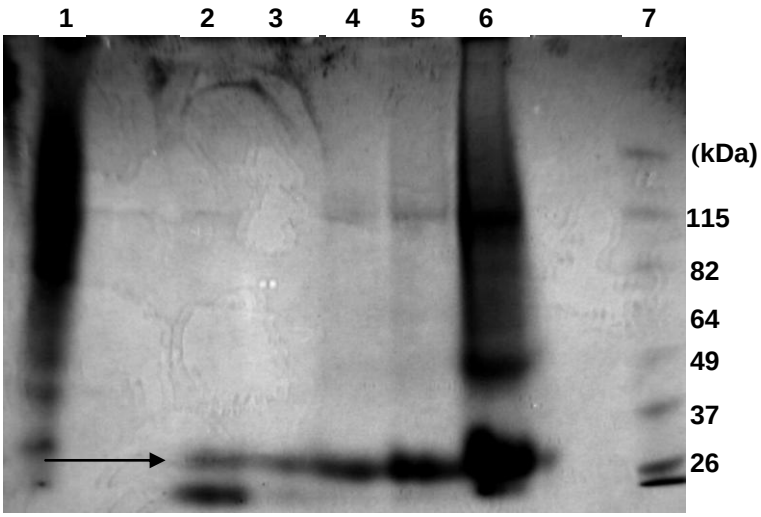

Figure 4.14 Analysis of STV/IL-2 solubilization in 100mM tris, 2M urea solubilization buffer by staining with Coomassie blue.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, visualized by Coomassie blue staining.
Lanes: (1) positive control - complete bacterial pellet from 4 hour incubation following induction of expression with IPTG; (2) inclusion bodies solubilized in buffer at pH 8.5; (3) inclusion bodies solubilized in buffer at pH 9.5; (4) inclusion bodies solubilized in buffer at pH 10.5; (5) inclusion bodies solubilized in buffer at pH 11.5; (6) inclusion bodies solubilized in buffer at pH 12.5; (7) MagicMark™ Protein standard. Numbers on the right hand side correspond to molecular mass (kDa) markers. The arrow on the left hand side highlights the protein bands which correspond approximately to the estimated recombinant STV/IL-2 mass.

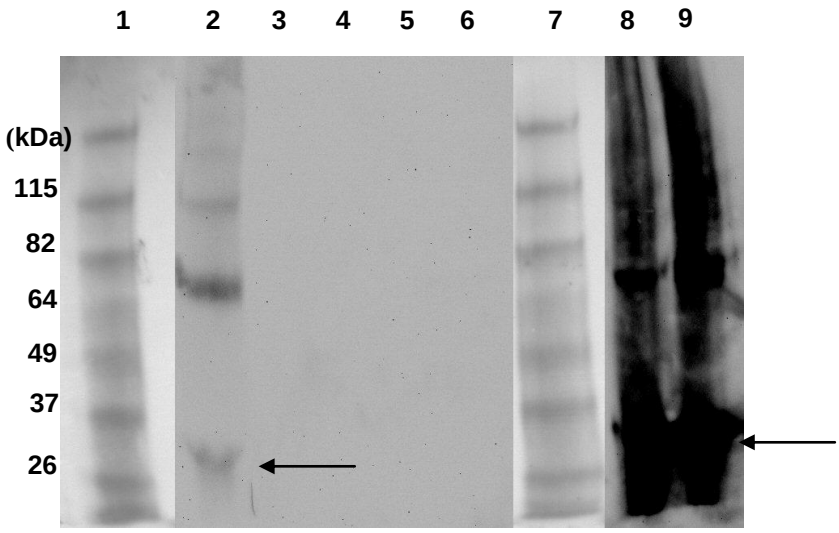

Figure 4.15 Analysis of STV/IL-2 solubilization in 100mM tris, 2M urea solubilization buffer by Western blot.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, visualized by Western blotting with an anti-streptavidin antibody.

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) inclusion bodies solubilized in buffer at pH 12.5; (3) inclusion bodies solubilized in buffer at pH 11.5; (4) inclusion bodies solubilized in buffer at pH 10.5; (5) inclusion bodies solubilized in buffer at pH 9.5; (6) inclusion bodies solubilized in buffer at pH 8.5; (7) BenchMark™ Pre-Stained Protein Ladder; (8) inclusion body pellet; (9) positive control – complete bacterial pellet from 4 hour incubation following induction of expression with IPTG. Numbers on the left hand side correspond to molecular mass (kDa) markers. The arrows highlight the protein bands which correspond approximately to the estimated recombinant STV/IL-2 mass

4.4.4 Refolding of Recombinant STV/IL-2

STV/IL-2 was successfully solubilized in 100mM tris, 2M urea buffer, however the high pH (pH 12.5) in order to undertake further work using the recombinant protein samples it was essential that the buffer containing the protein should contain a lower concentration of urea and have a lower pH than the resolubilization buffer (Section 4.4.3). Lowering the urea concentration and pH of the protein buffer is essential to correct refolding of the recombinant protein. Refolding of the proteins into their correct tertiary structure is a necessary step for the proteins to retain their biological function – for streptavidin, biotin binding, and for the cytokines, their *in vivo* immunological functions.

In order to produce a correctly folded recombinant protein exchange of the protein resolubilization buffer with PBS and refolding of the protein into its tertiary structure were attempted by dialysis. 3ml of resolubilized protein in 100nM tris 2M urea at pH 12.5 was dialyzed twice against 200 volumes of PBS for 2 hours at room temperature in a 2K molecular weight cut off Slide-A-Lyze® dialysis cassette with stirring. After the second dialysis step it was noted that the protein had precipitated out of solution inside the cassette. It was demonstrated that, after the sample was removed from the dialysis cassette, the precipitated protein could be pelleted by centrifugation and resolubilized in solubilization buffer at pH 12.5 following the protocol developed for solubilizing purified inclusion bodies (Section 2.7.6).

Attempts to lower the pH of this protein in solution by adding 1M HCl dropwise to the buffer likewise caused the protein to precipitate out of solution at around pH 9 to pH 10. Attempted refolding of STV/IL-2 by adding PBS or dH_2O to the solubilized protein

(thereby reducing the urea conentration and pH of the buffer) in a pulsatile manner likewise caused precipitation of the protein. However, slow pulsatile addition of the protein drop-wise (one drop every 5 seconds) to a 20 times volume of the diluents with constant stirring caused no precipitation, and reduced the pH of the sample to approximately pH 9 (Section 2.7.7).

The diluted protein was concentrated approximately 5 times using Microcon Centrifugal Filter Devices (Millipore) with a molecular weight cut-off of 10kDa. The samples, soluble protein in the original solubilization buffer (100mM tris, 2M urea) and the Microcon-concentrated soluble protein in diluted buffer (20mM tris, 100mM urea in PBS), were boiled in gel loading buffer containing β-mercaptoethanol and separated by electrophoresis on SDS-PAGE gel. SDS-PAGE gels were visualized by western blot analysis with a goat anti-human IL-2 IgG antibody (4.16a) and staining with Coomassie blue (Figure 4.16b) and. Distinct protein bands were visible on both the Coomassie blue stained gel and the western blot, in the diluted/refolded samples, at between 26 and 37kDa. Bands were also visible in the diluted/refolded sample at approximately the same size, although there was background smearing in these samples.

As well as the expected band of approximately 32.22kDa, 3 other bands are clearly visible in the diluted/refolded sample when visualized by Coomasie blue staining (Figure 4.16b). These bands represent protein with a molecular weight of approximately 60, 80 and 120kDa. Smearing in sample 2 could indicate overloading of protein onto the SDS-PAGE gel, or the presence of insoluble protein in the sample.

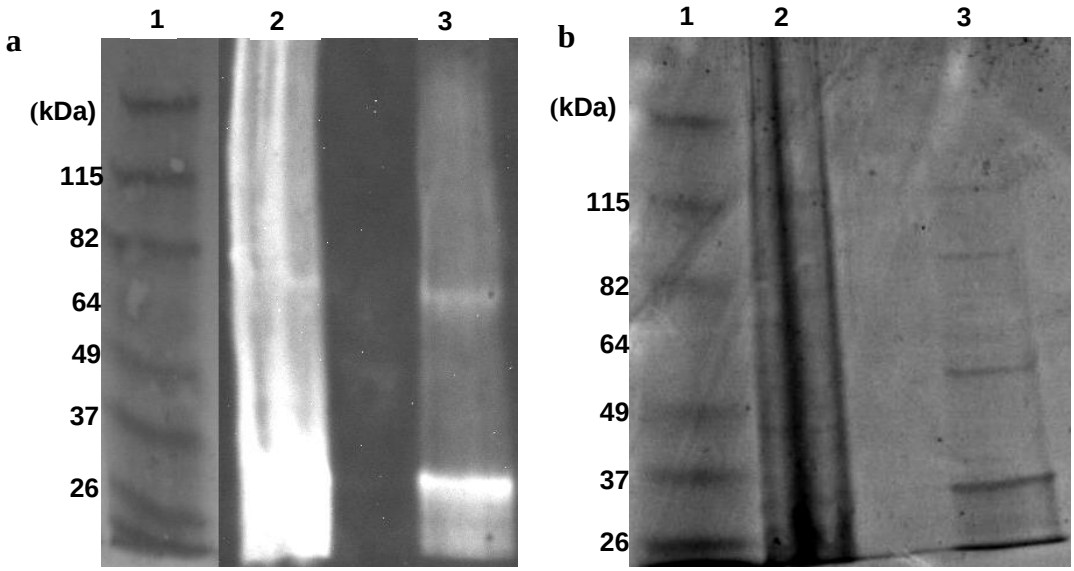

Figure 4.16 Comparison of soluble unfolded and refolded recombinant STV/IL-2.

a) 10 % SDS-PAGE analysis of solubilized STV/IL-2, visualized by probing with a mouse anti-histidine tag antibody.

b) 10% SDS-PAGE analysis of solubilized refolded STV/IL-2, visualized by Coomassie blue staining.

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) Protein in solubilization buffer; (3) refolded protein. Numbers on the left hand sides correspond to molecular mass (kDa) markers.

4.5 Removal of the Histidine Tag from STV/IL-2 using EKMax™ Enterokinase

4.5.1 Pilot Digestion of STV/IL-2 with EK Max™

A pilot reaction was undertaken to assess the optimum amount of EKMax™ required to digest the STV/IL-2 fusion protein at the enterokinase restriction site in order to remove the histidine tag, with little unspecific cleavage of the protein. To determine the optimal concentration of EKMax™ to be used, six reactions were set up with varying amounts of EKMax™, including a control reaction containing no EKMax™ (Section 2.7.8).

The digestion reactions were incubated overnight at 37^{o}C. Cell pellets from 1ml of cell culture were boiled in gel loading buffer containing β-mercaptoethanol. The samples were separated by electrophoresis on a 10% (w/v) SDS-PAGE gel and visualized by staining with Coomassie blue (Figure 4.17) and by western blotting with a mouse anti-histidine tag purified antibody (*data not shown*). Multiple bands were evident in the Coomassie blue stained gel, more than has been noted in previous STV/IL-2 samples, suggesting that the protein may be being unspecifically cleaved by the degrading or . Samples containing the highest amounts of EKMax™ contained no visible bands, even though samples intially contained the same protein amount, suggesting that, at these high concentrations, the enzyme may be digesting the protein. Western blot analysis revealed no bands in any samples, even in the digestion reaction control sample containing no EKMax™; however, a band could be seen in sample containing culture pellet which had not been treated overnight with EKMax™. This may be due to degradation of the protein from increased temperature, caused by incubation at 37˚C.

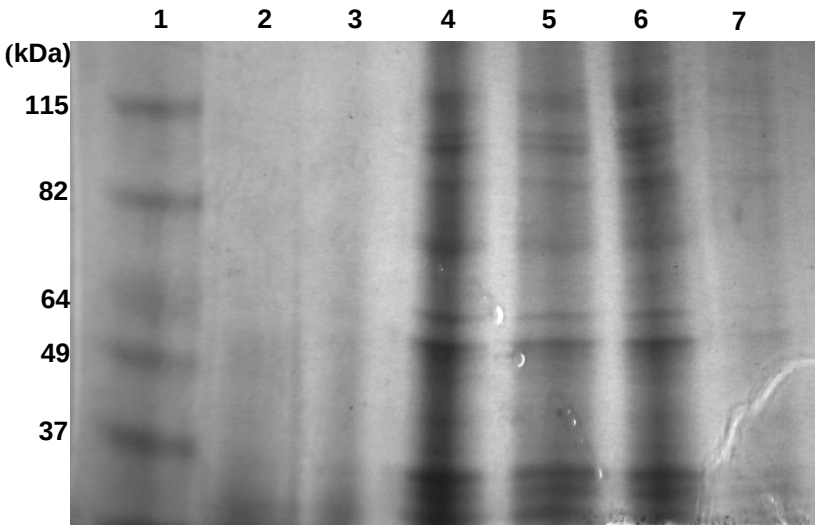

Figure 4.17 Analysis of the digestion of STV/IL-2 with EKMax™ enterokinase by Coomassie blue stain.

10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, digested with EKMax™ at 37°C visualized by Coomassie blue staining.

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2-7) STV/IL-2 protein samples containing 4, 1, 0.1, 0.01, 0.001 and 0units of EKMax™ per reaction; (7). Numbers on the left hand side correspond to molecular mass (kDa) markers.

As suggested by the manufacturers of EKMax™ (Invitrogen), the pilot reactions were repeated as described above with incubation overnight at room temperature instead of 37˚C. The samples were then boiled in gel loading buffer containing β-mercaptoethanol, and separated by electrophoresis on a 10% (w/v) SDS-PAGE gel. Visualization by western blot analysis was initially carried out using a mouse anti-histidine tag antibody (Figure 4.18). The Western blot membrane was then stripped by incubating with Restore™ Western Blot Stripping Buffer for 15 minutes at room temperature, and probed a rabbit anti-streptavidin polyclonal antibody (Figure 4.19).

Western blot analysis with a mouse anti-histidine tag antibody showed two bands at approximately 30kDa and 70kDa in the control sample (no EKMax™ digestion) and in the samples containing 0.1 units of EKMax™. The sample containing 1 unit of EKMax™ showed very faint bands at the same molecular mass. Protein bands should not be present on the western blot membrane when probing with an anti-histidine antibody if the histidine tag has been cleaved. This suggests that while 0.1 units of EKMax™ is not sufficient to cleave the histidine tag, 1 unit will cleave most of the histidine tags from the majority of protein. It is unclear why samples with less than 0.1 units of EKMax™ do not show visible bands, samples with more than 1 unit of EKMax™ may be subject to unspecific cleavage.

Western blot analysis with an anti-streptavidin antibody following stripping of the western blot membrane with Restore™ Western Blot Stripping Buffer showed greater apparent unspecific binding. However, the unspecific binding allows visualization of all the bands in the sample, and shows the bands to be clearer than those seen in samples incubated at 37^{o}C (Figure 4.19) suggesting that more unspecific cleavage or degradation

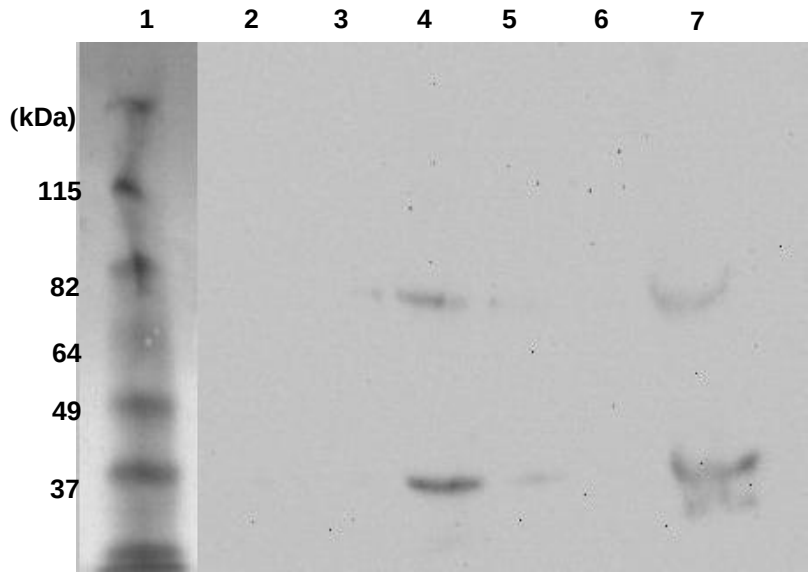

Figure 4.18 Analysis of the digestion of STV/IL-2 with EKMax™ enterokinase with a mouse anti-histidine tag antibody.

Photograph of a 10% SDS-PAGE/Western Blot analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, digested with EKMax™ at room temperature, visualized by probing with a mouse anti-histidine tag purified antibody.

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) Blank (3-7) STV/IL-2 protein samples containing 0, 0.01, 0.1, 1 and 4 units of EKMax™ per reaction respectively. Numbers on the left hand side correspond to molecular mass (kDa).

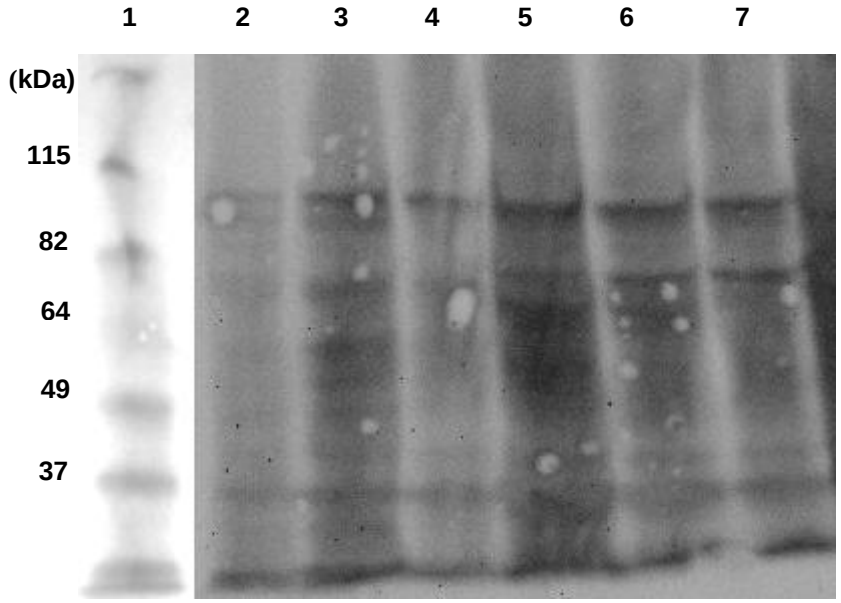

Figure 4.19 Analysis of the digestion of STV/IL-2 with EKMax™ enterokinase with a rabbit anti-streptavidin antibody.

10% SDS-PAGE/Western Blot analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-2 plasmid, digested with EKMax™ at room temperature, visualized by probing with a rabbit anti-streptavidin antibody.
Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2-7) STV/IL-2 protein samples containing 4, 1, 0.1, 0.01, 0.001 and 0 units of EKMax™ per reaction. Numbers on the left hand side correspond to molecular mass (kDa) markers.

of the protein is occurring at 37^{o}C.

4.6 STV/IL-18 Expression in *E. coli*

4.6.1 Transformation

Using the protocol optimized for expression of STV/IL-2 in BL21(DE3)PLysS Chemically Competent cells (Section 4.2), these cells were transformed with pCR®T7/NT-STV/IL-18 (pCR®T7/NT-TOPO® containing the STV/IL-18 insert) by heat shocking the cells at 42^{o}C. The transformed cells were added to 10ml LB liquid medium containing 100µg/ml ampicillin and 34µg/ml chloramphenicol, supplemented with 1% w/v glucose and grown overnight at 37^{o}C with shaking.

4.6.2 Pilot Protein Expression Study using LB Supplemented with Glucose

5ml of LB liquid medium containing 100µg/ml ampicillin and 34µg/ml chloramphenicol, supplemented with 1% w/v glucose, was inoculated with 250µl of overnight culture from the transformation of BL21(DE3)PLysS with pCR®T7/NT-STV/IL-18 (Section 2.7.1) and incubated for 2 hours at 37^{o}C after which the culture was induced with 0.8M IPTG and incubation resumed. 500µl samples of the culture were taken 2 hours, 3 hours, and 4 hours after induction, and after overnight expression. As previously (Section 4.2.3) pelleted cells from these cultures were reduced by resuspension and boiling in gel loading buffer supplemented with ß-mercaptoethanol. The samples were separated by electrophoresis on 10%w/v SDS-PAGE and subjected to western blot analysis with a mouse anti-histidine tag antibody (Figure 4.20). Bands corresponding approximately to the predicted molecular mass of STV/IL-18 (35.08kDa) (Section 2.8.6) are visible in all of the samples, but appear more intense in the 4 hour

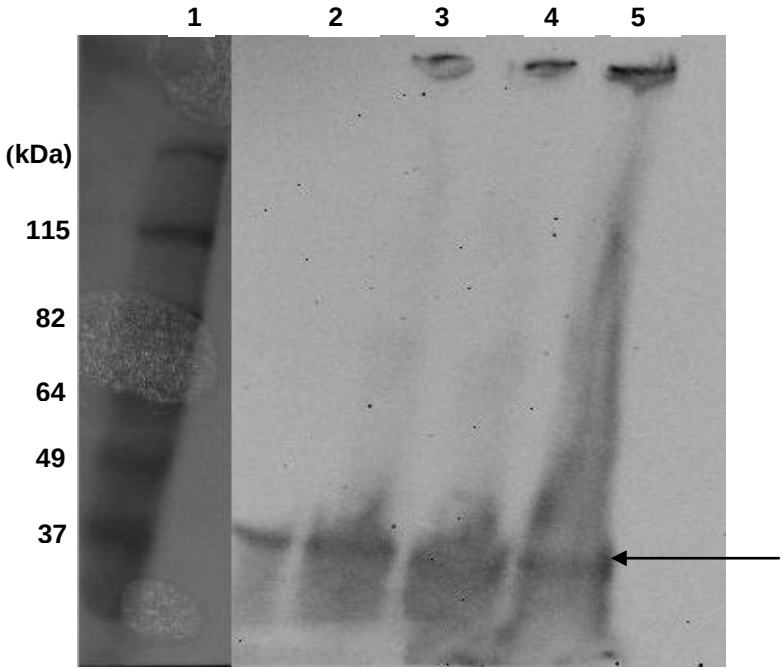

Figure 4.20 Analysis of recombinant STV/IL-18 expressed in BL21(DE3)PLysS cells.

10% SDS-PAGE/Western Blot analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-18 plasmid, grown in medium containing 1% w/v glucose, visualized by probing with a mouse anti-histidine tag purified antibody. Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2-5) culture samples induced with IPTG taken after 2, 3, 4 hours and overnight culture respectively. Numbers on the left hand side correspond to molecular mass (kDa) markers. . The arrow on the right hand side highlights the protein bands which correspond approximately to the estimated recombinant STV/IL-18 mass.

and overnight samples. Since this pilot expression study indicated that STV/IL-18 expression had been successfully induced following the same protocol as that used for the expression of STV/IL-2, the system was scaled up to increase the total yield of protein for further analysis.

4.6.3 Cell Lysis BugBuster® Protein Extraction Reagent

50ml culture pellets (prepared as in Section 4.6.2) were lysed by incubation at room temperature with 500µl BugBuster® Protein Extraction Reagent supplemented with Benzonase® Nuclease (Section 2.7.3). The insoluble and soluble fractions were separated by centrifugation in order to identify whether the STV/IL-18 protein was being expressed as a soluble protein, or in inclusion bodies.

4.6.4 Analysis of Soluble and Insoluble Protein Fraction

Solubilized protein in BugBuster® Protein Extraction Reagent was analysed for the presence of STV/IL-18 by SDS-PAGE and visualized by Western blotting (Figure 4.21a) and Coomassie blue staining (Figure 4.21b). A soluble sample and a control sample (1ml bacterial culture pellet as prepared in Section 4.6.2) were added to gel loading buffer containing β-mercaptoethanol and boiled to reduce the protein sample before loading onto a 10% SDS-PAGE gel.

A faint band could be seen in the soluble protein sample, when visualized by Western blot analysis using an anti-histidine antibody (Figure 4.21a), corresponding approximately to the estimated molecular mass of STV/IL-18 of 35.08kDa. The positive control showed significant background binding which may be due to very high

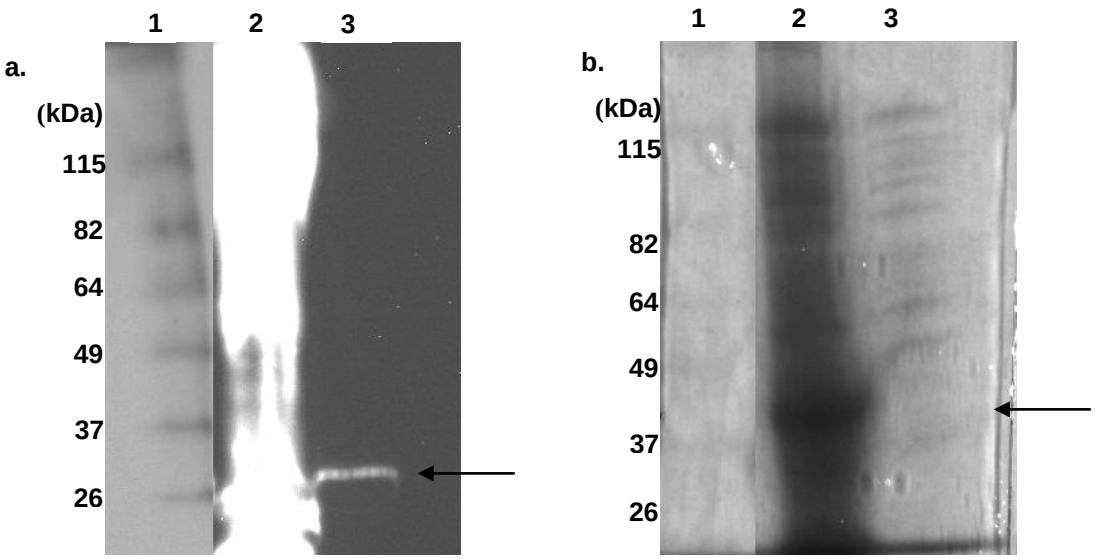

Figure 4.21 Analysis of the soluble and insoluble fractions prepared from *E. coli* cells expressing STV/IL-18.

a) 10% SDS-PAGE/Western blot analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-18 plasmid, visualized by probing with a mouse anti-histidine tag antibody.

b) 10% SDS-PAGE analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-18 plasmid, visualized by Coomassie blue staining.

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) complete bacterial pellet from 4 hour incubation following induction of expression with IPTG; (3) clarified soluble protein from bacterial pellet lysed with BugBuster®. Numbers on the left hand sides correspond to molecular mass (kDa) markers.

concentration of protein within the positive control sample. However, staining with Coomassie blue (Figure 4.21b), showed a strong band in the positive control sample which could not be seen in the solubilized protein sample, suggesting that the protein is largely insoluble.

Inclusion bodies isolated and purified from a 50ml culture (prepared as in section 4.6.2) (Section 2.7.5) was resuspended in gel loading buffer and β-mercaptoethanol, and boiled to reduce the sample. A 20µl sample was subjected to separation by electrophoresis on a 10% SDS-PAGE gel, along with a 20µl of a 1ml pelleted bacterial cell sample (as prepared in Section 4.6.2) as a positive control. Western blot analysis of the gel using a mouse anti-histidine antibody (Figure 4.22) showed an intense band in the sample containing the insoluble protein corresponding approximately to the estimated protein molecular weight of STV/IL-18 of 35.08kDa. A band at the same molecular weight could be seen in the positive control total protein sample. As the STV/IL-18 appears to remain in the insoluble pellet, this suggests that the majority of the STV/IL-18 protein is forming inclusion bodies and therefore must be solubilized prior to further analysis.

4.6.5 Solubilization and Refolding of Purified Inclusion Bodies with 100mM Tris, 2M Urea pH 12.5

Purified STV/IL-18 inclusion bodies were solubilized according to the protocol developed for the solubilization of STV/IL-2 inclusion bodies (Section 4.4.3). STV/IL-18 inclusion bodies (prepared from cultures as in Section 4.6.2) were isolated and purified from the insoluble fraction (Section 2.7.5) by incubation with BugBuster® and rLysozyme™, followed by several wash steps. Purified inclusion bodies were

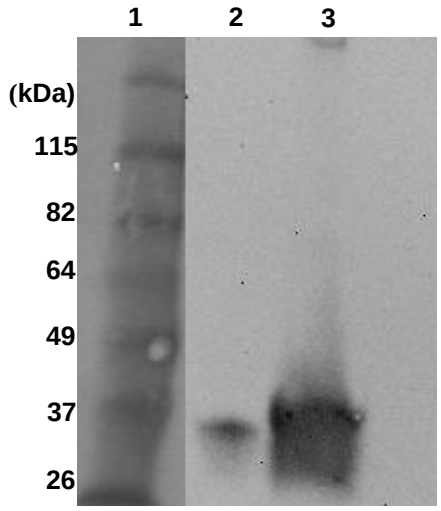

Figure 4.22 Identification of STV/IL-18 in inclusion bodies by western blot analysis.

10% SDS-PAGE/Western blot analysis of proteins produced by BL21(DE3)PLysS cells containing the pCR®T7/NT-STV/IL-18 plasmid visualized by probing with a mouse anti-histidine tag antibody.

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) complete bacterial pellet from 4 hour incubation following induction of expression with IPTG; (3) purified inclusion bodies. Numbers on the left hand side correspond to molecular mass (kDa) markers.

incubated with 100mM tris, 2M urea pH 12.5 for 30 minutes. Insoluble debris was removed from the sample by centrifugation at 16,000g for 20 minutes. 1ml of solubilization buffer was used to resuspend 0.8mg of inclusion bodies. The solubilized STV/IL-18 protein was then diluted into PBS in a pulsatile manner in order to reduce the pH and reduce the concentration of urea in the buffer. This was done using the same protocol as adopted for the solubilization of STV/IL-2. The sample was centrifuged again at 16,000g for 20 minutes to remove any insoluble debris. For samples stored for any length of time a cocktail of protease inhibitors was added to the final buffer to prevent protein degradation from occurring. STV/IL-18 appeared to solubilise using this protocol. However, after storage overnight protein precipitate could be seen in the samples.

4.7 Discussion

STV/IL-2 and STV/IL-18 were successfully expressed in OneShot®BL21(DE3)pLysS Chemically Competent *E. coli* transformed with pCR®T7/NT-STV/IL-2 and pCR®T7/NT-STV/IL-18. Supplementing the culture medium with 1% w/v glucose produced appeared to result in higher levels of expression than using medium with no added glucose (Sections 4.2.3 and 4.6.2) Toxic genes can be unstable in the BL21(DE3) cell strain used here for expression. Glucose helps to reduce basal levels of expression of T7 polymerase which is instrumental to the induction of expression of the pCR TOPO expression system. Lactose contamination of the media can cause leaky expression of the recombinant protein from this vector due to its induction of the *lac* operon. The inclusion of glucose in the culture medium prevents lactose inducing the *lac* operon by providing an alternative energy source for the cells (Berrow *et al* 2006), thereby reducing the expression of the recombinant fusion proteins prior to induction of

expression by the addition of IPTG, and preventing early death of the *E. coli* cells due to protein toxicity.

STV/IL-2 and STV/IL-18 fusion proteins were found to accumulate in inclusion bodies with the *E. coli* cells, which could be extracted and then solubilized with 100mM tris, 2M urea at pH 12.5. Since a high percentage of recombinant proteins, including interleukin-2 (Devos *et al* 1983) and streptavidin (Sano and Cantor 1990) are insoluble when expressed in *E. coli*, it is unsurprising to find that STV/IL-2 and STV/IL-18 were identified in greater quantities in inclusion bodies than as soluble proteins.

Interestingly, when a protein band of the recombinant STV/IL-2 protein was seen on a western blot at around 30kDa, it is often accompanied by a higher band at around the 64kDa marker on the protein ladder. This is approximately twice the predicted size of the monomeric fusion protein, suggesting that the STV/IL-2, like streptavidin (Green 1975), may be forming multimers (in this case, dimers) which had not been completely dissociated by the reduction of the protein with β-mercaptoethanol, or by dissociation using SDS prior to loading the protein samples onto SDS-PAGE for analysis.

Attempts to solubilize STV/IL-2 inclusion bodies by conventional methods using high concentrations of denaturant (6M urea) were not successful, and therefore an alternative method using mild denaturing conditions and high pH was followed. Bacterial inclusion bodies consist of densely packed protein aggregate which is usually highly homogeneous (Singh and Panda 2000). Because of the relative purity of expressed recombinant protein in inclusion bodies it can be advantageous for the isolation, extraction and purification of recombinant proteins. As well as high homogeneity,

inclusion bodies have other advantages over soluble proteins; they are easily separated from the rest of the bacterial cell and cellular proteins, and are less susceptible to digestion by proteases and degradation due to temperature (Morreale *et al* 2003).

Resolubilization of inclusion bodies using high concentrations of denaturant unfolds the protein to its primary structure, increasing the chance of the protein re-aggregating, or allows the protein to form coils exposing hydrophobic regions of the protein which are susceptible to aggregation (Bowden *et al* 1991). As proteins accumulating in inclusion bodies retain some secondary structure, solubilization using mild denaturants and a pH distinct from the isoelectric point of the protein retains this secondary structure, reducing the probability of protein aggregation. Patra *et al* (2000) developed a protocol for the solubilization of human growth hormone from inclusion bodies using 2M urea in 100mM tris buffer at a variety of pHs. They found that a pH of 12.5 was most effective, which was distant from the isoelectric point of human growth hormone of 4.9. A highly alkaline pH disrupts the ionic and hydrophobic bonds between proteins, both of which contribute greatly to aggregation. Following initial difficulties encountered in solubilising the recombinant fusion proteins from inclusion bodies this method was introduced and resulted in the successful solubilization of inclusion bodies from the expression of both STV/IL-2 and STV/IL-18; this however affects the folding of the recombinant fusion proteins.

To circumvent the need to resolubilize proteins which form inclusion bodies it is possible to tag the recombinant protein with fusion partner which native to the expression host organism; this can often lead to the expression of the insoluble protein in a soluble form. The fused tag can later be cleaved from the recombinant protein. De

Groot and Ventura (2008) studied several thermostable proteins isolated from *E. coli* for use as fusion partners for producing soluble recombinant proteins, and found that fusion of trigger factor (TF), a native *E. coli* protein, to amyloid-β via a ubiquitin linker allowed the normally insoluble amyloid-β protein to be expressed in a soluble form.

Refolding of STV/IL-2 by dialysis against PBS caused precipitation of the protein; removal of the denaturant and reduction of the pH of the buffer resulted in the protein forming aggregates. Thus STV/IL-2 and STV/IL-18 were refolded by dilution in a pulsatile manner into PBS which lowered the pH of the buffer from 12.5 to around 8 This refolding protocol was described by Rudolph and Fischer (1990), adapted and found to be effective in this study. Pulse dilution of small volumes of a concentrated protein into a large volume of diluents leads to rapid collapse of structure and causes refolding. Because of the small volume of protein added to the diluents and the time between additions of protein, the likelihood of aggregation of protein is much reduced, although the protein can misfold (reviewed by Tsumoto *et al* 2003). This may well be what has occurred in the case of the recombinant streptavidin-cytokine fusion proteins. Recombinant streptavidin has been shown to be insoluble when expressed in *E. coli.* Sørensen *et al* (2003) showed that the refolding of recombinant streptavidin posed problems, and subsequently they developed a gradual dilution dialysis method of refolding the protein. However, this method requires expensive custom-made equipment and is more time-consuming than the pulsatile dilution method that was

Protein aggregation does not necessarily imply loss of biological function (Thapa *et al* 2008) in fact, the production of proteins as inclusion bodies in bacterial expression systems may offer benifits, such as the resistance of the proteins to temperature or

proteolytic degradation (Bowden *et al* 1991), the relatively high percentage of recombinant protein contained within the inclusion body, compared to the heterologous protein expressed in soluble form, and the ease of isolation of the protein from the bacterial host cells. Both STV/IL-2 and STV/IL-18 were found to accumulate in bacterial inclusion bodies, they did not appear to retain their function, suggesting that these proteins did not refold correctly using the method employed.

Lack of aggregation of the proteins when the denaturant was diluted and the pH reduced indicated that protein had refolded; however, this does not indicate that correct refolding of the protein has necessarily occurred. The absence of disulphide bonds in the correctly folded proteins should assist in the refolding of the proteins into their native form (Vallejo and Rines 2004). Protein function assays are therefore the key to identify whether the recombinant STV/IL-2 had folded correctly or not (Chapter 5).

Chapter 5

Analysis of Recombinant STV/IL-2 and STV/IL-18 Fusion Proteins

5.1 Introduction

Cloning and expression are key steps along the way to producing a recombinant protein; however, before it can be said to have been successfully produced it must be shown to be biologically active. With fusion proteins the confirmation of bioactivity is especially important, as the folding of one moiety may interfere with the folding of another, and overall folding of the molecule may be compromised, thereby reducing or eliminating protein function (Georgiou and Valax 1996). All moieties of the fusion protein therefore need to be assessed for bioactivity.

IL-2 induces the proliferation of T cells (Morgan *et al* 1976). Therefore a T cell proliferation assay has been developed using the cytotoxic T cell line CTLL-2 which was transformed from murine spleen cells and constitutively expresses IL-2R. CTLL-2 cells are entirely dependent on human or murine IL-2 or murine IL-4 for their growth and proliferation (Davis *et al* 2001). CTTL-2 proliferation has been measured using a range of techniques including tritiated thymidine ($^{[3H]}$TdR) incorporation into DNA during cell mitosis (Mosmann 1983), MTT cell proliferation assays (Tada *et al* 1986) or the more recent improvement of the MTT cell proliferation assay which uses Alamar Blue (Kwack and Lynch 2000). The Alamar Blue based assay has become the preferred method of assessing the bioactivity of IL-2 (Torres *et al* 2006, Jones *et al* 2007). This is less laborious than the MTT or $^{[3H]}$TdR incorporation assay and does not produce the radioactive waste of the $^{[3H]}$TdR incorporation assay, nor need the solubilization of formazan crystals required in the MTT assay, which is an error-prone, time consuming step. The IL-2 bioactivity described by Kwack and Lynch (2000) was shown to be accurate, and as the Alamar Blue reagent is not sample destructive, it can be used

throughout growth of the cell line in culture. This CTLL-2 assay can detect recombinant IL-2 in the range 16IU/ml to 2,000IU/ml.

Many IL-2 fusion proteins have been produced, and the CTLL-2 assay is commonly used to assess the biological activity of the IL-2 moiety. Some studies have shown that fusion of IL-2 with another protein can reduce its bioactivity (Xiang *et al* 1994, Gillies *et al* 2002, Jordon *et al* 2003, Zhang *et al* 2004) and this was hypothesised to be due to steric hindrance in these cases, while some concluded that IL-2 fusion with another molecule does not affect IL-2 bioactivity (Xu *et al* 2000, Yao *et al* 2004).

IL-18 induces IFN-γ production in combination with IL-12 (Okamura *et al* 1995b). The ability of recombinant human IL-18 to induce the production of IFN-γ was originally assayed using human PBMC (Ushio *et al* 1996). Because the preparation, propagation and maintenance of PBMC are time consuming and variability exists between cells from different donors, and because PBMC also produce IFN-γ in response to other cytokines such as IL-12, Konishi *et al* (1997) investigated many cell lines for the ability of IL-18 to induce IFN-γ. A human myelomonocytic cell line named KG-1 was found to produce IFN- γ in response to human IL-18 in a dose-dependent manner and was also shown to be independent of IL-12.

Konishi and colleagues developed a bioassay based on the treatment of KG-1 cells with human IL-18 to induce secretion of IFN-γ into the cell culture medium. The level of IFN-γ in the culture medium can reliably be assayed using an IFN-γ ELISA. The advantages of this method of investigating human IL-18 levels over a standard quantitative IL-18 ELISA lie in the confirmation of the bioactivity of recombinant

human IL-18, which can be inferred from the induction of IFN-γ production by KG-1 cells.This assay has been used previously to assess the biological activity of IL-18 in fusion proteins, including an IL-18-Green Florescence Protein fusion protein and an epidermal growth factor-IL-18 fusion protein. Both of the IL-18 fusion proteins were shown to retain the same activity as recombinant IL-18 (Kim *et al* 2004, Omoya *et al* 2004, Lu *et al* 2005).

Streptavidin has a high binding affinity ($K_d \approx 1\times10^{-14}$) for biotin (Chaiet and Wolf 1964) which is dependent on the correct folding, of the protein (Weber *et al* 1989). Native and recombinant streptavidin proteins bind one molecule of biotin per monomer of streptavidin (Green 1975). The biotin binding function of streptavidin can be assessed using biotinylated molecules which can be detected as complexes. The biotin binding capacity of streptavidin fusion proteins has been assayed in a variety of ways, including the binding of the streptavidin to a biotinylated fusion protein, a biotinylated fluorescence marker, biotinylated glass beads, radioactively labelled biotin and detection by gel filtration or streptavidin-biotin binding ELISA (Sano *et al* 1992, Steidler *et al* 1998, Dubel *et al* 1995, Reznik *et al* 1998, Clare *et al* 2001, Moll *et al* 2002). These studies suggest that the biotin binding activity of streptavidin is not compromised by its fusion to another fusion partner, with most showing the same or similar activity to commercial recombinant streptavidins.

This work described in this chapter was conducted with the aim of confirming the identity of the recombinant streptavidin-cytokine fusion proteins, study them in their native form by PAGE and mass spectrometry (sample testing undertaken by Kevin Welham, Department of Chemistry, University of Hull), and assessing the moieties of

the fusion proteins for functional activity. Limited time and the time-consuming protocol involved in producing batches of solubilized STV/IL-2 and STV/IL-18 meant that repeats of these experiments were often not possible, and a number of different studies were undertaken, rather than performing several repeats of one study.

5.2 Results

5.2.1 SDS-PAGE Analysis of Possible Solubilized Recombinant Fusion Proteins

In order to further investigate the identity of the recombinant proteins SDS-PAGE analysis was undertaken using Coomassie blue staining of SDS-PAGE gels and Western blot analysis with antibodies directed against the different moieties of the fusion proteins; anti-histidine tag, anti-streptavidin, anti-IL-2 and anti-IL-18.

IL-2/STV and IL-18/STV expressed using the PCR®T7 TOPO® bacterial expression system, were solubilized in solubilization buffer containing 100mM tris and 2M urea at pH 12.5 (Section 2.7.5), and reduced by boiling with β-mercaptoethanol and SDS in gel loading buffer. 20µl of each sample were loaded onto a 10% SDS-PAGE gel and separated by electrophoresis (Section 2.8.2).

(i) Staining with Coomassie blue

Analysis of the separated samples by staining with Coomassie blue (Figure 5.1) identified a number of bands in the lanes contaning STV/IL-2 sample at approximately 30-40kDa and 50-60kDa. The lower molecular weight band corresponds to the estimated product size of the recombinant STV/IL-2 fusion protein as well as to

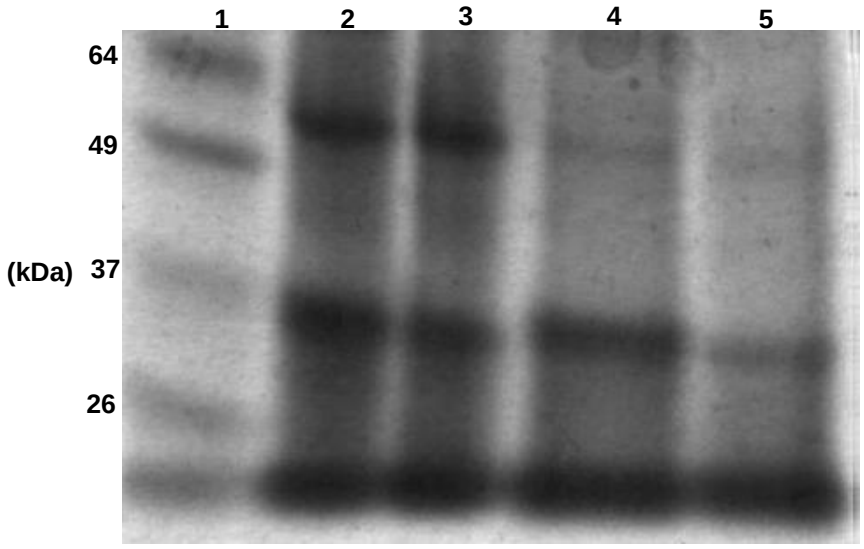

Figure 5.1 10% SDS-PAGE/Western blot analysis of solubilized STV/IL-2 and STV/IL-18 proteins visualized by Coomassie blue staining.

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) 20µl solubilized STV/IL-2; (3) 20µl solubilized STV/IL-2 diluted tenfold in dH2O; (4) 20µl solubilized STV/IL-18; (5) 20µl solubilized IL-18 diluted tenfold in dH2O. Numbers on the left hand side correspond to molecular mass (kDa) markers.

appearing to be the same molecular weight as the STV/IL-2 fusion protein in total bacterial pellet and inclusion bodies protein preparations detected by Western blot and Coomassie blue staining seen as previously (Chapter 4). The higher molecular weight band which may be a mulitmer of the STV/IL-2 protein, has also been previously observed and discussed in Chapter 4. Similarly, staining of a disrete band was seen as previously (Chapter 4) in lanes containing STV/IL-18 samples at 30-40kDa with a fainter band at 50-60kDa.

(ii) Protein Detection by Western Blot Analysis

Western blotting of protein samples separated by SDS-PAGE with i) a mouse anti-histidine tag antibody, ii) a goat anti-human IL-18 polyclonal antibody raised against a C-terminal IL-18 peptide, iii) a goat anti-human polyclonal antibody raised against *E. coli* derived rhIL-2 and iv) a rabbit anti-streptavidin polyclonal antibody (Figure 5.2) showed bands in lanes containing STV/IL-2 when stained with the anti-his tag, anti-streptavidin and anti-IL-2 antibodies. The bands show a protein of approximately 30kDa, which corresponds well to the estimated STV/IL-2 protein size of 32.2kDa. As has been seen in previous SDS-PAGE analysis (Chapter 4), there is a higher band visible. The Western blot nitrocellulose membrane used in this experiment was accidentally cut through lane 9 (Figure 5.2), and the STV/IL-2 sample in this lane was therefore visualized with both the anti-streptavidin and the anti-IL-2 antibodies, fortuitously showing that the protein bands detected by these antibodies are exactly the same protein band.

There were no bands observed in the STV/IL-18 samples stained with any of the antibodies. Faint high molecular mass bands around 115kDa were visible in the

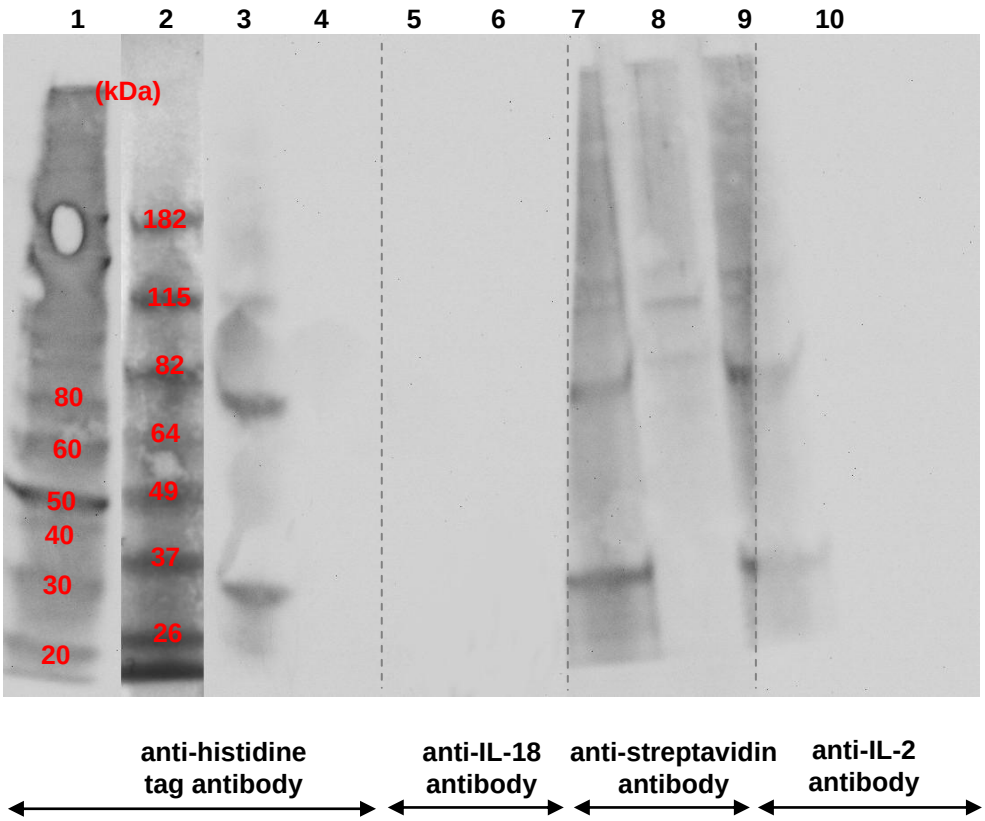

Figure 5.2 10% Western blot analysis of solubilized STV/IL-2 and STV/IL-18 proteins.

Western blot analysis of soluble STV/IL-2 and STV/IL-18 separated by gel electrophoresis on 10% SDS-PAGE gel, visualized by probing with a mouse anti-histidine tag antibody, anti-IL-18 antibody, anti-streptavidin antibody and anti-IL-2 antibody

Lanes: (1) BenchMark™ His-tagged Protein Standard (stained with the anti-histidine antibody); (2) BenchMark™ Pre-Stained Protein Ladder; (3, 5, 7, 9) 20µl STV/IL-2 (4, 6, 8, 10) 20µl STV/IL-18. Numbers on the left hand sides correspond to molecular mass (kDa) of each of the bands in the molecular weight protein ladder. Dotted lines show where the membrane was cut in order to be stained with the different antibodies (antibodies used are marked below each section of membrane).

STV/IL-18 sample which was detected with an anti-streptavidin antibody. A BenchMark™ His-tagged Protein Standard was used alongside the BenchMark™ Pre-Stained Protein Ladder; this acts as another protein marker as well as a positive control for the anti-histidine tag antibody. Repeated attempts to visualize solubilized samples of STV/IL-18 by SDS-PAGE/Western blot analysis were unsuccessful.

5.2.2 Native PAGE Analysis of Solubilized Recombinant Fusion Proteins

Native PAGE gels contain no detergent to dissociate the protein and so allow the protein to be analysed in its native configuration. Non-reducing gel loading buffer was added to samples of solubilized STV/IL-2 (Section 4.4.3), solubilized STV/IL-18 (Section 4.6.5), recombinant streptavidin, recombinant human IL-2 and recombinant human IL-18. The samples were loaded onto a native 10% polyacrylamide gel (containing no SDS) and separated by electrophoresis.

(i) Staining with Coomassie blue

As expected, analysis of the native gel by staining with Coomassie blue showed a higher level of protein staining in the samples containing recombinant fusion proteins (STV/IL-2 and STV/IL-18) which had not been diluted (Figure 5.3). However, in the lanes which contained the fusion proteins diluted a hundredfold, no bands could be seen in the STV/IL-18 sample, and the STV/IL-2 sample showed a stained band at approximately 70-80kDa. A fainter band may also be visible in this sample at the lower molecular weight of approximately 30kDa which would correspond with the predicted molecular mass of the STV/IL-2 recombinant fusion protein. These bands are highlighted on Figure 5.3 with yellow arrows. Staining of the recombinant human IL-2

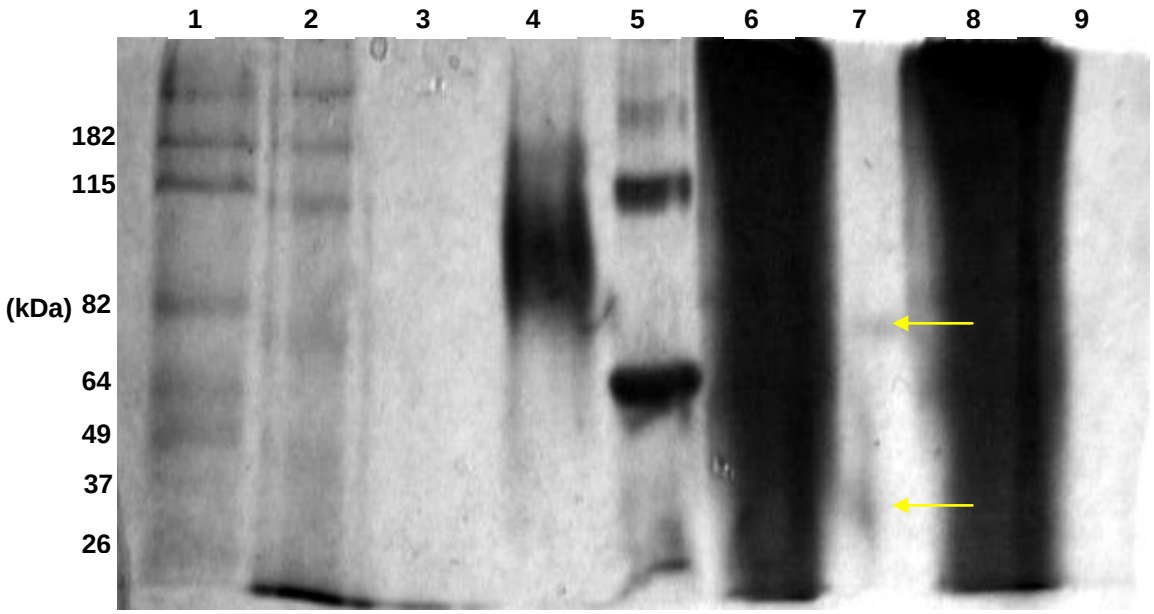

Figure 5.3 Comparison of STV/IL-2 and STV/IL-18 with recombinant IL-2, IL-18 and streptavidin.

10% native PAGE analysis of solubilized STV/IL-2 and STV/IL-18 compared with recombinant human IL-2 and IL-18, and recombinant streptavidin visualized by Coomassie blue staining.

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) BenchMark™ His-tagged Protein Standard (most bands are too faint to be useful); (3) 2µg recombinant human IL-2; (4) 2.5µg recombinant human IL-18; (5) 20µg recombinant streptavidin; (6) 20µl solubilized STV/IL-2; (7) 20µl solubilized STV/IL-2 100× dilution in dH2O; (8) 20µl solubilized STV/IL-18; (9) 20µl solubilized IL-18 100× dilution in dH2O. Numbers on the left hand side correspond to molecular mass (kDa) markers.

sample (2μg), however, showed no bands; it is possible that the concentration of the IL-2 was too low to be detected by Coomassie blue staining.

Staining of recombinant human IL-18 showed a high intensity band between approximately 115 and 82kDa. Human IL-18 is approximately 18kDa. The band observed (Figure 5.3) may be an aggregate of IL-18 which is appearing as a band at a higher than expected molecular weight because the gel is not a dissociating gel, although IL-18 is not expected to form multimers or aggregates (Ushio *et al* 1996). Recombinant streptavidin shows a strong band at approximately 60kDa which corresponds to the expected size of the streptavidin tetramer (60kDa). A smaller band appears at approximately twice this size, and a further faint band at a high molecular mass which may again be due to protein aggregation.

(ii) Analysis by Western blotting

Western blotting of the samples subjected to PAGE as described above, was undertaken using anti-IL-2, anti-IL-18, anti-histidine tag and anti-streptavidin antibodies to further identify and characterise the protein bands seen in Figure 5.3

STV/IL-2 and STV/IL-18 in solubilization buffer were added to non-reducing gel loading buffer and loaded onto a native PAGE gel. The samples were then separated by electrophoresis. Recombinant human IL-2 (2μg), streptavidin (20μg) and IL-18 (2.5μg) were also analysed in the same way. Western blot analysis of protein samples was carried out using a goat anti-human polyclonal antibody raised against *E. coli*-derived

rhIL-2 (Figure 5.4), a polyclonal rabbit anti-streptavidin antibody (Figure 5.5), a goat anti-human IL-18 polyclonal antibody raised against a C-terminal IL-18 peptide (Figure 5.6) and a polyclonal mouse anti-histidine tag antibody (Figure 5.7).

Detection of recombinant IL-2 with the anti-IL-2 antibody was unsuccessful and no protein bands were visible (Figure 5.4). Although IL-2 protein could not be visualized in native gels stained with Coomassie blue stain (Figure 5.3), Western blotting is more sensitive than Coomassie blue staining and the absence of a visible band in the IL-2 sample is surprising; however, this may be due to the concentration of IL-2 in the sample being low and below the level of sensitivity of detection using this antibody in this protocol. Protein bands can be seen in the samples analysed by Western blot when probed with the anti-streptavidin antibody (Figure 5.7); however, this can be seen in all samples and recombinant streptavidin from an adjacent well may have contaminated this sample. As expected, recombinant IL-2 was not detected by probing with an anti-histidine tag antibody (Figure 5.5) or an anti-IL-18 (Figure 5.6).

Recombinant IL-18 could not be seen in any of the samples analysed using native gels. Because IL-18 could be seen in sample separated by native gel stained with Coomassie blue (Figure 5.3), it is more likely that there is a problem with the IL-18 antibody not binding to the recombinant IL-18 that is present in this sample.

STV/IL-2 could be visualized with an anti-IL-2 antibody (Figure 5.4) but showed a band at a high molecular weight (70-120kDa); this could be due to the protein separating differently than would on a SDS-PAGE gel, because it is in the native form, or it could be due to aggregation of the protein. Visualization with an anti-histidine tag

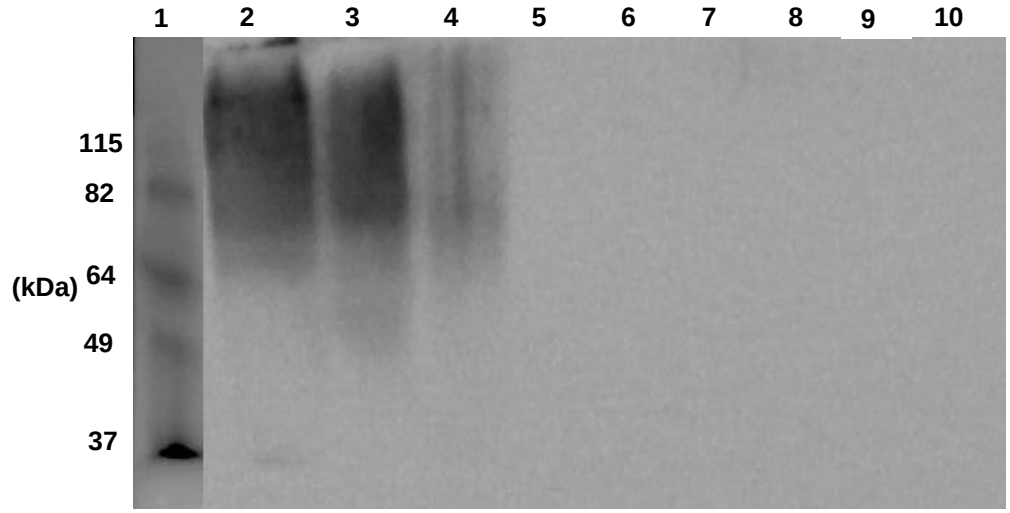

Figure 5.4 Western blot analysis of solubilized STV/IL-2 and STV/IL-18, visualized by probing with an anti-IL-2 antibody.

Western blot analysis of soluble STV/IL-2 and STV/IL-18 along with recombinant IL-2, IL-18 and streptavidin, separated by gel electrophoresis on 10% SDS-PAGE gel, visualized by probing with an anti-IL-2 antibody

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) 20µl solubilized STV/IL-2; (3) 20µl solubilized STV/IL-2 10× dilution in dH2O; (4) 20µl solubilized STV/IL-2 100× dilution in dH2O; (5) 20µl solubilized STV/IL-18; (6) 20µl solubilized STV/IL-18 10× dilution in dH2O; (7) 20µl solubilized STV/IL-18 100× dilution in dH2O; (8) 2 µg recombinant human IL-2; (9) 20µg recombinant human streptavidin; (10) 2.5µg recombinant IL-18. Numbers on the left hand side correspond to molecular mass (kDa) markers.

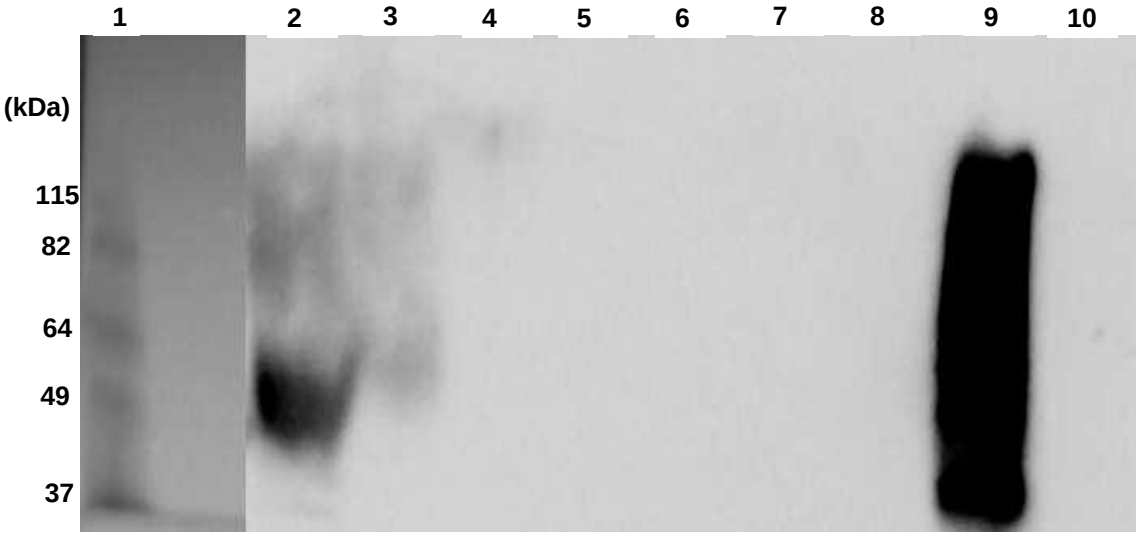

Figure 5.5 Western blot analysis of solubilized STV/IL-2 and STV/IL-18, visualized by probing with an anti-histidine tag antibody.

Western blot analysis of soluble STV/IL-2 and STV/IL-18 along with recombinant IL-2, IL-18 and streptavidin, separated by gel electrophoresis on 10% SDS-PAGE gel, visualized by probing with an anti-histidine tag antibody

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) 20µl solubilized IL-2/STV; (3) 20µl solubilized IL-2/STV 10× dilution in dH2O; (4) 20µl solubilized IL-2/STV 100× dilution in dH2O; (5) 20µl solubilized IL-18/STV; (6) 20µl solubilized IL-18/STV 10× dilution in dH2O; (7) 20µl solubilized IL-18/STV 100× dilution in dH2O; (8) 2µg recombinant human IL-2; (9) 20µg recombinant human streptavidin; (10) 2.5µg recombinant IL-18. Numbers on the left hand side correspond to molecular mass (kDa) markers.

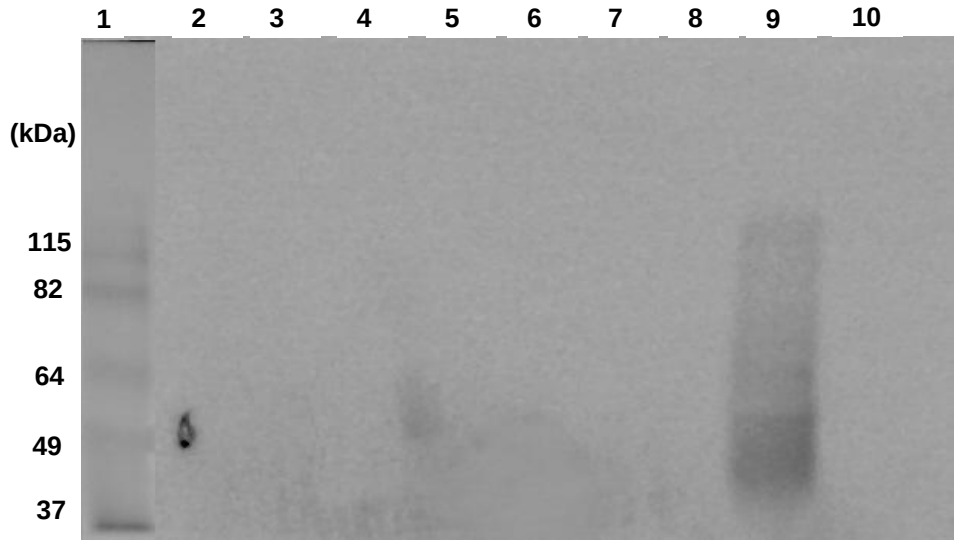

Figure 5.6 Western blot analysis of solubilized STV/IL-2 and STV/IL-18, visualized by probing with an anti-IL-18 antibody.

Western blot analysis of soluble STV/IL-2 and STV/IL-18 along with recombinant IL-2, IL-18 and streptavidin, separated by gel electrophoresis on 10% SDS-PAGE gel, visualized by probing with an anti-IL-18 antibody

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) empty; (3) 20µl solubilized IL-2/STV; (4) 20µl solubilized IL-2/STV 10× dilution in dH2O; (5) empty; (6) 20µl solubilized IL-18/STV; (7) 20µl solubilized IL-18/STV 10× dilution in dH2O; 8) 2µg recombinant human IL-2; (9) 20µg recombinant human streptavidin; (10) 2.5µg recombinant IL-18. Numbers on the left hand side correspond to molecular mass (kDa) markers.

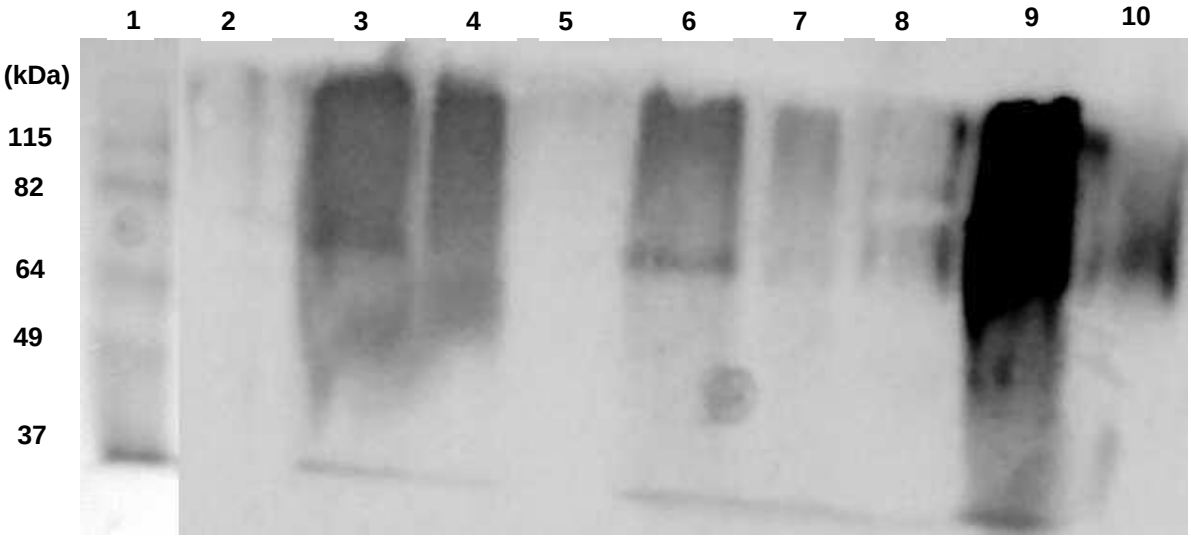

Figure 5.7 Western blot analysis of solubilized STV/IL-2 and STV/IL-18, visualized by probing with an anti-streptavidin antibody.

Western blot analysis of soluble STV/IL-2 and STV/IL-18 along with recombinant IL-2, IL-18 and streptavidin, separated by gel electrophoresis on 10% SDS-PAGE gel, visualized by probing with an anti-streptavidin antibody

Lanes: (1) BenchMark™ Pre-Stained Protein Ladder; (2) empty; (3) 20µl solubilized IL-2/STV; (4) 20µl solubilized IL-2/STV 10× dilution in dH_2O; (5) empty; (6) 20µl solubilized IL-18/STV; (7) 20µl solubilized IL-18/STV 10× dilution in dH2O; 8) 2µg recombinant human IL-2; (9) 20µg recombinant human streptavidin; (10) 2.5µg recombinant IL-18. Numbers on the left hand side correspond to molecular mass (kDa) markers.

antibody (Figure 5.5) showed a band at a slightly higher than expected molecular weight (approximately 50kDa); the protein appears at a lower molecular weight here than in the gel stained with anti-IL-2 antibody. Consistent with Figure 5.2 and staining with the anti-IL-18 antibody, no bands were seen in the gel visualized by probing with an anti-IL-18 antibody (Figure 5.6) and as with other samples non-specific staining was visible in the sample probed with anti-streptavidin antibody (Figure 5.7).

STV/IL-18 could not be detected with any of the antibodies tested here. As with all samples tested, staining was visible when probed with an anti-streptavidin antibody, which may be non-specific binding of the antibody (Figure 5.7).

To assess the binding of the anti-IL-18 antibody to STV/IL-18 a comparison of the binding of anti-IL-18 and anti-streptavidin antibodies to STV/IL-18 was undertaken. STV/IL-18 in solubilization buffer was diluted by 10, 100 or 1000 times in dH_2O to reduce the concentration of urea in the samples. Each of the dilution samples were added to non-reducing loading buffer, loaded onto a native PAGE gel and separated by electrophoresis. Western blot analysis was carried out using a rabbit anti-streptavidin polyclonal antibody and a goat anti-human IL-18 polyclonal antibody raised against a C-terminal IL-18 peptide (see Figure 5.8). Bands were visible in the samples stained with anti-streptavidin antibody at molecular weights of approximately 65kDa, 80kDa and 110kDa. The bands at around 65 and 110kDa could correspond to multimers of the IL-18 protein. No bands were visible in the sample stained with an anti-IL-18 antibody. This may be because the IL-18 antibody is not binding to the STV/IL-18 sample, or because the protein seen in the STV/IL-18 is not the desired recombinant STV/IL-18 protein. As recombinant human IL-18 could be visualized (but at a higher molecular

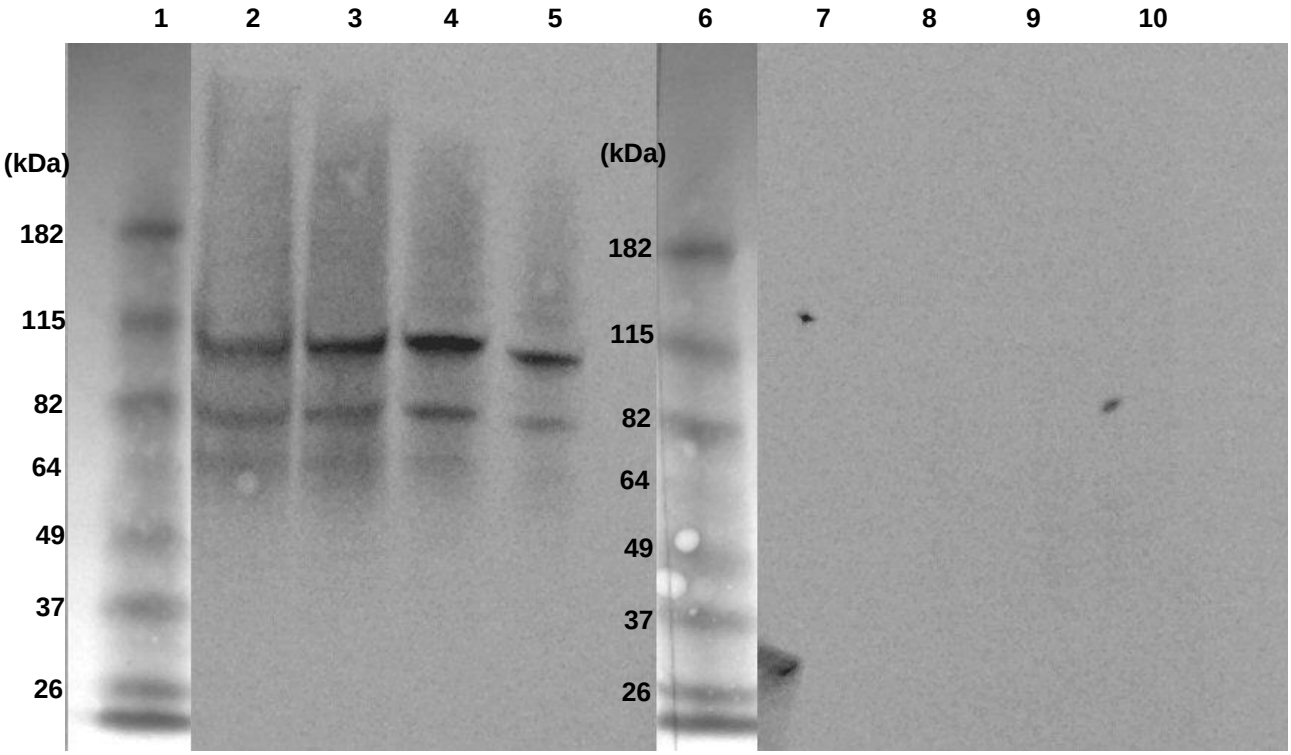

Figure 5.8 Western analysis of solubilized STV/IL-18 proteins, visualized by probing with an anti-streptavidin antibody and an anti-human IL-18 antibody.

Separated by gel electrophoresis on 10% SDS-PAGE gel

Lanes: (1 and 6) BenchMark™ Pre-Stained Protein Ladder; (2-5) STV/IL-18 at 1×,10×,100× and 1000× dilution respectively, visualized by probing with a rabbit anti-streptavidin antibody; (7-10) STV/IL-18 at 1×,x10×,100× and 1000× dilution respectively, visualized by probing with a goat anti-human IL-18 antibody. Numbers on the left hand side and centre of the figure correspond to molecular mass (kDa).

weight than expected) by staining with Coomassie blue stain, but could not be visualized by probing with the anti-IL-18 antibody even though the antibody is designed for western blotting, it is likely that the IL-18 antibody is not recognizing the specific epitope in either recombinant IL-18 or STV/IL-18.

5.2.3 Protein Quantification with Bio-Rad Protein Assay

After solubilization in solubilization buffer, and attempted refolding by twentyfold dilution in PBS (Section 2.7.7), samples of protein extracted from *E. coli* cells expressing STV/IL-2 or STV/IL-18 were assayed using Bio-Rad protein quantification assay (Section 2.8.1).

Solubilization was performed after each batch of recombinant fusion protein was produced (Section 2.7.5); the yield of soluble protein obtained from each batch of expression culture varied between 0.1 and 1mg/ml for a total of four batches of STV/IL-2 and STV/IL-18. Generally the concentration of protein in the STV/IL-18 samples was slightly higher than in the STV/IL-2 samples.

5.2.4 IL-2 Enzyme Amplified Sensitivity Immunoassay (EASIA)

The concentration of protein in a STV/IL-2 sample which had been solubilized and diluted twentyfold in PBS to refold the protein and reduce pH was assayed using an IL-2 EASIA (Section 2.8.9). STV/IL-2 was serially diluted twofold from undiluted to 128 times dilution in PBS. Recombinant streptavidin (10mg/ml) in dH_2O was included as a negative control to confirm no unspecific binding of the IL-2 antibody with streptavidin, and solubilized, refolded STV/IL-18 was used as a negative control to determine whether there was any unspecific binding of the antibody to any contaminating bacterial

proteins which may be present in the sample due to the process of producing the recombinant fusion proteins.

Recombinant human IL-2 protein standards at known concentrations and activities were used in order to create a standard concentration curve (Figure 5.9) which then permits the concentration of the samples to be determined by comparison to the IL-2 recombinant standards (Figure 5.10). An average of '*calculated concentration of the samples* × *dilution factor'* for samples diluted 1:4, 1:8 and 1:16 (3-6), which are within the detectable concentration of the assay, demonstrated a concentration of 121IU/ml to 3 significant figures. No significant activity was seen in the recombinant streptavidin or STV/IL-18 samples.

The concentration of STV/IL-18 could not be assayed using an ELISA in the same way as the only assay available for human IL-18 (Human IL-18 ELISA Kit – MBL International) showed significant cross-reactivity with recombinant streptavidin (*data not shown*), and therefore a measure of STV/IL-18 in comparison to recombinant IL-18 could not assayed using this method.

5.2.5 Functional Analysis of Recombinant Streptavidin by Native PAGE

Functional analysis of recombinant streptavidin in its native form was carried out by native PAGE (Section 2.8.2). Non-reduced streptavidin was run on a native PAGE gel and visualized by western blotting (Section 2.8.4) and detection using a biotinylated primary rabbit anti-streptavidin antibody that was detected with secondary goat anti-rabbit antibody conjugated to HRP (Section 2.8.5). In order to determine the optimum

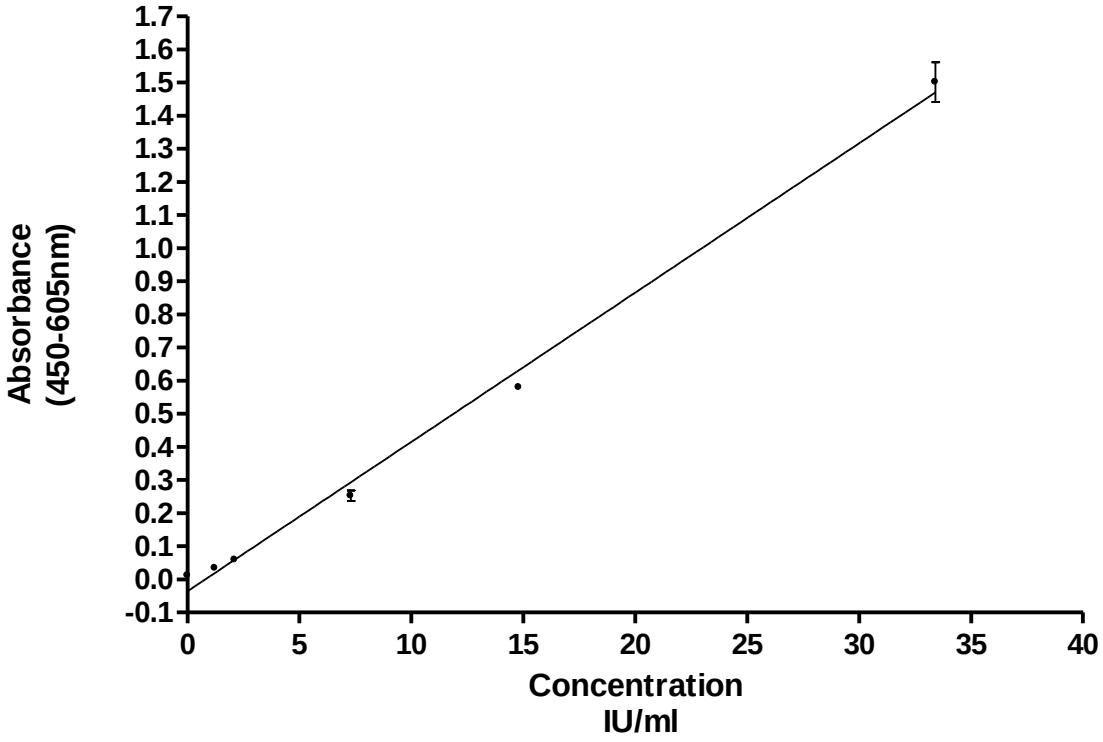

Figure 5.9 Concentration curve of IL-2 protein standards against absorption at 450nm (subtracting absorbance at 605nm).

The results shown are the mean of duplicate samples from one experiment.

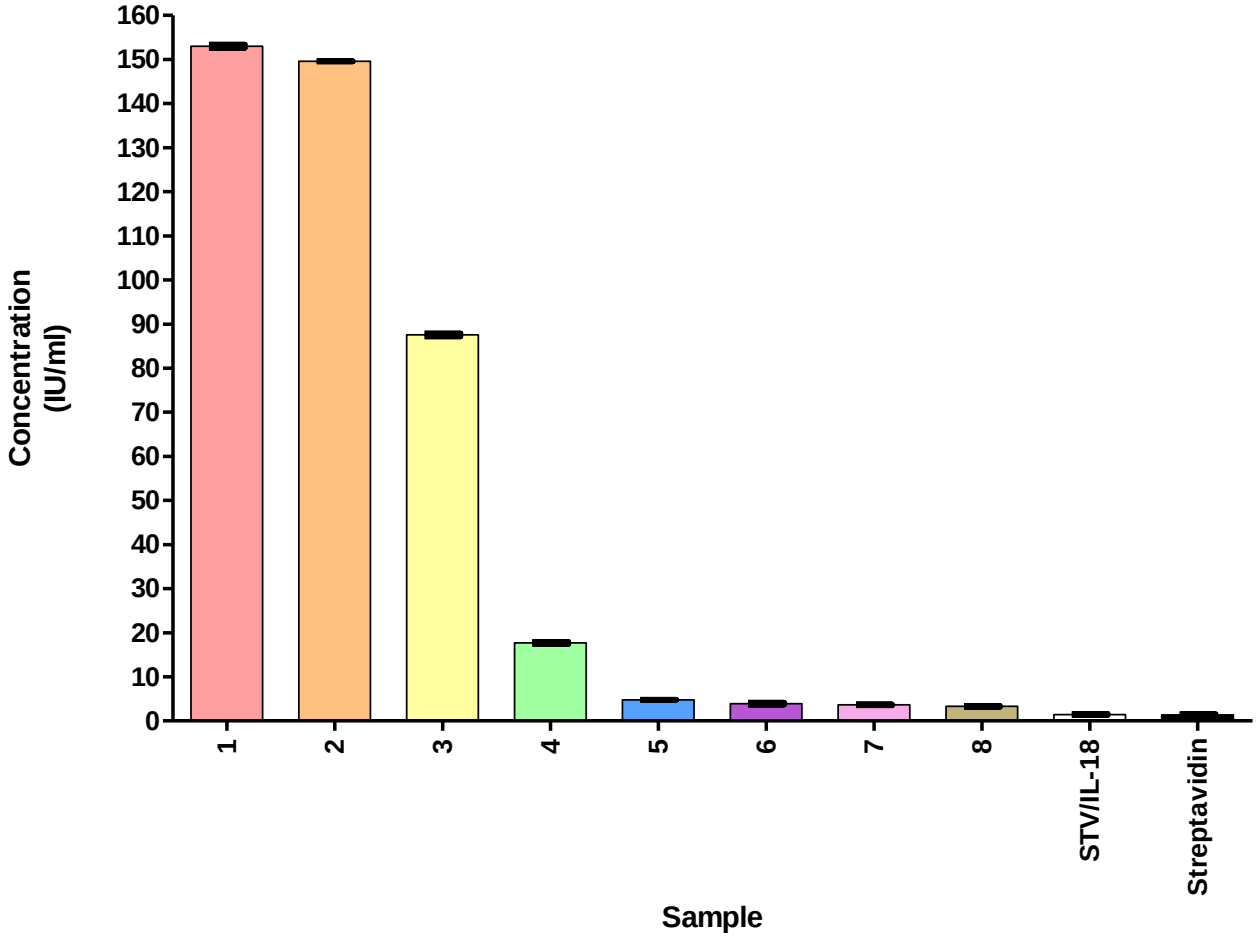

Figure 5.10 Histogram showing the determined concentration of STV/IL-2 samples as well as STV/IL-18 and recombinant streptavidin negative controls.

Samples: (1) Undiluted folded STV/IL-2 sample; (2) 1:2 folded STV/IL-2; (3) 1:4 folded STV/IL-2; (4) 1:8 folded STV/IL-2; (5) 1:6 folded STV/IL-2; (6) 1:32 STV/IL-2; (7) 1:64 folded STV/IL-2; (8) 1:128 folded STV/IL-2; (STV/IL-18) undiluted folded STV/IL-18; (Streptavidin) 0.1mg/ml recombinant streptavidin; all dilute samples were diluted with PBS. The results shown are the mean of duplicate samples from one experiment.

blocking buffer to be used in the study. This experiment was repeated twice, once using 10% w/v milk as a blocking agent, and once using 10% w/v BSA as a blocking agent. When milk was used to block the membrane, no bands were observed on the western blot. When BSA was used to block the membrane, non-specific binding of the antibody to the membrane was too high to allow the detection of bands; consequently, it was not possible to utilize this method to measure the biotin binding capacity of STV/IL-2 and STV/IL-18 (*data not shown*) and an alternative method for analysis of the biotin binding capability of the recombinant streptavidin component of the fusion proteins was developed.

5.2.6 Analysis of the Biotin Binding Activity of STV/IL-2 and STV/IL-18 Samples by ELISA

Optimization of a streptavidin ELISA using recombinant streptavidin (Section 2.8.7) showed that streptavidin will stick to a MaxiSorb™ plate, and can be detected using a biotinylated antibody such as rabbit anti-goat IgG-Biotin as a first layer followed by an HRP-linked secondary antibody such as sheep anti-rabbit IgG-HRP (Figure 2.2). Using milk as a blocking agent does not appear to reduce the binding of the antibody to the streptavidin (Table 5.1).

A variety of primary and secondary antibody pair combinations were tried (Table 5. Antibody pair A provides a good result (absorbance of 0.986), and showed little unspecific binding (antibody pair E, absorbance 0.115) and so was used to compare the biotin binding ability of the streptavidin-cytokine fusion proteins to recombinant streptavidin (Table 5.1).

Table 5.1 Optimization of an ELISA to assess the biotin binding activity of streptavidin proteins.

1°Antibody + 2° Antibody	**2% w/v Milk in PBS** Absorbance (450nm)	**2% w/v BSA in PBS** Absorbance (450nm)
A. Goat anti-rabbit IgG-Biotin + donkey anti-goat IgG-HRP	1.124	0.986
B. Goat anti-mouse IgG-Biotin + donkey anti-goat IgG-HRP	1.202	1.4995
C. Rabbit anti-goat IgG-Biotin + Sheep anti-rabbit IgG-HRP	0.6955	1.456
D. Rat anti-mouse IgG-Biotin + Sheep anti-rabbit IgG-HRP	0.0035	0.002
E. Goat anti-mouse IgG + Donkey anti-goat IgG-HRP	0.2095	0.115
F. Rabbit anti-goat IgG + Sheep anti-rabbit IgG-HRP	-0.0035	-0.0005

Antibody combinations used are shown in the first column, followed by the absorbance at 450nm when using either 2% w/v milk in PBS or 2% w/v BSA in PBS as a blocking buffer.

Recombinant streptavidin, and solubilized refolded STV/IL-2 and STV/IL-18 were used to coat the well surfaces of a MaxiSorb™ 96 well plate (Section 2.8.8). Recombinant streptavidin was used at known concentrations (Table 5.2) in order to create a standard concentration curve. STV/IL-2 and STV/IL-18 were used undiluted, and serially diluted tenfold in order for sample concentration to be in the range of detection for the ELISA.

Although points for the standard concentration curve for streptavidin were not able to provide a good fit, high concentrations of streptavidin in the range 5-100μg/ml gave positive results compared with zero (Figure 5.11). The original data for the standard concentration curve is also included (Table 5.2). STV/IL-2 and STV/IL-18 samples tested at six dilutions in the ELISA did not generate results significantly above zero (a mean of 0.225 and 0.229 OD respectively), suggesting that STV/IL-2 and STV/IL-18 do not bind biotin.

5.2.7 Mass Spectrophotometry

STV/IL-2 and STV/IL-18 samples were prepared for mass spectrophotometry using a standard in-gel trypsin digestion protocol (Rosenfeld *et al* 1992) with modifications provided by Kevin Welham, (Department of Chemistry). Mass spectrophotometry of the samples was inconclusive. Both fusion proteins both gave a mix of digested fragments which could not be conclusively identified as a single protein when searched against protein databases. Time only permitted the mass spectrophotometry of STV/IL-2 and STV/IL-18 to be undertaken on one occasion.

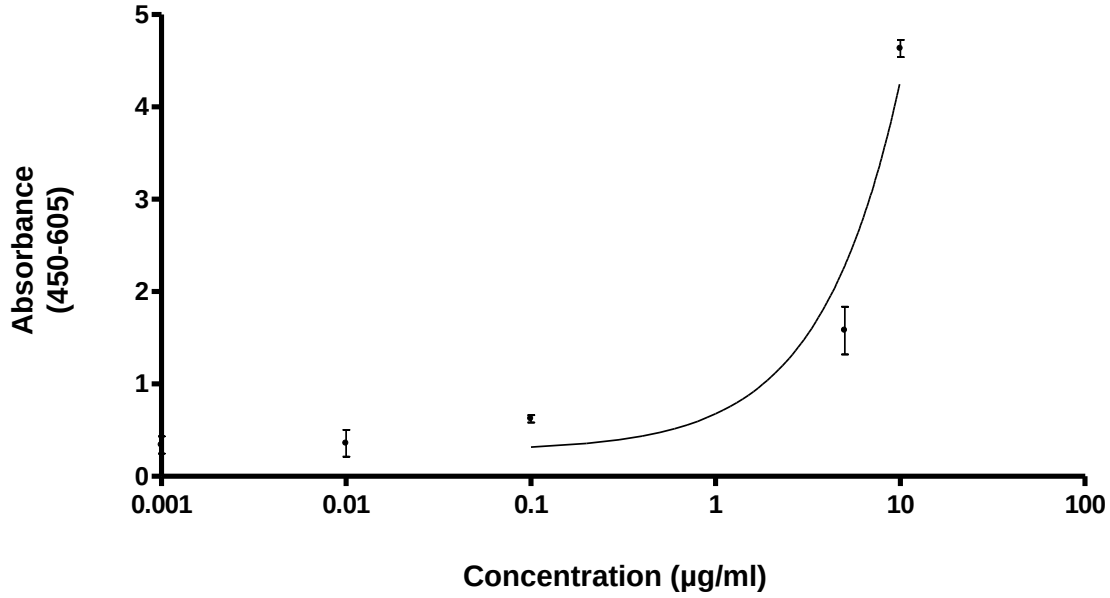

Figure 5.11 Concentration curve of recombinant streptavidin protein standards against absorption at 450nm (subtracting absorbance at 605nm).

The results are expressed as the mean +/- the standard error of the mean of three sample triplicates from one experiment.

Table 5.2 Raw data showing OD at 450nm of recombinant streptavidin protein standards used to create standard curve.

Concentration (µg/ml)	1	2	3	Average	Standard Deviation(+/-)
50	4.546	4.620	4.732	4.633	0.094
10	1.400	1.460	1.875	1.578	0.259
5	0.584	0.661	0.622	0.622	0.039
0.1	0.215	0.506	0.352	0.358	0.105
0.0	0.158	0.170	1.164	0.164	0.106

5.3 Discussion

5.3.1 SDS-PAGE Analysis of Solubilized Recombinant Fusion Proteins STV/IL-2 and STV/IL-18

Solubilized samples of protein from *E. coli* containing the expression vector encoding STV/IL-2 and STV/IL-18 were analysed by Coomassie blue staining (Figure 5.1 show protein bands of the correct estimated size of the STV/IL-2 and STV/IL-18 proteins suggesting these proteins are present in their respective samples. The results of Western blot analysis of the same samples (Figure 5.2) show convincing results for the presence of STV/IL-2 by binding of anti-IL-2 anti-streptavidin and anti-histidine tag antibodies (whilst not binding to an anti-IL-18 antibody) to a protein band of approximately the estimated size for STV/IL-2. The binding of STV/IL-2 to both the anti-IL-2 and anti-streptavidin antibodies (as highlighted in Figure 5.2, lane 9) due to cutting of the membrane through the sample shows that the same protein band is binding both these antibodies, further suggesting that this band contains the STV/IL-2 protein.

Western blot analysis of STV/IL-18 did not show any binding to the relevant antibodies against IL-18 and leaves some doubt as to whether the expressed proteins purified from the cells do in fact comprise the STV/IL-18 fusion protein. Although a protein band is clearly seen in the Coomassie blue stained samples, it is possible that this protein is an unrelated protein native to *E. coli* although the recovery of recombinant protein from inclusion bodies, as was preformed here, is thought to produce less contamination of the protein by native proteins than recovery of recombinant protein by other means (Singh and Panda 2005). Another possibility is that STV/IL-18 is present in the expressed protein samples but is not binding to the antibodies, which could be due to incorrect

folding unless misfolding is resulting in the binding epitope for the anti-IL-18 antibody becoming inaccessible, however, misfolding of the protein should not be an issue on an SDS-PAGE gel using samples that have been reduced by boiling with β-mercaptoethanol). In order to be sure that the STV/IL-18 was present in the sample, binding to the anti-IL-18 antibody would be necessary, and it is unclear whether the anti-IL-18 antibody was functional using the protocol employed, since it failed to bind to recombinant IL-18 (Figure 5.6) in another experiment. However, as the anti-histidine tag antibody and anti-streptavidin antibody can be shown binding to STV/IL-2 and not STV/IL-18 (Figure 5.2) in this experiment, the presence of STV/IL-18 can not be confirmed. It is possible that a combination of the discussed issues may be responsible for the difficulties encountered in detecting and confirming the presence of STV/IL-18 fusion protein in the samples expressed during this investigation.

5.3.2 Native PAGE Analysis of Solubilized Recombinant STV/IL-2 and STV/IL-18

Non reduced protein samples subjected to electrophoresis on a native PAGE gel often appear at a different molecular weight than if they are reduced and subject to electrophoresis on SDS-PAGE gels (Wittig and Schägger 2005). Very high molecular weight bands or smears apparent on PAGE gels may be caused by protein aggregation, where bonds between protein molecules have not been broken by detergent or reducing agents used during sample preparation or in the gel and buffers. Proteins may display at a molecular weight which is somewhat higher than that predicted on native gels, due to the folded protein containing polar regions which affects the rate of separation on the PAGE gel by electrophoresis. Thus native PAGE gel is not useful for separation and analysis of proteins by molecular weight. However, as native PAGE gel does not contain detergent, which disrupts the secondary and tertiary structure of proteins and the

samples are not reduced, the proteins tend to retain their native conformation, useful for detection and analysis of proteins which exist as multimers or multi-subunit proteins, and can also be potentially used for the assessment of protein function such as the biotin binding function of streptavidin. These factors could explain why several of the recombinant proteins which were analysed on native PAGE gels e.g STV/IL-2(Section 5.2.2) appeared at higher molecular weight than expected when compared to a protein ladder.

STV/IL-2 was detected using an anti-IL-2 antibody, which revealed a large quantity of protein at a range of high molecular weights (Figure 5.2), suggesting possible aggregation of the protein which is broken down when the protein is reduced and analysed by electrophoresis on SDS-PAGE gel. Visualization of STV/IL-2 with anti-histidine tag and anti-streptavidin antibodies detected protein bands at a lower molecular weight (approximately 60kDa), but still higher than estimated. Undiluted samples of STV/IL-2 revealed a protein band corresponding to the sample running front which may contain non-aggregated protein; this is supported by the absence of these bands in samples containing recombinant human IL-2 and recombinant human IL-18. This band was visible when visualized with anti-IL-2, anti-histidine tag and anti-streptavidin antibodies. Although recombinant human IL-2 could not be detected with the anti-IL-2 antibody, the detection of STV/IL-2 by a combination of antibodies against different moieties of the fusion protein indicates that a protein contained in the sample contains a histidine tag, IL-2 component and streptavidin component, which strongly supports STV/IL-2 as the identity of the protein.

The evidence to confirm the presence of STV/IL-18 in these samples is very inconclusive. Native PAGE gel analysis by Coomassie blue staining (Figure 5.3) shows large amounts of protein in undiluted samples of solubilized STV/IL-18 at approximately the predicted sizes, and protein bands were seen in samples probed with an anti-streptavidin antibody (Figure 5.7). However, recombinant IL-2 and recombinant IL-18 used as negative controls also bound the anti-streptavidin antibody (Figure 5.7), and anti-histidine tag (Figure 5.5) whereas anti-IL-18 antibodies did not bind to the sample (Figure 5.6). As the anti-IL-18 antibody was not found to bind recombinant human IL-18, the function of this antibody under the conditions employed is in doubt. An experiment comparing the binding of samples of STV/IL-18 to the anti-streptavidin antibody or the anti-IL-18 antibody showed only bands of high molecular weight binding to the anti-streptavidin antibody, but not to the anti-IL-18 antibody. These observations suggest that the bands seen bound to the anti-streptavidin antibody (Figure 5.8) may contain an unrelated *E. coli* expressed protein which is binding to the anti-streptavidin antibody in a non-specific manner.

5.3.3 Protein Quantification using Bio-Rad Protein Assay and IL-2 EASIA

The Bio-Rad protein assay measures the total soluble protein content of samples. The values given for the samples by this assay are between 0.1-1mg/ml. Patra *et al* (2000) reported using a similar low denaturant, high pH solubilization buffer to resolubilize recombinant human growth hormone isolated from bacterial inclusion bodies and found that a maximum of 6.5mg/ml of recombinant protein could be achieved using the pQE-60 expression vector (Qiagen) in *E. coli.* Thus the yield of protein seen in these studies is disappointing.

High level expression of proteins in *E. coli* which are not of bacterial origin can be achieved by the creation of synthetic DNA which is designed to take account of the codon usage of *E. coli* (Burgess-Brown *et al* 2008). Use of arginine codons AGA and AGG is rare in *E. coli* and their presence in recombinant proteins has been shown to cause mistranslation of proteins and lower levels of protein expression (Calderone *et al* 1996). The IL-2 sequence incorporated into the STV/IL-2 coding sequence contains four of these rare codons, with seven found in the IL-18 coding sequence and one in the streptavidin coding sequence. These codons may well be responsible, at least in part, for the poor level of fusion proteins observed. Altering these codons, using site directed mutagenesis to replace then with those the more commonly found in E. coli, or co expression of genes that encode tRNAs specific for these rare codons could help to produce a higher yield of recombinant protein (Burgess-Brown *et al* 2008).

Interestingly, it was noted that storage of the solubilization buffer resulted in a reduction of pH over time, and the denatured recombinant fusion proteins stored in solubilization buffer appear to precipitate after several days, even when stored at -80˚C. Agitation of the precipitated samples resulted in resolubilization. Therefore it was concluded that fresh solubilization buffer was prepared before use, and protein was only stored in this buffer for short periods of time.

An IL-2 EASIA was used to measure the concentration of STV/IL-2 relative to the concentration and activity of recombinant IL-2. EASIA results for the solubilized STV/IL-2 sample that had been refolded by diluting twentyfold in PBS gave an approximate STV/IL-2 concentration comparable to 121IU/ml, suggesting that STV/IL-2 is being solubilized at a concentration equivalent to approximately 2000IU/ml activity

of human IL-2. Clinical doses of IL-2 used for injection (Aldesleukin, Novaris) are at a concentration of 1.1mg/ml or 18×10^6 IU/ml (*http://www.pharma.us.novartis.com/product/pi/pdf/proleukin.pdf*). Thus, the protocol developed here clearly does not produce STV/IL-2 in the concentration required for clinical use. The total protein concentration measured using the Bio-Rad protein assay was found to between 0.1 and 1mg/ml (Section 5.2.3). This assay therefore suggests that STV/IL-2 is less active per mg of protein than IL-2, which is to some extent unsurprising as the STV/IL-2 has a higher molecular weight than IL-2. However, as the Bio-Rad protein assay measures total protein, this may suggest some contamination of the sample with protein other than STV/IL-2.

5.3.4 Analysis of the Biotin Binding Activity STV/IL-2 and STV/IL-18 Samples by ELISA

Since analysis of recombinant streptavidin using PAGE and Western blotting (Section 5.2.5) to assess the biotin-binding function was not satisfactory, an alternative method using ELISA was established. A method was devised in order to optimize an ELISA for the comparison of the biotin binding activity of generated fusion proteins with the biotin binding activity of recombinant streptavidin from a commercial source (Section 5.2.6). This ELISA assay optimization study showed that streptavidin-biotin binding can be investigated by coating wells with recombinant streptavidin and binding 1° biotinylated antibody and 2° HRP-linked antibody. Several unspecific biotinylated antibody and secondary antibody pairs were compared to find an appropriate combinantion that provided good results with minimal levels of unspecific binding of the antibodies. A goat anti-rabbit IgG conjugated to biotin and a donkey anti-goat IgG conjugated to HRP provided good results and showed very little evidence of unspecific binding, and so

were used to compare the biotin binding ability of the streptavidin-cytokine fusion proteins to recombinant streptavidin. Comparisons using BSA in place of milk powder as blocking agents in the ELISA experiments showed that there is little difference in the results between the two, suggesting that milk is not interfering with the binding of the biotinylated antibody to streptavidin in this case. However, since milk has been reported to interfere with the binding of biotin to streptavidin (Hoffman and Jump 1989), and was the case when milk was used to block Western blot membrane BSA was used as the blocking agent in this study.

Comparison of recombinant streptavidin with the STV/IL-2 and STV/IL-18 recombinant fusion proteins showed that while recombinant streptavidin would bind a biotinylated antibody, neither STV/IL-2 nor STV/IL-18 were captured by the same antibody. This could be due to incorrect folding of the recombinant fusion proteins which would cause the activity of the proteins to be compromised. If this is in fact the case further activity assays conducted to analysed the proteins would be of little value, and a more comprehensive folding strategy would need to be considered before further work on these fusion proteins could be undertaken. There are several further strategies which can be considered for this end.

The most commonly used method of refolding proteins is dialysis of the protein in solution against decreasing concentrations of the solubilisation buffer; however, this method resulted in STV/IL-2 and STV/IL-18 precipitating out of solution. A modified dialysis procedure is described by Sørensen *et al* (2003) for the refolding of recombinant streptavidin expressed in *E. coli*, where gradual buffer exchange in a dialysis cylinder and gradual feeding of the protein into the cylinder allowed the

percentage of refolded protein to increase to 77.7%, compared to 43.9% when the protein was subjected to direct dialysis against refolding buffer, or 47.6% when the protein was subjected to gradual dialysis against decreasing concentration of solubilisation buffer. As this method was successful in increasing the percentage refolding of recombinant streptavidin, it is potentially applicable for achieving refolding of STV/IL-2 and STV/IL18, whereby correct folding of the streptavidin moiety susequently lead to the correct folding of the cytokine moiety.

Once the streptavidin-cytokine fusion proteins have been correctly folded, the function of the streptavidin and cytokine moieties of the protein must be assessed. IL-2 function may be assessed using a CTLL-2 cell-based proliferation assay. IL-2 promotes the proliferation of CTLL-2 cells which can be quantified with a metabolic dye, such as Alamar Blue (Kwack and Lynch 2000). Comparison of recombinant IL-2 obtained from a commercial source versus the streptavidin-IL-2 fusion protein produced here will give a comparative measure of the functional activity of the IL-2 portion of the fusion protein. Similarly, the activity of IL-18 can be assessed using an assay incorporating KG-1 cells. IL-18 induces the production of IGIF in KG-1 cells, which can be measured using an IGIF ELISA (Konishi *et al* 1997).

Chapter 6

General Discussion

6.1 Discussion

Much of the research into cancer immunotherapy in recent years has focused on vaccines which induce and promote a TH1-type immune response, as this type of response is considered to be the most effective in inducing an anti-tumour response in patients (Berzofsky *et al* 2004). Many immunoadjuvants are used to boost the weak immune responses to cancer by provoking a non-specific immune response. When immunoadjuvants are used in conjunction with a cancer vaccine it is anticipated that they will act to augment the immune reaction, and lead to an enhanced anti-tumour response. The aim of the work in this thesis was to produce recombinant streptavidin-IL-2 and IL-18 fusion proteins, in order to investigate their potential as candidates for use as immunoadjuvants

Protein expression constructs were generated consisting of a truncated core-streptavidin (STV) sequence fused to the IL-2 or IL-18 coding sequences via a short polypeptide linker. The recombinant fusion proteins were expressed in *E. coli* containing a PlysS plasmid using the bacterial expression plasmid pCRT7-NT® TOPO®. Analysis of expressed STV/IL-2 and STV/IL-18 suggested that the fusion proteins may be forming multimers. Formation of multimers in this way is not entirely surprising, since native streptavidin is a tetrameric protein and while STV/IL-2 could be bound by an anti-IL-2 antibody in an EASIA, assessing the biotin binding of the fusion proteins suggested that the streptavidin moiety was not active, indicating that the recombinant molecule may be folded incorrectly. It is therefore likely that any protein multimers formed are interacting by chaotic aggregation (Friedan 2007), rather than by normal multimerization of the streptavidin moiety. Difficulties with the solubilization of STV/IL-2 and STV/IL-18, and ease of it's precipitation from solution support the

proposed theory that aggregation is taking place. Before further analysis of recombinant STV/IL-2 and STV/IL-18 can be undertaken they must be produced in a soluble refolded form which is biologically active.

Further studies to investigate the potential clinical therapeutic application of the recombinant STV/IL-2 and STV/18 proteins would include investigation of the immunogenic response to the fusion proteins *in vitro* using mammalian cell lines, mammalian blood and animal models, alone or in combination with a breast cancer vaccine such as a vaccine specific to the HER2/neu TAA.

Trastuzumab is a monoclonal antibody targeted against the HER2/neu TAA that is over-expressed in approximately 25-30% of breast tumours (Liu *et al* 1992). However, not all patients who are treated with trastuzumab respond to the treatment, and of those that do respond a number become resistant to this antibody. Therefore, there is much interest in the development of other treatment strategies targeted to HER2/neu. One of the approaches investigated is the boosting of the anti-tumour CTL-cell response by injection of the HER2/neu antigen to stimulate the immune system to produce specific antibodies against this TAA.

Renard and Leach (2007) developed a modified HER2/neu vaccine that incorporated a T-helper epitope into the HER2/neu recombinant protein in order to increase the immunogenicity of the vaccine. The T-helper epitope constituted a foreign promiscuous T-helper epitope from tetanus toxin designed to overcome tolerance to HER2/neu by providing exogenous T cell help to HER2/neu specific B and T lymphocytes and elicit and antibody mediated immune response to HER2/neu. Studies in mice transgenic for

the activated form of HER2/neu under the MMTV promoter found that this vaccine in would protect against transplanted HER2/neu expressing tumours in 87.5% of mice, after one dose, while an unmodified HER2/neu vaccine had no effect i.e. 100% of mice developed tumours. Additionally, significant anti-HER2/neu antibody titres were only found in those mice injected with the modified antigen. Transfer of sera from vaccinated to unvaccinated mice also conferred protection against HER2/neu expressing tumours, showing a key role for anti-HER2/neu antibodies. The vaccine designed for use in humans incorporated the two tetanus toxin epitopes to maximise MHC coverage and included either Alhydrogel™ (aluminium hydroxide adjuvant) or QS-21 (purified saponin) adjuvants, both of which are non specific immunogenic molecules. Initial studies in primates were promising as significant HER2/neu antibody titres were seen in animals receiving 500μg of vaccine. However, evaluation of the HER2/neu vaccine with Alhydrogel™ adjuvant in phase I clinical trials on disease free patients previously suffering from tumour expressing HER2/neu with no indication for trastuzumab, showed that immune response in these patients was slower to develop and of a lower magnitude than that seen in mice and primates. Addition of immunoadjuvant QS-21 to the vaccine formulation was tested in a third trial in which patients with metatstatic breast cancer expressing HER2/neu recived 1250μg of vaccine over several time points until disease progression. Antibody responses after 8 weeks (4 administrations) showed antibody responses of approximately 10 times that seen in the first trial.

The argument for the addition of the novel immunoadjuvants STV/IL-2 or STV/IL-18 to a vaccine of this type, where a specific immune response against a TAA can be elicited, is attractive. Like streptavidin, aluminium hydroxide adjuvant is known to provoke a non-specific immune response, however the immune response which is stimulated by

aluminium hydroxide tends towards a TH2-type response (Petrovsky and Aguilar 2004). The novel immunoadjuvants STV/IL-2 and STV/IL-18 are designed to drive the provoked immune response towards the TH1 response, thus the addition of this immunoadjuvant in to the HER2/neu-P30 vaccine described by Renard and Leach (2007) may well result in the increased immune response to the HER2/neu antigen which they conclude to be lacking.

Another recent vaccine targeting tumours which express HER2/neu is the E75 HER2/neu peptide vaccine (Mittendorf *et al* 2008). The E7 HER2/neu epitope is a short immunogenic peptide (amino acids 369-377 of the HER2/neu protein, which correspond to a section of the extracellular HER2/neu protein) and is recognised by tumour-specific CTLs. The E75 HER2/neu vaccine was administered in combination with an immunoadjuvant, either Freund's incomplete adjuvant or GM-CSF (Leukine®), to patients with metastatic breast cancer expressing the HER2/neu antigen (Zaks 1998, Knutson 2002, Murray 2002) and was demonstrated to be capable of provoking a E75 specific immune response in these patients.

The study by Mittendorf and colleagues investigated the use of E75 as a preventative vaccine for patients at a high risk of relapsing having previously been successfully treated for HER2/neu expressing breast tumours, working on the hypothesis that the induction of CTLs recognizing tumours early may be effective in preventing or containing tumourigenesis or progression. Initial studies in node positive and node negative patients showed in increase in E75 specific CTL, with a tumour recurrence rate of 5.6% in the vaccinated group compared to 14.2% in the unvaccinated group after 18 months. This recurrence rate was increased to 8.3% in the vaccinated group and 14.8%

in the unvaccinated group, losing the significant benefit of this vaccine in the long term (over 5 years), suggesting the need for booster inoculations. In addition to a specific response to the E75 peptide, epitope spreading to the GP2 epitode (HER-2/neu residues 654-662) was noted in a high percentage of patients (all node postitive and 85% of node negative patients). The authors of this study suggested combining the E75 peptide with the GP2 peptide, to produce a multi-epitope vaccine.

The studies by Mittendorf and colleagues demonstrate the ability of the immune system to be stimulated against tumour cells. Unlike treatment with the monoclonal HER2/neu antibody trastuzumab, the body's own immune system is recruited to produce antibodies against TAA, potentially producing longer term immunity and the possibility of using these vaccines in prevention of relapse. Although STV/IL-2 and STV/IL-18 were originally designed with monoclonal antibody vaccines such as trastuzumab in mind, there is equally scope for their addition to antigenic peptide vaccines, such as E75 HER2/neu, which require an immunoadjuvant and provoke a CTL response that may be enhanced by stimulating a TH1 response.

The biotin-binding capability of streptavidin gives the fusion molecules further potential in targeted therapy in the future. Theoretically biotinylation of a monoclonal antibody directed against a TAA (such as trastuzumab (HER2/neu), rituximab (CD20), cetuximab (EGFR) or bevacisumab (VEGF)) could direct the STV/IL-2 or STV/IL-18 to the tumour site via streptavidin-biotin binding to capture the immunoadjuvant and localize its immunostimulatory effects.

STV/IL-2 and STV/IL-18 have exciting potential as novel immunoadjuvants. Although a great deal of work is still needed before the protein can be produced in a soluble, functionally active form which can be investigated in animal models, I am hopeful that this can be achieved and that the work presented in this thesis can be carried forwards to produce a worthy addition to currently available cancer therapies.

6.2 Future Work

Assuming that the recombinant fusion proteins can be refolded and produced in suitable quantities for therapeutic use the following steps can then be taken:

Both immunogenicity and toxicology of the fusion proteins must be investigated. *In vitro* assays involving mammalian cell lines and human blood may provide the first indication of the effect of STV/IL-2 and STV/IL-18 on immune cells. Following this, *in vivo* studies firstly in mouse models and then in primates should provide a more complete picture of the immunogenic effects of the recombinant fusion proteins, as well as the tolerated dose. Important areas to investigate will be the cytokine repertoire that is activated by the STV/IL-2 and STV/IL-18 as well as the T cell, B cell and antibody response. Further investigation could investigate the response of animal models to combination doses of STV/IL-2 or STV/IL-18 with TAA peptide vaccines, or single monoclonal antibodies directed at TAA.

Having investigated the toxicology and optimum dosage of STV/IL-2 and STV/IL-18, It is hoped that clinical trials would be undertaken to investigate the potential of the immunoadjuvant to elicit and immune response in humans, firstly alone and then in combination with a vaccine.

It is hoped that within 10 years the immunoadjuvants will enter phase III clinical trials comparing the use of vaccines containing these fusion proteins to a current cancer immunotherapy such as trastuzumab.

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Appendix

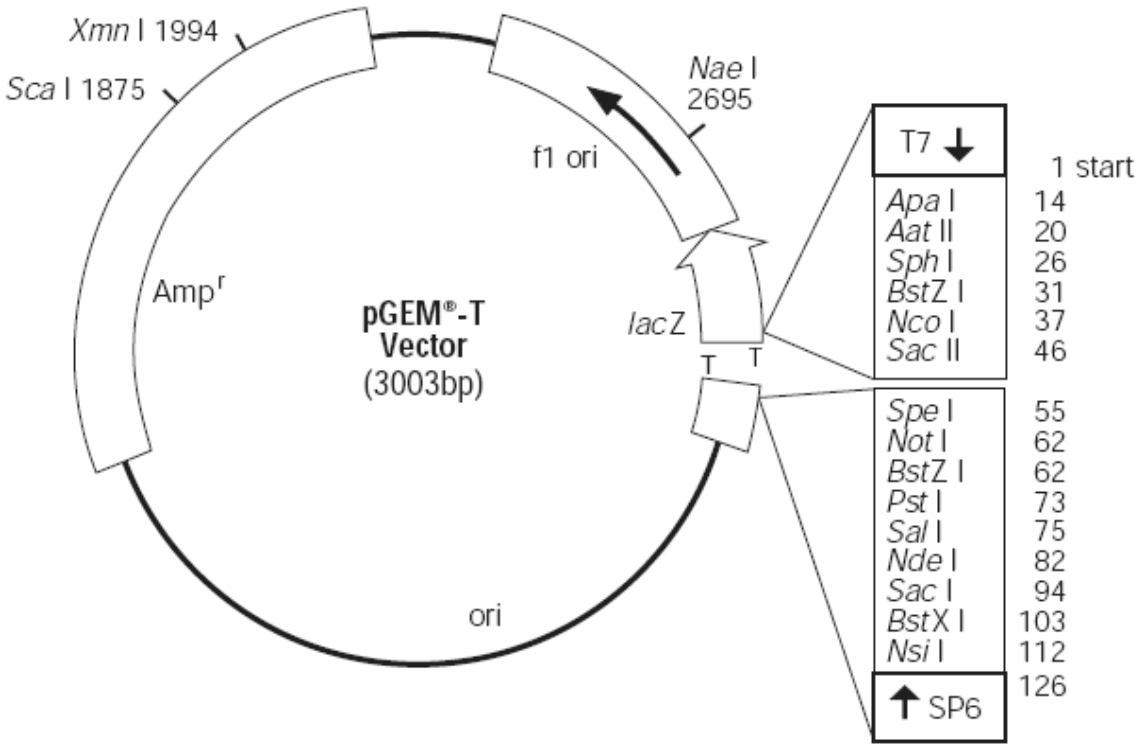

Figure A.1 pGEM® Vector circle map.

pGEM®-T Vector Sequence reference points:
T7 RNA Polymerase transcription initiation site 1
SP6 RNA Polymerase transcription initiation site 126
T7 RNA Polymerase promoter 2987-6
SP6 RNA Polymerase promoter 121-143
multiple cloning site 10-113
lacZ start codon 165
lac operon sequences 2824-2984, 151-380
lac operator 185-201
β-lactamase coding region 1322-2182
phage f1 region 2368-2823
binding site of pUC/M13 Forward Sequencing Primer 2944-2960
binding site of pUC/M13 Reverse Sequencing Primer 161-177

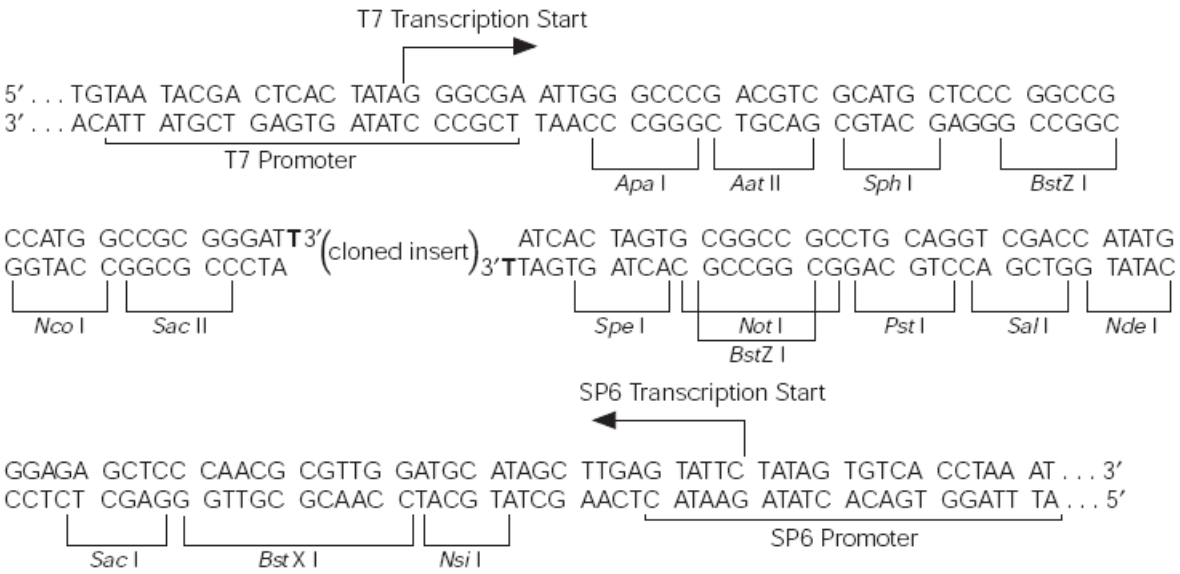

Figure A.2 The promoter and multiple cloning sequence of the pGEM®-T Vector

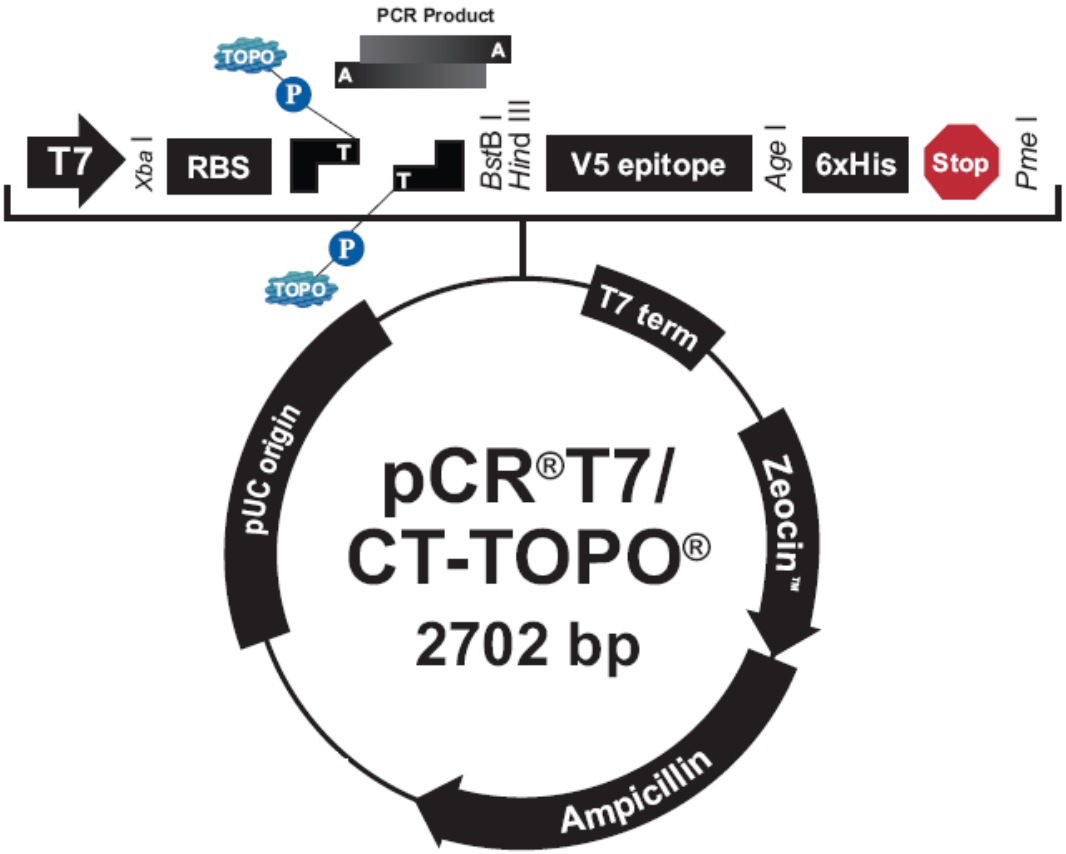

Figure A.3 pCR®T7/CT-TOPO Vector circle map.

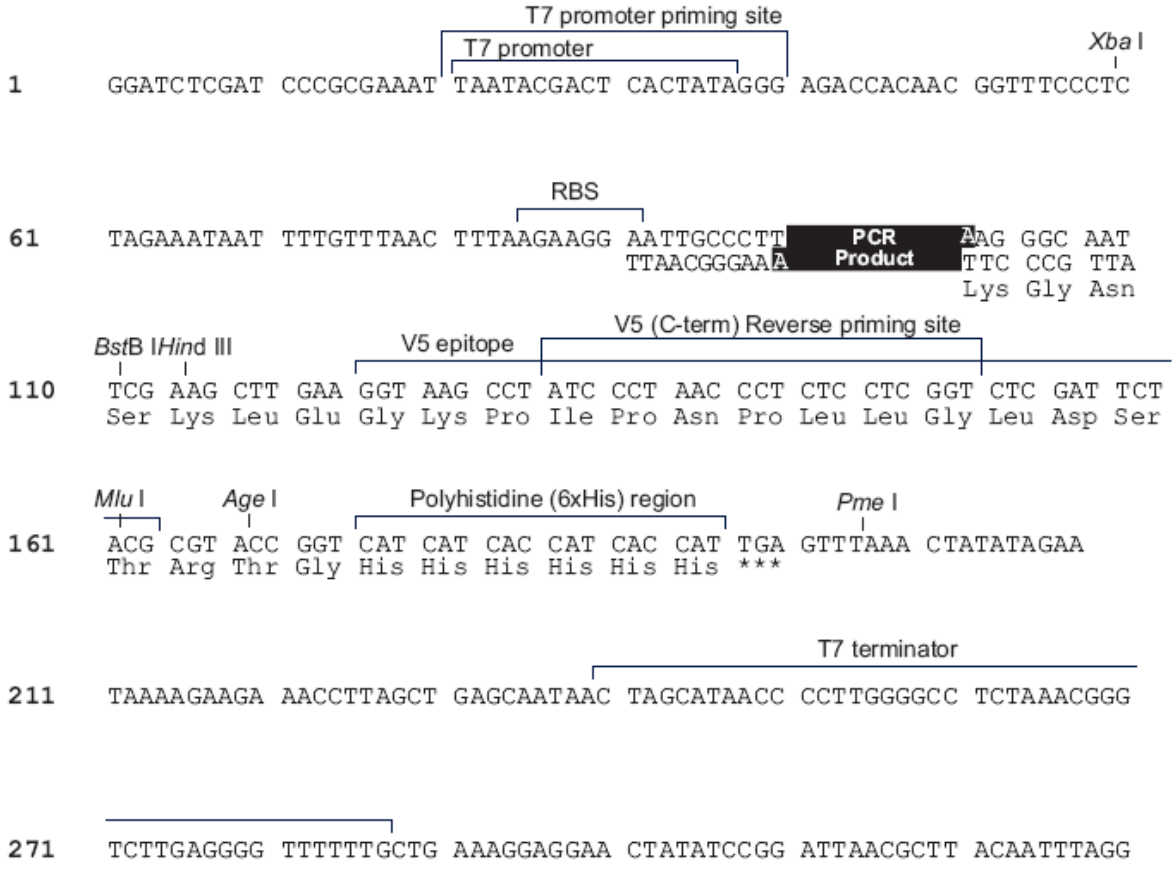

Figure A.4 The promoter and multiple cloning sequence of the pCR®T7/CT-TOPO® Vector

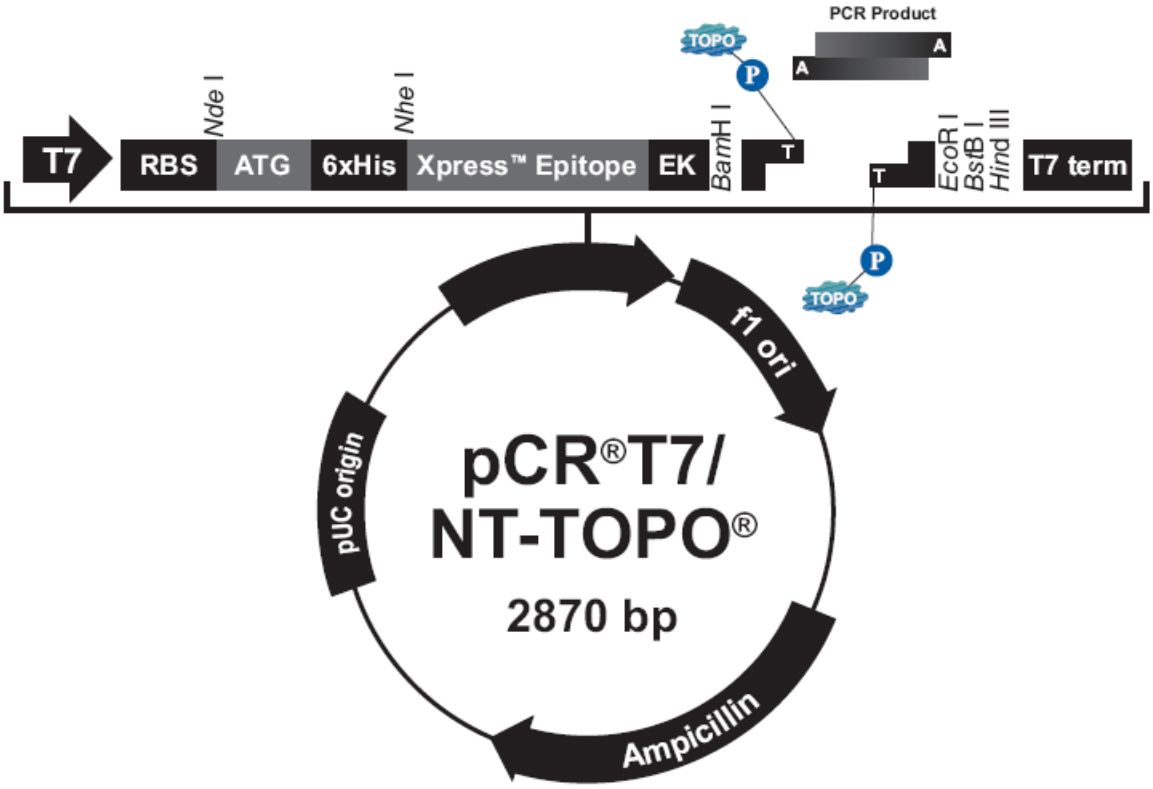

Figure A.5 pCR®T7/NT-TOPO Vector circle map.

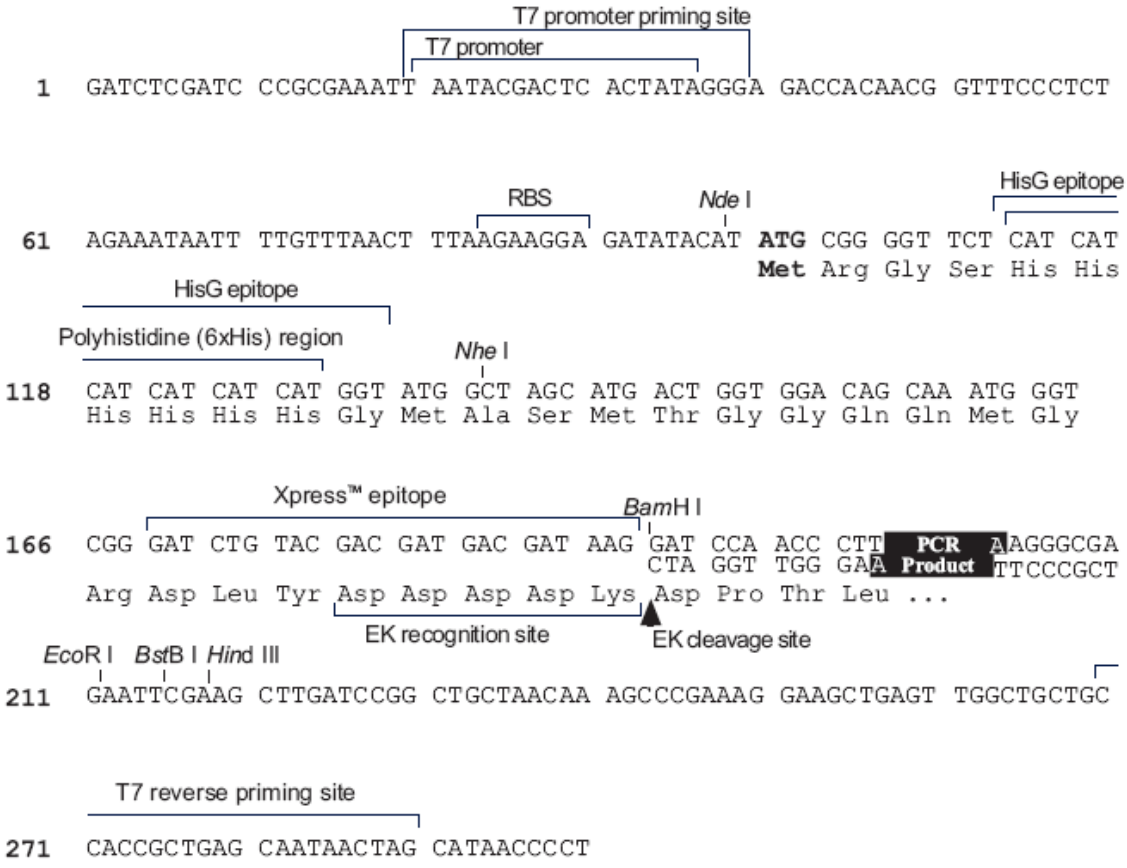

Figure A.6 The promoter and multiple cloning sequence of the pCR®T7/NT-TOPO® Vector

```
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 K  G  S  E  T  T  F  M  C  E  Y  A  D  E  T  A  T  I  V  E
tttctgaacagatggattacctttgtcaaagcatcatctcaacactgacttga
 F  L  N  R  W  I  T  F  C  Q  S  I  I  S  T  L  T  -
```

Figure A.7 the streptavidin-IL-2 fusion protein coding sequence and amino acid translation

```
ggcatcaccggcacctggtacaaccagctcggctcgaccttcatc
 G  I  T  G  T  W  Y  N  Q  L  G  S  T  F  I
gtgaccgcgggcgccgacggcgccctgaccggaacctacgagtcg
 V  T  A  G  A  D  G  A  L  T  G  T  Y  E  S
gccgtcggcaacgccgagagccgctacgtcctgaccggtcgttac
 A  V  G  N  A  E  S  R  Y  V  L  T  G  R  Y
gacagcgccccggccaccgacggcagcggcaccgccctcggttgg
 D  S  A  P  A  T  D  G  S  G  T  A  L  G  W
acggtggcctggaagaataactaccgcaacgcccactccgcgacc
 T  V  A  W  K  N  N  Y  R  N  A  H  S  A  T
acgtggagcggccagtacgtcggcggcgccgaggcgaggatcaac
 T  W  S  G  Q  Y  V  G  G  A  E  A  R  I  N
acccagtggctgctgacctccggcaccaccgaggccaacgcctgg
 T  Q  W  L  L  T  S  G  T  T  E  A  N  A  W
aagtccacgctggtcggccacgacaccttcaccaaggtgagttct
 K  S  T  L  V  G  H  D  T  F  T  K  V  S  S
ggtggaggtggatactttggcaagcttgaatctaaattatcagtc
 G  G  G  G  Y  F  G  K  L  E  S  K  L  S  V
ataagaaatttgaatgaccaagttctcttcattgaccaaggaaat
 I  R  N  L  N  D  Q  V  L  F  I  D  Q  G  N
cggcctctatttgaagatatgactgattctgactgtagagataat
 R  P  L  F  E  D  M  T  D  S  D  C  R  D  N
gcaccccggaccatatttattataagtatgtataaagatagccag
 A  P  R  T  I  F  I  I  S  M  Y  K  D  S  Q
cctagaggtatggctgtaactatctctgtgaagtgtgagaaaatt
 P  R  G  M  A  V  T  I  S  V  K  C  E  K  I
tcaactctctcctgtgagaacaaaattatttccttaaggaaatg
 S  T  L  S  C  E  N  K  I  I  S  F  K  E  M
aatcctcctgataacatcaaggatacaaaaagtgacatcatattc
 N  P  P  D  N  I  K  D  T  K  S  D  I  I  F
tttcagagaagtgtcccaggacatgataataagatgcaatttgaa
 F  Q  R  S  V  P  G  H  D  N  K  M  Q  F  E
tcttcatcatacgaaggatactttctagcttgtgaaaaagagaga
 S  S  S  Y  E  G  Y  F  L  A  C  E  K  E  R
gacctttttaaactcattttgaaaaaagaggatgaattgggggat
  D  L  F  K  L  I  L  K  K  E  D  E  L  G  D
agatctataatgttcactgttcaaaacgaagac
 R  S  I  M  F  T  V  Q  N  E  D
```

A.8 The streptavidin-IL-18 fusion protein coding sequence and amino acid translation.